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In vitro bioactivity and osteoblast-like cell test of zinc containing fluoridated hydroxyapatite films

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Abstract Zinc containing fluoridated hydroxyapatite (ZnFHA) films on Ti6Al4V substrates was prepared using sol–gel dip-coating method. The release of zinc ions from ZnFHA film was controlled mainly by the zinc content in the film. The release behavior showed an initial rapid increase release followed by a tapering-off and directed to a constant value at longer time. After soaking in SBF for 8 days, a layer was deposited and completely covered the original surface of the ZnFHA film, indicating good in vitro ''bioactivity.'' The osteoblast-like MG63 cells were seeded on the ZnFHA films; FHA film and Ti6Al4V substrate were used as control. The cell culture result showed that cell adhesion and proliferation on ZnFHA films were significantly increased compared with the controls. The results in this work suggest that ZnFHA films on Ti6Al4V substrates can function as an implant with good bioactivity and cytocompatibility.

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Introduction

As the main inorganic phase in bone, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HA] has been recognized as substitute materials for bone and teeth. However, the poor mechanical property of HA ceramics limits their application as load-bearing prosthesis. HA coatings on titanium have been applied as orthopedics and dental implants because of the perfect integration between good bioconductivity and mechanical property [1, 2]. Nevertheless, the resorption and biodegradation of HA in biological environment could lead to deterioration of the long-term stability of the coating, and even cause failure of implantation.

In comparison with HA, fluoridated hydroxyapatite [$Ca_{10}(PO_4)_6F_x(OH)_{2-x}$, $0 < x < 2$, FHA,] with lower solubility is feasible to improve the long-term stability of the implants [3, 4]. Furthermore, FHA retains comparable biocompatibility and bioactivity to pure HA in terms of the tissue and cell response [5, 6]. However, osteoconductivity, biocompatibility and cell response of FHA at early stage of implantation still needs to enhance.

In vitro and in vivo studies revealed that reactions such as dissolution, precipitation, ion exchange and structural rearrangement occurred at the material-tissue interface [7], where the released ions can create solution-mediated effects on cellular activity. As an essential trace element in body, zinc is found to have positive roles in promoting bone formation and inhibiting bone resorption [8–10]. Several studies have been carried out on bulk zinc containing materials based on co-precipitated HA [11–13], the dissolution or biodegradation of zinc containing HA could release zinc ions which have beneficial effects on bone formation.

In this study, we attempted to evaluate the effect of zinc incorporation on the in vitro properties of FHA film. Zinc containing fluoridated hydroxyapatite (ZnFHA) films on

Ti6Al4V substrates was prepared using sol–gel dip-coating method. The zinc release behavior, in vitro apatite deposition ability in SBF solution, and osteoblast-like cells response of ZnFHA films were measured, assayed and discussed.

Materials and methods

Film preparation

ZnFHA Films were prepared using sol–gel dip-coating method described in previous work [14]. In brief, the Ca ($Ca(NO_3)_2 \cdot 4H_2O$ (AR)) and P precursor (P_2O_5 (AR)) ethanol solutions were mixed in Ca/P ratio of 1.67 before HPF_6 (AR) was added, the mixture was refluxed for 24 h to form an initial sol. Different designed amount of $Zn(NO_3)_2$ ethanol solution was added into the initial sol to form dipping sols. The Zn/Ca molar ratios of the dipping sols for ZnFHA-1 and ZnFHA-2 in this work are 0.00775 and 0.0155, respectively. In preparation of ZnFHA films, cleaned Ti6Al4V substrates were immersed into the dipping sols and withdrawn at a speed of 8 cm/min. The as-dipped films were dried at 150 °C for 15 min and fired at 600 °C for another 15 min. Such dipping-drying-firing procedure was repeated twice.

Zinc release test

The specimens (10 × 10 mm) were immersed in 5 mL Tris buffered solution (0.05 mol/L) at pH 7.25 in sealed polyethylene tubes. After dwelling in oscillating water bath at 37 °C for 16, 24, 48, 168 h, respectively, the concentrations of zinc in the solution was determined by atomic absorption spectrophotometry (HITACHI 180-50).

SBF immersing test

Supersaturated simulated body fluid (1.5 SBF) solution was prepared with 1.5 times of ion concentrations than the normal SBF, with pH of 7.4 at 36.5 °C. The specimens were soaked in 1.5 SBF solutions in sealed polyethylene bottles (filled with 35 mL 1.5 SBF solution) at 37 °C with refreshing the solution every other day. After 8 days, the specimens were taken out, rinsed with deionized water and dried in the air. The surface morphology variation was observed by Field Emission Scanning Electron Microscopy (FE-SEM, Model FEI SIRION).

Cell culture assay

Human osteoblast-like MG63 cells were obtained from ATCC (Manassas, VA, USA). The cells were thawed from a frozen stock and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin and streptomycin in a standard incubator (37 °C, 5% CO_2 atmosphere and 100% relative humidity). The media were refreshed every 2–3 days. When the cells reached 90% confluence, they were subcultured routinely.

The films were cut into 10 × 10 mm pieces and sterilized in 120 °C water steam for 20 min. The samples were then placed in 24-well plates for osteoblast-like cells implantation at a set density of 1×10^4 cells/cm^3. The cell viability was evaluated by MTT-assay. Briefly, 100 mL MTT solution (5 mg/mL in PBS solution) and 900 mL media were added to each well and allowed to incubate for 4 h at 37 °C. About 400 μL dimethyl sulphoxide (DMSO) was added to each sample and surged for 15 min to dissolve formazan. The tests were performed on three replicate samples. The absorbance was determined at 570 nm in microplate reader (BIO-RAD Model550).

For cell morphology observation, the osteoblast-like cells attached on the Films were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 4 h at 4 °C, 1% osmium tetroxide in Veronal buffer, and critical point dry with CO_2. Finally the cells were gold coated by sputtering and observed in FE-SEM.

Statistical analysis

Results were expressed as mean ± SD of triplicate measurements. Comparative studies of means were performed using one-way ANOVA followed by LSD test with SPSS software. The bar graphs showed mean values and SD for each dataset.

Results

For the zinc release behavior of ZnFHA film in Tris solution (Fig. 1), the zinc ions released fast in the initial 16 h and then tapered off. The zinc concentration in Tris solution of ZnFHA-2 was higher than that of ZnFHA-1, which was consistent with the designed amount in the films.

For all the specimens, continuous layers deposited and completely covered the original surfaces of the films after soaking in SBF for 8 days, as shown in Fig. 2. At higher magnification, the deposited layer demonstrates to have a dune-like morphology with nano-sized plate-like microstructure (Fig. 2a–c), which is typical of SBF derived apatite [15].

The biological properties of ZnFHA films and the controls were evaluated through in vitro osteoblast-like cells response. Figure 3 shows the cell viability on the films

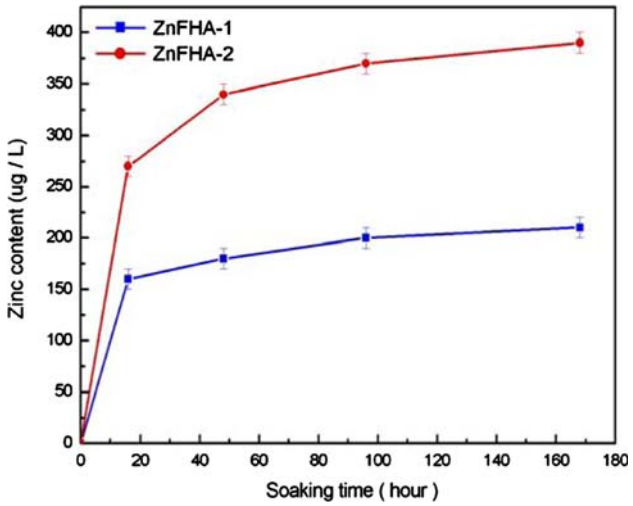

Fig. 1 The in vitro zinc release profile of ZnFHA coating

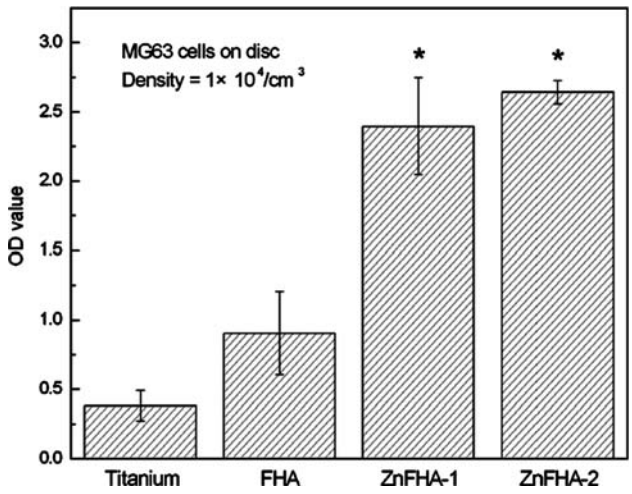

Fig. 3 MTT results of cells cultured on the surface of the different films at day 5. *Significantly different from the controls (ANOVA, LSD $p < 0.05$)

after 5 days in term of MTT absorbance (at $p < 0.05$). Cell proliferation was evaluated by optical density (OD) value. Comparatively, the cell counts on ZnFHA-1 and ZnFHA-2 films show statistically significantly higher OD than that on titanium substrate and FHA film. No significant difference is observed between ZnFHA-1and ZnFHA-2.

The typical morphology of attached osteoblast-like cells on pure titanium substrate, FHA film, and ZnFHA films are showed in Fig. 4. Only a small amount of sphere-like cells sporadically attached on the surface of pure titanium substrate (Fig. 4a), while irregularly spread and grew favorably cells were observed on FHA and ZnFHA films (Fig. 4b and c). For ZnFHA films, the cells even covered almost the whole film surface and formed several cell tiers (Fig. 4c).

Discussion

The capability of zinc release for the ZnFHA film is priority for applications since only released zinc ions can create solution-mediated effects on cellular activity.

Our previous work found that sol–gel derived ZnFHA films had a characteristic: the surface of the top layer of the film has much higher in zinc content than inside [14]. This implies that the sol–gel derived ZnFHA films could be

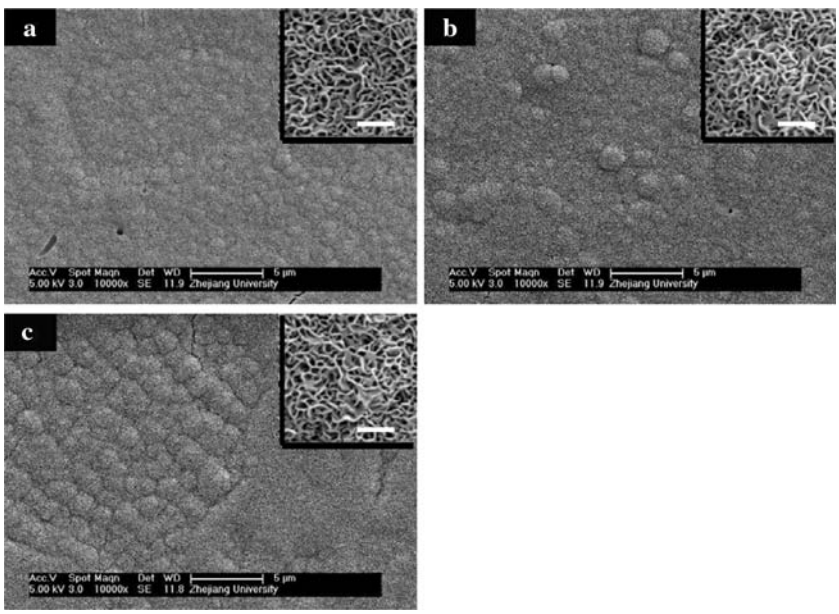

Fig. 2 SEM micrographs of the coatings after soaking in SBF for 8 days: (**a**) FHA, (**b**) ZnFHA-1 (**c**) ZnFHA-2. Insect: higher magnification. Scale bars: all 500 nm

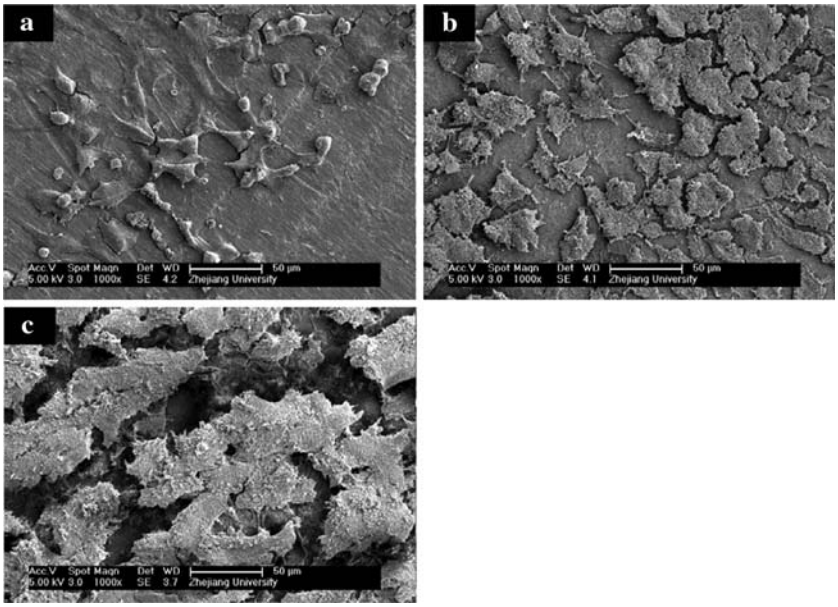

Fig. 4 Morphology of 5-day cultured cells attached on the surface: (**a**) uncoated Ti6Al4V, (**b**) FHA, (**c**) ZnFHA-2

capable of releasing zinc as well as having good bioactivity.

The soaking test results (Fig. 1) prove the ZnFHA films to be able to release zinc. The zinc release profile of the ZnFHA films shows that Zn initially releases fast within 16 h, and then the release slows down. The zinc concentration of ZnFHA-2 in Tris solution is higher than that of ZnFHA-1, which is consistent with the designed amount. This indicates that the release of zinc can be controlled by zinc amount in the films. After soaking for 168 h, the zinc of ZnFHA-1 almost released out, while, the ZnFHA-2 continuously released zinc ions slowly.

The existence of zinc in HA is usually considered to have two ways. Most of zinc exist on crystal surface or grain boundary [16], and only a small amount of zinc can be incorporated into HA lattice [17]. The two existence states can cause different probability for zinc to leave from HA. Hence, it is reasonable that zinc comes mainly from the surface of the film in the fast release stage, then from HA lattice in the stable release stage.

Biomimetic apatite layer with dune-like morphology covers the film's surface after soaking in 1.5 SBF for 8 days (Fig. 2). At higher magnification (the insets in Fig. 2a–c), the microstructure of the deposited layer demonstrates to be nano-sized plate-like. These results are quite typical for biomimetic apatite. The easy and fast deposition of such apatite layer implies the ZnFHA films have good in vitro ''bioactivity.''

Biomaterial surface chemical properties can influence protein adsorption and elicit diverse cellular responses. Our studies highlight the obvious differences between FHA Films with and without zinc in modulating cellular responses. The results demonstrate that the cell proliferation on the ZnFHA films is significantly increased compared with a control of FHA film (Fig. 3). Moreover, cell attachment and spread show better livelihood on ZnFHA films than on FHA film as shown in Fig. 4. Based on the above analysis, the enhanced osteoblast-like cell response to ZnFHA film is most probably due to the release of zinc ions, which induces positive solution-mediated effects on cellular activity.

Conclusion

The release of zinc ions from the present sol–gel derived ZnFHA film can be controlled through the variation of Zn concentration in the film. The Zn release process underwent an initial rapid increase release followed by a tapering-off and directed to a constant value at longer time. After soaking in SBF for 8 days, the ZnFHA film showed good in vitro ''bioactivity.'' The in vitro osteoblast-like cells response to the ZnFHA films showed that the cells spread and grew favorably and the proliferation of osteoblast-like cells was significantly enhanced on ZnFHA films. In view of both the long-term stability and high biological response of the films, the present ZnFHA film is just used as an outer layer, a dense and stable FHA layer should exist as a bottom layer on titanium substrate, thus, a high quality implant surface will be constructed.

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