Nano-scaled hydroxyapatite/polymer composite I. Coating of sintered hydroxyapatite particles on poly(γ-methacryloxypropyl trimethoxysilane)-grafted silk fibroin fibers through chemical bonding

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The inorganic–organic composite consisting of nano-scaled hydroxyapatite (HAp) and silk fibroin (SF) fibers was prepared through covalent linkage to develop a novel biomaterial for a soft-tissue-compatible material. The preparation of the composite was conducted through the three-step procedure consisting of chemical modification using 2-methacryloxyethyl isocyanate (MOI) monomer to introduce vinyl groups on SF, poly(γ-methacryloxypropyl trimethoxysilane) (MPTS) graft-polymerization on SF, and coupling process between the surface of polyMPTS-grafted SF and HAp nano-particles. The amount of the graft-polymerization of polyMPTS through vinyl groups was well controlled by the reaction time. The nano-crystals were subsequently coated on the grafted fibers by heating at 120 °C for 2 h in a vacuum. The crystalline structure of the SF substrate did not change in the procedure. In the SEM observation of the composite surface, it was found that the bonded nano-crystals were separated and partially aggregated with several crystals attached on the SF fiber surface. The HAp particles adhered more strongly on the SF surface with separation or aggregation of several crystals than on the surface of the original SF after ultrasonic treatment.

1. Introduction

Hydroxyapatite (HAp) has unique properties for biomaterials such as hard-tissue-compatible material for bone and tooth [1] and also soft-tissue-compatible material for skin tissue [2]. From the point of view of soft-tissue-compatible material made up of HAp, although the reasons for the compatibility have not been manifested completely, one of them might be favorable to adsorption of adhesion molecules or growth factors *in vivo* on the HAp surface [3]. Aoki *et al*. first developed a percutaneous device clinically using a ceramic disk made of HAp in catheters, blood pressure transducers, leads, and electrodes [2]. A rigid ceramic disk that partially protrudes through the skin, however, limits a patient's mobility and causes discomfort [4]. The hard and brittle nature of HAp limits the development of a percutaneous device. To overcome this problem, a novel inorganic–organic composite consisting of micro- or nano-scaled HAp particles and polymer substrate via a covalent linkage has been designed.

An unique composite of sintered HAp micro-particles covalently coupled to a silicone elastomer as soft-tissue-compatible material was developed recently [5, 6]. The HAp particles were attached to the silicone substrate covalently without damaging the mechanical properties of the polymer substrate. In this way, the adhesion strength between the HAp particle and polymer substrate, however, was believed to be unsatisfactory because a commercial grade of a HAp spherical particle with an average diameter of 2.0 μm was used. To increase the interaction between the HAp particle and substrate surface, a nano-scaled HAp particle with a larger surface-area of adhesion on a polymer substrate has been developed by an emulsion system [7, 8]. It is expected

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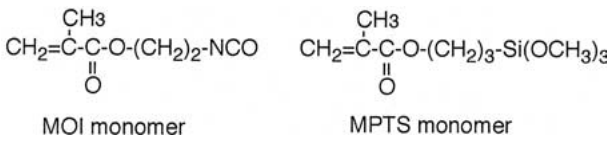
that the inorganic–organic composite can be satisfactorily developed using the nano-scaled HAp particles.

In this report, we develop a novel composite consisting of a nano-scaled HAp particle and a silk fibroin (SF) fiber as a polymer substrate through covalent linkage. 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. However, there is a drawback, for SF sometimes causes inflammation in a living body. If HAp is coated on SF, the inflammation is expected to be reduced compared with the original SF. 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.

2. Materials and methods

2.1. Materials

For the preparation of the nano-scaled HAp, calcium hydroxide [$Ca(OH)_2$], potassium dihydrogen phosphate (KH_2PO_4), dedecane as a continuous oil phase, and pentaethylene glycol dodecyl ether as a nonionic surfactant were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dimethyl sulfoxide (DMSO) and ethanol as solvents, di-n-butyltin (IV) dilaurate as a catalyst, and hydroquinone as a polymerization inhibitor were also purchased from the same chemical company. Degummed habutae fabric (Fujimura-Seishi Co., Kochi, Japan), which is the textile name, made of silk from *Bomboyx mori* was cleaned by soxhlet extractor with acetone for 24 h to remove the wax, rinsed with distilled water, and freeze-dried for 24 h. 2-methacryloxyethyl isocyanate (MOI) and γ-methacryloxypropyl trimethoxysilane (MPTS) monomers were donated by Showa Denko Co. (Tokyo, Japan) and Shin-Etsu Chemical Industries Co. (Tokyo, Japan), respectively (Scheme 1).



Scheme 1 Chemical structures of MOI and MPTS monomers.

2.2. Measurements

An attenuated total reflection (ATR) and diffuse reflectance Fourier transform infrared spectrometry (FT-IR) was recorded using a Spectrum One (Perkin-Elmer Inc., MA, USA). The resolution of each spectrum was $4\,cm^{-1}$ with 16 s scan times ranging from 4000 to $400\,cm^{-1}$. The HAp and the composite were observed with a scanning electron microscope (SEM, JSM-6301F, Jeol Ltd., Tokyo, Japan). X-ray diffraction measurements were conducted at 2θ (varying from 2° to 75°) using an automatic diffractometer (RAD-X, Rigaku Inter-national Co., Tokyo, Japan) with CuKα radiation ($\lambda = 0.154\,nm$) and an Ni filter generated at 45 kV and 25 mA. Data were collected using a receiving slit of 0.15 mm, 2θ scanning at 2°/min, and a 2θ-scan step of 0.02°.

2.3. Sample preparation

Nano-particles of HAp were prepared by the emulsion system described in the former reports [7, 8]. Briefly, $10\,cm^3$ of an aqueous suspension of $2.5\,mol\,dm^{-3}$ of $Ca(OH)_2$ was poured into $40\,cm^3$ of the dodecane containing 0.5 g of the surfactant. After rapidly stirring the W/O emulsion, $10\,cm^3$ of $1.5\,mol\,dm^{-3}$ KH_2PO_4 aqueous solution was added into the system, and reacted at 50 °C for 24 h. After purification by centrifugation with ethanol and water, the powder was calcined at 800 °C for 1 h at a 10 °C/min heating rate. Finally, truncated rod-shaped HAp particles below 200 nm in length were obtained. Fig. 1 shows the HAp particles by SEM observation. These particles possess a large surface area with an a-plane and show a slight calcium deficiency (Ca/P = 1.61) containing carbonate [7].

Before the graft-polymerization of polyMPTS, an MOI monomer was reacted on the SF due to the donation of vinyl bonds according to the former literature [9]. The SF fabrics, 1.8 cm in diameter, were used in this reaction. The MOI-modified SF with 7.0–8.0 wt % of weight gain was used in the graft-polymerization of polyMPTS. MPTS grafted via the vinyl double bond of MOI was reacted on the SF using AIBN as an initiator. 2.0 mmol of the MPTS monomer and 0.4 mmol of AIBN were dissolved in $5.0\,cm^3$ of DMSO. Six pieces of the SF were immersed in the reaction solution in $50\,cm^3$ thick-walled polymerization tubes. The tubes were degassed by freezing and evacuating four times and sealed. Graft-polymerization was conducted at 60 °C for different periods. PolyMPTS-grafted fabrics were collected from the reaction system, washed with DMSO and methanol, and finally dried by vacuum for 24 h at room temperature. Weight gain was calculated from the increase in weight of the dried MOI-modified SF after graft-polymerization with MPTS as follows;

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

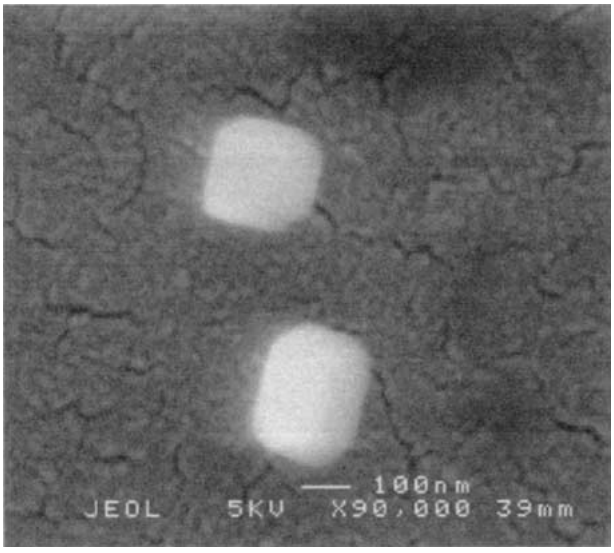


Figure 1 SEM photograph of the nano-scaled HAp particles.

where W_1 and W_2 are the dried MOI-modified SF and polyMPTS-grafted SF, respectively.

After HAp nano-scaled particles were suspended in toluene/methanol (9/1), a polyMPTS-grafted SF of 1.5 cm in diameter 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 methanol. This fabric with HAp was heated at 120 °C for 2 h in vacuum at 1 mmHg to react between the HAp particle and alkoxysilyl group of the grafted polymer. The composite was washed by using an ultrasonic generator for 3 min (output: 20 kHz, 35 W) to remove excess adsorbed HAp particles attached to those 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 using the synthetic process.

To estimate the siloxane bond existing between HAp particles and the grafted substrate, a mixture product consisting of 90 mg of MPTS monomer and 10 mg of HAp particles was prepared. The mixture was treated at 120 °C for 2 h in vacuum (1 mmHg), according to the HAp coating process.

3. Results and discussion

The composite was prepared through a three-step procedure – the donation of vinyl groups on the SF as the first step, graft-polymerization of polyMPTS on the SF via the vinyl bonds as the second step, and coupling between HAp and the modified SF as the final step. The target of the amino acid in the SF for the donation of vinyl groups is hydroxyl residues because there are 10.6 mol % of Ser, 5.0 mol % of Tyr and 0.9 mol % of Thr in the amino acids of the SF. The isocyanate group of the MOI monomer predominantly reacts with primary alcohols using a di-n-butyltin (IV) dilaurate. Fig. 2 shows the ATR FT-IR spectra of the original SF, the MOI-modified SF and the polyMPTS grafted SF of 37.6 wt % of weight gain. Peaks at 1621, 1514 and 1260/1230 cm^{-1} were attributed to amide I, II, and III, respectively, which are the typical absorbance of the SF

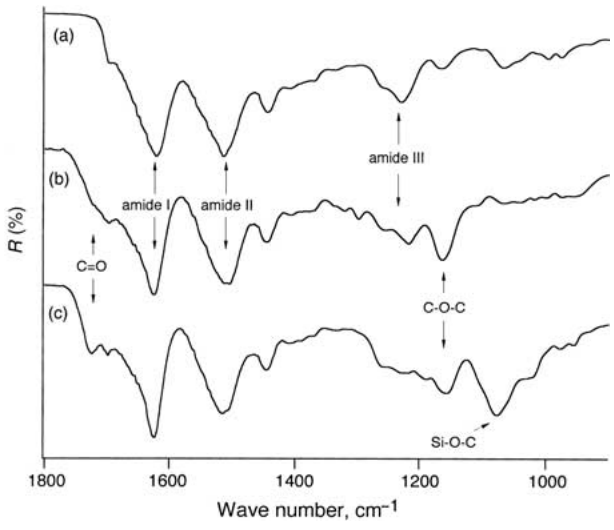


Figure 2 ATR FT-IR spectra of (a) original SF, (b) MOI-modified SF with 8.0 wt % of weight gain, and (c) polyMPTS-grafted SF with 33.8 wt %.

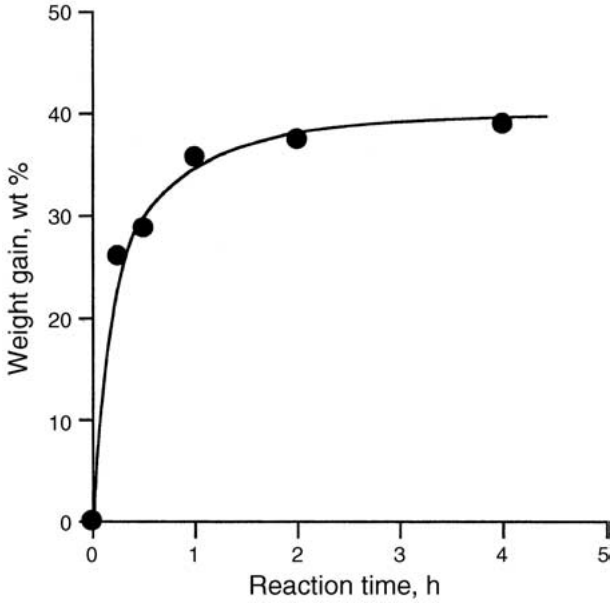


Figure 3 Weight gain of polyMPTS-grafted SF as a function of reaction time.

substrate in Fig. 2(a). After the modification with the MOI monomer, new peaks appeared at 1721 and 1163 cm^{-1} contributing carbonyl and ether groups of the monomer. The weak bands belonging to vinyl groups at 3095–3010 and 1690–1635 cm^{-1} cannot be observed in the FT-IR spectrum due to overlapping with the stronger bands of the SF substrate in Fig. 2(b) [9]. It has already been stated that the amount of MOI monomer donation could be well controlled by the reaction time [9]. Finally, additional peak at 1076 cm^{-1} attributed to Si–O–C appeared due to the graft-polymerization with polyMPTS in Fig. 2(c). The weight gain of polyMPTS on the SF was plotted as a function of the reaction time (Fig. 3). The weight gain of polyMPTS increased with increase in the reaction time, eventually reaching a plateau value of about 38 wt %. Compared to the amount of MOI monomer donation of 7.0–8.0 wt % to the original SF in the first step, the degree of the graft-polymerization in the next step is estimated relatively low by a simple calculation. The donated MOI monomer on SF fibers with large surface area was reasonable from the previous report [9]. There might be considerable vinyl groups that are unable to contribute to the graft-polymerization reaction in narrow spaces among fibers in the SF fabric. In the previous study, the weight gain of poly(2-methacryloyloxyethyl phosphorylcholine)-grafted on the SF through the same reaction system shows a plateau value that was about 26 wt % in a water/DMF mix solvent system [9]. The difference between these graft-polymerization efficiencies might depend on the solvent effect. The amount of the graft-polymerization on the SF could actually be well controlled in this system.

The existence of covalent bonds between the HAp particle and the polyMPTS-grafted SF was indirectly estimated by FT-IR (Fig. 4). The peak at 1076 cm^{-1} of the FT-IR spectrum was attributed to an Si–O–C stretching vibration of an alkoxysilyl group in the MPTS monomer (Fig. 4(a)). The peaks at 963 cm^{-1} reflected ν_1 PO_4^{3-} and 1100/1135 cm^{-1} for ν_3 PO_4^{3-} in

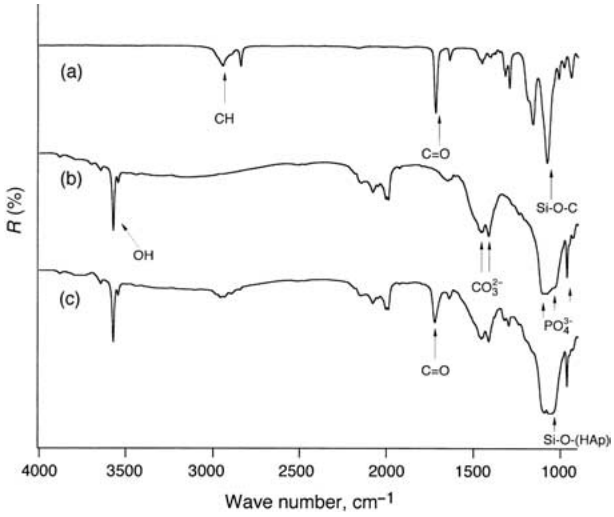


Figure 4 FT-IR spectra of (a) MPTS monomer, (b) original HAp particles, and (c) a heating product of a mixture of the HAp particles and MPTS monomer. The (a) and (b)/(c) spectra were analyzed by ATR and diffuse reflectance FT-IR, respectively.

Fig. 4(b). The strong peak at 3572 cm^{-1} was attributed to an OH stretching vibration which showed a well-crystallized product. The peaks at 1456 and 1412 cm^{-1} can be attributed to ν_3 CO_3^{2-}, which is known to be a better biomaterial due to its similarity to the composition of biological apatite. In the FT-IR spectrum (Fig. 4(c)) of the heating product of the mixture with the HAp particles and the MPTS monomer at 120 °C for 2 h in vacuum, a new peak appeared at 1043 cm^{-1}. This band is known as the sign belonging to an Si–O stretching vibration from the covalent bond between the HAp and the silane coupling agent [10]. The nano-particles actually remained on the surface of the SF substrate, even if the composite sustained considerable shear stress by an ultra-sonic vibration.

Fig. 5 shows the XRD patterns of the original SF (a) and HAp coating SF (b). The sharp peaks at 32° and 33° (2θ), attributed to (2 1 1) and (3 0 0) planes of HAp particles, newly appear in the XRD pattern (b) after the coating. This means that the highly crystalline inorganic material exists on the substrate surface. The peak at around 20° (2θ) shows the silk II form, β-sheet structure, in which crystalline regions of the SF fibers have an

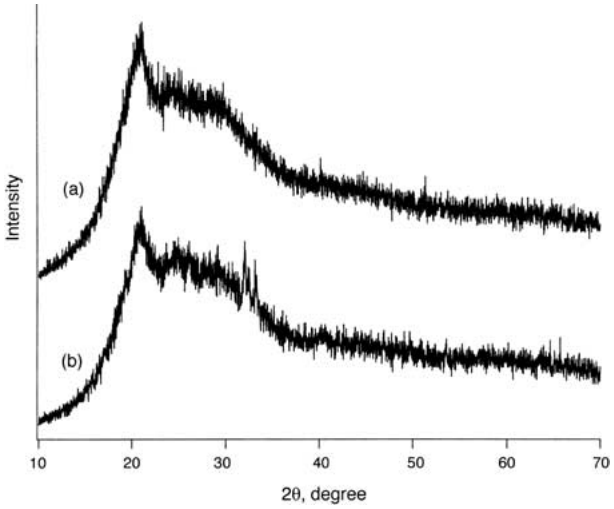


Figure 5 XRD patterns of (a) original SF and (b) the composite.

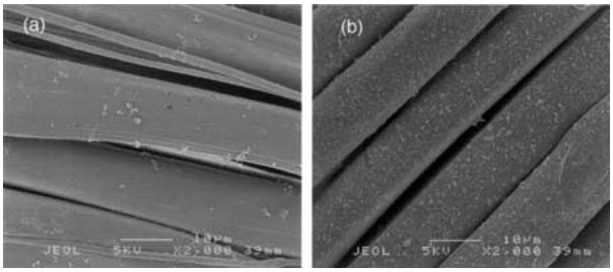


Figure 6 SEM photographs of (a) original SF surface after HAp adsorption and ultrasonic treatment, and (b) the composite surface.

identical orientation. There was no change in XRD patterns between the original SF and the composite except for the patterns of the HAp. This result shows that the crystalline structure of the SF substrate did not change after the heat applied to the HAp coating nor by the process of the graft-polymerization. This also means that the SF is able to withstand the severe condition of the reaction procedure, such as 120 °C for 2 h in vacuum. This phenomenon depends on the crystalline structure of the strong hydrogen bonding in antiparallel chain-pleated sheet structure consisting of a sequential polypeptide $[Ala–Gly]_n$ in the SF [11].

Fig. 6 shows the SEM photograph of the composite surface as well as the original SF surface conducted HAp adsorption and subsequent ultrasonic treatment. SF fibers of about 10 μm in width formed a parallel line. On the original SF, HAp particles were rarely observed due to the removal by ultrasonic treatment (Fig. 6(a)). The nano-particles separated or aggregated with several crystals, meanwhile, strongly remained on the treated SF surface, though some larger aggregates were removed by shear stress using an ultrasonic generator. It was very difficult to prepare a mono-layer adsorption of HAp crystals on the SF because the particles easily aggregate each other due to having ionic phases on the surface. A HAp crystal has two planes – the a- and c-plane. It is well known that the a- and c-plane show cationic and anionic charge, respectively [12]. We are now examining the adsorption behavior using a quartz crystal microbalance technique. Ionic charges of the HAp surface, moreover, might complexly influence against its bioactivity. In the former literature, the sintered HAp particle with an average diameter of 2 μm, that is the crystal aggregation, was used for cell adhesion and animal implantation [6]. The results show, eventually, the good bioactivity. The nano-HAp coating material thus presumes to possess good biocompatibility for soft tissue.

4. Conclusions

HAp nano-crystals below 200 nm in diameter were coated on the SF fibers, which were modified by polyMPTS-grafted polymers through vinyl groups. The graft-polymerization with polyMPTS was well controled by the reaction time. The nano-crystals were subsequently coated on the grafted fibers by heating at 120 °C for 2 h. The crystalline structure of the SF substrate did not change in the synthetic procedure. It was found that the bonded nano-crystals were separated and partially aggregated with several crystals on the fiber surface by SEM observation. The HAp crystals coated on the fibers

were not removed on the modified SF compared to the original one by ultrasonic treatment. The bioactivity of HAp could be applied to the polymer surface since this HAp resembles biological inorganic material. We are now developing three-dimensional material made of the HAp/SF composite aiming at a novel soft-tissue-compatible material.

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Received 20 January
and 29 July 2003