Preparation and Characterization of Regenerated *Bombyx mori* Silk Fibroin Fiber Containing Recombinant Cell-Adhesive Proteins; Nonwoven Fiber and Monofilament

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ABSTRACT: To improve the cell-adhesive character of *Bombyx mori* silk fibroin fiber, the regenerated *B. mori* silk fibroin fibers with recombinant cell-adhesive proteins were prepared. The recombinant proteins were originally designed in our laboratory and expressed in *Eschericchia coli*; one is the protein with the primary structure of $(TGRGDSPAS)_8$ which was designed based on the cell adhesive sequence in fibronectin and the other is $(GERGDLGPQGIAGQRGVVGERGERGERGAS)_8$GPPGPCC GGG originated from the cell adhesive sequences in collagen. The nonwoven silk fibroin nano-fibers including these recombinant proteins were prepared by electrospinning method from the hexafluoroisopropanol (HFIP) solutions of mixture of *B. mori* silk fibroin and the recombinant proteins. The cell-adhesive characters of such nonwoven silk fibers were much improved compared with that of the nonwoven silk fibroin fibers without the recombinant proteins. In addition, the degradation rates of the nonwoven fibers were accelerated by mixing of the recombinant proteins into silk fibroin. Here, we also prepared the regenerated silk fibroin monofilaments including the recombinant proteins by wet spinning from the HFIP dope solution. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 2956–2963, 2008

Key words: regenerated *Bombyx mori* silk fibroin; fibronectin cell-adhesive sequence RGD; collagen cell-adhesive sequence; nonwoven nano-fiber; electrospinning; silk fibroin monofilament

INTRODUCTION

Bombyx mori silk fibroin is of practical importance because of its excellent intrinsic properties utilized in biotechnological and biomedical fields as well as the importance of silkworm silks in the manufacture of high quality textiles.[1–4] However, their utilization has sometimes encountered some difficulty due to the lack of cell adhesive character and also the lack of biodegradation of the silk fibroin. Therefore it is important to improve these characters of the silk fiber in the application to the biomedical fields.

One of the most commonly used in biomaterials and the most physiologically ubiquitous binding motif is the short peptide containing, arginine-glycine-aspartic acid (RGD) which was identified as the active sequence of cell-adhesive protein, fibronectin, of the extracellular matrix (ECM). Actually, to promote the cellular adhesion character of silk fiber, RGD peptides have been covalently immobilized on the polymeric supports[5] or introduced in the backbone chain[6,7] directly those have been used in cell cultivation.

In this article, regenerated *B. mori* silk fibroin fibers were prepared after mixing the silk fibroin with the recombinant proteins, $(TGRGDSPAS)_8$ which was originally designed and produced from *E. coli* in our laboratory to improve the cell-adhesive character of *B. mori* silk fibroin fiber or after mixing the silk fibroin with the recombinant protein based on the cell adhesive sequences derived from collagen, $(GERGDLGPQGIAGQRGVVGERGER\text{-}GERGAS)_8$ GPPGPCCGGG which was also originally designed in our laboratory. For preparation the regenerated *B.mori* silk fiber, we used two methods; electrospinning and wet spinning. As a common solvent of both silk fibroin and recombinant proteins, hexafluoroisopropanol (HFIP) was used. HFIP dissolves both silk fibroin and recombinant proteins and thus, the mixing of them is achieved. In addition, the regenerated silk fiber from HFIP shows comparable strength to the natural silk fibroin fiber.

The electrospinning experiments of *B. mori* and other silk fibroin solutions with certain concentrations in aqueous solution or organic solvents have been reported.[8–10] Electrospun nonwoven fibers have

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super specific surface area and the highly porous 3-D structure is utilized for high-density cell and tissue cultivations.[11,12] For these reasons, the nonwoven silk nano-fiber was prepared after mixing the recombinant protein with original *B. mori* silk fibroin in HFIP. The preparation of the regenerated single long silk fibroin fiber with the recombinant protein was also attempted using HFIP as dope solution and methanol as coagulation solvent.[13] As mentioned earlier, the stronger regenerated silk fiber has been developed by using HFIP. Moreover, it is possible to add functional agents or recombinant proteins to fiber by wet spinning method. Thus it is expected to obtain functionalized silk fiber to mix the cell-adhesive proteins into silks.

The cell-adhesive characters of the nonwoven fiber obtained by electrospinning were evaluated by cultivation of normal human dermal fibroblasts (NHDFs) *in vitro*. The degradation experiments of the fibers in the enzyme solutions were also tried to aim the application of the materials for a scaffold of tissue-engineering.

MATERIALS AND METHODS

Production of the recombinant proteins, $(TGRGDSPAS)_8$, and $(GERGDLGPQGIAGQRG VVGERGERGERGAS)_8$GPPGPCCGGG

After polymerization of DNA sequences which were inserted into the oligonuleotides of $(TGRGDSPAS)_8$ (Hereafter, we call this recombinant protein as RP1) or $(GERGDLGPQGIAGQRGVVGERGERGERGAS)_8$ GPPGPCCGGG (RP2), the inserts of the target DNAs were subcloned directionally into expression vector pET30a (+), and transformed into BL21(DE3)pLysS cells (an *E. coli* bacterial cell strain useful for protein expression). The cells were cultivated in TB broth at 37°C. Large scale cell cultivation was performed using a fermentor (Marubishi BioEng. Japan). Protein expression was induced by addition of 1 m*M* IPTG (isopropyl-thio-β-D-galactopyranoside) when the OD_{600} was between 0.8 and 1.0 at which time the temperature was reduced to 33°C. After ~2 h of protein expression (the $OD_{600} \approx 1.3$), the cells were harvested by centrifugation at 8000 rmp for 20 min and then stored at −70°C before purification. The frozen cells were thawed on ice and re-suspended in the lysis buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 20 m*M* imidazole for RP1 or 30 m*M* imidazole for RP2) at 5 mL per gram wet weight, and cells were lysed by gently vortex until the solution became translucent. To purify the proteins using His-tags, after loading the column was washed with wash buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 30 m*M* imidazole for RP1 or 40 m*M* imidazole for RP2, pH 8.0). The proteins were eluted with elution buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 250 m*M* imidazole, pH 8.0). The elute was dialyzed against distilled water for 3 days and then was lyophilized. SDS-PAGE and western-blot analysis were utilized to confirm the constituents in the elute.

Preparation of Hfip solution of silk fibroin

Silk fibroin fibers from cocoons of *B. mori* were degummed three times with 0.25% (w/v) Marseilles soap and Na_2CO_3 solution at 80°C for 30 min and washed with 60°C distilled water to remove another silk protein, sericin, from the surface of silk fibers. Resulting silk fibroin fibers were then dissolved in 9*M* LiBr (Wako Pure Chemical Industries, Ltd.) for 30 min at 37°C. After dialysis against distilled water for 3 days, the solution was lyophilized and sponges of the silk fibroin proteins were obtained. The sponges were then dissolved in HFIP to prepare 5 w/v% and 12 w/v% solutions for electrospinning and wet spinning, respectively. The recombinant proteins were mixed into the silk fibroin/HFIP solutions with different percentage, 10 w/w% or 30 w/w% to the weight of silk fibroin sponges.

Preparation of nonwoven silk fibroin nano-fiber by electrospinning method

Silk fibroin/HFIP solutions were extruded with the aid of a syringe pump (KDS100, Harvard Apparatus) through a metal blunt tipped needle (18G) at room temperature and at a flow rate of 3 mL/h. An aluminum board coated with Teflon was used as a collector for the fibers because it is easy to remove the nonwoven silk fibers from the board after spinning. The collector was placed at distance of 23 cm from the needle tip. The needle axis was set in the horizontal direction. A voltage of 17 kV was applied to the needle through a low current, high voltage power supply (AKT-030k033PS, Japan), while the collector was grounded. After spinning, the nonwoven fibers were immersed to 80% methanol/H_2O at room temperature resulting in water-insoluble materials. Scanning electron microscope (SEM, KEYENCE VE-7800, Japan) was used to investigate the macroscopic morphology and surface texture of electrospun fibers. FTIR spectrometer (FT/IR-4100, JASCO, Japan) was used to elucidate the secondary structure of silk fibroin.

Preparation of single long silk fiber by wet spinning method

A flow rate of 149 mL/h was used for the preparation of the single long silk fiber by wet spinning. The diameter of the spinneret was 0.8 mm. The as-spun filament passes through a 2-cm air gap before

flowing into the methanol coagulation bath at room temperature. The as-spun filaments were soaked in the methanol coagulation bath for 3 h to remove HFIP. To improve the mechanical properties of the fibers, the filament was then drawn to three times its original length in 40–50°C water. After drawing, the filament was fixed at the length after elongation for overnight to prevent the contraction of fibers. The SEM pictures and FTIR spectra were obtained. Stress–strain curves of the regenerated silk fibers were recorded on a tensile testing machine (EZ Graph, SHIMADZU, Japan) at room temperature with 5 N load under the head speed of 10 mm/s.

Cell adhesive experiment of nonwoven nano-fiber

Normal human dermal fibroblasts (NHDFs) were purchased from Kurabo Industries, Osaka. NHDFs were subcultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% heat-inactivated FBS (fetal bovine serum), 20 m*M* HEPES (*N*-2-hydroxyethlpiperadine-*N*′-2-ethansulfonic acid), 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air. The nonwoven silk sheet prepared by the electrospinning method after treating with methanol was aseptically cut into a square shape ($\sim 7 \times 7$ mm^2), transferred to a polystyrene culture dish with a negative charge surface (Falcon no. 3001; 35 mm in diameter), and immersed completely in 2.0 mL of distilled water under a reduced pressure condition at 0.07 MPa. The wet sheet, after removing the water, was spread in a center region of the dish and dried under clean air (class 100) at 10°C with 40% humidity to yield a sheet-attached culture dish. Similarly, the nonwoven sheets of silk fibroin fibers involving 10 or 30% RP1, and 10 or 30% RP2 were prepared via the treating process with methanol and subjected to the preparation of culture substrata to yield a sheet-attached culture dish. NHDFs were seeded on each culture substratum at the initial cell density of 0.7×10^4 cells/cm^2 and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. After culturing the cells for 24 and 48 h on each substratum, the culture medium was removed and the cells were incubated with HBSS (Hanks' Balanced Salt Solution) containing 2*M* calcein-AM (Molecular Probes TM) and 4*M* ethidium homodimer (Molecular Probes TM) for 15 min. Subsequently, the cells were observed by a fluorescent microscope to examine their viability and also by a phase-contrast microscope to investigate their morphology under an identical visual field. The viable cell numbers on each substratum were calculated by counting the calcein-positive cells in the three independent areas of square (0.5×0.5 mm^2).

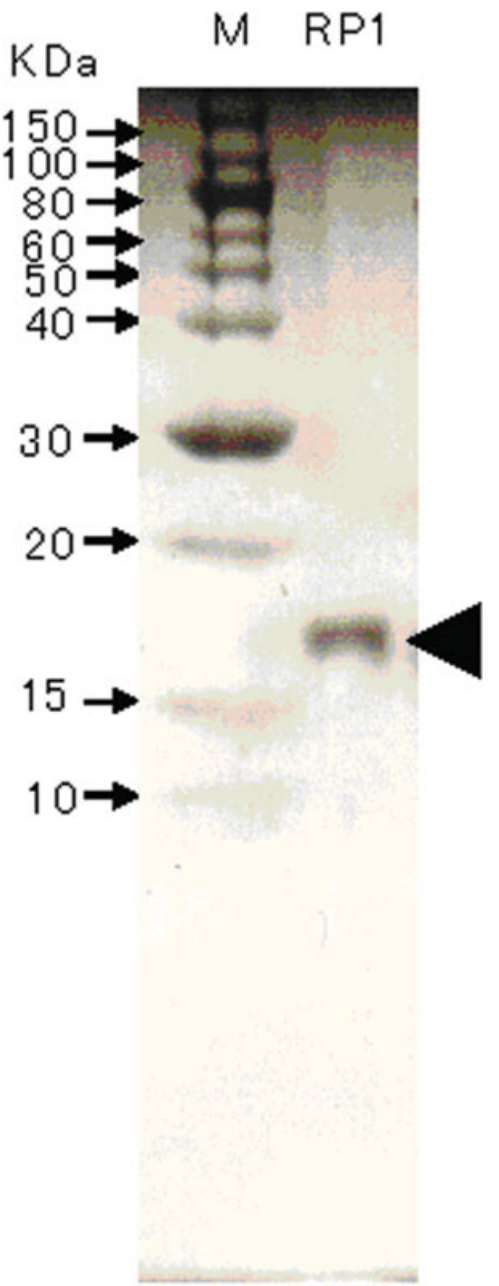

Figure 1 The SDS-PAGE of RP1, (TGRGDSPAS)8. Lane M: molecular markers; ◀: target protein PR1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Degradation experiments of the nonwoven nano-fiber and regenerated monofilaments

Five milligram of protease XIV (SIGMA) was dissolved in 20 mL of phosphate buffered saline (PBS, DANIBON) solution. Fifteen milligram of nonwoven fibers was immersed into the enzyme solution in a tube. PBS solution without the enzyme was used as control. Then the tubes were put into the incubator at 37°C. After 24 h, the nonwoven fibers were washed in MilliQ water and PBS solution for three times. Finally, the samples were dried in vacuum. Solutions were

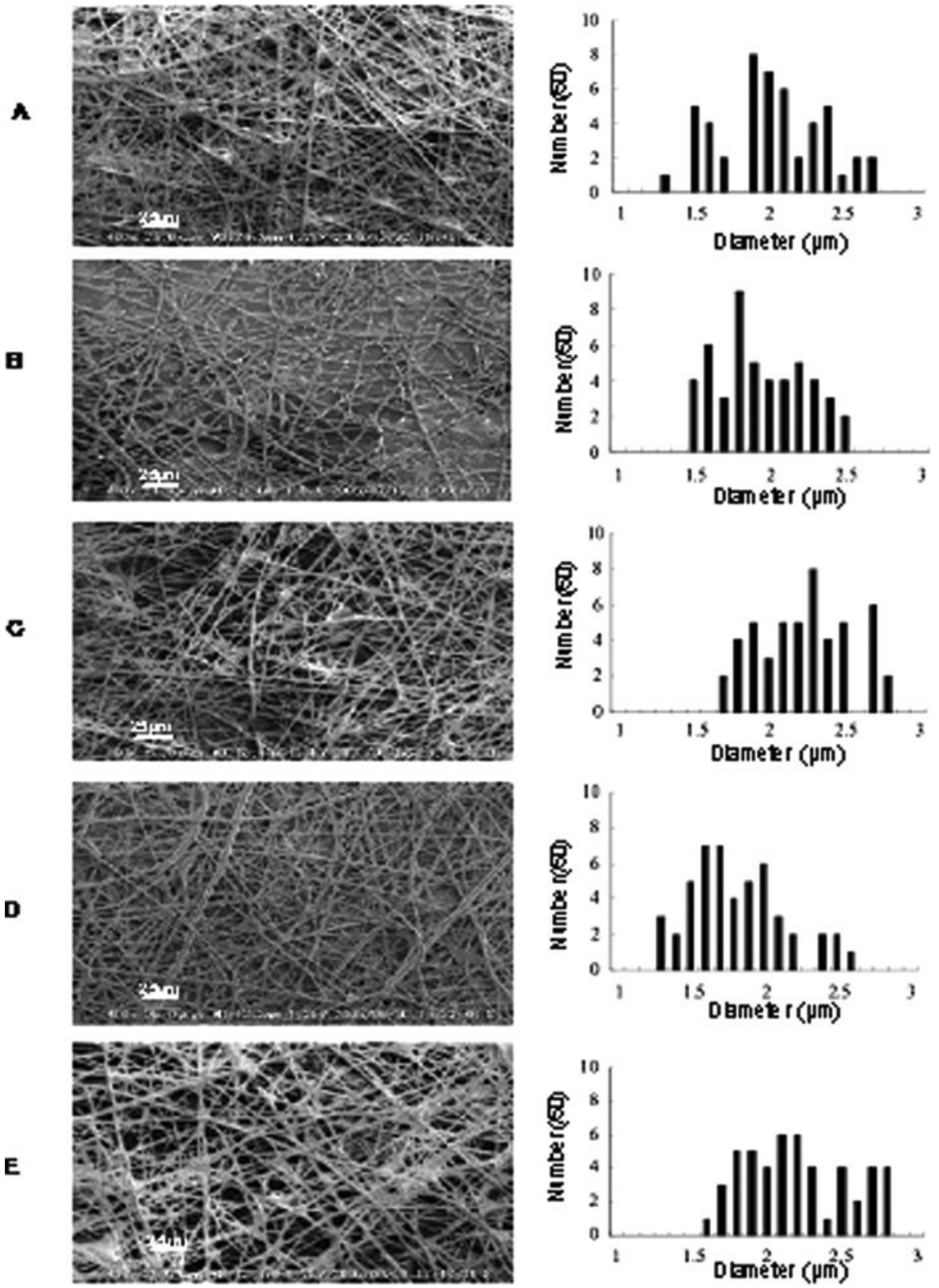

Figure 2 Scanning electron microscope images of nonwoven silk fibroin sheets from the HFIP solution. A: silk fibroin without the recombinant proteins. B–E: mixtures of 10 wt % RP1, 30 wt % RP1, 10 wt % RP2, and 30 wt % RP2, respectively. Histograms in the right column indicates the distribution of the fiber diameter in the nonwoven sheets.

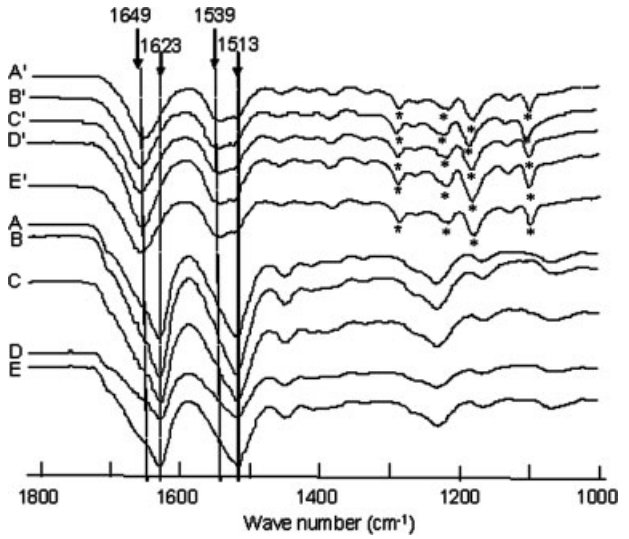

Figure 3 FTIR spectra of nonwoven silk fibroin sheets. A, A′: silk fibroin without the recombinant proteins; B, B′: silk fibroin mixed with 10 wt % RP1; C, C′: silk fibroin mixed with 30 wt % RP1; D,D′: silk fibroin mixed with 10% wt % RP2; and E, E′: silk fibroin mixed with 30 wt % RP2. A′–E′ and A–E are corresponding to the samples before and after methanol treatment, respectively. Asterisks indicate the HFIP peak.

changed and collected every 24 h. A similar degradation experiment was performed for single long silk fibroin fiber prepared with wet-spinning method.

RESULTS AND DISCUSSION

Production of the recombinant proteins, RP1, and RP2

Figure 1 shows the result of SDS-PAGE for RP1. The molecular weight of RP1 was estimated as 17.5 kDa compared with the markers, which is in agreement with the predicted molecular weight from the primary sequence. The RP2 was produced under the similar condition and its molecular weight was confirmed by SDS-PAGE as reference.[7] These results suggest that the target recombinant proteins were successfully synthesized and purified. The yields of the proteins after purification were 25 mg/L (RP1) and 68 mg/L (RP2), respectively.

Characterization of nonwoven silk fibroin fibers

The nonwoven silk fibers with the recombinant proteins were prepared from the HFIP solution by electrospinning method. Figure 2 shows the SEM pictures and diameter distributions of nonwoven silk fibroin fibers. There are no significant changes among SEM pictures, indicating that the presence of the recombinant proteins does not affect to the fiber formation and the morphology of nano-fibers through the elec-

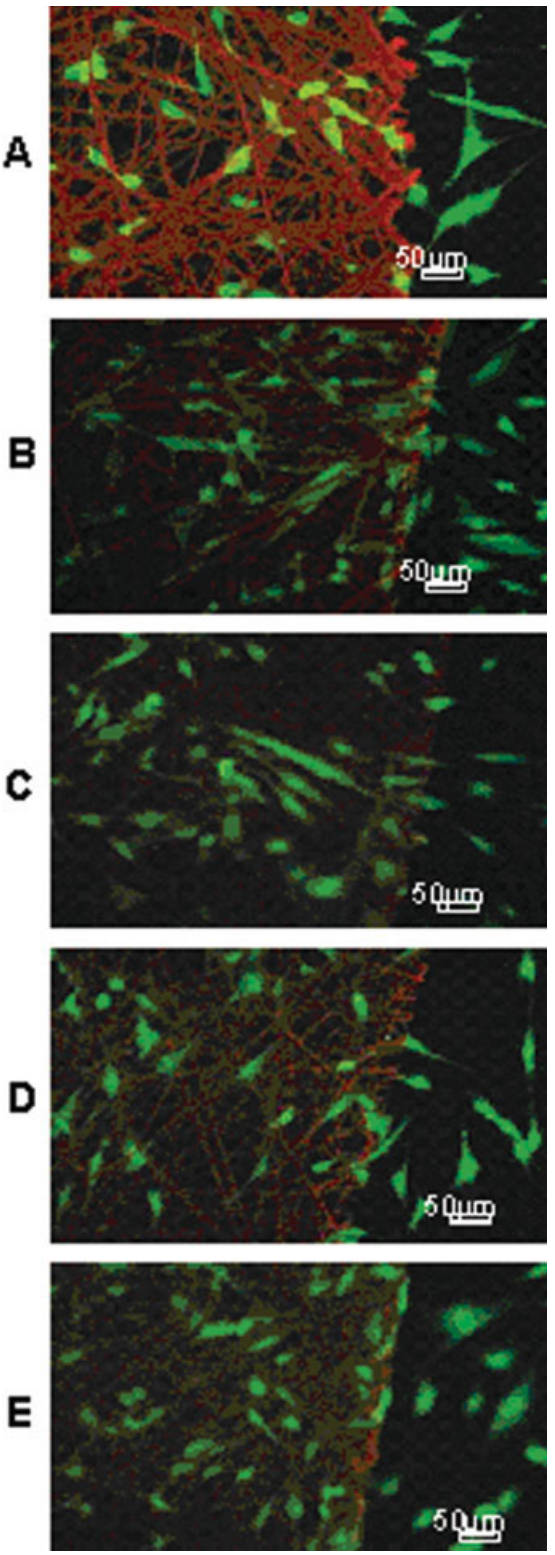

Figure 4 Images of cell adhesion and spreading on nonwoven silk sheets. A: 100% silk fibroin; (B–E) mixtures of 10 wt % RP1, 30 wt % RP1, 10 wt % RP2, and 30 wt % RP2, respectively. The active cells and the nonofibers emit green and red lights respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

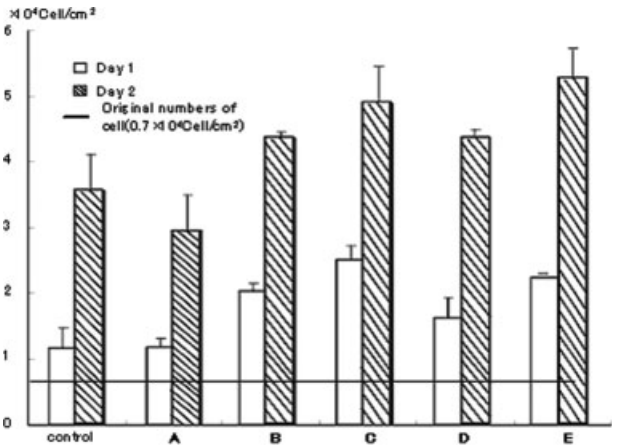

Figure 5 Cell adhesion assays of nonwoven silk fibroin sheets. A: 100% silk fibroin; (B–E) mixtures of 10 wt % RP1, 30 wt % RP1, 10 wt % RP2, and 30 wt % RP2, respectively.

trospinning process. The diameter of nano-fiber tends to increase slightly with increasing the fraction of recombinant proteins, but the change is not so large. The averaged diameter was about 2 μm.

We previously reported that *B. mori* silk fibroin took the conformation of 3_{10} helix in HFIP from the CD pattern.[13] During the electrospinning process, the structural transition of the silk fibroin to β-sheet does not occur and therefore it is necessary to promote structural transition to β-sheet for obtaining the nonwoven silk fiber with refractoriness to water. For that, we used 80% methanol/H_2O for the coagulation. Figure 3 shows a structural change from 3_{10} helix to β-sheet by methanol treatment detected by FTIR spectra. Before methanol treatment, all of the FTIR spectra for the nonwoven silk fibroin fibers with and without the recombinant proteins indicate typical helical patterns (1649 cm^{-1} of amide I band).[14] Moreover the presence of remaining HFIP molecules in the fibers before methanol treatment can be pointed out from the presence of several bands in the range of 1100-1300 cm^{-1} indicated by asterisks in Figure 3(A′–E′).[15] After methanol treatment, the amide I band shifted to 1623 cm^{-1}, indicating that the conformation changed to β-sheet structure.[16] It is also suggested that the HFIP molecules are removed by methanol treatment. These changes in the conformation of silk fibroin are independent of the presence of recombinant proteins and their fraction.

Cell adhesion and growth activities of the nonwoven silk fibroin fibers

Figure 4 shows the active cell distribution and cell shapes of NHDFs on the surface of the nonwoven silk fibers with and without recombinant protein after 1 day cultivation. The active cells and the nanofibers are shown as green and red, respectively, in the Figure. The active cells were distributed uniformly on the surface of the nonwoven fibers and were kept the original spindle shapes independent of the presence of recombinant proteins. The pictures show that the cells extend along the fiber direction, which means that these nonwoven silk fibroin sheets are no toxic for NHDFs. Figure 5 summarizes the numbers of active cells on the nonwoven silk sheets after incubating 1 and 2 days. The numbers of the active cells exhibit increase from 1 day to 2 days. In addition, the number increases slightly for the nonwoven silk fiber containing 30% recombinant protein compared with the case of nonwoven silk fibroin containing 10% recombinant protein. This tendency is independent of the recombinant proteins, PR1 and PR2. To conclude,

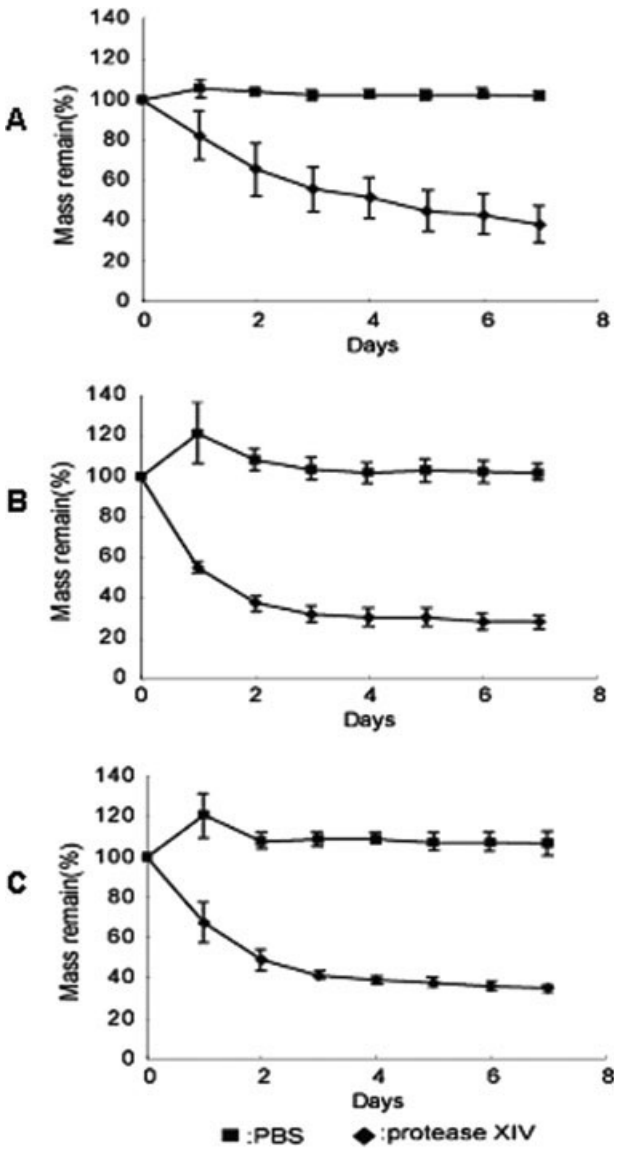

Figure 6 Mass remain of *B.mori* silk fibroin nonwoven sheets for protease treatment and PBS treatment, (A) 100% silk fibroin; (B) and (E) mixtures of 10 wt % RP1 and 10 wt % RP2.

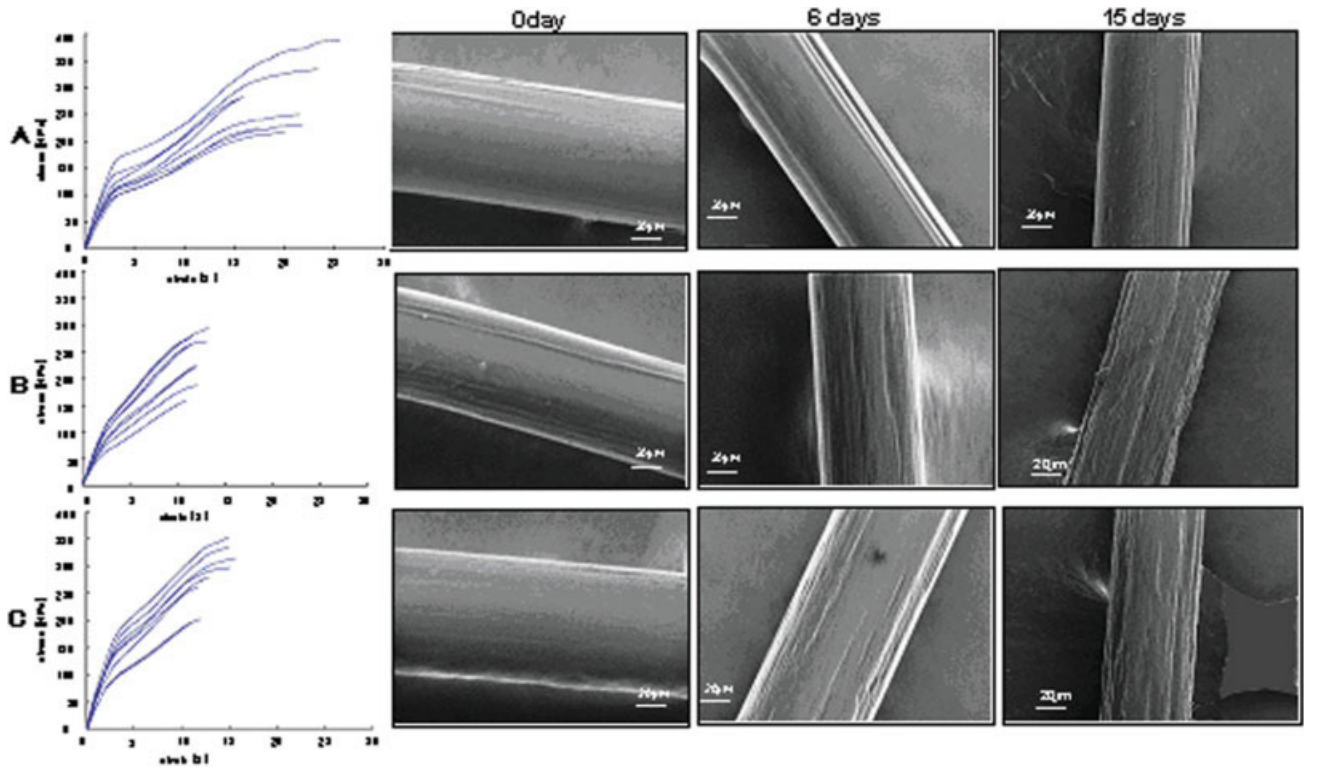

Figure 7 SEM and strain-stress curve of the regenerated long silk fibroin fibers (A: regenerated silk fibroin fiber, Stress: 329 ± 59 MPa; Elongation: 21.6 ± 3% B: regenerated silk fibroin fiber mixed with 10% RP1 of fibroin weight, Stress: 259 ± 48 MPa; Elongation: 12.9 ± 2%; C: regenerated silk fibroin fiber mixed with 10% PR2 of fibroin weight; Stress: 311 ± 65 MPa; Elongation: 14.1 ± 2%,) and kept degrading for 6 and 15 days in the protease solution, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the nonwoven silk fibroin fibers containing the recombinant protein, $(TGRGDSPAS)_8$ or (GERGDLGP QGIAGQRGVVGE-RGERGERGAS)$_8$GPPGPCCGGG are suitable for the cultivation of fibroblasts compared with the case of nonwoven silk fibroin only.

The degradation of nonwoven silk fibroin fibers

Figure 6 shows the aspect of degradation of the nonwoven silk fibroin fibers by protease XIV. These fibers do not show biodegradable behavior in only PBS solution, but bio-degradable in protease XIV. The mass loss of nonwoven fibers containing RP1 [Fig. 6(B)] is slightly larger than other two cases. Thus the nonwoven silk fibroin fiber containing recombinant proteins can be used for excellent biomaterials in viewpoint of cell-adhesive and biodegradable characters. By changing condition of methanol treatment, it seems possible to change the characters of nonwoven silk fibroin fiber largely.

The preparation and characterization of the regenerated silk fibroin monofilament mixed with recombinant proteins

Silk fibroin has been used as fiber in the textile and medical fields. Therefore, we attempted to prepare the regenerated silk fibroin monofilament with wet-spinning methods. In Figure 7, SEM pictures and stress–strain curves of regenerated monofilaments containing 10w/w% RP1 [Fig. 7(B)] and 10 w/w% RP2 [Fig. 7(C)] were shown together with that of silk fibroin only [Fig. 7(A)]. We previously reported the stress–strain curve of regenerated *B. mori* silk fibroin fiber without including recombinant protein prepared by using similar wet spinning process.[13,17] The stress–strain curve for the regenerated silk fibroin monofilament without the recombinant proteins is almost the same as the previous date within experimental error. It is noted that the stress–strain curves change slightly when the recombinant proteins are included. Especially, the elongations of the regenerated silk fibroin fibers mixed with RP1 or RP2 clearly decreased. This may be due to the interference of these recombinant proteins in the stretching process of silk fibroin monofilament. The biodegradation of these fibers was also slightly promoted by the presence of these recombinant proteins. This is clear in the pictures of silk fibroin monofilaments after 15 days in the degradation experiment. Thus, the presence of recombinant proteins change character of stress–strain curve and biodegradation compared with those of silk fibroin monofilament with-

out recombinant protein, but the degree of the change is not so much.

CONCLUSIONS

The regenerated nonwoven silk fibroin fibers with the cell-adhesive proteins prepared by electrospinning are excellent for cell cultivation as biomaterials. Adhesive properties of the recombinant proteins improved the biocompatibility and degradation rate of these silk fibroin fibers. This means that RP1 and RP2 produced by transgenic biotechnology in *E.coli* are well integrated with the silk fibroin and promote cell adhesion and growth. These materials are useful as biomaterials such as scaffold, matrix and suture.

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