**Construction of surface HA/TiO₂ coating on porous titanium scaffolds and its preliminary
biological evaluation**

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Abstract

A simple approach to fabricating hydroxyapatite/titanium dioxide (HA/TiO₂) coating on porous titanium (Ti) scaffolds was developed in the present study. Surface TiO₂ layer was firstly formed on porous Ti scaffolds with multi-scale pores by acid-alkali (AA) treatment. The outer HA layer was then formed on the TiO₂ layer by subsequent pulse electrochemical deposition (ED) technique. All the three main process parameters, i.e. deposition times, current density and mass transfer mode affected the properties of the HA coating notably. Under the conditions of 90 deposition cycles, -10 mA/cm² of pulse current density and stirring, a thin layer of homogeneous and nanorod-like HA sediments were formed on the substrate surface of porous Ti scaffolds. The results of protein adsorption and cellular experiments showed that compared to the single TiO₂ surface, the HA/TiO₂ surface allowed more adsorption of serum proteins and further enhanced the alkaline phosphatase (ALP) activity of MC3T3-E1 osteoblasts.

Keywords: porous titanium; HA/TiO₂ coating; electrochemical deposition; protein adsorption; osteoblasts

1. Introduction

It is known that appropriate porous structure is one of the essential conditions for the ideal bone repair or substitute materials. On one hand, three dimensionally interconnected macropores can provide space for vascular and bone tissue ingrowth, which help the fixation of the implant to the surrounding bone and increase its mechanical stability [1, 2]. On the other hand, micropores occurred on the walls of the macropores have been confirmed to play an active role in enhancing the bioactivity and even osteoinduction of the implant [3-5]. Titanium (Ti) and its alloys have been widely used for load-bearing bone defect repair because of their superior mechanical property, corrosion resistance and good biocompatibility. Recently, In order to overcome their over high elastic modulus and thus triggered stress shielding effect [6], various methods including selective laser melting method [7], replication of polymer sponge [8], powder stack sintering [9] and so forth have been used for fabrication of porous Ti scaffolds. The mechanical properties of them could be adjusted to match with native bone by controlling the porosity and pore size distribution of the scaffolds [10, 11].

Ti metal is a biologically inert material in nature and can't have effective osteointegration with the host bone when implanted into human body [12]. In order to better meet the clinical requirements for orthopedic implants, two main methods have been used to improve the bioactivity of Ti implant. One method was surface chemical treatment, such as acid-alkali [13], alkali-heat [14], H_2O_2 treatment [15] and so forth. Another method was surface coating technique,

especially the bioactive calcium phosphate (Ca-P) coating [16]. So far, hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA) coating has been clinically applied on orthopedic and dental implants, owing to its excellent biocompatibility and osteointegration [17-19]. However, by traditional plasma spray method, HA coating can't be formed in the inner pores of a porous scaffold. In addition, the formation of amorphous Ca-P compounds resulted from plasma spraying process would adversely affect the long-term reliability of an implant [19].

Recently, as a potential method to fabricate bioactive Ca-P coating, ED has been widely investigated [20-22]. This method has three major advantages. Firstly, the coating can be formed on the irregular inner surfaces and its property can be well controlled by altering the parameters. Secondly, the relatively rapid deposition process can be easily carried out at low temperature. Finally, some other bioactive compounds can be co-deposited on the surface to further enhance the biological performance of the coating. However, most of the related reports focused on the dense substrate materials, such as Ti sheets and cylinders [23, 24], and ED Ca-P coating on porous Ti scaffolds has not been adequately studied [25]. The major challenges are that the trapped hydrogen could impede Ca-P deposition onto the inner surface [26] and the inadequate solutes exchange could lead to the formation of uneven coating during the course of ED [27].

In our previous study, a very simple method, i.e. microporous Ti spheres stack sintering process was developed to produce porous Ti scaffolds with uniform porous structure and high mechanical strength [28]. In this work, we further presented a simple approach to fabricating HA/TiO₂

bioactive coating on porous Ti scaffolds, based on the combination of chemical treatment and ED methods. A rough and microporous surface TiO_2 layer on the scaffolds was made by acid-alkali (AA) treatment. The outer Ca-P layer was deposited on the surface TiO_2 layer by the subsequent ED method. As a result, a surface HA/ TiO_2 coating was formed on the scaffolds. Influences of current density, deposition time and mass transfer mode on the morphology, thickness and uniformity of the coating were investigated. The biological performance of the HA/ TiO_2 coating was evaluated by in vitro protein adsorption and cellular experiments.

2. Materials and Methods

2.1. Preparation of HA/ TiO_2 coating on porous Ti scaffolds

Porous Ti scaffolds with uniform macropores and abundant micropores were fabricated according to our previous study [28]. Briefly, a centrifugal granulation technology was used to prepare microporous Ti spheres. Next, the Ti spheres ($\sim 900\ \mu\text{m}$) were closely packed in a cylindrical mold and sintered in a vacuum of 1×10^{-3} Pa at $1300\ ^\circ\text{C}$ for 2 hours (ZR-50-21, Shanghai Cheng Hua Electric Furnace Co. Ltd., China). The sintered porous Ti samples were in turn cleaned by petroleum ether, acetone, alcohol and deionized water, and then dried at $50\ ^\circ\text{C}$. Subsequently, the samples were subjected to AA treatments based on the previous method [29] to form the surface TiO_2 layer. The acidic etching was carried out in the mixture of 48% H_2SO_4 and 18% HCl for 1 hour at $70\ ^\circ\text{C}$. The alkaline treatment was conducted in 6M NaOH solution for 5 hours at $70\ ^\circ\text{C}$. Finally, ED for the AA treated samples were made to form the outer Ca-P coating.

The electrolyte containing 2.5 mM $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 1.5 mM $\text{NH}_4\text{H}_2\text{PO}_4$, and 0.15 M NaCl was used.

The AA treated samples were placed into a container, in which the air were pumped out to a negative pressure state by a vacuum pump. The electrolyte was then added to the container so that the electrolyte could penetrate into the inner pores of the samples completely. ED was carried out in an electrochemical workstation (PARSTAT 2273, Princeton Applied Research, USA). Pulsed current method and three-electrodes were adopted. AA treated porous Ti sample, platinum plate and saturated calomel electrode were used as the cathode, anode, and reference electrode, respectively. The electrolyte temperature was controlled at about 85 °C during the ED process.

2.2. Characterization of HA/ TiO_2 coating

Morphology, thickness and chemical composition of the surface coating on the samples were investigated by field emission scanning electron microscopy (FE-SEM, S4800, Hitachi, Japan), thin-film X-ray diffraction (TF-XRD, X'pert pro-MPD, PANalytical, Netherland), and transmission electron microscope (TEM, Tecnai G^2 F20 S-Twin, FEI, USA), respectively. Because of the limitation of TF-XRD analysis for porous Ti samples, the dense Ti disks ($\Phi 14 \times 1$ mm) with the same AA treatment and ED process as porous Ti samples were used in the tests.

2.3. Protein adsorption tests

The Ti spheres (or beads) sintered porous Ti samples with AA treatment and/or ED treatment were used for protein adsorption experiments. Four types of samples ($\Phi 4 \times 8$ mm) in total were used, as summarized in Table 1. Protein adsorption experiments were performed according to our

previous method [28]. Simply, samples were placed into the 24-wells plates, and then added 1 mL of human serum (Beijing Minhai Biotechnology, China) per well. After incubated for 4 hours at 37 °C in an incubator, the loose-binding proteins were removed by washing the samples with 0.01M phosphate buffer solution (PBS). Proteins adsorbed on the samples were eluted by 2% sodium dodecyl sulfonate (SDS) solution, and the protein quantitative analysis (BCATM protein assay kit, Pierce, USA) was then performed using desorbed fractions.

2.4. Cellular experiments

The Ti spheres sintered porous Ti samples ($\Phi 14 \times 3$ mm) with AA treatment and/or ED treatment, i.e. TiO₂/Ti-900/54 and HA/TiO₂/Ti-900/54 were used for cellular experiments. Mouse pre-osteoblast cell line MC3T3-E1 cells were cultured in α -MEM medium supplemental with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin in an incubator at 37 °C with 5% CO₂. 5×10^4 /well MC3T3-E1 cells were seeded onto the samples and cultured in 24-well culture plates. The culture medium was renewed every 2 days. The morphology of cells growth on the scaffolds was observed using an inverted confocal laser scanning microscope (CLSM, TCS SP 5, Leica Microsystems, Germany). Simply, after culturing for 1, 4, and 7 days, the attached cells were stained with 0.1% fluorescein diacetate (FDA, Topbio Science, China) for 15 minutes, and visualized as green fluorescence. The alkaline phosphatase (ALP) activity of the cells was tested by SensoLyte® pNPP Alkaline Phosphatase Assay Kit (ANASPEC, USA). Simply, after culturing for 1, 4, and 7 days, the attached cells was lysed by repeated freezing and

thawing using 1% Triton X-100 solution. The supernatants were then collected by centrifugation, and measured according to the specifications. Osteocalcin (OCN) secretion of the cells was also tested by Mouse Osteocalcin ELISA Kit (Shanghai BlueGene Biotech Co. Ltd., China). Simply, after culturing for 1, 4, and 7 days, the supernatants were collected and analyzed according to the specifications. All above experiments were carried out with 3 replicates. T-test statistical analysis was used and the statistical differences were defined as $p < 0.05$.

3. Results and Discussion

3.1. Effect of ED parameters on HA deposition on AA treated porous Ti scaffolds

Figure 1 shows the SEM images of the porous Ti scaffolds before and after AA treatment. Without any treatments, the scaffold had a relatively smooth surface (Figure 1a and c). According to our previous report, the porosity of the scaffold was $41.2 \pm 2.3\%$, and the macropore and micropore sizes were $234.1 \pm 12.2 \mu\text{m}$ and $9.5 \pm 0.8 \mu\text{m}$, respectively [28]. After AA treatments, a typical microporous network was formed on the surface of the scaffold (Figure 1b and d). The results were well in accordance with the previous reports [30, 31], which confirmed that the main composition of this network was titanium oxide and usually in TiO_2 form.

ED process for AA treated porous Ti scaffolds was performed by a pulse current method according to the cycle as shown in Figure 2. The cycle was composed of a deposition time ($t_d = 1 \text{ s}$) and a break time ($t_b = 10 \text{ s}$). Same base current density (0 mA/cm^2), different pulse current densities and different deposition cycle were used. Figure 3 shows the effect of deposition time on

Ca-P coating at -10 mA/cm^2 of pulse current density with electromagnetic stirring (200 rpm). It could be found that the amount of the sediments gradually increased with the increase of the deposition cycle. At 30 cycles, the filamentous sediments were formed on the substrate surface, but there were still large blank areas without any sediment (Figure 3a and b). At 90 cycles, a layer of uniform sediments with nanorod-like structure occurred on the substrate surface (Figure 3c and d). At 180 cycles, the amount of the sediments continued to increase. Some micropores on the scaffolds were almost closed by the sediments, and some cracks existed on the sediments could be seen clearly (Figure 3e and f). However, compared to those occurred at 90 cycles, the smaller sediments were formed at 180 cycles. This could be ascribed to the heavily consumed calcium and phosphorus ions during ED process, which inhibited the growth of the deposits in the outermost layer. Therefore, the deposition time has a significant effect on Ca-P coating, and 90 cycles could be more appropriate to not only produce uniform sediments, but also retain the original microporous structure of the scaffolds.

Figure 4 shows the effect of pulse current density on Ca-P coating at 90 cycles with electromagnetic stirring (200 rpm). At -5 mA/cm^2 of pulse current density, the substrate surface was completely covered by the sediments with plate-like structure, and no micropores were closed (Figure 4a and b). At -10 mA/cm^2 of pulse current density, the sediments presented nanorod-like shape, as described above (Figure 4c and d). However, at -20 mA/cm^2 of pulse current density, almost all micropores on the scaffolds were closed by the sediments (Figure 4e and f). It is

indicated that pulse current density has great influence on the morphology of Ca-P coating.

Besides deposition time and pulse current density, we also investigated the influence of mass transfer mode of the electrolyte on Ca-P coating. Three mass transfer modes, i.e. no stirring, electromagnetic stirring (200 rpm) and ultrasonic agitation (50 kHz of output frequency) were used. Figure 5 shows the effect of mass transfer mode on Ca-P coating at -10 mA/cm^2 of pulse current density and 90 cycles. At low magnification, all samples were covered by a layer of uniform coatings, and no obvious difference among them could be observed (Figure 5a, c and e). However, when further observed at high magnification, it was clearly seen that the coating under no stirring condition was uneven. Partial surface had not the sediments, but some petal-shaped sediment occurred on the top of the firstly formed nanorod-like deposits at other areas (Figure 5b). Under no stirring condition, diffusion is the main driving force for mass transfer, and it may be too weak to drive the electrolyte to the inner surface of the scaffolds timely. Therefore, newly formed crystal nuclei could deposit and grow on the originally formed sediments, leading to the above results. On the contrary, the substrate surface was completely covered by a layer of uniform coating under electromagnetic stirring or ultrasonic agitation, only the ultrasound-assisted deposits had some cluster-like protrusions with finer crystals (Figure 5d and f). This proves that mass transfer mode of the electrolyte is very important for Ca-P coating, adequate and timely exchange of the solutes could promote the formation of uniform sediments.

3.2. Confirmation of HA/TiO₂ coating formed on porous Ti scaffolds

Figure 6 shows the cross-section images of Ca-P coatings prepared at 90 cycles and -5 mA/cm^2 or -10 mA/cm^2 of pulse current densities. The double layer of coating could be seen clearly. The thickness of outer Ca-P layer about $1 \text{ }\mu\text{m}$, and no obvious difference could be found between the sediments at two pulse current densities. The thickness of underlying Ti oxide layer was about 500 nm. This suggests that pulse current density has little effect on the thickness of Ca-P coating within a certain range. Furthermore, the results of thin-film XRD patterns confirmed the phase composition of the double layer of coating, as shown in Figure 7. All characteristic peaks were well in agreement with the JCPDS X-ray powder standards. The peaks corresponding to (110) β -Ti (JCPDS 05-0682), (101) TiO_2 anatase (JCPDS 21-1272) and (002), (211), (213), (202), and (222) HA (JCPDS 09-0432) were detected at both samples deposited at -5 mA/cm^2 and -10 mA/cm^2 of pulse current densities.

In order to further confirm the phase composition of Ca-P coating, TEM analysis for the crystals obtained by ultrasonic agitation was performed. Figure 8 shows the typical TEM images of the crystals formed at -10 mA/cm^2 of pulse current density. In the crystallized domains, the measured interplanar spacing was 0.339 nm and 0.286 nm, which corresponds to (002) and (211) planes of HA, respectively. The result was well in accordance with above XRD analysis and further confirmed that the main phase of Ca-P coating is HA. The HA/ TiO_2 double coating could endow the porous Ti scaffolds with better bioactivity. Some previous reports found that the oxide nanotubes could provide good osteointegration with bone cells and the nanoscale HA layer

exhibited better bioactivity [32, 33].

3.3. Preliminary biological evaluation for HA/ TiO₂ coated porous Ti scaffolds

As an initial process occurring at the interface of the implant and tissue, the protein-surface interaction strongly influenced the biological property of a biomaterial, such as biocompatibility and bioactivity [34, 35]. It is known that protein adsorption is related to surface property of an implant, such as surface roughness, specific surface area and so on [35-38]. In this work, adsorption of rat serum proteins on porous Ti scaffolds with single TiO₂ coating or double HA/TiO₂ coating were evaluated, and the results are shown in Figure 9. It could be seen that the scaffolds with double HA/TiO₂ coating (HA/TiO₂/Ti-900 and HA/TiO₂/Ti-900/54) adsorbed more serum proteins than those with single TiO₂ coating (TiO₂/Ti-900 and TiO₂/Ti-900/54), meaning that double HA/TiO₂ coating could allow more cells adhered to the surface and thus have better biological performance.

In order to verify the above assumptions, further in vitro cellular experiments were carried out using MC3T3-E1 osteoblastic cell line. The CLSM observation of the cells grown on the scaffolds with single TiO₂ coating or double HA/TiO₂ coating is shown in Figure 10. On day 1, the cells were randomly distributed on the substrate surface. After 3 days of consecutive cell culture, the osteoblasts were observed to align themselves on the edge of the microporous Ti spheres. On day 7, only a few cells could be observed on the surface of the outmost layer Ti spheres, while a large number of cells were found to aggregate on the inner surface of the Ti spheres (Figure 10e and f,

blocks marked position). Compared with day 3, it seems that lesser cells resided on the surface of two samples on day 7. One possible explanation could be the migration of osteoblasts into the inner pores of the scaffold. In this experiment, the thickness of the samples was 3 mm, while the scan depth of CLSM is limited to 500 μm . From day 1 to day 7, cell area spread on the HA/TiO₂ surface was consistently larger than that on the TiO₂ surface ($p < 0.05$, data not shown). Typical spindle-shaped osteoblasts were visualized in the CLSM images of HA/TiO₂/Ti-900/54 throughout day 1, 4 and 7, whereas rounded shape of osteoblasts were mainly seen in TiO₂/Ti-900/54.

ALP activity was used as biochemical marker for determination of osteoblast phenotype and was considered as an important surrogate in bone differentiation and mineralization [39, 40]. OCN is a non-collagenous protein, which is specifically synthesized and secreted by osteoblasts. Therefore, it is often regarded as a highly predictive marker for phenotype of osteoblasts [41, 42]. Figure 11a shows ALP activity of MC3T3-E1 cells cultured on the scaffolds. With prolonged culture time, ALP activity of the cells increased on both samples. Besides, at each time point, ALP activity of the cells on HA/TiO₂/Ti-900/54 was higher than that on TiO₂/Ti-900/54. Figure 11b shows OCN secretion of MC3T3-E1 cells cultured on the scaffolds. Likely, the secreted OCN by the cells increased on both samples with the increasing of culture time. However, no significant difference in OCN secretion was found between HA/TiO₂/Ti-900/54 and TiO₂/Ti-900/54. The above results confirmed that double HA/TiO₂ coating on porous Ti scaffolds could further

promote the early osteogenic differentiation of MC3T3-E1 cells, showing better bioactivity than single TiO₂ coating.

4. Conclusions

By AA treatment and subsequent ED process, a HA/TiO₂ double bioactive coating was formed on porous Ti scaffolds. At 90 cycles, a uniform HA layer was formed on the underlying TiO₂ layer. Morphology of the outer HA deposits could be controlled from plate-like to nanorod-like structure by altering pulse current density from -5 mA/cm² to -10 mA/cm². Phase composition of the surface coating was confirmed to be mainly HA and TiO₂ anatase by XRD and TEM analysis. Compared to single TiO₂ surface, HA/TiO₂ surface allowed more adsorption of serum proteins and further enhanced the ALP activity of MC3T3-E1 cells. In conclusion, porous Ti scaffolds with a HA/TiO₂ double bioactive coating could be potential to biomedical application.

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Figure Legends

Figure 1 Macroscopic appearance and microstructure of porous Ti scaffolds: Digital photos of the scaffolds without any surface treatment (a) and after AA treatment (b); SEM images of the scaffolds without any surface treatment (c) and after AA treatment (d).

Figure 2 Schematic diagram of pulse ED process.

Figure 3 SEM micrographs of Ca-P coating deposited by ED process at different deposition cycles: (a, b) 30 cycles; (c, d) 90 cycles; (e, f) 180 cycles.

Figure 4 SEM micrographs of Ca-P coating deposited by ED process at different current densities: (a, b) -5 mA/cm^2 ; (c, d) -10 mA/cm^2 ; (e, f) -20 mA/cm^2 .

Figure 5 SEM micrographs of Ca-P coating deposited by ED process at different mass transfer modes: (a, b) no-stirring; (c, d) electromagnetic stirring; (e, f) ultrasonic agitation.

Figure 6 Cross-section images of Ca-P coating deposited by ED process at different current densities: (a, b) -5 mA/cm^2 ; (c, d) -10 mA/cm^2 .

Figure 7 Thin-film XRD pattern of the Ti samples with AA treatment and ED process at different current densities: (a) -10 mA/cm^2 ; (b) -5 mA/cm^2 .

Figure 8 TEM images of the particles dropped from Ca-P coating deposited by ED process at -10 mA/cm^2 of current density.

Figure 9 Adsorption of serum proteins on different porous Ti scaffolds with surface TiO₂ or HA/TiO₂ coating.

Figure 10 CLSM observation for MC3T3-E1 cells growth on porous Ti scaffolds with surface TiO₂ or HA/TiO₂ coating.

Figure 11 ALP activity (a) and OCN secretion (b) of MC3T3-E1 cells cultured on porous Ti scaffolds with surface TiO₂ or HA/TiO₂ coating for 1, 4, and 7 days. * $p < 0.05$.

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Table 1 Designation for various porous Ti scaffolds.

Designation	The particles for stacking	Surface modified method
TiO ₂ /Ti-900	Commercial Ti beads	AA
HA/TiO ₂ /Ti-900	Commercial Ti beads	AA+ED
TiO ₂ /Ti-900/54	Microporous Ti spheres	AA
HA/TiO ₂ /Ti-900/54	Microporous Ti spheres	AA+ED