Formation of hydroxyapatite within porous TiO$_2$ layer by micro-arc oxidation coupled with electrophoretic deposition

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Abstract

Micro-arc oxidation (MAO) is commonly used to modify the surface of Ti-based medical implants with a bioactive and porous titanium oxide (TiO$_2$) layer. This study reports a novel method of incorporating hydroxyapatite (HA) within the TiO$_2$ layer by coupling MAO with an electrophoretic deposition (EPD) process. A HA-incorporated, porous TiO$_2$ layer was produced successfully on the Ti substrate using the EPD-coupled MAO treatment, as confirmed by electron microscopy observations. Addition of ethanol to the electrolyte solution containing the fine HA particles was essential to reduce the level of gaseous emission on the anode, which obstructs the attachment of HA particles. In vitro cellular assays showed that the incorporation of HA significantly improved the osteoblastic activity on the coating layer.

Keywords: Titanium; Micro-arc oxidation (MAO); Electrophoretic deposition (EPD); Hydroxyapatite; Titanium oxide

1. Introduction

Titanium (Ti) and its alloys are suitable materials for dental and orthopedic implants on account of their outstanding chemical stability, mechanical properties and biocompatibility, which are mainly due to the surface oxide layer formed naturally in air or in many aqueous environments [1,2]. Therefore, the properties of the surface oxide layer, such as roughness, topography and composition, play an important role in the biocompatibility of a Ti implant. Thus far, a number of techniques have been developed to improve the surface properties of Ti implants. These include blasting with hard particles, etching in an acidic solution, coating with bioactive materials and electrochemical treatments [3–6].

Micro-arc oxidation (MAO) is considered one of the most useful methods for surface modification because it can produce porous and firmly adherent TiO$_2$ films on Ti implants, which can not only enhance the fixation of the implants to the bone, but also improve their in vivo corrosion behavior [6–11]. This technique basically makes full use of the anodic oxidation of Ti by applying a positive voltage to a Ti substrate used as the anode immersed in an electrolyte. In particular, when an applied voltage is increased beyond a certain point, micro-arcs are generated as a result of the dielectric breakdown of the surface TiO$_2$ layer, whereupon Ti ions in the Ti implant and OH ions in the electrolyte move in opposite directions very quickly to form TiO$_2$ again.

Furthermore, bioactive materials or antibiotics can be incorporated into the coating layer during the MAO process by tailoring the composition of the electrolyte solution [6,7,12,13]. For example, Ca and P ions have been...
incorporated successfully into the TiO$_2$ layer using an electrolyte solution containing Ca and P sources, which resulted in a considerable improvement in the osseointegration ability of the implant in vivo tests [6,12,13]. In addition, these incorporated Ca and P ions can be crystallized to form hydroxyapatite (HA) or other calcium phosphate phases using a hydrothermal treatment [14–16]. More recently, it has been demonstrated that a thin calcium phosphate layer could be directly deposited onto a micro-arc oxidized Ti substrate using electron beam evaporation, which could enhance osseointegration of Ti implants [17]. However, it is still challenging to develop new methods that can allow the incorporation of bioactive materials, particularly in the form of crystalline phase, into the TiO$_2$ coating layer in an in situ manner.

Therefore, in this study, a new simple method was proposed to directly incorporate well-crystallized HA particles into the TiO$_2$ layer on Ti without significantly altering the microporous coating morphology. The strategy combined the principles of the MAO process with an electrophoretic deposition (EPD) process, which is often used to coat the surface of Ti materials with a HA layer [18–20]. In particular, a porous TiO$_2$ layer was formed via the MAO process, while, at the same time, negatively charged HA particles migrate toward the Ti anode through the EPD process, as illustrated in Fig. 1. These particles then become incorporated into the pores formed in the TiO$_2$ layer, resulting in the formation of a bioactive HA-incorporated TiO$_2$ coating layer on the Ti substrate. For the successful use of the newly developed MAO-EPD treatment, ethanol was added to the electrolyte containing the fine HA particles in order to retard the evolution of the gas at the anode, which would otherwise obstruct the attachment of HA particles [21]. The morphology, composition and phase of the coating layer were examined at different ethanol concentrations in the electrolyte and applied voltages during the MAO-EPD treatment. The biological properties of the coating layers were evaluated using in vitro cell growth and osteoblastic differentiation assays.

2. Materials and methods

2.1. Preparation of electrolytic solution containing HA particles

For successful use of the MAO-EPD treatment, it is essential to prepare a suitable electrolyte solution, in which nanosized HA particles are dispersed homogeneously without severe agglomeration. To achieve this, commercially available HA powder (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$; Alfa Aesar Co., Milwaukee, WI, USA) was calcined at 900 °C for 3 h to improve the rheological behavior of the suspension [22]. Subsequently, the calcined HA powders were ball-milled for 48 h in a mixture of deionized water and ethanol with various ethanol concentrations ranging from 0 to 50 vol.%. The amount of HA powder in the slurry was 20 g l$^{-1}$. After achieving a stable solution with fine HA particles for the EPD process, the suspension was transferred to a beaker and mixed with 0.08 mol l$^{-1}$ disodium β-glycerophosphate pentahydrate (C$_3$H$_7$Na$_2$O$_6$P$_5$H$_2$O, β-GP, Tokyo Kasei, Japan) for 1 h using a magnetic stirrer to prepare the electrolyte solution. In addition, ammonium hydroxide (NH$_4$OH, Sigma–Aldrich, St. Louis, MO, USA) was used to adjust the pH to 11. Before each coating treatment, the solutions were agitated using an ultrasonic cleaner for 1 h to prevent settling and agglomeration of the HA particles.

2.2. MAO-EPD treatment

Commercially pure (CP) Ti (Grade 2, Ka-Hee Metal Industry Co., Seoul, Korea) was used as the substrate. Plates, 10 × 10 × 1 mm$^3$ in size, were ground using 2000 grit SiC sandpaper and cleaned ultrasonically in acetone, ethanol and deionized water in series. The MAO-EPD treatment was carried out in the prepared electrolyte solutions by applying a pulsed DC field to the specimen using a pulsed DC power supply (Model-P6214, Auto Electric Co., Seoul, Korea). The frequency and duty cycle of the pulsed DC power were 660 Hz and 60%, respectively. The roughness and thickness of the oxide layer were controlled by applying a wide range of DC fields (300, 350, 375 and 400 V) to the specimens for either 3 or 10 min.

2.3. Characterization of oxide layer

An electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Co., Japan) was used to examine the zeta
potential, electromobility and size distribution of the HA particles in the various electrolytic solutions containing the suspended particles. The phase and microstructure of the specimens were examined by X-ray diffraction (XRD, M18XHF-SRA, MacScience Co., Yokohama, Japan) and field-emission scanning electron microscopy (FE-SEM, JSM-6330F, JEOL, Tokyo, Japan), respectively. The composition of the coating layer was analyzed using energy dispersive spectroscopy (EDS, ISIS 300 system, Link-analytical, Oxford Instruments, USA). The structure and composition of the coating layer were further analyzed using transmission electron microscopy (TEM, JEM-3000F, JEOL, Tokyo, Japan).

2.4. Characterization of biological properties

The biological properties of the specimens (pure Ti substrate, Ti substrate after the MAO treatment and Ti substrate after the MAO-EPD treatment in the electrolyte solution containing 25 vol.% ethanol) were examined using in vitro cell tests. The MC3T3-E1 cell line (ATCC, CRL-2593) was used to characterize the proliferation and differentiation behavior of the cells. The pre-incubated cell lines were placed onto the specimens with a cell density of 10^4 cells/cm^2, and cultured in a humidified incubator containing 5% CO_2 at 37°C. Minimum essential medium (α-MEM, Welgene Co., Ltd., Seoul, Korea) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., USA) was used as the culturing medium.

The level of cell attachment after culturing the specimens for 1 day was examined by SEM and confocal laser scanning microscopy (CLSM, Zeiss-LSM510, Carl Zeiss Inc., Jena, Germany). The proliferation behavior was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, Madison, WI, USA) for mitochondrial reduction. This assay is based on the ability of metabolically active cells to reduce a tetrazolium-based compound, MTS, to a purple formazan product. The quantity of the formazan cells to reduce a tetrazolium-based compound, MTS, to a purple formazan product. The quantity of the formazan product, which is measured by the absorbance at 490 nm using a micro-reader (Biorad, Model 550, USA), is directly proportional to the number of living cells in the culture.

The alkaline phosphate (ALP) activity, which corresponds to the degree of cell differentiation, was measured as an early marker of the maintenance of the osteoblastic phenotype using p-nitrophenyl phosphate (pNPP) (Sigma-Aldrich, UK). This colorimetric assay is based on the conversion of pNPP to p-nitrophenol (pNP) in the presence of ALP, where the rate of pNP production is proportional to the ALP activity. The production of pNP was determined by the absorbance at 405 nm measured using a micro-reader.

2.5. Statistical analysis

The experimental data is expressed as the mean ± standard error deviation (SED) of three specimens. Statistical analysis was carried out using a one-way analysis of variance (ANOVA). P values <0.05 (○), <0.01 (●) and <0.001 (▴▴) were considered significant.

3. Results

3.1. Stability of solutions

In order to achieve the effective movement of HA particles, the electrolyte should have a high zeta potential, high electromobility and high dielectric constant as well as low viscosity. The addition of ethanol to the aqueous solution makes the EPD process more effective by preventing gaseous emission at the anode caused by the electrolysis of water. However, this inevitably decreases the particle mobility by decreasing the zeta potential and increasing the viscosity of the suspension [21]. Nevertheless, the electrolyte solution containing 25 vol.% ethanol showed a reasonably high zeta potential (~16 mV) and electromobility (~0.125 μm cm V^-1 s^-1). The zeta potential and electromobility of the HA suspensions without the electrolyte and ethanol were ~23 mV and ~0.19 μm cm V^-1 s^-1, respectively. This suggests that when an electric field is applied, the HA particles suspended in the electrolyte solution become negatively charged and move efficiently toward the anode, where they are deposited on the Ti substrate [22]. The sizes of HA particles in the electrolyte were in the range of 0.3–0.6 μm, which would be expected to be very useful for the EPD process.

3.2. Morphology and composition of coating layer

The surface morphology of the specimens after the MAO-EPD treatment in the electrolyte solutions containing ethanol concentrations ranging from 0 to 50 vol.% was examined by SEM. Fig. 2A–H shows typical SEM micrographs of the surfaces formed on the specimens treated with a pulsed DC field of 375 V. When no ethanol was added, a highly porous layer, presumably a TiO_2 phase, was observed on the specimen. This layer was formed as a result of MAO of the Ti substrate, as shown in Fig. 2A. Some of the HA particles were deposited onto the porous coating layer but they detached easily from the surface during ultrasonic cleaning. This suggests that they had merely deposited on the coating layer formed on the specimen surface, as shown in Fig. 2B. On the other hand, the addition of ethanol to the electrolyte solution changed the surface morphology of the specimens significantly, as shown in Fig. 2C–H. Tiny HA particles were observed on the surface, and some of the pores were clogged (Fig. 2D). The deposition of HA particles became more evident as the ethanol concentration was increased to 25 vol.% (Fig. 2E and F). However, additional small pores appeared on the coating layer when a higher ethanol concentration (50 vol.%) was used (Fig. 2G and H).

Fig. 3A–C shows XRD patterns of the crystalline phases of the coating layers formed by the MAO-EPD treatment.
Without the addition of ethanol to the electrolyte, the sample showed only the peaks associated with anatase and Ti (Fig. 3A). No trace of HA was detected. On the other hand, when ethanol was added to the electrolyte solution, peaks associated with crystalline HA were detected, along with anatase (Fig. 3B–D). This indicates that a considerable number of HA particles were incorporated into the coating layer. The relative intensity of the HA peak increased slightly with increasing amount of ethanol from 10 to 25 vol.%. However, a slight decrease in HA and TiO\textsubscript{2} peaks was observed when a higher ethanol concentration of 50 vol.% was employed (Fig. 3D).

Fig. 4A–D shows the chemical compositions of the coating layers according to EDS. Interestingly, even without the addition of ethanol, the specimen showed peaks associated with Ca and P, as well as those for Ti and O (Fig. 4A). This implies that a small number of HA particles are incorporated into the coating layer though less than the...
detection limit of XRD (see Fig. 3A). On the other hand, the intensities of the Ca and P peaks for the specimens produced with the addition of ethanol were higher (Fig. 4B and D). In addition, the relative intensities of the Ca and
P peaks increased significantly with increasing amounts of ethanol. This suggests that there was an increase in the number of HA particles in the coating layer. Therefore, it can be concluded that the addition of 25 vol.% ethanol during the MAO-EPD treatment is quite useful because it allows the efficient incorporation of HA particles into the TiO₂ coating layer.

Fig. 5 shows TEM images of the coating layer produced using MAO-EPD treatment in the electrolyte solution containing 25 vol.% ethanol. Some internal pores were formed inside the coating layer. The chemical composition of the coating layer was examined by EDS line scan analysis along the arrow marked in Fig. 5A. The typical EDS line scan crossing the coating layer is shown in Fig. 5B. At the starting point near the Ti substrate, relatively strong peaks for Ti and O were observed along with weak peaks for P and Ca, indicating that this region consists mainly of a TiO₂ phase without the incorporation of the HA particles. However, the relative intensity of P increased gradually as the distance from the Ti substrate was increased to a certain point marked by the dashed line, which might be attributed to the incorporation of P ions from disodium β-glycerophosphate pentahydrate, while there were negligible changes observed for Ti, O and Ca. Beyond this region, a peak corresponding to Ca appeared and its intensity remained constant, indicating the presence of HA particles in the coating layer.

3.3. In vitro tests

An in vitro test with MC3T3-E1 cells was used to examine the biocompatibility of the specimens produced using the MAO-EPD process in the electrolyte solution containing 25 vol.% ethanol. For the purpose of comparison, two substrates were also tested: a pure Ti substrate and a Ti substrate treated by MAO at 270 V in an electrolyte solution without HA particles. Fig. 6A–F shows the morphology of the cells grown on the samples for 1 day. All the prepared specimens showed a similar morphology. In other words, the cells were in close contact with the specimens and spread out uniformly over the entire surface. This suggests that all the prepared specimens provide a biocompatible environment for favorable cell attachment.

The attachment behavior of the cells was further characterized by CLSM, as shown in Fig. 7A and B. A number of cells were well attached to both surfaces, i.e. the Ti substrate treated by MAO and the Ti substrate treated by MAO-EPD. However, the specimens after the MAO-EPD treatment showed better cell spreading (Fig. 7B).

The cell viability on the specimens was measured using an MTS assay after culturing for 3 days, as shown in Fig. 8. The level of cell growth on the sample after the MAO treatment was similar to that on the pure Ti substrate. However, the cell growth level on the Ti substrate after the MAO-EPD treatment was higher than that on the pure Ti substrate, (P < 0.05).

The differentiation behavior of the cells on the specimens was investigated further by measuring their ALP activity, as shown in Fig. 9. Although all the prepared specimens showed similar cell attachment and proliferation behavior, the differentiation behavior of the cells was strongly affected by the type of coating treatment. In particular, when the sample was treated using the MAO process, the ALP activity of the Ti substrate was improved significantly compared with that of a pure Ti substrate. The ALP activity was increased further when the sample was treated using the MAO-EPD process. This improvement was attributed to the successful incorporation of bioactive HA particles into the coating layer.

4. Discussion

This study demonstrated the possibility of using a MAO-EPD treatment to improve the biocompatibility of Ti implants, in which bioactive HA particles are incorporated into the coating layer. This method fundamentally combines the advantages of MAO with those of EPD. In particular, at the early stage of the treatment, a porous TiO₂ layer is produced using the MAO process. When the arc is reduced, HA particles are also incorporated into
the TiO$_2$ layer through the EPD process [7]. This is a simple and controllable method for producing a bioactive TiO$_2$/HA coating layer on the surface of a Ti implant. It should also be noted that the fine HA particles were incorporated into the TiO$_2$ layer in an in situ manner.

The properties of the coating layer, such as the porous microstructure and number of HA particles incorporated, were strongly influenced by the ethanol concentration in the electrolyte solution. The addition of ethanol allowed the efficient incorporation of HA particles into the coating layer (Fig. 2C–H). Hence, at the early stage of the MAO-EPD treatment, a porous TiO$_2$ layer is formed as a product of the MAO treatment of the Ti substrate. At the same time, negatively charged HA particles migrate toward the anode, i.e., the porous TiO$_2$ coating layer, via the EPD process. However, most of the particles subsequently become detached from the coating layer due to gaseous emission generated by the electrolysis of water at the anode [21]. This gaseous emission can be reduced effectively by adding ethanol to the electrolyte solution, thereby allowing the HA particles to become attached to the coating layer. It should be noted that the incorporated HA particles can be bonded strongly to the surrounding TiO$_2$ phase, due to the locally high temperature caused by micro-arcs, which is one of the striking advantages of the incorporation of HA particles into the porous TiO$_2$ coating layer in situ via the MAO-EPD treatment.

The crystalline phases and compositions of the coating layers are also related to the ethanol concentration in the electrolyte solution (Figs. 3 and 4). When no ethanol was added, only the peaks associated with the anatase phase of TiO$_2$ and Ti crystalline phases were observed, without any traces of a crystalline HA phase (Fig. 3A). However, EDS analysis showed not only Ti and O peaks, but also Ca and P peaks (Fig. 4A). These results suggest that only a small number of the HA particles were incorporated into the TiO$_2$ coating layer. In order to overcome this limitation, ethanol was added to the electrolyte solution since it...
was expected to retard the gaseous emission caused by the electrolysis of water [21]. Regardless of the ethanol concentrations used, all the specimens produced showed strong peaks associated with crystalline HA and anatase (Fig. 3B–D). This suggests that a considerable number of crystalline HA particles had been incorporated effectively into the coating layer. The use of higher ethanol concentrations led to an increase in the intensities of the Ca and P peaks (Fig. 4B and C).

The composition of the coating layer, which consisted of TiO$_2$ and HA phases, was examined more closely by EDS line scan analysis (Fig. 5B). The relative intensities of the peaks for P and Ca increased significantly with increasing distance from the point at which the TiO$_2$ coating layer had formed on the Ti substrate, which indicates the presence of an increasing amount of HA particles in the coating layer. These observations demonstrate that

![CLSM micrographs showing MC3T3 cells after culturing for 1 day on (A) Ti substrate after the MAO treatment and (B) Ti substrate after the MAO-EPD treatment.](image)

**Fig. 7.** CLSM micrographs showing MC3T3 cells after culturing for 1 day on (A) Ti substrate after the MAO treatment and (B) Ti substrate after the MAO-EPD treatment.

![Cell viability measured by MTS assay after culturing for 3 days on the samples (pure Ti substrate, Ti substrate after the MAO treatment and Ti substrate after the MAO-EPD treatment).](image)

**Fig. 8.** Cell viability measured by MTS assay after culturing for 3 days on the samples (pure Ti substrate, Ti substrate after the MAO treatment and Ti substrate after the MAO-EPD treatment).
the present method is very useful for incorporating bioactive HA particles into the porous TiO$_2$ coating layer in situ.

The incorporation of HA particles into the coating layer significantly improved the biocompatibility of the Ti implant compared with that of the pure Ti substrate and the Ti substrate treated by MAO at 270 V in an electrolyte solution without HA particles. All the specimens showed excellent cell attachment and proliferation (Fig. 6), indicating a biologically favorable environment. On the other hand, the ALP activity was strongly affected by the microstructure and chemical composition of the coating layer (Fig. 9). The ALP activity was increased by coating the surface of the Ti substrate with a porous TiO$_2$ layer using the MAO process due to the creation of a rough, biocompatible surface [6,10]. This ALP activity was increased further by incorporating bioactive HA particles into the coating layer. In addition, it should be noted that the HA-incorporated TiO$_2$ coating layer would be expected to provide excellent corrosion resistance [23].

5. Conclusion

This paper demonstrated the possibility and effectiveness of a MAO-EPD treatment for the production of a bioactive HA-incorporated TiO$_2$ coating layer on the surface of Ti implants. The addition of ethanol to the electrolyte solution inhibited gaseous emission at the anode generated by the electrolysis of water, and allowed the efficient incorporation of HA particles into the porous TiO$_2$ coating layer during deposition. This in situ coating method is expected to provide strong bonding between the incorporated HA particles and TiO$_2$ phase formed by MAO of the Ti substrate. The in vitro tests showed a considerable increase in ALP activity after the MAO-EPD treatment compared with the specimens before and after the MAO treatment.

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References


