Journal of Asian Ceramic Societies xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Asian Ceramic Societies

journal homepage: www.elsevier.com/locate/jascer

Full Length Article

Effects of pore distribution of hydroxyapatite particles on their protein adsorption behavior

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ARTICLE INFO

Article history:
Received 28 October 2016
Received in revised form 16 January 2017
Accepted 29 January 2017
Available online xxx

Keywords:
Hydroxyapatite
Protein adsorption
Nanostructure
Surface structure

ABSTRACT

The relationship between protein adsorption on hydroxyapatite (HAp) particles and their surface structure was investigated. Because the various crystal planes of HAp have been reported to exhibit selective adsorption, numerous studies have focused on developing methods to control HAp morphology for selective adsorption. However, few studies have examined the systematic adsorption of proteins on the HAp particles. We firstly synthesized HAp particles under various aging times and mild reaction conditions intending to obtain HAp particles having various surface structures despite similar morphology, chemical composition, and crystallinity. The aging time affected the pore size distribution of the HAp particles. A peak indicating pores with a diameter of approximately 2.5 nm was observed in the pore size distribution plots of the HAp particles prepared using aging times of 48 h or less. The adsorption of proteins on HAp particles with different surface structures was studied. The bovine serum albumin (BSA) adsorption behavior was influenced by the presence of pores on the HAp surface. The amount of BSA adsorbed on the HAp particles aged 72 h having no pores was nearly 1.5 times that of the other HAp particles having pores. These results indicated that the pore size distribution of HAp particles is one of the most important factors in controlling their protein adsorption behavior.

1. Introduction

The relationship between protein molecules and inorganic materials has attracted extensive interest in many fields, including biomaterials [1,2], biomineralization [3], biosensors [4], and biochemistry [5]. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) is the most well-known crystalline phase of calcium phosphate salts. Because HAp is both biocompatible and osteoconductive, it has been widely used in various applications such as orthopedic and dental materials and packing column materials for affinity chromatography for the separation of various proteins [6–8].

HAp has been reported to exhibit selective adsorption on different crystal planes. HAp crystallizes in the $P6_3/m$ space group has unit-cell parameters of $a = b = 0.943$ nm and $c = 0.688$ nm. The HAp particles possess two different charge planes, C-sites and P-sites, on their surface. They also possess binding sites capable of adsorbing different proteins [9,10]. The calcium atoms in HAp include two screw-axis calcium ions (Ca^{2+}) at the particle surface. The C-sites are arranged on the *a*-plane of particles. Therefore, the *a*-plane is rich in Ca^{2+} ions, whose positive charge facilitates adsorption of the acidic groups of proteins. The P-sites are composed of oxygen ions of PO_4^{3-} group. The P-sites, which are negatively charged, are arranged hexagonally on the *c*-plane. Therefore, positively charged proteins tend to adsorb on the *c*-plane [11,12].

Improvements in the control of the crystal and morphological properties of synthesized HAp crystals are important for their utility as general adsorbents, affinity chromatographic solid supports, drug delivery systems, and protein carriers [13–15]. Therefore, numerous researchers have developed methods to control the HAp morphology to influence its protein adsorption behavior.

Yoshimura et al. have reported the preparation of HAp whiskers with a sharp-faced hexagonal morphology by hydrothermal treatment of HAp with nitric acid, urea, and cetyltrimethylammonium bromide (CTAB). They have also reported the syntheses of HAp particles with plate-, hexagonal-, prism-, and needle-like morphologies by hydrothermal synthesis [16,17]. Aizawa et al. have reported the syntheses of calcium-deficient apatite fibers by a homogeneous precipitation method. The fibers had long axes of 60–100 μm, and elongated along the *c*-axis of the crystal structure with a wide *a*-plane [18,19]. Nagata et al. have reported the synthe-

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http://dx.doi.org/10.1016/j.jascer.2017.01.005

sis of HAp plate-like particles with a wide *a*-plane by hydrothermal treatment with alcohol or ethylamine [20,21]. Sadat-Shojai et al. systematically correlated various parameters (e.g., pH, temperature, and time) related to the hydrothermal treatments with the shape of HAp nanoparticles using a Taguchi experimental design approach [22]. Han et al. have reported a method for controlling the HAp nanoparticle morphology under hydrothermal treatment in the presence of urea and gelatin. They synthesized various HAp morphologies by altering the phase transition route by varying the initial pH and hydrothermal treatment time [23]. Kawachi et al. have reported the selective BSA adsorption on needle-shaped hydroxyapatite under appropriate concentration of phosphate in the solution [24].

Many researchers have demonstrated the ability to control HAp morphology, although only a few studies have examined the ability of HAp to selectively adsorb proteins. Numerous researchers have, however, reported studies detailing the relationship between protein adsorption behavior and HAp properties such as carbonate content, crystallinity, etc.

Kandori et al. have reported the effects of HAp chemical composition on the adsorption of bovine serum albumin (BSA) by synthesized carbonate calcium hydroxyapatites [25]. Nagata et al. have reported that HAp with high crystallinity is strongly affected by its specific binding sites [26]. Fuji et al. and Dasgupta et al. have reported on the highly selective protein adsorption properties of zinc-containing hydroxyapatite crystals [27,28].

As previously described, the protein adsorption behavior of HAp has been investigated using various types of HAp particles. However, it is also known that HAp particles synthesized with different methods show different protein adsorption behaviors, even if the HAp particles have similar morphologies, chemical compositions, and crystallinities. Therefore, we have focused on surface structure of HAp particles which is the interface structure that contacts proteins. Few studies have investigated the surface structure of HAp particle from the view point of protein adsorption on HAp particles. In this study, two different sized proteins, BSA (molecular weight 66,000) and lysozyme (LSZ, molecular weight 15,000), were used to examine their effects on the HAp surface structure. We firstly synthesized HAp samples with different surface structures despite their similar morphologies, chemical compositions, and crystallinities. Our aim is to evaluate the relationship between the HAp particle surface structure and the proteins adsorbed on the HAp. We prepared HAp under mild conditions to prevent changes in its morphology, chemical composition, and crystallinity. In this paper, we report the synthesis of HAp with a controlled surface structure and discuss in detail the relationship between the HAp particle surface structure and proteins that adsorb on the HAp.

2. Material and methods

2.1. Materials

Calcium acetate solution [$(CH_3COOH)_2Ca{\cdot}H_2O$] (99.0%), diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] (99.0%), and phosphate buffer (11 mM, pH 7.4) were procured from Wako Pure Chemical Industries, Japan. BSA [isoelectric point (pI) = 4.7, molecular weight 66,000] and LSZ (pI = 11.1, molecular weight 15,000) were procured from Sigma-Aldrich. All materials were of analytical grade and were used without further purification.

2.2. Preparation of HAp

HAp was prepared from a mixture of a 50 mM solution of $(CH_3COOH)_2Ca{\cdot}H_2O$ and a 30 mM solution of $(NH_4)_2HPO_4$. Two-hundred milliliters of the 30 mM $(NH_4)_2HPO_4$ solution was added to 200 mL of the 50 mM $(CH_3COOH)_2Ca{\cdot}H_2O$ solution. The resulting mixture was subsequently stirred for 10 min at 20 °C. The temperature of the mixture was increased at 1 °C/min using a cool stirrer from NISSIN (SWC-9000, series D). The mixture was further stirred at 60 °C for 3, 5, 24, 48, and 72 h. The time for which the mixture was maintained at 60 °C is hereafter referred to as the aging time. The products of various aging times were recovered by filtration, washed repeatedly with distilled water, and freeze–dried.

In order to compare the crystallinity of the products with highly crystalline HAp in the XRD analysis, highly crystalline HAp was prepared using a hydrothermal method at 180 °C for 3 h.

2.3. Characterization of synthesized HAp

The morphology of the samples was studied by field-emission scanning electron microscopy (FE-SEM) using a Hitachi S-4300 electron microscope (Japan) operated at 20 kV.

Fourier-transform infrared spectroscopy (FT-IR) was performed on a JASCO MTF-2000 (Japan). The synthesized products (approximately 2 mg) were thoroughly ground with potassium bromide powder (approximately 150 mg, IR-grade KBr, Wako) using an agate mortar and pestle to give a fine mixture that was subsequently subjected to IR analysis. Finely ground KBr powder was used for the background spectrum.

The products were characterized by powder X-ray diffraction (XRD) using a Rigaku RINT2000/PC equipped with a Cu–Kα radiation source operated at 30 mA and 40 kV. Data were collected in the 2θ range from 3.0 to 60.0° at a scan speed of 2.000°/min.

The values of the ζ-potential of the samples were measured using an ELS-Z (Otsuka Electronics Co., Japan).

All the samples were characterized by nitrogen gas adsorption/desorption isotherms using a Shimadzu TriStar3000 system (Japan). The pore volume of the samples was calculated using the BJH method.

2.4. Protein adsorption on HAp

The protein adsorption behavior was studied using BSA and LSZ. Five milligrams of HAp particles were mixed in 1 mL of a 0.5 mg/mL protein solution dissolved in a 11 mM phosphate buffer solution. The mixture was stirred overnight at 4 °C. The mixture was then centrifuged at 12,000 rpm for 12 min. The amount of unadsorbed protein in the supernatant was determined using the Bradforf protein assay. The amount of protein adsorbed on the HAp was calculated on the basis of the amount of proteins in the supernatant.

The equation used to calculate the protein adsorption per unit surface area is

$$q = \frac{(C_p - C_s)V}{a_{HA}M}$$

where q ($g\,m^{-2}$) is the amount of protein adsorbed per unit surface area, C_p ($mg\,mL^{-1}$) is the concentration of the initial protein solution, C_s ($mg\,mL^{-1}$) is the concentration of protein in the supernatant, V (mL) is the volume of the protein solution, a_{HA} ($m^2\,g^{-1}$) is the specific surface area of the sample, and M (mg) is the mass of the sample used in the adsorption experiment.

3. Results and discussion

3.1. Properties of HAp particles

Fig. 1 shows the XRD patterns of the products synthesized using different aging times. As a reference pattern of highly crystalline HAp, the XRD pattern of the product synthesized hydrothermally is shown in [Fig. 1(a)]. The peaks associated with HAp (JCPDS: 90432) were observed in the pattern for the product synthesized

Please cite this article in press as: T. Nagasaki, et al., Effects of pore distribution of hydroxyapatite particles on their protein adsorption behavior, J. Asian Ceram. Soc. (2017), http://dx.doi.org/10.1016/j.jascer.2017.01.005