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Comparative study of silk fibroin porous scaffolds derived from salt/water and sucrose/hexafluoroisopropanol in cartilage formation

Kumiko Makaya,[1] Shinichi Terada,[2] Kosuke Ohgo,[1] and Tetsuo Asakura[1,*]

Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan[1] *and Tokyo Women's Medical University Yachiyo Medical Center, Yachiyo, Chiba 276-8524, Japan*[2]

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The purpose of this study is to create a new silk fibroin scaffold with sufficient three-dimensional morphology and porous structure for cartilage formation. We have applied sucrose particles sized around 300 to 500 μm as porogens compared to equal-sized salt particles. After the porogen was leached out with water, scaffolds were prepared with fibroin derived from sucrose/hexafluoroisopropanol (Su/H) or salt/water (Sa/W) based composites. A compression test indicated that the Sa/W fibroin was much harder than the Su/H fibroin, but a protease enzyme digested the Sa/W fibroin more quickly than Su/H fibroin. Rabbit ear chondrocytes were seeded onto the scaffolds for 4–8 week *in vitro* culture and histological analyses were performed. The distribution of cartilage formation in Safranin O staining was more homogenous in Su/H fibroin than that of Sa/W fibroin. The overall amount of cartilage was significantly better in the Su/H fibroin than that in the Sa/W fibroin. However, the inner structure of pore wall in the Sa/W fibroin was rough and microporous with cartilage matrix deposition, while that in the Su/H fibroin was thin and homogenous. Since mature cartilage gradually regenerates to fill the porous space, slowly degradable Su/H fibroin should be a better candidate for cartilage formation.

[**Key words:** Biomaterial; Tissue engineering; *Bombyx mori*; Silk fibroin; Cartilage; Scaffold]

Pursuit of tissue regeneration for congenitally impaired, traumatically injured, or naturally aged organs and soft tissue in the human body has been a challenge for decades in the plastic surgery field. Surgeons continue to be faced with several choices including the use of the patient's own tissue, another person's organ, and/or artificial implants (1, 2). Although the autograft has the highest compatibility, patients are often suffered from donor site morbidity such as pain, scars and deformities. To overcome the autograft problems, allograftic and alloplastic implants could be better candidates if risks of resorption and infection in long term could be accepted. Therefore, a new implant material combining both the advantages of autologous tissue and alloplastic material is of great significance.

Tissue engineering technology is still seeking a new implant material, because most degradation products of synthetic biodegradable polymers have acidic properties and cause a long-term thicker granulomatous inflammation reaction including monocytes and macrophages migration, and subsequent multinucleated giant cell induction (3) until the polymers completely absorbed. Additionally, synthetic biodegradable polymers were reported to have acute toxicity *in vitro* (4). On the other hand, silk proteins are of practical interest because of their excellent intrinsic properties that are utilizable in biomedical fields and the quality control inherent in the manufacture of silk textiles (5, 6). Silk threads without sericin were once the most popular in the surgery field, which requires stable, safe conditions for use in the human body. *In vivo* tissue reactions of silk film were reported as forming a milder and thinner reactive layer of macrophages and fibroblasts than those of poly (lactic acid) films (3). More recently, porous silk scaffolds have been developed by freeze-drying, salt leaching, and gas foaming for bone and cartilage repairs (7–9).

Kim et al. showed better cartilage formation with larger pores of fibroin scaffolds using the salt leaching technique for the aqueous fibroin solution (9). However, larger pores have several disadvantages on cellular involvement, extracellular matrix deposition and morphological accuracy comparing to smaller pores. In this study, we have applied smaller salt or sucrose particles as porogen to generate porous structure of silk fibroin scaffolds. Smaller particles around 250–500 μm in size were expected to form a better interconnected porous structure with adequate spatial dimensions for cell growth and cartilage matrix deposition (10). We performed a comparative study of the newly developed sucrose/hexafluoroisopropanol (HFIP) and salt/water-derived fibroin scaffolds to investigate the optimal conditions in fibroin microporous scaffolds for cartilage tissue engineering.

MATERIALS AND METHODS

A schematic diagram of fibroin scaffold processing is shown in Fig. 1. Briefly, *Bombyx mori* cocoons were placed in boiling water at 95 °C after which the threads were reeled. The dried silk threads were degummed in a mixture of sodium carbonate (0.08 w/v%) and Marseille soap (0.12 w/v%) solution at 95 °C for 120 min in order to remove sericin protein from the surface of the raw silk fibers. The degummed silk fibers were dissolved in a 9 M lithium bromide solution to a concentration of 20 w/v% at 60 °C

* Corresponding author. Tel./fax: +81 042 383 7733.
E-mail address: asakura@cc.tuat.ac.jp (T. Asakura).

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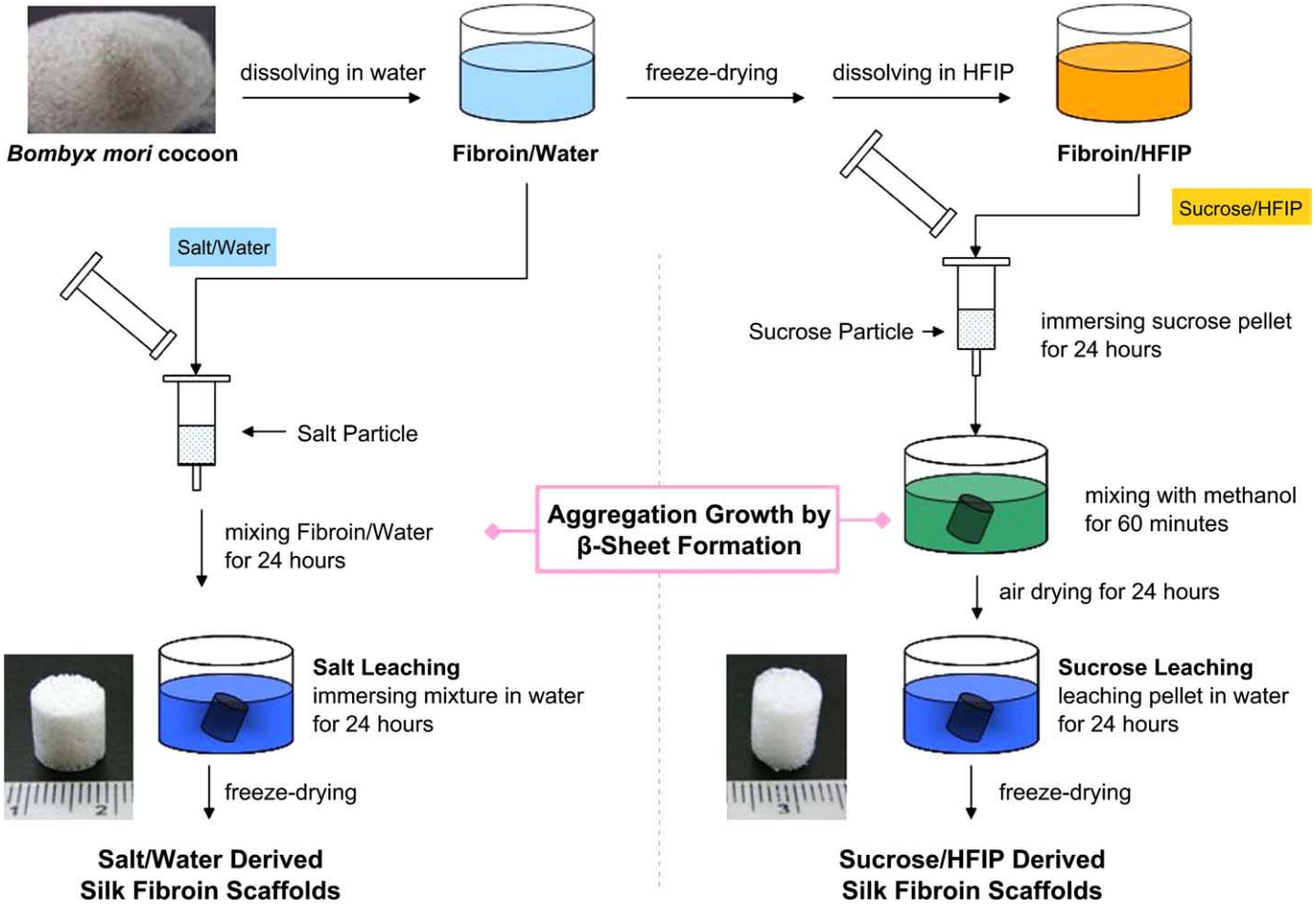

FIG. 1. Schematic diagram of the preparation for the sucrose/HFIP (Su/H) and salt/water (Sa/W) derived fibroin scaffolds.

for 4 h and then dialyzed against distilled water for 3 days at 4 °C using a cellulose membrane (MWCO 14,000). The final concentration of the fibroin/water solution was 6.0 w/v%. For preparation of the fibroin/HFIP solution, the concentration of dialyzed aqueous fibroin solution was adjusted to 2.0 w/v% and then lyophilized.

Sucrose/HFIP-derived fibroin scaffolds (Su/H fibroin) were prepared as follows. HFIP was used as the solvent of the fibroin. The lyophilized silk cake was dissolved in HFIP, yielding 4.0 w/v% solution. Sucrose particles were sieved with both 300 and 500 μm meshes and mixed with 3.1 wt.% water droplets for partial dissolution of the sucrose surfaces that stuck together. Immediately after adding each 50 μl water droplet, a 25 ml tube was shaken vigorously. The sucrose particles were packed into a plastic syringe (9.5 mm in diameter) and compressed into a cylindrical shape. After removing the constructs from the syringe, they were air-dried and then placed in a vacuum dryer for 24 h. They were then immersed into fibroin/HFIP solution for 24 h to allow the solution to penetrate into the center of the construct. The fibroin/sucrose composites were placed into methanol for 60 min to induce a beta-sheet structure, which we confirmed later using the ^{13}C CP/MAS NMR. After immersion, the composites were removed from the methanol and allowed to air dry for 24 h. The composites were then placed into water for 24 h to leach out any sucrose particles. Su/H fibroin scaffolds were obtained after 36 h of freeze-drying. While the columnar scaffolds were frozen, they were sectioned perpendicular to the longitudinal axis using a razor blade to obtain an 8 mm height short column.

On the other hand, salt/water-derived fibroin scaffolds (Sa/W fibroin) were prepared as follows [7]. Briefly, salt particles were sieved with both 300 and 500 μm meshes and packed into a plastic syringe. Immediately after pouring a 4.0 w/v% fibroin aqueous solution into the syringe, the piston was pressed as quickly as possible to avoid from being stuck due to the beta-sheet formation. Any air between the particles was eliminated from a small opening opposite the needle. After 24 h, the silk/salt composites were processed in the similar way.

The surface morphology of the raw scaffolds was observed in both groups and compared to that of the previous study (9). The diameters of the scaffolds ($n=5$) were measured and directly compared with the template diameter (9.5 mm) to calculate the mold shrinkage ratio. As the inner structural survey, we prepared the raw scaffolds frozen to cut with a razor blade, operated scanning electron microscope (VE-7800, Keyence, Osaka, Japan) at 1.2 kV without any metal coatings and analyzed pore shape, size, and interconnectivity in the cross sections of the raw scaffolds. Seven pores in randomly selected areas of the SEM images were measured at the largest diameter of the approximately circular pores. In addition, interconnecting smaller pores inside the pores in the same images were also measured. The ^{13}C CP/MAS NMR spectra of each Sa/W and Su/H fibroin scaffold were obtained on a Chemagnetics CMX 400 MHz (JEOL Ltd., Tokyo, Japan) spectrometer using a cross-polarization pulse sequence and magic-angle spinning at 9 kHz. The experimental conditions were as follows: 1 ms contact time and 3 s pulse delay. ^{13}C chemical shifts were calibrated indirectly through the adamantane methine peak observed at 28.8 ppm relative to tetramethylsilane at 0 ppm. Force exerted in resistance to mechanical compression was measured using a 100 N load cell in the tensile testing machine (EZ-Graph, Shimadzu, Kyoto, Japan) at 21 °C and 50% humidity. The crosshead speed was 2 mm/min. The compression tests were conducted using the conventional open-sided/unconfined method. The dry columnar-shaped scaffolds ($n=3$) in both groups were measured in the diameter and the height. The compressive stress and the strain were automatically recorded, and calculated on the compressive strength and the compressive modulus. In this study the compressive strength was determined by the point at the end of the elastic region. The compressive modulus was defined as the slope of the initial linear section of the stress-strain curve. The *in vitro* degradation times of the fibroin scaffolds ($n=3$) were examined using enzymatic digestion of protease XIV. 10 U/ml protease XIV was dissolved into phosphate buffered saline (PBS) and incubated with the scaffolds on a shaker at 37 °C for 4 days. After cleansing thoroughly with distilled water, samples were freeze-dried and measured the dry weight every day.

Our animal experiment was approved under the Guidelines for Proper Conduct of Animal Experiments from the Science Council of Japan by the Institutional Research Council at the National Hospital Organization Disaster Medical Center where co-author, S. Terada, was previously working.

Under 1% xylocaine local injection to a rabbit ear in a restraining cage for 10 min, a small piece (3 × 2 mm) of elastic cartilage was quickly harvested in a sterilized manner from the crus helicis, where the cartilage is the thickest, of a rabbit ear. After removal of the perichondrium, the ear cartilage was minced into fragments to digest with 0.2 w/v% type II collagenase PBS solution (Worthington Biochemical, Lakewood, NJ, USA) for 3 h in a shaker at 37 °C. After filtration through a 100-μm cell strainer, isolated chondrocytes were spun down, counted, and plated. The chondrocyte proliferation medium consisted