

## Interaction between Nanometer-sized Hydroxyapatite Particles and Amino Acid

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A nanometer-sized hydroxyapatite powder ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ) with  $104 \text{ m}^2/\text{g}$  of specific surface area (equivalent diameter 18.5 nm) was synthesized by the reaction between  $\text{Ca}(\text{OH})_2$  suspension and  $\text{H}_3\text{PO}_4$  solution at room temperature. The zeta potential and dispersibility of the calcined hydroxyapatite powder (specific surface area  $55 \text{ m}^2/\text{g}$ ) were studied with 2 vol% hydroxyapatite suspensions with glycine ( $\text{H}_2\text{NCH}_2\text{COOH}$ ), phenylalanine ( $\text{C}_6\text{H}_5\text{CH}_2\text{CHNH}_2\text{COOH}$ ), leucine ( $(\text{CH}_3)_2\text{CHCH}_2\text{CHNH}_2\text{COOH}$ ), glutamic acid ( $\text{HOOC}(\text{CH}_2)_2\text{CHNH}_2\text{COOH}$ ), and lysine ( $\text{H}_2\text{N}(\text{CH}_2)_4\text{CHNH}_2\text{COOH}$ ) of 0.5 mass% against hydroxyapatite at pH 5-9. The hydroxyapatite powder dissolved in the acidic suspensions at pH below 4. In the pH range from 5 to 9, addition of phenylalanine, leucine and lysine increased the zeta potential of negatively charged hydroxyapatite toward the positive values. On the other hand, glycine and glutamic acid gave a very little influence on the zeta potential. The dispersibility of hydroxyapatite with and without the amino acid was low at pH 5 and 7. However, a high dispersion state was measured in the hydroxyapatite suspension at pH 9. The dispersibility-zeta potential relationship indicated the strong steric stabilization effect of glutamic acid, lysine, leucine and phenylalanine on the dispersion of hydroxyapatite particles. The rheological properties of the suspensions with amino acid were also measured.

Key words: hydroxyapatite, amino acid, nanometer-sized particles, dispersibility

### 1. Introduction

Hydroxyapatite (HAp,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ) ceramics has been used as a prosthesis of human bone or teeth because HAp is an inorganic component of the human bone and has a high bioactivity and biocompatibility [1]. HAp ceramics has a comparable flexural strength (100–200 MPa) to human bone. However, the fracture toughness is significantly higher for human bone ( $2\text{--}6 \text{ MPa}\cdot\text{m}^{1/2}$ ) than for HAp ceramics ( $\sim 1 \text{ MPa}\cdot\text{m}^{1/2}$ ) [2-4]. This improvement may be achieved by making the fibrous collagen-reinforced HAp matrix composite whose structure is very similar to that of human bone. HAp matrix should contain micropores to run small blood vessels after the implant in human body. To maintain the mechanical strength of HAp matrix with micropores, the shape and size of the pores are to be strictly controlled. That is, the purpose of this study is to develop a fine-grained strong hydroxyapatite ceramics with controlled micropores. In this paper, the preparation and dispersibility of nanometer-sized hydroxyapatite particles were studied to produce the improved bioceramics with the micropores. Low temperature sintering is effective to produce a fine-grained hydroxyapatite ceramics because of a low grain growth rate. In addition, the dispersion of fine ceramic particles in a liquid medium and subsequent consolidation of the colloidal particles [5-8], assist in producing a uniform microstructure and high packing density for the powder compact. When the size of colloidal particles becomes smaller, the interparticle interaction increases, i.e., the high viscosity at a low

solid content, the low dispersibility of colloidal particles due to increased attractive force and the large amount of dispersant required to cover the high specific surface area of the colloidal particles [9-11]. The following criteria were looked into a possible dispersant for the nanometer-sized particles: (1) The size ratio of the established dispersant to the submicrometer-sized particles in a well-dispersed suspension should be kept in a nanometer-sized particle suspension (steric stabilization effect) [12-14] and (2) the low molecular weight dispersant should be highly charged compared to the nanometer-sized particles to give a strong electrostatic stabilization effect [15, 16]. Thus, amino acid with the two types of ionizable groups (carboxyl and amino groups) can be a good choice as the dispersant. These ionized groups provide the adsorption onto particle surfaces and the electrostatic repulsion. Here, a nanometer-sized hydroxyapatite powder was dispersed in the aqueous solutions with glycine, phenylalanine, leucine, glutamic acid and lysine to investigate their interactions.

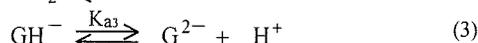
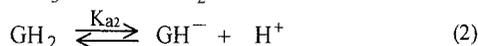
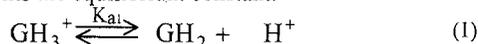
### 2. Experimental procedure

In this experiment,  $\text{Ca}(\text{OH})_2$  and  $\text{H}_3(\text{PO}_4)$  were used as raw materials to produce hydroxyapatite particles according to the reaction,  $10 \text{ Ca}(\text{OH})_2 + 6 \text{ H}_3(\text{PO}_4) \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 18 \text{ H}_2\text{O}$ . Calcium hydroxide with specific surface areas of  $69.4 \text{ m}^2\cdot\text{g}^{-1}$  (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was dispersed to make the suspension of 0.5 M  $\text{Ca}(\text{OH})_2$ . A 0.3 M orthophosphoric acid solution of 200 ml was added to

the calcium hydroxide suspension of 200 ml at one time. The produced hydroxyapatite powder was aged for 24 h. Then, the hydroxyapatite particles in the suspension were filtrated through 0.1  $\mu\text{m}$  pores, washed with ethanol three times to prevent the flocculation of the particles, and dried at 100  $^{\circ}\text{C}$  for 24 h in air. The dried powder was calcined at 600  $^{\circ}\text{C}$  for 1 h in air. The phases produced in the synthesized and calcined powders were identified by X-ray powder diffraction (RINT 2200HK/PC, Rigaku Co., Tokyo, Japan). The specific surface areas of as-produced and calcined powders were measured by Brunauer-Emmett-Teller (BET) method (Flowsorb 2300, Shimadzu Co., Kyoto, Japan).

#### Dispersant

In Table I the chemical properties of the amino acid used are shown [17]. The five kinds of amino acid have the molecular length of 0.64 – 1.26 nm and provide a size ratio of 0.018 – 0.036 to the diameter of calcined hydroxyapatite particles. The isoelectric point for equal charge of  $\text{NH}_3^+$  and  $\text{COO}^-$  of the amino acid used was in the range from pH 3.22 to 9.74. The dissociation of amino acid ( $\text{GH}_2$ ) is seen in Eqs. (1) (2) and (3).  $K_a$  represents the equilibrium constant.



From Eqs. (1)-(3), the dissociated fraction ( $\alpha$ ) of  $\text{GH}_3^+$  to the total concentration of neutral and ionized amino acid is related to  $K_a$  and  $[\text{H}^+]$  by Eq. (4).

$$\begin{aligned} \alpha_{\text{GH}_3^+} &= \frac{[\text{GH}_3^+]}{[\text{GH}_3^+] + [\text{GH}_2] + [\text{GH}^-] + [\text{G}^{2-}]} \\ &= \frac{[\text{H}^+]^2}{[\text{H}^+]^2 + K_{a1}[\text{H}^+] + K_{a1}K_{a2}} \end{aligned} \quad (4)$$

Similarly,  $\alpha_{\text{GH}_2}$ ,  $\alpha_{\text{GH}^-}$  and  $\alpha_{\text{G}^{2-}}$  are given by Eqs. (5), (6) and (7), respectively.

$$\alpha_{\text{GH}_2} = \frac{\alpha_{\text{GH}_3^+} K_{a1}}{[\text{H}^+]} \quad (5)$$

$$\alpha_{\text{GH}^-} = \frac{\alpha_{\text{GH}_2} K_{a1} K_{a2}}{[\text{H}^+]^2} \quad (6)$$

$$\alpha_{\text{G}^{2-}} = \frac{\alpha_{\text{GH}^-} K_{a1} K_{a2} K_{a3}}{[\text{H}^+]^3} \quad (7)$$

Figure 1 shows the fractions of dissociated and neutral phenylalanine. With an increase of pH, the dominant species changes as follows: positively charged amino acid  $\rightarrow$  neutral amino acid  $\rightarrow$  negatively charged amino acid. A similar calculation is also possible for the other amino acid.

#### Dispersibility of hydroxyapatite particles

Dispersibility of the calcined hydroxyapatite particles in 2 vol% suspension with and without amino acid at pH 5-9 were evaluated by measuring the length of the phase separation into a clear solution and a concentrated suspension as a function of settling time [16]. The amino acid of 0.5 mass% against calcined

Table I Chemical properties of the amino acid used.

Amino acid	Molecular weight	Isoelectric point	Dissociation constant		
			pK <sub>a1</sub>	pK <sub>a2</sub>	pK <sub>a3</sub>
Glycine	75.1	5.97	2.35	9.78	—
Phenylalanine	165.2	5.48	2.16	9.18	—
Leucine	131.2	6.02	2.36	9.60	—
Glutamic acid	147.1	3.22	2.19	4.25	9.67
Lysine	146.2	9.74	2.20	8.90	10.28

hydroxyapatite was added to the suspensions. The zeta potential of colloidal hydroxyapatite was measured at pH 5-9 at a constant ionic strength of 0.01 M  $\text{NH}_4\text{NO}_3$  (Rank Mark II, Rank Brothers Ltd., Cambridge, UK). Rheological properties of 15 vol% hydroxyapatite suspensions with amino acid (0.5 mass%) at pH 7 and 9 were measured by viscometer at 20-25  $^{\circ}\text{C}$  (Visconic EHD, Tokimec Inc., Tokyo, Japan).

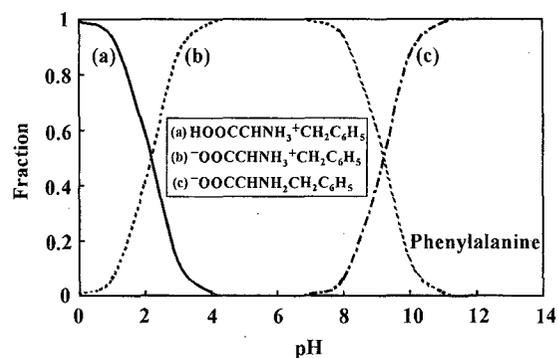


Fig.1 Fraction of dissociated phenylalanine as a function of pH.

### 3. Results and discussion

#### 3.1 Formation of hydroxyapatite

The diffraction patterns of the synthesized and calcined powders were in accordance with those of hydroxyapatite in JCPDS card (No. 9-432). The size of hydroxyapatite crystallite, measured on the diffraction line (002), was 17.1 and 22.5 nm for the as-produced and calcined powders, respectively. The synthesized and heat-treated hydroxyapatite gave the specific surface areas of 103.7 and 55.1  $\text{m}^2/\text{g}$ , respectively, corresponding to the particle diameter of 18.5 nm for synthesized hydroxyapatite and 34.8 nm for the heat-treated hydroxyapatite. This tendency of particle diameter agreed with the change in crystallite size of hydroxyapatite with heating.

#### 3.2 Zeta potential of hydroxyapatite particles

Figure 2 shows the zeta potential of the calcined hydroxyapatite powder. In the acidic solutions at pH below 4, the hydroxyapatite powder dissolved in the solutions. The hydroxyapatite powder was charged negatively in the pH range from 5 to 9. Addition of 0.5 mass% glutamic acid to the hydroxyapatite increased slightly the zeta potential toward the negative values. On the other hand, the other four kinds of amino acid shifted the zeta potential toward the positive values.

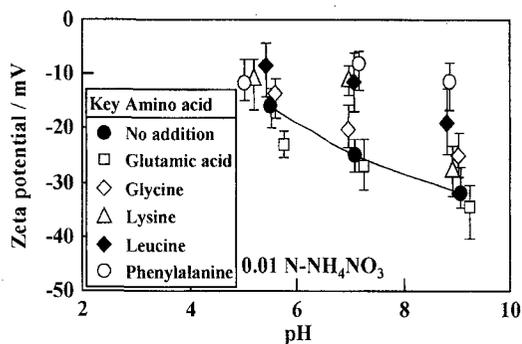


Fig.2 Zeta potential of hydroxyapatite powder in the suspensions with and without amino acids as a function of pH.

Figure 3 shows the possible interaction between phenylalanine and hydroxyapatite surface at pH 7.0. The surface of the calcined hydroxyapatite is covered with  $H^+$  and  $OH^-$  ions to form  $Ca-OH$ ,  $PO_4-H$  and  $OH-H$  sites. In the suspension at pH 5-9,  $PO_4-H_2^+$  and  $OH-H_2^+$  sites are formed by the reaction,  $PO_4-H + HCl \rightarrow PO_4-H_2^+ + Cl^-$  and  $OH-H + HCl \rightarrow OH-H_2^+ + Cl^-$ . The hydroxyapatite surface also reacts with  $OH^-$  ions to produce  $Ca-O^-$  sites ( $Ca-OH + OH^- \rightarrow Ca-O^- + H_2O$ ). The number of  $Ca-O^-$  sites is greater than that of  $PO_4-H_2^+$  and  $OH-H_2^+$  sites, explaining the negatively charged surface at pH 5-9. Phenylalanine dissociates into 99.8 and 99.3 % GH at pH 5 and 7, respectively. The adsorption of electrically neutral GH with equal number of  $COO^-$  and  $NH_3^+$  groups onto hydroxyapatite surface results in no change in the surface charge. However, the zeta potential of hydroxyapatite particles changed to the positive values with the addition of phenylalanine. This discrepancy may be explained by the shield effect of phenyl group with no charge, which covers the charged surface of hydroxyapatite particles. A similar shield effect by methyl group in leucine on the negatively charged hydroxyapatite can explain the change of the zeta potential toward the positive values with the addition of electrically neutral leucine at pH 5-7. The fraction of  $G^-$  ion in phenylalanine and leucine increases more at pH 9.0. Electrostatic repulsion between the negatively charged hydroxyapatite surface and  $G^-$  ion suppresses the adsorption of those ions at pH 9.0. On the other hand, glycine dissociates into 99.8 % GH at pH 5-7. The adsorption of electrically neutral GH

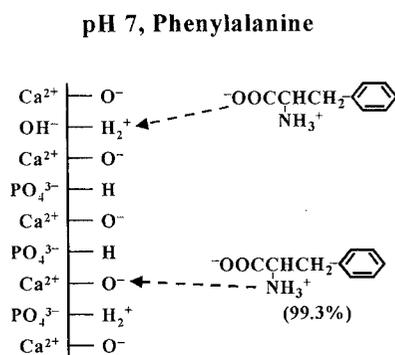


Fig.3 Schematic illustration of possible interactions between hydroxyapatite surface and phenylalanine at pH 7.

with no shield effect on the negatively charged hydroxyapatite surface leads to no change of zeta potential of hydroxyapatite particles. In a suspension at pH 7.0, lysine dissociates into 98.9%  $GH_2^+$ . The adsorption of  $NH_3^+$  in  $GH_2^+$  onto negative sites of hydroxyapatite surface causes an increase in positive charge on the hydroxyapatite surface. In a suspension at pH 9, lysine dissociates into 43.0 %  $GH_2^+$  and 54.2 % GH. The increase of the interaction between electrically neutral GH and the negatively charged hydroxyapatite surface suppresses the change in the zeta potential of hydroxyapatite particles.

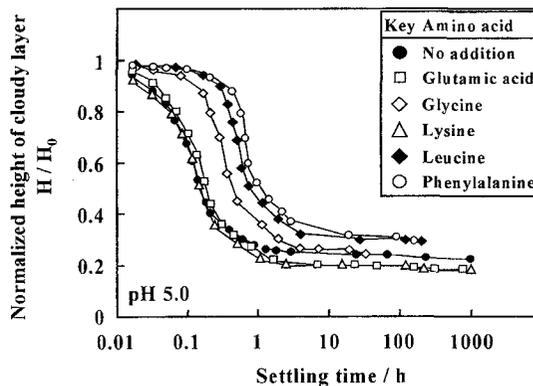


Fig.4 Length of phase separation in the sedimentation test for hydroxyapatite suspensions at pH 5.

### 3.3 Dispersibility of hydroxyapatite particles

Figures 4 and 5 show the height of cloudy layer of 2 vol% hydroxyapatite suspensions as a function of settling time at pH 5.0 and 9.0, respectively. As seen in Fig.2, the hydroxyapatite particles were charged with  $-16.0$  and  $-24.9$  mV at pH 5.0 and 7.0, respectively. However, the negatively charged hydroxyapatite particles sedimented rapidly. The decrease of particle size is accompanied by the decrease of the repulsive energy between charged particles, resulting in the decrease of primary maximum of interaction energy (summation of repulsive energy and van der Waals energy) as a function of distance between two particles [15]. The result in Fig.4 suggests the difficulty of dispersion of 35 nm-hydroxyapatite particles with  $-24.9$  mV of zeta potential. Addition of the five kinds of amino acid suppressed the phase separation of

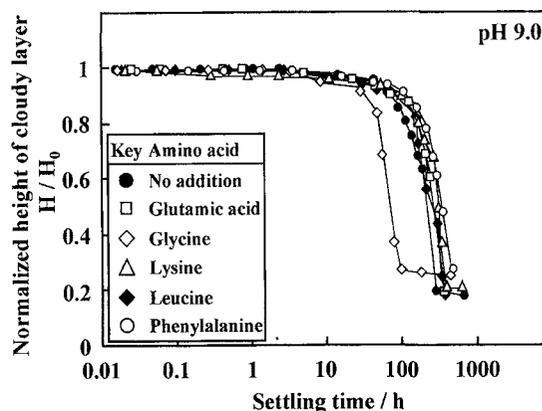


Fig.5 Length of phase separation in the sedimentation test for hydroxyapatite suspensions at pH 9.

hydroxyapatite suspension into a clear solution and a concentrated suspension. Since the magnitude of the zeta potential was reduced with the addition of amino acid, the improvement of the dispersibility of hydroxyapatite particles was due to the steric stabilization effect of amino acid adsorbed. When the suspension pH was increased to 9.0, the dispersibility of hydroxyapatite particles with  $-31.9$  mV zeta potential was greatly improved. A well-dispersed state was kept for the period longer than 100 h. The suspensions with the amino acid showed also the high dispersibility.

Figure 6 shows the relationship between zeta potential of hydroxyapatite particles and the height of cloudy layer after 120 h of settling time at pH 5-9. Increase of the zeta potential with increasing pH leads to the high dispersion of hydroxyapatite particles without amino acid due to electrostatic stabilization effect. Addition of the amino acid provides the high dispersibility due to steric stabilization effect for nanometer-sized hydroxyapatite particles with low zeta potential. This steric stabilization effect increased in the following order of the amino acid: glutamic acid < lysine < leucine < phenylalanine. The influence of glycine on the dispersibility of hydroxyapatite particles was small.

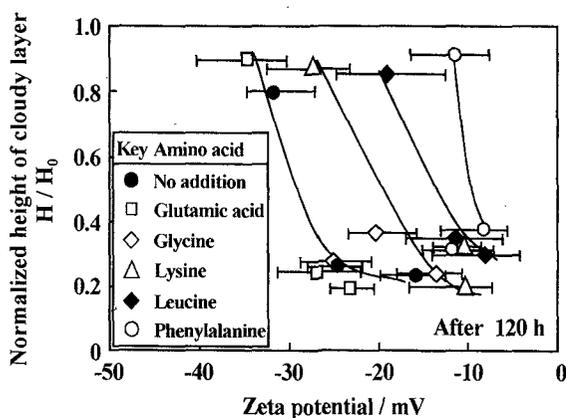


Fig.6 Relationship between the length of phase separation after 120 h of settling time and zeta potential of hydroxyapatite particles with and without amino acids.

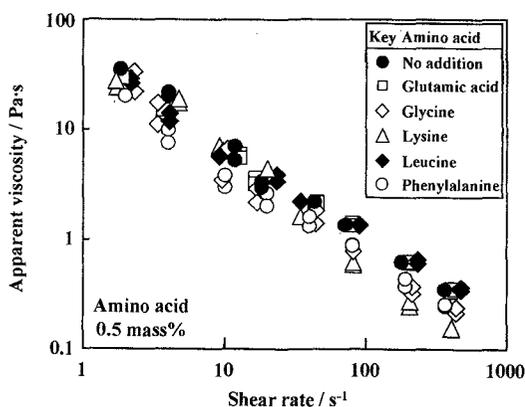


Fig.7 Rheological properties of 15 vol% hydroxyapatite suspensions with and without amino acids at pH 9.

Figure 7 shows the apparent viscosity of 15 vol% hydroxyapatite suspensions with 0.5 mass% amino acid against hydroxyapatite at pH 9. The apparent viscosity was lower for the suspensions with lysine and phenylalanine than for the suspension without amino acid.

#### 4. Conclusions

- (1) The dispersibility of 35 nm-size hydroxyapatite particles was improved by increasing the zeta potential to  $-32$  mV in the suspension at pH 9.0.
- (2) The addition of phenylalanine, leucine and lysine shifted the zeta potential of hydroxyapatite particles to the positive values at pH 5-9. The addition of glycine and glutamic acid resulted in a very little change in the zeta potential of hydroxyapatite particles.
- (3) Adsorbed amino acid provided the high dispersibility of nanometer-sized hydroxyapatite particles due to steric stabilization effect, reducing the suspension viscosity.

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