Research Article

Surface Modification of Porous Titanium with Microarc Oxidation and Its Effects on Osteogenesis Activity *In Vitro*

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Microarc oxidation (MAO) is a method about surface treatment that can provide nanoporous pits and thick oxide layers. A kind of porous metal-entangled titanium (Ti) wire material was treated with MAO process, resulting in a homogeneous rough TiO₂ layer, which helped facilitate MG-63 cell growth, cell viability, early cell differentiation, and cell mineralization *in vitro*. In addition, the MAO-treated Ti surfaces could promote the proliferation of MG-63 cells without sacrificing differentiation *in vitro*, which would benefit *de novo* bone formation around MAO-treated titanium at the early stage. The transcription levels of the extracellular matrix genes of osterix (OSX), collagen type I (Col I), bone sialoprotein (BSP), alkaline phosphatase (ALP), osteocalcin (OC) and osteopontin (OPN) and their protein expression levels were measured, suggesting that the cocultured cells with MAO titanium maintained the osteoblastic phenotype and that the MAO-treated titanium surface greatly stimulated osteoblast cell proliferation and differentiation compared to the untreated titanium. In conclusion, MAO technique can improve the surface of titanium and can contribute to the osseointegration process.

1. Introduction

A number of surgical implants have shown the ability to induce bone formation, of which the success or failure of surgical implants can be related to chemical and biological properties of their surfaces as well as to their micromorphology [1, 2]; that is, the implant surface plays an important role in the bone repair [3]. Part of the reason is the differences in the microstructure of implant surfaces that influence stress distribution, bone retention, and cellular response on its surface [4, 5]. In recent years, porous implants have been developed in bone repairing [6] although the ideal porous requirements for surgical implants have not yet been reached [7]. The most used ideal biomaterial for fabrication of surgical implants is porous titanium (Ti) thanks to its excellent physicochemical properties, biocompatibility, resistance to corrosion, and acceptability by human tissues [8, 9].

The biomaterial-bone tissue interface is one of the key factors in that it would promote a better tissue response and it would be adequate for the proliferation of bone cells [10].

However, Porous Ti without any surface treatment is bioinert [11], so it has to have surface modification for implant fixation by tissue ingrowth [12]. The surface modification can bring a formation of a stable and protective layer of titanium dioxide (TiO₂) [13] that avoids direct contact between the implants and its milieu and also reduces the reactivity of the metal [14, 15]. The TiO₂ layer can aid in connecting extracellular matrix to the implant surface [8], that is, to improve the biocompatibility of titanium. Up to now, there are various types of surface modification methods that have been explored to determine the optimum surface to further improve the bioactivity and biocompatibility of titanium [16]. Jäger et al. [17, 18] reported that the modification of the surface at the micronanoscale level is generally considered to be more conducive to the attachment, spread, and proliferation of osteoblast-like cells.

Microarc oxidation (MAO), also known as plasma electrolytic oxidation (PEO), is an electrochemical surface treatment process for generating oxide coatings on metals. MAO is similar to anodizing, but it employs higher potentials, so that discharges occur and the resulting plasma modifies

the structure of the oxide layer to present high hardness to protect against wear and corrosion [19]. MAO also provides nanoporous pits and the incorporation of calcium and phosphorus into the biocompatible TiO₂ coating layer which results in improved osteoblast cell responses [20, 21]. Therefore, the purpose of present study was to treat the pure Ti with MAO method to have nanoporous TiO₂ surface and to investigate its effects on osteogenesis activity.

2. Materials and Methods

2.1. Microarc Oxidation of Porous Titanium. A kind of porous metal-entangled titanium wire (porosity, 44.7%; yield strength, 75 MPa; tensile strength, 108 MPa; elastic modulus, 1.05 GPa) [22] (State Key Laboratory of Metal Matrix Composites, Shanghai Jiaotong University) was used as base material in this study. The titanium surface was ultrasonically cleaned with acetone, absolute ethanol, and distilled water for 15 min in series and then was treated by MAO in an aqueous electrolyte solution containing 3.5% glycerophosphate disodium salt pentahydrate and 1.2% calcium acetate monohydrate (voltage: 350 V, frequency: 800 Hz) for 30 seconds and again ultrasonically rinsed with distilled water for 15 min.

2.2. Ti Morphology and Chemical Composition Characterization. The surface morphology and chemical composition of the untreated and treated titanium wires were studied by scanning electron microscopy (SEM) with electron diffraction X-ray (EDX) system by FEI Nova NanoSEM230 scanning electron microscope.

2.3. Cell Culture. MG63 cells (Institute of Biochemistry and Cell Biology, SIBS, CAS), a line derived from a human osteosarcoma, were incubated with standard DMEM culture medium containing 10% (v/v) fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells at passage 2 or 3 were used for the following experiments. Cells were randomly divided into MAO group and control group, and each experiment was repeated 3 times.

2.4. Cell Morphology Observation. 1×10^4 cells/well was incubated with sterilized MAO titanium in a 24-well plate for one day, and then the MAO titanium with or without cells was observed by FEI Nova NanoSEM230 scanning electron microscope.

2.5. Cell Proliferation. The proliferation of MG63 cells was evaluated using a cell counting kit-8 (CCK-8) assay (Dojindo, Japan). 1×10^4 cells/well was incubated with sterilized MAO titanium in a 24-well plate under normal conditions for eight hours. After that, the samples were moved to a new 24-well plate (500 μ L per well) to culture for 1, 4, 8, and 16 days, and then 50 μ L CCK-8 solution was added to each well for 4 h. 100 μ L of the supernatants was transferred to a 96-well plate and the optical density was determined using a microtiter plate reader (Thermo LabSystems) at 450 nm.

2.6. Alkaline Phosphatase Assay. The alkaline phosphatase activity was determined by Alkaline Phosphatase Assay Kit (Abcam, USA), following the manufacturer's protocol. In brief, 1×10^4 cells/well was incubated with sterilized MAO titanium in a 24-well plate under normal conditions for eight hours, and then the samples were moved to a new 24-well plate and to culture for 1, 4, 8, and 16 days. The harbored cells were homogenized with 100 μ L assay buffer after cold PBS washing. For each sample, 80 μ L of supernatant was transferred into a 96-well plate, followed by adding 20 μ L of stop solution for all reactions. Standard curves were generated as manufacturer's guide. The alkaline phosphatase enzyme activity of each sample was calculated based on the comparison between standard curve and sample curve and was shown as Unit/mL (U/mL).

2.7. Measurement of Intracellular Ca²⁺. Intracellular Ca²⁺ levels were determined with a Becton Dickinson FACSCalibur flow cytometer. Cells were incubated with 3 μ M Fluo-3/AM dye at 37°C for 30 min in the dark. After gently rinsed three times with D-Hanks' solution, cells were resuspended in Krebs-Ringer-HEPES (KRH) buffer. The fluorescence was analyzed by flow cytometry with excitation and emission wavelengths of 488 nm and 526 nm every one minute.

2.8. Western Blotting. 1×10^4 cells/well was incubated with sterilized MAO titanium in a 24-well plate under normal conditions for eight hours, and then the samples were moved to a new 24-well plate to culture for 1, 4, 8, and 16 days. The total protein was extracted from the cells using M-PER mammalian protein extraction reagent (Pierce, USA). Equal amounts of protein (10 μ g per lane) estimated by a bicinchoninic acid (BCA) protein assay kit (Pierce, USA) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes and immunoblotting was performed using primary antibodies (Santa Cruz, USA), beta actin (1:800), OSX (1:500), OC (1:600), collagen I (1:300), OPN (1:500), and BSP (1:400). Antibodies were visualized using secondary HRP-conjugated anti-mouse/anti-rabbit antibody (CST, USA). After washing, the bands were detected by chemiluminescence (ECL detection kit) and imaged with X-ray films. Beta actin was used as an endogenous reference for normalization.

2.9. Quantitative Real-Time Polymerase Chain Reaction. The cells were treated according to the procedure described above. Total RNA samples were prepared from MG63 cells using Trizol (Invitrogen, USA) according to the manufacturer's instruction and reversely transcribed into cDNA using M-MLV reverse transcriptase (Takara BIO, Japan) and oligo (dT) 18 primer (Takara BIO). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by using a SYBR premix Ex Taq kit (Takara BIO) and TP800 system (Takara BIO). DNA from 500 ng total RNA was used as the template. The PCR reactions were carried out under the following conditions: 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. The primers

TABLE 1: Primers used in quantitative real-time PCR.

Gene/GenBank number	Forward primer (5'-3')	Reverse primer (5'-3')
Beta actin/NM_001101.3	CCCAAGGCCAACCGCGAGAAGATG	GTCCCGGCCAGCCAGGTCCAGA
OSX/NM_001173467.1	GCTGCCACCTACCCATCTGACTT	CTGCCCCCATATCCACCACTACCC
ALP/NM_014476.5	GACAATCGGAATGAGCCACAC	GTACTTATCCCGCGCCTTCACCAC
Collagen I/NM_000088.3	ACCTCCGGCTCCTGCTCCTCTTAG	GCGCCGGGGCAGTTCTTGGTCT
OC/NM_199173.4	AGCCCAGCGGTGCAGAGTCCA	GCCGTAGAAGCGCCGATAGG
OPN/NM_001040058.1	TCTGATGAATCTGATGAAGTGGTC	GGTGATGTCCTCGTCTGTAGCA
BSP/NM_004967.3	AAGAAGGGGAAGAAGAAAGTGTCA	GTATTCATTGGCGCCCGTGTATTC

(a1)

(a2)

(a3)

(a)

(b1)

(b2)

(b3)

(b)

FIGURE 1: SEM images of Ti samples before and after MAO process. (a) MAO titanium surface ((a1) 500x; (a2) 1000x; (a3) 3000x); (b) untreated titanium surface ((b1) 500x; (b2) 1000x; (b3) 3000x).

were listed in Table 1. Relative quantifications were calculated with the $2^{-\Delta\Delta C_t}$ method. Beta actin was used as housekeeping genes, due to its unchanged expression during treatment.

2.10. Statistical Analysis. Quantitative data were expressed as the mean $\pm$ sd. Statistical analysis was conducted using the two-sample *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Surface Analysis of Ti Samples before and after MAO Process. Figure 1 presents the SEM images of the morphology and microstructure of the samples. A homogeneous rough appearance could be seen on the surface of the MAO porous titanium sample (Figure 1(a)). Under higher magnification, a porous structure, in which grain and pore sizes were in

FIGURE 2: EDX micrographs of the Ti samples before and after MAO process. (a) MAO titanium surface; (b) untreated titanium surface.

nanoscale, formed by partially melted particles, was observed on the titanium wire surfaces. Compared to MAO process porous titanium sample, the appearance of untreated titanium porous samples (Figure 1(b)) exhibited a relatively smooth surface with some pits; no nanoscaled grains could be observed on their surfaces, which represented the typical morphology of native oxide film, with thin and nonporous structure. Figure 2 presented the energy dispersive X-ray (EDX) spectrum of untreated and treated titanium samples, which indicated that they formed a rough TiO_2 layer on the MAO-treated titanium surface compared to the untreated Ti surface. The surface chemical characterization also showed higher peaks of calcium and phosphate on the MAO-treated titanium surface compared to the untreated Ti surface.

3.2. Cell Morphology on Ti Surface. The morphology of the MG-63 cells cultured on the titanium surfaces with or without MAO process for 24 hours is shown in Figure 3. MG-63 cells were found growing on the titanium surfaces; in addition, there were more cells adhered to the MAO-treated surface than to the untreated Ti surface. The majority of the cells displayed a similar morphology and showed some slender filopodia or lamellipodia.

3.3. Assessment of Cell Proliferation. MG-63 cells proliferation was assessed using CCK-8 assay. As shown in Figure 4, the cell proliferation increased over time on the different surfaces. No significant differences were found among the samples at day 1 and day 4, but at day 8 and day 16 there were significant differences ($P < 0.05$ or < 0.01) between the two groups, suggesting that anodization facilitates cell growth and can promote cell viability.

3.4. Alkaline Phosphatase Activity. Alkaline phosphatase activity is an indicator of early osteogenic cell differentiation,

bone formation, and matrix mineralization [23]. As shown in Figure 5, the ALP activities for the two samples increased with time, and at day 8 and day 16 there were significant differences ($P < 0.05$ or $P < 0.01$) between them, suggesting that MAO facilitates early cell differentiation and bone formation.

3.5. Intracellular Ca^{2+} Assay. The intracellular Ca^{2+} levels at day 8 and day 16 were analyzed by flow cytometry (Figure 6). It was noted that the intracellular Ca^{2+} level in the experimental group significantly increased with time compared to that in the control group ($P < 0.01$), which suggested that MG-63 cells had definite osteoblastic phenotype to get mineralization on MAO-treated titanium surface.

3.6. Osteoblastic Phenotype Analysis. MG-63 cells were incubated with sterilized MAO titanium in a 24-well plate for 1, 4, 8, and 16 days, and these cocultured cells were found to express the transcripts for the extracellular matrix genes of OSX, Col I, BSP, ALP, OC, and OPN (Figure 7), the later and earlier markers of osteoblastic phenotype, based on the quantitative time-PCR results. The OSX, Col I, BSP, OC, and OPN protein expression (Figure 8) were further confirmed with western blotting, which suggested that the cocultured cells with MAO titanium maintained the osteoblastic phenotype. Moreover, the expression levels demonstrated differences between two groups, which suggested that the MAO-treated titanium surface greatly stimulated osteoblast cell proliferation and differentiation compared to the untreated titanium.

4. Discussion

Commercial pure titanium and its alloys are considered ideal materials because they have shown better acceptability by human tissues than other metals under diverse circumstances

FIGURE 3: SEM images of the titanium surfaces with or without MAO process after coculture with MG-63 cells. (a) MAO titanium surface ((a1) 500x; (a2) 1000x; (a3) 3000x); (b) untreated titanium surface ((b1) 500x; (b2) 1000x; (b3) 3000x).

FIGURE 4: The cell viability was measured by the CCK-8 assay versus control, * $P < 0.05$; ** $P < 0.01$.

due to their excellent mechanical properties, biocompatibility, and resistance to corrosion [8, 9, 24]. However, titanium implant failures still remain in some cases [25], and this is partly because Ti without any surface treatment is bioinert. In fact, surface chemistry, roughness, and topography are all

FIGURE 5: ALP activity of MG-63 cells on the titanium surfaces with or without MAO process. * $P < 0.05$.

FIGURE 6: Intracellular Ca^{2+} of MG-63 cells on the titanium surfaces with or without MAO process.

parameters that influence both the osseointegration and biocompatibility [26]. Hence, some types of surface modification method have been explored to further improve the bioactivity and biocompatibility of Ti and minimize the risk of implant failures [16].

MAO is a promising technology that can produce porous, rough, and firmly adherent TiO_2 coatings on titanium surfaces [27]. It is well known that TiO_2 is an attractive semiconductor for certain photocatalytic applications, such as decontamination and bactericidal effects. In addition, TiO_2

FIGURE 7: The quantitative time-PCR results versus control on the same day. * $P < 0.05$; ** $P < 0.01$. OSX, osterix; COL I, type I collagen; BSP, bone sialoprotein; ALP, alkaline phosphatase activity; OC, osteocalcin; OPN, osteopontin.

could help increase incorporates calcium or phosphorus ions into the surface layer and is beneficial to osteoblastic cell activity. In this paper, the titanium treated with MAO was a kind of porous metal-entangled titanium wire material [22], of which the porosity, yield strength, ultimate tensile strength, and elastic modulus are 44.7%, 75 MPa, 108 MPa, and 1.05 GPa, respectively. This kind of material is very

promising for implant applications because of their very good toughness, perfect flexibility, high strength, adequate elastic modulus, and low cost [22].

MAO-treated titanium surface showed a homogeneous rough TiO_2 layer on it (Figure 1(a)) compared to the untreated Ti surface; in fact, the roughness TiO_2/Ti surface is better than the smooth for biocompatibility. With further studies,

FIGURE 8: The western blotting results. OSX, osterix; COL I, type I collagen; BSP, bone sialoprotein; OC, osteocalcin; OPN, osteopontin.

we found that there were more MG-63 cells adhered to the rougher surface, and the cck-8 test and ALP assay suggested that MAO-treated outfaces facilitate cell growth, cell viability, and early cell differentiation. Alborzi et al. [28, 29] reported that an inverted correlation exists between osteoblastic proliferation and differentiation; however, it was found in this

paper that the MAO-treated Ti surfaces promote the proliferation of MG-63 cells without sacrificing differentiation, which would benefit *de novo* bone formation around MAO-treated titanium at the early stage. The intracellular Ca^{2+} level in the MAO-treated titanium group significantly increased with time compared to that in the control group ($P < 0.01$),

suggesting that MG-63 cells had definite osteoblastic phenotype to get mineralization on MAO-treated titanium surface.

Biologic and morphologic responses to osteoblast cell lines (MG-63) were further examined by measuring the transcription levels for the extracellular matrix genes of osterix (OSX), collagen type I (Col I), bone sialoprotein (BSP), alkaline phosphatase (ALP), osteocalcin (OC), and osteopontin (OPN) and their protein expression levels (Figures 7, 8, and 5). ALP, COL1, and OSX are known to be early markers of osteoblastic differentiation, whereas BSP, OC, and OPN are expressed later in the differentiation process [30]. These proteins of osteoblastic phenotype play major roles in osteoblast differentiation by promoting the differentiation of undifferentiated mesenchymal cells into osteoblasts [31, 32]. The results indicated that the cocultured cells with MAO-treated titanium maintained the osteoblastic phenotype and could greatly stimulate osteoblast cell proliferation and differentiation compared to the untreated titanium.

In conclusion, MAO technique can improve the surface of titanium and stimulate cell proliferation and early cell differentiation, which may provide a new approach to the osseointegration of surgical implants. On the other hand, we noted a limitation to this paper that this was a study *in vitro* on cells and MAO-treated titanium, so more studies are needed to determine the biocompatibility and the osseointegration *in vivo*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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