ADSORPTION BEHAVIOR OF PROTEINS ON Mg(II)-DOPED CALCIUM HYDROXYAPATITE PARTICLES AT VARIOUS Mg(II) CONTENTS

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Abstract: In this study, fundamental experiments on the adsorption behavior of proteins on synthesized magnesium-doped calcium hydroxyapatite particles (MgHap) at various Mg/(Ca + Mg) molar ratios (abbreviated as X_{Mg} : 0–0.3) were examined. Rod-like calcium hydroxyapatite particles, with a width of 24 nm and length of 180 nm, were obtained in the absence of Mg^{2+} ($X_{\text{Mg}} = 0$). With the increase in X_{Mg} up to 0.10, the width and length of MgHap particles decreased to ~10 and 50 nm, respectively. Agglomerates of small thin particles were obtained at $X_{\text{Mg}} \geq 0.15$. All of the synthesized particles exhibited single phase of calcium hydroxyapatite particles (CaHap). The X_{Mg} values for the particles precipitated at $X_{\text{Mg}} \geq 0.04$ were less than those of the corresponding solutions. Hence, almost all of the MgHap particles are deficient in magnesium. We propose that to compensate for the cation deficiency, PO_4^{3-} is incorporated as HPO_4^{2-} and H_2PO_4^- and thus decreases the particle negative charge. In addition, all of the adsorption isotherms of typical acidic and basic proteins such as bovine serum albumin (BSA) and lysozyme (LSZ) were compared. The isotherms obtained from a 1×10^{-4} mol/dm³ KCl solution were of the Langmuirian type. The saturated amount of adsorbed BSA (n_s^{BSA}) strongly depended on the particle length rather than on the X_{Mg} value. At a particle length of greater than 150 nm, n_s^{BSA} values abruptly increased. This result strongly suggests that the effects of the C sites produced on the *ac* and *bc* faces are advantageous for BSA adsorption. In other words, the adsorption of BSA on MgHap particles was considerably dependent on the length at a critical value of greater than 150 nm. However, the saturated amount of adsorbed LSZ (n_s^{LSZ}) exhibited no remarkable variation with the particle length.

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INTRODUCTION

Calcium hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ or CaHap) particles are the primary constituents of hard tissues in animal organisms. Synthetic CaHap is used in bone implant surgery¹. In addition, CaHap, with a space group of P6₃/m and unit-cell parameters $a = b = 0.943$ nm and $c = 0.688$ nm, exhibits two binding sites, viz. C and P sites, on the *ac* or *bc* and *ab* particle surfaces, respectively. Thus, CaHap exhibits multiple-site binding characteristics for proteins^{2–4}. Hence, CaHap is widely used as a column in high-performance liquid chromatography (HPLC) apparatuses with applications for the separation of various proteins^{2,5,6}.

Meanwhile, the CaHap structure is extremely tolerant to ionic substitution; several studies have reported the occupation of the Ca sites by various divalent (Ca(II), Mn(II), Mg(II), Sr(II), Cd(II), Pb(II), and Ba(II))^{7–14}, as well as trivalent (Al(III), Fe(III)) [15] and tetravalent (Ti(IV))^{16,17} cations. Not only

cations but also anionic PO_4^{3-} can be exchanged with anions such as CO_3^{2-} , SO_3^{2-} , AsO_4^{3-} , and VO_4^{3-} . In addition, OH^- can be substituted with F^- and Cl^- ¹⁸. Typically, such modification of the CaHap structure is carried out by the incorporation of certain chemical dopants via coprecipitation¹⁵. Current studies have focused on the search for dopants that would not cause a disadvantageous effect of lowering the biocompatibility of CaHap, but instead would improve the biological and chemical characteristics of these compounds. Manganese (Mn), among others, is an additive proposed for this purpose. The presence of Mn ions in the CaHap structure leads to changes in the biological and chemical properties of CaHap materials^{18,19}. Previously, the adsorption behavior of BSA and LSZ on Mn(II)-doped CaHap (MnHap) with plate- and rod-like particle morphologies has been investigated by our group²⁰. Our results revealed that the saturated amounts of adsorbed BSA (n_s^{BSA}) increase with the Mn/(Ca + Mn) atomic ratio (X_{Mn}) of plate-like MnHap, while the saturated amounts of

adsorbed LSZ (n_s^{LSZ}) decrease. This result is explained by the fact that a large fraction of positively charged adsorption sites produced on the *ac* and *bc* faces (C sites) of plate-shaped particles is advantageous for the adsorption of negatively charged BSA.

The effect of magnesium (Mg) on bone growth is of interest for biological studies. Magnesium deficiency can delay osteogenesis by lowering the osteoblast activity, possibly leading to bone deformation and growth inhibition, e.g., osteoporosis²¹. Mg(II)-doped CaHap (MgHap) particles have characteristic of bioapatite in tooth and bone tissues²². In addition, these characteristic effects resulting from trace Mg^{2+} are strongly correlated with the adsorption behavior of proteins on CaHap surfaces. Hence, it is crucial to disclose the adsorption properties of proteins on MgHap particles with various Mg/(Ca + Mg) atomic ratios (X_{Mg}), in addition to the different sizes obtained as a result of coprecipitation, through a series of fundamental investigations for examining the protein adsorption behavior. One of our researchers, Yasukawa, has produced MgHap particles by the addition of an H_3PO_4 solution into a mixed solution of $Mg(NO_3)_2$ and $Ca(OH)_2$ without pH control and reported the surface structure and properties of these MgHap particles¹⁰. However, she has not investigated on the adsorption studies of proteins. Hence, the aim of this study is to clarify the fundamental adsorption behavior of typical acidic and basic proteins on sufficiently characterized MgHap particles with various sizes and X_{Mg} atomic ratios, which were produced by the new coprecipitation method with pH control. The results obtained herein may not only provide fundamental results for the protein–MgHap interaction but also aid investigations of the effects of trace Mg^{2+} in biological fields.

EXPERIMENTAL

Materials and Methods

MgHap particles were synthesized by coprecipitation, which was similar to that reported previously for producing rod-like MnHap particles²⁰. First, $Ca(NO_3)_2$ (0.32–0.40 mol) and $Mg(NO_3)_2$ (0–0.08 mol) were dissolved in 20 dm³ of pure water (free from CO₂) at X_{Mg} values between 0 and 0.3. The total amounts of both Ca and Mg in the solutions were maintained at 0.4 mol. Second, H_3PO_4 (0.24 mol) was added to the solution, and the solution pH was adjusted to 9.5 by the addition of a 15 M NH_4OH solution. Third, the resulting suspension was aged in a Teflon-capped vessel at 100°C for 48 h. The generated particles were filtered, thoroughly washed with 10 dm³ of pure water, and finally dried at 60°C in an air oven for 24 h. All of the reagent-grade

chemicals supplied by Wako Chemical Co. were used without further purification. Pure water from Elix (Millipore) was used to prepare all solutions. Freshly purified water exhibited a conductivity of ~0.06 $\mu S/cm$.

Characterization

Transmission electron microscopy (TEM; JEOL JEM-2100), N₂ and H₂O adsorption, and X-ray diffraction (XRD; Rigaku Miniflex 600) measurements were carried out to determine the shape, specific surface area (SSA), and crystal phase of the obtained compounds. N₂ adsorption isotherms were obtained at liquid-N₂ temperatures by the ordinary automatic volumetric flow method using a Baratron diaphragm-type manometer with an attached mass flow meter that was constructed in-house. The degree of confidence for the SSA measured by this apparatus was within 2%. In addition, adsorption isotherms of H₂O at 25°C were determined using a gravimetric apparatus constructed in-house. Prior to these gas adsorption measurements, the samples were evacuated at 100°C for 2 h. SSAs were obtained by fitting the Brunauer-Emmett-Teller (BET) equation to these N₂ and H₂O adsorption isotherms, which were abbreviated as S_N and S_W , respectively. XRD patterns were recorded using Ni-filtered CuK α radiation (40 kV, 120 mA). The concentrations of Ca, Mg, and P in CaHap and MgHap were estimated by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Hitachi Hightech SPS3520UV-S) within a precision of 2% after the samples were dissolved in the HCl solution.

Protein Adsorption Measurements

The adsorbed amounts of BSA and LSZ molecules on the particles were measured by the batch method used in our previous studies^{23–31}, which were purchased from Sigma Co. (BSA: A-7030 and LSZ: L-6876). To prevent protein decomposition, this measurement was conducted at 15°C using a 1×10^{-4} mol/dm³ KCl solution of the protein containing 100 mg of MgHap in 10 cm³ Nalgene polypropylene centrifuge tubes. The centrifuge tubes were gently rotated end-over-end at 15°C for 48 h in a thermostat. The concentrations of each protein in the supernatant after centrifugation during rotation were determined by HPLC with UV monitoring at 220 nm using a YMC-Pack C₄-AP column. Precise HPLC measurements have been reported previously²⁰. A majority of the HPLC experiments were carried out in triplicate. The experiments were reproducible within 2%, indicative of an uncertainty of 2×10^{-2} mg/m² for the adsorbed amounts of protein. In addition, the zeta potential (ζp) of the protein-adsorbed particles was estimated using an electrophoresis apparatus (PEN KEM Model 501) within an accuracy of 2%. The diameter of the plotted

circles in adsorption isotherms presented in this study also expressed each standard deviation.

RESULTS AND DISCUSSION

Properties of the particles

Figure 1 shows the typical TEM images of the produced MgHap. Rod-like CaHap ($X_{Mg} = 0$) particles with a width of 24 nm and length of 180 nm were observed. The particle size was determined by counting greater than 300 particles in numerous TEM images that were captured for each system by using WinRoof 2015 software. With the increase in X_{Mg} up to 0.10, the particle width and length decreased to ca. 10 and 50 nm, respectively. As the particles produced at $X_{Mg} \geq 0.15$ were agglomerates of small thin particles, their particle sizes were not determined. Table 1 summarizes the sizes of the obtained particles. Figure 2 shows the XRD patterns of the synthesized particles: All of the particles exhibited single phase of CaHap (JCPDS 9-432), indicating that the incorporated Mg^{2+} does not inhibit the CaHap crystal structure due to its rod-like morphology; however, the degree of crystallinity decreased. Similar results have been reported previously³²⁻³⁴. Mg^{2+} inhibits the formation of CaHap, creating amorphous CaHap crystals and increasing their solubility. Table 1 also

summarizes the SSA values estimated from adsorption measurements of N_2 and H_2O molecules, abbreviated as S_N and S_w , respectively, in addition to the S_w/S_N ratio. With the increase in the X_{Mg} values of the solution, the S_N and S_w values increased from 76 and 48 to 296 and 317 m^2/g , respectively. This result corresponds to the decrease in their particle size (Fig. 1). The obtained results were similar to those reported by Yasukawa et al.¹⁰. Figure 3 shows the lattice constants of the a - and c -axes for the MgHap particles estimated from the XRD patterns. The a - and c -axis lengths were almost constant (~ 0.9420 and 0.6880 nm, respectively) in the entire range of X_{Mg} , as has been reported previously for single-crystal CaHap⁸. However, Yasukawa et al. reported that a - and c -axis lengths decreased with the increase in X_{Mg} , i.e., the incorporation of Mg^{2+} (0.072 nm), rather less than that for Ca^{2+} (0.100 nm)³⁵, into Ca(I) sites may lead to column disorder, leading to the destruction of the Hap structure¹⁰. This difference between the results reported by Yasukawa and those reported here is related to the difference in the solution pH in coprecipitation. As the solution pH was adjusted to 9.5 herein, the particle growth of MgHap in this study proceeded smoothly.

FIGURE 1 TEM images of the particles produced at various X_{Mg} values.

Table 1 Properties of CaHap and MgHap particles produced at various X_{Mg} values.

$X_{Mg}^a)$	Particle size[nm] Width × Length	S_N [m ² /g]	S_w [m ² /g]	S_w/S_N	$X_{Mg}^b)$ in particle	(Ca+Mg)/P ^{b)}
0.00	24 × 180	76	48	0.63	0.00	1.59
0.01	25 × 155	79	55	0.70	0.01	1.55
0.02	21 × 149	87	57	0.65	0.02	1.60
0.04	19 × 134	114	79	0.70	0.03	1.62
0.06	12 × 97	137	91	0.66	0.05	1.60
0.08	13 × 50	157	122	0.78	0.06	1.54
0.10	12 × 53	189	163	0.86	0.07	1.60
0.15	-	191	228	1.18	0.12	1.45
0.20	-	222	276	1.24	0.16	1.40
0.30	-	296	317	1.07	0.21	1.47

a) Atomic ratio of Mg/(Ca+Mg) in solution.

b) Assayed by ICP-AES measurement.

Table 1 summarizes the X_{Mg} values and (Ca + Mn)/P ratios of the synthesized particles estimated by ICP-AES. The X_{Mg} values produced from the assays of the particles precipitated at $X_{Mn} \leq 0.02$ were in fair agreement with those obtained for the solution although the X_{Mg} values in particles were less than those of the corresponding solutions, indicative of the difficulty associated with the incorporation of Mg^{2+} into the CaHap crystal structure. This fact corresponds well with the XRD results. Bigi et al. have reported that the total concentration of Mg^{2+} in the solid phase is significantly greater than the amount of Mg^{2+} incorporated into the MgHap structure³⁶. They concluded that the excess Mg^{2+} is located in the amorphous phase and/or on the crystalline surface. MgHap particles were synthesized by the addition of $Ca(NO_3)_2$ and $Mg(NO_3)_2$ solutions to the $NH_4H_2PO_4$ solution at neutral pH without aging, which could produce a high content of the amorphous phase in the precipitates.

Figure 4 shows the concentrations of calcium, magnesium, and phosphorus (i.e., [Ca], [Mg], and [P], respectively) in the obtained MgHap particles. These values determined from the ICP-AES measurements were plotted as a function of X_{Mg} in the solution. [Ca] and [Mg] linearly decreased (with a slope of -12.5) and increased (with a slope of +6.0), respectively, with an X_{Mg} value of up to $X_{Mg} = 0.2$. However, [P] was almost constant. This result reveals that only 50% of Mg^{2+} is exchanged with Ca^{2+} and that particles exhibit cation deficiency. This fact is further confirmed by the (Ca + Mg)/P values summarized in Table 1. All of these values were less than the theoretical value of 1.67. To compensate for the

cation deficiency, PO_4^{3-} is probably incorporated as HPO_4^{2-} and $H_2PO_4^{2-}$ to reduce the particle negative charge. In addition, the presence of HPO_4^{2-} was identified by TG-DTA measurements (data not shown). The weight losses in TG and endothermic peaks, corresponding to the elimination of H_2O molecules from the dehydration of HPO_4^{2-} , were observed at 700–900°C.

FIGURE 2 XRD patterns of the particles produced at various X_{Mg} values.

FIGURE 3 Changes in the lattice constants of a - and c -axes of the particles produced at various X_{Mg} values.

FIGURE 4 Changes in $[Ca]$, $[Mg]$ and $[P]$ in the particles produced as a function of X_{Mg} values. The black dot straight line reveals the $[Mg]$ line when $Mg(II)$ is completely exchanged with $Ca(II)$.

Adsorption Behavior of Proteins on MgHap Particles

Figure 5 shows the adsorption isotherms of BSA on MgHap particles, in addition to their corresponding z_p values. Here the MgHap particles with $X_{Mg} \leq 0.2$ were employed because MgHap particles produced at $X_{Mg} = 0.3$ were considerably amorphous. All of the adsorption isotherms for BSA obtained from the 1×10^{-4} mol/dm³ KCl solution were of the Langmuirian type, as well as the previously reported adsorption systems for CaHap and MnHap particles^{20,23–31}. The saturated amount of adsorbed BSA (n_s^{BSA}) on CaHap ($X_{Mg} = 0$) was 1.50 mg/m². The adsorption coverage of BSA (θ_{BSA}) in this system, which is defined as the ratio of the experimental n_s^{BSA} to its theoretical value, was 0.60. The theoretical value used in the aforementioned calculation was estimated to be 2.52 mg/m² by

assuming the side-on adsorption of globular BSA molecules, which were prolate ellipsoids with dimensions of 14×4 nm²³⁷. As the solution pH of the system was ~ 6 , the BSA molecules were negatively charged. Hence, the negative values obtained for the z_p of this system increase with the amount of adsorbed BSA. However, a relationship between the saturated positive z_p value and X_{Mg} values, except for $X_{Mg} = 0.10$ and 0.20 , which exhibited higher negative z_p values, was not observed. Nevertheless, currently, there is no explanation for this result, and a detailed understanding of this result needs additional investigation.

Figure 6 shows the data obtained for the adsorption of LSZ. Langmuirian-type isotherms, as well as the BSA system in Fig. 5, can be recognized. The saturated amount of adsorbed LSZ (n_s^{LSZ}) slightly decreased with the increase in X_{Mg} , but we did not observe a large difference for the MgHap particles with $0.02 \leq X_{Mg} \leq 0.20$. All of these systems exhibited n_s^{LSZ} values of 0.08–0.12 mg/m². As LSZ is positively charged, LSZ molecules are preferentially adsorbed on negatively charged **P** sites exposed on the ab particle surface. A relationship was not observed between n_s^{LSZ} and X_{Mg} values because the degree of reduction of the particle width was less than the particle length. A low fraction of **P** sites intrinsic to the rod-like particles was observed. Hence, the effect of **C** sites on the LSZ adsorption is regarded to be negligible. We observed positive z_p values for these systems as LSZ molecules were positively charged under the experimental conditions. The saturated positive z_p value for the CaHap ($X_{Mg} = 0$) system was the highest (+10 mV), while the other systems exhibited considerably lower, but almost equal, values (+4 to +6 mV). This decrease in the absolute value of the positive z_p of the systems for $X_{Mn} = 0.01$ – 0.20 is related to the shift in the slipping plane caused by the adsorption of the LSZ molecules³⁸.

Several studies have previously reported the driving forces for protein adsorption, including coulombic electrostatic interactions, van der Waals interactions (depending on the particle size), hydrophobic interactions, and structural rearrangement on adsorption (especially in the case of BSA)^{39–50}. As the difference in terms of the S_w/S_N ratio was not observed for the surface hydrophobicity of the particles (Table 1), a discussion regarding the hydrophobic interactions of these systems can be omitted herein. The n_s^{BSA} values for the MgHap particles were greater than those of n_s^{LSZ} . This result can be explained by the structural rearrangement of the soft protein BSA with a large molecular weight; the particles used herein exhibited a relatively large, flat surface (Fig. 1).

To describe the dependence of protein adsorption on the MgHap particle structure used for adsorption, the n_s^{BSA} and n_s^{LSZ} values were plotted as a function

of the X_{Mg} values of MgHap particles in Fig. 7(a). The n_s^{BSA} and n_s^{LSZ} values decreased up to $X_{Mg} = 0.04$ and reached ~ 0.45 and ~ 0.1 mg/m², respectively. Although there was no large difference in the (Ca+Mg)/P values for the MgHap particles produced at $0.04 \leq X_{Mg} \leq 0.20$, the (Ca + Mg)/P values were in

the range of 1.40–1.62. In addition, low amounts of Mg²⁺ were incorporated into the particles, i.e., the fraction of incorporated Mg²⁺ against Ca²⁺ was ~ 0.5 (Fig. 4). Hence, the reduction in the n_s^{BSA} and n_s^{LSZ} values cannot be attributed to the chemical composition of MgHap particles.

FIGURE 5 Adsorption isotherms of BSA on MgHap particles produced at various X_{Mg} values and their zp values.

FIGURE 6 Adsorption isotherms of LSZ on MgHap particles produced at various X_{Mg} values and their zp values.

FIGURE 7 Saturated amounts of BSA and LSZ as a function of (a) X_{Mg} and (b) particle length.

Next, the alteration in particle morphology should be considered. To clarify this point, the n_s^{BSA} and n_s^{LSZ} values were further plotted as a function of the particle length of MgHap particles in Fig. 7(b). Notably, a critical point was clearly observed for n_s^{BSA} (Fig. 7(b)). The n_s^{BSA} values abruptly increased at a particle length of greater than 150 nm. This result can be explained by the variation in length of these particles, i.e., there is an increase in the large fraction of positively charged C sites on the rod-like particles, which is advantageous for the adsorption of the negatively charged BSA at pH 6. In other words, the adsorption of BSA dictates a MgHap particle length of greater than 150 nm. In a previous study, the author has reported that the n_s^{BSA} values are strongly dependent on the length of the CaHap particles between 60 and 5210 nm (0.06–5.21 μm)²⁸. However, CaHap particles with a length of greater than 60 nm exhibit n_s^{BSA} values of 1.0–1.8 mg/m^2 , and we did not observe a critical point. This result leads the conclusion that particle length is among the most important properties on the adsorption of BSA onto MgHap particles, though the detailed mechanism is left to subsequent papers.

In this section, the adsorption behavior of proteins on rod-like MgHap particles with various X_{Mg} values was elucidated. The obtained results not only provided fundamental results for the protein–MgHap interactions, but also extended to the investigation of the effects of trace Mg^{2+} in biological fields. A critical particle length (150 nm) for the adsorption of BSA was noted. The valuable results obtained herein reveal that the adsorption of BSA leads to MgHap particles with a length of greater than 150 nm.

CONCLUSIONS

Fundamental experiments on the adsorption behavior of proteins on MgHap synthesized at various Mg(II) contents ($X_{\text{Mn}} = 0\text{--}0.3$) were carried out. Rod-like CaHap particles with a width of 24 nm and a length of 180 nm were obtained. With the increase in X_{Mg} up to 0.10, the particle width and length decreased to ~10 and 50 nm, respectively. All of the synthesized particles afforded peaks characteristic of CaHap, but almost all of the MgHap particles were deficient in magnesium. All of the adsorption isotherms of BSA and LSZ obtained from a 1×10^{-4} mol/dm^3 KCl solution were of the Langmuir type. The saturated amount of adsorbed BSA (n_s^{BSA}) strongly depended on the particle length rather than the X_{Mg} value. The n_s^{BSA} values abruptly increased with a particle length of greater than 150 nm. In other words, the BSA adsorption leads to a MgHap particle length of greater than 150 nm. However, the saturated

amount of adsorbed LSZ (n_s^{LSZ}) did not exhibit any remarkable variation with the particle length. The results obtained herein can possibly aid investigations of the effects of trace Mg^{2+} ions in biological fields.

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