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Increase in Cell Adhesiveness on a Poly(ethylene terephthalate) Fabric by Sintered Hydroxyapatite Nanocrystal Coating in the Development of an Artificial Blood Vessel

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Nano-scaled sintered hydroxyapatite (HAp) crystals were covalently linked onto a poly(ethylene terephthalate) (PET) fabric substrate chemically modified by graft polymerization with γ-methacryloxypropyl triethoxysilane (MPTS) for development of an artificial blood vessel. The weight gain of graft polymerization with poly(MPTS) on PET in benzyl alcohol containing H_2O_2 as an initiator increased as increasing the reaction time and finally reached a plateau value of about 3.5 wt%. The surface characterization of surface modification with poly(MPTS)-grafting was conducted by x-ray photoelectron spectroscopy. HAp nanocrystals of approximately 50 nm in diameter, monodispersed in pure ethanol, were coupled with alkoxysilyl groups of the poly(MPTS)-grafted PET substrate. The HAp nanocrystals were uniformly and strongly coated on the surface of the PET fabrics, although HAp particles adsorbed physically on the original PET without poly(MPTS) grafting were almost removed by ultrasonic wave treatment. More human umbilical vein endothelial cells adhered to the HAp/PET composite fabric compared with original PET after only 4 hours of initial incubation, and the same was observed on the collagen-coated PET. The coating of sintered HAp nanocrystals imparted bioactivity to the polyester substrate, which is a widely used biomedical polymer, without a coating of adhesion proteins derived from animals, such as collagen or gelatin. A prototype of an artificial blood vessel was finally fabricated by use of HAp/PET composite. *ASAIO Journal* 2006; 52:315–320.

Hydroxyapatite (HAp) has attracted considerable attention as hard-tissue–compatible material for implants and bone augmentation procedures, because it bonds directly to bone when implanted[1–5] and results in the formation of a strong bone-implant interface. In addition, HAp ceramics are compatible with soft tissue such as skin through the development of a percutaneous device.[6] We have developed an inorganic-organic composite consisting of nano-scaled sintered HAp crystals[7–9] and biomedical polymers (such as silk fiber) via covalent bonding at the interface.[10–12] Recently, a novel percutaneous device was developed using that inorganic-organic composite.[13] The actual effectiveness as a percutaneous device was evaluated by animal implant experiment. In these continuous studies, one of the reasons for using silk fiber as a polymer substrate is simply for surface modification because of the large number of functional groups on the polymer surface.

If our HAp nanocrystal coating technique can be applied to other medical polymers as well as silk fiber, the uses are expected to spread widely in medical fields. In this regard, polyester has been used as a typical and popular biomedical polymer, such as in artificial blood vessels[14] and ligaments.[15] Medical devices made of polyester (*e.g.*, artificial blood vessels) are generally coated with collagen or gelatin in order to increase interaction with living cells or tissue. Although the use of animal-derivative proteins is feared due to the possible outbreak of infectious diseases such as bovine spongiform encephalopathy (BSE), the HAp coating is biologically safe because it has no biologic derivative substances.

In this study, we developed a novel composite consisting of nano-scaled HAp crystals and a poly(ethylene terephthalate) (PET) fabric as a polymer substrate through covalent linkage for the purpose of development of an artificial blood vessel. Donation of covalent boding between HAp and the substrate was performed by a coupling reaction between hydroxyl groups on a HAp crystal and alkoxysilyl groups of the graft-polymer on the PET. Surface modification of polyester is relatively more difficult compared with a silk substrate, because functional groups seldom exist on the polyester surface. Hydrophilic moieties were, therefore, introduced on the surface by alkaline hydrolysis as a pretreatment for graft polymerization on PET.

Materials and Methods

Materials

The PET fabric (NBC Inc., Tokyo, Japan) and fiber (1.2 *d*, Teijin Fiber Ltd., Osaka, Japan) selected as typical polyesters were cleaned by soxhlet extraction with methanol for 24 hours, rinsed with distilled water, and dried at 60°C for 24 hours. γ-Methacryloxypropyl triethoxysilane (MPTS) was kindly donated by Shin-Etsu Chemical Co. Ltd. of Tokyo,

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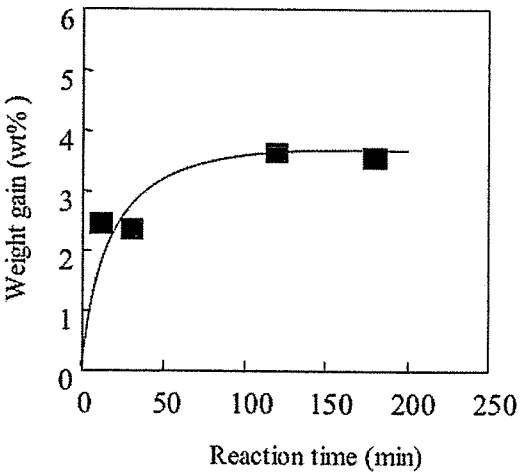

Figure 1. Weight gain of poly(MPTS)-grafted PET as a function of reaction time. Weight gain data were calculated as means of quadruplicate determinations. Error bars represent the standard deviation from the mean, and are included in the closed squares.

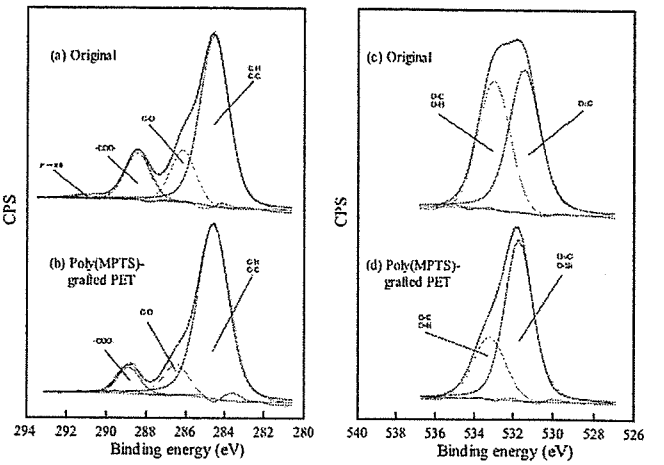

Figure 2. The XPS spectra of (a) original PET and (b) poly(MPTS)-grafted PET.

Japan. Benzyl alcohol (guaranteed reagent; Nacalai Tesque Inc., Kyoto, Japan), methanol (superior quality of reagent; WAKO Pure Chemicals Industries Ltd., Osaka, Japan), and 3% content H_2O_2 (superior quality of reagent; Sigma, Tokyo, Japan) were used without further purification. Water was purified with a Milli-Q system (Millipore Corp., Bedford, MA). HAp crystals with an average diameter of 50 nm were prepared by an alternating emulsion system and subsequently calcinated at 800°C for 1 hour, using a sintering agent such as calcium hydroxide as described in our previous reports.[7–9]

Graft Polymerization

Graft polymerization of MPTS onto alkaline-hydrolyzed PET fabric[16] was conducted using H_2O_2 as an initiator.[17] The PET fabric was carefully immersed in a 0.2 N aqueous NaOH solution for 30 minutes at 60°C and then rinsed with Milli-Q water in order to generate carboxyl groups on the surface. Carboxylate-functionalized PET fabric (0.03 g) was added into a 100-ml flask equipped with an inlet of N_2, a reflux condenser, and a stirrer, and then purged with N_2 for 30 minutes. As an initiator, 40 ml benzyl alcohol containing 785 μl H_2O_2 (100 mEq/l) was added to the flask. Subsequently, 10 ml (50 v/v%) MPTS monomer in benzyl alcohol was added, and the content was stirred occasionally during polymerization for a defined period. After the polymerization, the PET fabric was washed with ethanol several times to stop polymerization in order to remove ungrafted homopolymers formed during polymerization, and then dried under reduced pressure at 1.0 mm Hg for 24 hours at 70°C. The weight gain after polymerization was calculated from the following equation:

$$\text{Weight gain} = (W_2 - W_1)/W_1 \times 100$$

Table 1. Atomic Compositions on PET Surfaces Measured by XPS

Sample	C	O	Si
Original PET (atom%)	69.5	29.5	0.64
Poly(MPTS)-grafted PET (atom%)	60.7	29.5	9.76

where W_1 and W_2 are the weight of the dried original PET and the PET after the polymerization, respectively.

Coating of HAp Nanocrystals on PET

The poly(MPTS)-grafted PET fabric was soaked in the HAp suspension (2.0 wt/v%) in ethanol for 1 hour at room temperature to adsorb the crystals on the grafted PET. The fabric adsorbed with HAp particles was strongly washed by stirring in ethanol, and heated at 80°C for 2 hours under vacuum (1 mm Hg) in order to achieve the reaction between OH groups on the HAp crystals and ethoxysilyl groups on the poly(MPTS)-grafted PET. The composite was washed in ethanol using an ultra sonic generator for 1 minute (output: 20 kHz and 35 W) to remove unreacted HAp particles physically adsorbed on other particles. The composite was finally washed in a large amount of ethanol and pure water for 1 day to remove the residual organic solvents used in polymerization.

Cell Adhesiveness

Human umbilical vein endothelial cells (HUVEC) were incubated in endothelial cell basal medium-2 (EGM-2, supplemented with heat-inactivated 5% FBS, 1 mg/ml gentamicin/amphotericin B) in air containing 5% CO_2 at 37°C. The HUVEC were plated onto the HAp/PET composite fabric in 24-well multiplates at 1×10^5 cells/ml in EGM-2, and incubated at 37°C for 4 hours. After the fabrics adhered to the cells, they were washed twice in phosphate-buffered saline [PBS (–)], fixed with 2.5% buffered glutaraldehyde for 20 minutes at 30°C, and then rinsed with PBS(–) three times. The cells were dehydrated with aqueous ethanol (50–100%) and 100% *n*-butanol for 5 minutes at room temperature step by step. The samples were lyophilized and coated with gold. The morphology of the cells on the samples was observed by scanning electron microscopy (SEM). In the fluorescence observation, the nuclei of HUVEC on the fabric were stained with 1 μM Hoechst 33342 (Wako Pure Chemical Industries Ltd., Tokyo, Japan), and observed by fluorescence microscopy (Eclipse TE300, Nikon Co., Tokyo, Japan). In the cell adhesion experiment, 0.03; collagen-

Table 2. Results of the Peak Separations for C_{1s} and O_{1s} Spectra Measured by XPS

		C_{1s}				O_{1s}	
Binding energy	(eV)	284.6	286.2	288.5	291.0	531.5	533.0
Chemical state		C-H C-C	C-O	-COO-	$\pi \rightarrow \pi^*$	O=C O-Si	O-C O-H
Original PET	(atom%)	67.7	16.2	15.1	1.0	55.4	44.6
Poly(MPTS)-grafted PET	(atom%)	81.5	10.6	7.9	0.0	72.0	28.0

coated (Cellmatrix Type I-C, Nitta Gelatin Inc. Osaka, Japan) PET fabric was used as a positive control.

Measurements

Weight gain was measured by using a high precise balance (GR-202, A&T Co. Ltd., Tokyo, Japan), which shows 0.01 mg of the minimum weight (±0.02 mg). The HAp/PET composite and its cell morphology were observed with a 5 kV scanning electron microscope (SEM; JSM-6301F, JEOL, Tokyo, Japan). To characterize the surface-modified samples, x-ray photoelectron spectroscopy (XPS, 1600S type, PHI Inc., Tokyo, Japan) was used. The power of the nonmonochromated MgKα source was 100 W with an investigated size of 0.8 × 2.0 mm. Tensile properties were measured with the use of a Tensilon RTC-115 OA (Orientic Co. Tokyo, Japan) at an elongation of 50 mm/min. Measurement was conducted with 60 pieces of fiber samples of 40 mm long at 20°C and relative humidity of 65%. Data of weight gain and tensile properties were calculated as means of quadruplicate determinations. Error bars represent standard deviation from the mean. Statistical comparisons were performed with the use of the Student's *t* test, and *p* values less than 0.01 were considered significant.

Results and Discussion

Graft Polymerization

The HAp nanocrystals were covalently coated on the PET fabric through poly(MPTS)-grafted polymers. Alkoxysilyl groups of MPTS can be coupled with OH groups on HAp surfaces. Before coating the HAp crystals, it was necessary to chemically modify the PET surface by radical donation. Many methods of radical donation on a polymer surface are used to graft polymerize with vinyl monomers on PET, such as high-energy radiation of j-rays,[18] benzoyl peroxide,[19] hydrogen peroxide,[20] and persulfate.[21] For graft polymerization of MPTS on PET, we used H_2O_2 in benzyl alcohol as an initiator according to the method of Hebeish *et al.*,[17] because H_2O_2 treatment is easy to handle and a large facility is not necessary. The graft polymerization of MPTS on PET after the radical donation with H_2O_2 could not be accomplished, although the graft polymerization procedure was, at first, faithfully performed. The process of weak hydrolysis of the 0.2 N aqueous NaOH solution used to donate carboxylate functional groups, therefore, was added before the H_2O_2 treatment. **Figure 1** shows the weight gain of poly(MPTS) on the PET fabric, which was plotted as a function of the reaction time. The error bars representing standard deviation of quadruplicates determinations are included in closed squares in the figure. The weight gain of poly(MPTS) increased with increasing reaction time, and eventually reaching a plateau value of about 3.5 wt% using PET fabric consisting of a fiber diameter of 30 μm. However, Hebeish *et al.* reported a of 4.0 wt% maximum weight gain of graft polymerization of polyacrylic acid (AAc) on 1.2-denier PET consisting of a 11 μm fiber diameter in the same reaction solvent.[17] Compared with our simple calculation, the existing monomer ratios of poly(MPTS) and poly(AAc) per a unit of PET surface are 0.9×10^{-3} and 1.4×10^{-3} mol/m^2, respectively. The difference might depend on monomer reactivity for the solvent.

In the characterization of graft polymerization of poly(MPTS) on PET using attenuated total reflection Fourier transform infrared spectrometry (ATR FT-IR), the sign of the grafting with 3.5 wt% was not observed because the band of the Si-O-C stretching vibration of the alkoxysilyl groups overlapped with strong adsorption of an ester (C-O) stretching vibration of the PET substrate at around 1,100 cm^{-1}. Thus, XPS measurement, which enables a more sensitive surface analysis, was performed to characterize the graft surface (3.5 wt%) of PET. **Table 1** shows the change in the atomic proportions on the outermost surface of the original and poly(MPTS)-grafted PET. Although the atomic ratio of the carbon of poly(MPTS)-grafted PET was lower than that of the original PET, the silicon of the grafted PET was higher than that of the original one. The original PET initially contained a very small quantity of silicon contamination. This impurity is assumed to get mixed in during the manufacturing process of PET. **Figure 2** shows XPS C_{1s} and O_{1s} spectra of the original and grafted PET, and **Table 2** shows the quantified data from the area of the curve fitting these XPS signals. With high resolution of XPS C_{1s} spectra, they were resolved at 284.6, 28.62, 288.5, and 291.0 eV due to the C-C/C-H, C-O, -COO-, and $\pi \rightarrow \pi^*$ components, respectively.

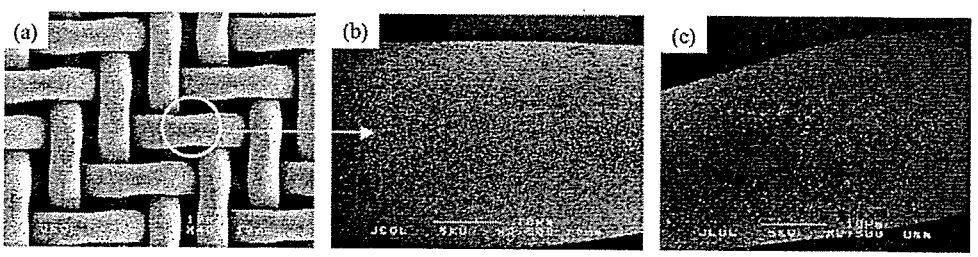

Figure 3. SEM photographs of HAp particles covalently coated on a PET fabric. Lower magnification of HAp/PET composite surface after ultrasonic treatment, (b) higher magnification of HAp/PET composite surface of (a), (c) higher magnification of HAp nanocrystals adsorbed on original PET surface after ultrasonic treatment.

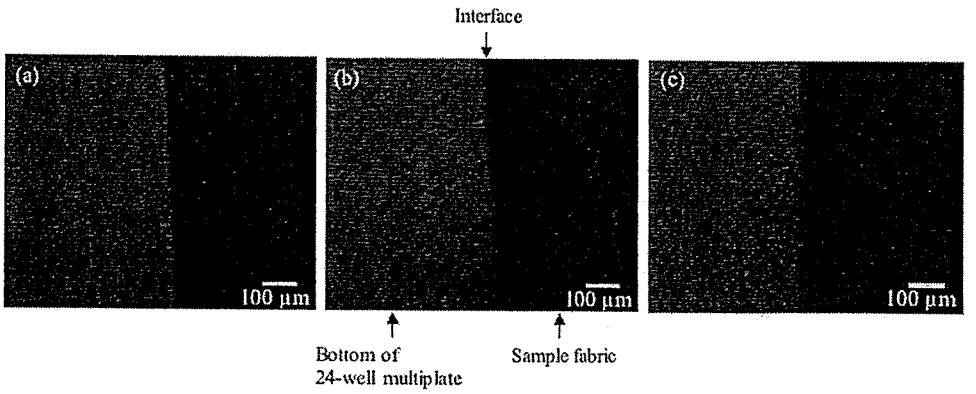

Figure 4. Optical microscopic images of cell toxicity on (a) original PET, (b) poly(MPTS)-grafted PET and (c) HAp/PET composite were incubated after 24 hours.

Although the area of the C-C/C-H component of the grafted PET increased compared with that of the original PET, the area of the C-O and –COO- components decreased. In O_{1s} spectra, however, they were resolved at 531.5 and 533.0 eV due to the O = C(O-Si) and O-C/O-H components, respectively. After graft polymerization of poly(MPTS), the area of O = C(O-Si) and O-C/O-H increased and decreased, respectively. For the control of poly(MPTS)-grafted PET, XPS measurement was conducted against the sample fabric that was adsorbed with MPTS homopolymers and washed strongly with a large amount of ethanol. The value of the silicon component was almost the same as the original PET in the XPS spectra. From the XPS results, it was clear that graft polymerization on PET was well conducted by our process.

Coating of HAp Nanocrystals on PET

The HAp crystals were coated on poly(MPTS)-grafted PET fabric through covalent bonding. Because the covalent bonding could not be observed directly, the chemical bonding was estimated indirectly using a FT-IR analysis that has been described in previous literature.[10] **Figures 3a** and **3b** show SEM photographs of HAp/PET fabric after treatment with an ultrasonic generator. As for the negative control, an SEM image of the original PET surface after ultrasonic treatment on the nongrafted PET surface adsorbed with HAp nanocrystals is shown in **Figure 3c**. Although the HAp crystals adsorbed on the original fabric were almost removed by the ultrasonic treatment, they strongly adhered to the poly(MPTS)-grafted PET surface. The crystals could be uniformly coated with almost every crystal without severe aggregations, because almost all single-dispersed nanocrystals in a medium can be developed using antisintering agents.[9]

For tensile properties measurement, a PET fiber was used because a fabric was very difficult for determination of exact values. Tensile strength and elongation at break were measured with the use of HAp crystal coated 60 pieces of PET fiber by means of quadruplicate determinations. The values of tensile strength of original PET and HAp/PET showed at 468 ± 5 and 417 ± 13 MPa, respectively. The tensile strength of HAp/PET was lower statically by about 10% than that of original PET by using the Student's *t* test. On the other hand, the values of the elongation at break indicated at 24.4 ± 0.8 and 23.0 ± 3.0%, respectively. Although there was no difference statically between the values, it showed a tendency to slightly decrease by HAp coating. In previous studies, HAp coating did not damage the mechanical strength of the substrates, such as silicone sheet[22] and silk fiber.[11] Before HAp coating, in this case, PET was hydrolyzed by 0.2 N aqueous NaOH solution to introduce functional groups on the surface. The alkaline-hydrolysis in the pretreatment process assumes to affect the decrease of mechanical properties. We are now trying new coating process to present no mechanical disadvantage. Actually, the HAp coating PET fabric remained as flexible as the original fabric.

Cell Adhesiveness

To evaluate cell toxicity simply, at first the interface of cells/fabric of the original PET, poly(MPTS)-grafted PET and HAp/PET composite incubated for 2 days were observed by using an optical microscope (**Figure 4**). The sample fabrics were cut in half and put into the bottom of a 24-well multiplate. The cells around the interface of the fabrics of poly(MPTS)-grafted PET and the HAp/PET composite adhered and proliferated well, the same as those on the original PET. Therefore, it was clear that the composite material did not express cell toxicity. **Figures 5** and **6**, respectively, show SEM images of HUVEC morphologies and fluorescence images of stained nuclei of HUVEC on sample substrates after 4 hours of

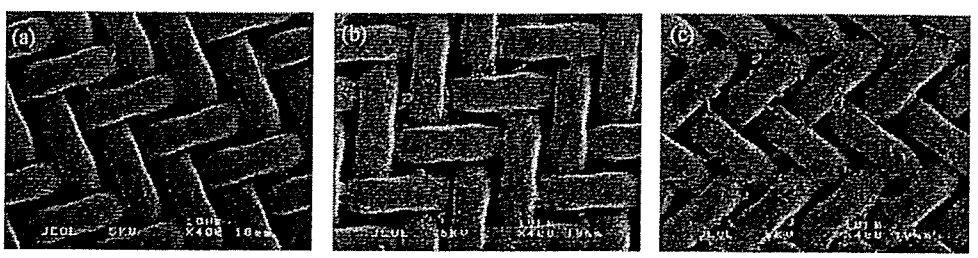

Figure 5. Scanning electron microscopy photographs of HUVEC adhering to (a) original PET, (b) collagen-coated PET, and (c) HAp/PET composite.

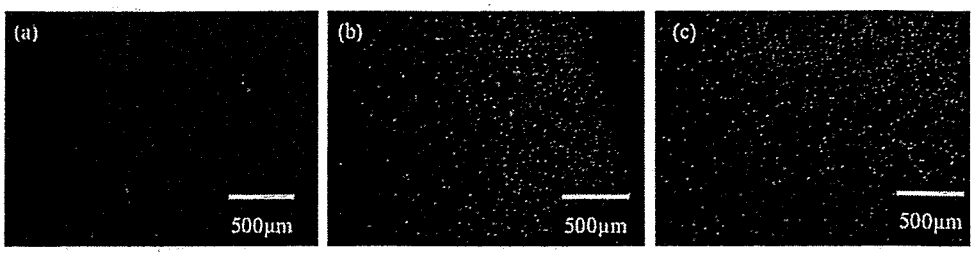

Figure 6. Fluorescence photographs of HUVEC nuclei on (a) original PET fabric, (b) collagen-coated PET, and (c) HAp/PET composite.

incubation. The initial interaction of HUVEC on substrates was evaluated after 4h of incubation, according to several reports.[23,24] In the SEM images, it seemed that many cells adhered on HAp/PET fabric as well as collagen-coated PET, while only a few cells adhered on the original fabric. The difference in the number of cells which adhered could not be distinguished by SEM observation, since HUVEC were flattened and spread over the substrate. The cells which adhered were then stained by fluorescent stain and observed by a fluorescence microscope. It was found that the number of cells which adhered on HAp/PET was qualitatively the same as that of collagen-coated PET, although the cells seldom adhered on the original PET for such a short period of incubation. This phenomenon may be explained by the fact that cell adhesion proteins, such as fibronectin, vitronectin, etc. in FBS of a culture medium, may be favorably adsorbed on the HAp surface.[13] In other words, it is clear that calcinated HAp coating on popular biomedical substrates is effective to obtain the affinity of cells without using biological scaffold proteins such as collagen or gelatin. It can be said that the HAp/PET composite is very meaningful for biological safety in medical fields today when the danger of BSE infection by using proteins derived from a bovine animal is trumpeted loudly. A prototype of artificial blood vessel made of the HAp/PET composite was fabricated (**Figure 7**), and the effect of HAp nanocrystals on it through animal implantation experiments *in vivo* is being evaluated now.

In conclusion, a novel composite consisting of nano-scaled HAp crystals and PET through covalent linkage was developed. In a cell toxicity test, it was confirmed that the HAp/PET composite was nontoxic, and HUVEC adhered more plentifully on the HAp/PET composite compared to the original PET and to the same degree as collagen-coated PET after only 4 hours of incubation. This result demonstrates that the coating of sintered HAp nanocrystals is a simple method for making a polyester substrate bioactive, and that the coating of HAp nanocrystals is superior in the terms of biologic safety.

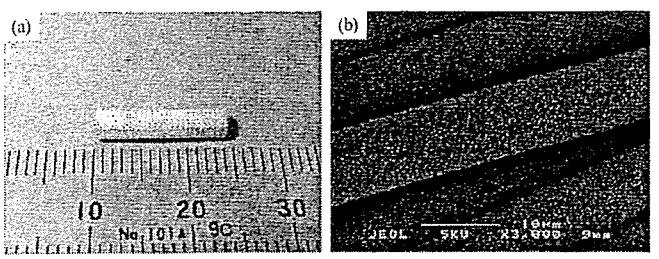

Figure 7. The images show a prototype of an artificial blood vessel made of HAp/PET composite. (a) External view of the prototype. (b) SEM image of HAp/PET fibers inside of the prototype.

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Development of Nano-Ceramic Coating on a Substrate through Covalent Linkage and Its Biological Properties

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Keywords: hydroxyapatite, coating, covalent bonding, biological property

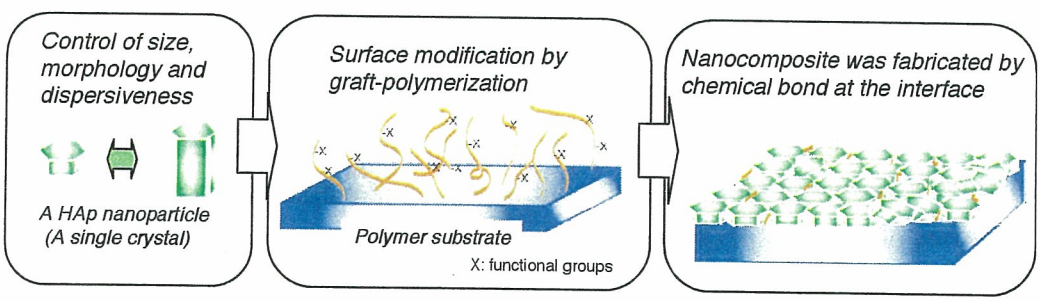
Abstract. Hydroxyapatite (HAp) has unique properties for biomaterials such as hard-tissue-compatible material for bone and tooth and also soft-tissue-compatible materials for skin tissue. However, the hard and brittle nature of HAp limits spreading many medical devices. Recently, a unique composite of sintered HAp nano-crystals -- ceramics -- covalently coupled to polymer substrates was developed -- *Nano-Ceramic Coating* --. The development was depended on the two original technologies: (1) control of size/morphology of high-dispersed sintered nano-HAp, (2) donation of covalent bonding between nano-HAp and substrates to coat strongly on the surface. The nano-composite material holds not only the mechanical properties of the substrate but also biological properties of the ceramics coated on the surface. In this report, the method of synthesis of high-dispersed nano-HAp, the preparation of the nano-composite, the biological properties with cells or animal tissue, and especially, the development of medical devices, such as percutaneous device or blood vessel and so on, made of the composite will be presented.

Introduction

Hydroxyapatite (HAp: $Ca_{10}(PO_4)_6(OH)_2$) has unique properties for biomaterials such as hard-tissue-compatible material for bone and tooth also soft-tissue-compatible material for skin tissue. From the point of view of tissue-compatible material made up of HAp, The hard and brittle nature of HAp limits spreading over many medical applications. To overcome the defects of ceramics maintaining the nature of HAp, we developed a novel inorganic-organic composite, which calcined HAp nanoparticles

(crystals) [1] coated on polymer substrate by chemical bonding, such as covalent or ionic linkage [2-5]. The significant feature of our fabrication is formation of a nano-scaled ceramic layer on substrate surface without damage to the mechanical properties of the polymer substrate. The technology of nano-ceramic coating can be applying to increase of adhesiveness between hard materials and soft tissue in living body, and developing novel biomaterials which is compatible for soft-tissue, such as percutaneous device, artificial blood vessel, or a scaffold for regenerative medicine, and so on.

The design of the nano-composite consisting of calcined HAp nanoparticles and polymer substrates depends on two key technologies: (I) control of size, morphology and dispersiveness of calcined HAp nanoparticles, (II) fabrication of inorganic-organic composite by chemical bonding between the interface as shown in **Scheme 1**. In this report, the method of synthesis of high-dispersed nano-HAp, the preparation of the nano-composite, the biological properties with cells or animal tissue, and especially, the development of medical devices, such as percutaneous device or blood vessel and so on, made of the composite were presented.



Scheme 1 Schematic presentation of an inorganic-organic composite material by chemical bonding between the interface.

Calcined HAp Nanoparticles for Nanofabrication

This subsection describes the preparation of HAp nanoparticles by calcination with an anti-sintering agent interspersed between the particles and the subsequent removal of the agent [6-8]. There was no contact between the particles during calcination. $Ca(OH)_2$ was selected as an anti-sintering agent because it would not melt at the calcination temperature (800 °C), presumably not dissolve the HAp, and could be removed by washing with water after calcination. The HAp nanoparticles obtained

here should be suitable for the surface coating described the later section owing to their high dispersibility in liquid media and high thermal and chemical stability.

Starting HAp particles with low crystallinity were prepared by a modified emulsion system at 25°C. The resulting product was centrifugally washed and redispersed in water (solid content: 5 wt%). In order to intersperse $Ca(OH)_2$ -- an anti-sintering agent -- between the particles, the HAp aqueous dispersion was added into a saturated aqueous $Ca(OH)_2$ solution (0.17 wt%), and the mixture was dried under reduced pressure at 40°C. The resultant HAp/$Ca(OH)_2$ (1/1, w/w) mixture was calcined at 800°C for 1 h in air (heating rate: 10°C/min). After calcination, the mixture was centrifugally washed with water to remove the $Ca(OH)_2$. As a control, HAp was calcined using the same procedure without adding $Ca(OH)_2$.

The sizes of the crystals dispersed in ethanol measured by dynamic light scattering were quite different between the two kinds of the calcined HAp with and without $Ca(OH)_2$ as shown in **Fig. 1**. In the case of calcination without $Ca(OH)_2$ shown as the solid columns, the mean size of the HAp crystals dispersed in HAp crystals were dispersed as agglomerates consisting of sintered polycrystals whose mean size indicated about 600 nm. On the other hand, calcining with the anti-sintering agent, the mean size of the crystals was much smaller than that of without the agent. The average size of the HAp nanoparticles shows about 80 nm. The results indicate that the sintering among HAp nanocrystals could be avoided by calcination with $Ca(OH)_2$ interposed among the crystals, followed by removing of $Ca(OH)_2$ after calcination. It was clear that the HAp nanoparticles were scattered by a single particle on a SEM mount.

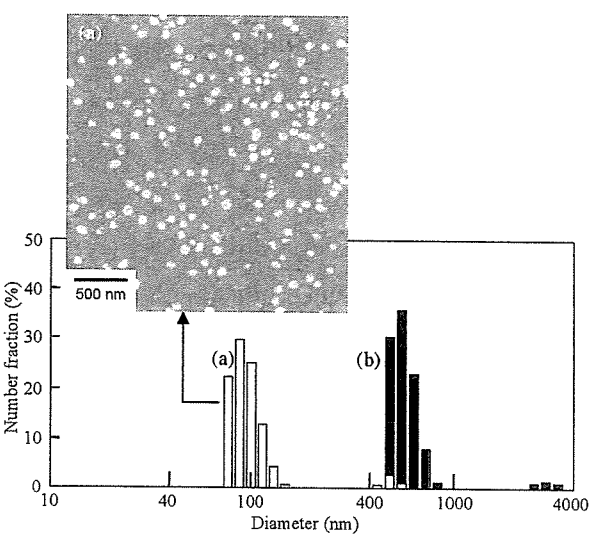


Fig. 1 Size distributions of HAp particles calcined at 800°C for 1 h with (a) and without (b) an anti-sintering agent, The size distribution was measured in ethanol as a medium by using dynamic light scattering.

Nano-HAp coating by chemical bonding

Graft-polymerization with γ-methacryloxyethyl trimethoxysilane(MPTS) monmer having an alkoxysilyl group on SF fibers with 100 μm of length was conducted by free radical initiation. After the HAp particles were suspended in a toluene/methanol (9/1) mixture solvent, a poly(MPTS)-grafted SF was soaked in the suspended solution for 1h at room temperature to be adsorbed on the SF. The SF adsorbed with the HAp particles was washed by stirring in methanol and filtered by a filter with a 5-μm cut-off point to remove unreacted HAp particles. The fibers adsorbed with HAp were heated at 120 °C for 2h in vacuum at 1mmHg for a reaction between the HAp surface and the alkoxysilyl groups of the graft polymers. The composite was washed by using an ultrasonic generator for 3 min (output: 20 kHz and 35W) to remove excess adsorbed HAp particles attached to ones in ethanol. Finally, the composite was washed in a great amount of distilled water for 1 day to remove the residual organic solvents used in the synthetic process. **Fig. 2** shows an SEM photograph of the HAp-coated SF fiber with covalent bonding. A fiber cut approximately 100 μm in length was more effective for the coating than that of a fabric-form. The HAp particles separated and slightly aggregated into several crystals under SEM observation. Generally, aggregation is easy because the HAp mono-particle has an a-plane with a cationic charge and a c-plane with an anionic charge in a mono-crystal. Our high-dispersed HAp nano-crystals were suitable for preparation of mono-layer-like nano-HAp coating.

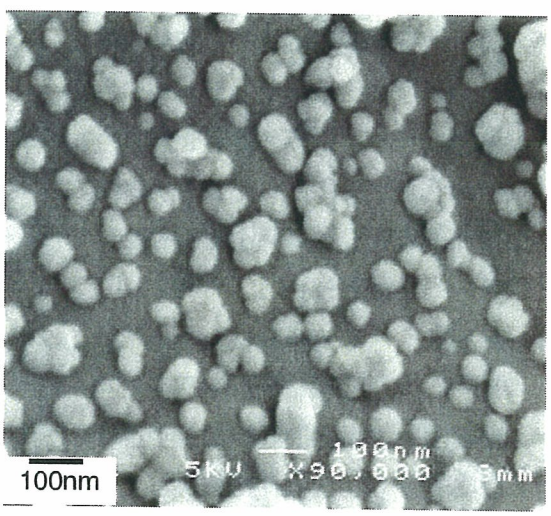


Fig. 2 SEM image of surface of HAp-coated SF by covalent linkage.

Cell Interaction

To evaluate the cell adhesiveness on the HAp-coated silk fibroin (SF), the morphologies of L929 fibroblast cells incubated on sample fabrics were observed by

SEM. **Fig. 3** shows SEM observations of the surfaces of sample fabrics --- gelatin-coated glass as a positive control (a), untreated SF (b), hydrolyzed poly(MPTS)-grafted SF (c) and HAp-coated SF (d) --- incubated with L929 cells for 24 h. The cells hardly adhered on the hydrolyzed poly(MPTS)-grafted SF as well as the untreated SF in **Fig. 3 (b, c)**. Although it has been known that the initial cell adhesion on intact SF is actually not good, the reason has not been thoroughly manifested. It is presumed to depend on high surface wettability due to containing many hydrophilic amino acid residues[17] --- the hydroxyl group: Ser 10.63 mol%, Tyr 4.97 mol%, Thr 0.89 mol%; the carboxyl group: Asp 1.65 mol%, Glu 1.21 mol%; the amino groups: Lys 0.33 mol%, His 0.18 mol%, Arg 0.49 mol% --- and peptide bonds without an arrangement of Arg-Gly-Asp (RGD) as a cell-adhesion molecule, or probably the existence of a

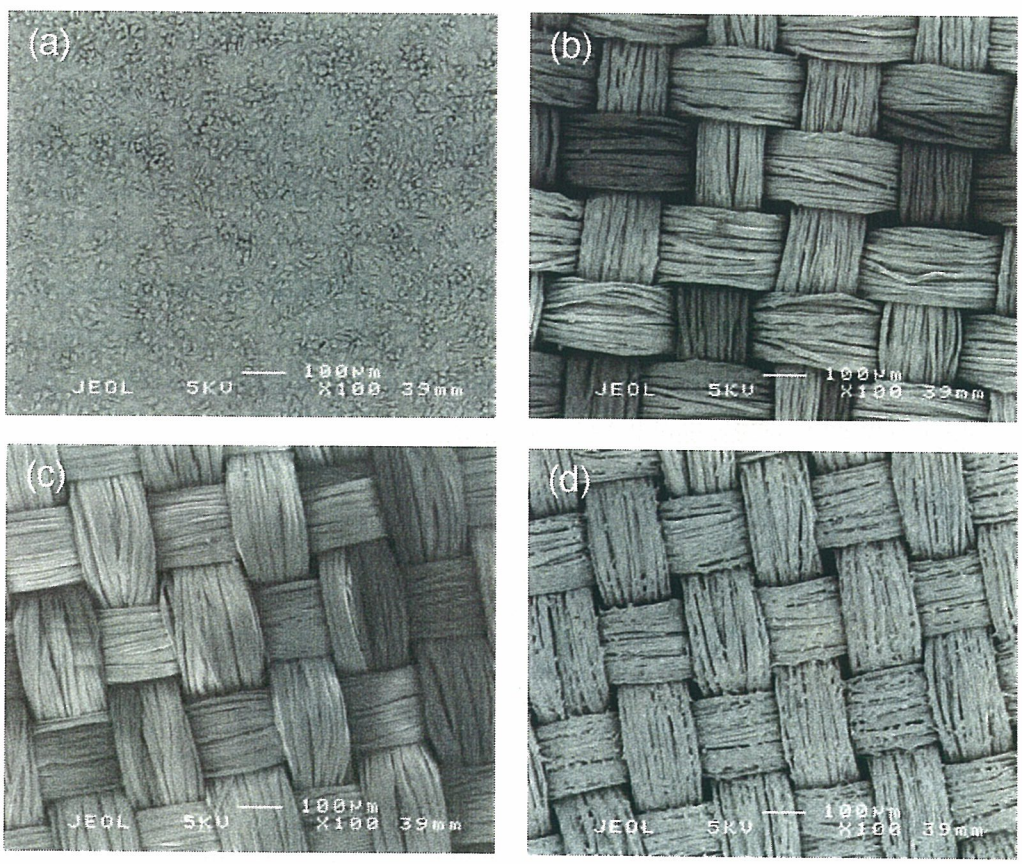


Fig. 3 SEM photographs of cell morphologies on (a) gelatin-coated grass, (b) original SF fabric, (c) hydrolysed poly(MPTS)-frafted SF fabric, and (d) calcined HAp nanopartilces covalently coated on an SF fabric.

microdomain structure consisting of crystalline and amorphous regions attributed to an arrangement of $(Gly\text{-}Ala\text{-}Ser)_n$ and the other residues in SF . The cells indicated weak interaction with the hydrolyzed poly(MPTS)-grafted surface during the initial incubation period because of the surface hydrophilicity belonging to Si-OH moieties on Si-O-Si cross-linking networks produced by hydrolysis of the poly(MPTS)-grafted SF substrate. Meanwhile, the cells adhered well on HAp-coated SF as shown in **Fig. 3 (d)**. It is clear that cells, thus, favorably adhere only on the HAp surface of the composite but not on the dehydrated grafted-surface without HAp particles on the SF substrate. It is estimated that cell-adhesion proteins in serum, such as fibronectin, vitronectin, bFGF, etc., prior to cell adhesion, adsorb on a HAp surface much better than on an area of dehydrated graft-polymer. That is to say, the surface of the HAp nanocoating can provide bioactivity to a polymer substrate.

Fabrication of Medical Devices

A Percutaneous Device. To fabricate a prototype for a percutaneous device, the HAp-coated SF fibers were transplanted onto a button-shaped substrate made of silicone via an adhesive agent. The percutaneous device was implanted in back of a rabbit for 3 months according to the Guideline for Animal Experimentation National Cardiovascular Center. The skin tissue was adhered on the device without a gap between the tissue and the material surface, and severe inflammation and abscess was not observed from the external view (**Fig. 4**).

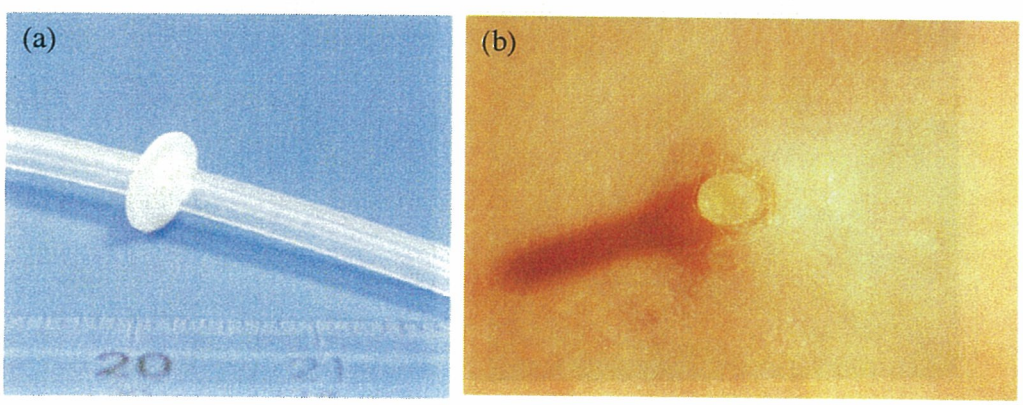


Fig. 4 External views of the button prototype of a percutaneous device (a) and the surrundings of the device implanted into back of a rabbit for 3 months.

An Artificial Blood Vessel. We developed a novel inorganic-organic composite consisting of calcined HAp nanoparticles chemically bonded on polymer substrate. HAp nanoparticles were covalently linked onto a poly(ethylene terephthalate) (PET) fabric substrate chemically modified by graft polymerization with MPTS for development of artificial blood vessel. A prototype of artificial blood vessel made of the HAp/PET composite was fabricated (**Fig. 5**). The calcined HAp nanoparticles were thoroughly coated on PET fibers of inside and outside of an artificial blood vessel [9]. The effect of HAp nanocrystals on it through animal implantation experiments *in vivo* are evaluating now.

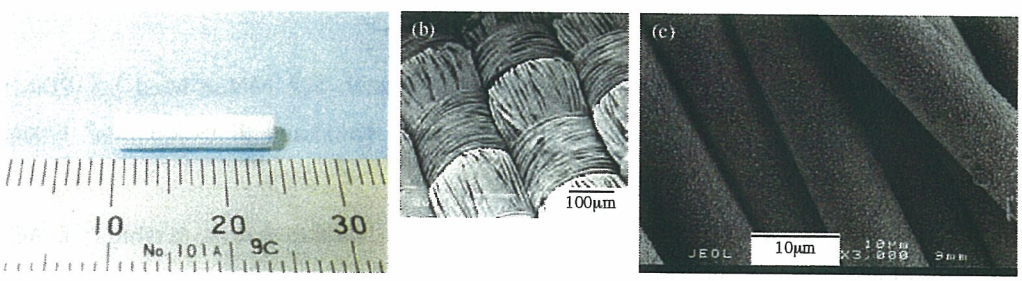


Fig. 5 Images show prototype of an artificial blood vessel made of HAp/PET composite. (a) External view of the prototype. (b, c) lower and higher magnification of SEM images of HAp/PET fibers of inside of the prototype.

Conclusions

The inorganic-organic composite consisting of calcined HAp nanoparticles and polymer substrates were prepared through chemical bonding, such as covalent or ionic bonding. Preparation of composites consisting of calcined HAp nanoparticles and polymer substrates, for examples, mixture, *in situ* or nano-chemical bonding methods, is necessary for nano-scaled observation from the points of view of the bulk structures, surface properties, biological interactions, etc. For our composite, especially, the size, coverage ratio or strength of chemical bonding of sintered HAp nanoparticles assumed to be very important to know interactions with biomolecules such as proteins, cells, tissues to develop medical devices. This composite material is expected to establish a novel concept for fabrication of an inorganic-organic composite

as biocompatible materials for hard and soft tissue.

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LETTER

Fabrication of high-dispersibility nanocrystals of calcined hydroxyapatite

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Hydroxyapatite (HAp) is a major inorganic component of bone and teeth. Artificially synthesized HAp has been extensively used in a variety of applications, such as biomaterials, ion exchangers, adsorbents, and catalysts, by exploiting its biocompatibility and adsorbability of many compounds. When low-crystallinity HAp nanoparticles are calcined to increase thermal and chemical stability, the particles typically sinter into a large agglomerate consisting of polycrystal [1–5]. Thus, calcined HAp crystals dispersed in liquid medium on a nanoscale have been difficult to obtain. This paper describes the fabrication of HAp nanocrystals by calcination with an anti-sintering agent interspersed between the particles and the subsequent removal of the agent. The HAp nanocrystals obtained here should be suitable for the above applications owing to their high dispersibility in liquid media, high specific surface area, and high thermal and chemical stability.

We have recently developed a novel inorganic/organic composite for a soft-tissue-compatible material: a flexible silicone elastomer [6] or a silk fibroin [7], whose surface was modified with calcined HAp crystals. After the HAp crystals dispersed in a liquid medium were adsorbed on the substrate, chemical reaction at their interface between the HAp crystals and the substrate was conducted to connect them through covalent bonding. The novel composite retained flexibility of the polymer substrate and showed improved tissue adhesion with the HAp crystals on the surface [8]. Throughout these studies, the HAp crystals were used after calcination at 800 °C to reduce in vivo absorbability. As mentioned above, HAp nanoparticles mostly sinter into large agglomerates of polycrystals during calcination. This made it difficult to control the surface morphology of the composite because the agglomerates had poor dispersibility in liquid media and large size distribution.

Hydrothermal treatment of HAp particles in water medium under high pressure is known to enable the preparation of agglomerate-free HAp crystals [9–11]. However, this treatment generally leads to an increase in crystal size due to Ostwald ripening [12, 13], and is restricted to laboratory-scale products as it is a high-pressure process.

The present study reports the fabrication of nanosized and calcined HAp crystals protected against calcination-induced sintering using an anti-sintering agent interspersed between the particles. Thus, there was no contact between the crystals during calcination. Calcium hydroxide [$Ca(OH)_2$] was selected as an anti-sintering agent because it would not melt at the calcination temperature (800 °C), presumably not dissolve HAp, and could be removed by washing with water after calcination.

Starting HAp particles with low crystallinity were prepared with a modified emulsion system at 25 °C [14]. The resulting product was centrifugally washed and redispersed in water (solid content: 5 wt%). In order to intersperse $Ca(OH)_2$ between the particles, the HAp aqueous dispersion was added into a saturated aqueous $Ca(OH)_2$ solution (0.17 wt%), and the mixture was dried under reduced pressure at 40 °C. The resultant

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$HAp/Ca(OH)_2$ (1/1, w/w) mixture was calcined at 800 °C for 1 h in air (heating rate: 10 °C/min). After calcination, the mixture was centrifugally washed with water to remove the $Ca(OH)_2$. As a control procedure, the same starting HAp particles were calcined without adding $Ca(OH)_2$.

First, in order to examine the effect of $Ca(OH)_2$ on the crystal phase and composition of HAp, X-ray diffraction (XRD; RAD-X, Rigaku International Co., Tokyo, Japan) with CuKα radiation and Fourier-transform infrared (FTIR) spectroscopy (Spectrum One, Perkin-Elmer Inc., MA, USA) were performed as shown in Fig. 1. In Fig. 1a and b, both XRD profiles showed highly crystalline HAp, and no other calcium phosphate phases could be detected. In the FTIR spectrum of HAp calcined with $Ca(OH)_2$ shown in Fig. 1d, a peak at 3,640 cm^{-1} due to stretching of OH in $Ca(OH)_2$ was not observed, indicating complete removal of $Ca(OH)_2$. Bands at 877 and 1,413/1,456 cm^{-1} observed in both the FT-IR spectra are attributed to CO_3^{2-} substituting phosphate positions in HAp lattice [15], and came from atmospheric carbon dioxide under high solution pH during the preparation of the starting HAp. A new peak at 3,544 cm^{-1} in Fig. 1d seems to be due to the formation of calcium-rich apatite (Ca/P atomic ratio > 1.67) [16]. It is worth pointing out that the Ca/P atomic ratio of the HAp calcined with $Ca(OH)_2$ (Ca/P = 1.58) slightly increased as compared with that without additives (Ca/P = 1.56) measured by inductively coupled plasma-atomic emission spectrometry (SPS4000, Seiko Instrument Inc., Chiba, Japan). These results might suggest the formation of calcium-rich apatite from the crystal surface during calcination, by migration of calcium ions from $Ca(OH)_2$ on the calcium-deficient crystal.

The HAp crystals were observed by scanning electron microscopy (JSM-6301F, JEOL Ltd., Tokyo, Japan) and transmission electron microscopy (JEM-2000 EXII, JEOL Ltd.) as shown in Fig. 2. Single-crystal size (or grain size of polycrystal) was measured from the micrographs, and is presented as "mean ± SD" (N = 100). The crystal sizes were not statistically different between the HAp crystals calcined without and with $Ca(OH)_2$ (52.5 ± 15.4 nm for calcination without additives; 55.7 ± 15.1 nm for calcination with $Ca(OH)_2$). Although some agglomerates were observed in Fig. 2b, it is difficult to judge whether the agglomerates were polycrystals or not. This is because the micrograph was taken under dry condition and nano-sized particles generally tend to gather due to a capillary force of the medium between the particles during the drying of the medium.

Therefore, the dispersibility of the HAp crystals was evaluated form the dispersed-particle size, which was measured in ethanol medium by dynamic light scattering (DLS; ELS-8000, Otsuka Electronics Co., Ltd., Kyoto, Japan) at 10-ppm concentration and a light-scattering angle of 90° (Fig. 3). In the case of calcination without

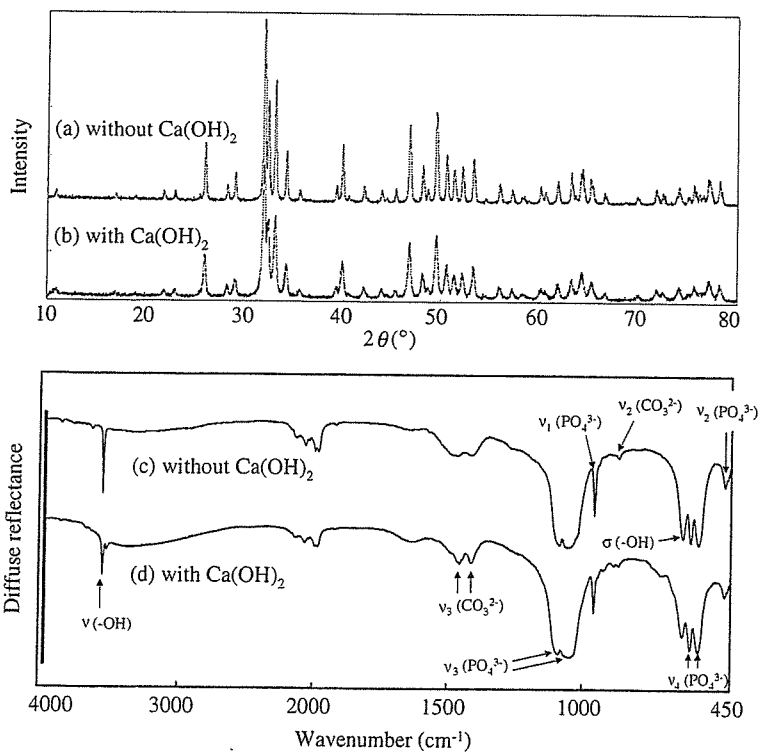


Fig. 1 X-ray diffraction patterns (**a**, **b**) and FTIR spectra (**c**, **d**) of hydroxyapatite (HAp) particles calcined at 800 °C for 1 h without (**a**, **c**) and with (**b**, **d**) $Ca(OH)_2$ (HAp/$Ca(OH)_2$ = 1/1 w/w) interspersed between the particles. $Ca(OH)_2$ was centrifugally washed off with an aqueous solution after calcination

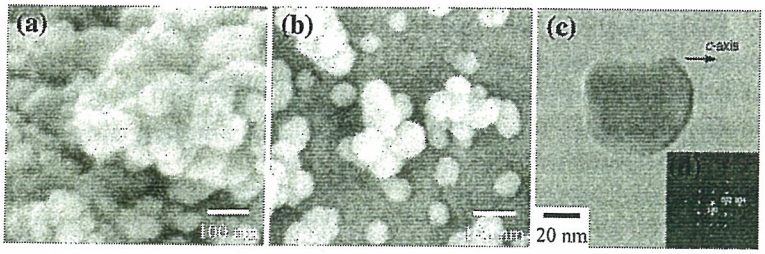


Fig. 2 SEM photographs (**a**, **b**) of HAp crystals calcined without (**a**) and with (**b**) $Ca(OH)_2$. A TEM photograph (**c**) and the associated electron diffraction pattern corresponding to the [210] zone (**d**) of a HAp crystal calcined with $Ca(OH)_2$, showing that the crystal consisted of a single HAp phase

additives, the dispersed-particle size (664.0 ± 382.0 nm) was much larger than the crystal size (52.5 ± 15.4 nm), which indicates the formation of polycrystals of sintered HAp. On the other hand, the dispersed-particle size of the HAp calcined with $Ca(OH)_2$ was 126.3 ± 79.0 nm. This indicates that sintering between the HAp nanocrystals can be mostly prevented by interspersing $Ca(OH)_2$ between the crystals prior to calcination.

However, the dispersed-particles size (126.3 ± 79.0 nm) was larger than the crystal size (55.7 ± 15.1 nm) measured by electron microscope. This suggest that some particles contacted with each other before the deposition of $Ca(OH)_2$ from the aqueous solution. In order to avoid the contact, poly(acrylic acid) (PAA) was used. PAA can act as a dispersion stabilizer by adsorbing on HAp surfaces [17, 18], and an addition of calcium ions into an aqueous PAA solution induces a rapid precipitation of poly(acrylic acid calcium salt) (PAA-Ca). Therefore, it is expected that PAA-Ca precipitates onto the separated HAp particle by addition of calcium ions into the PAA-adsorbed HAp particles. Although the organic component of PAA-Ca will be decomposed during calcination at 800 °C, the thermally decomposed product (CaO) remains on the crystals and presumably acts as an anti-sintering agent.

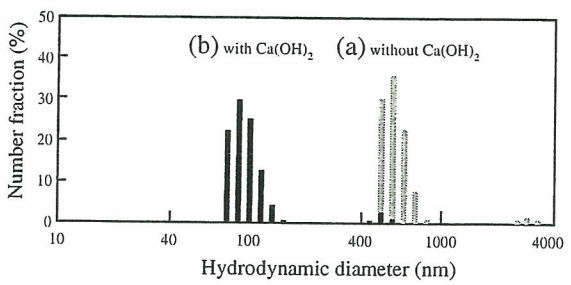
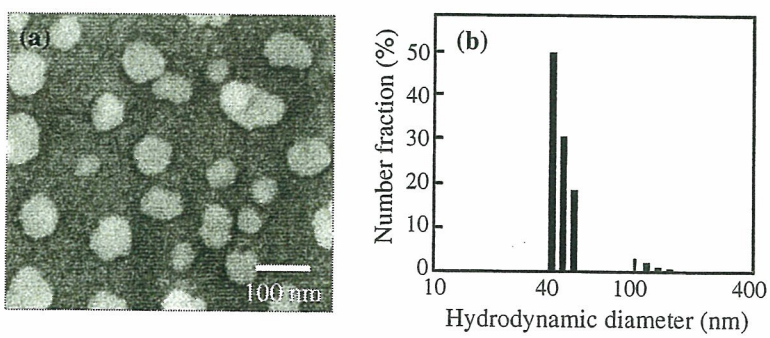


Fig. 3 The dispersed-particle sizes of HAp crystals calcined without (**a**) and with (**b**) $Ca(OH)_2$, measured in ethanol medium

In the present study, PAA (Sigma-Aldrich Co.; weight-average molecular weight = 15,000; HAp/PAA = 1/1 w/w) was added at pH 10, and $Ca(OH)_2$-saturated aqueous solution ($Ca(OH)_2$/COOH in PAA = 1/1 molar ratio) was added to precipitate PAA-Ca. The resultant HAp/PAA-Ca mixture was calcined in the same manner described above, and then washed with water to remove CaO.

The resultant HAp crystals are shown in Fig. 4. The dispersed-particle size of the HAp crystals in ethanol (45.0 ± 14.9 nm) was not statistically different from the crystal size (53.4 ± 16.2 nm) measure by electron microscope. This result indicates that HAp nanocrystals calcined with PAA-Ca can be dispersed as single crystals. IR spectrum and XRD pattern of the HAp single crystals calcined with PAA-Ca were almost the same as those shown in Fig. 1b and d, and Ca/P atomic ratio was 1.58. The achieved high dispersibility of HAp single crystals should be due to the absence of the calcination-induced sintering and might be due to cationic charge of the calcium-rich surface.

Fig. 4 A SEM photograph (**a**) and the dispersed-particles size (**b**) of HAp crystals calcined at 800 °C for 1 h with poly(acrylic acid calcium salt) (PAA-Ca) surrounding the particles. CaO, the thermally decomposed product of PAA-Ca, was centrifugally washed off with an aqueous solution after calcination



In summary, calcined HAp nanocrystals were successfully fabricated by calcination using an anti-sintering agent interspersed between or surrounding the particles, followed by removal of the agent. The HAp nanocrystals obtained here should be suitable for the various applications mentioned above, and also as dental and orthopedic ultrafine fillers for microporosity owing to their high dispersibility in liquid media and high thermal and chemical stability. Calcination with an anti-sintering agent has a potential application to a wide range of calcined nanoceramic powders, such as alumina, titania, and magnesia, and offer significant benefits over existing technologies because the technique is simple, inexpensive, and amenable to scale-up and processing.

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Nano-Sized Ceramic Particles of Hydroxyapatite Calcined with an Anti-Sintering Agent

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Nano-sized crystals of calcined hydroxyapatite (HAp) having spherical morphologies were fabricated by calcination at 800 °C for 1 h with an anti-sintering agent surrounding the original HAp particles and the agent was subsequently removed by washing after calcination. The original HAp particles were prepared by a modified emulsion system, and surrounded with poly(acrylic acid, calcium salt) (PAA-Ca) by utilizing a precipitation reaction between calcium hydroxide and poly(acrylic acid) adsorbed on the HAp particle surfaces in an aqueous medium. In the case of calcination without PAA-Ca, micron-sized particles consisting of sintered polycrystals were mainly observed by scanning electron microscopy, indicating the calcination-induced sintering among the crystals. On the other hand, most of the crystals calcined with the anti-sintering agent were observed as isolated particles, and the mean size of the HAp crystals was around 80 nm. This result indicates that PAA-Ca and its thermally decomposed product, CaO, surrounding the HAp crystals could protect them against calcination-induced sintering during calcination at 800 °C. The HAp crystals calcined with PAA-Ca showed high crystallinity, and no other calcium phosphate phases could be detected.

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1. INTRODUCTION

Hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) ceramic is an important biomaterial and has been widely used by taking into account its biocompatibility, because its constituent is chemically similar to those of the mineral of bones and teeth. HAp also has been extensively used in a variety of applications, such as adsorbents, carrier for drug delivery system, ion exchangers, and catalysts, by exploiting its adsorbability of many compounds and biocompatibility. However, owing to its mechanical weakness and brittleness,[1–3] applications of HAp have been confined to those with low mechanical stress. We recently developed a novel inorganic/organic composite:[4,5] a flexible polymer substrate, whose surface was modified with calcined HAp nanoparticles through covalent bonding. The novel composite retained the flexibility of the polymer substrate and showed surface properties of HAp such as tissue adhesion in a living body.

Throughout these studies, the HAp particles were prepared by a modified emulsion system[6,7] and used after calcination at 800 °C to reduce sorbability *in vivo*. When low-crystallinity HAp nanoparticles are calcined to increase thermal and chemical stability, the particles sinter randomly into large agglomerates consisting of polycrystals.[8–12] Therefore, calcined HAp crystals on a nanoscale have been difficult to obtain. Because the sintered polycrystals has poor dispersibility in liquid media and a large size distribution, it was difficult to control the surface morphology (covering ratio, surface roughness) of the above-mentioned composite. Hydrothermal treatment of HAp particles in an aqueous medium under high pressure is known to enable the preparation of agglomerate-free HAp crystals.[13–15] However, this treatment generally leads to an increase in crystal size due to Ostwald ripening,[16,17] and is restricted to laboratory-scale products as it is a high-pressure process.

In this article, nano-sized and calcined HAp crystals protected against calcination-induced sintering will be fabricated by using an anti-sintering agent surrounding the HAp particles. Thus, there was no contact between the particles during calcination. Calcium hydroxide [$Ca(OH)_2$] was selected as a component of the anti-sintering agent because it would not melt at the calcination temperature (800 °C), presumably not dissolve the HAp, and could be removed by washing with water after calcination. In order to surrounding the HAp particles efficiently with the anti-sintering agent, a precipitation reaction between $Ca(OH)_2$ and poly(acrylic acid) (PAA) adsorbed on the HAp particles in an aqueous medium was utilized. The obtained

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