Tailored Surface Treatment of 3D Printed Porous Ti6Al4V by Microarc Oxidation for Enhanced Osseointegration via Optimized Bone In-Growth Patterns and Interlocked Bone/Implant Interface

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ABSTRACT: 3D printed porous titanium (Ti) holds enormous potential for load-bearing orthopedic applications. Although the 3D printing technique has good control over the macro-structures of porous Ti, the surface properties that affect tissue response are beyond its control, adding the need for tailored surface treatment to improve its osseointegration capacity. Here, the one step microarc oxidation (MAO) process was applied to a 3D printed porous Ti6Al4V (Ti64) scaffold to endow the scaffold with a homogeneous layer of microporous TiO2 and significant amounts of amorphous calcium-phosphate. Following the treatment, the porous Ti64 scaffolds exhibited a drastically improved apatite forming ability, cyto-compatibility, and alkaline phosphatase activity. In vivo test in a rabbit model showed that the bone in-growth at the untreated scaffold was in a pattern of distance osteogenesis by which bone formed only at the periphery of the scaffold. In contrast, the bone in-growth at the MAO-treated scaffold exhibited a pattern of contact osteogenesis by which bone formed in situ on the entire surface of the scaffold. This pattern of bone in-growth significantly increased bone formation both in and around the scaffold possibly through enhancement of bone formation and disruption of bone remodeling. Moreover, the implant surface of the MAO-treated scaffold interlocked with the bone tissues through the fabricated microporous topographies to generate a stronger bone/implant interface. The increased osteointegration strength was further proven by a push out test. MAO exhibits a high efficiency in the enhancement of osteointegration of porous Ti64 via optimizing the patterns of bone in-growth and bone/implant interlocking. Therefore, post-treatment of 3D printed porous Ti64 with MAO technology might open up several possibilities for the development of bioactive customized implants in orthopedic applications.

KEYWORDS: 3D printing, porous Ti6Al4 V, microarc oxidation, osseointegration, contact osteogenesis

1. INTRODUCTION

Management of a major-sized load-bearing bone defect has been a challenge to physicians. The recent advancement of 3D printing techniques, such as selective laser melting (SLM) and electron beam melting (EBM), might provide a novel approach to solve this problem through the fabrication of customized porous metallic scaffold.1,2 This approach can simultaneously circumvent stress shielding of solid implant, meet the needs for patient-specific design and promote osseointegration by bone in-growth.3−5 Like other endosseous implants, the key for success of 3D printed implant is achievement of osseointegration. Macro-porous architectures and surface properties are two of the most important aspects affecting the osseointegration of a metallic scaffold.6 Although the 3D printing technique has good control over the macrostructures of the scaffold, the surface properties that determine the tissue response are beyond its control.7 Therefore, the tailored surface treatment may be required to enhance the osseointegration of 3D printed scaffolds further in several situations, such as applying the implants in the bone bed of poor quality or in bone defects of major size.7−9

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Unlike the solid implants, surface treatments of 3D printed porous metals have several critical issues to be addressed. First, in endosseous integration of implants, there are two patterns of osteogenesis which exhibit distinct processes of bone regeneration either from the implant to the adjacent bones (contact osteogenesis) or from the host bones to the implant surface (distance osteogenesis), depending on the surface properties of the implant. Although this concept has not been applied to a porous metal before, it could be inferred from the literature that bone in-growth at the as-printed porous Ti exhibits a pattern of distance osteogenesis by which bone grows from the periphery toward the inner scaffold. Since it has been proved that contact osteogenesis is 30% faster than distance osteogenesis, bone in-growth by distance osteogenesis can be substantially slower than that of contact osteogenesis. Second, another important issue regarding the metallic scaffold is that the ingrown bone may undergo remodeling due to the stress shielding by the scaffold itself. In contrast to contact osteogenesis, bone in-growth by distance osteogenesis is generally lacking bone contact with the inner surface of the scaffold. As a result, the ingrown bones would be more likely to be remodeled because mechanical stimulus cannot be transduced from the scaffold to the bones within it. Despite several studies on large animal models demonstrating satisfactory bone in-growth at the porous Ti scaffold, this problem could happen when porous metal is applied to bone defects of great size or in bones with poor quality. A typical scenario can be found in a study of bone in-growth at porous Ti scaffolds using a rabbit model where most of the ingrown bones at 3 weeks were substantially remodeled at a later stage, leading to rather poor bone in-growth at 8 weeks. On the basis of these aspects, it would be possible for the choice of proper surface treatment to solve these problems through optimizing the patterns of bone in-growth at the scaffold.

Third, the major goal of applying a porous metallic scaffold for load-bearing bone replacement is to achieve regional stability by strong osseointegration. However, it has been revealed that bone in-growth alone does not necessarily guarantee a better biomechanical strength. Besides bone in-growth, another key for osseointegration strength is the stability of the bone/implant interface. With the help of atom probe tomography, Karlsson et al. have recently determined that bone bonds chemically to Ti implant through direct contact between calcium atoms and titanium oxide surface after osseointegration. Nevertheless, since the strength at the basic level of biochemical bonding is inherently weak, a more powerful mechanism is required to achieve a higher strength at the functional level. It has been suggested that the synergistic role of surface chemistry and microtopography would be more beneficial for the achievement of higher bone/implant stability. Therefore, the rationale for an optimal design of the surface properties of a metallic scaffold should be aimed toward (1) optimizing the patterns of bone in-growth by contact osteogenesis and (2) achieving functional bone bonding to the implant surface via elaborated chemical and topographic modifications.

Figure 1. Surface characteristics of the porous Ti64 scaffold before and after MAO treatment. (A) CAD model of multiplanar hexagonal unit cell structures; macroscopical view of the untreated scaffold (B) and MAO treated scaffold (E); scanning electron microscopic (SEM) images and EDS analysis (inset image) of the untreated scaffold (C) and the MAO-treated scaffold at the (D) outer surface and (F) central surfaces. Cross-sectional image of the MAO coatings at the (G) outer and (I) inner surface of the scaffold. (H) Scout view shows the positions of the observed struts of the scaffold.
However, surface modification of 3D porous metal is more challenging than that of solid implant because the widely used line-of-sight techniques such as plasma spraying are technically inapplicable, and the choice of available techniques is very limited. Although several methods have been reported in the literature, they exhibited limitations as well (Table S1). Among these methods, the biomimetic coating proved ineffective and either the chemical treatments or Mg-doped hydroxyapatite (HA) coating resulted in slightly enhanced bone in-growth; even the complex anodizing and heat treatment process yielded suboptimal bone ingrowth. The recently reported polydopamine-assisted biomimetic HA coating is effective but time-consuming. In contrast, microarc oxidation (MAO) is an electrochemical surface treatment technique for generating oxide coatings on valve metals (e.g., Ti, Al, Mg, and Ta) with complex geometry. This process involves anodic oxidation of the implants in electrolyte under high potentials, so that discharges occur at the implant surface and produce numerous microporous structures on the oxide. During this process, bioactive elements such as calcium and phosphate can be simultaneously incorporated into the coating from the electrolyte. Previous studies have demonstrated that the bone healing around the oxidized implant had a significantly higher level of osteogenic gene expression than that of the machined one, and that the oxidized implants can more rapidly and strongly integrate in bone. Considering the versatility of MAO treatment in the chemical and topographic modifications of implant surface and its positive role on the bone regeneration and remodelling, we hypothesized that the MAO may be a promising candidate in the realization of the optimal designs as mentioned above. Therefore, we carried out this study to (1) evaluate the characteristics of MAO coatings on a 3D printed porous Ti6Al4V (Ti64) scaffold; (2) investigate the effects on in vitro bioactivity, cell proliferation, and osteoblast differentiation; (3) probe the osseointegration of the scaffold in a rabbit bone defect model; and (4) identify the bone in-growth patterns and features of bone/implant interface of MAO scaffold.

2. EXPERIMENTAL SECTION

2.1. Sample Fabrication. Cylindrical porous Ti64 scaffolds (diameter 5 mm, length 6 mm) were designed using computer-assisted design (CAD) software (Magics, Materialise, Belgium), and the data were stored in STL file format. The porous architecture was designed based on a dodecahedron unit cell with a pore size of 640 μm, strut diameter of 400 μm and porosity of 73% (Figure 1). This architecture was adopted because previous study demonstrated that the pore size at this range is beneficial for in-growth of bone and vessels. Then the implants were rapidly prototyped using EBM S12 system (Arcam AB, Sweden) as described previously. The nominal surface area of the scaffold was about 2.25 cm² using micro-CT evaluations.

2.2. MAO Treatment. Before treatment, all the samples were ultrasonically washed with acetone, alcohol, and distilled water and dried at 60 °C overnight. The porous Ti64 was used as an anode, and a stainless steel plate was used as the cathode. The MAO process was performed in an aqueous electrolyte containing 0.065 M Ca(CH₃COO)₂, 0.03 M Na,HPO₄, 0.065 M EDTA-2Na, and 0.5 M NaOH at a working voltage of 350 V, a pulse frequency of 500 Hz and a duty ratio of 10% for 5 min. The bath temperature was maintained below 40 °C via cooling water.

2.3. Surface Characterizations. The surface morphology and cross-sectional features of the outer and inner surface of the MAO-treated scaffold was characterized using scanning electron microscopy (SEM, Hitachi, Japan) coupled with energy-dispersive X-ray spectrometry (EDS). The chemical composition was further tested on polished plate samples that were treated under the same MAO process using X-ray photoelectron spectroscopy (XPS, Kratos, U.K.), and the crystal phase of the coating was characterized using X-ray diffractometer (XRD, D8 Focus, Bruker) equipped with Cu–Kα radiation source. The release characterization of Ca²⁺ from the scaffold/coating system was examined in vitro at 37 °C for 5, 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, KH₂PO₄, 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 to adjust the pH to 7.4, as described by Kokubo et al. The formation of apatite on the surface was observed using SEM and EDS.

2.6. Cell Proliferation on Scaffold. After sterilization of the scaffolds using ethylene oxide gas, primary human mesenchymal stem cells (hMSCs, passage 6, Lonza, Walkersville) were cultured and incubated on the untreated and MAO-treated samples (n = 4) as described previously. Generally, 5 × 10⁴ cells in suspension were drop-seeded on the scaffold in 24-well plates (Ultra Low Cluster Plate, Costar, Corning), and incubated for 1 h to allow cell attachment. The samples were then supplemented with 1.0 mL Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum, 1% sodium pyruvate, and 1% antibiotics. The cells were cultured at 37 °C with 5% CO₂, as described previously. The absorbed was measured at 450 nm.

2.7. Alkaline Phosphatase (ALP) Activity. Osteoblast differentiation ability was assessed in vitro by testing the ALP activity of the harvested cells with a colorimetric assay (Pierce). The ALP activity was normalized to the amount of Ca²⁺ per surface area of the scaffold (with a nominal surface area of 2.25 cm²).

2.8. Animal Experiments. In Vivo Implantation and Fluorescent Labeling. To evaluate the in vivo osseointegration of the
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were determined in the workstation to characterize the on-growth and fi

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the push-out force. The end point of the test was the presence of an abrupt push-out procedure was carried out with a constant rate of 1.5 mm/min. The end point of the test was the presence of an abrupt drop of the push-out force, and the maximal load was documented as


determined as the ratio of bone volume to the total volume of

2.8.3. Histological Examination. Twelve samples were retrieved for fluorescence and histological analysis at 8 weeks postsurgery. Samples were fixed in 10% formalin for 14 days, and dehydrated in serial concentrations of ethanol (70, 85, 95, and 100%) for 3 days each. The specimens were embedded in methyl methacrylate and cut using EXAKT systems (EXAKT Apparatebau, Norderstedt, Germany). Ground sections of 40 to 50 μm were prepared as described previously.34 The unstained sections from 6 samples of each group were analyzed under the fluorescent microscope. The bones formed at the third and seventh week were marked by a green label from calcine green and an orange label from tetracycline, respectively. The sections from another 6 samples of each group were stained with methyl blue and basic fuchsin, by which the bone tissues were stained red. Quantitative analysis was performed using the Image-Pro Plus software based on 2 middle longitudinal sections of each blocks regarding the bone in-growth (BI) and bone-implant contact ratio (BICR). Thus, 12 slices were analyzed for each group. The BI was defined as the percentage of new bone within the pores. The BICR was measured as the faction of the surface area of the implant in contact with the bone. 2.8.4. Analysis of Bone/Implant Interface. To investigate the features of bone/implant interface, we performed a separation test to expose the implant surface of the untreated and MAO-treated scaffold. Specifically, 3 histological blocks of the samples from each group were fixed and dehydrated and embedded into the epoxy, and then the implant together with the surrounding bone was sliced into sections using the EXAKT cutting system. Thereafter, the strut of the implant was levered from the underlying bone tissues using a sharp needle to expose the surface of the implant. The implant surface of the strut was sputtered with gold and observed with SEM. 2.8.5. Push out Test. The universal testing machine (Landmark, MTS Inc. MN, U.S.A.) was used to measure the push-out force between the implant and the bone. Twelve samples from each group were carefully cut at a tangent angle to the long axis of the implant to expose the inner side of the implant, and the periosteal bone at the outer side was removed prior to test. A custom-designed special holder was applied to fix the sample to ensure the loading alignment and then, the push-out procedure was carried out with a constant rate of 1.5 mm/min. The end point of the test was the presence of an abrupt drop of the push-out force, and the maximal load was documented as the push-out force. 2.9. Statistical Analysis. For all experiments, values for statistical analysis were reported as Mean ± SD. An independent-sample t test was performed to test the significant differences regarding the intrav

Figure 2. XPS survey spectrum of the MAO coatings (A) and the high resolution spectra of elements Ti 2p (B), Ca 2p (C), P 2p (D), and O 2s (E). (F) XRD pattern of the MAO-treated specimen.

porous Ti64 implants, 27 MAO-treated implants, and 27 untreated implants were implanted in the bilateral femur condyles of 27 healthy and mature male New Zealand rabbits. The animals were anesthetized by intramuscular injection of Ketamine (50 mg/kg). The skin was sterilized with 0.5% povidone iodine, and the bilateral medial femur condyles were surgically opened. A bone defect was created by a 5 mm diameter drill under irrigation with saline, and then the implant was inserted on the medial condyle by pressing (Figure S1). The incision was closed in layers with absorbable thread (PDS II, ETHICON). Intramuscular injections of penicillin were administered by intramuscular injection of Ketamine (50 mg/kg). The skin was sterilized with 0.5% povidone iodine, and the bilateral medial femur condyles were surgically opened. A bone defect was created by a 5 mm diameter drill under irrigation with saline, and then the implant was inserted on the medial condyle by pressing (Figure S1). The incision was closed in layers with absorbable thread (PDS II, ETHICON). Intramuscular injections of penicillin were administered at a dose of 0.1 g/kg during surgery and postoperatively for 3 days. To determine the process of osteogenesis on the scaffold following implantation, in vivo sequential fluorescent labeling was performed to label the regenerated bone at different time points. Generally, calcine green (10 mg/kg, Sigma, U.S.A.) and tetracycline (30 mg/kg, Sigma, U.S.A.) were injected subcutaneously at 3 and 7 weeks post-implantation, respectively. After 8 weeks of implantation, all the animals were euthanized and the samples with surrounding tissues were excised. All the experimental protocols were approved by the Institutional Animal Ethics Committee of our university, and all animals were housed according to the national guidelines for care and use of laboratory animals. 2.8.2. Micro-CT Analysis. Six specimens from each group were scanned by micro-CT (InveonTM, Siemens Medical Solutions U.S.A., Inc.) with a scanning rate of 6°/min and a resolution of 9 um. The X-ray source voltage was 80 kV and beam current was 80 mA using filtered Bremsstrahlung radiation. A 1 mm aluminum filter was used during the scanning. The micro-CT images were then reconstructed using Inveon Acquisition Workplace. The in-grown new bone was distinguished from soft tissue and metal implant by partition of different Hounsfield units (HU). The phase of the bone was defined in the range from 1000 to 2250 HU. Two regions of interest (ROIs) were determined in the workstation to characterize the on-growth and in-growth of new bone to the scaffold. They included (1) the peri-implant region at the peripheral 500 μm around the scaffold and (2) the intraporous region within the scaffold. The peri-implant bone fraction was defined as the ratio of bone volume to the total volume of the region, while the intraporous bone fraction was the ratio of bone volume to the total volume of the pores.

2.8.3. Histological Examination. Twelve samples were retrieved for fluorescence and histological analysis at 8 weeks postsurgery. Samples were fixed in 10% formalin for 14 days, and dehydrated in serial concentrations of ethanol (70, 85, 95, and 100%) for 3 days each. The specimens were embedded in methyl methacrylate and cut using EXAKT systems (EXAKT Apparatebau, Norderstedt, Germany). Ground sections of 40 to 50 μm were prepared as described previously.34 The unstained sections from 6 samples of each group were analyzed under the fluorescent microscope. The bones formed at the third and seventh week were marked by a green label from calcine green and an orange label from tetracycline, respectively. The sections from another 6 samples of each group were stained with methyl blue and basic fuchsin, by which the bone tissues were stained red. Quantitative analysis was performed using the Image-Pro Plus software based on 2 middle longitudinal sections of each blocks regarding the bone in-growth (BI) and bone-implant contact ratio (BICR). Thus, 12 slices were analyzed for each group. The BI was defined as the percentage of new bone within the pores. The BICR was measured as the faction of the surface area of the implant in contact with the bone. 2.8.4. Analysis of Bone/Implant Interface. To investigate the features of bone/implant interface, we performed a separation test to expose the implant surface of the untreated and MAO-treated scaffold. Specifically, 3 histological blocks of the samples from each group were fixed and dehydrated and embedded into the epoxy, and then the implant together with the surrounding bone was sliced into sections using the EXAKT cutting system. Thereafter, the strut of the implant was levered from the underlying bone tissues using a sharp needle to expose the surface of the implant. The implant surface of the strut was sputtered with gold and observed with SEM. 2.8.5. Push out Test. The universal testing machine (Landmark, MTS Inc. MN, U.S.A.) was used to measure the push-out force between the implant and the bone. Twelve samples from each group were carefully cut at a tangent angle to the long axis of the implant to expose the inner side of the implant, and the periosteal bone at the outer side was removed prior to test. A custom-designed special holder was applied to fix the sample to ensure the loading alignment and then, the push-out procedure was carried out with a constant rate of 1.5 mm/min. The end point of the test was the presence of an abrupt drop of the push-out force, and the maximal load was documented as the push-out force. 2.9. Statistical Analysis. For all experiments, values for statistical analysis were reported as Mean ± SD. An independent-sample t test was performed to test the significant differences regarding the in vitro
results, whereas the significant differences of the in vivo results were determined by a nonparametric test (Mann–Whitney) using SPSS (17.0 version). \( P < 0.05 \) was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Physi-chemical Characterization of the TiO2/CaP Coating. Inspired by the hierarchical structure of natural bone, we proposed a strategy of MAO post-treatment that simultaneously endowed the surfaces of the macro-porous scaffold with micro/nanoporous TiO2/CaP coatings. A specific Ca-EDTA-P electrolyte was chosen for this process, which resembled the electrolyte of Frauchiger et al., who termed it "electrolyte for osteointegrative coatings, ESOC". The surface morphologies of the porous Ti64 scaffold before and after MAO treatment are shown in Figure 1. After reaction for several minutes, the Ti64 scaffold loses its metallic luster and is oxidized into a brown color; the coating process has little influence on its macro-porous architectures. SEM images reveal that a homogeneous layer of microporous titanium oxides coating containing a significant amount of Ca and P is fabricated on the surface of the scaffold. The thickness of coating at the inner surface (4.4 \( \mu m \)) was only slightly smaller than that of the outer surface (4.8 \( \mu m \)) (Figure 1G, I). As revealed by EDS analysis, the coatings contained a high Ca/P ratio on the scaffold surface (1.67 to 1.98), which was close to the one found in the mineral phase of bone (1.67 for HA).

The chemical composition and state of the components in the MAO coatings are investigated by XPS, as shown in Figure 2. According to the survey spectrum, the major surface elements are O, Ti, Na, Ca, and P (Figure 2A). The doublet in the Ti 2p core-level spectrum is ascribed to the spin−orbit splitting of Ti 2p\textsubscript{3/2} (458.4 eV) and Ti 2p\textsubscript{1/2} (464.1 eV) in TiO\textsubscript{2} (Figure 2B). Similarly, doublet signals can be expected in the Ca 2p core-level spectrum (Figure 2C). In the fitted P 2p spectrum in Figure 2D, it displays multiple peaks associated with penta-valent (P\textsuperscript{5+}) oxidation state at binding energies 134.2 eV (P\textsubscript{2}O\textsubscript{7}\textsuperscript{4−}), 133.5 eV (HPO\textsubscript{4}\textsuperscript{2−}), and 132.8 eV (PO\textsubscript{4}\textsuperscript{3−}). Putatively, the above Ca (II) and P (V) in the MAO-treated samples should create a favorable chemical environment for the formation of calcium phosphate compounds, such as Ca\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, CaHPO\textsubscript{4}, and Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}. The O 1s high-resolution spectrum can be deconvoluted into three peaks centered at 529.9, 531.2, and 531.3 eV (Figure 2E), assigning to Ti−O−Ti, Ti−OH (or H\textsubscript{2}O from adsorbed water\textsuperscript{41}), and P=O− bond, respectively. Taken together, it suggests that the dominate coating compounds can be titania and apatite consisting a mixture of hydrated Ca\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, CaHPO\textsubscript{4}, and Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}. In order to reveal the phase state of the above components, XRD is performed, and the results are given in Figure 2F. For the as-oxidized titanium samples, apart from diffraction peaks arising from substrate Ti, relatively sharp peaks corresponding to well crystallized anatase and rutile are clearly present on the surface. However, the peaks for planes like (203), (222), and (213) are weak and difficult to be identified, mainly due to the low degree of crystallinity of the apatite. Hence the coating is composed primarily of anatase and rutile crystals, and partly of low-crystallized or amorphous apatite.

3.2. In Vitro Results. 3.2.1. Ca\textsuperscript{2+} Dissolution from the Coating in PBS. By one-step MAO process, the macro-porous scaffold immersed in simulated body fluid (SBF) for 14 days and (D) SEM image of the MAO treated scaffold immersed in SBF for 3 days.

Figure 3. (A) Illustrative diagram shows the simultaneous generation of microporous topography and bioactive elements on the macro-porous scaffold by MAO treatment in a Ca-EDTA containing electrolyte and (B) the noncumulative and cumulative release curves of Ca\textsuperscript{2+} in PBS for 28 d; SEM images of the implant surface before and after immersion for 28 d are shown at the right side. (C) SEM image of the untreated porous Ti64 scaffold and (D) SEM image of the MAO treated scaffold immersed in SBF for 3 days.
scaffold was endowed with simultaneous microporous topography and CaP coating on its surface, and thereby a bioactive hierarchical scaffold system was developed through this process (Figure 3A). A major aim of the developed system was directed toward increased CaP incorporation and improved biological performance of the scaffold. Of note, the implanted scaffolds would not only play the role of bone replacement but also acted as a delivery platform for bioactive ions. We performed an immersion test to characterize the release behavior of Ca\(^{2+}\) from the coatings. Figure 3B depicts the release profiles of Ca\(^{2+}\) from the coated scaffold in PBS for varied periods of times. The total content of Ca\(^{2+}\) was (13.3 ± 2.4) μg/cm\(^2\). In the first 1 day, 4.2 μg/cm\(^2\) of Ca\(^{2+}\) was leached into PBS. After a week, that value reached 7.9 μg/cm\(^2\) (59.5% of total), and it kept growing slowly during subsequent periods. By the end of the test, there was still 21.6% of remnant, confirming a durable delivery. In addition, SEM-EDS analysis of the soaked sample surface was done, and revealed that the atomic content of Ca at the surface declined from 9.27 at. % to 3.16 at. % following immersion in PBS for 28d. The sustained release of Ca\(^{2+}\) from the coating is probably due to the chelation of EDTA that prevents fast leakage as well as the incorporation of a large quantity of CaP within the coatings. As a result, the sustained release of Ca\(^{2+}\) can lead to a saturated ion environment around the scaffolds, which may help accelerate the nucleation of apatite in SBF, yielding significantly higher bioactivity.

### 3.2.2. Apatite Forming Ability

Kokubo et al. have proposed the use of SBF for the in vitro test of bioactivity of the biomaterials for bone regenerations.\(^{36}\) They found that the ability of apatite to form on biomaterials in SBF correlates well with the in vivo bone bioactivities. Recently, its role has been further validated by the atomic probe tomographic findings that bone bonds chemically to a Ti implant at the bone/implant interface.\(^{18}\) Figure 3C, D depicts the morphologies of the samples immersed separately in SBF for a period of time. After being soaked in SBF, the apatite layer were deposited over the surface of the MAO-treated scaffold within 3 days. High magnification images showed that the morphology of apatite exhibited a nanoflake shape. EDS analysis suggested the formation of apatite on the scaffold surface where the Ca/P ratio was about 1.75, close to that found in the mineral phase of HA (1.67) (Figure S2). In contrast, apatite was not observed on the untreated scaffold after immersion in SBF for up to 14 days.

### 3.2.3. Cyto-compatibility and ALP Activity

Besides the apatite forming ability, the release of Ca\(^{2+}\) from the scaffold into the extracellular environment is expected to promote the growth and osteogenic differentiation of MSCs on the scaffold.\(^{32}\) We evaluated the proliferation of hMSCs on the 2 types of scaffolds by measuring the cellular metabolic activity at different time points (Figure 4A). Generally, the MAO group exhibited a higher level of cell proliferation than the control group, especially on day 1 and day 14 (p < 0.05). On day 7, though the proliferation appeared higher on the MAO scaffold, there was no significant difference between the 2 groups (p = 0.08). In addition, we compared the osteoblastic differentiation of hMSCs on the 2 types of scaffolds using ALP as an early phase marker. It was found that the treated group exhibited significantly higher activity of ALP than the control group after 7 days of culture (Figure 4B).

The cell viability and morphology on these scaffolds on day 14 were also assessed using both Live/Dead staining and SEM analysis (Figure 4C). Overall, the cells survived on all the scaffolds, as observed by their staining patterns on the scaffolds where no staining of dead cells (red staining) could be visualized. The cell density on day 14 was higher on the MAO scaffold, which was consistent with the cell proliferation data. Additionally, improved cell morphology was evident on the MAO treated scaffold, where the cells spread out more evenly on the implant surface compared with the untreated one. The above results suggested that the MAO-treated scaffold possessed favorable cyto-compatibility and enhanced osteoblastic activity compared with the untreated one.
3.3. In Vivo Results. 3.3.1. Micro-CT Analysis. To explore the effect of MAO treatment on the in vivo osseointegration of porous Ti64 scaffold, we implanted the scaffolds into the rabbit condyles for 8 weeks by which bone remodeling has already occurred.\(^\text{12}\) We quantified the bone formation both around the scaffold and within it by micro-CT analysis. As shown in Figure 5A, B, the implants, bones in the peri-implant region (outer circle), and those within the scaffold (inner circle) were labeled white, green, and pink, respectively. Generally, the MAO group had more extensive bone formation at both regions. The quantitative analyses of bone fraction at the peri-implant region and intraporous region of the scaffold were displayed in Figure 5C, D, respectively. The bone fraction at the peri-implant region was 35.6 ± 4.0% for the MAO group and was 29.0 ± 3.7% for the control group (Figure 5C). The MAO treated scaffold had a significantly higher bone formation at this region than the untreated scaffold (\(p < 0.05\)). Besides, as shown in Figure 5D, the MAO treated scaffold had a significantly higher bone fraction within the scaffold as well (25.7 ± 3.7% vs 10.8 ± 3.4%) and, a larger quantity of bone formation within the MAO treated scaffold was observed (\(p < 0.05\)). According to these results, the MAO process not only enhances bone in-growth but also the bone on-growth at the porous Ti64 scaffold.

3.3.2. Quantitative and Qualitative Histological Results. Figure 6 shows representative histological images of cross-sectioned implants in the femoral condyle defects where the struts of the implant are shown in black, while mineralized trabecular bones are red. For the control group, only the peripheral area was apposed by mineralized bones, and the ingrown bone was usually juxtaposed to the implant without a tight contact (Figure 6A, C). In contrast, the bone in-growth on the MAO-treated implant was more extensive, and nearly every strut of the scaffold was apposed by bones (Figure 6B). The regenerated bone arranged alongside the strut of the scaffold and even bridged adjacent Ti64 struts (Figure 6D). Figure 6E, F shows the quantitative analysis of the BI and BICR at the scaffolds, respectively. Significantly higher BI and BICR were found in the MAO group. Compared with the control group, the BI of the MAO group was enhanced by 209% (16.3 ± 2.7% vs 6.9 ± 3.7%, \(p < 0.05\)), and the percentage of bone in contact with the MAO treated implant was enhanced by 407% (66.1 ± 14.7% vs 16.3 ± 4.3%, \(p < 0.05\)).

The poor bone in-growth at the untreated scaffold appears not in line with several previous studies which generally reported a significantly higher bone in-growth. For example, it was reported that the BI of EBM porous Ti at the frontal skull of pigs was 30% and 46% at 30 and 60 days, respectively;\(^\text{13}\) long-term studies of osseointegration of porous Ti in a sheep model also demonstrated a significantly higher BICR compared with the current study.\(^\text{3,15,44}\) Nevertheless, our results were in line with previous studies where a similar animal model was used. Takemoto et al. reported a similar BI (9.8 ± 4.2%) on the untreated implants in a rabbit model.\(^\text{45}\) Putatively, since the rabbit bones inherently lack trabecular bones, the bone quality is relatively poor compared with other larger animal models (e.g., pig, sheep) so as to result in significantly less bone formation within the inserted porous implant. In addition, Lopez-Heredia et al. have investigated the bone in-growth of rapid prototyped porous Ti in a similar rabbit model for 3 and 8 weeks.\(^\text{12}\) Interestingly, the bone in-growth was more extensive at 3 weeks; however, the ingrown bones were substantially remodeled at 8 weeks by which the bone in-growth was only found at the periphery of the scaffold, a result similar to the present study (Figure 6A). Therefore, the poor bone in-growth at the control group in our study would have been due to the excessive remodeling of the ingrown bones as well. Clearly, the use of different animal models and the effect of bone remodeling within the scaffold should explain the mismatch between these studies.
3.3.3. Osseointegration Patterns As Revealed by Fluorescent Labeling. The fluorescent labeling indicated different patterns of bone in-growth between the 2 types of scaffolds. Figure 7A, B shows the representative fluorescent micrographs of the MAO-treated and untreated scaffold, respectively. In the MAO-treated scaffold, nearly the entire surface of the scaffold was surrounded by fluorescent stains (Figure 7A). In the untreated group, however, fluorescent stain was only observed at the periphery of the scaffold (Figure 7B). According to the magnified images (Figure 7C), the early osteogenesis on the MAO treated scaffold occurred predominantly on the implant surface (stained green), followed by bone expansion toward the outer space (stained orange). Clearly, the osteogenesis mainly exhibited a pattern of contact osteogenesis by which the bone formed directly on the implant surface during the osseointegration process (Figure 7D). For the untreated scaffold, as tracked by the fluorescent labels, there was little attachment of bone (green stains) to the implant surface at the early stage of osseointegration. The bone formed primarily adjacent to the host bone and expanded toward the implant surface, exhibiting a pattern of distance osteogenesis. Therefore, the 2 types of scaffolds exhibit different patterns of bone in-growth after intraosseous implantation.

Contact osteogenesis may offer an explanation to the drastically enhanced osteogenesis at the MAO treated scaffold. As revealed by micro-CT analysis, besides bone in-growth, the bone formation at the peri-implant region was significantly higher at the MAO treated scaffold, which is rather beneficial for earlier fixation of the implant (Figure 5). This result is corroborated with a previous study in which an alendronate-CaP-coated porous tantalum exhibited an enhanced gap filling through this pattern of osteogenesis. Moreover, as mentioned above, the ingrown bone at the porous Ti scaffold will undergo bone remodeling after 3 weeks in the rabbit bone. Therefore, it is reasonable to suggest that the MAO treatment should have enhanced the bone in-growth through disruption of bone remodeling within the scaffold. The possible mechanism would be that the healing at the MAO-treated scaffold was more focused on the implant surface from the beginning (contact osteogenesis) so that biological or mechanical stimulus can be transduced from the implant surface to the attached bones and maintain the bone formation on its surface (Figure 7D). Omar et al. have previously revealed that the anodically oxidized implant can rapidly recruit MSCs on its surface and trigger a higher level of osteoblastic gene expressions in the surrounding tissues compared with the machined implant. This fact can be partially confirmed in this study by the findings that hMSCs on
the MAO-treated scaffolds are associated with a higher level of proliferation and ALP activity (Figure 4). Notably, it was recently reported that MAO treated implant has a direct effect on the bone remodeling as well.33 Receptor activator of nuclear factor kappa-B (RANK), receptor activator of nuclear factor kappa-B ligand (RANKL), and osteoprotegerin (OPG) have been suggested as important regulatory molecules in the process of bone remodeling. During the remodeling process, RANKL binds to RANK on osteoclasts to promote osteoclast maturation, and OPG works as a feedback factor to block the activation of osteoclasts. It was revealed that the MAO treated implant can induce a higher RANKL/OPG ratio at the early healing process which could promote the maturation of bone on its surface and at later stage, a high level of OPG expression follows and slows down the osteoclast activity.33 On the basis of these aspects, the MAO treated porous Ti scaffold may have enhanced the osteogenesis through the promotion of mature bone formation and inhibition of bone resorption on its surface. In contrast, since the bone in-growth at the untreated group was primarily distant to the implant surface at the inner scaffold, the earlier ingrown bone would more likely be remodeled due to the stress shielding by the scaffold (Figure 7D).

3.3.4. Features of Bone/Implant Interface. SEM images of the implant surface following separation of the bone from the implant are shown in Figure 8. After separation of the implant from the underlying bone, the implant surface of the untreated scaffold was lack of bone attachment, whereas significant amounts of bones remained attached to the MAO-treated implant surface (Figure 8A, B). Image with a higher resolution revealed that numerous ruptured bone tissues, which could be judged from their whiter appearance under SEM observation,17 were bonded to the micropores of the MAO coating (Figure 8C). This finding was similar to previous study reporting an anodically oxidized dental implant, TiUnite, which exhibited bone/implant interlocking after intraosseous implantation.47 However, there was lack of bone bonding on the flat surface of MAO treated implant. Therefore, the micropores should have played an essential role in the generation of stronger bonding force during the separation process. This assumption can be inferred from the oblique observation of the implant surface, which revealed that numerous globular bone matrixes were exclusively located in the micropores with a diameter of 1 to 2 μm, and were frequently attached by ruptured bone tissues (Figure 8D). We have tried to perform cross-sectional analysis of this feature at the bone/implant interface. However, because

![Figure 7](image_url)

**Figure 7.** Parts (A) and (B) show the fluorescent image of the MAO treated and untreated scaffold, respectively. Red dotted line (“---”) indicates the scaffold/tissue margin. (C) Magnified unstained and fluorescent images of the scaffolds. The bone-formation fronts at the 3rd and 7th week are marked by a green label from calcein green and an orange label from tetracycline, respectively. (D) Cartoon illustrates the different patterns of bone in-growth in the two types of porous Ti64 scaffolds. In the pattern of distance osteogenesis, the ingrown bones (red color) without contact with the strut surface are likely to be resorbed during bone remodeling.

![Figure 8](image_url)

**Figure 8.** SEM image of the (A) untreated implant surface and (B) MAO-treated implant surface following forced separation of bone from the implant surface. Inset image in (A) shows the lack of bone attachment on the untreated implant. (C) Some ruptured bone tissue remained attached to the micropores of the MAO-treated implant. (D) The globular bone matrix (asterisk) is generally settled in the micropores with a diameter of 1 to 2 μm, and attached by ruptured bone tissues (inset image). (E) A globular bone matrix partially disrupted from the implant surface displays connections with the implant surface by some nanoscale band structures (black arrow). (F) Cartoon illustrates how the microporous topography of the MAO coating cooperates with the globular bone matrix (GBM) to generate a higher bonding strength at the bone implant interface.
of the technical difficulty in preparing the samples, we failed to get qualified cross-sectional images where the bone in-growth into the pores of the surface could be visualized. Nevertheless, close observation of a partially disrupted bone matrix from the micropore revealed that the globule itself was connected with the residing wall by nanoscale band structures. This phenomenon was in line with the recent findings that bone bonds chemically to implant surface through direct contact between Ca atoms and titanium oxide surface (Figure 8E). The mechanism for higher bonding force at the MAO treated implant surface was illustrated in Figure 8F. At the bone implant interface, each micropore with a corresponding globular bone matrix may constitute a functional complex which can act as an anchorage point for the bonding of overlying bone tissues. It is noteworthy that the assumption of this complex may have a biological basis. We suspected that the globular bone matrix may be an analogue of the globular cement line matrix, which is about 1 μm in diameter and plays important role in the process of natural bone remodeling and implant osseointegration. The micropores of the implant surface may have accommodated the globular cement line matrix during the osseointegration process and provided significantly larger contact area in between. Compared with the bonding force at the flat surface, this complex can provide a much stronger mechanism for bone bonding to occur (Figure 8F).

3.3.5. Biological Fixation Strength. The aim of surface modification of porous metallic scaffold is to enhance its osseointegration strength. We performed a push out test to compare the biological fixation strength of the 2 types of scaffolds with bone (Figure 9). Figure 9D shows the typical displacement curves during the push-out tests. It was found that MAO treated scaffolds had a steeper curve than that of the control group, indicating that the MAO treated scaffolds required a higher force to generate a similar displacement. Figure 9E shows the comparison of the push out force between the 2 groups. It was calculated that the push-out force for the control group was about 380 ± 19.6 N, and it was enhanced to 520 ± 15.0 N after MAO treatment (p < 0.001). This significant improvement in biological fixation strength should have resulted from the drastically enhanced bone formation as well as bone bonding to the implant surface.

4. CONCLUSIONS

As a first attempt to determine the benefits of MAO technology in surface modifying a 3D printed porous Ti64 scaffold, its significance lies not on the technical novelty but on the ingenious combination of MAO technology and 3D printing with the yielding results. The significant improvement in bone in-growth capacity and bone bonding to implant surface will guarantee a more rapid and durable osseointegration of the porous implants. Notably, these improved biological performances are obtained through one-step MAO process. Compared with the other reported methods (Table S1), MAO technology appears to be a favorable choice regarding the high treatment efficiency and excellent convenience for industrial application. Therefore, post-treatment of 3D printed porous Ti64 implants with MAO technology may open up several possibilities for the development of bioactive customized porous metallic implants for orthopedic applications. Besides, to our knowledge, this is the first study that carefully evaluates the effect of surface treatment on the patterns of osteogenesis on a porous metallic scaffold. By doing this, we disclose an important phenomenon that surface treatment with one-step MAO process can enhance the bone in-growth through optimized bone in-growth patterns and disrupted bone remodeling within the scaffold. These findings may provide insight into the study of bone tissue engineering using other surface modified metallic scaffolds. Future studies can also be directed to incorporate extra biologics (e.g., strontium and bisphosphonates) into the coating and explore their effects on the osteogenesis and bone remodeling within the scaffold under different physiological conditions.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b05893.

Table S1. Summary of the reported coating methods on 3-D printed porous metallic scaffold; Figure S1. An intraoperative photograph indicates the site of the bone defect after insertion of the porous Ti64 implant (arrow); and Figure S2. EDS of the MAO-treated scaffold surface after immersion in SBF for 3 days (PDF)

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