Osseointegrating and phase-oriented micro-arc-oxidized titanium dioxide bone implants

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Abstract
Here, we present a bone implant system of phase-oriented titanium dioxide (TiO₂) fabricated by the micro-arc oxidation method (MAO) on β-Ti to facilitate improved osseointegration. This (101) rutile-phase-dominant MAO TiO₂ (R-TiO₂) is biocompatible due to its high surface roughness, bone-mimetic structure, and preferential crystalline orientation. Furthermore, (101) R-TiO₂ possesses active and abundant hydroxyl groups that play a significant role in enhancing hydroxyapatite formation and cell adhesion and promote cell activity leading to osseointegration. The implants had been elicited their favorable cellular behavior in vitro in the previous publications; in addition, they exhibit excellent shear strength and promote bone–implant contact, osteogenesis, and tissue formation in vivo. Hence, it can be concluded that this MAO R-TiO₂ bone implant system provides a favorable active surface for efficient osseointegration and is suitable for clinical applications.

Keywords
Osseointegration, rutile titanium dioxide, micro-arc oxidation, in vivo

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Introduction
Osseointegration is the formation of a direct and active interface between an artificial implant and the surrounding bone tissue without intervening soft tissue.1 An osseointegrated implants, such as endosteal implants, contain pores that allow osteoblasts and to migrate into the implant and form connective tissue that is integrated with the surrounding tissue.2 Certain essential criteria must be satisfied before an implant can be classified as an osseointegrated implant: it must have a supporting base of an appropriate material, a biomimetic porous structure, and a bioactive contact interface. Supporting base materials are initially chosen for their mechanical properties, which should be similar to those of human bone; widely used materials include stainless steel,3,4 commercial pure Ta,5 Ti6Al4V,6,7 TiNbTaZr (TNTZ),8–10 and commercially available pure Ti (cp-Ti).5,11–13 The architecture of the implant is then designed based on the intended application. A porous structure is then formed to provide interlocking sites and a biomimetic microenvironment to promote cell adhesion during the initial period of cell growth.13–15 Finally, the active surface is designed with specific characteristics (e.g. chemical composition, surface

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chemistry, and biocompatibility) to facilitate osseointegration at the interface with the surrounding tissue.\textsuperscript{11,12}

The aforementioned factors are significantly correlated with the new bone growth rate and the recovery rate, which highlights the importance of the careful design of osseointegrated implants. The base materials must be biocompatible to keep them from being ingested by macrophages, which ultimately destroy the base material, causing the implant to separate from the healthy tissue and preventing the desired growth.\textsuperscript{16,17} Biocompatible base materials allow osteoprogenitor cells to migrate into the implant, differentiate into osteoblasts and generate more new bone tissue. Titanium (Ti) alloys effectively promote the differentiation of osteoblasts, thus facilitating tissue mineralization and healing; however, the thin oxide layer\textsuperscript{18} that can naturally form on Ti alloys causes it separate from the bone tissue and, thereby, inhibits bone tissue adhesion. Therefore, surface modification is indispensable to enhance the biocompatibility of Ti implants.

Surface modification can introduce various chemical compositions,\textsuperscript{11,12,19–23} microstructures,\textsuperscript{22,24–28} and topographies.\textsuperscript{29–32} For example, the chemical composition of the surface can be modified with titanium dioxide (TiO\textsubscript{2}),\textsuperscript{11,12} apatite,\textsuperscript{19,20} or hydroxyapatite (HA)\textsuperscript{21–23} to actively stimulate bone tissue adhesion and induce new bone growth. The amorphous and crystal phases of HA\textsuperscript{22,24,25} and the amorphous, anatase, and rutile phases of TiO\textsubscript{2}\textsuperscript{26,27,28} promote cell growth. Furthermore, crystal structures (such as the anatase and rutile phases of TiO\textsubscript{2} and the crystal phase of HA) provide more favorable outcomes than amorphous structures.\textsuperscript{22,24,25}

Micro-arc oxidation (MAO) is a simple single-step process based on the use of one of a wide variety of electrolyte solutions\textsuperscript{23} to form the desired coating phase with a natural porous structure that promotes strong film adhesion.\textsuperscript{33–35} Thus, this method is ideal for TiO\textsubscript{2}-based surface modification for osseointegration. We hypothesize that MAO rutile-phase-dominant (R-TiO\textsubscript{2}) bone implant systems with preferential orientation along the (101) plane can promote in vivo osseointegration compared to raw \(\beta\)-Ti (as a control) and MAO anatase-phase-dominant (A-TiO\textsubscript{2}) bone implant systems. Herein, experiments are conducted to elucidate the osseointegrational mechanism of MAO TiO\textsubscript{2} implants, validate the osseointegration performance of MAO TiO\textsubscript{2} implants, and demonstrate the potential for clinical applications of this material.

**Materials and methods**

**Sample preparation**

In this study, \(\beta\)-type Ti–13Cr–3Al–1Fe alloy (cp-Ti from Japan Daido Steel) was utilized as the substrate. The substrates were manufactured as plates with dimensions of 10.0 mm \(\times\) 10.0 mm \(\times\) 1.0 mm for microstructural characterization of the TiO\textsubscript{2} coatings and in vitro cellular tests and into cylindrical bullet-shaped samples of 4.0 mm in diameter and 6.0 mm in height for in vivo animal tests. The parameters for the MAO process are shown in Table 1. In our previous reports,\textsuperscript{11,12} it was demonstrated that the crystal structures and microstructures of the TiO\textsubscript{2} coatings can be adjusted by varying the applied voltage during the MAO process. In this study, two crystal structures, anatase and rutile TiO\textsubscript{2}, were formed for comparison with bare \(\beta\)-Ti alloy surface (i.e. the control sample).

**In vivo animal testing**

The animal experiments conducted in this study were approved by the China Medical University Institutional Animal Care and Use Committee according to ISO 10993-6.\textsuperscript{36} The IACUC approval number is NO. 98-88-N. Fifteen white male specific-pathogen-free (SPF) New Zealand rabbits (weighing 3.6 kg on average) were randomly divided into three groups for embedding three different implants into their distal femora: raw \(\beta\)-Ti, A-TiO\textsubscript{2}, and R-TiO\textsubscript{2} implants. The implants were cylindrical in shape (4.0 mm in diameter and 6.0 mm in height). First, the rabbit was anesthetized and its skin was disinfected and shaved. An incision was made to expose the distal femur, and a hole (4.0 mm in diameter and 6.0 mm in depth) was drilled for insertion of the prepared implant. Finally, the wound was closed, and the animal was allowed to recover naturally for 4, 8, or 12 weeks.

**Table 1. Micro-arc oxidation parameters.**

| Parameters                  | Values                                |
|----------------------------|---------------------------------------|
| TiO\textsubscript{2} phase  | Anatase                              |
| Electrolyte composition    | 0.05 M NaH\textsubscript{2}PO\textsubscript{4} (aq) | Rutile |
| Applied voltage (V)        | 350                                   |
| Oxidation time (min)       | 20                                    |
| Anode material (substrate) | cp-Ti plate (10 \(\times\) 10 \(\times\) 1 mm) |
| Cathode material           | Stainless steel (4 \(\times\) 9 cm\textsuperscript{2}) |
| Bath temperature (°C)      | 25                                    |
| Bath stirring               | Magnetic stirrer                      |
After the implantation period (4, 8, or 12 weeks), each rabbit was euthanized and its implant-containing femur was excised from the surrounding soft tissue. The excised samples were photographed, placed in formaldehyde (4%), then cold-mounted in EpoFix and coagulated at room temperature. The bone sections were then sliced at a thickness of 1 mm using an Accutom-50 machine (Struers A/S, Denmark). The sections were then progressively grounded and polished to a thickness of 80–100 µm. Each section was stained with hematoxylin and eosin staining to observe the histomorphology under an optical microscope.

Push-out tests

After the specific implantation period (i.e. 4, 8, and 12 weeks), the rabbits were sacrificed for push-out tests. The excised implant-containing femora samples were soaked in a 37% formaldehyde solution for bone tissue fixation. Each sample then fixed on the sample holder of a JSV-H1000 push-out tester using epoxy so that a uniform loading could be applied. The push-out tester applied linear loading with a constant stepping speed of 1.0 mm/min to push-out the implant from the bone; the shear force and displacement were recorded simultaneously. The shear strength was calculated according to equation (1).

\[
\text{Shear strength} = \frac{\text{critical loading}}{\text{BIC area}} = \frac{\text{critical loading}}{\text{cortical bone thickness} \times \text{implant circumference}}
\]

Here, the load at which the connecting interface between the femur and implant surface was broken was defined as “critical loading” in equation (1). The bone-to-implant contact (BIC) area is equal to the product of the cortical bone thickness and the implant circumference. In addition, the type of microstructural deformation in each sample was evaluated by SEM, and the element distributions were measured by electron-dispersive spectroscopy (EDS) mapping.

Results and discussion

In vivo shear strength

It was hypothesized that the MAO R-TiO\(_2\) implant system promotes desirable in vitro cellular performance with MC3T3-E1 cells and in vivo osseointegration compared to raw β-Ti and MAO A-TiO\(_2\) implant systems. In the in-vitro cellular performance point of view, MAO R-TiO\(_2\) implant system indeed acquires the optimal achievements, compared to the other two systems. This study thus focuses the in vivo osseointegration performance among three systems demonstrated herein. Push-out tests imitating the forces applied to bone and implants in the human body were used to measure the push-out loadings and displacements of the implants embedded in the distal femora of SPF rabbits. Subsequently, the adhesive forces (critical loading) between newly grown appositional bone tissue and the fabricated MAO TiO\(_2\)-coated implants were evaluated.

Figure 1 shows the correlations among the implant displacement, shear force, and implantation period. Here the shear force represents the resistance against push-out loading, which is the optimal shear force (critical loading) required to loosen the supports from the newly grown bone that has interlocked with an implant. Each implant reached its relatively highest peak shear force during the fourth week; at this time point, the supports on the β-Ti, R-TiO\(_2\), and A-TiO\(_2\) implants separated from the newly grown bone tissue after displacements of 1.3, 2.5, and 3.2 mm, respectively. Similar trends were observed during the eighth and the twelfth weeks following implantation (Figure 1). These consistent results among these three implants are attributed to the observed surface roughness and correspond to the osteogenic performance observed in-vitro. The surface roughness of the raw β-Ti substrate was reduced by mechanical polishing and flattening (Ra < 400 nm), while the porous MAO TiO\(_2\) implants exhibited rough surfaces (Ra = 0.39–1.6 µm).

The covered area influences how much force is required to loosen the support from the newly grown bone tissue on the implants and is correlated with the optimal force (critical loading), which represents how firmly the bone tissue has attached to the implant. The results the BIC of the R-TiO\(_2\) implant was the greatest, indicating that the R-TiO\(_2\) implant resulted in the highest density of newly grown appositional bone tissue and the greatest covered area attaching to the R-TiO\(_2\) implant, which is expected to allow it to withstand to higher shear forces.

The shear strength of each implant was calculated based on their critical loadings and BIC areas according to equation (1) (Figure 2). These shear strengths reflect the level of interlocking that occurs as the bone tissue grows onto the implants. The shear strengths of the three implants increased proportionally with the implantation period, suggesting that the growth and firm attachment of appositional bone tissues onto the implant surfaces progress after the implantation. Strong interlocking contributes to the increased BIC areas, which eventually results in higher shear strength. The β-Ti implant exhibited the lowest shear strengths for all implantation periods while the R-TiO\(_2\) implant produced the highest shear strengths (4.86, 6.95, and 11.71 MPa at the fourth, eighth, and twelfth implantation weeks, respectively), which can be attributed to the relatively high specific surface area of the R-TiO\(_2\) implant due to its flat and porous surface structure and preferentially oriented microstructure. Furthermore, the shear strength of the R-TiO\(_2\) implant was 73.5% greater than that of the A-TiO\(_2\) implant at the twelfth implantation week.
Bone-implant failure model

Figure 3 shows the microstructural characteristics and EDS mapping results for the (a) $\beta$-Ti, (b) A-TiO$_2$, and (c) R-TiO$_2$ implant-containing femoral sections after the push-out tests conducted after twelve weeks of implantation in rabbit femora. Each implant was loosened and exhibited a certain level of displacement after the push-out test. An and Draughn$^{37}$ established a Bone-implant failure model based on the connections between newly grown appositional bone tissue and implant after a push-out test, classifying failure into three major types: (i) interface failure at the bone-coating interface, (ii) failure at the bone, and (iii) failure at the coating. The failure model was determined by qualitative observations, as shown in Figure 3(a) to (c). The red, purple, and light blue arrow and dashed lines indicate the distributions of $\beta$-Ti, epoxy and newly grown bone tissue, respectively.

Interface failure at the bone-coating interface was associated with relatively weak adhesive strengths (Figure 2) between the newly grown appositional bone tissue and the implant. The newly grown appositional bone tissue is entirely detached from the $\beta$-Ti implant and formed the gap been filled with epoxy (found in Figure 3(a) purple dashed line region), which was classified as interface failure. Both the A-TiO$_2$ and R-TiO$_2$ implants exhibited failure at the bone, as shown in Figure 3(b) and (c), which suggests that the strength of the bone was less than those of the interfaces between the bone and the TiO$_2$ implants. Noticeably, a small portion of the A-TiO$_2$ coating was slightly detached from the $\beta$-Ti substrate (Figure 3(b) left side of the white dashed line region), while the R-TiO$_2$ coating remained fully adhered to the $\beta$-Ti substrate. This
difference is consistent with the shear strength test results, which indicated that the R-TiO$_2$ implant had greater strength. As bone tissue penetrates into the porous structure of an MAO TiO$_2$ implant to form interlocking sites and achieve higher adhesion strengths, failure at the bone indicates that the implant provided a suitable optimal microenvironment for bone growth and that the newly formed bone tissue was strong and adhered well to the TiO$_2$ implant. However, failure at the coating model, which represents the worst adherent strength of the three failure models, was not observed in this study, which indicates that the MAO process guaranteed strong film adhesion.

Next, EDS was used to map the elementary distributions of calcium (cyan), titanium (red), carbon (purple), and oxygen (green), as shown in Figure 3. The EDS mapping profile for the $\beta$-Ti implant clearly shows the detachment between the newly grown bone tissue (represented by the high Ca signal) and the Ti substrate (represented by the high Ti signal). A gap separates the newly grown bone tissue and Ti substrate, which is then filled with epoxy (represented by the high C signal). The A-TiO$_2$ implant exhibited a small degree of detachment, with epoxy occupying the detachment location, indicating that the newly grown bone tissue did not fully adhere to the A-TiO$_2$ coating. The R-TiO$_2$ implant exhibited obvious failure at the bone, further confirming that the newly grown bone tissue forms a strong and intact adhesion to the R-TiO$_2$ coating, with epoxy occupying only the region outside the bone fracture gap. These failure models reflecting the quality of the newly formed bone tissue and its adherence to the three implants in vivo are consistent with the results of the push-out tests (Figures 1 and 2).

**Histological observations of Bone-implant sections**

Histological observations of the (a) $\beta$-Ti (b) A-TiO$_2$, and (c) R-TiO$_2$ implants embedded in rabbit femora were performed twelve weeks after implantation, as shown in Figure 4. Isolated and disconnected mature compact bone (pink region) was observed on the $\beta$-Ti (Figure 4 (a)). However, large amounts of dense and continuous mature compact bone were observed on the MAO TiO$_2$-coated implants (A-TiO$_2$ in Figure 4(b) and R-TiO$_2$ in Figure 4(c)), particularly on the R-TiO$_2$ implant. This result again indicates that the MAO TiO$_2$ implants can efficiently promote mature bone tissue growth on their surfaces. Only a small portion of appositional trabecular bone adhered to the TiO$_2$ surface initially; however, the osteogenesis continued over time, and the immature appositional trabecular new bone transformed into laminated compact bone then irregular compact bone, which finally penetrated into the porous structure of the MAO TiO$_2$ coating and became incorporated firmly in the porous cavities.

MAO TiO$_2$ implants promoted osseointegration compared to the $\beta$-Ti implant due to their high surface roughness, material properties, and crystalline contact surfaces. The MAO TiO$_2$ coatings also exhibited multi-scale pore cavities due to the dielectric layer breakdown procedure involved in the MAO process, which, combined with the high surface roughness, facilitated osteoblast adhesion.
and cell interlocking better than the relatively smooth and non-porous surface of the β-Ti implant. Therefore, the R-TiO₂ and A-TiO₂ coatings attracted more cells than the raw β-Ti substrate, and the cells adhered to and grew on the coated surfaces, leading to the observed high shear strengths. In addition, the cells underwent increased cell differentiation (mineralization) on the coated implants due to the microstructure. The cavities in the MAO TiO₂ surfaces provided many effective sites for interlocking with bone cells in vitro and bone tissue in vivo, which further enhanced the shear strength between the bone tissue and the implant.

However, the large surface area and roughness did not guarantee osseointegration, as the R-TiO₂-coated implant was superior to the A-TiO₂-coated implant, indicating that the implant material is another important factor. The MAO surface modification process can be used to form TiO₂ coatings with unique preferential orientations, crystallinities, porous topographies, and surface roughness on the raw β-Ti. TiO₂ is regarded as antimicrobial, non-cytotoxic, and biocompatible, serves as a potential surface-modified material. In this study, direct MAO treatment of β-Ti was conducted with different working voltages (350 and 450 V) to synthesize phase-dominant and preferentially oriented crystal (100) A-TiO₂ (metastable anatase phase) and (101) R-TiO₂ (stable rutile phase) coatings, respectively. These two TiO₂ phases readily react with water molecules from the surrounding air and liquid, forming hydroxyl groups on the surface. These hydroxyl groups may shorten the initial recognition period (i.e. time for cell adhesion and bone tissue interlocking); they also facilitate absorption of calcium and phosphorus from the microenvironment to gradually form bone-like apatite structures, which promote osteogenesis.

Interestingly, the A-TiO₂ and R-TiO₂ coatings generated via the MAO process shared similar topographies and surface roughness but very different in-vitro cellular performances and in vivo osseointegration. Thus, the difference in the cell performance was not attributed to the surface topography but rather to the phase and preferential orientation of the TiO₂. The crystallinity of the TiO₂ coatings is another important factor in osseointegration. The rutile phase (R-TiO₂) allows abundant negative hydroxyl bonding on the surface, which guides cell adhesion, while the anatase phase (A-TiO₂) provides less hydroxyl bonding, which limits cell adhesion. Furthermore, the MAO R-TiO₂ coating contains a (101) facet, which is higher in energy than that of the (100) A-TiO₂ coating; this preferentially oriented R-TiO₂ coating demonstrated better apatite-forming ability due to the lattice matching between the (101) rutile phase and the (100) apatite phase.

Thus, surface modification by MAO accelerates the growth of new bone tissue, and the porous structure contributes to higher shear strength between the TiO₂-coated implants and bone tissue. The osteogenic performances of the A-TiO₂ and R-TiO₂-coated implants were comparable in terms of the histological observations, which demonstrated similar levels of bone growth on these implants; this may be due to the similar ratios of anatase and rutile in the coatings. However, the (101) R-TiO₂-coated implant exhibited optimal osteocompatibility and osteogenic performance and was superior to the (100) A-TiO₂-coated implant in terms of the in-vitro cellular performance and in vivo osseointegration results.

In summary, the MAO (101) R-TiO₂ implant system was found to be superior due to its lattice matching to apatite, abundant hydroxyl groups, rutile phase TiO₂, and roughened and porous morphology, effectively enhances cell adhesion and differentiation, which subsequently promotes the formation of laminated compact bone, irregular compact bone in the porous structure, and finally mature compact bone.
Conclusions
In this study, porous TiO₂-coated implants ((100) anatase and (101) rutile phases) were successfully prepared from raw β-Ti implants by MAO with different working voltages. Our previous publications had proved the A-TiO₂ and R-TiO₂ coatings owing to their similar micro/nano-porous structures and surface roughness exhibit the superior in-vitro cellular performances compared with the raw β-Ti implant. The porous MAO TiO₂-coated implants promoted dense and abundant mature compact bone growth on the surfaces and penetrating into the porous cavities; this formed bone tissue firmly interlocked with the implants. Further, the in vivo osseointegration of the MAO (101) R-TiO₂-coated implant were superior to those of the MAO (100) A-TiO₂-coated implant due to its unique phase and preferential orientation. The R-TiO₂-coated implant exhibited the highest shear strength with the newly grown bone tissue, which was higher than that of the MAO A-TiO₂-coated implant; both were superior compared with the raw β-Ti implant. Both the MAO R-TiO₂ and A-TiO₂ implants underwent failure at the bone, which reflects the extremely strong adhesion between the newly grown bone tissue and the implant surface. However, the raw β-Ti implant exhibited failure at the bone-implant interface. These results demonstrate that the MAO TiO₂-coated implants facilitate better bone induction compared to the raw β-Ti implants. Overall, the MAO (101) R-TiO₂-coated implant exhibited excellent osteogenic performance due to its lattice matching to apatite and abundant hydroxylation layers, demonstrating its promise for future clinical applications.

In the future, we are going to invest our resources and formulate our MAO (101) R-TiO₂-coated system toward titanium-based and non-titanium-based clinical devices. The titanium-based clinical devices can be easily performed the MAO (101) R-TiO₂-coated implant system; however, non-titanium-based one will generate new challenges of material types, working voltage/current variations and surface morphological performance. Thus, a titanium-based high entropy alloy, which can be easily tuned their shape, mechanical modulus and materials to meet criteria of the stem materials, integrates our phase-selective MAO TiO₂-coated system is our optimal target.

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Ethical statement
The animal experiments conducted in this study were approved by the China medical university institutional animal care and use committee according to ISO 10993-6. The IACUC approval number is NO. 98-88-N.

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