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Fabrication of Ag doped Hydroxyapatite and its Antimicrobial Effects with the Particle Size

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Abstract Ag doped Hydroxyapatite powder in nano-scale was successfully synthesized either by co-precipitation or by ion exchange route. The fabricated powder was successfully dispersed through freeze drying due to the prevention of secondary particles. The antimicrobial effects of nano-HAp against *E.coli* was superior to micron ones not only in its strength but also in duration.

Keywords : Nano-scale powder, Ag dopted Hydroxyapatite, powder synthesis, antimicrobial effect

1. Introduction

Microorganism is a kind of prokaryote with the size range from 0.2 to 80 μm. It multiplies geometrically when proper temperature, humidity and nutrition for proli-feration are provided. When the number of microorganism exceeds a critical limit against the resistance of leukocytes, an infection is caused to human body. In case of *Staphylococcus aureus,* 10^6 organisms were reported to result in an infection for a patient free of wound, though the number depends on individuals.[1] However for those patients with a silk suture, the number of infection-producing organism decreases drastically to only 100. Therefore sterilization is inevitable in the wounded parts to prevent the infection.

For the sterilization of medical devices, high temperature, pressure and chemical treatments are generally used. Such treatments can be effective for instant use of medical devices, but the effects do not last for such a long time. The microorganisms are known to have the strong tendency of adhering[2] on the surface of inanimate object and even makes the dense layer called biofilm. Once the biofilm is formed, the effect of antibiotics is very limited and difficult to sterilize.[3] Medical devices such as catheters are used to connect the organ inside the body to outside and thus always exposed to the danger of the proliferation of microorganisms.

Antimicrobial effects of transient metal ions such as silver were well known[4,5] from the experiences. Silver ion reacts with microorganism and inactivate the metabolism.[4] Maintaining the ionic state is required and thus, the metal ions should be reserved in polar liquid like water or in ion exchangeable solids. The benefit of ion exchangeable solid is that antimicrobial effects can be integrated in any kind of medical devices by simple incorporation. Hydroxyapatite(HAp) is not only a excellent biocompatible biomaterial but also ion exchangeable. The role of such a carrier is important, because antimicrobial effects are determined not only by the types of agent but also by the capacity of the carrier. Thus the antimicrobial effects might be improvable by using the nano-scaled carrier in a well dispersed state. In this work, HAp powder of nano-size was prepared and techniques of incorporating silver ions were explored. With the use of freeze drying, dispersion of nano-powder was achieved. The antimicrobial effects were observed for the prepared nano-HAp powders.

2. Experimental Procedure

HAp($Ca_{10}(PO_4)_6(OH)_2$) powder was precipitated by mixing the solution of $Ca(NO_3)_2 4H_2O$ and $(NH_4)_2 HPO_4$. Both of the solution had the concentration of 0.5 M and weighed so that the ratio between Ca and P is 1.667 : 1. At 80°C, NH_4OH was added so that the pH of 10 is attained for both of the solution. The solutions were mixed instantaneously under vigorous stirring and kept for six hours. The pH and temperature of solution chosen in the experiment was the necessary conditions for the successful fabrication of the HAp powder.[6,7] The precipitated powder was filtered and washed repeatedly until pH smaller than 9 is attained from the

cleaning water. The filtered cream was demoistured either in the oven at 120°C for 24 h or in the freeze drier at -50°C under vacuum. After complete drying, X-ray diffraction(XRD) analysis was carried out. Transmission Electron Microscopy(TEM) sample was prepared during the precipitation by dipping the Cu grid in the solution.

Silver ion was introduced in HAp either by co-precipitation or ion exchange. $AgNO_3$ was weighed so that the Ag ion occupies 0.1 to 10% of all cations (Ag and Ca) and dissolved with $Ca(NO_3)_2 4H_2O$. The pH and the temperature of the solution were kept at 10 and 80°C respectively as before. During the ion exchange, HAp powder was immersed in $AgNO_3$ solution whose concentration was prepared so that the silver ion occupies 0.1 to 10% of total cations(Ag and Ca) in the same manner. The slurry was stirred slowly at room temperature for 24 h and filtered for separation from the solvent. The Ag content in either co-precipitated or ion exchanged HAp was analysed using Inductively Coupled Plasma(ICP).

The antimicrobial effects of Ag doped nano-HAp were measured and compared with that of coarse (~1 μm) HAp powder. The coarse HAp powder was prepared by heat treating nano powders at 1200°C for 1 h. For both nano and micron powders, 3 mol% of Ag ion was introduced through ion exchange technique. *E.Coli* was selected for antimicrobial tests. The micro-organisms were cultured in the nutrient agar badge and sterilized subsequently. The amount of nutrient was 8 g in a liter of badge. Both nano and micron powders were mixed in nutrient agar badge in amounts of 0.001 g (0.1% sample hearafter) and 0.005 g (0.5% sample) in a milli liter of a badge. The mixed badge was put in contact with *E.coli* for 5, 30, and 60 minutes and cultured in petri agar dish at 30°C for 24 h. The number of colony was counted using colony counter.

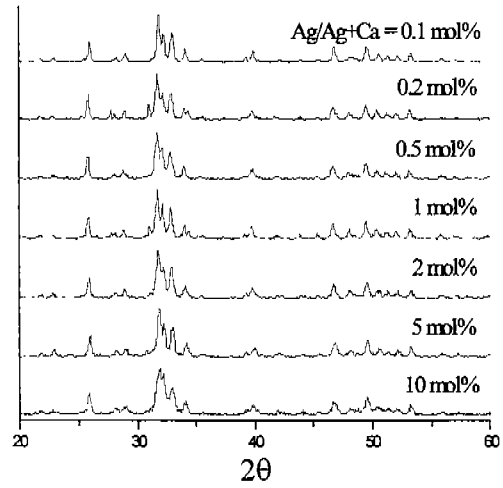

Fig. 1. XRD pattern of hydroxyapatite powders coprecipitated with various amounts (0.1-10 mol%) of Ag.

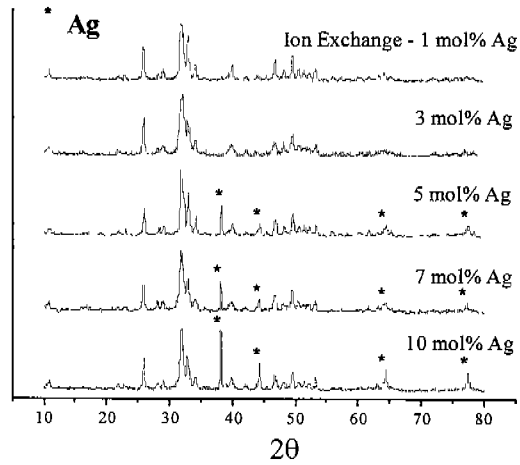

Fig. 2. XRD pattern of hydroxyapatite powders ion-exchanged with various amounts (0.1-10 mol%) of Ag.

3. Results and Discussion

Generally, HAp has a nonstoichiometry between Ca/P range of 1.5 and 1.67. When the powders are heated higher than 700°C, Ca/P of 1.5 evolves to $Ca_3(PO_4)_2$ while HAp is obtained from that of 1.67. Therefore, it is necessary for the precipitated powders to be heat-treated for identification of phase and furthermore its Ca/P ratio. Fig. 1 is the XRD patterns from the powders heated at 700°C for 1 h varying Ag content from 0.1 to 10 mol% of the total cations. It can be noticed that only HAp phase is present regardless of the introduced Ag contents except minor tricalcium phosphate peak near 31° in 0.2 and 1 mol% Ag sample. The ICP analysis from the powders showed that the maximum amount of Ag does not exceed 3 mol% even though more than 3 mol% were dissolved. The Ag concentration detected increased with introduced amount only in very small doping region and showed a saturated behaviour.

Figure 2 is the XRD patterns obtained from the powders where Ag ion was introduced through the ion exchange method. For 1~3 mol% of Ag doping, only HAp phase was found, but for more Ag, metallic Ag was precipitated. The intensity of metallic Ag increases

with the concentration of Ag solution, and thus it means that the amount of precipitation also increases. With the increase in the amount of precipitation, there was a change in the color of powder. White powders were obtained for the Ag contents below 3 mol% and it gets darker and darker with the increase of Ag content from 3 mol%.

It is worthwhile to take notice that the saturation of silver in HAp takes place near 3 mol% both in co-precipitation and ion exchange. HAp is known to have a hexagonal crystal structure with the space group of $P_{63/m}$. Ag^+ ion has a similar ionic radius(1.15Å) with Ca^{2+}(1.00Å) in a octahedral site of oxygen.[8] Indeed, it was reported that Ag^+ is substituted in Ca^{2+} site.[9] Such a substitution generates a -1 effective charge. It is also proposed that the generated charge can be compensated by the formation of vacancy in OH sites. Hence the chemical equation of Ag incorporation in HAp can be written as Eq.(1)

$$Ca_{10}(PO_4)_6(OH)_2 + xAg \Rightarrow Ca_{10-x}Ag_x(PO_4)_6(OH)_{2-x} \quad (1)$$

Eq.(1) shows that concentration of vacancy x can not be greater than 2. It means that the theoretical limit of the Ag substitution of Ca is 20%. However, due to the limit of the diffusion distance, the substitution is confined in the surface region of HAp powder. Furthermore, incorporation of Ag ion and formation of vacancies can cause a stability problem in the crystal structure. Thus, the practical limit of substitution must be much lower than the theoretical one as ~3%.

To improve the antimicrobial effects, it is believed that the HAp powders should be fine enough and well dispersed at the same time. In this work, nano-HAp powders prepared under the high stirring rate were used.[10] Fig. 3 shows the HAp powders used had a length around 100 nm and a width of 10 nm.

When the filtered cream was dried in the oven at 120°C, the cream shrank to less than half of its original dimension, and hard agglomerates are formed. These hard agglomerates were not only difficult to be crushed, but also detrimental for successful dispersion. To avoid the formation of such hard agglomerates, the cream was freeze-dried. Fig. 4 is the TEM micrograph of the powder dispersed with the addition of 0.01 ml of Darvan C in a gram of powder. When the cream was dried at 120°C as shown in Fig. 4(a), it is found that the hard agglomerates are still remaining even though some primary particles are well dispersed. On the contrary, when the cream was freeze-dried as shown in Fig. 4(b), good dispersion of powder can be seen.

By freeze drying, the formation of secondary particles is supposed to be minimized. In this case, the solvent is eliminated through sublimation without forming the droplets which is responsible for the formation of secondary particles. The successful dispersion

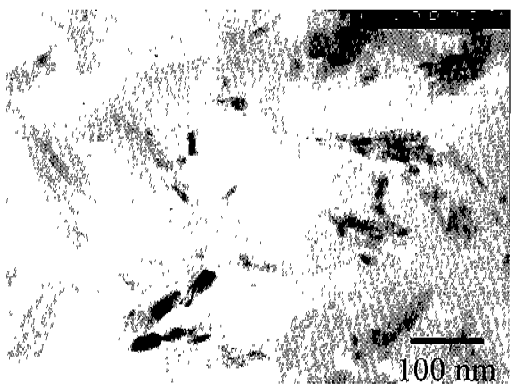

Fig. 3. Nano-HAp powders precipitated.

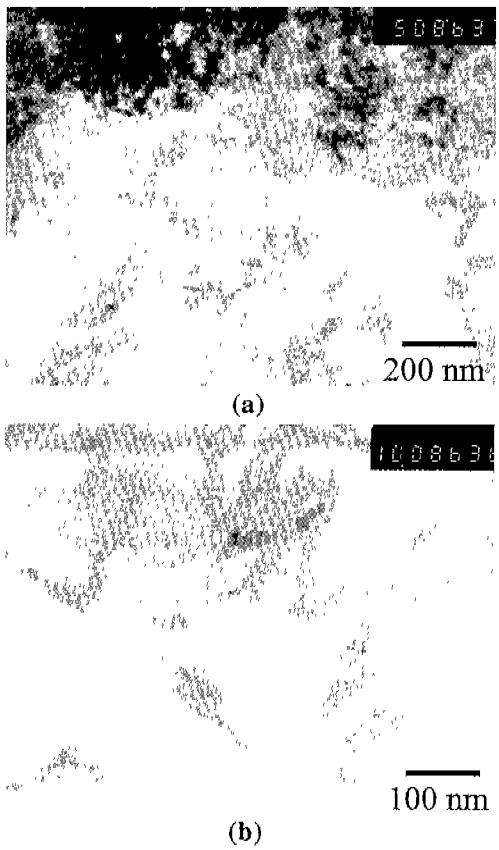

Fig. 4. Hydroxyapatite powders dispersed with Darvan C after (a) drying at 120°C for 24 h and (b) freeze drying.

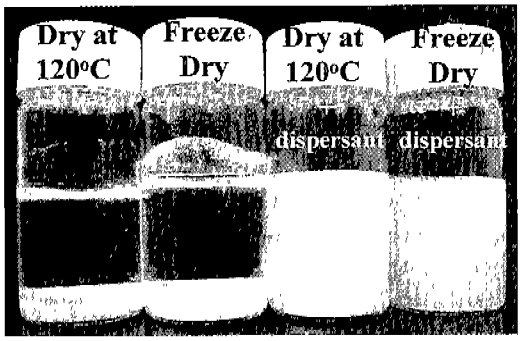

Fig. 5. Slurries of fabricated HAp powders.

Table 1. Number of *E.Coli* colony with time and size of particles

sample	0.1%			0.5%		
	5 min	30 min	1 h	5 min	30 min	1 h
Micron	410^4	+	+	210^3	+	+
Nano	310^2	+	+	210^2	1.210^4	+

(+ : Indistinguishable in number with the references(1.28×10^8))

can be seen in Fig. 5 also. The slurries in Fig. 5 was taken after keeping the bottles calmly for 24 h. When dispersant was not added, it can be seen that the powder was completely sedimented regardless of the drying method. When dispersant was added, it looks like that successful dispersions were attained for both of the slurries. However, in the slurry dried at 120°C, some sediment is observed while it was absent in freeze-dried powder.

Table 1 shows the antimicrobial effects of Ag doped HAp powders against *E.Coli*. The antimicrobial effects of well dispersed nano-HAp powders in Fig. 3 were compared with those of micron-HAp powders in Fig. 6. The micron powder was obtained through heat-treating at 1200°C for 1 h. The numbers of organisms alive after the contact with 0.1% powders for 5 min were 4×10^4 and 3×10^2 for micron and nano powders respectively. These are the numbers much smaller than the number of organisms (1.28×10^8) found from the references without any antimicrobial agent. When the 0.5% diluted powders were used, the numbers of remaining microrganisms decreased to 2×10^3 and 2×10^2 for micron and nano, respectively.

In case of contact for 30 min, antimicrobial effects are decreased for both powders perhaps due to the exhaust of Ag ions. For 0.1% samples, proliferation of *E.Coli* was so active that they were indistinguishable with the references. However in the 0.5% samples,

Fig. 6. Coarse HAp powder synthesized by heat treating at 1200°C for 1 h.

active proliferation was found only in micron powders, and number of microorganisms were limited to 1.2×10^4 for nano-powders. Hence it can be found that antimicrobial effects are sustained for longer time in nano powders. These results shows that nano powders have stronger and longer antimicrobial effects due to the larger specific surface area.

4. Summary

By either co-precipitation or ion exchange route, Ag doped HAp powder was successfully synthesized. For improved antimicrobial effects, the processing conditions were optimized to obtain nano-particles with good dispersion. Freeze drying was a effective technique for successful dispersion due to the minimization of the secondary particles. The antimicrobial characteristics of nano-HAp was superior to micron ones not only in its strength but also in duration.

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This paper is dedicated to Professor Dr. Koichi Niihara on the occasion of his 60th birthday

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