Chem. Res. Chinese Universities, 2019, 35(4), 686—692 doi: 10.1007/s40242-019-9101-x

Fabrication and *In vitro* Bioactivity of Robust Hydroxyapatite Coating on Porous Titanium Implant

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Abstract Compared with the traditional dental implant, TixOs® manufactured by direct laser metal forming(DLMF) technology exhibits improved capability for bone osteointegration due to its porous surface structure, and has achieved remarkable clinical effect. However, like the traditional titanium and other alloyed implants, the porous titanium implant TixOs® also has relatively weak bioactivity. To address this issue, a proper surface modification method may be needed. Hydroxyapatite(HA) has been widely used in implant surface coating for its similar chemical composition to bone tissue and its osteoconductive properties. Thus, combining TixOs® implants with hydroxyapatite can be an efficient way to enhance their bioactivity. We herewith reported a competent pulsed laser deposition(PLD) method of coating nano-sized HA thin film onto the porous TixOs® implant. The HA coatings were characterized by means of scanning electron microscopy(SEM), energy dispersive X-ray spectroscopy(EDS), X-ray photoelectron spectroscopy(XPS) and focused ion beam(FIB) method, and nanocrystal sized thin HA films were identified on the surface of TixOs® implants. The low cytotoxicity and improved cell proliferation ability of HA coated implants were further tested and verified using MC 3T3 E1 cells with the consideration of the controlling group. Our results show that a stable and bioactive HA thin film is able to form on the surface of the porous titanium implant by PLD method. This may benefit the further clinical application of TixOs® implants.

Keywords Hydroxyapatite; Pulsed laser deposition; Porous titanium; Bioactivity

1 Introduction

With the development of fundamental researches on the mechanism of dental implant and the progress of material science, implants have been widely applied in oral clinic[1]. Titanium and its alloys(*e.g.*, Ti_6Al_4V and Ti_6Al_7Nb) are commonly used as implant materials in modern orthodontics due to their prominent mechanical properties, corrosion resistance, and low toxicity[2,3]. However, it is difficult for the commercial pure titanium to form tight integration with host tissue because of its biological inertia, which may eventually lead to the failure of titanium-based denture implantation and other series problems. Another important issue is the mismatch between the elastic modulus of the titanium implant and that of the surrounding bone tissue. This mismatch may lead to the stress shielding effect, which can finally affect the stability of long-term fixation. Therefore, many researches have attempted to perform surface treatments or coatings that may improve the bone-bonding ability of titanium dental implants.

Rough surfaces have been a critical characteristic for demonstrating better biomolecule adsorption of biological fluids and better bone response. This is because that comparing with a relatively smooth surface, a rough surface exhibits the ability to influence cellular behavior, including cytoskeletal organization and cellular differentiation with matrix deposition[4—7]. On the other hand, porous structures are able to help reducing the elasticity mismatch between an implant and the bone tissue[8]. Taking together, there is a demand for bulk porous titanium fabrication methods that can control porosity, pore size, and mechanical properties.

Rapid prototyping is a way to directly fabricate physical objects with defined structure and shape on the basis of virtual 3D data[8,9]. With the assistance of a computer assisted design, direct laser forming of metal powders has been applied to produce laser-sintered titanium implants, such as the well-known commercial implants TixOs®(Leader-Novaxa, Milan, Italy). The TixOs® with 200—400 μm micropores can closely combine with the bone tissue through mechanical intercalation.

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Received April 10, 2019; accepted May 5, 2019.

Supported by the Jilin Provincial Health and Family Planning Young Science and Technology Backbone Training Program, China(No.2016Q022) and the Jilin Provincial Department of Education Science and Technology Program, China (No.JJKH20190104KJ).

Moreover, its porous structure can provide sufficient 3D spaces for cell proliferation[10,11]. Interestingly, the surface elastic modulus of the TixOs® is similar to that of the alveolar bone, which provides a suitable microenvironment for osteoblasts to infiltrate into the surface of the implant. Consequently, as a kind of successful implants, TixOs® have shown a high survival rate[12] in the clinical study. However, the porous titanium implant also exhibits a rather weak bioactivity and might release harmful elements(Ni, V, etc.) into the bone tissue around the implant. Thus, in order to enhance its bioactivity and improve the bone response to the implant surface, the fabrication of a bioactive film onto the TixOs® may be an effective and widely used approach.

Hydroxyapatite[HA, $Ca_5(PO_4)_3OH$] is the main chemical constituent of the bony matter(more than 70%)[13]. It has been developed and made available for experimental or clinical application because of its excellent biocompatibility, faster bone regeneration, and bonding to regenerate bone directly without intermediate connection tissue[14,15]. Until now, various deposition techniques, including plasma spraying[16], radio-frequency magnetron sputtering[17], sol-gel[18], ion beam sputtering[19], electrophoretic deposition and pulsed laser deposition (PLD)[20,21] have been applied for the preparation of HA thin films on titanium substrates. Experiments have proved that the implant with low crystallinity HA coating is prone to biodegradation, leading to the failure of the implant[22]. Therefore, the PLD method, which may provide an excellent adherence of the deposited structures to substrate and restore the complex HA stoichiometries to produce crystalline, outperforms other techniques in versatility[17,23].

The PLD method, first used by Smith and Turner in 1965[24], is a very competitive technique for preparing a wide range of well-defined thin films with its original stoichiometry using appropriate pulsed lasers[25]. The target material is vaporized from a rotating disk placed inside a reaction chamber and projected onto the surface of a metallic substrate[26]. Several kinds of metallic substrates, such as Ti and Mg, are able to be coated with HA thin films by PLD method[15,27,28]. However, the HA thin film fabricated by PLD onto the TixOs® implant has not been investigated to the best of our knowledge. Thus, in this work we have attempted to address the process of HA adhesion onto the porous TixOs® with stoichiometry and crystallization. We herewith reported fundamental characterization of HA thin films synthesized on TixOs® implants by PLD. The bioactivity of the HA/TixOs® was tested against MC 3T3 E1 cells. Comparing to traditional electrochemical deposition method, our results show that a stable and high crystallinity HA thin film is formed on the surface of the porous titanium implant by PLD. The biocompatibility tests have quantativly demonstrated that the film significantly raises the level of cell proliferation on the surface of TixOs®. Thus, the biological inertia of the implant can be effectively improved. The aim of this study is to identify the optimal processing conditions for preparing the HA thin film with bioactivity on the porous surface of TixOs® by PLD and consequently and improve the biocompatibility of TixOs® implants.

2 Experimental

2.1 TixOs® Implant and HA

The commercial TixOs® implants were obtained from the Company of Leader-Novaxa(Italy). The implant, which was a circular sample with a porous reticular surface, a diameter of 14 mm and a thickness of 2 mm, was treated by ultrasonic cleaning with acetone, ethanol and distilled water, respectively, for 15 min, and then dried in compressed N_2 atmosphere flow for subsequent use. HA nano-grade powder(10 g, Macklin, China) was adequately grinded in an agate mortar and pressed into a disc with a diameter of 2.54 cm. The target was sintered in an airtight corundum crucible at 600 °C for 10 h, cooled at a rate of *ca*. 2 °C/min, and hermetically stored.

2.2 PLD Deposition of HA Coating

PLD was performed inside the deposition chamber (Pioneer 180, Neocera Inc., USA). The deposition of HA thin films was conducted by ablation of target with an excimer laser(COMPex Pro 205, CoherentInc., USA) under the protection of high purity argon. The laser wavelength was 248 nm, the output energy was set at 300, 350 and 420 mJ, respectively, and the repetition rate was of 5 Hz. The laser pulses of 5000(5k), 10000(10k), 15000(15k) and 20000(20k) were selected for the deposition of films with different thicknesses. After the deposition, the resultant films were annealed *in situ* for 30 min under 10^{-4} Pa pressure to further stable the crystallinity of HA films.

2.3 Sample Characterization

The surface morphology of the HA film deposited TixOs® was investigated by scanning electron microscopy(SEM) with a focused ion beam(FIB) system(FEI Inc., USA) at an acceleration voltage of 20 kV and in high vacuum. Energy dispersive X-ray(EDX) spectral mapping and compositional measurements of HA films on the TixOs® were carried out by energy dispersive X-ray spectroscopy(EDS, EDAX Apollo X) and X-ray photoelectron spectroscopy(XPS, Thermo ESCALab 250). The EDS analyses were conducted in duplicate on film regions having area of 250 μm×250 μm, and operated at 20 kV. Both sets of experiments lead to comparable results and for that reason, only the results from one of the two quantitative analyses are presented in the paper. For XPS, a spectrometer using monochromatic Al $K\alpha$($h\nu$=1486.92 eV) radiation was employed, and the scanning area was set to 500 μm×500 μm on the implant.

2.4 Thickness Evaluation

The HA thin film was prepared by PLD at 20k laser pulse and 300 mJ output energy on the surface of titanium sheet instead of the porous TixOs® implant, and the thickness of the films on different substrates under the same condition using the same method was considered to be equivalent. Then the titanium sheet coated with HA was placed vertically and the film thickness was evaluated by SEM.

2.5 *In vitro* Stability of HA Film

To evaluate the stability of the HA film, the TixOs® coated with HA was immersed in culture medium at a constant temperature of 37 ℃. The solution was refreshed every 2 d. The pH value of the solution was set as 7.4 at 37 ℃ by adding 1 mol/L HCl-Tris[29]. Samples were taken out after 3, 7, and 15 d and gently rinsed with deionized water. At last, the changes in their surface morphology were studied by SEM at an accelerating voltage of 20 kV after gold sputtering.

2.6 Bioactivity Characterization

MC 3T3 E1 cells were used to assess the bioactivity of HA films in terms of cellular viability and proliferation. Cells were cultured in a Dulbecco's modified Eagle medium(DMEM) supplemented with 10%(volume ratio) fetal bovine serum(FBS), 2 mol/L glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin in a humidified and sterile atmosphere with 5% CO_2 at 37 ℃. The medium was changed every 3 d, allowing the cells to grow and reach confluency in the tissue culture plate.

The cellular viability(that is cytotoxicity) of the samples was tested according to ISO 10993-5: 2009. Briefly, the samples were immersed in DMEM solution(1.25 cm^2/mL) at 37 ℃. Solutions were then extracted at 1, 2, 3 and 4 weeks. The extracts(100 μL) with FBS, a fresh medium(as negative control), and a solution of phenol at a concentration of 6.4 g/L(known to be cytotoxic, as positive control) were added to the corresponding wells of a 96-well plate. Cells were subsequently seeded with a concentration of 10000 cells per well and cultured for 24 h. After another 24 h incubation, the cell viability was quantitatively analyzed by cell counting kit-8(CCK-8, DOJINDO, Japan) reagent.

The proliferation of cells on the samples was also investigated using CCK-8 reagent. After culturing cells on the surface of HA films for 2, 4, 6 and 8 d, the CCK-8 reagent was then added to medium. After 3 h incubation, the absorbance was read at 450 nm with a multimode microplate reader(Zenyth 3100, Anthos, USA).

All experiments were carried out in triplicate in order to address the statistical significance. The statistical analysis was performed using unpaired Student's *t*-test and differences were considered significant at $P<0.05$.

3 Results

3.1 Characterization of HA Thin Films on the Porous TixOs®

The morphologies of TixOs® implant, HA and HA-coated TixOs® samples with various laser pulse and output energy were investigated by SEM. As shown in Fig.1, the surface of TixOs®[Fig.1(A)] is relatively smooth with randomly distributed metallic balls with a diameter of 30—50 μm(red arrow) and the interconnecting micropores with a diameter of 10—80 μm(green arrow). Morphology of HA shows a typical fibrous-like shape with interconnected structures[Fig.1(B)].

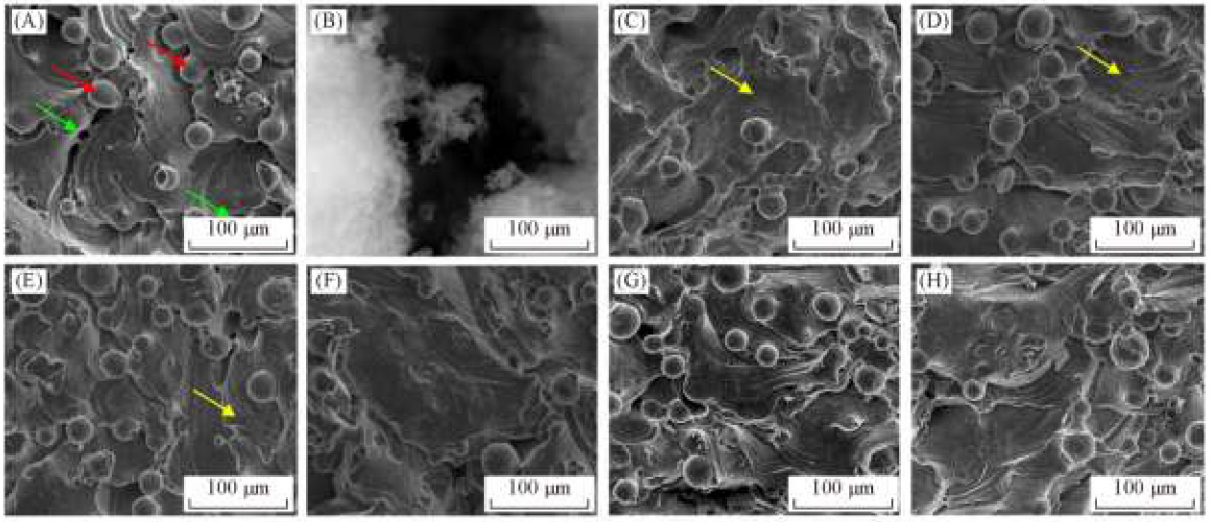

Fig.1 SEM images of TixOs® implant surface(A), HA powder(B), HA coated TixOs® by PLD at 5k(C), 10k(D), 15k(E) and 20k(F) laser pulse under 300 mJ output energy, and HA coated TixOs® at 20k laser pulse under 350(G) and 420 mJ(H) output energy

The HA coating on TixOs® was deposited by PLD with 5k[Fig.1(C)], 10k[Fig.1(D)] or 15k[Fig.1(E)] laser pulse, respectively. The deposited HA coatings on the surface of TixOs® show needle-like morphology as indicated with yellow arrows. While with 20k laser pulses, the HA crystal presents relatively uniform thin sheet and micrometer-sized crystal interleaving arrangement[Fig.1(F)]. The enhancement of output energy at the optimal 20k laser pulse cannot significantly affect the morphology of HA films[Fig.1(G) and (H)], but may block the microporous structure of TixOs®. This is also the usual cases in a traditional electrochemical method[30], in which the surface morphology of Ti implants may be significantly changed due to the unbalanced deposition process. Therefore, the most suitable conditions for preparing HA film coated porous TixOs® by PLD are 20k laser pulses and 300 mJ output energy.

EDS spectra of the HA films indicated the presence of typical apatite elements(only Ca and P), along with the signal originating from the TixOs® implant(Al, Ti). The EDS

quantitative results are listed in Table 1. The measured atomic ratio of Ca/P in the pure HA films approaches that of the theoretical value(Ca/P=1.67) with slight divergence within the allowed error limit of EDS analysis technique.

Table 1 Percentages of all the elements on the surface of the porous TixOs® deposited with HA

Condition	Atomic ratio(%)				Ca/P
	Al	P	Ca	Ti	
TixOs®	12.90	—	—	87.10	—
HA	—	14.43	23.83	—	1.65
5k@300 mJ	10.30	0.58	1.18	87.94	2.03
10k@300 mJ	7.56	0.77	1.44	90.23	1.87
15k@300 mJ	7.53	1.12	1.98	89.36	1.77
20k@300 mJ	9.21	2.31	3.88	84.61	1.68
20k@350 mJ	9.53	5.14	8.89	76.44	1.73
20k@420 mJ	6.06	3.67	6.52	83.76	1.78

When the output energy of PLD remained unchanged, the Ca/P atomic ratio decreased from 2.03 to 1.68 in HA films with the number of laser pulses increasing from 5k to 20k. Since under the 20k laser pulse condition, the Ca/P atomic ratio(1.68) in the HA film deposited on the surface of TixOs® implant is closest to that of the pure HA film(1.65), the output energy of PLD was further optimized under this condition. As the output energy increased from 300 mJ to 450 mJ, the Ca/P atomic ratios in the HA films increased slightly. Therefore, according to the results of EDS, we summarized that the most suitable experimental conditions for depositing the HA thin film on the TixOs® using PLD method were the laser pulse at 20k and output energy at 300 mJ, which was consistent with the SEM images.

Subsequently, various characterization methods, including XPS, FE-SEM and FIB were employed to investigate the properties of HA coating TixOs® fabricated by PLD with the optimal conditions(20k, 300 mJ). The general XPS spectrum is shown in Fig.2(A), and all the peaks were identified and coincided to the elements of HA. There was no detectable signal of Ti from the substrate, which indicated perfect coating was formed on the TixOs® surface by dense HA films. The narrow scan for Ca_{2p} and P_{2p} is shown in Figs.2(B) and (C), respectively. The Ca/P atomic ratio calculated by the Ca_{2p} and P_{2p} peak area integration was 1.67, which was completely consistent with the HA stoichiometric ratio of 1.67. The O_{1s} peak [Fig.2(D)] was well deconstructed into three Gaussian components corresponding to the binding energy positions of 530.46, 531.23 and 532.41 eV, which can be assigned to the CaOH, PO_4^{3-} and absorbed oxygen or carbon dioxide. It was worth mentioning that the calculated OH^-/PO_4^{3-} ratio for the film was closed to that of the starting material. The highly existed surface OH groups are crucial to enhance the cell adhesive ability of HA coated TixOs® implant, which might probably further improve the biological activity performance[31].

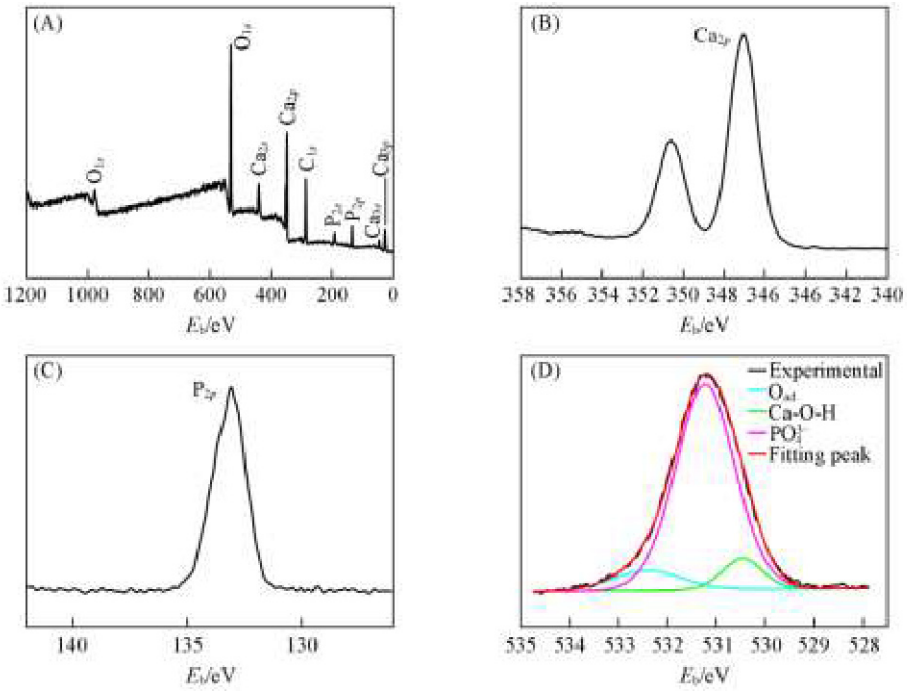

Fig.2 Survery(A) and high-resolution XPS spectra of Ca_{2p}(B), P_{2p}(C) and O_{1s}(D) of the resultant HA films deposited on the porous TixOs®

Due to the porous and rough surface of the TixOs® implant, it is not easy to directly evaluate the thickness of the HA film coated on the implant. Therefore, the HA thin film was prepared on the surface of a titanium sheet instead of the TixOs® under the same PLD experimental conditions. The thickness of the films on these two kinds of substrates was

considered equivalent. In Fig.3, the thickness of an HA film on the uniform titanium sheet was about 135 nm, which was equivalent to the thickness of an HA film coated on the TixOs®, indicating the thickness of the HA film formed with PLD method is in the range of nano-scale.

Fig.4 shows the element distribution, including Al, P, Ca and Ti of the TixOs® surface coated with HA. Among them, Al[Fig.4(B)] and Ti[Fig.4(E)] elements were the original component of TixOs®. P[Fig.4(C)] and Ca[Fig.4(D)] elements were not present in the TixOs®, but they were clearly observed and evenly distributed on the surface of TixOs® deposited with an HA film. These findings indicated that the HA coating on the porous TixOs® was prepared by PLD.

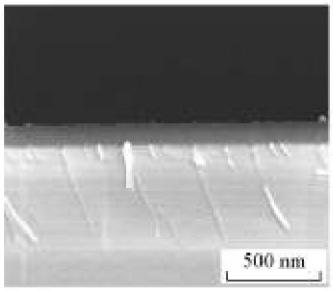

Fig.3 Cross-section view of the as-deposited HA coating on the implant by PLD method with 20k laser pulses and 300 mJ output energy

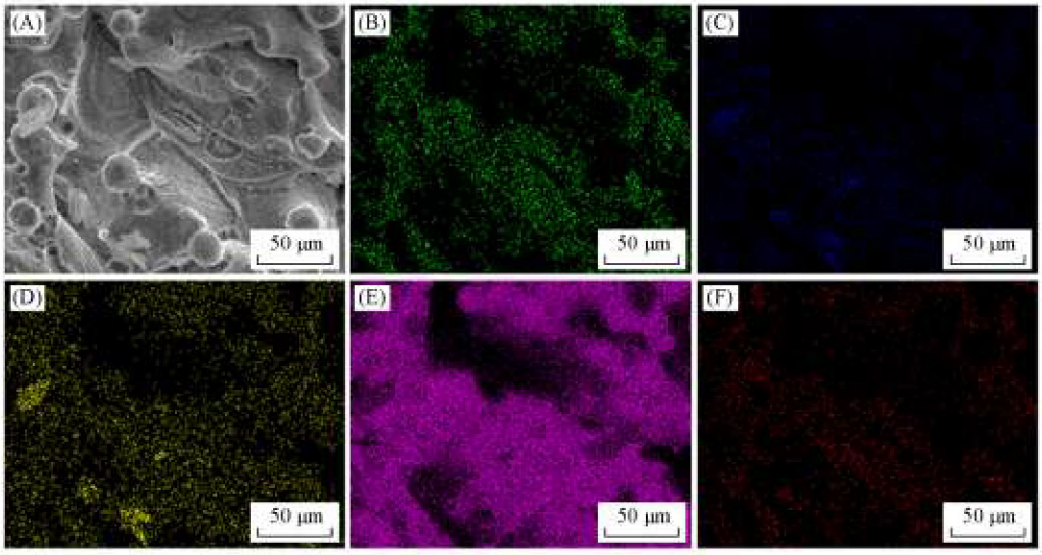

Fig.4 Element distribution of the surface of the TixOs® deposited with HA

(A) SEM image of the porous TixOs® surface. Al(B), P(C), Ca(D), Ti(E) and O(F) elements distributed on the surface of the TixOs® implant deposited with a HA thin film, respectively.

3.2 Stability of HA Thin Films on the TixOs®

The stability of HA coatings on TixOs® was measured with the acidic solution immersion experiments that characterized by SEM with controlled immersion duration of 0, 3, 7, and 15 d, respectively(Fig.5). Interconnected crook structures could be found on the surface of HA-coated TixOs® for the as-deposited sample[Fig.5(A)]. It was obvious that within our experimental period, the surface morphologies of these coatings changed little comparing with its original state. Even after 15 d of immersion in the medium, there were rarely cracks in the HA film on the TixOs®[Fig.5(D)], and the HA coating remained intact. This result illustrated that HA thin film fabricated by PLD on the porous TixOs® was stable and resistant to acidic medium.

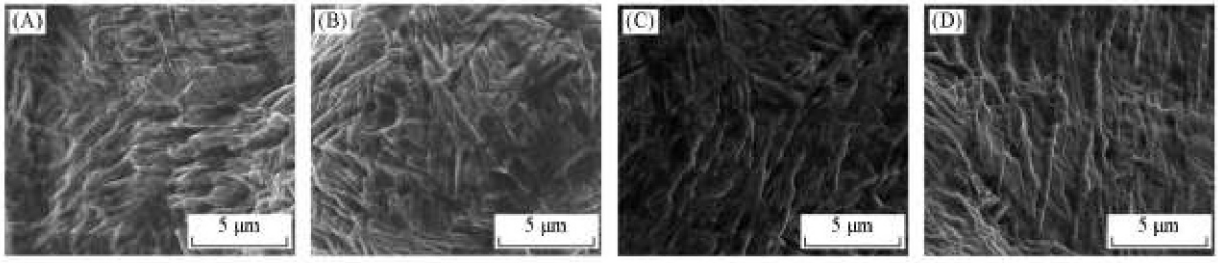

Fig.5 Surface morphology of the HA coated samples after immersion in culture media for 0(A), 3(B), 7(C) and 15 d(D)

3.3 Bioactivity of HA Thin Films on the TixOs®

The results of bioactivity tests of HA film coated TixOs® implants are summarized in Fig.6. The influences of the chosen DMEM media on MC 3T3 E1 cells have been tested[Fig.6(A)]. Phenol was added to DMEM as a control group. According to our result, the tested biomaterials did not influence the cellular viability. The relative growth rate(RGR) of MC 3T3 E1 cells showed significantly difference to that of the negative control(DMEM) group even at week 4[Fig.6(A)], while the phenol

group hardly showed cell growth rate.

For both TixOs® implants and HA film coated TixOs® implants, the cellular proliferation rate on the surface of the materials increases with prolonging the culture time[Fig.6(B)]. The cell proliferation capacity of HA coated TixOs® implants is significantly stronger than that of the TixOs® implants(about 1.73 times higher on day 8). These results evidently indicate that cells are more favored to grow on the surface of HA film coated TixOs® implants, which means that coating HA film onto TixOs® implants with PLD method can significantly improve the biological activity of TixOs® implants.

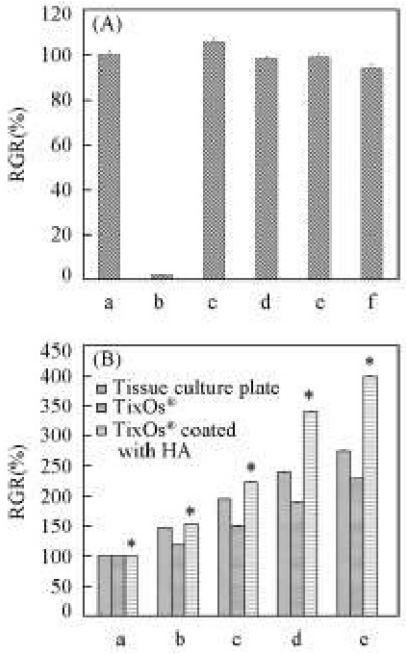

Fig.6 Bioactivity of HA thin films on the TixOs® implants

(A) Cytotoxicity of the TixOs® implants deposited with HA thin film(compared with phenol group). a. DMEM; b. phenol; c. DMEM, week 1; d. DMEM, week 2; e. DMEM, week 3; f. DMEM, week 4. (B) cellular proliferation of the TixOs® implants and the HA film coated TixOs® implants(at a significant of $P<0.5$). Time/d: a. 0; b. 2; c. 4; d. 6; e. 8

4 Discussion

As a promising dental implant material, TixOs® with porous reticular structure closely combined with the bone tissue and provided sufficient 3D space for new host growth due to the microporous structure. The implant surface area might also lead to a stronger osteointegration response[32—34]. However, similar to the conventional dental implants, TixOs® showed relatively weak bioactivity and biocompatibility, which were expected to be improved by HA coating. However, the HA film prepared by traditional processes(*e.g.*, electrophoretic deposition)[17,23] showed relatively low crystallinity and large elastic modulus, resulting in low binding strength between the coated film and the substrate. These problems might lead to the falling off or the breaking off for the HA films on the implants. Therefore, it showed a practical method to deposit an HA thin film on the porous TixOs® by PLD.

According to the mechanism of PLD method, sintered HA particles were either expulsed directly from target by phase explosion, plasma recoil and surface instabilities or formed by coalescence of particles due to intense collisions during the transit from target to substrate[35]. Consequently, quantity and output energy of the laser pulses could affect the crystallization and formation of HA thin films on the TixOs®. By tailoring these parameters, we have obtained the optimum PLD condition for HA film coating[Fig.1(F)]. Considering the morphology and crystallization of HA films, the optimum deposition conditions were 20k laser pulses and 300 mJ output energy. As shown in Fig.1(F), the morphology of HA film coating on TixOs® exhibited layered structure with different micrometric size superimposed on the uniform thin film. Unlike the traditional electrochemical method, in which the microstructure of implant might be overlapped by ununiformed film[30], this deposition condition has been identified to cover the surface of implant with uniform amorphous and micro crystalline combined film. We have noted that the Ca/P atomic ratio of the HA film increased as the laser pulse decreased, this behavior could be explained by the considerably low HA formation and the difficulty in crystallization at low laser pulse[36]. XPS(Fig.2), SEM(Fig.3) and FIB(Fig.4) also confirmed that the HA films on TixOs® implants deposited by the above PLD conditions had appropriate thickness, fine crystallinity and uniform distribution. Besides, the microstructure of the porous TixOs® was preserved under the conditions.

While the usual HA material is hydrophilic and prone to crack in aqueous solution[37,38], an advantage of our method was that one may achieve stable and bioactive HA thin films. We have tested the stability of our synthesized HA film coated TixOs® implant, and no obvious crack was observed after immersing into culture medium for 15 d[Fig.5(D)]. This result was in consistent with the previous report and it was attributed to the good crystallinity of HA on the implant surface[39,40].

In our study, we have demonstrated that osteoblasts grew better on the surface of HA film coated TixOs® implant than that of pure TixOs® implant. This is because HA is more bioactive than TixOs® and the microporous structure left on the TixOs® may let osteoblasts go inside to hold and adhere to the implant. According to Ball *et al*.[41], the osteoblasts growing on the crystalline surfaces of HA film are more bioactive with the production of alkaline phosphatase. This mechanism might also be important in our experiment. The explicit mechanism of osteoblasts growing will be included in our future studies.

5 Conclusions

HA thin films coating onto the porous TixOs® using PLD were performed. According to the results of morphology(SEM) and quasi-stoichiometry EDS of the HA on the implant, the optimal conditions for coating were 20k laser pulse and 300 mJ output energy. The biostability and bioactivity of the obtained HA film coated on TixOs® were subsequently tested, and the results showed that our PLD coating method might be promising in improving both of these qualities of TixOs®. Further studies are expected to elaborately address the detailed mechanism of osteoblasts growing on HA coated implants.

Acknowledgments

The authors would like to thank the Company of Leader-Novaxa(Italia) for providing TixOs® implants for free.

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