JOURNAL OF MATERIALS SCIENCE **39** (2004) 3221–3225

Nano-scaled hydroxyapatite/polymer composite II. Coating of sintered hydroxyapatite particles on poly(2-(o-[1′-methylpropylideneamino] carboxyamino) ethyl methacrylate)-grafted silk fibroin fibers through covalent linkage

A. KOREMATSU
Department of Biomedical Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan

T. FURUZONO*
Department of Biomedical Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan; PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, 332-0012, Japan
E-mail: furuzono@ri.ncvc.go.jp

S. YASUDA
Department of Biomedical Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan

J. TANAKA
Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan

A. KISHIDA
Department of Biomedical Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan

Hydroxyapatite (HAp) has been applied to various biomaterials because of its good biocompatibility for hard and soft tissue. A percutaneous device with sintered HAp was developed by Aoki *et al.* but had the disadvantage of limiting a patient's mobility due to the rigid ceramic disc [1, 2]. We have developed nano-scaled HAp particles and a HAp/polymer composite to add further improvement in the application of HAp to this device [3–6]. It is expected that this HAp/polymer composite can be developed as percutaneous implant material.

Silk fibroin (SF) shows good qualities for biomaterials because of its unique physicochemical properties [7]. It is expected that SF can be applied to a polymer substrate of the composite with HAp since SF can add appropriate flexibility to the inorganic material. In our former paper [8], we reported the coupling between a nano-scaled HAp and a poly(γ-methacryloxypropyl trimethoxysilane)-grafted SF through vinyl bonds of 2-methacryloxyethyl isocyanate (IEM). This synthetic way requires, however, a three-step procedure and is complicated. In this report, to improve the method of synthesizing a novel composite consisting of HAp particles and SF through covalent linkage, therefore, we adopted the graft-polymerization with 2-(o-[1′-methylpropylideneamino] carboxyamino) ethyl methacrylate (IEM blocked with oxime; IEM-oxime) onto the SF by free radical initiation. This is because blocked isocyanate groups combining with HAp can be introduced onto the SF by only one step.

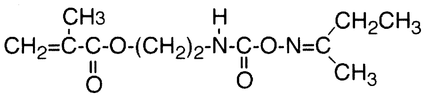
The composite was prepared through a two-step procedure—the graft-polymerization of poly(IEM-oxime) on the SF as the first step, and coupling between HAp and the modified SF as the second step. This synthetic method is simpler and more reasonable compared to our previous three-step procedure [8]. IEM 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 IEM derivative, for example IEM-oxime, allows the benefit of IEM being extended into a water-based system [9].

Nano-particles of HAp were prepared by the emulsion system using calcium hydroxide and potassium dihydrogen phosphate, described in the former reports [3, 4]. IEM-oxime [Showa Denko Co., Tokyo, Japan (Scheme 1)] was grafted on the SF using ammonium peroxodisulfate (APS, Wako Pure Chemical Industries, Ltd., Osaka, Japan) as an initiator, and pentaethylene glycol dodecyl ether as a nonionic surfactant. Graft-polymerization with IEM-oxime onto the SF was conducted by free radical initiation [10]. 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. 339×10^{-6} m^3 (1.8 mmol) of the IEM-oxime monomer, 41 mg (0.18 mmol) of APS and 73 mg (0.18 mmol) of the surfactant were mixed in 6.0×10^{-6} m^3 of distilled water. Five pieces of the SF (18 mm in diameter)

---

*Author to whom all correspondence should be addressed.

0022–2461



Scheme 1 Chemical structure of IEM-oxime monomer.

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(IEM-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 IEM-oxime as follows;

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

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

After HAp nano-scaled particles were suspended in toluene/isopropanol (9/1), a poly(IEM-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 water. This fabric with HAp was heated at 140 °C for 20 min in vacuum at 133.3 Pa for the deblocking of IEM-oxime and the reaction between the HAp particles and the isocyanate group of the grafted polymer [9]. The composite was washed by using an ultrasonic generator for 3 min (output: 20 kHz, 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.

Attenuated total reflection (ATR) and diffuse reflectance Fourier transform infrared spectrometry (FT-IR) were recorded by a Spectrum One (Perkin-Elmer Inc., MA, USA). The HAp and the composite were observed with a scanning electron microscope (SEM, JSM-6301F, Jeol Ltd., Tokyo, Japan). The tensile properties were measured by using Tensilon RTC-115 OA (Orientec Co., Tokyo, Japan) at an elongation rate of 5 mm/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 [11].

$$A_0 = d/(9 \times 10^6 \rho)$$

A_0: cross-section area (m^2),
d: fineness [the experimental value = $97D$(denier) (=1.078×10^{-5} kg/m)],
ρ: density (1,333 kg/m^3)

$$S = P/A_0$$

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

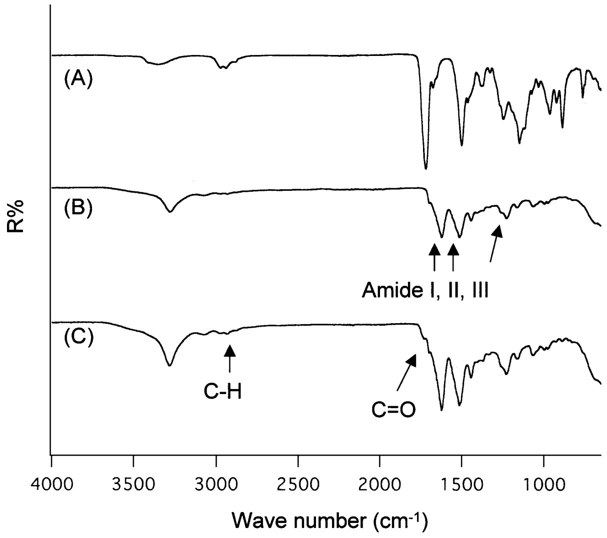


Figure 1 ATR FT-IR spectra of (a) IEM-oxime monomer, (b) the original SF, and (c) the (IEM-oxime)-modified SF.

D(denier) is a general unit of fineness of SF fiber and based on a standard mass per length of 1 g per 9000 m of SF fiber. Data from the tensile tests are presented as ± standard deviation from the mean. Statistical comparisons were performed with the use of the Student's t test and p values <0.01 were considered.

The cell culture procedure using L-929 cells was according to our former report [6]. Briefly, L-929 cells were placed onto 24-well multiplates with samples at 1×10^5 cells/well in an α-minimum essential medium with 10% fetal bovine serum, and incubated at 37 °C for 24 h.

Fig. 1 shows the ATR FT-IR spectra of the IEM-oxime monomer, the original SF, and the (IEM-oxime)-modified SF. 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. 1B. After the modification with the IEM-oxime monomer, new peaks appearing at 2936 and 1716 cm^{-1} contributed hydrocarbon and carbonyl groups of the monomer. The weight gain of poly(IEM-oxime) on the SF was plotted as a function of the reaction time (Fig. 2). The weight gain of poly(IEM-oxime) increased with increasing the reaction time, eventually

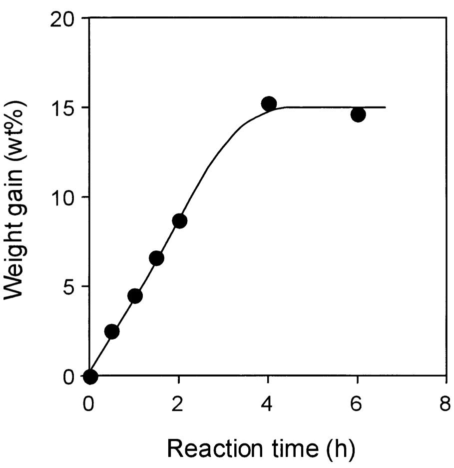


Figure 2 Weight gain of IEM-oxime on the SF as a function of reaction time.

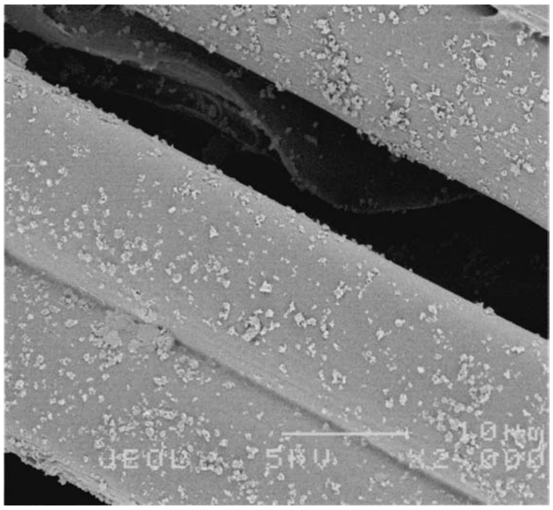
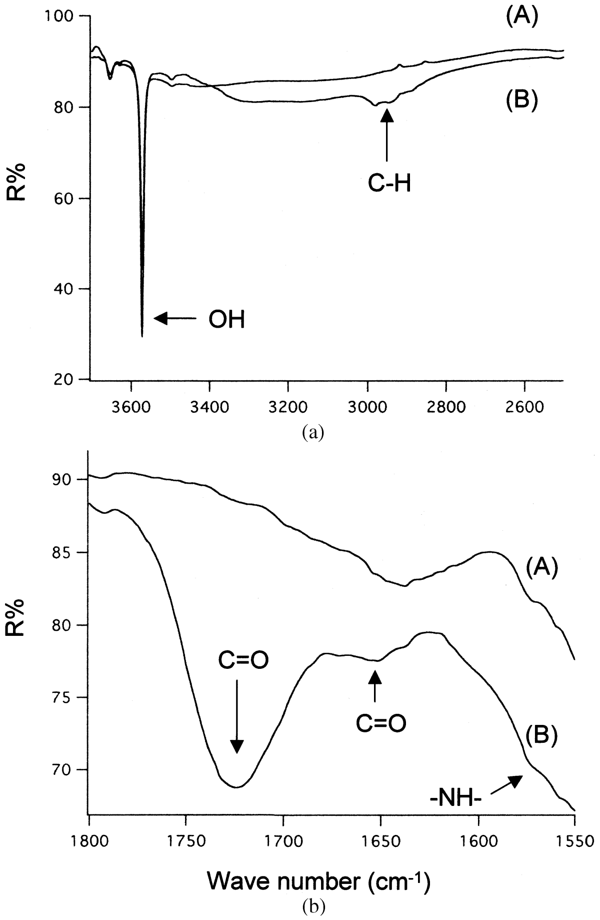


Figure 3 SEM photograph of the HAp/SF composite surface.

reaching a plateau value of about 15 wt%. In our graft-polymerization, it is theorized that graft-efficiency is low and does not increase beyond a certain value due to steric hindrance between the side chains of the IEM-oxime and the silk substrate [10]. Graft-polymerization with IEM-oxime onto SF can thus be well controlled. In all experiments of this report, we used 15 wt%-grafted SF.

Fig. 3 shows the SEM photograph of the composite surface conducted HAp adsorption and subsequent ultrasonic treatment. SF fibers of about 10 μm in width formed a parallel line. The nano-particles separated or aggregated with several crystals strongly remaining on the treated SF surface. It seems that it is very hard for HAp particles to bond on the SF fiber in mono layers. The HAp particles easily aggregate due to ionic phases on the HAp surfaces.

To prove indirectly the formation of the urethane linkage between HAp particles and IEM-oxime, the reaction of the particles with the IEM-oxime monomer was carried out. Briefly, the HAp particles adsorbed with IEM-oxime were heated at 140 °C for 20 min in vacuum at 133.3 Pa and the product was washed by acetone to remove the unreacted reagents. Fig. 4 shows the diffuse reflectance FT-IR spectra of the original and (IEM-oxime)-modified HAp particles. The spectrum of the (IEM-oxime)-modified HAp (Fig. 4B) 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 cm^{-1}. This result is in accord with Liu's paper [12]. By heating in vacuum, the formation of the urethane linkage follows the release of oxime from IEM-oxime to generate the isocyanate groups [9], because the deblocking temperature of IEM-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 the (IEM-oxime)-modified SF by the urethane linkage. It is possible to lower the reaction temperature by using IEM blocked with phenol or imidazole, for the deblocking temperature of these agents is 110–130 °C [9]. This reaction system is, therefore, a unique method of fixing HAp on the polymer substrate.



(a)

(b)

Figure 4 Diffuse reflectance FT-IR spectra of (a) the original and (b) the (IEM-oxime)-modified HAp particles.

Fig. 5 shows the tensile properties of three types of SF fiber [original SF, (IEM-oxime)-modified 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 (IEM-oxime)-modified SF was somewhat higher but Young's modulus thereof was somewhat lower, statistically. These results suggest that the difference of the elasticity may depend on the crystallinity on the surface. By means of the graft-polymerization of poly(IEM-oxime) on the SF, the crystallinity on the surface of SF decreases. The elasticity of (IEM-oxime)-modified SF, therefore, decreases. After the coupling between HAp and the modified SF, it seems that the crystallinity is compensated by the covering with the inorganic substrate on the surface of SF and the elasticity of the HAp/SF composite is apparently equal to that of the original SF. These results show that this HAp/SF composite maintains flexibility equivalent to the original SF.

To examine the bioactivity of the composite, a preliminary cell adhesion test was conducted. The morphology of L-929 cells on two sample substrates (original SF and HAp/SF composite) incubated for 24 h was observed by SEM (Fig. 6). Observing L-929 cell morphology on the HAp/SF composite, the cells adhered to the substrate uniformly, although few were found

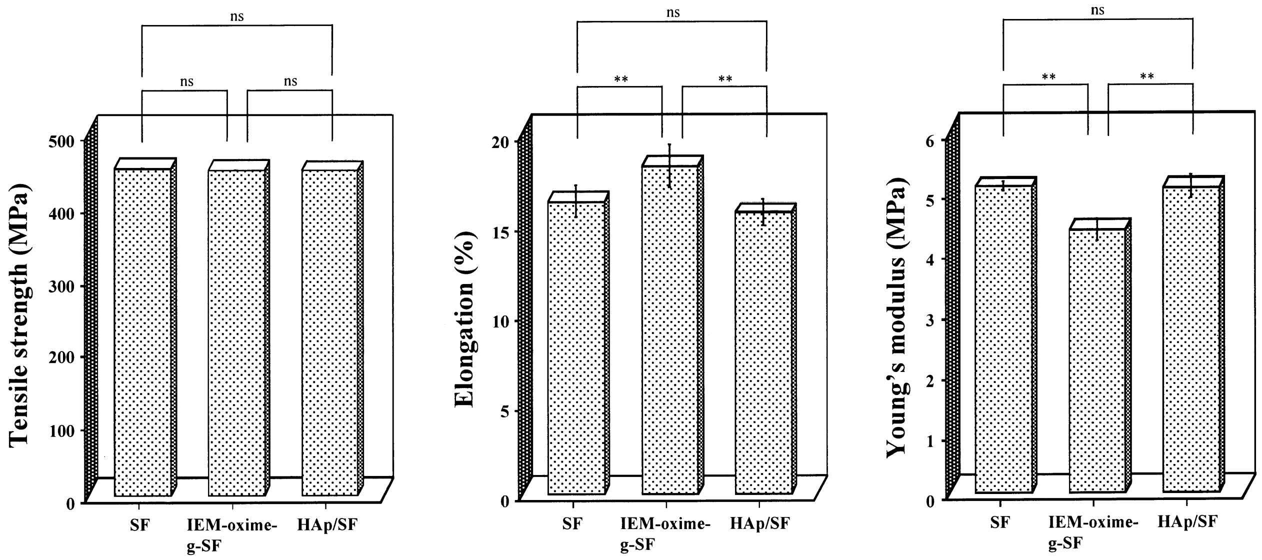


Figure 5 Tensile properties of three types of SF [original SF, (IEM-oxime)-modified SF, and HAp/SF composite]. Data were calculated as means of six-time determinations. Error bars represent standard deviations of six-time determinations. [**means an existence of a significant difference ($p < 0.01$) between two samples.]

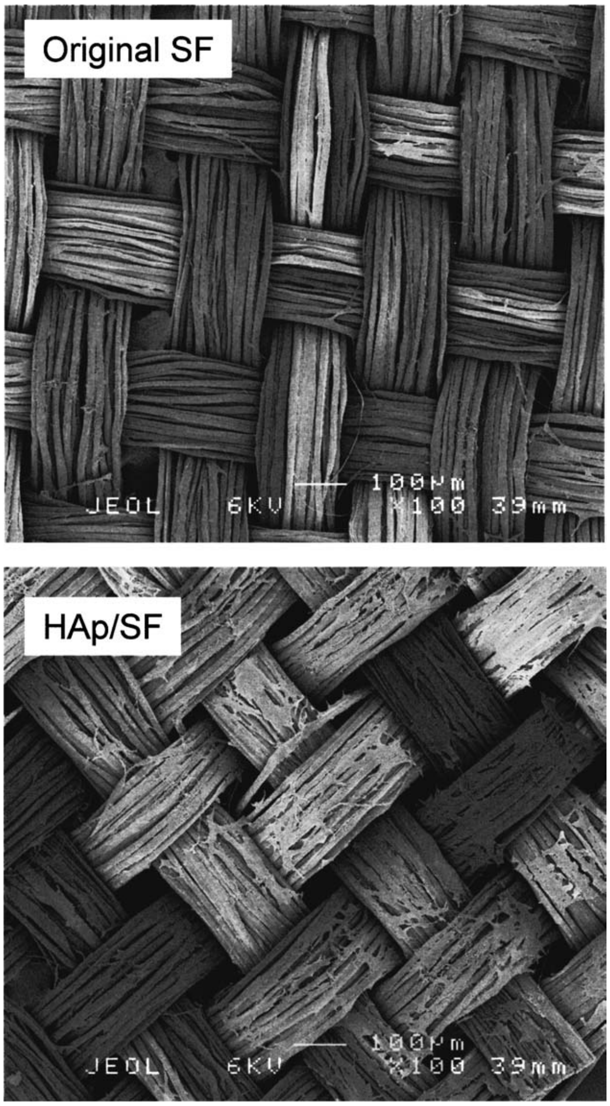


Figure 6 SEM photographs of L-929 cells cultured on the original SF and HAp/SF composite for 24 h.

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. In addition, these results demonstrate that the HAp/SF has no cytotoxicity.

In conclusion, a novel composite consisting of nano-scaled HAp particles and SF through covalent linkage was synthesized. This synthetic method is simpler and more reasonable in terms of requiring only a two-step procedure for obtaining the HAp/SF composite. Graft-polymerization with IEM-oxime onto the SF is well controlled. The tensile test shows that the elasticity of the HAp/SF composite is equivalent to that of the original SF. The cell adhesion test shows that the HAp/SF composite improves bioactivity compared to the original SF and has no cytotoxicity. This HAp/SF composite will be applicable to implant material having good biocompatibility for soft tissue and make a contribution to the development of percutaneous devices.

Acknowledgment

This work was partially supported by CREST, JST.

References

1. H. AOKI, "Medical Applications of Hydroxyapatite" (Ishiyaku EuroAmerica Inc., 1994).
2. M. TANIGUCHI, H. TAKEYAMA, I. MIZUNO, N. SHINAGAWA, J. YURA, N. YOSHIYAMA and H. AOKI, *Jpn. J. Artif. Organs* **20** (1991) 460.
3. T. FURUZONO, D. WALSH, K. SATO, K. SONODA and J. TANAKA, *J. Mater. Sci. Lett.* **20** (2001) 111.
4. K. SONODA, T. FURUZONO, D. WALSH, K. SATO and J. TANAKA, *Solid State Ionics* **151** (2002) 321.
5. T. FURUZONO, K. SONODA and J. TANAKA, *J. Biomed. Mater. Res.* **56** (2001) 9.
6. T. FURUZONO, P. L. WANG, A. KOREMATSU, K. MIYAZAKI, M. OIDO-MORI, Y. KOWASHI, K. OHURA, J. TANAKA and A. KISHIDA, *J. Biomed Mater. Res.* **65B** (2003) 217.
7. Y. GOTOH, M. TSUKADA and N. MINOURA, *ibid.* **39** (1998) 351.

8. T. FURUZONO, J. TANAKA and A. KISHIDA, *J. Mater. Sci. Mater. Med.* (in press).
9. P. E. CRANLEY, *Polym. Sci. Technol.* **29** (1984) 765.
10. T. FURUZONO, K. ISHIHARA, N. NAKABAYASHI and Y. TAMADA, *J. Appl. Polym. Sci.* **73** (1999) 2541.
11. NIPPON-SEN'I-KIKAI-GAKKAI (ed.), "Sangyoyo-sen'i-sizai Handbook" (Nippon-sen'i-kikai-gakkai, Tokyo, 1979) p. 8.
12. Q. LIU, J. R. DE WIJN and C. A. VAN BLITTERSWIJK, *J. Biomed. Mater. Res.* **40** (1998) 257.

Received 11 June
and accepted 24 November 2003