Hierarchical Micropore/Nanorod Apatite Hybrids In-Situ Grown from 3-D Printed Macroporous Ti6Al4V Implants with Improved Bioactivity and Osseointegration

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The advent of three-dimensional (3-D) printed technique provides great possibility in the fabrication of customized porous titanium (Ti) implant. However, the bioinert property of the printed Ti poses an outstanding problem. Hybrid micro-arc oxidation and hydrothermal (MAO–HT) treatment on porous metals is able to produce multi-scaled hierarchical orthopedic implant, showing great potential for surface modification of 3-D printed implant. In this study, cylindrical porous Ti6Al4V (Ti64) scaffolds with pore size of 640 μm, porosity of 73% were 3-D printed by electron beam melting process, and their surfaces were left untreated or treated by a combined MAO–HT procedure. In vitro bioactivity was tested by immersion in simulated body fluid for different time points. Then, 12 scaffolds in each group were implanted into the femoral condyles of New Zealand rabbit for 8 weeks. Osseointegration was evaluated by qualitative and quantitative histological analysis, and the bone ingrowth features were probed by sequential fluorescent labeling at 3 and 6 weeks post-surgery. Following the MAO–HT treatment, the porous Ti64 scaffold was endowed with multi-scaled micro/nano-topographies and high amounts of CaP on its surface. The treated scaffold exhibited drastically enhanced apatite forming ability compared with the untreated one. In vivo test revealed significantly that a higher amount of bone ingrowth and bone implant contact at the treated scaffold. The 2 types of scaffolds had different patterns of bone ingrowth: the treated scaffold exhibited a pattern of contact osteogenesis, by which bone formed directly on the treated implant surface, whereas bone formed distal to the implant surface of the untreated scaffold. MAO–HT treatment can significantly enhance the in vitro apatite-inducing ability and in vivo osseointegration capacity of 3-D porous Ti64 scaffold and may provide as a viable approach for the fabrication of bioactive 3-D printed porous implant for orthopedic applications.


1. Introduction

Currently, the newly-developed three-dimensional (3-D) printed techniques, such as electron beam melting (EBM) and selective laser melting (SLM), provide new insight in the fabrication of sophisticated and controllable porous titanium (Ti) scaffolds with completely interconnecting pores, which allows the ingrowth of bony tissues[1,2].

Due to the adjustable mechanical properties (e.g., lower elastic modulus), excellent biocompatibility, as well as high adaptability to host site anatomy, rapid prototyped porous Ti scaffold holds the potential in the manufacturing of customized porous orthopedic implants for the management of complex bone defect reconstructions[3]. As a metallic material, however, porous Ti scaffold still exhibits a biopassive property compared with bioglass or calcium phosphate based materials[4,5]. Despite the reported capacity of osseointegration of 3-D printed porous Ti in animal studies, there may be situations when surface modification is needed for rapid osseointegration[2,6]. Such situations include applying porous Ti implant in bone bed of poor quality or for large bone defect reconstruction, e.g., following total spondylectomy[3,7].

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Micro-arc oxidation (MAO), also known as plasma electrolytic oxidation, is an electrochemical surface treatment technique for generating micro-porous oxide coatings on valve metals (e.g., titanium, aluminum, and magnesium). During this process, bioactive elements such as strontium (Sr), calcium (Ca), phosphate (P) and zinc (Zn) can be simultaneously incorporated into the coating from the electrolyte. This technique enables simultaneous chemical and morphological modifications of the implant surface through a single process. MAO is usually combined with subsequent activation methods, such as sol–gel and hydrothermal (HT) treatment to further improve its bioactivity. Previous studies have suggested that the combined MAO–HT treatment can produce nano-scaled crystals on the micro-porous oxidized coatings and yield enhanced biological performance. In particular, when combined the MAO–HT treatment is applied to a metallic scaffold, a multi-scaled hierarchical orthopedic implant can be fabricated, comprising nano-scale fibers on the micro-porous walls of the macro-porous Ti scaffold. To our knowledge, application of MAO–HT treatment to a 3-D printed porous Ti64 scaffold has not been reported.

In this work, we reported the fabrication of multi-scale bioactive coating on an EBM-fabricated porous Ti64 scaffold via sequential MAO–HT treatments. All the samples were ultrasonically washed with acetone, alcohol and distilled water and dried at 60 °C overnight. For MAO treatment, the porous Ti64 scaffolds were used as an anode, and a stainless plate was used as a cathode in an electrolytic bath. The electrolyte was prepared by dissolving 11.4 g/L Ca-(CH3COO)2·H2O, 4.7 g/L NaH2PO4·2H2O, 15 g/L EDTA, and 10 g/L NaOH into deionized water. It was treated with a working voltage of 350 V, a pulse frequency of 500 Hz and a duty ratio of 10% for 5 min. The samples were then placed in the bottom of a Teflon-lined autoclave, in which 20 mL of distilled water was adjusted to pH 12 by NaOH, and then the samples were hydrothermally treated at 180 °C for 12 h. The treated samples were ultrasonically rinsed with distilled water for 10 min, and dried at room temperature. Scanning electron microscopy (SEM, S-4800, Hitachi) was performed to observe the surface morphology of the implant, and energy-dispersive X-ray spectroscopy (EDS) was used to evaluate the chemical composition of the implant surfaces. X-ray diffraction (XRD, D8 Focus, Bruker) was used to analyze the phase structures of the coatings between 20° and 60°. The scratched nanorod coatings were also examined by transmission electron microscopy (TEM, H-9000, Hitachi).

Fig. 1. Schematic presentation of the two-step surface treatment for obtaining the hierarchical micropore/nanorod apatite hybrids on macroporous Ti64 scaffolds.

2. Materials and Methods

2.1. Implant fabrication

Cylindrical porous Ti64 scaffolds with 6 mm in height and 5 mm in diameter were rapidly prototyped using EBM S12 system (Arcam AB, Sweden) from a standard Arcam Ti64 powder with a particle size of 45–100 μm. The porous structure was designed based on a dodecahedron unit cell using computer-assisted design (CAD) software. The porous Ti64 scaffold was printed with pore size of 640 μm, strut diameter of 400 μm and porosity of 73%, as described previously.
2.3. Apatite forming ability

To test the in vitro bioactivity, the samples were immersed in a 30 mL simulated body fluid at 37 °C for 3, 7 and 14 days, and the SBF was refreshed every other day. The SBF was prepared by dissolving reagent-grade chemicals of NaCl, NaHCO3, KCl, K2HPO4·3H2O, MgCl2·6H2O, CaCl2, and Na2SO4 into deionized water and buffering at pH 7.4 with tris-hydroxymethylaminomethane ((CH2OH)2CNH2) and 1.0 mol/l HCl at 37 °C, as described by Kokubo and Takadama.[20]

The formation of apatite on the surface was observed by SEM.

2.4. Animal surgery and fluorescent labeling

Prior to conducting the animal studies, all the experimental protocols were approved by the Institutional Animal Ethics Committee. All animals were housed according to the national guidelines for care and use of laboratory animals. After sterilization of the scaffolds using ethylene oxide gas, 12 untreated implants and 12 oxidized implants were implanted at the bilateral femoral condyles in 12 healthy and mature male New Zealand rabbits. The rabbits were immobilized on operating table under general anesthesia with 0.5% povidone iodine and the bilateral medial femoral condyles were surgically opened. A bone defect was created by a 5-mm diameter drill under irrigation with saline, and the implants were inserted by press fitting (Fig. S1). Then the incision was closed in layers. Intramuscular injections of penicillin were administered at a dose of 0.1 g/kg during surgery and postoperatively for 3 days. Sequential fluorescent labeling was performed to label the regenerated bone at different time points. Calcein green (10 mg/kg, Sigma, USA) and alizarin (30 mg/kg, Sigma, USA) were injected subcutaneously at weeks 3 and 6 post-implantation, respectively. After 8 weeks of implantation, all the animals were euthanized by an overdose of phenobarbital and then the samples and surrounding tissues were excised.

2.5. Histological and histomorphometric evaluations

After euthanasia, all samples from each group underwent fixation, serial dehydration, and were embedded in methyl methacrylate. The ground sections with 40 to 50μm were prepared using EXAKT systems (EXAKT Apparatebau, Norderstedt, Germany) as described previously.[19] Sections from 6 samples were analyzed under the fluorescent microscope, and the bones fronts formed at the 3rd and 6th weeks were marked by a green label from calcein green and a red label from alizarin, respectively. The unstained sections were then sputtered with gold and observed under SEM. Another 6 samples were prepared in similar procedures; two longitudinal middle sections from each sample were stained with methyl blue and basic fuchsin for histological analysis. Histomorphometric analysis of the sections was performed using the Image-Pro Plus software. Quantitative assessments were performed on 12 histological sections of each group regarding the bone in-growth and bone-implant contact ratio. The bone in-growth was defined as the percentage of new bone within the pores. The bone-implant contact ratio was measured as the faction of the surface area of the implant in contact with the bone.

2.6. Statistical analysis

For all experiments, values were reported as mean ± SD. Statistical analysis was determined by a non-parametric test (Mann–Whitney) using SPSS (17.0 version). Significance was defined as a p value of less than 0.05.

3. Results

3.1. Characterizations of the coatings

In this study, a facile and versatile strategy involving the novel combination of MAO and HT treatments was applied to 3D-printed Ti64 scaffolds, as illustrated in Fig. 1. At first, micro-scaled porous structures were formed uniformly on the strut surfaces of the treated scaffolds after minutes’ treatment with MAO. Then, the resulting scaffolds were subjected to HT modification, by which nanorod-like structures were further developed around and within the micropores (Figs. 1 and 2). These “rods” were in hundreds of nanometers in length and dozens of nanometers in diameter. According to EDS analysis, compared with the chemical compositions of the as-printed Ti64, which mainly consisted of Ti, Al and V (Fig. S2), a significant amount of elements Ca (10.9 at.%), P (4.37 at.%), O (59.7 at.%) were incorporated with the MAO–HT process (Fig. 2(d)), indicating the apatite nature of the coatings.

To clarify the change in phase state of the hydrothermally treated specimens, XRD analysis was performed (Fig. 3). For the MAO-oxidized Ti64 samples, beside peaks belonging to metal Ti, fairly strong peaks assigned to well crystallized anatase and rutile were clearly observed. Although it was largely reported, MAO in Ca- and P-containing electrolytes will result in hydroxyapatite. The XRD patterns here did not show obvious presence of apatite crystals. This is probably due to the lack of crystallization within such a short treatment (5 min), leaving the major composition still amorphous. Nevertheless, the subsequent HT process can overcome this limitation. As is evident, representative diffraction peaks of apatite, i.e., 2θ of 26.1°, 30°–33° and 57.5° (002), the combined peaks of (211), (112), and (300) planes, and the (500) plane appeared or were strengthened by this post-treatment.[11,22] Hence, the MAO–HT coatings were a mixture of anatase, rutile, and apatite crystals.

Moreover, TEM observation of the scratched nanorod coatings was performed, as shown in Fig. 4. It can be seen that the TEM images had both high and low density regions, corresponding to the underlying titania and superficial apatite, respectively (Fig. 4(a)). Particularly, a closer examination disclosed that the parts with low electron density were nanorod shaped (Fig. 4(b)), correlating well with the SEM results in Fig. 2. High-resolution transmission electron microscopy (HRTEM) investigation (Fig. 4(c)) provided more evidence that these regions were made of high crystallized apatite, with a typical lattice space of 0.345 nm (i.e. (0002) facet of apatite).[17] In addition, the distinct rings in its fast Fourier transformation (FFT) pattern (Fig. 4(d)) verified similar fact.

3.2. Apatite forming ability

Fig. 5 depicts the morphologies of samples immersed separately in SBF for a period of time. After being soaked in SBF, apatite layers were deposited over the entire surface of the MAO–HT treated scaffold within 7 days (Fig. 5(b)). High magnification images showed that the morphology of apatite exhibited a nanoflake shape. In contrast, apatite was not observed on the untreated scaffold after immersion in SBF for up to 14 days (Fig. 5(a)).

3.3. Histological and histomorphometric results

Fig. 6(a, b) shows the representative light optical micrographs of the cross-sectioned implants in the femoral condyle at 8 weeks. It could be observed that significantly more mineralized bone formed in the MAO–HT treated scaffold as compared to the untreated one. For the control group, only the periphery of the scaffold was deposited by bone, and the inner surface of the implant was generally lack of bone apposition (Fig. 6(a)). In contrast, the osteogenesis on
the treated scaffold was more extensive, and even the inner struts of the scaffold were deposited by mineralized bone. Further, the regenerated new bone was found in close contact with the implant surface and following the irregular contours of the implant surfaces (Fig. 6(b)). SEM images confirmed the histological evaluations, where a significantly larger amount of bone tissue was observed around the implant surface of the treated scaffold (Fig. 6(c, d)).

The results of histomorphometric evaluations are shown in Fig. 7. The bone in-growth in the control group was 8.2% ± 2.3%, and it was 16.3% ± 2.6% in the treated group. Significantly higher bone in-growth at the treated scaffolds was observed (p < 0.0001) (Fig. 7(a)). For bone implant contact ratio, it was 22.3% ± 6.6% in the untreated scaffolds. For the treated scaffolds, the percentage of bone deposited on the implant surface was 66.1% ± 14.0%. Clearly, the treated scaffold had significantly more bone in contact with the implant surface than the untreated one (p < 0.0001) (Fig. 7(b)).

### 3.4. Fluorescent labeling

The patterns of osteogenesis on the implant were analyzed by sequential fluorescent labeling (Fig. 8). The fluorescent labeling revealed that the 2 types of implant exhibited different patterns of bone in-growth. In the control group, there was bone growth into the porous implant with little attachment to the implant surface of the Ti64 strut itself, as tracked by the fluorescent labels (Fig. 8(a)). In contrast, in the treated group, early bone formation occurred primarily on the implant surface (stained green), followed by bone expansion toward the porous space (stained red). The osseointegration of the treated implant was in a pattern of contact osteogenesis, by which bone formed directly on the implant surface during endosseous integration (Fig. 8(b)).

### 4. Discussion

Scaffolds for osteogenesis should mimic natural bone morphologies in order to optimize osseointegration. The bone tissue, particularly the spongy bone, is inherently a macro-porous structure with a porosity of 50%–90%. The advent of 3-D printed technique triggered the interest of fabrication of trabecular metals to mimic the structures of bone. In fact, at the microscopic level,
natural bone exhibits a hierarchical architecture that comprises nano-scale collagen fibers and hydroxyapatite crystals on the trabecular struts of the bones\(^{[17]}\). However, although 3-D printing technique has good control over the macro-porous architectures of porous metal, the surface properties that directly affect the bone response are beyond the control of this technique\(^{[25]}\). The printed Ti scaffold exhibits a bioinert property as well. Based on this aspect, surface treatment may be a plausible approach to speed up the osseointegration process by endowing the scaffold surface with bioactive property\(^{[26]}\). In this study, we introduced a hybrid MAO–HT process for the surface treatment of 3-D printed porous Ti64 implant and evaluated its osseointegration in a rabbit model. This combined treatment process successfully fabricated a multi-scaled hierarchical porous metallic implant based on the 3-D printed porous Ti64, which comprised nano-scaled needle-like structures on the micro-porous coatings of the scaffold (Fig. 2).

![TEM images displaying the morphology of a cluster of nanorods scratched from the coatings. Here, (b) is enlarged observation of area 1 in (a) and the “rods” were false-colored pink for visual identification. (c) HRTEM image taken from area 2 in (b). (d) Fast Fourier transformation (FFT) image of the lattice fringes in (c).](image1)

![SEM images of the surface morphologies of the porous Ti64 scaffold immersed in stimulated body fluid (SBF) for a given period: (a) untreated scaffold at day 14; (b) MAO–HT treated scaffold at day 3.](image2)
enhancement in bioactivity of implant surface was obtained as indicated by the in vitro apatite forming assays. Moreover, in vivo test revealed that the treated porous Ti64 implant was associated with significantly enhanced osseointegration capacity compared with the untreated implant.

The hybrid MAO–HT process has long been proposed for the surface modification of nonferrous metals with the aim of enhancing the surface bioactivity of the implant\cite{14–18}. Kokubo and Takadama\cite{20} have initiated the use of SBF for the in vitro test of bioactivity of the biomaterials for bone regenerations. They found that the ability of apatite to form on biomaterials in SBF correlates well with the in vivo bone bioactivities. In this study, it was shown that the MAO–HT treatment drastically enhanced the apatite forming ability of the porous Ti64 scaffold (Fig. 5). The underlying mechanism would be that MAO–HT process can provide abundant Ti—OH groups on the implant surface\cite{16}. Another reason is that the incorporated Ca, P ions can increase the degree of oversaturation of SBF, promoting the nucleation of apatite on its surrounding environment\cite{27}. Though it has been well established that the MAO–HT treatment can enhance the in vitro bioactivity of the Ti implant\cite{14,16–18}, the effect of this process on the in vivo osseointegration of metallic implant has been less reported\cite{12}. In particular, its effect on the in vivo osseointegration of 3-D printed porous Ti64 implant has not been determined. In this study, we demonstrated that MAO–HT treatment can substantially enhance the osseointegration of the porous Ti64 scaffold (Fig. 6). Specifically, the treated scaffold had significantly higher bone in-growth and bone implant contact ratio compared with the untreated one (Fig. 7).

In addition to the bone formation extent, the patterns of bone formation varied significantly between the 2 groups. The bone in-
growth in the untreated scaffold was rather limited and only the periphery was integrated by bone tissue (Fig. 6(a)). In contrast, bone formed extensively on the entire surfaces of the MAO–HT treated porous Ti64 scaffold (Fig. 6(b)). This altered exhibition of bone formation at the treated scaffold could be dynamically revealed from the in vivo sequential fluorescent labeling using Ca-binding dyes. As revealed by fluorescent tracking, the bone formed first on the implant surface of the treated scaffold whereas bone formed distal to the implant surface at the untreated scaffold (Fig. 8). The patterns of osteogenesis on the treated and untreated scaffolds were termed contact and distance osteogenesis, respectively [28]. Davies has indicated that the surface topographies of the implant play a critical role in the generation of contact osteogenesis [29]. It is emphasized that the micro/nano-scale topographies can drastically enhance the osteo-conductivity of the implant surface and thus facilitate the formation of contact osteogenesis [29]. Based on these aspects, the generation of contact osteogenesis on the porous Ti64 scaffold should have been related to the fabrication of numerous micro/nano-topographies on the implant surface by this hybrid treatment.

It has been demonstrated that contact osteogenesis is significantly faster than the osteogenesis from the host bone toward the implant [30]. Therefore, the significantly improved osteogenesis on the treated implant may have resulted from the enhanced osteo-conductivity of the implant surface with the associated altered osseointegration pattern. It is noteworthy to mention that to achieve contact osteogenesis is of particular importance in the setting of porous metallic implant. Since the inner surface of the implant can integrate directly in bone from the beginning of bone regeneration, the osseointegration process can be dramatically expedited. This dramatic improvement of bone in-growth and the generation of contact osteogenesis have important implications in orthopedic applications. In orthopedic practice, reconstruction of complex macro/micro-porous metal implants is used to reconstruct major bone defects. The treated scaffold is associated with significantly enhanced in vitro apatite-inducing ability and in vivo osseointegration capacity. MAO–HT treatment may provide as a facile approach in the fabrication of bioactive 3-D printed porous implant for orthopedic applications.

5. Conclusion

A combined MAO–HT treatment is able to fabricate a multi-scaled hierarchical orthopedic implant which comprises nano-scale fibers on the micro-porous walls of the macro-porous Ti64 scaffold. The treated scaffold is associated with significantly enhanced in vitro apatite-inducing ability and in vivo osseointegration capacity. MAO–HT treatment may provide as a facile approach in the fabrication of bioactive 3-D printed porous implant for orthopedic applications.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jmst.2016.05.013.

References
