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Cytotoxicity of stoichiometric hydroxyapatites with different crystallite sizes

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ABSTRACT

Hydroxyapatite (HAp) samples were synthesized by a solution reaction method followed by heat-treatments at three different temperatures. Special attention was given to optimizing the processing parameters to obtain the chemical composition near to the stoichiometry of HAp. No trace of secondary crystalline phase was found from powder X-ray diffraction in all samples. X-ray fluorescence measurements found that the Ca/P ratio was 1.68 ± 0.02, which is close to the stoichiometry of HAp, i.e., 1.67. Electron microscope observations revealed that the grain size was uniform within a sample, which was dependent on the heat treatment temperature. Dissolution rates in acid solution and cytotoxicity of the samples were measured. Tendency to decrease both the dissolution rates and the cytotoxicity with increasing crystallite size was observed. After heat-treatment at 1000 °C, the cytotoxicity of the sample was found to be minimal, which had uniaxial and faceted grains with a mean diameter of 200 nm.

1. Introduction

Hydroxyapatite (HAp, $Ca_5(PO_4)_3OH$) is a main inorganic component of human bones and teeth. HAp is used as a main component or a surface coating of artificial bones and implant materials because of its biological compatibility. HAp in the human body contains various kinds of atomic scale defects, such as Ca vacancies, OH vacancies, excess protons and other trace impurities, which strongly affect its biological properties [1,2]. Among trace impurities, Zn is known to activate osteoblasts and promote bone formation [3,4]. A molar ratio of Ca to P in HAp is known to affect dissolution rates of HAp into acid solutions and the biological properties [5]. Although first principal studies on defects and solute atoms in HAp have been systematically made [6–9], roles of such atomic scale defects are mostly unknown by experiment.

In order to elucidate defect-related mechanisms behind properties of HAp by experiments, it is essential to have a series of model samples of HAp. There are many synthesis methods of HAp: solution reaction method [10–12], solid state reaction method [13,14], hydrothermal method [15,16], sol–gel method [17], etc. Among them, a solution reaction method can synthesize HAp at low temperatures close to a body temperature. This is a great advantage in mimicking biological HAp with low crystallinity involved in tooth dentines and bones that is naturally formed in the human body.

Many works have reported biological properties of synthesized HAp [3–5,18,19]. Although biological and other properties were investigated in some detail, little attention has been paid to the magnitude of the off-stoichiometry of samples synthesized by a solution reaction method. Consequently, the roles of atomic scale defects on the properties have not been clearly revealed.

The present study aims for the synthesis of undoped HAp with a stoichiometric composition (Ca/P = 1.67) and the measurement of biological properties of samples. Heat-treatment is employed to obtain samples with different crystallite sizes. Their dissolution rates into acid solutions and cytotoxicity are comparatively examined. This should provide a good starting point for further studies on more complicated HAp issues by experimental methods.

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2. Experimental procedure

2.1. Sample preparation

HAp samples were synthesized by a solution reaction method using 0.1 mol/L $Ca(NO_3)_2{\cdot}4H_2O$ and 0.1 mol/L $(NH_4)_2HPO_4$ solutions. A series of preliminary experiments found that crystallinity and chemical compositions of products were dependent on many factors, such as mixing ratios of the two starting solutions, solution-reaction temperature, maturation time and solution pH value. Under our optimized conditions, the phosphate solution was poured into the calcium solution and stirred for 5 h at 80 °C under an argon atmosphere to prevent carbonate ions from being mixed into the samples. The solution pH was kept at 10 using 5 mol/L KOH solution. After the maturation, the powders thus obtained were filtered, washed by ultrapure water, and dried at 50 °C for 24 h. Then, some of the samples were heat-treated for 3 h at 500 and 1000 °C.

Phase contents of samples were analyzed by X-ray diffraction (XRD) method using the Rigaku Ultra IV (Rigaku) and Cu-Kα radiation. X-ray fluorescence analysis (XRF) by EDX-800 (Shimadzu) with Rh target was used to examine the chemical composition. Microstructures of the samples were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) using S-800 (Hitachi High-Technologies) and JEM-2010HC (JEOL), respectively. TEM samples were prepared by evaporation of methanol suspension with HAp samples.

2.2. Property characterization

Dissolution rates into acid solution were investigated by immersion of the samples in 0.08 mol/L CH_3COOH–CH_3COONa buffer solution at pH = 5.5. Samples were pelletized and put into 50 mL of the buffer solution at 37 °C for 2, 7 and 14 days. After the immersion, the samples were dried at 50 °C for 24 h. The amount of dissolution was determined by weighing samples before and after the immersion. The dissolution experiments were performed for three times at each condition.

Cytotoxicity of HAp samples was examined in the following way. MDA-MB-231 cells procured from Prof. Hisataka Sabe (Osaka Bioscience Institute) were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37 °C under an atmosphere of 5% CO_2. The cells (1.0×10^4 cells) were incubated for 1 day in 48-well plates. Then, 300 μL of HAp sample dispersions in culture medium (1 mg/mL) was added to the cells. After 72-h incubation, the fraction of surviving cells was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay [20]. Before the measurement of the absorbance at 490 nm using a multiplate reader (Thermo Multiskan spectrum reader, Thermo Scientific), centrifugation (3000 rpm, 20 °C, 60 min) was performed to remove the insoluble part of HAp samples. The experiments were performed three times for each sample.

3. Results and discussion

3.1. Characterization of HAp

Fig. 1a shows XRD patterns of HAp samples after drying at 50 °C and subsequently heat-treated at 500 and 1000 °C. These samples were obtained by the solution reaction method using two starting solutions with the mixing ratio of Ca/P = 2.00. All diffraction peaks can be assigned to those of HAp, implying the absence of secondary phases. The samples showed Ca/P ratios of 1.68 ± 0.02 by XRF composition analyses, which indicate the formation of stoichiometric HAp. Similar synthesis experiments were made by varying the mixing Ca/P ratio of the two starting solutions from 1.50 to 2.50. Fig. 1b shows XRD patterns of HAp samples heat-treated at 1000 °C with three mixing ratios of Ca/P. When the mixing Ca/P ratio is smaller than 1.90, formation of β-tricalcium phosphate (β-TCP) was detected after the heat-treatment at 1000 °C, as clearly seen in Fig. 1b for the case of Ca/P = 1.67. It has been established that β-TCP with Ca/P = 1.50 appears when the overall Ca/P content is smaller than the stoichiometric ratio of HAp, Ca/P = 1.67 [21]. The presence of β-TCP is evident only after the heat-treatment of the corresponding samples at 1000 °C, since the low Ca/P ratio may be accommodated in the defective HAp formed at temperatures lower than 500 °C. The reason for the lower Ca/P ratio in the synthesis product than that in the supplied mixing ratio may be ascribed to residual Ca ions in the aqueous solution during the solution reaction process, which are disposed of after the process. On the other hand, when the mixing Ca/P ratio in the initial solutions is larger than 2.00, formation of CaO was detected after the heat-treatment at 1000 °C as seen in Fig. 1b for the case of Ca/P = 2.50, implying the presence of excessive Ca in the samples. According to these results, it can be said that a ration of Ca/P = 2.00 for the starting solutions is optimum for the preparation of stoichiometric HAp samples.

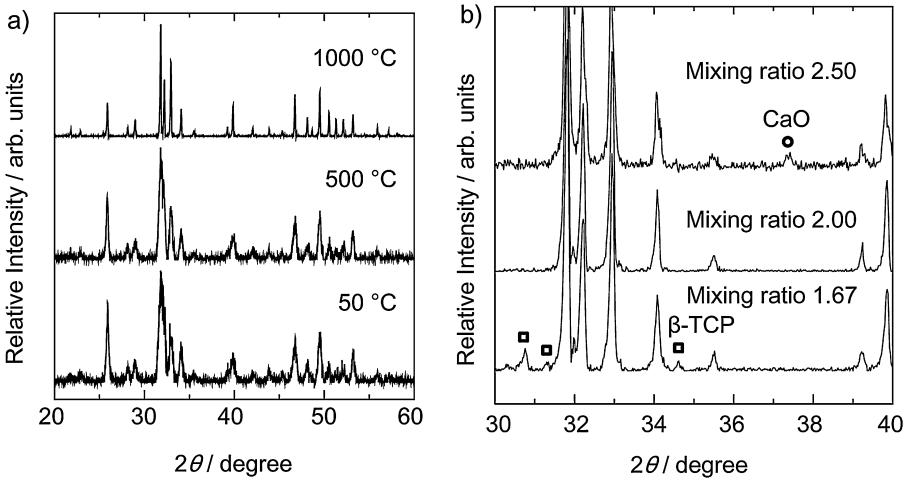

Fig. 1. (a) XRD patterns (Cu-Kα) of stoichiometric HAp samples (supplied mixing ratio of Ca/P = 2.00) treated at three temperatures. All peaks can be assigned to HAp crystal. (b) XRD patterns of HAp samples heat-treated at 1000 °C with several mixing ratio of Ca/P. Additional peaks of β-TCP and CaO are detected on the samples with mixing ratio of Ca/P = 1.67 and 2.00, respectively.