

was centrifugally washed with water to remove the  $\text{Ca(OH)}_2$ . As a control, HAp was calcined using the same procedure, but without adding  $\text{Ca(OH)}_2$ .

X-ray diffraction (XRD) measurement was carried out for the calcined HAp particles at 800 °C for 1 h with  $\text{Ca(OH)}_2$  interposed among the crystals. After calcination,  $\text{Ca(OH)}_2$  was removed by centrifugal washing with water. The XRD profiles showed highly crystallized HAp, and no other calcium phosphate phases could be detected. This result indicates that the heat treatment with  $\text{Ca(OH)}_2$  at 800 °C for 1 h did not influence the crystal phase of HAp. 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  $\text{Ca(OH)}_2$  as shown in **Figure 5**. In the case of calcination without  $\text{Ca(OH)}_2$  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  $\text{Ca(OH)}_2$  interposed among the crystals, followed by removing of  $\text{Ca(OH)}_2$  after calcination. From electron microscope observations, the size of the HAp crystals was ranged from 50 to 100 nm, which was irrespective of the calcination with or without  $\text{Ca(OH)}_2$  shown in Figure 6 as well as **Figure 5(a)**. It was clear that the HAp nanoparticles were scattered by a single particle on a SEM mount. The higher magnification TEM image of a particle calcined with  $\text{Ca(OH)}_2$  and its electron diffraction pattern [**Figure 6 (b, c)**] confirmed that the particle was a single HAp crystal with an irregular spherical morphology.

Calcined HAp nanocrystals with a rod-like morphology were also obtained with poly(acrylic acid–Ca salt) (PAA-Ca) as the anti-sintering agent surrounding the particles followed by removal of the agent. The SEM and TEM/ED images were shown in **Figure 7**. The HAp nanoparticle which showed also a single crystal (**Figure 7(c)**) definitely elongated to c axis and was scattered by almost a single one in SEM image [**Figure 7(a)**].

the HAp nanocrystals calcined with PAA-Ca showed high dispersibility in liquid media and a large specific surface area due to the anti-sintering effect of PAA-Ca

surrounding the particles. Also, the HAp crystals calcined with PAA-Ca showed highly crystallinity, and no other calcium phosphate phases could be detected. The HAp nanocrystals obtained here should be suitable for various applications, such as biomaterials, ion exchangers, adsorbents, catalysts, and 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 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 scaling-up and processing.

### **3. HAp Nanocoating by chemical bonding**

#### **3.1 Background**

The surface of HAp crystal possesses hydroxyl groups, calcium and phosphate ionic sites to react with chemical functional groups of organic molecules. Some reports exist that describe the donation of organic functional groups to calcium phosphate ceramics through covalent linkages with functional-group-terminated organic

reagents. Nishizawa et al. reported that the surface hydroxyl groups of calcium phosphate ceramics formed covalent bonds with silane coupling [52]. Labella et al. speculated that the silane coupling agents could react with surface hydroxyl groups of HAp, and they developed a silanized HAp dental filler [53]. Dupraz et al. characterized the surface properties of silane-treated HAp powder with XPS and  $\zeta$ -potential measurement [54]. Liu et al. first showed that the surface hydroxyl groups of HAp nanoparticles are reactive with isocyanate groups in organic reagents with solid  $^1\text{H}$  Magic-Angle Spinning Nuclear Magnetic Resonance [55].

In this section, we demonstrated preparation methods of a novel composite consisting of a nano-scaled HAp particle and a polymer substrate -- a silk fibroin (SF) fiber -- through covalent linkage and ionic interaction. Some methacrylate monomers -- 2-(*o*-[1'-methylpropylideneamino] carboxyamino) ethyl methacrylate (MOI-oxime) that is 2-methacryloxyethyl isocyanate (MOI) blocked by oxime,  $\gamma$ -methacryloxyethyl trimethoxysilane (MPTS), and 4-methacryloyloxyethyl trimellitate anhydride (4-META) -- which have telechelic functional groups, such as alkoxysilyl, isocyanate and anhydride groups have been used for reaction with HAp surface by chemical

bonding as shown in **Scheme 2**.

The SF fiber shows good qualities for implant materials, such as good mechanical properties, moldability, possessing many functional groups on the surface, and actual results as a suture in the medical field for a long period. We selected SF as a polymer substrate to develop a composite with HAp since the SF fiber can add mechanical strength and flexibility to the inorganic material. The chemical modification of the HAp surface in advance is not necessary for the preparation of the composite.

## **3.2 HAp coating by covalent bonding**

### **3.2.1 Isocyanate Group**

The composite was prepared through a two-step procedure -- the graft-polymerization of poly(MOI-oxime) (Showa Denko Co., Tokyo, Japan) on the SF as the first step, and coupling between HAp and the modified SF as the second step. This synthetic method is simpler and more reasonable compared to our previous three-step procedure [18]. MOI is a difunctional monomer with an isocyanate group

and a vinyl double bond. The isocyanate group reacts easily with active hydrogen compounds, such as water. The blocked MOI derivative, for example MOI-oxime, allows the benefit of MOI being extended into a water-based system [56].

MOI-oxime was grafted on the SF using ammonium peroxodisulfate (APS) as an initiator, and pentaethylene glycol dodecyl ether as a nonionic surfactant. Graft-polymerization with MOI-oxime onto the SF was conducted by free radical initiation [57]. The SF fabric used in this reaction was degummed habutae fabric (Fujimura-Seishi Co., Kochi, Japan) made of silk from *Bomboyx mori* and was cleaned by a soxhlet extractor. 339 ml (1.8 mmol) of the MOI-oxime monomer, 41 mg (0.18 mmol) of APS and 73 mg (0.18 mmol) of the surfactant were mixed in 6.0 ml of distilled water. Five pieces of the SF (1.8 cm in diameter) were immersed in the reaction mixture in 50-ml thick-walled polymerization tubes. The tubes were degassed by freezing and evacuating three times and then sealed. Graft-polymerization was conducted at 45 °C for different periods. Poly(MOI-oxime)-grafted fabrics were collected from the reaction system, washed with acetone followed by washing with distilled water to remove unreacted monomers and homopolymers, and finally dried by

vacuum for 24 h at room temperature. The weight gain was calculated from the increase in weight of the dried original SF after graft-polymerization with MOI-oxime as follows;

$$\text{weight gain (wt\%)} = (W_2 - W_1) / W_1 \times 100$$

where  $W_1$  and  $W_2$  are the dried weight of the original SF and poly(MOI-oxime)-grafted SF, respectively.

**Figure 8** shows the ATR FT-IR spectra of the MOI-oxime monomer, the original SF, and the poly(MOI-oxime)-grafted SF. Peaks at 1621, 1514 and 1260/1230  $\text{cm}^{-1}$  were attributed to amide I, II, and III, respectively, which are the typical absorbances of the SF substrate as shown in **Figure 8 (b)**. After the modification with the MOI-oxime, new peaks appearing at 2936 and 1716  $\text{cm}^{-1}$  contributed hydrocarbon and carbonyl groups of the monomer. It is clear that graft-polymerization of MOI-oxime is well conducted.

After HAp nano-scaled particles were suspended in toluene/isopropanol (9/1), a poly(MOI-oxime)-grafted SF was soaked in the suspended solution for 1 h at room temperature to be adsorbed on the SF. The SF adsorbed with the particles was washed

by stirring in ethanol. This fabric with HAp was heated at 140 °C for 20 min in vacuum at 1 mmHg for the deblocking of MOI-oxime and the reaction between the HAp particles and the isocyanate group of the grafted polymer. The composite was washed by using an ultrasonic generator for 3 min (output: 20kHz, 35W) to remove excess adsorbed HAp particles attached to ones in the same solution. 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.

The surface morphologies of HAp nanoparticles coated on a SF fiber explained in the next paragraph. To prove indirectly the formation of the urethane linkage between HAp particles and MOI-oxime, the reaction of the particles with the MOI-oxime monomer was carried out. Briefly, the HAp nanoparticles adsorbed with MOI-oxime were heated at 140 °C for 20 min in vacuum at 1 mmHg and the product was washed by acetone to remove the unreacted reagents. **Figure 9** shows the diffuse reflectance FT-IR spectra of the original and (MOI-oxime)-modified HAp nanoparticles. The spectrum of the (MOI-oxime)-modified HAp [**Figure 9 (b)**] shows additional absorption bands, with respect to the original particle spectrum, due to C-H stretch at



2978, C=O stretch of the ester group at 1724, C=O stretch of the urethane group at 1653, and N-H bending at 1575  $\text{cm}^{-1}$ . This result is in accord with Liu's paper [58]. By heating in vacuum, the formation of the urethane linkage follows the release of oxime from MOI-oxime to generate the isocyanate groups [56], because the deblocking temperature of MOI-oxime is 140 °C and the boiling point of oxime is 60 °C. From the spectrum, it is estimated indirectly that HAp particles might be coupled with the isocyanate groups in poly (MOI-oxime)-grafted SF by the urethane linkage. It is possible to lower the reaction temperature by using MOI blocked with phenol or imidazole, for the deblocking temperature of these agents is 110-130 °C [56]. This reaction system is, therefore, a unique method of fixing HAp on the polymer substrate.

### **3.2.2 Alkoxysilyl Group**

Graft-polymerization with MPTS monmer having an alkoxysilyl group on SF fibers with 100  $\mu\text{m}$  of length was conducted by free radical initiation. Initially, 1.8 mmol of the MPTS and 0.18 mol of a nonionic surfactant, pentaethylene glycol dodecyl ether, were thoroughly mixed and added to 50-ml thick-walled polymerization tubes.

Subsequently, 0.18 mmol of APS in 6.0 ml of distilled water and 600 mg of SF fibers was added to the tubes. The tube was degassed by freezing, evacuated three times and then sealed. Graft-polymerization was conducted at 50 °C for different periods. Poly(MPTS)-grafted SF were collected from the reaction system, washed with 300 ml of dry-methanol and filtrated by a filter with a 5-  $\mu$ m cut-off point to remove unreacted monomers and homopolymers, and dried by vacuum for 1h at 60 °C. 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-  $\mu$ m 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 alkoxyisilyl 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.

Graft-polymerization with MPTS possessing alkoxyethyl groups coupled with hydroxyl groups on the HAp surface on the SF substrate through a one-step procedure was conducted. **Figure 10** shows the ATR FT-IR spectra of the poly(MPTS) homopolymer, unreacted SF and the poly(MPTS)-grafted SF having 14.3 wt% of weight gain against intact SF. Typical peaks in the poly(MPTS) homopolymer can be seen at 1725 and 1076  $\text{cm}^{-1}$  ascribed the C=O stretching vibration of ester groups and the Si-O-C stretching vibration of alkoxyethyl groups, respectively [**Figure 10 (a)**]. The peaks at 1621, 1514 and 1260/1230  $\text{cm}^{-1}$  were attributed to amide I, II and III, respectively, which are the typical absorbances of the SF substrate, as shown in **Figure 10 (b)**. After graft-polymerization with poly(MPTS), a peak contributing to Si-O-C of the graft-polymer remains at 1076  $\text{cm}^{-1}$  in **Figure 10(c)**. **Figure 11** shows ATR FT-IR spectra of the poly(MPTS)-grafted SF just after preparation and the sample treated by hydrolysis with water in an autoclave. In the spectrum of the poly(MPTS)-grafted sample (a), the Si-O-C stretching vibration attributed to the graft-polymer at 1076  $\text{cm}^{-1}$  and the OH stretching vibration belonging to the SF substrate at 926  $\text{cm}^{-1}$  were

recognized. On the other hand, two new peaks appear at 1068/922  $\text{cm}^{-1}$  belonging to the Si-O-Si and Si-OH stretching vibrations, respectively, in the spectrum of the hydrolyzed sample (b). This means that the alkoxyethyl groups of the graft-polymers just after preparation avoided hydrolysis, and maintained their activity in coupling with the hydroxyl groups on the HAp surface regardless of using water as the reaction solvent. The surfactant in the reaction solvent was effective in protecting the active groups from hydrolysis. In our previous study, graft-polymerization having alkoxyethyl groups was achieved through a vinyl bond of 2-methacryloxyethyl isocyanate (MOI) bonded on the SF substrate in anhydrous organic solvents via a two-step procedure [19]. It can be mentioned that the alternative graft-polymerization system in this section is technically easier and more advantageous compared to the former method due to using water as the solvent and the single-reaction procedure.

**Figure 12** shows the ATR FT-IR spectrum of the calcined HAp nonoparticles coated on poly(MPTS)-grafted SF. The weight gain of HAp particles coated on SF was 13.2 wt% determined by a thermogravimetric analysis (TGA). The peak at 3572  $\text{cm}^{-1}$  belongs to the OH stretching vibration of highly crystalline HAp coated on SF.

This peak is very small because a signal intensity of the ATR FT-IR spectrum shows the specific character that is weaker in a higher wave-number region than that in a lower region. The peaks at 1091 and 1052  $\text{cm}^{-1}$  reflect  $\nu_3(\text{PO}_4^{3-})$  of the HAp crystals. The peak at 3281  $\text{cm}^{-1}$  shows OH stretching of amino acids in SF because there are many hydroxyl residues such as 10.63 mol% of Ser, 4.97 mol% of Tyr and 0.89 mol% of Thr in SF. Although the bands attributed to the Si-O-Si stretching vibration at 1068  $\text{cm}^{-1}$  or Si-OH at 922  $\text{cm}^{-1}$  generated by hydrolysis of poly(MPTS) can't be seen due to an overlap of the peaks attributed to the  $\text{PO}_4^{3-}$  stretching vibration of HAp at around 1100 ~ 900  $\text{cm}^{-1}$ , the peaks of amide I/II and two C=O from poly(MPTS) and the SF substrate at 1621/1514 and 1725/1696  $\text{cm}^{-1}$ , respectively, can be clearly observed. This means the coating of HAp particles did not fully and thickly cover the surface of grafted SF. The existence of covalent bonds between the HAp particles and the polyMPTS-grafted SF was estimated by an indirect method using an FT-IR measurement [18].

As the evidence of existence of covalent linkage, in the FT-IR spectrum of the heating product of the mixture with the HAp nanoparticles and the MPTS monomer at 120 °C for 2h in vacuum, a new peak appeared at 1043  $\text{cm}^{-1}$  [18]. This band is known

as the sign belonging to an Si-O stretching vibration from the covalent bond between the HAp and silane coupling agent [52].

**Figure 13** shows an SEM photograph of the HAp-coated SF fiber with  $13.1 \pm 1.2$  wt% (n=4) in the composite determined by TGA. The weight gain of HAp particles on the SF fiber increased about 5 times compared to that on the SF fabric,  $2.8 \pm 0.5$  wt% by the same preparation conditions. This means that it is hard for nano-HAp particles to penetrate into and coat on gaps between fibers in the SF fabric. In other words, a fiber cut approximately 100  $\mu\text{m}$  in length was more effective for the coating than that of a fabric-form. The HAp particles separated or aggregated into several crystals under SEM observation. Aggregation was 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 [59]. It was difficult, therefore, to prepare perfect mono-layer with HAp nano-particles separately on the SF surface.

### 3.3 HAp Coating by Ionic Bonding

#### 3.3.1 Carboxyl Group

In the former subsections, the coupling method between HAp nanoparticles and a polymer substrate through covalent linkage was mentioned. This synthetic way needs, however, one more step, which is thermal treatment above 100 °C in a vacuum in order to connect HAp to SF by covalent linkage, besides the physical adsorption of HAp on the SF. This system is not useful when applying it to the non-heat-resistant biomedical material such as polyethylene and is industrially disadvantageous. In this subsection, in order to couple HAp particles and SF under a non-heat condition, therefore, we applied graft-polymerization with 4-methacryloyloxyethyl trimellitate anhydride (4-META) onto the SF by free radical initiation. It is well known that 4-META has already been applied to resin monomers for dental surgery and is harmless to a living body. It was reported, moreover, that the methyl methacrylate (MMA) resin with 4-META had larger tensile bond strength compared with the MMA resin without 4-META because of the strong affinity between HAp and 4-META [60]. Here we report on the synthesis and bioactivity of the novel HAp/SF composite using 4-META.

Graft-polymerization with 4-META onto the SF was conducted by free radical initiation [57]. 273.80 mg (0.9 mmol) of the 4-META monomer, 41 mg (0.18

mmol) of APS and 73 mg (0.18 mmol) of the surfactant were mixed in 6.0 ml of distilled water. 74.94 mg of the SF fabric (18 mm in diameter) was immersed in the reaction mixture in 50-ml thick-walled polymerization tubes. The tubes were degassed by freezing and evacuating three times and then sealed. Graft-polymerization was conducted at 50 °C for different periods. The poly(4-META)-grafted fabrics were collected from the reaction system, washed with acetone followed by washing with distilled water to remove unreacted monomers and homopolymers, and finally dried by vacuum for 24 h at room temperature.

The poly(4-META)-grafted SF was immersed in a 0.01 M potassium hydroxide aqueous solution for 10 min and the five-member ring of this SF was opened and ionized. After HAp nano-scaled particles were suspended in toluene/methanol (8.8/1), an ionized poly(4-META)-grafted SF was soaked in the suspended solution for 1 h at room temperature in order to be adsorbed on the SF. The SF adsorbed with the particles was washed by stirring in acetone to remove the solvent. The composite was washed in distilled water by using an ultrasonic generator for 3 min (output: 20 kHz, 35 W) to remove excess adsorbed HAp particles. Finally, the composite was freeze-dried



by vacuum for 1 day to evaporate the residual water.

**Figure 14** shows the ATR-FTIR spectra of the 4-META monomer, the original SF, the poly(4-META)-grafted SF, and the ionized poly(4-META)-grafted SF. The peaks at 1621, 1514 and 1260/1230  $\text{cm}^{-1}$  were attributed to amide I, II, and III, respectively, which are the typical absorbances of the SF substrate as shown in **Figure 14 (b)**. After modification with the 4-META monomer [**Figure 14 (c)**], new peaks contributing to the monomer appeared due to the C=O stretching vibration of  $-\text{CO-O-CO}-$  at 1724  $\text{cm}^{-1}$  and ester groups at 1696  $\text{cm}^{-1}$ , and the C-O stretching vibration of ester groups at 1158  $\text{cm}^{-1}$ . After ionization of the grafted SF [**Figure 14 (d)**], the peak of the C=O stretching vibration of  $-\text{CO-O-CO}-$  disappeared and new peaks at 1392 and 1367  $\text{cm}^{-1}$  attributed to the symmetric vibration of  $-\text{COO}^-$  appeared.

To estimate indirectly the formation of ionic interaction, HAp particles connected with ionized 4-META were prepared. The HAp particles were adsorbed with 4-META, ionized by a 0.1 M potassium hydroxide aqueous solution in toluene/methanol (8.8/1) for 1 h. The reactant was washed with distilled water to remove the unreacted reagents and dried by heating at 120 °C under vacuum.

In **Figure 15**, the solid line shows the difference FT-IR spectrum which subtracts the original HAp from the HAp particles with ionized 4-META. The peak of  $1368\text{ cm}^{-1}$  demonstrates the existence of  $\text{-COO}^-$ . This absorption band shifted by  $6\text{ cm}^{-1}$  to a lower side of wave number in comparison with ionized 4-META (dotted line,  $1374\text{ cm}^{-1}$ ). This shift suggests that carboxylate groups in ionized 4-META interacted with  $\text{Ca}^{2+}$  ions on the surface of HAp [61, 62]. From these spectra, it is estimated indirectly that HAp nanoparticles might be coupled with  $\text{-COO}^-$  groups in the ionized poly(4-META)-modified SF by the ionic interaction. This synthetic procedure of the composite is outlined in **Scheme 3**. By using the mixture of toluene and methanol, the HAp nano-particles are equally dispersed in the medium and are adsorbed physically and dispersively on the SF surface because of the strong affinity between HAp and 4-META [8]. The SF, after ring-opening using a potassium hydroxide aqueous solution, moreover, has carboxylate groups on the surface. It is well known that the a-plane of HAp has a cationic surface which consists of  $\text{Ca}^{2+}$  ions [59]. It seems, therefore, that the a-plane of HAp was adsorbed on the SF covered with carboxylate groups. When the SF adsorbed with particles is washed in distilled water,  $\text{K}^+$  ions are

released from the SF, and  $\text{-COO}^-$  in the ionized SF is coupled with the  $\text{Ca}^{2+}$  ions of HAp particles by ionic interaction due to the ionization tendency. In the series of our papers on the HAp/SF composite [18, 19, 21], the hydroxyl groups of HAp were needed in order to couple between the HAp nanoparticles and the SF substrate. In this synthetic method, however, these groups are not necessarily required, because the HAp particles are connected with the SF substrate by ionic interaction. By using 4-META, it is expected that not only HAp but also other inorganic compounds, such as  $\beta$ -tricalcium phosphate ( $\beta$ -TCP), can be adsorbed and connected on the SF fiber surface. In this way, HAp nanoparticles can be connected with modified SF under a non-heat condition, compared with the conventional way. This condition is useful when using non-heat-resistant biomedical material, such as polyethylene, as a polymer substrate for synthesizing the HAp/polymer composite.

### **3.4 Mechanical Properties**

The tensile properties were measured by using TENSILON RTC-115 OA (ORIENTEC Co., Tokyo, Japan) at an elongation rate of 5mm/min and 25 °C. To determine the mechanical properties of the composite, a suture-like composite made of

Kinsyu x Syowa (a variety of silk) was adopted. The unit of stress value was changed from  $g/d$  to  $Pa$  as shown by the equations described below [63].

$$A_0 = d / (9 \times 10^5 \rho)$$

$A_0$ : cross-section area ( $cm^2$ ),

$d$ : denier (the experimental value=97),

$\rho$ : specific gravity ( $1.33 g/cm^3$ )

$$S = P / (A_0 \times 10^{-4})$$

$S$ : stress (Pa),  $P$ : experimental value of stress (N)

Data from the tensile test are presented as  $\pm$  standard deviation for the mean. Statistical comparisons were performed with the use of the Student's  $t$  test and  $p$  values  $< 0.01$  were considered.

**Figure 16** shows the tensile properties of three types of SF fiber [original SF, graftpolymer-grafted SF, and HAp/SF composite]. The tensile strengths, the elongation at break, and Young's modulus between the original SF and the HAp/SF composite were unchanged. Compared with the original SF and HAp/SF composite, the elongation at break of graftpolymer-grafted SF was somewhat higher but Young's