CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Raw Silk and Shrimp Shells

Raw silk fibers, *Bombyx Mori*, were kindly supplied from Queen Sirikit Sericulture Center, Saraburi Province (Thailand). The shells of *Penaeus merguiensis* shrimps were kindly provided by Surapon Foods Public Co., Ltd., Thailand.

3.1.2 Model Drugs

Phenol red (dye content 90%, MW 376.36, Sigma-Aldrich), Chromotrope 2R (dye content 75%, MW 468.37, Aldrich), Crystal violet (dye content 90%, MW 407.98, Sigma-Aldrich), and Indoine blue (dye content 70%, MW 506, Aldrich) were used as the low-molecular weight model compounds. Human recombinant bFGF with an isoelectric point of 9.6 was supplied by Kaken Pharmaceutical Co., Tokyo, Japan.

3.1.3 Radionuclidic Compounds

Radioisotope $Na^{125}I$ (740 MBq/mL in 0.1 N NaOH aqueous solution) and N-succinimidyl-3-(4-hydroxy-3-di[^{125}I]iodophenyl) propionate (^{125}I-Bolton-Hunter Reagent, NEX-120H, 147 MBq/ml in anhydrous benzene) was purchased from NEN Research Products (DuPont, Wilmington, Del).

3.1.4 Other Chemicals

Sodium hydroxide (NaOH) 50% w/w aqueous solution was kindly supplied by KPT Cooperation Co., Ltd., Thailand. Sodium hydroxide anhydrous pellets (NaOH), and monochloroacetic acid were analytical grade purchased from Carlo Erba Co., Ltd. Protease (EC 3.4.24.31, 4 Umg^{-1}) from *Streptomyces griseus* was purchased from Sigma. All other chemicals were reagent grade and used as received.

3.2 Equipment

3.2.1 Fourier Transform Infrared Spectrophotometer

A Thermo Nicolet Nexus 671 Fourier-transform Infrared (FT-IR) Spectrophotometer was used to characterize the chemical structure and conformation of silk fibroin, chitin and CM-chitin.

3.2.2 Scanning Electron Microscope

A JEOL JSM 5410LV scanning electron microscope (SEM) was used to observe the morphology of the surface and cross-section of the films and scaffolds

3.2.3 Wide-angle X-ray Diffractometer

A Rigaku Rint2000 wide-angle X-ray diffractometer (WAXD) was used characterize the conformation of silk fibroin and corresponding blend films.

3.2.4 Differential Scanning Calorimeter

A Mettler-Toledo DSC 822e/400 differential scanning calorimeter (DSC) was used to evaluate the thermal properties of silk fibroin, chitin derivative and their blend materials.

3.2.5 Transmission Electron Microscope

A JEOL JEM-1230 transmission electron microscope (TEM) was used to observe the morphology and sizes of the chitin whisker and the dispersion of the whisker in silk fibroin matrix.

3.2.6 UV-vis Spectrophotometer

A Perkin Elmer Lambda 10 UV-vis spectrophotometer was used to examine a concentration of the dye solutions.

3.2.7 Gamma Counter

A ARC-301B Aloka gamma counter was used to measure a radioactivity of the ^{125}I-labelled bFGF solutions or ^{125}I-labelled silk fibroin materials.

3.3 Methodology

3.3.1 Preparation of Regenerated Silk Fibroin Solution

The raw silk fibers of *Bombyx mori* were boiled for 15 min in an aqueous solution of 0.05% Na_2CO_3. The boiling process was repeated two times to remove sericin. The fibers were then rinsed thoroughly with hot water and dried at 40°C overnight. After drying, the degummed silk was dissolved in a solvent mixture of $CaCl_2$:Ethanol:H_2O (molar ratio = 1:2:8) at 78°C. The protein solution was filtered with filter cloth. The filtrated solution was subsequently dialyzed in distilled water for 4 days by changing the media everyday, followed by centrifugation at 10,000 rpm for 10 min. The as-prepared aqueous silk fibroin concentration was 6.32 wt%.

3.3.2 Preparation of Chitin

Chitin was prepared from shrimp shell by decalcification and deproteinization to remove calcium carbonate and protein, respectively. The shrimp shells were cleaned and dried under sunlight before grinding into small pieces. Shrimp shell chips were treated by immersion in 1 N HCl solution for 2 days with occasional stirring. The decalcified product was washed with distilled water until neutral to pH paper. Deproteinization was followed by boiling the decalcified product in 4% w/w of NaOH solution at 80-90°C for 4 h. After NaOH solution was decanted, the chips were washed with deionized water until neutral to pH paper. The product obtained was dried at 60°C in a convective oven for 24 h. The obtained chitin was determined by Fourier-transformed infrared spectroscopy (FT-IR) following the method of Baxter *et al.* (1992) and was found to be 20%.

3.3.3 Preparation of CM-chitin

Alkaline chitin was prepared by suspending chitin power (4 g) in 42% NaOH solution (80 ml). After the suspension was allowed in desiccator for 30 min under reduced pressure, crush ice (160 g) was added and the mixture was mechanically stirred for 30 min in an ice bath to dissolve chitin. A viscous alkaline chitin solution was obtained. For successful synthesis of CM-chitin, the concentration of NaOH solution should not less than 14 %. Monochloroacetic acid solution was prepared by dissolving in 14% NaOH solution in an ice bath and added dropwise into the alkaline chitin solution with stirring over 30 min. After standing overnight at room temperature, the mixture was neutralized with acetic acid under cooling in an ice bath and dialyzed against running water for 2 days, followed by dialysis against distilled water for 1 day. The dialysate was centrifuged at 5000 rpm for 20 min in order to remove insoluble material, and the supernatant was added to 3 volumes of acetone. After standing overnight, the precipitate was collected by centrifugation and washed with acetone. The product was resuspended in ethanol and collected by filtration. After drying at room temperature, CM-chitin Na salt was obtained.

3.3.4 Preparation of Chitin Whisker Suspension

The preparation of chitin whisker was modified from the method of Morin & Dufresnè (2002). Briefly, chitin whisker suspension was prepared by hydrolyzing chitin flake (< 2 mm) with 3 N HCl at its boiling point (i.e. 104°C) for 6 hours under vigorous stirring. The ratio of 3 N HCl to chitin sample was 30 $cm^3 \cdot g^{-1}$. After acid hydrolysis, the suspension was diluted with distilled water, followed by centrifugation at 10,000 rpm for 10 min. This process was repeated three times. The suspension was then transferred to a dialysis bag and dialyzed in running water for 2 h and later in distilled water until the pH of the suspension became neutral. The dispersion of the whisker in the suspension was completed by ultrasonication treatment for 10 min. The suspension was refrigerated at 4°C prior to further use. The solid content of the as-prepared chitin whisker suspension was 4.63 wt%.

3.3.5 Methanol Treatment

The silk fibroin matrix was immersed in an aqueous methanol solution at a concentration of 90% (v/v) for 30 min. After excessively washing with distilled water, the methanol-treated silk fibroin materials were subsequently dried at 40°C overnight.

3.3.6 Characterization of Silk Fibroin, CM-chitin and Their Blend films

3.3.6.1 Morphology, Thermal Properties, Chemical and Crystal Structures

Silk fibroin blend films were prepared by adding 1 wt% CM-chitin solutions into the 1 wt% regenerated silk fibroin solutions at the following silk fibroin/CM-chitin blend ratios; 10/0, 8/2, 6/4, 5/5, 4/6, 2/8, and 0/10. The solutions were slowly stirred for 12 h at room temperature and then cast on polystyrene Petri dish at 40°C. For the crosslinked silk fibroin/CM-chitin blend films, glutaraldehyde used as a crosslinking agent was added into the blend solution to achieve the concentration of 0.0075%.

A Mettler-Toledo DSC, moedel 822e/400 was used to evaluate the thermal properties of silk fibroin, CM-chitin, the blend films, and the corresponding methanol-treated films (about 3 mg) under N_2 atmosphere at a heating rate of 10 $K \cdot min^{-1}$ from 50 to 450°C.

Morphology of the fractured cross-sections and surfaces of silk fibroin, CM-chitin, the blend films, and the corresponding methanol-treated films were observed by a SEM (JSM-5410LV, Jeol, Japan) at a voltage of 15 kV. The fractured cross-section of the films was carried out by cracking them after immersion in liquid nitrogen. The further investigation of the miscibility between silk fibroin and CM-chitin was done by observation of films morphology after solvent extraction of water-soluble component from the blend films. Surfaces of the extracted films were observed by SEM after submerging the methanol-treated silk fibroin and the blend films in distilled water at room temperature for 48 h to extract the water-soluble fraction. All of samples were coated with gold on the ion sputter (SCD040, Lichtenstein) at 50 mTorr and 15 mA for 3 min.

FTIR spectroscopy was used to verify the chemical structure and conformation of silk fibroin, CM-chitin, the blend films and the corresponding

methanol-treated films. The measurements were carried out on a Thermo Nicolet Nexus 671 FT-IR (32 scans at a resolution of 4 cm^{-1}). A Rigaku Rint2000 XRD was used to investigate the crystal structure of the as-cast silk fibroin, CM-chitin, the blend films and the corresponding methanol-treated films. The X-ray source was Ni-filtered Cu-Ka radiation (40 kV, 30 mA).

3.3.6.2 In Vitro Biodegradation and Equilibrium Water Content

The crosslinked methanol-treated silk fibroin, CM-chitin and the silk fibroin/CM-chitin blend films at 9:1, 7:3, 4:6 blend ratios (circle-shaped films with diameter of 14 mm and thickness of ca. 30 μm) were incubated at 37°C for 2 and 6 days in a 1 $mg \cdot mL^{-1}$ protease in phosphate buffer saline (PBS) at pH 7.4. Samples without the enzyme served as a negative control. After reaching the desired time, the films were washed with distilled water for 3 times, then left to dry at room temperature under vacuum for at least 3 days and another 1 day in a desiccator before weighing.

Equilibrium water content (H) was determined by the following equation;

$$H = \frac{W_s - W_d}{W_s} \quad (1)$$

where W_s is the weight of the swollen hydrogel after submerging in PBS at pH 7.4 for 48 h and W_d is the weight of the dry hydrogel after extensively washed with distilled water to remove buffer salts. To measure W_s, the swollen hydrogels were removed and then gently blotted with lint-free wipe prior to weigh.

3.3.7 Preparation and Characterization of Chitin Whisker-reinforced Silk Fibroin Samples

3.3.7.1 Preparation of Chitin Whisker-reinforced Silk Fibroin Sponges

The as-prepared chitin whisker suspension were added to distilled water to achieve a chitin whisker concentration at 0, 0.125, 0.25 or 0.50 wt% (a concentration based on the final volume including distilled water, chitin whisker suspension, and silk fibroin solution) with a volume of 8.42 ml. The suspensions were then treated by ultrasonication for 10 min. After the suspension was kept at 4°C

for 10 min, 1.58 ml of 6.32 wt% aqueous silk fibroin solution was added to the suspension with slow mechanical stirring. The obtained chitin whisker/silk fibroin suspension was equivalent to chitin whiskers to silk fibroin weight ratio (C/S ratio) of 0, 1/8, 2/8, or 4/8. The mixture suspension was further stirred mechanically for 10 min before being poured with 1.6 ml into each well of a 24 multi-wells culture plate (COSTAR®, Corning Inc., NY, USA). The suspension was frozen at -40°C overnight under controlled cooling rate, followed by freeze-drying under vacuum condition of less than 10 Pa for 24 hours to obtain the nanocomposite sponges.

3.3.7.2 Characterization of Chitin Whisker-reinforced Silk Fibroin Samples

The volume of the sponges before and after methanol treatment was determined in order to calculate the percent shrinkage (i.e. shrinkage (%) = $(V_i-V_f)/V_i \times 100$; where V_i and V_f represent the volume of the sponges before and after methanol treatment, respectively). The sample number was five for each experimental group.

The compression test was performed for the dried sponges of the methanol-treated samples at room temperature on the Texture Analyzer (TA.XT2i, England) at a crosshead speed of 0.5 mm/min. The compressive modulus was calculated from the initial slope of the linear portion of the stress–strain curve. In order to eliminate the influence of additional weight of the sponges from mixing with chitin whiskers and the volume change after methanol treatment, the compressive modulus was then divided by the bulk density, namely the specific modulus. The experiment was performed for four sponges per sample.

FT-IR spectrophotometry was used to verify the chemical structure and conformation of the as-prepared silk fibroin sponge, methanol-treated sponges at various C/S ratios, and chitin whisker film. The measurements were carried out on a Thermo Nicolet Nexus 671 FT-IR (32 scans at a resolution of 4 cm^{-1}). A Mettler-Toledo DSC model 822e/400 was used to evaluate thermal properties of the methanol-treated silk fibroin sponges with and without chitin whisker, and the freeze-dried chitin whisker (about 3 mg) under N_2 atmosphere at a heating rate of 10 $K \cdot min^{-1}$ from 50 to 450°C.

The inner microstructure of the as-prepared silk fibroin sponges with and without chitin whisker, and the corresponding methanol-treated sponges was observed by a SEM (JSM-5410LV, Jeol, Japan) at a voltage of 15 kV. The sponge was cut with a razor blade. The cross-section of the sponges was coated with gold on the ion sputter (SCD040, Lichtenstein) at 50 mTorr and 15 mA for 4 min.

The morphological appearance and sizes of the as-prepared chitin whiskers were observed using a JEOL JEM-1230 TEM. Samples were prepared from a drop of a dilute chitin whisker suspension which was deposited and left to dry on a carbon coated copper grid. The dispersion of chitin whisker in the silk fibroin matrix was also observed with TEM by embedding the sponge at C/S ratio of 4/8 with Aradite resin. The sample was then cut to ultrathin sections (200 nm) and placed on a copper grid with a carbon coating. The observations were carried out at an accelerating voltage of 120 kV.

The universal hardness (H) of silk fibroin and chitin whisker/silk fibroin films at C/S ratio of 4/8 was carried out using Nanoscope (R) IV scanning probe microscope (Veeco/Digital Instruments, CA) in a nano-indentation mode in order to investigate the influence of hardness on cell spreading. The diamond-tipped probe made a prescan to measure the surface profile. The probe then applied the force at a trigger threshold of 3 V (230 μN) onto the scanned film surfaces (spring constant of 291 N/m specified by the manufacturer). The hardness were calculated from $H = Force/A$, where A is the projected area at peak load.

3.3.7.3 Cells and Cell Culture

A cell line of L929 (ECACC Cat. No. 85011425) mouse connective tissue, which is a fibroblast-like cell, was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, BIOCHROM AG), together with 100 units/ml penicillin (GIBCO) and 100 μg/ml streptomycin (GIBCO) at 37°C in a wet atmosphere containing 5% CO_2. When the cells reached 80% confluence, they were trypsinized with 0.25% trypsin containing 1 mM EDTA (GIBCO) and counted by a hemacytometer (Hausser Scientific, USA) before use in the experiments.

3.3.7.4 Cell Spreading Analysis

In these experiments, each prepared sponge was placed in a 24-well culture plate and sterilized by 70% ethanol solution for 30 min. Thereafter, it was washed twice with phosphate buffer saline (PBS, pH 7.4) and subsequently washed with culture media. Before cell seeding, 500 µl of culture medium was pipetted into each well of the 24-well plate. 1×10^5 of L929 cells were seeded into each well of the culture plate and allowed to attach to the methanol-treated silk fibroin and chitin whisker/silk fibroin sponges (at C/S ratio of 4/8) for 6 h or 24 h. After each sample was rinsed twice with PBS, they were fixed with 3% glutaraldehyde for 30 min, followed by two rinses with a 0.2 M phosphate buffer. Then the samples were dehydrated through a series of graded ethanol and subsequently dried in a critical point dryer with liquid CO_2. The samples were gold sputtered in a vacuum at 50 mTorr and 15 mA for 4 min.

Cell spreading was evidenced from SEM micrographs taken by a JSM-5410LV scanning electron microscope at a voltage of 15 kV. The cells that adopted a flattened, polygonal shape, with filopodia- and lamellipodia-like extensions were regarded as spreading cells. In contrast, the cells that resisted to washing and remained tethered to the surface were regarded as non-spreading cells. The percent cell spreading was quantified by dividing the number of spread cells by the total number of bound cells (N=100 cells). Standard deviations were calculated from two independent experiments.

3.3.8 Study on Influence of Charge Characteristic of Silk Fibroin on Sorption and Release of Charged Dyes

3.3.8.1 Preparation of Silk Fibroin Films

To prepare the film, the silk fibroin solution was diluted to 6 wt%, and 200 µl of the diluted solutions were subsequently pipetted into each well of a 48 multi-well plate (COSTAR®, Corning Inc., NY, USA). The solutions were dried overnight at 40°C. The as-cast films were then treated with 90% (v/v) aqueous methanol or were annealed with water. After 30 min, the methanol-treated or water-annealed silk fibroin films were left until dry at 40°C.

3.3.8.2 Dye Sorption

In the sorption test, the experiment was done by using the films which were still on the 48 multi-well plate throughout the study. In order to prevent the evaporation of water during the test, a rubber sheet (kindly supplied by Perfect Built Co., Ltd.) coated with aluminum foil and an additional two layers of flexible polyethylene film was attached to the cover to tightly close the plate.

In order to study the influence of conformations of the silk fibroin on the sorption behavior, the methanol-treated and water-annealed silk fibroin films were compared using Phenol red, Chromotrope 2R, Crystal violet, and Indoine blue as the model compounds. The methanol-treated or water-annealed silk fibroin films were pre-swelled in water for 24 h and then allowed to equilibrate to the aqueous dye solutions (0.6 ml) at an initial concentration of 132 μM. After 24 h, a remaining concentration of the dye solutions after sorption was analyzed using a UV-vis spectrophotometer (Perkin Elmer, Lambda 10). The λ_{max} for Phenol red, Chromotrope 2R, Crystal violet, and Indoine blue were 423, 530, 303 (using 587 nm for a lower concentration range — 0 to 24 μM) and 555 nm, respectively. The results were compared in terms of the partition coefficient, *K* (see section 2.4 for details).

To study the effect of the pH values of the media on the sorption of charged dyes to silk fibroin, Chromotrope 2R and Crystal violet were used because the absorbance of these compound solutions was not sensitive to the studied pH range. The Chromotrope 2R or Crystal violet was dissolved in a buffer solution of 0.04 M acetic/phosphoric/boric acid to achieve a dye concentration at 150 μM (the desired pH of a buffer solution was adjusted by a suitable volume of 0.2 M NaOH solution). After pre-swelling at the same pH value of sorptive conditions for 24 h, the methanol-treated silk fibroin films were allowed to equilibrate with the aqueous dye solutions for 24 h. Thereafter, a remaining concentration of the dye solutions was analyzed using UV-vis spectrophotometry. The results were expressed in terms of the partition coefficient, *K*.

3.3.8.3 Partition Coefficient Calculation

The partition coefficients, *K*, were calculated as the equilibrium ratio of the concentration of model dye in the film to the dye concentration in the solution using equation 1 (Hunt et al., 1990):

$$K = \frac{C_{film}}{C_{solution}} \quad (2)$$

$$= \frac{V_s(C_0 - C_{solution})}{V_m C_{solution}} \quad (3)$$

where C_{film} is the concentration of dye in the film, $C_{solution}$ is the concentration of dye after sorption, C_o is the initial concentration of dye, V_s is the volume of the dye solution, and V_m is the volume of the hydrated film.

The volume of the hydrated film was estimated from its weight and density. The density was determined in a separate experiment in which the diameter, thickness, and weight of a disk of the film were measured after hydration (Hunt et al., 1990).

3.3.8.4 Density

For determination of the density of the hydrated methanol-treated and water-annealed silk fibroin films, both kinds of films were submerged in water for 1 h and then punched out to obtain disks of ~6.4 mm in diameter. The disks were further submerged in a pH 7.0 buffer solution at 30°C for 2 days. The densities of the hydrated films were determined using equation 3:

$$D = \frac{W_s}{V_m} \quad (4)$$

where W_s is the weight of a hydrated film at 30°C and V_m is the volume of a hydrated film calculated from its thickness and diameter (N=15) (Hunt et al., 1990).

To measure W_s, the hydrated films were removed from the test conditions and then gently blotted with a lint-free wipe to remove surface moisture prior to weighing.

3.3.8.5 Dye Release

The release of dyes from the methanol-treated films was studied by adding the release media over the dye-loaded film, still on the well plate, under strong shaking at 30°C. Dye release was assumed to occur from one side of the film only, since the film adhered to the well plate throughout the experiment.

To study the effect of the pH value of the media on the release behavior, the methanol-treated silk fibroin films were loaded with dye at an appropriate pH. Then, the dye-loaded films were used for a release study. Briefly, the

methanol-treated silk fibroin films were pre-swelled with a buffer solution at the same pH value as the sorptive conditions for 24 h. Thereafter, the hydrated films were allowed to equilibrate with the dye solutions using Chromotrope 2R (Crystal violet) at 132 μM, pH 2.0 (pH 7.0) with a volume of 1.3 ml. This pH value was chosen because the highest loading was achieved. After 24 h, the remaining dye concentration in the solution was determined to calculate the amount of loaded dye in the films. The dye solution was then replaced by 1.0 ml of the release media of pH 3.0, 4.4, 5.4, or 7.0. The release media was periodically replaced with the 1.0 ml of fresh media and the concentration of the release dyes was analyzed using UV-vis spectrophotometry. Note that the release of dyes at those pH levels was investigated because the fibroin films exhibited different charges at different pH levels—a positive charge in pH 3.0, a (roughly) neutral charge in pH 4.4, and a negative charge in pH 5.4 and 7.0.

3.3.8.6 Characterization of Methanol-Treated and Water-Treated Silk Fibroin Films

Attenuated total reflectance-fourier-transform infrared (ATR-FTIR) spectroscopy was used to verify the conformation of as-cast, water-annealed, and methanol-treated silk fibroin films. The measurements were carried out on a Thermo Nicolet Nexus 671 FTIR. Each spectrum was acquired in absorbance mode on ZnSe ATR crystal by the accumulation of 256 scans with a resolution of 4 cm^{-1}

3.3.9 Study on In Vitro and In Vivo Release of Basic Fibroblast Growth Factor Using Silk Fibroin Scaffolds as Delivery Carrier

3.3.9.1 Preparation of the Silk Fibroin Scaffolds

The HFIP-derived silk fibroin scaffolds were prepared from a freeze-dried silk fibroin that was re-dissolved in HFIP to obtain a 9 wt% solution. The scaffolds were prepared in a disk-shaped perfluoroalkoxy (PFA) container with 1.8 cm in diameter by adding 1.8 grams of granular NaCl particles (500-600 μm) into 0.6 ml of 9 wt% silk fibroin in HFIP. The container was covered and left overnight at 4°C for a homogeneous salt distribution. The solvent was then evaporated at room temperature for 3 days. The silk/porogen matrix was treated in a 90% (v/v) methanol

solution for 30 min to induce the formation of the β-sheet structure. The matrices were immersed in DDW for 2 days to remove the salts, and were subsequently air-dried. The aqueous-derived silk fibroin scaffolds were prepared by adding 2.1 grams of granular NaCl particles (500-600 μm) into 0.9 ml of 12 wt% of aqueous silk fibroin solution in PFA container. The containers were covered and left at 30°C for 24 hours, followed by immersion in DDW to extract the salts for 2 days (Kim et al., 2005b).

The obtained silk fibroin scaffolds from both HFIP-derived and aqueous-derived processing were shaped into smaller pieces with 4 mm in height and 5 mm in diameter using metallic-hole puncher. The average pore size of HFIP-derived and aqueous-derived silk fibroin scaffold was 479±130 and 473±146 μm, respectively.

3.3.9.2 Morphology of the Silk Fibroin Scaffolds

To observe the cross-section of the scaffold, both HFIP-derived and aqueous-derived silk fibroin scaffolds were carefully cut with a razor blade. The cross-section of the scaffolds was coated with platinum on the ion sputter (E- 1010, Hitachi, Tokyo, Japan) at 50 mTorr and 5mA for 3 min. The inner microstructure of the scaffolds was observed by a SEM (S-2380N, Hitachi, Tokyo, Japan) at a voltage of 10 kV.

3.3.9.3 In Vitro Enzymatic Degradation

The degradation of the silk fibroin scaffolds was evaluated using protease type XIV from *Streptomyces griseus* (EC 3.4.24.31, Sigma-Aldrich) as an enzyme. The scaffolds were incubated in 1 ml of phosphate-buffer saline pH 7.4 (PBS) containing the protease at 37°C under strong shaking. The enzyme concentrations used in this test was 0.05 or 0.5 $mg \cdot ml^{-1}$. The enzyme solution was replaced with freshly prepared solution daily. The scaffolds submerged in PBS without enzyme were served as a control. After reaching the desired time, the scaffolds were washed twice with DDW, followed by air-drying at 30°C overnight. The dried scaffolds were kept in desiccators at least one day prior to weighing.

3.3.9.4 Radiolabeling of Silk Fibroin Scaffolds

Silk fibroin scaffolds were radiolabeled according to the method of Tabata et al. (1999b). Briefly, 100 μl of ^{125}I Bolton–Hunter reagent solution in anhydrous benzene was bubbled with dry nitrogen gas until benzene evaporation was complete. Then, 125 μl of 0.1 M sodium borate-buffered solution (pH 8.5) was added to the dried reagent, followed by pipetting to prepare aqueous ^{125}I Bolton– Hunter solution. The silk fibroin scaffolds were impregnated with the prepared aqueous solution at a volume of 25 μl per scaffold overnight. The radio-labeled scaffolds were then rinsed with DDW by exchanging it periodically at 4 °C for 7 days to exclude non-coupled, free ^{125}I-labeled reagent from ^{125}I-labeled silk fibroin scaffolds.

3.3.9.5 Radioiodination of bFGF

bFGF was radioiodinated according to a chloramine T method (Greenwood et al., 1963). Aqueous solution of bFGF at 0.5 mg/ml (450 μl) was added into 100 μl of 0.5 M potassium-phosphate buffered (KPB) solution (pH 7.5). Then, 5 μl of Na^{125}I solution and 100 μl of 0.05 M KPB solution (pH 7.2) containing 0.06 mg of chloramine T were added to the bFGF solution. After agitation at room temperature for 2 min, 100 μl of 0.01 M PBS containing 0.12 mg of sodium metabisulfate was added to stop the radioiodination. The mixture was passed through a column of Dowex resin to remove uncoupled, free ^{125}I molecules, followed by addition of 650 μl of water to obtain an aqueous solution of ^{125}I-labeled bFGF.

3.3.9.6 In Vitro Release of bFGF

In order to incorporate ^{125}I-labeled bFGF into both HFIP-derived and aqueous-derived silk fibroin scaffolds, 20 μl of ^{125}I-labeled bFGF solution was dropped onto the scaffolds. The absorption process was allowed to occur at 4°C overnight. The obtained ^{125}I-labeled bFGF-impregnated silk fibroin scaffolds were then used for *in vitro* and *in vivo* bFGF release studies.

In vitro release of ^{125}I-labeled bFGF from the ^{125}I-labeled bFGF-impregnated silk fibroin scaffolds was first conducted in PBS. After periodic replacement with PBS for 24 hours, the release behavior of bFGF in enzyme solution was then investigated. Briefly, ^{125}I-labeled bFGF-impregnated silk fibroin scaffolds were placed into 1 ml of PBS. The PBS was periodically replaced at 0.5, 1, 2, 4, 8 and 12 hours. At 24 hours, the release media, neat PBS, started to be replaced by

PBS containing protease at 0.05 or 0.5 $mg \cdot ml^{-1}$ in order to observe the influence of enzymatic degradation of the scaffolds on the release of bFGF. The enzyme solution was daily replaced with freshly prepared solution. The release of bFGF was calculated by measuring the radioactivity of release media divided by a total radioactivity of ^{125}I-labeled bFGF in the scaffolds using a gamma counter (ARC-301B, Aloka, Tokyo, Japan). The total radioactivity of ^{125}I-labeled bFGF in the scaffolds was obtained by the sum of the radioactivity remaining in the scaffolds plus cumulative radioactivity of bFGF released. The natural decay of the radionuclide was adjusted using a control solution of ^{125}I species.

3.3.9.7 Estimation of In Vivo Release of bFGF

To evaluate *in vivo* release of bFGF from the silk fibroin scaffolds, the ^{125}I-labeled bFGF-impregnated silk fibroin scaffolds were subcutaneously implanted into a pocket created in the back of 4-week-old female ddY mice under pentobarbital anesthesia (Tabata et a., 1999b). As a control, 100 µl of aqueous solution of ^{125}I-labeled bFGF was subcutaneously injected into the back of mice. The mice were sacrificed at 1, 3, 7, 14, 20, and 26 days. The radioactivity remaining in the scaffolds and local tissue were measured using a gamma counter (n=3 for each time point). The remaining radioactivity percentage was calculated by dividing the radioactivity remaining in the scaffold by its initial radioactivity.

3.3.9.8 Estimation of In Vivo Degradation of Silk Fibroin Scaffolds

In vivo degradation of the scaffolds was evaluated in terms of the radioactivity remaining in ^{125}I-labeled silk fibroin scaffolds after implantation in back of mice (Tabata et al. 1999b). At different time interval, the mice were sacrificed and the radioactivity remaining in the scaffolds and local tissue were measured using a gamma counter (n=3 for each time point). The percentage of radioactivity remaining was calculated by dividing the radioactivity remaining in the scaffold by its initial radioactivity.