

## Selective protein adsorption property and characterization of nano-crystalline zinc-containing hydroxyapatite

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### Abstract

Nano-crystalline Zn-containing hydroxyapatite (ZnHAp) was prepared by the wet-chemical method and the selective adsorption of essential proteins was examined, taking bovine serum albumin (BSA) and pathogenic protein such as  $\beta_2$ -microglobulin ( $\beta_2$ -MG) as model proteins. Transmission electron microscopy observation and X-ray diffraction analysis indicated that the increase of Zn content led to smaller crystallites and their specific surface area of ZnHAp increased with increasing Zn content, accordingly. Furthermore, the amounts of BSA adsorption on ZnHAp particles decreased with increasing Zn content in spite of the increase in the specific surface area. It is thus concluded that nano-crystalline ZnHAp had a highly selective adsorption property with regard to  $\beta_2$ -MG.

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### 1. Introduction

Hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ; HAp) is the major inorganic component of natural bone and has been used as an orthopedic and dental material [1,2], a column packing material for affinity chromatography to separate various proteins, and in industrial catalysts [3–7]. Recently, much attention has been focused on nano-crystalline HAp as an adsorbent for removing pathogenic proteins from blood for blood purification therapy [8–11], since HAp might have good blood compatibility as well as a superior ability for selective protein adsorption, depending on crystallite size, pore distribution and other structural factors, such as which ions occupy which sites on the HAp lattice. A preliminary study by Takashima et al. [8,9] indicated that carbonate–hydroxyapatite had good

blood compatibility and showed selective adsorption of pathogenic proteins, such as  $\beta_2$ -microglobulin ( $\beta_2$ -MG). Furthermore, Hayakawa et al. [10] and Takemoto et al. [11] pointed out that the carbonate ions substituted at either P-sites or OH-sites were enhanced and that smaller crystallite size and a higher carbonate ion content were responsible for better selectivity for  $\beta_2$ -MG adsorption. Therefore, it is expected that nano-crystalline HAp should very effectively remove  $\beta_2$ -MG from blood.

Thus, substituting some other ions for the HAp component ions,  $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-}$  and  $\text{OH}^-$ , should result in significant effects for the selective adsorption of physiologically active components in blood. In examining such effects, the size of substituting ions is an important factor. Among the cations known as substituents in the HAp lattice,  $\text{Zn}^{2+}$  was chosen in the present study because it is one of the essential trace elements in human bone and plasma [12,13]. Moreover, Ito et al. [14,15] and Miyaji et al. [16] reported that  $\text{Zn}^{2+}$  was able to substitute the  $\text{Ca}^{2+}$  site in the HAp lattice under

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some specific experimental conditions [17,18]. Therefore, nano-crystalline Zn-containing apatitic calcium phosphates (ZnHAp) were targeted and prepared through the wet-chemical process. The selective adsorption of  $\beta_2$ -MG was examined by an in vitro experiment similar to that of Takemoto et al. [11], where the ZnHAp prepared were soaked in mixed-protein saline solutions containing bovine serum albumin (BSA) and  $\beta_2$ -MG as typical examples of the essential proteins and pathogenic proteins in blood plasma, respectively. The ZnHAp particles were characterized by X-ray diffraction (XRD), Brunauer–Emmett–Teller specific surface area measurement (SSA), transmission electron microscopy (TEM), zeta potential measurement, and solid-state  $^{31}\text{P}$  magic angle spinning-nuclear magnetic resonance spectroscopy (MAS-NMR). The relation between the characterization of parameters such as the structure, shape and surface charge of ZnHAp, and the selective protein adsorption property was discussed in detail.

## 2. Materials and methods

HAp with a stoichiometric molar ratio of Ca/P (= 5/3) was prepared by a wet-chemical reaction between calcium nitrate tetrahydrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , Nacalai Tesque, Japan) and diammonium hydrogenphosphate ( $(\text{NH}_4)_2\text{HPO}_4$ , Nacalai Tesque, Japan) at pH 10 adjusted by the addition of  $\text{NH}_4\text{OH}$ . Zinc nitrate hexahydrate ( $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , Nacalai Tesque, Japan) was dissolved in the calcium nitrate solution so that the  $\text{Zn}^{2+}$  could be incorporated into the HAp lattice structure in the range from 0 to 6 mass%. The diammonium hydrogenphosphate solution was added dropwise to the Zn-containing calcium nitrate solution at 60 °C under stirring, and the solution was stirred for 24 h. Then, the calcium phosphate was separated from the suspensions by centrifuging, it was washed with distilled water, and then dried at 100 °C for 24 h. The Zn-containing calcium phosphate powder was denoted as  $n\text{ZnHAp}$  ( $n = 1\text{--}6$  mass%), depending on the nominal Zn content in the HAp.

The crystalline phases were identified by a powder X-ray diffractometer (XRD) (RINT2500, RIGAKU, Japan) operated at 40 kV and 200 mA. The  $\text{N}_2$  adsorption isotherms for dried calcium phosphate powder were obtained by multi-point  $\text{N}_2$  gas adsorption experiments (GEM-INI2370 Micromeritics, Shimadzu, Japan). The specific surface area (SSA) and the pore size distribution in a range of 2–30 nm were derived by the BET method and the Barrett–Joyner–Halenda (BJH) method, respectively. The zeta potential at pH 7.4 of the sample powder was measured by a ZETASIZER (ZETASIZER 3000HS<sub>A</sub>, Malvern, UK). The morphology of the sample particles was observed using TEM (JEM-2010, JEOL, Japan). The sample powder was chemically analyzed by inductively coupled plasma (ICP) emission spectroscopy (ICPS-7500, Shimadzu, Japan). The local chemical structure around the phosphorus atoms was examined by solid-state  $^{31}\text{P}$  magic angle spinning-nuclear magnetic resonance (MAS-NMR) spectroscopy.

$^{31}\text{P}$  MAS-NMR spectra were recorded at 7.05 T (tesla) on a Varian <sup>UNITY</sup>INOVA300 Fourier transform (FT)-NMR spectrometer, equipped with a CP/MAS probe. The samples were placed in a silicon-nitride sample tube 7 mm in diameter. The sample spinning speed at the magic angle to the external magnetic field was 6 kHz.  $^{31}\text{P}$  MAS-NMR spectra were taken at 121.4 MHz with 3.5  $\mu\text{s}$  pulse length (pulse angle,  $\pi/4$ ), 40 s recycle delays, where the signals from about 40 pulses were accumulated.  $^{31}\text{P}$  NMR chemical shifts are reported in ppm, with 85%  $\text{H}_3\text{PO}_4$  as the external reference (0 ppm). For comparison,  $^{31}\text{P}$  MAS-NMR spectra of tricalcium phosphate,  $\beta\text{-Ca}_3(\text{PO}_4)_2$  ( $\beta$ -TCP, Nacalai Tesque, Japan), monetite,  $\text{CaHPO}_4$  (DCPA, Aldrich, USA) and brushite,  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  (DCPD, Nacalai Tesque, Japan) were also measured.

The protein adsorption experiments were conducted after Takemoto et al.'s method [11]. Both BSA and  $\beta_2$ -MG were dissolved in saline to prepare mixed-protein solutions so as to involve BSA and  $\beta_2$ -MG in concentrations of 35 mg/mL and 15  $\mu\text{g}/\text{mL}$ , respectively. The pH of the mixed-protein solutions was adjusted to pH 7.4 with a Tris-buffer solution. Each calcium phosphate powder (0.1 g) was soaked in 1.0 mL of the mixed-protein solution and left in contact for 6 h. The optical absorptions of the filtrates were measured on the basis of  $\approx 420$  nm for  $\beta_2$ -MG and  $\approx 630$  nm for BSA. The fractions of BSA and  $\beta_2$ -MG adsorption in the filtrates were derived from the following equation:

$$C_{6\text{h}}/C_{0\text{h}} = A_{6\text{h}}/A_{0\text{h}},$$

where  $C$  and  $A$  stand for the concentration of the proteins and the absorbance of the peaks around 420 and 630 nm, respectively.

## 3. Results

Fig. 1 shows the XRD patterns of HAp and 1–6ZnHAp as dried at 100 °C for 24 h. All peaks were assigned to hydroxyapatite (JCPDS: 9-432). No secondary phases, such as calcium hydroxide and zinc phosphates, were observed, even by detailed scans. Note that the XRD peaks of ZnHAp became broader with increasing Zn content.

Table 1 indicates the atomic ratios Ca/P and (Ca + Zn)/P of the ZnHAp powder derived from ICP emission spectroscopy, or the stoichiometry of the synthesized HAp. It shows the calcium site deficiency of the HAp lattice. The Zn content was almost the same as the nominal content of zinc nitrate hexahydrate. Therefore, the added  $\text{Zn}^{2+}$  ions were all involved in the formation of the HAp lattice. So, the Ca/P atomic ratio naturally decreased with the increase in the Zn content, but the ratio (Ca + Zn)/P was smaller than 1.67, the theoretical or designed ratio of HAp. This indicates that the samples were all deficient in cations, Ca and Zn.

The  $^{31}\text{P}$  MAS-NMR spectra in Fig. 2 demonstrate the local chemical structure around P atoms in the HAp and 6ZnHAp powders. Other ZnHAp powders gave

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