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Preparation of an electrodeposited hydroxyapatite coating on titanium substrate suitable for in-vivo applications

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Abstract In this paper the porous hydroxyapatite coating on Ti implant materials was prepared by the process of electrodeposition, hydrothermal and sinter. The surface morphology, bond strength and thickness of HA coatings were investigated by SEM, AFM, and its biocompatibility was evaluated by cytotoxicity experiments and implant experiments, respectively. Results showed that (1) The HA coatings was 50 μm thickness and adhered on the Ti substrate strongly, which bond strength reached 38MPa. AFM analysis showed that the HA coating was porous structure, in which the mean pore size was 236.5 μm, (2) Cytotoxicity experiments and implant experiments showed that HA-coated Ti implant materials has little cytotoxicity in vitro and little inflammatory reaction in vivo, and there were no statistically disparity between HA-coated Ti implant and titanium implant materials of clinical application ($p > 0.05$), which demonstrated that HA-coated Ti has a good biocompatibility.

Introduction

Nowadays, the use of implanted devices is a well-acknowledged practice in the field of orthopaedic and dental surgery. Scientific research and clinical experience suggest that the successful exploitation of these devices mainly depends on integration with tissue, considered as both anatomical congruency and load-bearing capacity. Indeed, the integration process is influenced by a wide range of factors: anatomical location, implant size and design, surgical procedure, loading effects, biological fluids, age and sex, and, in particular, surface characteristics. For this reason, several attempts have been aimed at modifying implant surface composition and morphology to optimize implant-to- tissue contact and improve integration. Preliminary interactions between implanted materials and biological environment are deemed to be governed by the surface properties; they control the amount and quality of cell adhesion on the surface and, consequently, cell/tissue growth. Thus, surface properties govern new tissue formation and implant integration.

Titanium and its alloys are wildly used as implant materials because of their excellent mechanical properties and chemical stability in physiological environment. However, the difficultly to bond with living tissue and the release of corrosion product into surrounding tissues restrict their applications. Hydroxyapatite has the same structure as the major mineral constituent of human hard tissue and can form a very strong bond to the tissue. However, its poor sintering properties and low fracture strength limit its applications. Preparation of HA coatings on the surface of titanium implant materials would take advantage of both the biocompatibility of ceramics and the strength of metals [1–5].

It has been already proved that the surface roughness and the chemical composition of coating play a key role in determining tissue response [6]. So the porous surface morphology, high bond strength between HA coating and Ti substrate, and proper thickness for the HA coatings were

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necessary to enhances the corrosion resistivity of the Ti ions entering into biological environment of human body and to improve the implant integration with the living tissue [7–8]. Recently many researches had been done on the HA coating by different methods [9–15]. However, the relationship between the surface of an endosseous implant, its reactivity with biological tissue constituents, and its clinical efficiency are not clearly understood.

In order to prepare porous hydroxyapatite coatings and to achieving a deeper understanding of the phenomena involved in the interactions between tissue and implant materials, the process of electrodeposition, hydrothermal and sinter was applied. The bond strength, pore size and surface morphology of HA coatings were studied, and its biocompatibility was evaluated by cytotoxicity experiments and implant experiments according to GB/T16886.

Materials and methods

Pretreatment of titanium plate

The commercially titanium plates were metallographic ally polished using SiC emery paper to remove the oxide layer on the surface, and washed in the acetone, alcohol and deionized water by ultrasonic for 10 min, respectively. Then the Ti plates were immersed into the 1 mol/L NaOH solution. After 72 h. the Ti plates were taken out and rinsed in water

Preparation of HA coatings

The pretreated Ti plates acted as cathode, graphite acted as the anode, the electrolyte was prepared by dissolving the reagent $Ca(NO_3)_2$, KH_2PO_4 into deionized water, with molar ratio Ca:P = 5:3, $[Ca]=2\times10^{-3}$ mol/L. NH_4OH and HCl were used to adjusted pH = 4.7. The Ti plates and graphite were put into the electrolyte to electrodeposit for 3 h under the condition of 30 mV voltage DC. Then the coated Ti plates were taken out and put into autoclave to carry out hydrothermal treatment at 200 °C for 3 h. Subsequently the coated Ti plates were sintered at 700 °C for 2 h. After those the coated Ti plates were washed by distilled water and dried.

Characteristic of coatings

The crystal component of coatings was investigated by XRD after scrape coatings from the Ti substrate. The thickness, pore size and surface morphology of coatings were investigated by SEM and AFM directly. The bond strength between HA coating and Ti substrate was evaluated by Instron 2195 according to ASTM C-633. The bond strength can be calculated according to Eq. 1

$$\sigma_b = F/S \tag{1}$$

Where F is the max tensile strength, S is the area of samples. The data of bond strength was obtained by average the sum of five samples' data.

Biocompatibility experiments

Reagents and instruments

Fetal calf serum, Trypsinase and PI were purchased from Siji Qing Co., GIBCO Co. and sigma Co, respectively. The rest reagents were analytical pure. L929 mouse skin fibroblasts, six healthy young swine and the animal experimental protocol were provided by XiangYa Medical School of Central South University, the Experimental Animal Center of Xiangya Medical School, and the Academic Committee of Xiangya Medical School, China, respectively.

Ti implant materials were divided into two groups. One group was the Ti implant materials of clinical applied (without HA coatings), the other was Ti implant materials with HA coatings.

Preparation of leaching liquor

Two group of Ti implant materials were steam sterilized at137.3 kPa, 121 °C for 30 min and RPMI-1640 medium in culture flask according to the ratio of $0.2 g/mL^{-1}$between quality of materials and volume of culture medium, then the flask were cultured about 72 h in humidified incubator at 37 °C with 5% CO2 in air.

Cell culture

L929 mouse skin fibroblasts were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum in humidified incubator at 37 °C with 5% CO_2 in air, and passage after the cell overgrew culture flask.

Flow cytometry assay

L929 mouse skin fibroblasts were serial subcultivated 24 h, and the medium of each culture flask was replaced respectively with leaching liquor of two materials. These culture flasks were incubated 24 h again at the same condition and were observed by inverted microscope. After the cells attached on the bottom of the culture flask, living cells were collected by trypsinization and were washed twice with PBS buffer solution. Then, the living cells were fixed with 4 °C, 70% alcohol, centrifuged 5 min at 500–1,000 r/min, the supernatants were discarded, and the cells were suspended

5 min in buffer solution, centrifuged again and discarded supernatants. The cells were stained with PI to make the final concentration to 100 μg/ml, and then the cells were protected from light 30 min at 4 °C and were detected by flow cytometry after sieving with 400 nylon net.

Implant experiment in vivo

Six healthy young swine, weighing 20–25 kg,were anesthetized by administration of 2% Pentobarbital. The abdominal wall of the animals were shaved and disinfected with 5% iodine in ethanol. Cutting open from the animals' abdominal wall to abdominal wall muscular layer in 2 cm two sides of medico-ventral line, and choosing six implant spots and each spot between 2 cm, the materials were implanted into spots. Finally, the incisions were closed compactly. At the end of each period (14, 30, and 90 days), the animals were sacrificed by anodyne. Materials were removed along with 0.5–1.0 cm the surrounding tissue and immersed in 4% buffered formalin. After fixing for 24 h, the tissue was processed for paraffin embedding. A paraffin block was oriented and sections were stained with hematoxylin and eosin. The tissue responses were observed by light microscope.

Statistics

Results were reported as mean ±SD. The results were performed on a computer using the SPSS 11.0 package of statistical program. Student's *t*-test was performed for the paired data and results were considered significant difference at a $p < 0.05$.

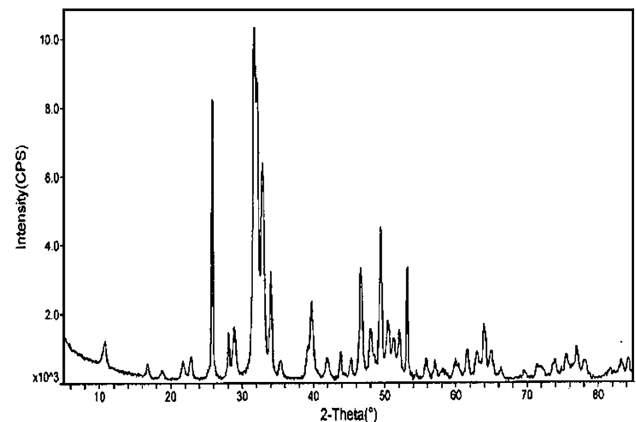

Fig. 1 The XRD graph of HA coatings brushed from the Ti substrate

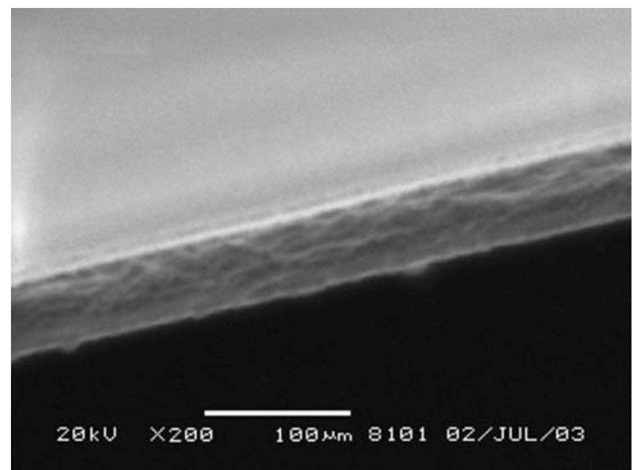

Fig. 2 The cross-section SEM paragraph of HA-coated Ti substrate

Results

Component and morphology of coatings

Figure 1 is the XRD paragraph of coatings scraped from the Ti substrate. It showed that the coating was pure HA, the sharp diffraction peaks indicated that HA was well crystallinity, which ensure that the HA coating had good stability in body fluid.

Figure 2 is the cross-section SEM paragraphs of HA coatings. From them we can see that the thickness of HA coatings was 50 μm. And the HA coating adhered to the Ti substrate strongly. The bonding strength tested was 38 MPa.

Figure 3 is the AFM paragraphs of HA coatings. From it no crack can be observed in a large range of the coatings. And the HA coatings was porous structure. Statistic carried out by program for 530 pores of Fig. 3, showed that the mean pore size of coating was 236.5 μm.

Cytotoxicity experiment

Figure 4 was the paragraph of S-period cells. The results demonstrated that the cells still overgrew on the bottom of flask after incubated leaching liquor of HA-coated Ti materials and the shapes of cells were polygon and fusiform, there were no died cells. The percentage of S-period cell of Ti materials with HA coatings was 49.86 ± 3.28, while the Ti materials without coatings was 52.13 ± 4.38. Statistic showed that there were no disparity between two groups ($p > 0.05$).

Implant experiment in vivo

Figure 5 showed the pathological sections after materials implanted. There were few neutrophil granulocytes, lymphocytes, macrophages or desmocytes on the surrounding tissue of both groups at early stage (14 days). neutrophil granulocytes infiltration extent of Ti implant

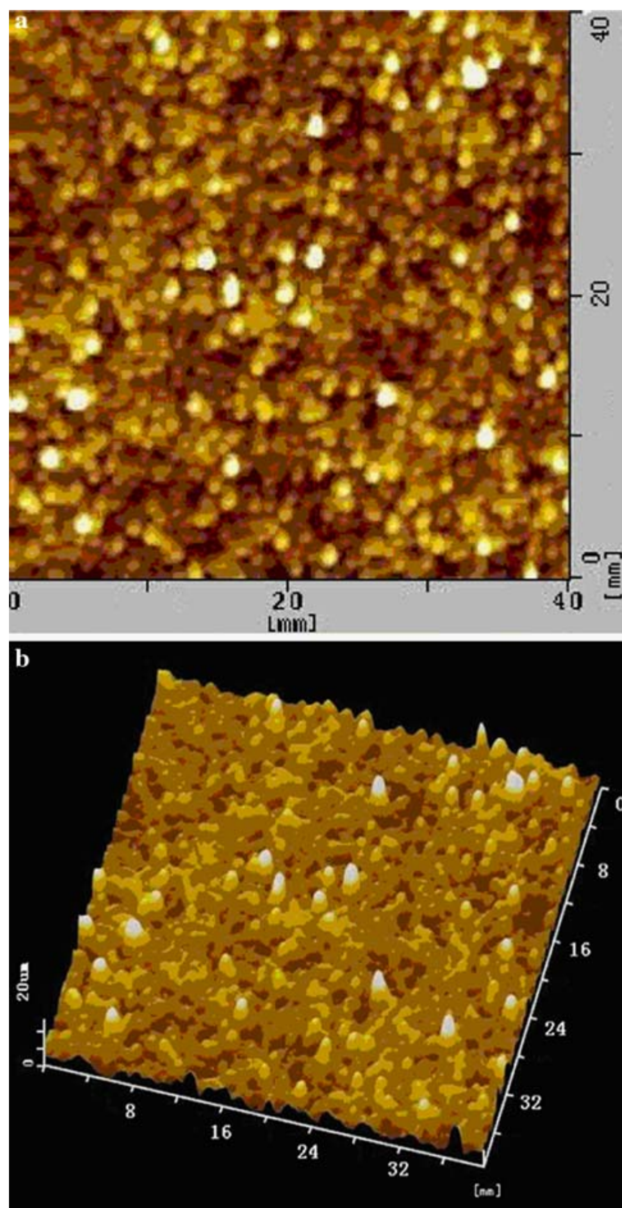

Fig. 3 The AFM paragraph of HA coatings (a) and its three-dimensional paragraph (b)

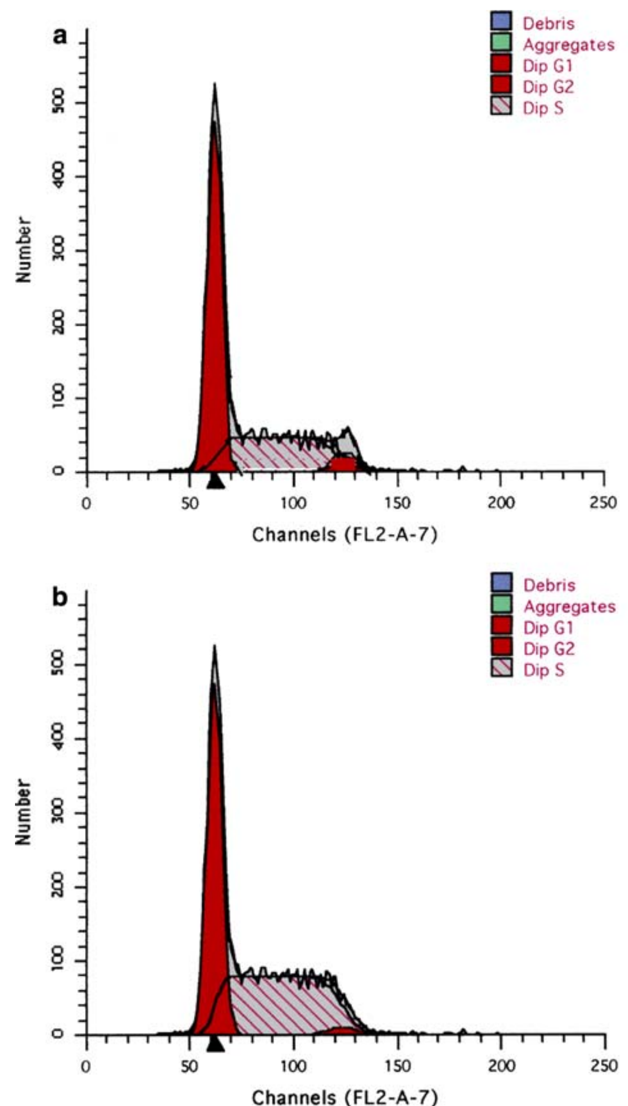

Fig. 4 Graphs of cell cycle in the extract of Ti materials with HA-coated (**a**) and without coatings (**b**) by flow cytometry

materials of clinical application was 23.5 ± 0.05, HA - coated Ti materials was 24.6 ± 0.36; No lymphocytes and fibrous capsule were composted on surrounding tissue of Ti materials at intermediate stage (30 days) (as shown in Fig. 5), lymphocytes' infiltration extent of Ti with and without coatings was 10.9 ± 0.81and 11.4 ± 0.55, respectively, There were no statistically disparity between two groups ($p > 0.05$); the layer of fibrocyte were tapering and becoming slender and compact fiber on the surrounding tissue of both group at 90 days, which have proved that Ti materials with HA coatings have a good biocompatibility.

Discussion

For the HA coatings on Ti substrate, proper thickness and high bond strength of coatings were the key factors to ensure the efficient implant and to prohibit the coatings brush off from the substrate. Studies showed that the necessary and optimized thickness of HA coatings was about 50 μm. Thinner coatings can't play the barrier roles to Ti ions entering into biological environment of human body, while thicker coatings will lead to high stresses on the substrate and result in coatings brushing off. Figures 1 and 2 showed that the coatings was pure, well crystallinity HA with 50 μm thickness, which can be stable in the environments of body fluid and meet the requirements for the coatings.

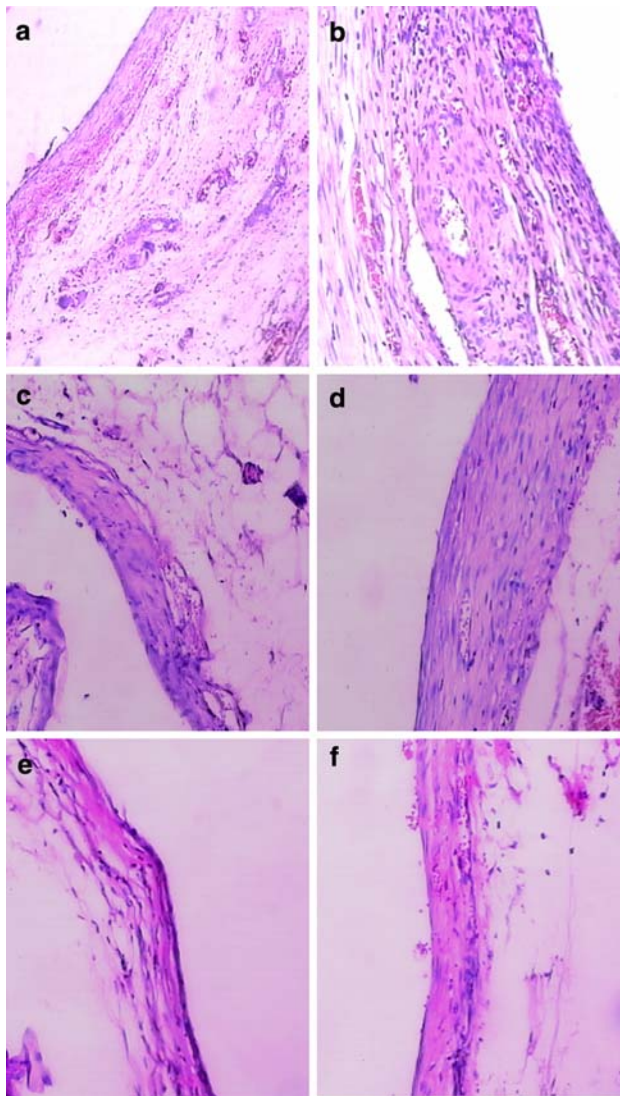

Fig. 5 Graphs of tissue pathological section of Ti materials and HA-coated Ti materials (**a**) Ti materials, 14 days; (**b**) HA-coated Ti materials, 14 days; (**c**) Ti materials, 30 days; (**d**) HA-coated Ti materials, 30 days; (**e**) Ti materials, 90 days; (**f**) HA-coated Ti materials, 90 days; (HEX20)

On the other hand, the coefficients of heat expansion of Ti substrate and hydroxyapatite (8.9×10^{-6} K^{-1} and 15×10^{-6} K^{-1}, respectively) was significant difference, which will caused generation of high stresses on the interface during cooling of sintered, and result in HA coatings cracking and even in complete brush off from the substrate. This may reduce the corrosion resistively of the Ti ions entering into biological environment of human body and arise the inflammatory reaction. Those problems can be eliminated when the porous HA coatings was formed. Furthermore, the porous structure attribute the tissues/cells grew into the pores and improve the coatings integrated with tissue, which can prevent some unwilling cases happen, such as biomaterial loosening, failure of translate implant, and even surgical removal of such failed implants.

The method of cell culture in vitro is a quick and effective toxicity screening program. It is a quick and precise method to evaluate the effect of different biomaterials on cytoactive by detecting S-period cells with flow cytometry after the cell cultured with materials [16–18]. The higher percentage of S-period cells, the less toxicity of biomaterials. Cytotoxicity results showed that the cells cultivated in leaching liquor of HA-coated Ti materials grew very well, and its proliferate rate was as high as that of titanium implant materials of clinical application, which demonstrated that the HA-coated Ti implant materials have little cytotoxicity in vitro.

Titanium implant materials are wildly used as implant materials because of their excellent mechanical properties and chemical stability in physiological environment. However, it is apt to release corrosion product into surrounding tissues, which would restrict their applications. Implant experiment in vivo showed that HA-coated Ti implant materials had little inflammatory reaction in vivo, which demonstrated that HA-coated Ti has a good biocompatibility.

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