Fabrication of bioactive implants through biomimetic deposition of hydroxyapatite onto the micro-arc oxidation-created porous TiO_2 coating

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Titanium (Ti) and its alloys are considered as candidate materials for bone implants. To improve the bioactivity of the implants, surface modifications are necessary. This study reports a new method to deposit hydroxyapatite (HA). It uses biomimetic deposition in simulated body fluid (SBF) within the TiO_2 coating created by micro-arc oxidation (MAO) treatment of a Ti plate. The morphology, chemical and phase composition of the coatings were examined by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and X-ray diffraction (XRD). The MTT assay was then used to determine the cell proliferation efficiency on different coatings. In vitro cellular assays showed that the incorporation of HA significantly improved the osteoblastic activity. In conclusion, we developed a simple, flexible and reliable method to grow the TiO_2/HA composite coatings, which opens up a new avenue toward designing Ti-based medical implants.

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1. Introduction

Titanium (Ti) and its alloys have great potentials as dental and orthopedic implants because of their high mechanical strength, low modulus and excellent corrosion resistance[1]. However, Ti and Ti-based alloys cannot be directly used as implants because they are bio-inert and have a poor adhesion with the living bone. If they are embedded in the body directly, a fibrous tissue capsule will isolate the implant from the surrounding bone, leading to poor chemical connection to the living bone and prohibiting the bone growth[2, 3]. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) is considered as one of the most important calcium (Ca) phosphate (P)-based ceramics, which has attracted extensive studies due to its good bioactivity, osteoconductive and osteoinductive[4, 5]. However, the bioactive HA ceramic is unsuitable to be used as implants usually subject to load-bearing conditions owing to its poor mechanical properties[6]. Taken together, one possible way to fabricating promising implant materials that can be perfectly "grafted" onto the living bone is through modifying the Ti and Ti-related alloys with bioactive ceramics such as HA ceramics.

Because of the huge chemical difference between Ti and HA, it is difficult to bind them together without introducing a buffer layer. Titanium oxide (TiO_2) is an appropriate buffer layer, which can be generated by microarc oxidation (MAO) process. MAO is a relatively simple and cost-effective wet-chemical method to produce a porous and rough TiO_2 layer tightly adhered to the Ti or Ti-based implants[7]. Although desired elements such as Ca and P can be introduced into the porous TiO_2 coating by diffusion and electrophoresis, it is difficult to form HA phase during MAO process because of a high temperature and a rapid cooling rate at anodic surfaces[8,9]. Thus, a subsequent HA growth process, such as sol-gel, ultraviolet irradiation, hydrothermal treatment or biomimetic deposition (BMD), has to be performed.

Among these techniques, the BMD can mimic the biological bone and tooth formation process by using a simulated body fluid (SBF) similar to biological fluids in composition, pH, and temperature[10]. The BMD technique principally allows any type of substrate, with any morphology and size, to be coated with a uniform layer of apatite with control over coating characteristics, such as thickness, composition, and crystallinity[11].

Here, we show the BMD growth of HA on MAO treated Ti surfaces. The Ca and P species introduced during the MAO treatment of Ti to create porous TiO_2 layer are essential for the subsequent BMD growth of HA. Without the existence of Ca and P in the porous TiO_2 layer, HA is unable to form on the Ti surfaces. The formation of the HA coatings have been verified using scanning electron microscopy (SEM) with energy dispersive X-ray spectrometer (EDS) and X-ray diffraction (XRD). The biological properties of the TiO_2/HA composite coatings are also studied in vitro.

2. Materials and methods

2.1 Sample preparation

Commercially available Ti plates (impurity content in wt.%: 0.10C, 0.30Fe, 0.03N, 0.25O, 0.015 H, bal. Ti.) with a size of 20 mm×10mm×2mm were used as substrates. They were first abrased with 600#, 800#, 1000#, 1200#, 1600# and 2000# waterproof abrasive papers, and then ultrasonically washed with acetone, ethyl alcohol, distilled water and dried by hot air.

The MAO process was carried out using a pulse power supply in an electrolyte containing calcium acetate and β-glycerol phosphate disodium salt pentahydrate. The Ti plate was used as the anode while the wall of the stainless steel container was used as the cathode. The MAO treatment was performed at a constant current density of 0.3A/dm^2 and the final voltage was 200-450V. The working frequency and duty cycle were 600 Hz and 20%, respectively. The bath temperature was maintained at 30℃ by a stirring and cooling system.

After the MAO pretreatment, the samples were immersed in SBF at 37℃ to allow biomimetic formation of HA coating on the TiO_2 surface. SBF was prepared by dissolving reagent grade $CaCl_2$, KH_2PO_4•$3H_2O$, NaCl, KCl, $MgCl_2$•$3H_2O$; $CaHCO_3$ and Na_2SO_4 in distilled water. The ion concentrations in SBF closely resemble those in human blood plasma, as shown in Table 1. The solution was buffered at physiological pH of 7.25 with 155.1mM Tris (hydroxymethyl) aminomethane and 75 mM hydrochloric acid (HCl). The HA growth time in SBF were 7, 14 and 21 days study the HA growth process. The SBF was refreshed every 2 days. The samples were carefully rinsed with deionized water after removing from the SBF, and then dried at room temperature.

Table 1. Ion concentrations in SBF in comparison with those in human blood plasma

Specie	Ion concentration (mmol/L)	
	Blood plasma	1×SBF
Ca^{2+}	2.5	2.5
HPO_4^{2-}	1.0	1.0
Na^+	142.0	142.0
Cl^+	148.0	148.8
Mg^{2+}	1.5	1.5
K^+	5.0	5.0
SO_4^{2-}	0.5	0.5
HCO_3^-	4.2	4.0

2.2. Structure characterization

The surface morphologies of the samples were observed by SEM (FE-SEM; JSM-6700F, JEOL, Japan). The elemental compositions were measured with EDS equipped on the SEM equipment. The phase composition of the films was analyzed by XRD (RTNT-1500; Rigaku, Tokyo) using CuKα radiation operating under 40 kV and 200 mA acceleration at a scanning speed of 6.0°/min with a scanning range (2θ) from 20° to 80°.

2.3. Biological properties

For the assessment of the cellular behaviors on the coating system, the MC3T3-E1 cell line was used. The culture medium consisted of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 50 IU/ml penicillin, and 50 ¹g/ml streptomycin. The culture was maintained at 37℃ in a humidified incubator with 5% CO2. The culture medium was changed every 3 days. A content MC3T3-E1 mono-layer was treated with a 0.25% trypsin plus 0.02% EDTA solution to achieve cell detachment. Finally, the cells were centrifuged at 400 g for 5 min and then re-suspended in DMEM for following examinations.

For cell proliferation assay, 2×104 cells/ml were seeded on each specimen in 24-well tissue culture plates. The cultures were incubated in a 37 °C, 5% CO2 environment for 1, 4, and 7 days. Cell proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assay. At the prescribed time point, 10 μl MTT solution (5 mg/ml) was added to the culture. Then the culture was incubated for an additional 3 h to form formazan, which was then dissolved using dimethyl sulfoxide (DMSO). The optical density (OD) was measured at wavelength of 570 nm using a microplate reader.

2.4 Statistical analysis

A statistical analysis was performed using the soft-ware package SPSS 16.0. The data was presented as mean ± standard deviation (SD) and was analyzed using a one-way analysis of variance (ANOVA). Differences were considered to be significant as p<0.05.

3. Results and discussion

3.1. Morphologies of the coatings

Fig. 1 shows surface morphologies of MAO-induced TiO_2 coatings formed in the electrolyte of sodium β-glycerophosphate and calcium acetate operated at 200V, 300V and 350V, respectively. It can be seen that the surfaces of the TiO_2 coatings were porous and rough. The pore size increase as the operating voltage increases. When the applied voltage is 200V, the coating has a fine porous structure with a small pore diameter. The coating is very thin as observed from the scratches formed during mechanical grounding (Fig. 1(a)). When the voltage was increased to 300V, the porous structure of the coating became uniform and the pore diameter became large. In addition, the scratches almost disappeared after the

mechanical grounding due to the increase of the coating thickness (Fig. 1(b)). At 450V, the number of the pores reduced greatly, which their size increased significantly, induced by the decrease of spark number and the increase of spark size (Fig. 1(c)). Distinct cracks could be observed on the surface of the coatings created at 450 V, which might be due to the growth stress of the coatings. Therefore, the applied voltage has a significant effect on the surface morphology of the MAO-created TiO_2 coating in the electrolyte used.

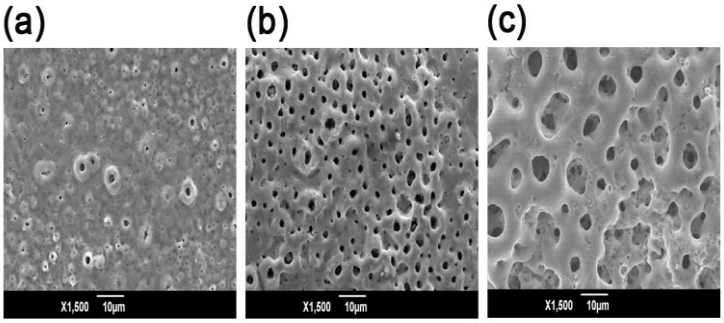

Fig. 1 Surface morphologies of the TiO_2 coatings formed at various applied voltages (MAO time is 5 min): (a) 200 V, (b) 300 V, (c) 450 V.

The formation process of the microporous structures is closely related to the discharge of the plasma. During the MAO process, the substances are melted inside the discharge channel to generate the plasma. Some of the plasma diffuses into the electrolyte and the rest of the plasma is condensed quickly on the electrode surface to form the oxide coating due to the rapid temperature drop, which makes the prepared coating layer with a crater-like structure. Clinical studies demonstrated that the bone-to-implant contact was significantly influenced by the surface properties of the implant, such as surface morphology, microstructure, etc[12].

As shown in Fig. 1, the MAO-generated TiO_2 coating is highly porous. The molten oxide particles with different size pores were uniformly distributed on the coating layer. The porous structure is conducive to cell attachment, propagation and bone-growth, which is important to prevent loosening of the implant from the living bone[13,14].

3.2. EDS analyses of the MAO-created TiO_2 coating

Fig. 2 shows the results of the EDS analysis after the MAO pretreatment of the Ti plate. It is clear that the coating was mainly composed of Ca, P, O and Ti. Obviously, Ca and P component in the coatings come from the electrolytes, underlying that the elements in an electrolytic solution can be introduced into the coating layer during MAO process.

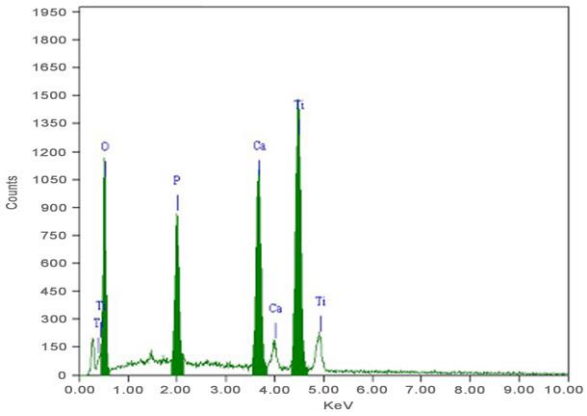

Fig. 2 EDS analysis of the TiO_2 coating formed in the Ca- and P-containing solution

Fig. 3 shows SEM images of the TiO_2/HA composite coatings deposited on the MAO pretreated Ti plate immersed in SBF for different times. After immersion in SBF for 7 days, apatite was observed to nucleate and grow on the porous TiO_2 coating. After immersion in SBF for 14 days, single and clustered ball-like particles were observed on the surface of the MAO pretreated TiO_2 coating. After immersion in SBF for 21 days, the MAO pretreated TiO_2 coating was fully covered by a dense apatite coating.

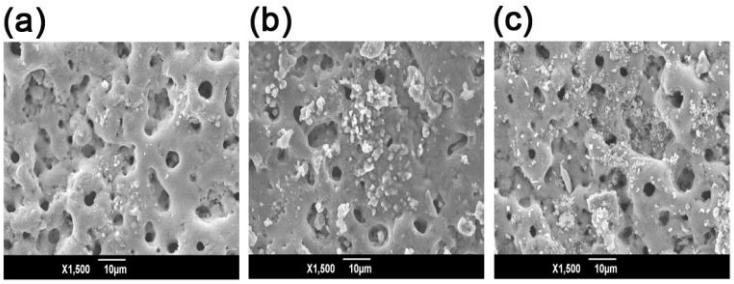

Fig. 3 Morphologies of biomimetic HA coating on the surface of TiO_2 coating formed after immersing in SBF for different times: (a)7 days, (b) 14 days, (c) 21 days.

It has been reported that Si-OH, Ti-OH and COOH groups can induce apatite formation in a biomimetic solution[15] since these functional groups are negatively charged in the body environment, which can bind Ca^{2+} in the surrounding liquid to form complexes and further induce apatite nucleation at the initial stage. Then the complexes incorporate PO_4^{3-} to form apatite nuclei. This initial stage may govern the heterogeneous nucleation of apatite on the substrate after being exposed to a solution mimicking the body environment. Consequently, functional groups with negative charge act as an inductive factor on apatite nucleation even on the surface of MAO pretreated pure Ti[16].

3.3. Phase composition of the coatings

Fig. 4 shows the XRD patterns of the composite coatings after BMD. According to XRD patterns, the composite coatings were constituted by anatase TiO_2, rutile TiO_2 and HA, The XRD results proved that HA crystals were formed on the surface of the MAO pretreated pure Ti.

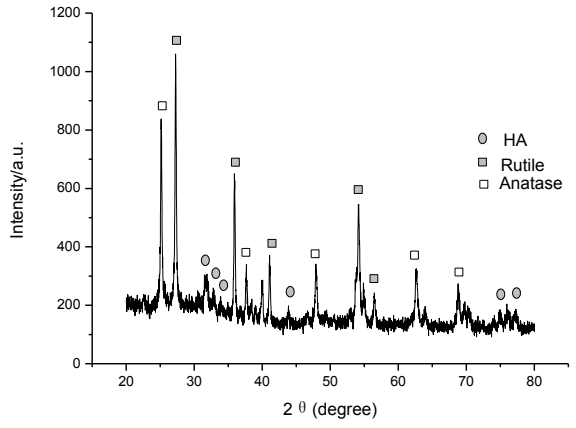

Fig. 4 XRD patterns of the TiO_2/HA composite coatings

The formation process of HA crystals was affected by the nucleation of HA in the porous and rough TiO_2 layer. Similarly to Si–OH, Zr–OH and other functional groups[17,18], the important functional groups facilitating the HA formation in our study must be associated with TiO_2-OH. TiO_2-OH groups have already proven to be crucial to the deposition of HA. Gu et al[19] showed that TiO_2 coatings prepared on NiTi alloy by heat treatments have excellent bioactivity by inducing apatite formation. Also, the porous structure of the TiO_2 surface also plays an important role in inducing HA nucleation. Once apatite nuclei are formed, they can spontaneously grow by consuming Ca^{2+} and PO_4^{3-} around the apatite nuclei in the SBF solution to form a dense and uniform apatite coating according to the following reaction[9]:

$$10Ca^{2+} + 6PO_4^{3-} + 2OH^- \leftrightarrow Ca_{10}(PO_4)_6(OH)_2$$

3.4 Cell proliferation

Fig. 5 shows the MC3T3-E1 cell proliferation efficiency on different coatings for 1, 4 and 7 days after initial culture. In all cases, the cell number attached on the samples increases as a function of culture time, indicating a profound proliferation of cells. After culturing for 1 day, no significant difference in cell proliferation for all of the three specimens can be observed (p>0.05). However, compared with the two groups, TiO_2/HA coatings showed a higher cell proliferation at 4-day and 7-day culture (p<0.05), and TiO_2 coatings was higher than pure Ti at 4-day and 7-day culture (p<0.05)

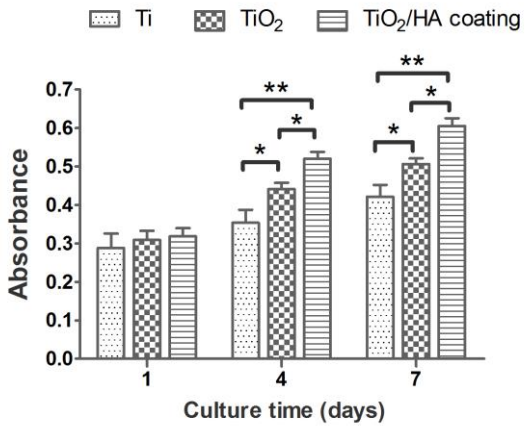

Fig.5 Proliferation of MC3T3-E1 cell on different surfaces determined by MTT

Using the MTT assay, we found that the growth rate

of MC3T3-E1 cells on the TiO_2 coating and the TiO_2/HA coating was significantly higher than that on the pure Ti after 4 days and 7 days incubation, which indicated that the MAO-treated Ti can accelerate the proliferation of MC3T3-E1 cells. On the one hand, the porous coating was tailored both physically and chemically to provide beneficial surroundings for the cell ingrowth and bone-bonding ability[20]. On the other hand, HA can improve the bioactivity of the implant surface, because the HA can keep the original morphology induce by the porous TiO_2 coating for cell adhesion and proliferation. In addition, the surface roughness may also contribute to the cell proliferation.

According to the above results, HA can be formed within the porous TiO_2 coating created by the MAO treatment of the Ti plate using the BMD growth. The HA modified Ti implant has a better bioactivity.

4. Conclusions

In summary, porous TiO_2 coating containing Ca and P on a Ti plate was prepared by the MAO treatment in an electrolyte containing sodium β-glycerophosphate and calcium acetate. After immersing the MAO-treated Ti plate into the SBF, a biomimetic apatite coating can form on the the surface of the Ti plate. The possibility and effectiveness of a MAO-BMD treatment for the production of a HA-incorporated TiO_2 coating on the surface of Ti implants have been proved. This in situ coating method is expected to provide strong bonding between the incorporated HA particles and the TiO_2 phase formed by MAO treatment of the Ti substrate.

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