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Preparation and Characterization of Blended *Bombyx mori* Silk Fibroin Scaffolds

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Abstract: The aim of this study was to compare physical, mechanical and biological properties of 3-dimensional scaffolds prepared from *Bombyx mori* silk fibroin (SF), fibroin blended with collagen (SF/C), and fibroin blended with gelatin (SF/G) using a freeze-drying technique. The prepared scaffolds were sponge-like structure that exhibited homogeneous porosity with highly interconnected pores. Average pore size of these scaffolds ranged from 65-147 μm. All biodegradable scaffolds were capable of water absorption of 90 %. The degradation behavior of these scaffolds could be controlled by varying the amount of blended polymer. The SF/C and SF/G scaffolds showed higher compressive modulus than that of SF scaffolds which could be attributed to the thicker pore wall observed in the blended constructs. The less crystalline SF structure was observed in SF/G scaffolds as compared to SF/C scaffolds. Thus, the highest compressive modulus was observed on SF/C matrix. To investigate the feasibility of the scaffolds for cartilage tissue engineering application, rat articular chondrocytes were seeded onto the scaffolds. The MTT assay demonstrated that blending collagen or gelatin into SF sponge facilitated cell attachment and proliferation better than SF scaffolds. The blended SF scaffolds possessed superior physical, mechanical and biological properties in comparison to SF scaffolds and showed high potential for application in cartilage tissue engineering.

Keywords: Silk fibroin, Collagen, Gelatin, Scaffolds

Introduction

Nowadays, millions of patients are suffering from cartilage defect caused by trauma, injury and age-related degeneration. Unfortunately, cartilage has a limit for self-repairing due to its avascular, aneural and alymphatic characteristics. Moreover, current treatments for cartilage repair are unsatisfactory and rarely return to the structure of native cartilage [1,2]. A new approach as an alternative treatment for repairing, maintaining, and improving tissue function is cartilage tissue engineering. In this technique, three dimensional (3-D) scaffolds are specially bioengineered constructs which mimic natural conditions in cartilage before implantation. To achieve this goal, biomimetic scaffolds with appropriate pore size, high mechanical properties, porosity, and interconnected pores are characteristics of interest for polymeric scaffold design that provides temporary framework for supporting cell attachment, proliferation, differentiation and extracellular matrix formation [3-8]. Several techniques have been developed to fabricate 3-D scaffold using different synthetic and natural materials [9-23]. Among scaffold fabrication methods, freeze drying is a very widely studied preparation method because of its simplicity and mild process.

Silk fibroin (SF), a natural fibrous polymer produced by the silkworm, *Bombyx mori*, has been used as biomedical sutures for centuries. It is a protein mainly comprised of amino acids glycine, alanine, and serine that form crystalline β-sheets in silk fibers, leading to the unique mechanical properties and hydrophobic domain structure. Additionally, silk fibroin has advantages of biodegradability, biocompatibility, and low inflammatory response; therefore, it has a potential as natural biomaterial for biomedical applications. However, several previous reports have shown that silk fibroin scaffold prepared by freeze-drying method possesses poor mechanical properties that could be improved by blending with natural or synthetic polymers [24].

Collagen (C), one of the major components of the extracellular matrix, has been reported that it could inhibit the unwanted aggregation in fibroin scaffold during the preparative processes and produce scaffolds with high porosity [25,26]. It is a biodegradable natural protein with low antigenicity. In addition, it contains the amino sequence, arginine-glycine-aspartic acid (RGD), which stimulates cell adhesion and also protein expression in cells [27-29].

Gelatin (G) is a partial derivative of collagen. It has been widely used in surgery as a wound dressing, and as biomaterials in the controlled drug delivery systems. With structure similar to collagen, gelatin retains cell-specific domains (RGD sequence), that helps to promote cell adhesion, proliferation, and differentiation [30].

In recent years, SF, collagen and gelatin were among the most extensively explored biomaterials for tissue engineering

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due to their impressive biocompatibility, biodegradation and mechanical properties. The biomaterial scaffolds made of these materials effectively provide temporary constructs for attachment and proliferation of fibroblasts, hepatocytes, chondrocytes, osteoblasts, and mesenchymal stem cells [3,4, 13,23,31-45]. However, there has been no report on the comparison of SF scaffolds and SF blended scaffolds in terms of physical, mechanical and biological properties for chondrocytes cultivation. Therefore, in this study, we attempted to compare the physical properties of scaffolds prepared from SF, SF blended with collagen, and SF blended with gelatin by freeze-drying method. The morphology, pore size, porosity and mechanical properties, as well as the water uptake and degradation rate of the scaffolds were examined. Moreover, biological properties of prepared scaffolds in terms of cell proliferation and biocompatibility were studied.

Materials and Methods

Materials

Raw silk yarns of *Bombyx mori* were purchased from Badint Thai-Silk Korat Co., Ltd., Nakhonratchasima, Thailand. Bovine collagen was purchased from Fluka, USA. Type A Gelatin (~300 bloom) was purchased from Sigma Chemical (St. Louis, MO, USA). Snakeskin pleated dialysis tube (10,000 MWCO) was purchased from Thermo Scientific, Rockford, IL, USA. All other chemicals and solvents were of analytical grade.

Preparation of Silk Fibroin Solutions

The soluble fibroin solutions were prepared according to methods reported in previous literature [46,47]. Raw silk yarns of *Bombyx mori* were degummed twice by boiling in a 0.5 % (w/v) sodium carbonate solution for one hour to remove sericin. The silk yarns were washed three times with warm reverse osmosis water and then dried overnight in a drying oven at 40 °C. Degummed silk fibroin was heated at 85-90 °C in a mixed solution of $CaCl_2$:H_2O:EtOH at 1:8:2 mole ratio until the solution became gel-like. After dialysis in a snakeskin pleated dialysis tube (10,000 MWCO) against continuous stirred distilled water at room temperature for 3-5 days to remove residue salts, fibroin gel was freeze-dried and kept in sealed plastic bags at controlled humidity until used.

Preparation of 3-D Scaffolds

Three dimensional scaffolds of silk fibroin (SF), silk fibroin/collagen (SF/C), and silk fibroin/gelatin (SF/G) were prepared using a freeze-drying technique. SF scaffolds were prepared from various concentrations of SF solution, 1 %, 2 %, 4 %, 6 % w/v, by dissolving SF in deionized (DI) water at room temperature. The method for SF/C blending were modified from Lv *et al.* [26]. SF/C scaffolds were prepared by adding different ratios of 0.5 and 1 % collagen solution into 2 % fibroin solution with the blending ratio of fibroin to collagen of 95:5, 90:10 and 75:25. Collagen solution was prepared by dissolving collagen in 5 % v/v acetic acid [27] at 4 °C, overnight, before used. The method for SF/G blending were modified from Mandal *et al.* [48]. SF/G scaffolds were prepared by adding different ratios of 4 % gelatin solution into 6 % fibroin solution with the blending ratios of fibroin to gelatin of 85:15 and 70:30. Gelatin was dissolved in DI water at 50 °C. Then, the blending solutions were mixed with mild stirring for 20 min. The resulting solutions were transferred into the polystyrene petri dishes and kept frozen at −20 °C overnight prior to lyophilization for 3 days (PowerDry LL3000, Heto, USA). The dry porous sponges were removed from the molds and treated with methanol for 30 min. Finally, methanol was evaporated at room temperature. All samples were prepared in triplicate.

Physical Characterization of 3-D Scaffolds

Morphology Observation and Mean Pore Size Measurement

The morphology of porous 3-D scaffolds was investigated using a scanning electron microscopy (SEM, 1455VP, LEO Electron Microscopy Ltd., Cambridge, UK). Freeze-dried sponge scaffolds were sectioned using a razor blade and then sputter coated with gold before SEM examination. The mean pore size of scaffolds was determined by randomly measuring at least 30 pores from the SEM micrographs using ImageJ program (Java image processing program, downloaded from http://rsb.info.nih.gov/ij/index.html)

Porosity

The porosity of the prepared scaffolds was determined using liquid displacement method [49]. Hexane was used as the displacement liquid as it permeates through porous sponge scaffolds without swelling or shrinking the scaffold. The scaffold was immersed in a known volume (V_1) of hexane in a graduate cylinder for 5 min. The total volume of hexane- impregnated scaffold was recorded (V_2). Then the volume of hexane in cylinder after removal of hexane-impregnated scaffold was recorded (V_3). Finally, the percentage of porosity (ε) of the scaffold was calculated by;

$$\varepsilon\,(\%) = (V_1 - V_3) / (V_2 - V_3) \times 100$$

Swelling Properties

The three dimensional scaffolds were immersed in distilled water at room temperature for 24 h. After excess water was removed, the wet weight of the scaffold was recorded (W_s). Then, the wet scaffolds were dried in an oven at 65 °C for 24 h and the dried weight of scaffolds was recorded (W_d). The percentage of water uptake was calculated as follows;

$$\text{Water uptake}\,(\%) = [(W_s - W_d)/W_s] \times 100$$

Mechanical Properties

Mechanical properties of the scaffolds were evaluated using an Instron-8872 (Instron corporation, MA, USA) equipped with a 0.25-kN load cell at room temperature. The

cross- head speed was set at 0.5 mm/min. The scaffolds were cut into a cylindrical shape 6-12 mm in diameter and 4-8 mm in height. Compressive load and compressive extension were determined. The compressive modulus values were obtained from the slope of the linear region in the stress-strain curve. Three samples were evaluated for each treatment.

Structure Analysis

Approximately 2-3 mg of freeze-dried scaffolds and 80 mg of potassium bromide were compressed into a pellet. The FT-IR spectra were obtained using a spectrum GX series (Perkin-Elmer, MA, USA) equipped with a mirtgs detector and an extkbr beamsplitter. Spectra were obtained at 4.00 cm^{-1} resolution, under a dry air purge, and accumulation of 16 scans. Each spectrum of the samples was acquired with a spectral range of 4000-400 cm^{-1}.

Powder X-ray diffraction patterns were recorded at a scanning speed of 0.02 ° $2\theta^{o}$ intervals using Philips X'pert diffractometer (X'pert, Philips, EA Almelo, Netherlands) with Cu Kα radiation (λ=0.154 nm). The voltage and current of the X-ray source were 30 kV and 20 mA, respectively.

In Vitro Degradation Studies of 3-D Scaffolds

The *in vitro* degradation of the prepared scaffolds was evaluated using protease XIV (Sigma, St. Louis, MO, USA) with an activity of 3.5 units/mg. The scaffolds were cut into a cylindrical shape of 10 mm in diameter and 5 mm in height. The dry weight of sample (W_0) was determined before the samples were immersed in 2 m*l* of phosphate buffered saline (PBS) with calcium (pH 7.4) containing protease 1 unit at 37 °C. The enzyme solution was replaced with freshly prepared solution every 24 h. After the determined time (1, 3, 5, and 7 days), the samples were removed and washed twice with distilled water. Then, they were dried at 65 °C to obtain constant weight. Finally, the samples were weighed (W_1) and the percentages of weight remaining was calculated as follows;

$$\%\text{Weight remaining} = (W_1/W_0) \times 100$$

For control, the samples were also immersed in PBS without enzyme.

In Vitro Cell Viability and Proliferation Studies

Cell viability and cell proliferation were determined by MTT assay. Chondrocytes were obtained from articular cartilage of 4-8 week old Spraque Dawley rats with an approval of Naresuan University Animal Ethic Committee. The method for chondrocytes isolation was modified from Mohan *et al*. [50]. Briefly, cartilage specimens were sliced and minced to small pieces and then washed three times in sterile PBS. The extracellular matrix was digested by 0.2 % (w/v) collagenase II at 37 °C, 5%CO_2, in sterile tube for 1 h or more until the tissue fragment dissolved. Finally, the isolated cells were collected by centrifugation and washed three times with serum free Dulbecco's modified Eagle medium (DMEM). The suspended chondrocytes were cultured in DMEM supplemented with 10 % FBS, and 1 % penicillin/ streptomycin. All cultures were maintained at 37 °C, 5 % CO_2, and the culture medium was changed every 2-3 days.

The second passage (P2) of chondrocytes was seeded on the 6 % SF, SF/C (75/25) and SF/G (70/30) scaffolds. The sterilized scaffolds were shaped into a cylinder of 5 mm in height and 10 mm in diameter and washed 2 times with sterile PBS pH 7.4. Then, they were placed in 500 μl culture medium in a 24-well plate. After incubated in CO_2 incubator for overnight, the medium was discarded before cell seeding. Then chondrocytes were seeded at a concentration of 1×10^6 cells per scaffold. After 3 h, 500 μl of fresh culture medium was added into each well, and cells were maintained at 37 °C, 5 % CO_2. The medium was replaced every 2-3 days over 28 days. Cell viability and proliferation were determined at time intervals, 0, 7, 14, 21, and 28 days. At determined time, the samples were removed and transferred into a new 24-well plate. After that, 1 m*l* of serum-free medium containing 0.5 mg/m*l* thizolyl blue tetrazolium bromide (MTT) was added and incubated at 37 °C, 5 % CO_2, for 4 h. Then, the medium was discarded and 2 m*l* of dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystals. The samples were shaken at 120 rpm for 30 min to ensure a homogeneous solution of the dye and then 200 μl of each sample was transferred to a 96-well plate. Optical density was read at 595 nm using a microplate reader. Control wells were SF, SF/C and SF/G scaffold without chondrocytes seeding.

Results and Discussion

Several studies have revealed that scaffolds prepared from pure SF using freeze-drying technique were unsuitable for cell cultivation because of its frangibility. To further improve mechanical and biological properties of SF scaffold suitable for chondrocyte cultivation, in this study, biocompatible natural protein, collagen and gelatin, were blended with silk fibroin solution. In the preliminary study, the SF and blended SF scaffolds prepared by freeze drying method without methanol treatment showed frangible and water soluble scaffolds. Thus, methanol treatment after freeze drying was used to create a water-stable construct and to enhance the mechanical properties by inducing β-sheet conformation of silk fibroin as evidence from FTIR.

Morphology and Mean Pore Sizes

3-D scaffolds of SF and blended SF were successfully constructed using a freeze-drying technique with methanol treatment. A sponge-like structure of 3-D scaffold was obtained after lyophilization. The morphology and the mean pore size of 3-D porous SF, SF/C and SF/G scaffolds were investigated using SEM, Table 1. The concentration of SF exerted major impact on the scaffold morphology and pore

Table 1. Average pore sizes of silk fibroin-based scaffolds

Samples	Polymer concentration	Polymer weight ratio	Average pore size (μm)±SD
SF	1 % w/v	100/0	147.69±65.61
	2 % w/v	100/0	82.92±28.26
	4 % w/v	100/0	71.51±17.72
	6 % w/v	100/0	65.33±15.95
SF/C	2% SF:0.5 % C (4:1)	95/5	76.22±20.56
	2 % SF:1 % C (4:1)	90/10	87.74±23.72
	2 % SF:1 % C (3:2)	75/25	92.79±20.93
SF/G	6 % SF:1 % G (4:1)	85/15	91.46±46.52
	6 % SF:1 % G (3:2)	70/30	80.02±27.72

Values are average±standard deviation (N=30).

size. Scaffold prepared from 1 % SF solution revealed a mean pore size of 147.69±66 μm with a leaf or sheet formation, Figure 1, that resulting in a poor mechanical properties and was unsuitable for cell migration [51,52]. However, a homogeneous porous structure with highly interconnected pores could be constructed by increasing SF concentration, Figure 1. The mean pore sizes of SF scaffolds prepared from 2, 4, and 6 % w/v SF solution were 82.92±28, 71.51±18 and 65.33±16 μm, respectively. A decrease in the mean pore may be a result from the increasing content of SF in the system lead to the more interaction between fibroin molecules; subsequently the formation of large ice crystals during the freezing process was inhibited [26,52]. Although, the leaf morphology of SF scaffold could be prevented by increasing the amount of SF, the SEM micrographs of scaffolds at various SF concentrations revealed a thin pore wall structure indicating low mechanical properties. Since the *in vivo* environment of cartilage is often subjected to mechanical compression, an ideal scaffold must be designed to withstand this force. Interestingly, when collagen and gelatin were added to the SF scaffold, the thicker pore wall was observed which may be attributed to the interaction between SF and collagen or gelatin via hydrogen bond and ionic interaction.

SEM micrographs of blended SF scaffold showed homogeneous porous structure with a thicker pore wall and lesser interconnected pores than those prepared from pure SF, Figure 1-3. The SEM micrograph of SF/C scaffold prepared from 2 % SF solution blended with different ratio of SF and collagen were shown in Figure 2. The results illustrated that with an increase in collagen content, the rough surface with a thicker pore wall was observed. Moreover, the mean pore size of SF/C scaffold tended to increase as the collagen content was increased. Scaffolds prepared with the different ratios of SF and collagen, 95:5, 90:10 and 75:25, showed average pore size of 72.22±21, 87.74±24 and 92.79±21 μm, respectively.

The morphology of SF/G scaffolds prepared from 6 % SF solution blended with different ratio of SF and gelatin was shown in Figure 3. As expected, gelatin promoted the porous structure and restrained the sheet formation of SF. The SEM

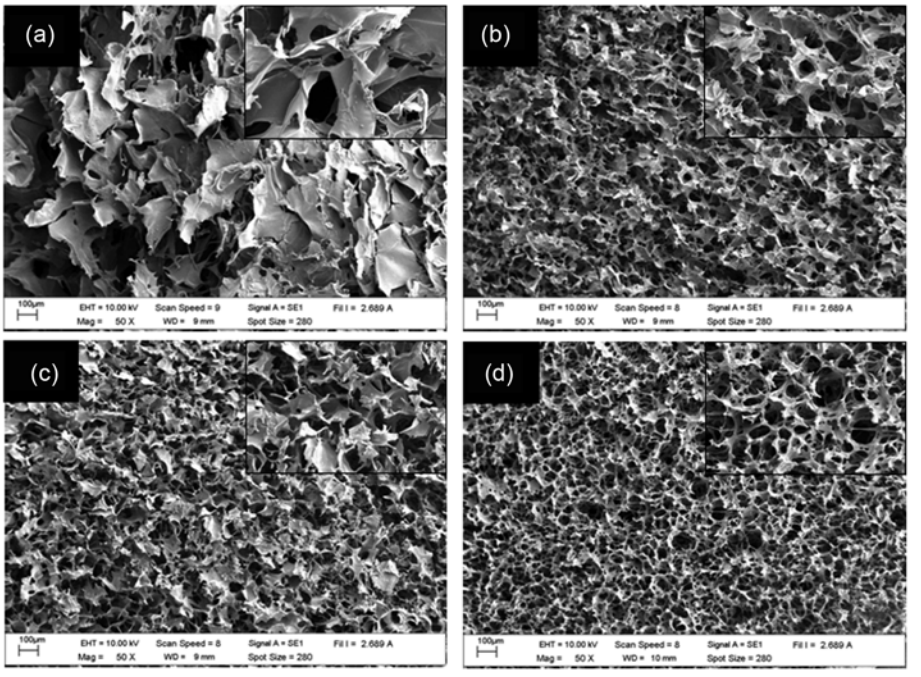

Figure 1. SEM micrographs of SF scaffolds prepared from different concentration of silk fibroin solutions; (a) 1 % w/v SF, (b) 2 % w/v SF, (c) 4 % w/v SF, and (d) 6 % w/v SF.

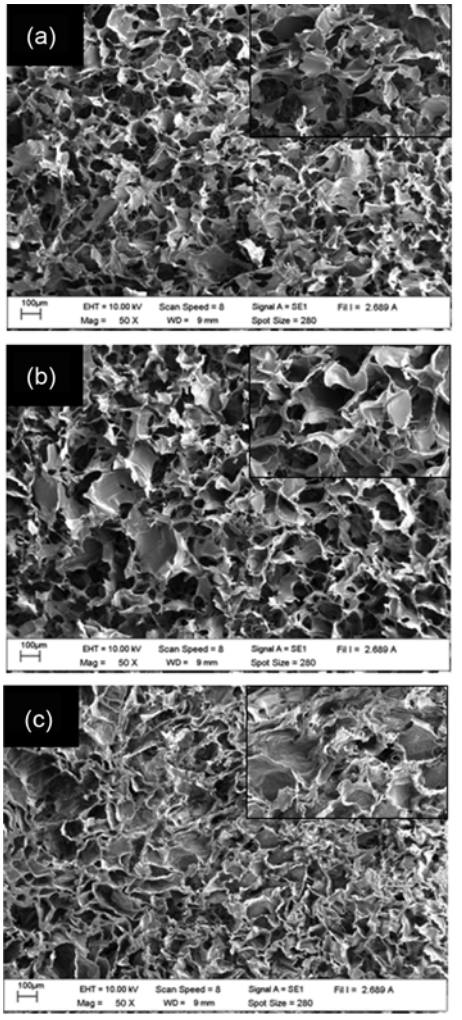

Figure 2. SEM micrographs of SF/C scaffolds prepared from different ratio of silk fibroin and collagen at (a) 95:5, (b) 90:10, and (c) 75:25.

micrograph revealed a smooth surface with a thick pore wall compared to those of pure SF. However, the mean pore size

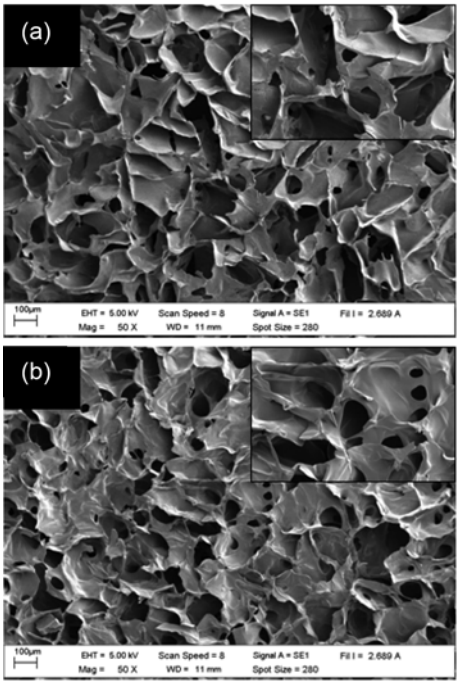

Figure 3. SEM micrographs of SF/G scaffolds prepared from different ratio of silk fibroin and gelatin at (a) 85:15 and (b) 70:30.

of SF/G scaffold tended to decrease as the gelatin content increased. As shown in Table 1, the mean pore size of SF/G scaffolds prepared with the different ratios of SF and gelatin at 85/15 and 70/30 was 91.46±47 and 80.02±28 μm, respectively.

Porosity and Swelling Properties

Porosity of the scaffold is an important property, which provides sufficient opportunity for cell migration and proliferation. The concentration of SF and blended polymer played a key factor affecting the porosity of prepared scaffolds. With increasing SF concentration ranging from 1-6 %, the porosity was increased from ~62 % to 89 %,

Table 2. Porosity and water up-take of different silk fibroin-based scaffolds

Samples	Polymer concentration	Polymer weight ratio	Porosity (%)±SD	Water up-take (%)±SD
SF	1 % w/v	100/0	61.67±12.58	94.42±1.65
	2 % w/v	100/0	77.78±9.62	93.45±0.19
	4 % w/v	100/0	86.31±1.03	93.40±0.93
	6 % w/v	100/0	88.89±0.00	91.97±0.94
SF/C	2 % SF:0.5 % C (4:1)	95/5	78.33±2.89	94.26±1.13
	2 % SF:1 % C (4:1)	90/10	75.00±0.00	95.21±1.06
	2 % SF:1 % C (3:2)	75/25	61.11±9.62	95.45±0.08
SF/G	6 % SF:4 % G (4:1)	85/15	69.84±2.75	90.81±0.93
	6 % SF:4 % G (3:2)	70/30	61.27±4.89	89.02±2.50

Values are average ± standard deviation (N = 3).

Table 2. These results were in agreement with SEM micrographs. A high degree of porosity was observed in the scaffold possessing highly interconnected pores with small pore size. On the contrary, by adding of collagen or gelatin to SF solution, the prepared scaffolds showed lesser interconnected pore, thus, leading to a decrease in porosity. In addition, the results showed that the porosity of SF/C and SF/G decreased with the increase of collagen and gelatin content.

Water uptake ability is an important parameter both in native tissue and biomaterials since the ability of water to diffuse freely would allow the transport of nutrients into the scaffold and the excretion of waste from the construct [26,49]. Regardless of pore size and porosity, the high water uptake ability, >90 %, of the prepared scaffold was evident, Table 2. This could be attributed to the highly porous structure of the construct. The water uptake slightly decreased with increasing SF content suggesting that the hydrophobicity of SF may play a role. As expected, the water uptake of SF/C scaffold increased slightly indicating that adding collagen slightly improved the hydrophilicity of SF scaffold. Unexpectedly, adding gelatin to SF scaffold should give a more hydrophilic scaffold than SF/C scaffold, but the water uptake of SF/G decreased slightly, ~90 %. This may be due to the weight loss of SF/G scaffold during the experiment as the gelatin could dissolve from the construct into the medium, thus, the lower water uptake was observed.

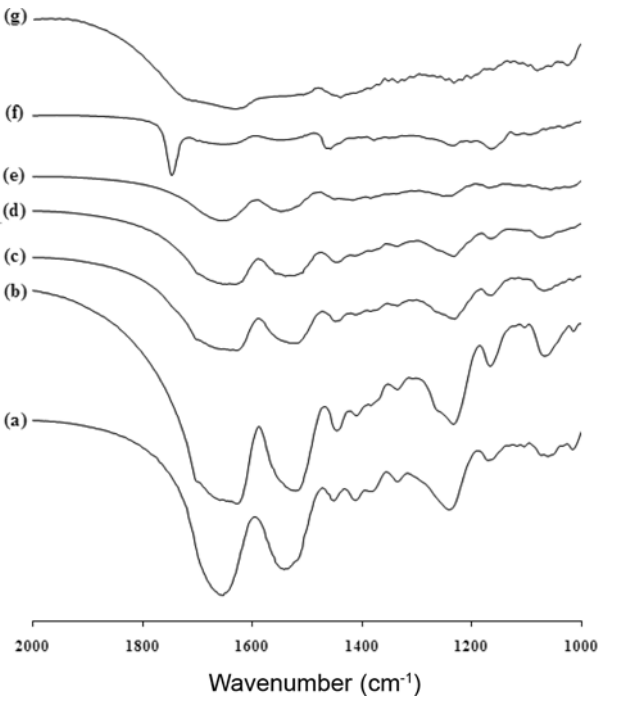

Figure 4. FTIR spectra of (a) silk fibroin powder, (b) SF scaffold with methanol treatment, (c) SF/C scaffold with methanol treatment, (d) SF/G scaffold with methanol treatment, (e) SF scaffold without methanol treatment, (f) lyophilized collagen, and (g) gelatin powder.

Structural Analysis

The conformation changes in the fibroin based scaffolds were determined by FTIR and X-ray diffraction (XRD). The FTIR spectra of SF scaffold without methanol treatment possessed unordered conformation as it showed characteristic peaks of SF at 1656, 1548, and 1252 cm^{-1} representing for amide I, amide II and amide III, respectively, Table 3 [53,54]. In contrast, the FTIR of SF scaffold treated with methanol showed β-sheet conformation as evident from the peaks shifted to the position of 1627, 1521, and 1233 cm^{-1}. Therefore, SF scaffold with methanol treatment could provide a more water-stable construct. The silk fibroin conformation changed from random coil to β-sheet during methanol treatment was reported as a result from the strongly intermolecular interaction of fibroin. This finding was in agreement with other researcher groups [55,56]. In addition, the SF/C scaffold treated with methanol also exhibited the β-sheet conformation. Interestingly, the SF/G scaffolds showed characteristic peaks at 1638, 1540, and 1233 cm^{-1}, suggesting that gelatin partially prevent SF conformation change from random coil to β-sheet as a result from the interaction of fibroin with gelatin via interchain H-bonding. These results also correspond with the compression test showing that SF/G scaffolds were more fragile than SF/C scaffolds.

Table 3. Characteristic peaks of prepared scaffolds determined by FTIR

	Formula	Methanol treatment	Characteristic peaks (cm^{-1})		
			Amide I	Amide II	Amide III
Fibroin powder	-	No	1653	1542	1241
SF scaffold	SF2	No	1656	1548	1252
	SF2	Treat	1627	1521	1233
SF/C scaffold	SF/25C	Treat	1628	1521	1231
SF/G scaffold	SF/30G	Treat	1638	1540	1233
Lyophilized collagen	-	No	1650	1550	1234
Gelatin powder	-	No	1634	-	1233

Table 4. Mechanical properties of SF scaffolds prepared from various fibroin concentration and silk fibroin-based scaffolds

Samples	Polymer concentration	Polymer weight ratio	Compressive properties (kPa)±SD	
			Compressive stress	Compressive modulus
SF	1 % w/v	100/0	34.16±16.94	45.16±28.91
	2 % w/v	100/0	37.09±1.85	47.75±3.09
	4 % w/v	100/0	47.59±4.37	54.78±3.92
	6 % w/v	100/0	134.91±9.31	147.97±11.50
SF/C	2 % SF:0.5 % C (4:1)	95/5	81.31±16.83	94.65±26.36
	2 % SF:1 % C (4:1)	90/10	72.05±17.05	107.07±47.09
	2 % SF:1 % C (3:2)	75/25	940.88±353.19	1531.71±697.41
SF/G	6 % SF:4 % G (4:1)	85/15	293.45±79.30	327.44±98.50
	6 % SF:4 % G (3:2)	70/30	332.62±80.95	363.56±46.73

Values are average±standard deviation (N=3).

X-ray diffraction was used to confirm the crystallinity of the prepared scaffolds. X-ray diffraction patterns of the scaffolds were shown in Figure 4. The SF, SF/C and SF/G scaffolds with methanol treatment exhibited a sharper and stronger diffraction peak at $2\theta=21^{\circ}$ than those scaffolds without methanol treatment, corresponding to increasing the β-sheet crystalline structure (silk II) of silk fibroin observed with FTIR.

Mechanical Properties

Mechanical properties of prepared scaffolds were presented in Table 4. The results indicated that the mechanical properties of the scaffold were affected by the mean pore size, the thickness of pore wall [24,26,49,57], and the structural of biomaterials [58]. The compressive stress of SF scaffolds slightly increased, 34-48 kPa, with an increase in SF concentration from 1-4 %. However, the compressive stress of 6 % SF scaffolds was 3 times, 135 kPa, larger than that of 4 % SF scaffold as a result from the decreased in pore size with uniform pore size distribution. As expected, SF/C and SF/G scaffolds showed higher compressive modulus than SF scaffolds. Compressive stress of SF/C (75/25) and SF/G (70/30) were 7 times, 941 kPa, and 2.5 times, 333 kPa, larger than that of 6 % SF scaffold. Considering that 6 % SF scaffold showed smaller pore size with high porosity than SF/C and SF/G scaffolds, the significant increased in compressive stress could be attributed to the increased thickness of pore walls in blended SF scaffolds as evident from SEM.

Interestingly, the SF/C scaffold possessed higher compressive stress than SF/G. This may be a result from SF/C scaffold containing more β-sheet conformation of SF than SF/G scaffold as confirmed by FTIR. In addition, the structure of collagen and gelatin might play a role. Ratanavaraporn *et al.* reported that collagen scaffolds, triple helix structure collagen, exhibited the higher compressive modulus than gelatin scaffolds, random-coil structure [58]. The compressive modulus of SF and blended SF scaffolds showed a similar trend.

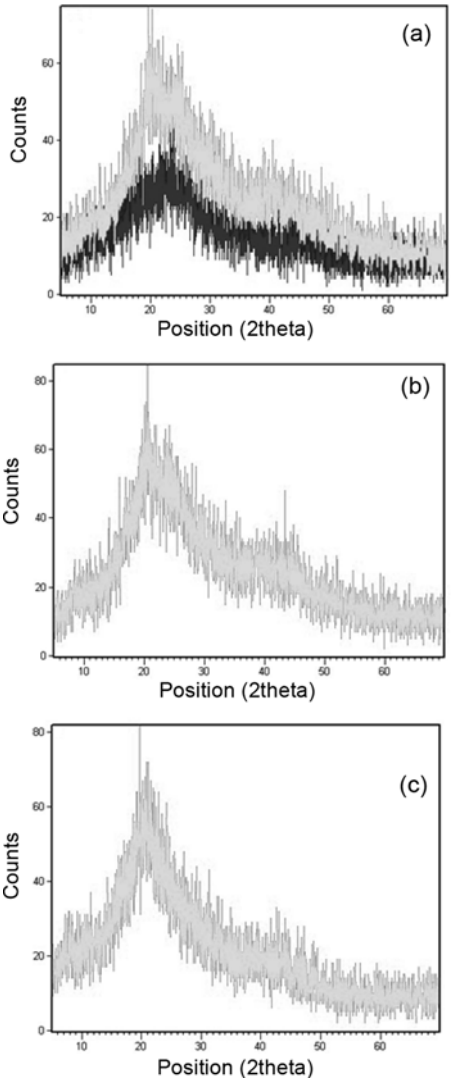

Figure 5. X-ray diffraction patterns of the 3-D porous scaffolds; (a) SF scaffold (black) without and (grey) with methanol treatment, (b) SF/C scaffold with methanol treatment, and (c) SF/G scaffold with methanol treatment.

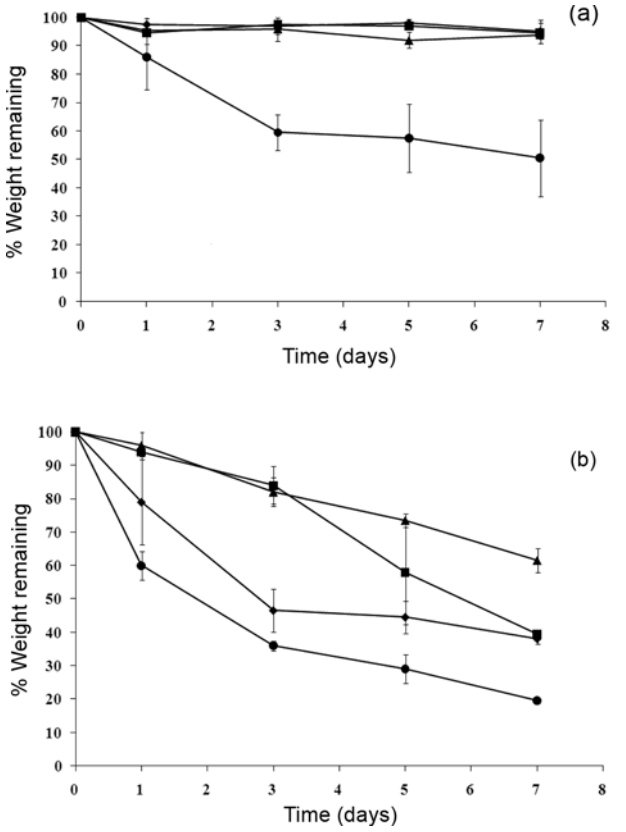

Figure 6. Mass of scaffolds remaining after immersed in PBS in (a) the absence and (b) presence of protease enzyme; (■) 2% SF, (▲) 6% SF, (◆) SF/C (75/25) and (●) SF/G (70/30).

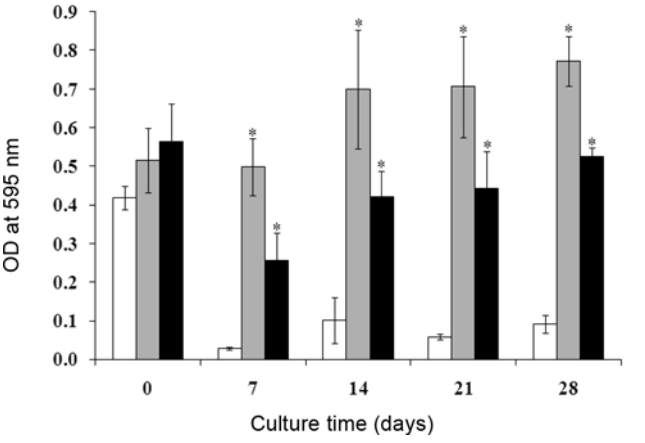

Figure 7. MTT assay after chondrocytes cultured in (□) 6 %SF, (▩) SF/C (75/25) and (■) SF/G (70/30) scaffolds for 0, 7, 14, 21, and 28 days. *Significant differences are from SF scaffold at $p < 0.05$.

Degradation of 3-D Sponges Scaffolds

The degradation behaviors of 3-D porous scaffold play a crucial role in the process of new tissue formation [49,59]. To assess their biodegradability, the remaining mass of prepared scaffolds was determined after incubated in PBS with and without protease enzyme at 37 °C. After being incubated in PBS without protease for 7 days, SF and SF/C scaffolds showed no degradation, while the SF/G scaffolds exhibited rapid degradation with a remaining weight at 50 %, Figure 6(a), indicating more hydrophilicity. For enzymatic degradation, after incubated in PBS with protease for 7 days, the SF scaffolds prepared from 2 and 6 % SF gradually degraded with time and the mass was reduced to 40 and 60 %, respectively, Figure 6(b). Additionally, the SF/C and SF/G scaffolds rapidly degraded and the remaining weight was reduced to 40 and 20 %, respectively, Figure 6(b). The difference in the degradation rate of SF, SF/C and SF/G scaffold may be a result from structural changes of fibroin based construct or the susceptibility to protease degradation. As confirmed by FTIR, SF/G scaffold showed less ordered structure than SF and SF/C scaffold resulting in the fastest degradation rate. Thus, these results indicated that the degradation rate of scaffold could be controlled by varying the amount of SF and blended polymers.

In Vitro Cell Viability and Proliferation Studies

MTT assay is a quantitative colorimetric assay used to access cell viability and proliferation. The purple formazan crystals created by metabolically active chondrocytes on the scaffolds indicated that cells were alive during the whole culture period and could be detected by UV-VIS spectrophotometer at 595 nm. Thus, it is an indirect method for determining cell growth and proliferation. Chondrocytes viability and proliferation on 3-D scaffolds were tested on different biomaterials; 6 % SF, SF/C (75/25) and SF/G (70/30). As shown in Figure 7, the OD value of SF scaffold decreased after 7 day cultivation and remained stable over 28-day culture period, suggesting that there may be cell death in the SF scaffold. On the other hand, the OD value of SF/C scaffolds showed a slight increase over 28-day cultivation period. The OD value of SF/G scaffolds slightly decreased after 7-day cultivation, but tended to increase over the remaining culture period. The OD values of blended scaffolds were significantly higher than those of SF scaffolds over cultivation time at $p < 0.05$.

This finding indicated that adding collagen or gelatin to SF scaffolds not only improved mechanical properties of the SF scaffold but also enhanced their biological properties. Wang *et al.* [44] reported that 3-D aqueous-derived silk fibroin provided a favorable environment for the proliferation of adult human chondrocytes (hCHs) when cultured in medium containing transforming growth factors β (TGF-β1). However, in this present study, the growth factor was not added into the culture medium and it was found that the SF scaffold showed a detrimental effect on chondrocyte attachment and proliferation. This may be because SF contains mainly neutral amino acid, such as glycine, alanine

and serine resulting in lower cell adhesion. Additionally, the SF scaffold possessed more hydrophobicity, thus creating unfavorable environment for cell adhesion. On the contrary, collagen and gelatin were composed of positively charged amino acid residues, such as lysine and arginine, and specific cell adhesion sites such as arginine-glycine-aspartate (RGD) groups [30,43,60]. In general, cells adhere much more strongly to substrate with basic groups than those with acidic or neutral groups due to negatively charged on the cell surface. Additionally, it is worth noting that higher cell proliferation found on SF/C scaffolds than that on SF/G scaffold may be associated with the rough surface of SF/C scaffold that tends to favor cell adhesion.

Conclusion

Freeze-dried SF, SF/G and SF/C scaffolds followed with methanol treatment exhibited sponge-like structure with homogeneous interconnected pores and high water adsorption. The methanol treatment caused changes in secondary structure of silk fibroin to be more crystalline beta sheet, resulting in water-stable constructs. The SF blended scaffolds exhibited showed superior mechanical properties and biocompatibility for chondrocytes attachment and proliferation to the blended SF scaffolds. The degradation rate of these scaffolds can be manipulated by controlling the amount of silk fibroin and blended polymers. Thus, the blended SF scaffolds have potential to be used as biomaterial constructs for tissue engineering application.

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