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Synthesis of plate-like single-crystal hydroxyapatite rods with c-axis orientation by biotemplate small intestinal submucosa

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ABSTRACT

Since hydroxyapatite crystals are characteristically c-axis orientation on the surface of vertebrate long bone, c-axis orientation HAP biomimetic synthesized will find extensive applications in long bone growth, remodeling and fracture healing. In this paper, plate-like single-crystal HAP rods with c-axis orientation was successfully synthesized at bone mineralization conditions in vivo, with small intestinal submucosa membrane as biomineralization template. The samples were characterized by XRD, FIIR, SEM, TEM and EDS to unveil the phase structure, composition, morphology, and a plausible growth mechanism was proposed. The results showed that morphology of samples changed from flower-like to plate-like with extension reaction time from 1 day to 10 days. The plate-like HAp rods were single-crystal with c-axis orientation. A unit of plate-like HAP rods is about 70 μm and the width is 4 μm. Phase composition transformed from octocalcium phosphate and HAp biphase to HAp phase with very little octocalcium phosphate phase. Finally, biocompatibility of the samples was evaluated by CCK8. The samples without significant cytotoxicity conformed to the need for substitute materials of bone regeneration.

1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAP), as a main mineral constituent of bone, is an attractive implant material promoting for bone regeneration with outstanding osteoconductivity, biocompatibility and bioactivity [1]. It is well known that HAP stimulates the proliferation and differentiation of osteoprogenitor cells without systemic toxicity or immunological reactions [2]. The close chemical similarity of HAP to natural bone has led to extensive research efforts to synthesize HAP as a bone substitute and/or replacement in biomedical applications [3,4]. As HAP crystals have a characteristic of c-axis orientation in vertebrate long bone surfaces [5], synthesis of c-axis oriented HAP crystals is of great importance. Up to now, c-axis oriented HAP crystals have been synthesized by homogeneous precipitation [5] and hydrothermal method [6–8]. However, these methods often require harsh reaction conditions like elevated temperature and/or extreme pH value. In contrast, There are a few methods which have also been entirely set at room-to-physiological temperature, such as the double diffusion technique [9] and the impregnation in simulated body fluid (SBF) [10,11]. A drawback of the double diffusion method was relatively stable brushite together with HA crystals. Although the SBF as a reservoir has the strong advantage of being the closest to physiological conditions, a tedious preparation procedure of the metastable solution increases the risk of obtaining nonreproducible results [12]. And the bone-like nanocrystalline hydroxyapatite was synthesized by using amorphous calcium-phosphate precursors. However, this approach needs a continuous computer-controlled [13].

In recent years, biomimetic template mineralization method was further studied. The available biotemplates are diverse. A variety of recombinant proteins (bovine serum albumin [14], egg-white protein [15,16], biodegradable plant proteins [17,18]) were used as biotemplate. However, it was difficult to extract and purify these proteins from the final products and to determine the properties and functions. Thus, it is imperative to come up with a simpler proven technique and biological templates with excellent performance. In such context, small intestinal submucosa (SIS, natural biological material) was obtained with standard procedures [19]. Its main constituents are type I collagen which is similar to the main organic constituents of bone [20]. And it has been proved that type I collagen can serve as the template for bone mineralization [21]. The literature about SIS as biotemplate is rarely reported [10,22]. Yang et al. [10] reported their preparation of HAP bone minerals by soaking SIS membrane as the biotemplate in 1.5 SBF and demonstrated the good performance of the mineralized of SIS membrane in promoting osteogenic differentiation of rat mesenchymal

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stem cells (MSCs). They also showed that in a HAP-supersaturated solution, the SIS membranes could promote the formation of HAP crystals along the collagen fibers to form biomineralized HAP-SIS scaffolds [22].

In this study, we employ low cost, no immunogenicity and a wide range of raw material source of SIS membrane as template, a simple experiment device and reproducible experiment process to synthesize biomimetic HAP. In addition, the plate-like HAP is desirable because it resembles the morphology as the inorganic phase of biological tissue [23]. Current toxicological knowledge about plate-like HAP is relatively limited. Based on these points, aligned plate-like, single-crystal and c-axis orientation HAP rods were synthesized in vitro. And the growth mechanism and biocompatibility are further analyzed and tested, respectively.

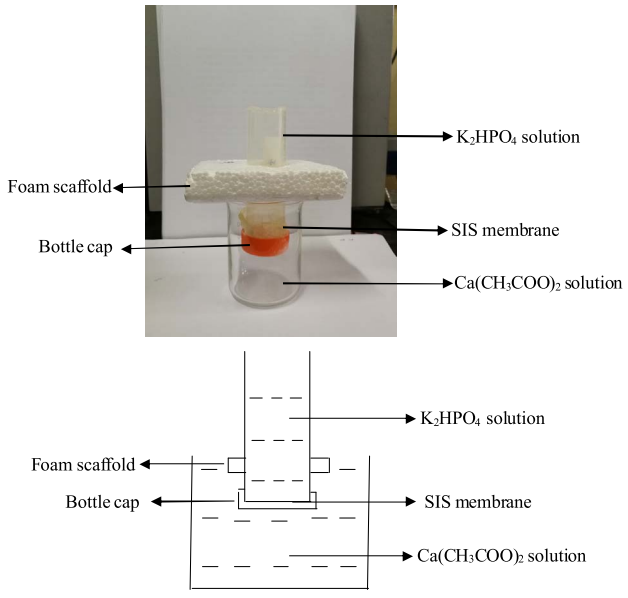
2. Experimental

2.1. Materials

Dipotassium phosphate (K_2HPO_4), (TianJin Chemical Regent Factory, China), and Calcium acetate ($Ca(CH_3COO)_2$), (Xi'an Chemical Regent Factory, China) were used as the raw materials. Ammonium hydroxide ($NH_3{\cdot}H_2O$), (Xi'an YueLai Medical Technology Co. Ltd., China) and Hydrochloric acid (HCl), (Xi'an Chemical Regent Factory, China) were used to adjust PH to 7.4 for reaction system. The chemicals were all analytical grade reagents in the experiment and were used without purification. Distilled water was used as an additional agent during biomimetic mineralization synthesis process and for the aqueous solutions and washing.

2.2. Synthesis procedure

Experiment device is a uniquely designed reaction vessel, as shown in Fig. 1. The important key is that SIS membrane sealed hole located the middle of the bottle cap, and it was ensured the top liquid (30 ml of 0.1 M K_2HPO_4 solution) should not to leak from the side of the SIS membrane. At the bottom, the beaker was filled with 30 ml of 0.1 M $Ca(CH_3COO)_2$. The reaction system simulated bone mineralization conditions (PH 7.4, 37 °C, SIS membrane as biomineralization template), and the reaction process was carried out without sitrring.



(a. Optical images of device, b. Schematic diagram)

Fig. 1. Self-design device.

During the process, the top or lower surface of SIS membrane always contacted the liquid in the beaker. The process last for 10 days and the crystal formed on the lower surface of the membrane was respectively peeled off and dried at 1st, 3rd, 5th, 7th and 10th days.

2.3. Characterization

X-ray powder diffraction (XRD) patterns of the samples were obtained by a Japanese Rigaku D/Max-IIIC diffractmeter at a voltage of 60 kV and a current of 80 mA with Cu Kα radiation (λ=1.546 Å), employing a scanning rate of 2 °min^{-1} in the 2θ ranging from 3.5° to 80°. Scanning electron microscopy (SEM) micrographs and the elements energy spectrum analysis were explored using a JEOL JSM-6700F microscope. IR specta was recorded on a SHIMADZU Pretige-21 FI-TR spectrometer. The measurements were performed on films containing KBr and the samples. Transmission electron microscopy (TEM) micrographs were taken using a JEOL JEM-3010 transmission electron microscope at an accelerating voltage of 300 kV.

2.4. Analysis of the process and mechanism of crystals growth

Analysis of the XRD, SEM and TEM results, the schematic diagram of the process and mechanism of crystals growth were drawn by ChemDraw.

2.5. Biological assessment

2.5.1. Cell culture

Mesenchymal stem cells (MSCs) were cultured at 37 °C in DMEM supplemented with 10% (v/v) FBS in a humidified atmosphere containing 5% CO_2. The medium was refreshed twice per week. Cells were harvested after 7 days. Prior to Cell Counting Kit-8 (CCK8) experiment, the samples for different biomimetic mineralization time were irradiated by short-wavelength UV-light for 30 min, sterilized with 70% ethanol overnight, and washed with sterile PBS three times. The samples were leached (ISO 10993–12:2012, Biological evaluation of medical devices) with DMEM containing 10% fetal bovine serum (FBS) the least three days before CCK8 experiment. the leach liquor was blended every day and stored at 4 °C until analysis.

2.5.2. Cell treatment procedure

The monolayer cells were detached with trypsin ethylenedaminetetraacetic acid (EDTA) to make single cell suspensions. The viable cells were counted with a hemocytocytometer and diluted with medium containing 5% FBS to give final density of 1×10^5 cells/ml. The cells were seeded in 96-well plates (100 μl per well) at plating density of 10,000 cells/well and incubated to allow for cells attachment at appropriate conditions. After 24 h, culture medium was removed and the cells were cultured with 100 μl 100% leach liquor for 24, 48, 72 h, respectively. 100 μl 100% leach liquor with cells served as experiment group. The wells containing 100% leach solution but no cells served as the blank control, and the cells cultured in the culture medium without 100% leach solution served as the positive control. Triplicate wells were maintained for each group all samples, and the plates were incubated in routine cell culture conditions.

2.5.3. CCK8 assay

Relative growth rate (RGR) of cells was checked by CCK8 assay. Samples toxicity level was evaluated according to the relative cell proliferation rate. The samples are not obvious cytotoxicity and in line with the requirements of biological applications if cell toxicity of material is not more than Grade 2 [24]. The culture medium was removed from each well after 24, 48 and 72 h of incubation respectively, then 100 μl fresh culture medium and 10 μl CCK8 solution were added to each well of the plate before CCK8 was checked. The plate was incubated for 2 h in the incubator, and the optical density (OD) at