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Nano-scaled hydroxyapatite/polymer composite III. Coating of sintered hydroxyapatite particles on poly(4-methacryloyloxyethyl trimellitate anhydride)-grafted silk fibroin fibers

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A novel composite coupling between nano-scaled hydroxyapatite (HAp) particles and poly[4-methacryloyloxyethyl trimellitate anhydride (4-META)]-grafted silk fibroin (SF) through ionic interaction was synthesized. The weight gain of poly(4-META) by graft-polymerization increased with increasing the reaction time, eventually reaching a plateau value of about 20 wt%. The HAp nano-particles were adsorbed equally and dispersively on the treated SF fiber surface. The HAp content in the composite was 4.554 wt% ± 0.098 ($n = 4$), confirmed by thermogravimetry (TG). This synthetic system requires no heat to connect HAp to SF and is useful when applying to non-heat-resistant polymers. The L-929 cell-adhesion test shows that the HAp/SF composite improves bioactivity compared to the original SF.

1. Introduction

Silk fibroin (SF) has been applied to biomedical use because of its outstanding physicochemical properties [1, 2]. For example, SF is used as a surgical suture. It has, however, the disadvantage of causing inflammation [3]. It is assumed that the reason for inflammation is the interaction of the SF surface and cells. If the biocompatibility of the SF surface is improved by chemical modification, the range of the application of SF as biomedical material will expand. To overcome this problem, we adopted hydroxyapatite (HAp) as the coating material on the SF surface. HAp has good biocompatibility for hard and soft tissue and has been studied as a biomaterial. When applied as a percutanaous device, however, the use of HAp only restricted the patient's mobility because of its rigidity [4, 5]. It is expected that SF can be applied to a polymer substrate of a composite with HAp since SF can add appropriate flexibility to the inorganic material. In the series of our papers on the HAp/SF composite [6, 7], we reported the coupling between a nano-scaled HAp and an SF through covalent linkage. 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 report, 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, Scheme 1) 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 [8]. Here we report on the synthesis and bioactivity of the novel HAp/SF composite using 4-META.

2. Materials and methods

2.1. Materials

Nano-particles of HAp were prepared by the emulsion system using calcium hydroxide and potassium

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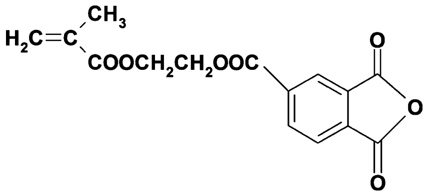


Scheme 1 Chemical structure of 4-META monomer.

dihydrogen phosphate, as described in former reports [9, 10]. The 4-META monomer was kindly donated by Sun Medical Co., Ltd. (Shiga, Japan). Ammonium peroxodisulfate (APS) as an initiator and pentaethylene glycol dodecyl ether as a nonionic surfactant were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Toluene, methanol, and acetone as solvents and potassium hydroxide as the ionized reagent of 4-META were also purchased from the same chemical company. The SF fabric used in this report was degummed *habutae* fabric (Fujimura-Seishi Co., Kochi, Japan) made of silk from *Bomboyx mori* and was cleaned by a soxhlet extractor.

2.2. Sample preparation

Graft-polymerization with 4-META onto the SF was conducted by free radical initiation [11]. 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 weight gain was calculated from the increase in weight of the dried original SF after graft-polymerization with 4-META as follows;

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

where W_1 and W_2 are the dried original SF and poly(4-META)-grafted SF, respectively.

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.

2.3. Estimation of ionic interaction

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.

2.4. Measurements

Attenuated total reflection (ATR) and diffuse reflectance Fourier transform infrared spectrometry (FT-IR) was recorded by a Spectrum One (Perkin-Elmer Inc., MA, USA). The HAp content in the HAp/SF composite was confirmed by thermogravimetry (TG, EXSTAR6000 TG/DTA6300, Seiko Instruments Inc., Chiba, Japan). The heating rate was 20 °C/min and the temperature range was from 30 to 1000 °C. The HAp and the composite were observed with a scanning electron microscope (SEM, JSM-6301F, JEOL Ltd., Tokyo, Japan).

2.5. Cell culture

The cell culture procedure using the mouse fibroblast cell line L-929 was referred to in our former report [12]. L-929 cells were plated onto 24-well multiplates with samples (18 mm in diameter) at 1×10^5 cells/well in an α-minimum essential medium with 10% fetal bovine serum, and incubated at 37 °C for 1 day. After incubation, the samples were washed two times in phosphate-buffered saline [PBS(-)]. The cells were fixed with 2.5% buffered glutaraldehyde for 20 min at 30 °C and were rinsed with PBS(-) three times. Cells were dehydrated with aqueous ethanol (50–100%) and 100% *n*-butanol for 5 min 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 SEM.

3. Results and discussion

Fig. 1 shows the ATR-FTIR spectra of the 4-META monomer, the original SF, the poly(4-META)-modified SF, and the ionized poly(4-META)-modified SF. The peaks at 1621, 1514 and 1260/1230 cm^{-1} were attributed to amide I, II, and III, respectively, which are the typical absorbances of the SF substrate as shown in Fig. 1(b). After modification with the 4-META monomer (Fig. 1(c)), new peaks contributing to the monomer appeared due to the C=O stretching vibration of —CO—O—CO— at 1724 cm^{-1} and ester groups at 1696 cm^{-1}, and the C—O stretching vibration of ester groups at 1158 cm^{-1}. After ionization of the grafted SF (Fig. 1(D)), the peak of the C=O stretching vibration of —CO—O—CO— disappeared and new peaks at 1392 and 1367 cm^{-1} attributed to the symmetric vibration of —COO$^-$ appeared. The weight gain of poly(4-META) on the SF was plotted as a function of the reaction time (Fig. 2). The weight gain of poly(4-META)

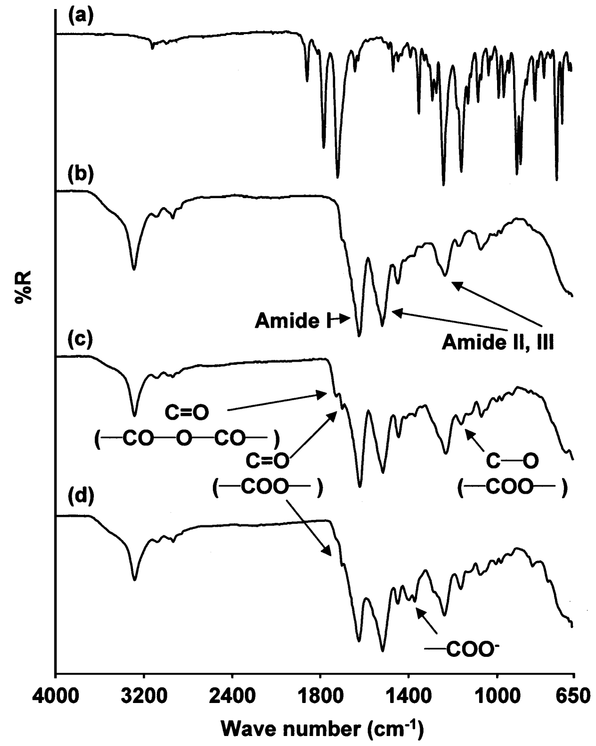


Figure 1 ATR-FTIR spectra of: (a) the 4-META monomer, (b) the original SF, (c) the poly(4-META)-modified SF, and (d) the ionized poly(4-META)-modified SF.

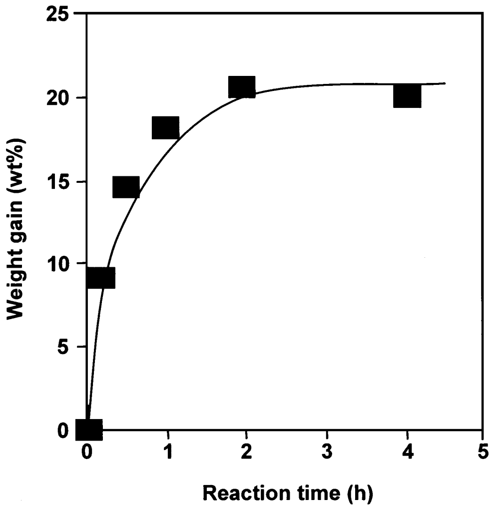
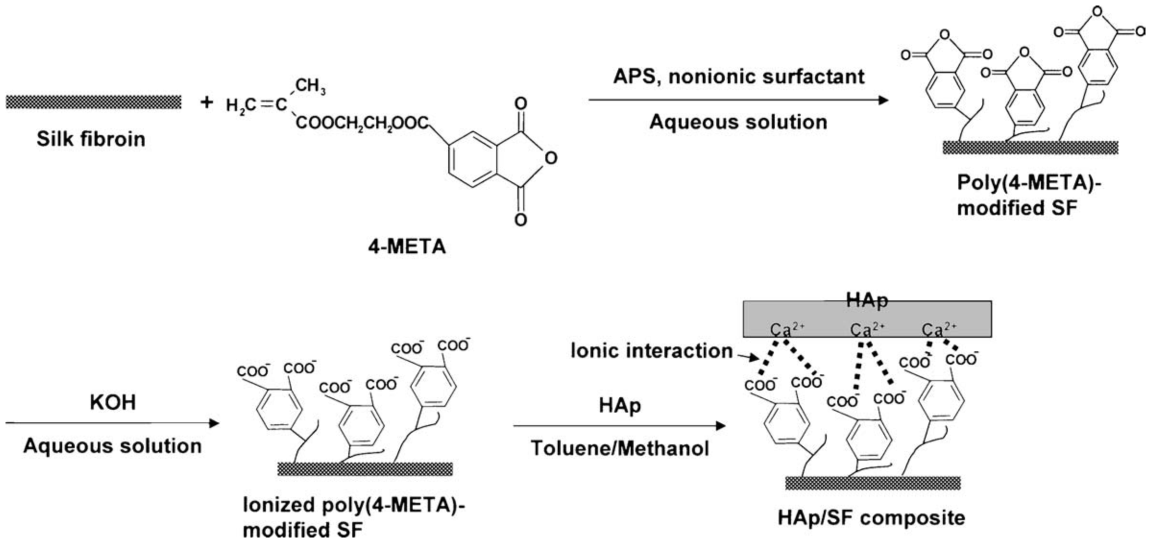


Figure 2 Weight gain of 4-META on the SF as a function of reaction time.

increased with increasing the reaction time, eventually reaching a plateau value of about 20 wt%. In our graft-polymerization, it is theorized that graft-efficiency is low and does not increase beyond a certain value owing to steric hindrance between the side chains of 4-META and the silk substrate [11]. Graft-polymerization with 4-META onto the SF can be well controlled. In all experiments of this report, we used 20 wt%-grafted SF.

Fig. 3 shows an SEM photograph of the composite surface which conducted HAp adsorption and subsequent ultrasonic treatment. This figure demonstrates that the nano-particles were adsorbed equally and dispersively on the treated SF fiber surface. To prove indirectly the formation of the ionic interaction between HAp particles and ionized SF, a reaction of the particles with the ionized 4-META monomer was carried out. In Fig. 4, 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 cm^{-1} demonstrates the existence of $-\mathrm{COO}^-$. This absorption band shifted by 6 cm^{-1} to a lower side of wave number in comparison with ionized 4-META (dotted line, 1374 cm^{-1}). This shift suggests that carboxylate groups in ionized 4-META interacted with Ca^{2+} ions on the surface of HAp [13, 14]. From these spectra, it is estimated indirectly that HAp particles might be coupled with $-\mathrm{COO}^-$ groups in the ionized poly(4-META)-modified SF by the ionic interaction. This synthetic procedure of the composite is outlined in Scheme 2. 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



Scheme 2 Schematic presentation of the synthesis of the HAp/SF composite.

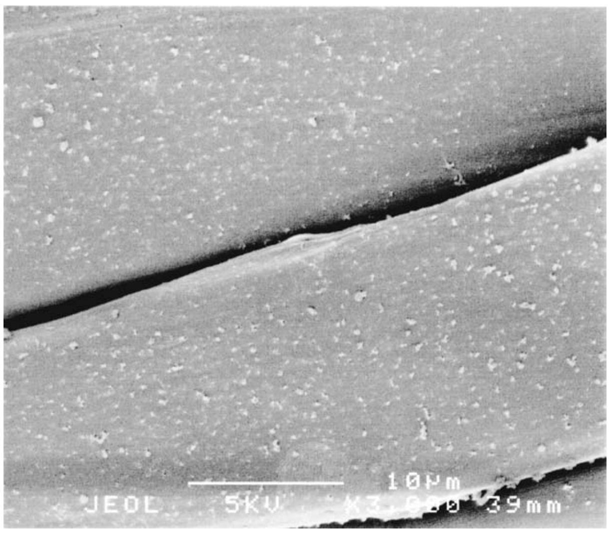


Figure 3 SEM photograph of the HAp/SF composite surface.

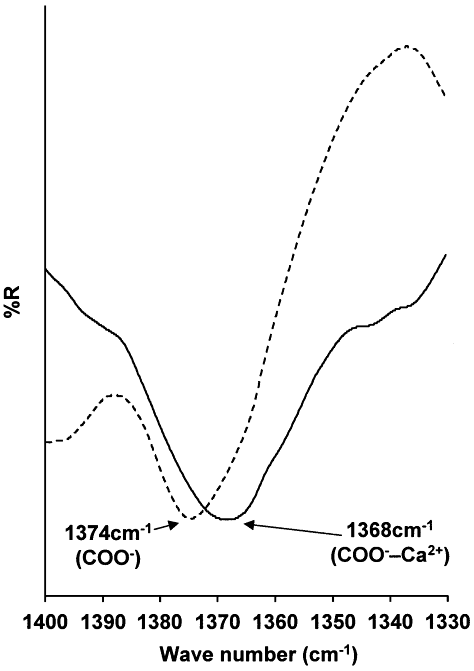


Figure 4 Difference FT-IR spectrum of diffuse reflectance which subtracts the original HAp from the HAp particles with ionized 4-META (—) and FT-IR spectrum of the ionized 4-META monomer (---) at 1400–1330 cm^{-1}.

surface which consists of Ca^{2+} ions [15]. 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, K^+ ions are released from the SF, and $-COO^-$ in the ionized SF is coupled with the 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 [6, 7], the hydroxyl groups of HAp were needed in order to couple between the HAp particles 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 β-tricalcium phosphate, can be adsorbed and connected on the SF fiber surface. In this way, moreover, HAp particles 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

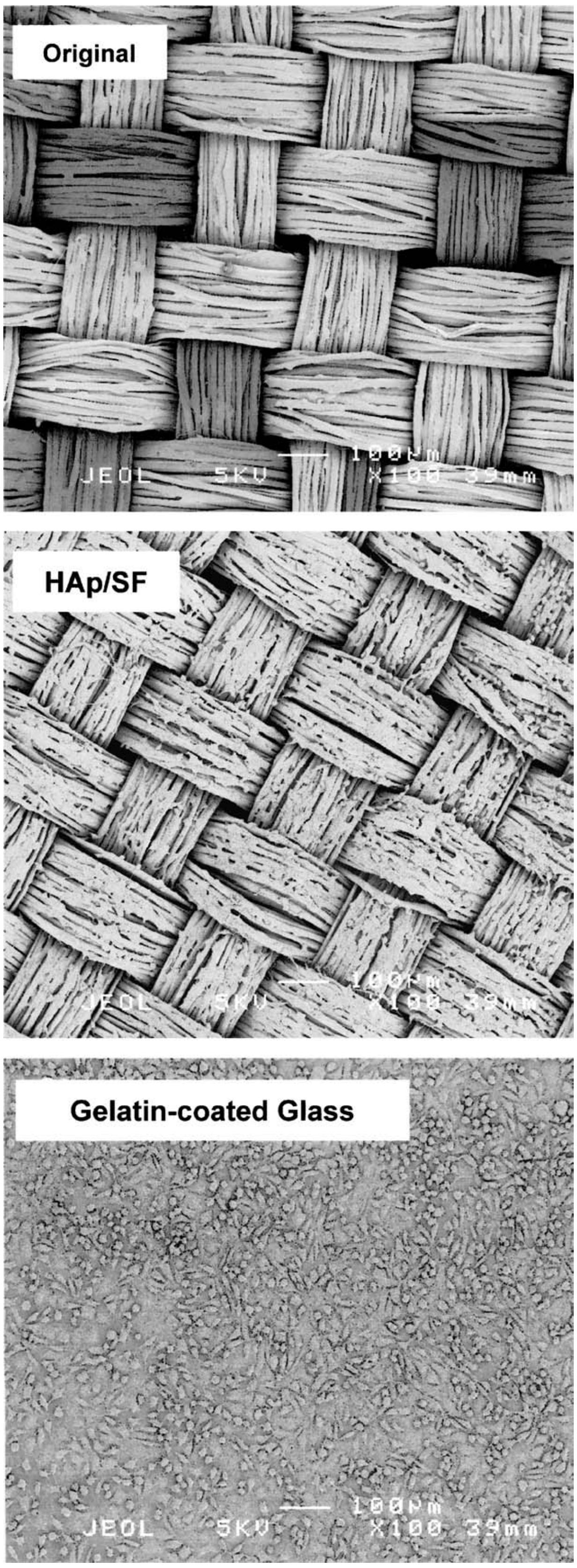


Figure 5 SEM photographs of L-929 cells cultured on the original SF, HAp/SF composite, and gelatin-coated glass for 24 h.

a polymer substrate for synthesizing the HAp/polymer composite.

To calculate the HAp content in the HAp/SF composite, TG analyses were conducted. The residual weight of the composite after heating to 1000 °C is equal to the weight of HAp, because SF and 4-META are burned out. The HAp content was calculated as a percentage of the residual weight compared to the weight of the composite before analysis. The mean value was 4.554 wt% ± 0.098 ($n = 4$).

To examine the bioactivity of the composite, a preliminary cell-adhesion test was conducted. The morphology of L-929 cells on three sample substrates incubated for 1 day was observed by SEM (Fig. 5). Gelatin-coated glass was used as a positive control showing similar cell adhesion on tissue-culture plastic. From these SEM photographs, the cells adhered to the HAp/SF composite homogeneously, although few were found on the original SF surface. It is well known that HAp has good bioactivity. The results show that this composite also has good bioactivity due to the presence of HAp on the surface of SF, compared to the original SF. Generally, HAp does not cause inflammation in living bodies. Therefore, it is estimated that this HAp/SF composite will not bring about inflammation. Further investigation about inflammation is under way by animal test.

In conclusion, a novel composite consisting of nanoscaled HAp particles and SF was synthesized. Graft-polymerization with 4-META onto the SF is well controlled. HAp nano-particles were adsorbed equally and dispersively on the treated SF fiber surface by ionic interaction. This synthetic system requires no heat to connect HAp to SF and is useful when applying to non-heat-resistant biomedical material. The cell-adhesion test shows that the HAp/SF composite improves bioactivity compared to the original SF. It is assumed, moreover, that this composite does not cause inflammation in living bodies. This HAp/SF composite will allow the range of SF application as a biomedical material to expand. This composite, moreover, will make a contribution to the development of implant devices.

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