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Tissue response and biodegradation of composite scaffolds prepared from Thai silk fibroin, gelatin and hydroxyapatite

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Abstract This work aimed to investigate tissue responses and biodegradation, both in vitro and in vivo, of four types of *Bombyx mori* Thai silk fibroin based-scaffolds. Thai silk fibroin (SF), conjugated gelatin/Thai silk fibroin (CGSF), hydroxyapatite/Thai silk fibroin (SF4), and hydroxyapatite/conjugated gelatin/Thai silk fibroin (CGSF4) scaffolds were fabricated using salt-porogen leaching, dehydrothermal/chemical crosslinking and an alternate soaking technique for mineralization. In vitro biodegradation in collagenase showed that CGSF scaffolds had the slowest biodegradability, due to the double crosslinking by dehydrothermal and chemical treatments. The hydroxyapatite deposited from alternate soaking separated from the surface of the protein scaffolds when immersed in collagenase. From in vivo biodegradation studies, all scaffolds could still be observed after 12 weeks of implantation in subcutaneous tissue of Wistar rats and also following ISO10993-6: Biological evaluation of medical devices. At 2 and 4 weeks of implantation the four types of Thai silk fibroin based-scaffolds were classified as "non-irritant" to "slight-irritant", compared to Gelfoam® (control samples). These natural Thai silk fibroin-based scaffolds may provide suitable biomaterials for clinical applications.

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1 Introduction

Bone tissue engineering is an approach that has developed to heal bone loss from trauma or bone diseases without the limitations of current clinical autografting and allografting treatments [1]. One of the key components in bone tissue engineering is the scaffold, which functions as a structural support and delivery vehicle to provide cells and bioactive molecules necessary for new bone formation. Useful scaffolds should possess mechanical properties adequate to support bone tissue growing, degrade upon new bone tissue growth, demonstrate good biocompatibility, and have high porosity to enable bone tissue ingrowth. Polymeric scaffolds used for bone tissue engineering, such as poly (lactic-co-glycolic acid) or poly-lactic acid, can induce inflammation due to the acidity of their hydrolysis products [1]. Therefore, there is a need to identify alternative biomaterials to overcome these limitations.

In the world of natural fibers, silk has long been recognized as a remarkable fiber for its unique combination of high strength and toughness [2]. Moreover, core silk fibroin fibers exhibit comparable biocompatibility with other

commonly used degradable polymeric biomaterials such as poly-lactic acid and collagen [3]. Silk fibroin has recently been explored as a biomaterial for orthopedic applications [4]. Kim et al. [5] revealed that osteoblast-like cells increased with culture time in porous silk fibroin scaffolds seeded initially with human bone marrow derived mesenchymal stem cells (hMSCs) and exposed to osteogenic conditions. Meinel et al. [1] reported that implantation of silk scaffolds seeded with hMSCs into calvarial critical size defects in mice induced advanced bone formation within 5 weeks when compared to the implantation of silk scaffolds alone.

In our previous work, Chamchongkaset et al. [6], we developed three-dimensional salt-leached Thai silk fibroin scaffolds were formed from cocoons of Nangnoi Srisaket 1, one of *Bombyx mori* silk. Gelatin conjugation was introduced to modify the surface of Thai silk fibroin scaffolds, enhancing cell proliferation and mechanical properties. In addition, hydroxyapatite deposition via an alternate soaking method was employed to grow hydroxyapatite crystals into the porous structure of Thai silk fibroin-based scaffold. Scaffolds prepared from gelatin-conjugated silk fibroin with hydroxyapatite enhanced osteogenic differentiation of both MC3T3-E1 and rat bone marrow-derived stem cells [7]. In an attempt to apply Thai silk fibroin-based scaffolds in bone tissue engineering, tissue response and biodegradation of these composited scaffolds had to be explored.

The aim of the present work was to investigate tissue responses in vivo and the biodegradation in vitro and in vivo of Thai silk fibroin-based scaffolds conjugated with gelatin and with deposited with hydroxyapatite. The tissue response was evaluated according to the methods described by the international organization for standardization 10993-Part 6: tests for local effects after implantation (ISO 10993-6) [8].

2 Materials and methods

2.1 Materials

Bombyx mori Thai silk from yellow cocoons of Thai silkworm "Nangnoi-Srisaket 1" obtained from Queen Sirikit Sericulture Center, Nakhonratchasima province, Thailand, and type A gelatin from Nitta Gelatin Co., Osaka, Japan were used as raw materials. Other chemicals used were analytical grade.

2.2 Preparation of four types of Thai silk fibroin-based scaffolds

2.2.1 Thai silk fibroin scaffold (SF)

Thai silk fibroin scaffolds were prepared using a salt porogen leaching method as described previously [9]. The cocoons were boiled in 0.02 M Na_2CO_3 solution to extract sericin and other contaminating proteins. The silk fibroin fiber was dissolved in 9.3 M LiBr solution at 60°C for 4 h. The solution was dialyzed in deionized water using a dialysis tube (MWCO 12,000–16,000) for 2 days to obtain 6.5 wt% silk fibroin solution. The silk fibroin scaffold was prepared by adding granular NaCl (600–710 μm) into silk fibroin solution. The solution was left at room temperature to allow gelation prior to washing with deionized water to leach out the salt. Next the silk scaffold was left to dry overnight. The silk scaffold was punched into discs, 11 mm in diameter and 2 mm in thickness.

2.2.2 Conjugated gelatin/Thai silk fibroin scaffold (CGSF)

Conjugated gelatin/Thai silk fibroin scaffolds were prepared following our prior methods [6]. Thai silk fibroin scaffolds obtained from salt leaching were immersed in 0.5 wt% gelatin solution under vacuum and then freeze dried. Silk fibroin scaffolds coated with gelatin were crosslinked by dehydrothermal treatment (at 140°C for 48 h under vacuum pressure of −0.4 bar) and then conjugated using carbodiimide solution (14 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 5.5 mM *N*-hydroxy-succinimide (NHS)) for 2 h. Conjugated scaffolds were rinsed with deionized water to remove excess EDC, NHS and other reaction products. The treated scaffolds were left to dry at room temperature.

2.2.3 Hydroxyapatite/Thai silk fibroin scaffold (SF4)

Hydroxyapatite/Thai silk fibroin scaffolds were prepared using an alternate soaking method [10]. They were soaked in 0.2 M $CaCl_2$ solution under vacuum for 30 min and washed in deionized water. They were transferred to 0.12 M Na_2HPO_4 solution and were soaked under vacuum for 30 min and then washed in deionized water. This was considered as one cycle of alternate soaking. The soaking process was performed for four cycles to allow deposition of hydroxyapatite on the surface of Thai silk fibroin scaffold. The hydroxyapatite/Thai silk fibroin scaffolds were left to dry at room temperature.

2.2.4 Hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffold (CGSF4)

The conjugated gelatin/Thai silk fibroin scaffolds were used as the substrate for the deposition of hydroxyapatite by the alternate soaking process using the same procedure described in the case of SF4.

2.3 In vitro biodegradation of Thai silk fibroin-based scaffolds

The four types of scaffolds were sterilized with ethylene oxide and left at room temperature for a few days prior to further investigation. To perform in vitro biodegradation studies, Thai silk fibroin-based scaffolds were incubated at 37°C, pH 7.4 in 1.5 ml solution of 1 U/ml collagenase [11] and sodium azide 0.01% w/v [12] for 1, 7, 14, 21 and 28 days. The solution was changed every 2 days. After each time interval, Thai silk fibroin-based scaffolds were removed from the solution, rinsed with deionized water, centrifuged at 5,000 rpm (3,214 g) and freeze dried. The remaining weight, morphology and structure were examined and compared to the original material prior to enzymatic digestion.

2.3.1 Dried weight of Thai silk fibroin-based scaffolds

The dried weight of scaffolds before and after in vitro biodegradation was measured. The remaining dried weight of the scaffolds was calculated using the following equation

$$\text{Remaining weight } (\%) = \frac{W_t}{W_O} \times 100$$

where W_O was the initial dried weight of scaffold and W_t was the remaining dried weight of the scaffold after biodegradation in collagenase. The values were reported as mean ± standard deviation ($n = 3$).

2.3.2 Morphological observation of Thai silk fibroin-based scaffolds

Changes in the physical appearance and morphology of the scaffolds, before and after biodegradation, were observed and compared. The physical appearance (macrostructure) of scaffolds was assessed with a digital camera. The microstructure of scaffolds was examined by scanning electron microscopy (SEM, JEOL JSM 5410LV). The samples were sputter-coated with a thin gold layer in order to provide sample conductivity prior to SEM.

2.3.3 Conformational structure of Thai silk fibroin-based scaffolds

Before and after in vitro biodegradation, X-ray diffraction patterns of scaffolds were analyzed using a X-ray diffractometer (XRD, Siemens D5000) with CuK_α radiation. The voltage of the X-ray source was 30 kV at 30 mA. The scan speed used was 2.0 s/step with the step size of 0.04°.

2.4 Subcutaneous implantation

In vivo tests were approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (No. 09/52). The animal experiments were performed according to Chulalongkorn University Animal Care and Use Committee (CU-ACUC). The scaffolds, 11 mm in diameter and 2 mm thick, were implanted into the subcutaneous tissue of female Wistar rats (8 weeks old, 200–300 g). The rats were anaesthetized by intraperitoneal injection of thiopental sodium (60 mg/kg body weight) [13]. Animals were shaved and cleaned with betadine solution and then ethanol at the lumbar region. Using sterile techniques, a 1 cm skin incision was made to form four pockets in the subcutaneous tissue. The skin was closed with 6-0 prolene suture and cleaned with betadine solution. Both in vivo biodegradation and tissue response were performed in the subcutaneous implantation.

2.4.1 In vivo biodegradation of Thai silk fibroin-based scaffold

A total of 48 scaffolds were implanted into 12 female rats (4 scaffolds per rat). The rats were divided into 3 groups. After 2, 4, and 12 weeks of implantation, the animals were sacrificed with an overdose of thiopental sodium. The scaffolds and surrounding tissue were retrieved. The physical appearance and morphology of scaffolds were evaluated. The dorsal skin of the rats where the samples were placed was removed for photography. The scaffolds and surrounding tissue were removed and fixed in formalin for 1 day. Then, the samples and tissue were dehydrated with a series of ethanols at different concentrations. Finally, samples were treated with hexamethyldisilazane and air dried [14]. Cross-sections of dried samples were mounted onto a stub, sputter-coated with gold and observed under SEM.

2.4.2 Tissue response

For each rat, the sample and control were placed into the left and right sides of the back. Gelfoam®, a medical device used as a hemostatic material for bleeding surfaces, served as a control. A total of 32 female rats used in the experiment were divided into two groups (2 and 4 weeks). After 2 and 4 weeks of implantation, the animals were sacrificed with an overdose of thiopental sodium. Scaffolds and surrounding tissue were retrieved, formalin-fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) for histologic evaluation. Both macro- and microscopic assessments were carried out. For histologic assessment, the H&E slides were semi-quantitatively scored following ISO10993-6 [8]. Inflammatory cell types,

neovascularisation, fibrosis, and fatty infiltrate were scored by one pathologist (Somruetai Shuangshoti) at two different times. Intensity of inflammatory cells was recorded using 0–4 scales (0 = not observed, 1 = rare, 2 = minimal, 3 = heavily infiltrate, and 4 = packed infiltrate). Neovascularisation, fibrosis, and fatty infiltrate, were recorded using a scale ranging from 0 to 4. The final score was calculated according to Eq. 1 and classified as follows: 0.0–2.9 (sample is non-irritant), 3.0–8.9 (sample is slight-irritant), 9.0–15 (sample is moderate-irritant), and >15 (sample is severe-irritant). The level of irritation was compared to that of the control (Gelfoam®).

$$\text{Final score} = [2I_t + N_t] - [2I_c + N_c] \quad (1)$$

where I_i is the total number of polymorphonuclear cell, lymphocytes, plasma cells, macrophages, giant cells, and necrosis of sample i (i = test sample (t) and control (c)), N_i is the total number of neovascularisation, fibrosis, and fatty infiltrate of sample i (i = test sample (t) and control (c)).

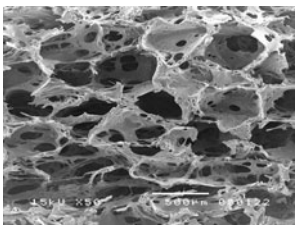
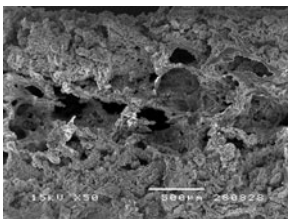
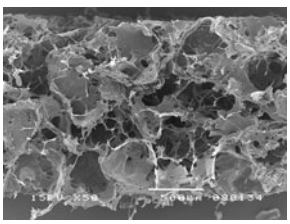
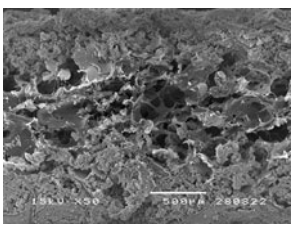
2.5 Statistical analysis

The significant levels of data were determined by an independent two-sample t-test. All statistical calculations were performed on the SPSS system for Windows. A P-value of <0.05 was considered significant.

3 Results and discussion

Table 1 presents the characteristics of four Thai silk fibroin-based scaffolds. The pore sizes of pure Thai silk fibroin (SF) scaffolds was around 580 ± 40 μm diameter, which was slightly smaller than the size of NaCl crystals used (600–710 μm). In the case of CGSF, conjugated gelatin fibers formed inside the pores of silk fibroin scaffold. For SF4 and CGSF4 scaffolds, pores were filled with deposited hydroxyapatite crystals, resulting in rough surfaces with smaller pore sizes, lower porosity and higher

Table 1 Characteristics of all Thai silk fibroin-based scaffolds

Scaffold type	Organic:inorganic	Morphology	Properties of scaffold
Thai silk fibroin scaffold (SF)	100:0		Pore size: 580 ± 40 μm Density: 0.081 ± 0.005 mg/mm^3
Hydroxyapatite/Thai silk fibroin scaffold (SF4)	52 ± 0.73:48 ± 0.73		Pore size: 300 ± 58 μm (determined from the middle part of the cross-sectioned scaffolds) Density: 0.130 ± 0.009 mg/mm^3
Conjugated gelatin/Thai silk fibroin scaffold (CGSF)	100:0		Pore size: 485 ± 42 μm Density: 0.093 ± 0.006 mg/mm^3 Porosity: 90.7 ± 0.6%
Hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffolds (CGSF4)	55 ± 0.40:45 ± 0.40		Pore size: 286 ± 82 μm (determined from the middle part of the cross-sectioned scaffolds) Density: 0.146 ± 0.009 mg/mm^3

density compared to the SF and CGSF scaffolds. It was noted that the pore sizes of SF4 and CGSF4 scaffolds was measured from the middle part of scaffolds. Near the edge of cross-sectioned scaffolds, the pore size was much smaller due to more deposited hydroxyapatite from the alternate soaking method.

3.1 In vitro biodegradation of Thai silk fibroin-based scaffolds

3.1.1 Physical appearance of Thai silk fibroin-based scaffolds

Physical appearance of the four types of Thai silk fibroin based-scaffolds, before and after degradation in collagenase for 1, 7, 14, 21 and 28 days, is presented in Fig. 1. The original SF and CGSF scaffolds before degradation were

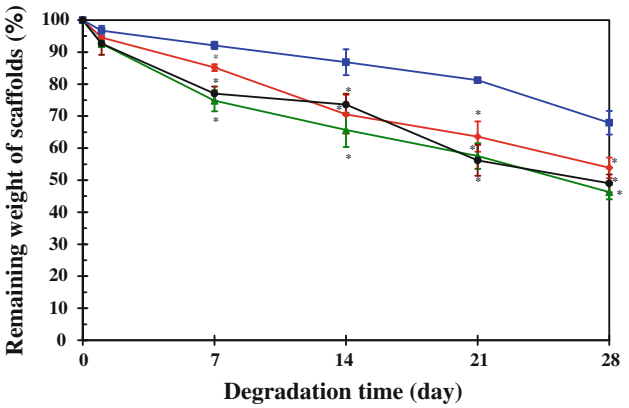


Fig. 2 Remaining weight (%) of Thai silk fibroin-based scaffolds at each period of degradation time in collagenase: 1, 7, 14, 21 and 28 days: SF (*diamond*), SF4 (*triangle*), CGSF (*square*) and CGSF4 (*circle*) scaffolds. *Asterisk* represented the significant difference ($P < 0.05$) relative to CGSF scaffolds in each periods

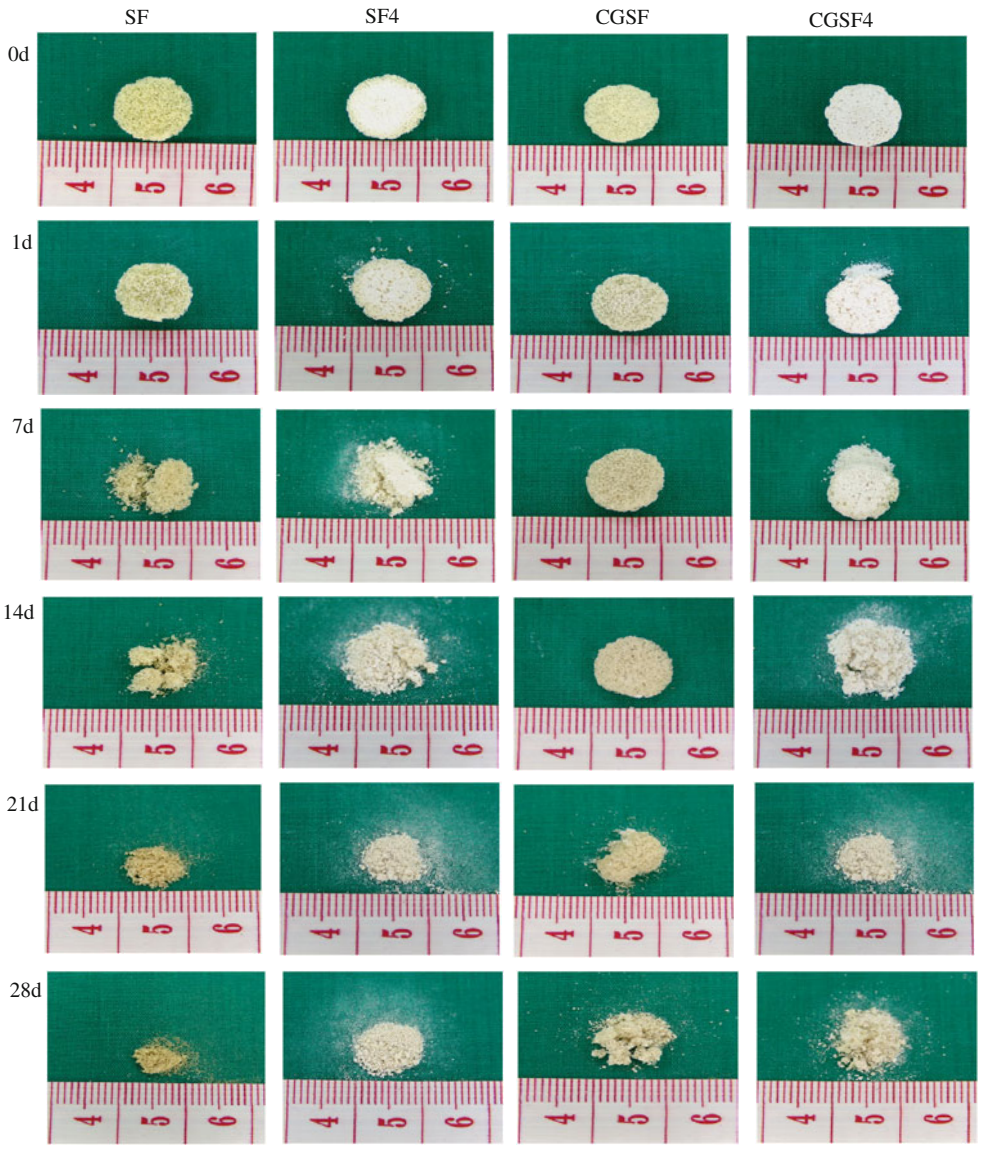


Fig. 1 Physical appearances of dried SF, SF4, CGSF, CGSF4 scaffolds after incubation in 1 U/ml collagenase for 1, 7, 14, 21 and 28 days

light yellow while SF4 and CGSF4 scaffolds were white due to the hydroxyapatite deposition. After 1 day of incubation in collagenase, SF and CGSF scaffolds remained whole. For SF4 and CGSF4 scaffolds, some small pieces of the scaffolds broke apart from the main scaffold. These small pieces were the hydroxyapatite deposited on the scaffold surface. After 14 days of incubation, only CGSF scaffolds still remained intact. From the physical appearance, SF, SF4 and CGSF4 scaffolds were easier to break apart than CGSF scaffold.

Figure 2 illustrates the remaining weight of the four types of Thai silk fibroin-based scaffolds after incubation in collagenase solution. After 7 days of incubation, it was noticed that the remaining weights of SF, SF4 and CGSF4 scaffolds were significantly lower than those of the CGSF scaffolds. The lower remaining weights of scaffolds containing hydroxyapatite was the result of the degradation of the protein portion by the collagenase. Because hydroxyapatite crystals deposited into the porous scaffolds were separated from the surface, the collagenase solution could penetrate inside the scaffolds, leading to more rapid degradation of the proteins, especially gelatin. The time point where half the original weight of scaffolds containing hydroxyapatite (SF4, CGSF4) and pure silk fibroin scaffold

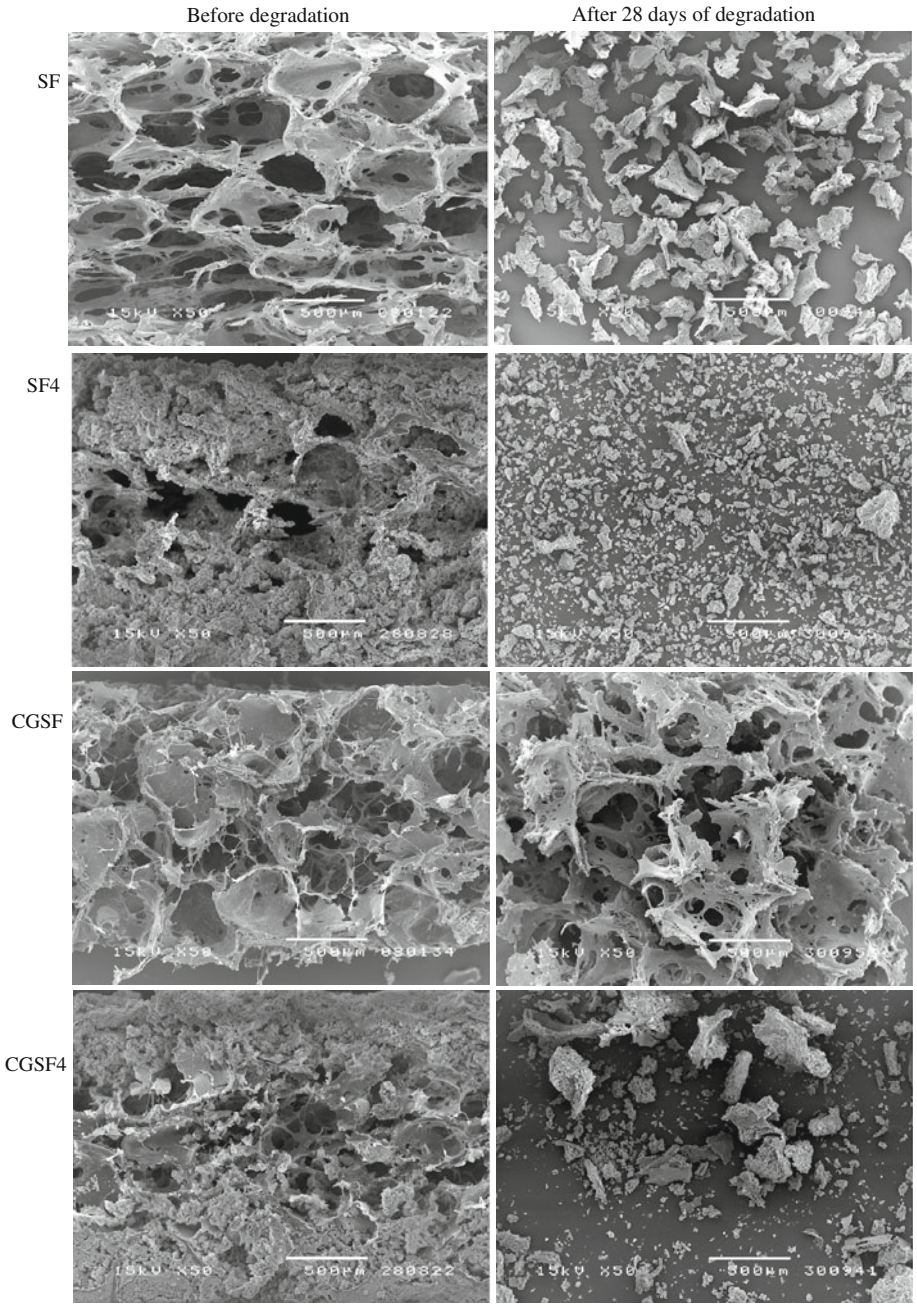


Fig. 3 SEM micrographs of SF, SF4, CGSF and CGSF4 scaffolds before and after incubation in collagenase for 28 days (scale bar = 500 μm)

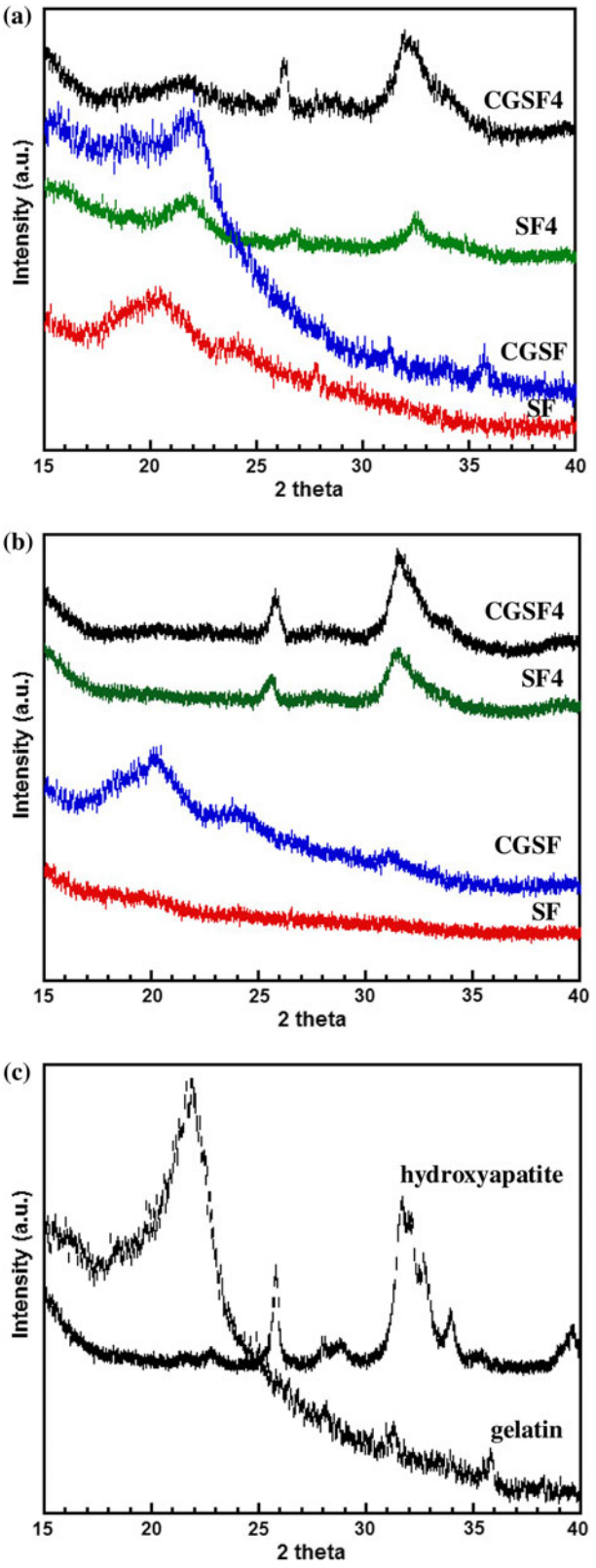


Fig. 4 X-ray diffraction patterns of SF, SF4, CGSF and CGSF4 scaffolds **a** before biodegradation test, **b** after 28 days of incubation in collagenase solution, and **c** hydroxyapatite (Fluka, Germany) and type A gelatin (Nitta, Japan)

(SF) were reached was after 21 and 28 days, respectively, after incubation in collagenase solution, while for CGSF scaffolds longer than 28 days was required. The in vitro biodegradation results suggested that among the four types of scaffolds, CGSF scaffold showed the slowest degradability. This could be due to the double crosslinking (dehydrothermal treatment followed by EDC/NHS crosslinking). Dehydrothermal treatment generates chemical bonds between the carboxyl groups of gelatin and silk [15]. EDC/NHS treatment was further crosslinked via activated carboxyl groups and amine groups of silk and conjugated gelatin molecules [16].

3.1.2 *Morphology of Thai silk fibroin-based scaffolds*

When comparing the morphology of the four types of Thai silk fibroin-based scaffolds before and after degradation, SF, SF4 and CGSF4 could not maintain their original structures (Fig. 3). Only small particulate debris was found for these three scaffolds. In contrast, the porous morphology of CGSF scaffold was retained. This implied that the gelatin conjugated on silk fibroin scaffold could resist the enzymatic degradation due to the chemical conjugation by dehydrothermal and EDC/NHS treatments resulting in more stable structures and slowing the rate of degradation [15]. The morphological changes of the four scaffolds corresponded to the results for weight change, i.e. the degradability of CGSF scaffolds was the slowest compared to the other scaffolds.

3.1.3 *Conformational structure of Thai silk fibroin-based scaffolds*

X-ray diffraction patterns of the four types of Thai silk fibroin-based scaffolds before degradation are presented in Fig. 4a. The broad diffraction peaks of SF scaffold were found at $2\theta = 20.7°$ and 24.6°. These peaks represented the β-sheet crystalline structure of silk protein [9, 11, 17]. This implied that the Thai silk fibroin scaffold prepared using salt leaching had assumed a β-sheet crystalline structure. The other peak indicating the β-sheet crystalline structure ($2\theta = 8.8°$) was not observed due to limitations with the equipment.

After hydroxyapatite was deposited on SF scaffolds via alternate soaking, the peaks for the SF4 scaffolds were observed at 21.8°, 26.7° and 32.5°. The peaks indicating β-sheet crystalline structure of silk fibroin in the SF4 scaffolds (21.8°) were shifted from those in the SF scaffold. The other peaks at 25.8° and 31.8° were the hydroxyapatite crystals, which were confirmed with the diffraction pattern of commercial hydroxyapatite particles (Fluka, Germany) as seen in Fig. 4c. The characteristic diffraction peaks at around 26° and 32° were assigned to

the (002) and (211) planes of hydroxyapatite crystals [18–20]. However, peaks of deposited hydroxyapatite in SF4 scaffolds were slightly broader and weaker as compared to those of the commercial hydroxyapatite, implying lower crystallinity and a smaller crystal size for the deposited hydroxyapatite. The XRD results of SF4 scaffolds ensured the crystallinity of the hydroxyapatite and silk fibroin in the SF4 scaffolds.

Considering the XRD pattern of CGSF scaffolds in Fig. 4a, a broad diffraction peak appeared at $2\theta = 22°$ and small diffraction peaks were found at $2\theta = 31.4°$ and 36.0°. Comparing to the diffraction patterns of type A gelatin (Nitta, Japan) in Fig. 4c [21], the XRD pattern of CGSF scaffolds revealed that type A gelatin was dominant. The X-ray diffraction pattern of CGSF4 scaffolds shown in Fig. 4a indicated that the hydroxyapatite component appeared as dominant, similar to the case of SF4 scaffolds. The result for CGSF4 scaffolds corresponded to the combination of the characteristic peaks of SF4 and CGSF scaffolds.

Figure 4b illustrates the XRD patterns of Thai silk fibroin-based scaffolds after degradation in collagenase. After 7 days of incubation and thereafter, Silk II crystalline structure ($2\theta = 20.7°$, 24.6°) disappeared while the Silk I crystalline structure ($2\theta = 19.7°$) was observed. The XRD results indicated that the structure of pure silk fibroin scaffolds (SF) changed from β-sheet structure (Silk II) to random coil structure (Silk I) after 7-days of incubation in collagenase. The structure of all small pieces of SF4 scaffolds incubated for 14–28 days exhibited only characteristic peaks of hydroxyapatite. This implied that the silk fibroin portion completely degraded after 14 days of incubation. In the case of CGSF scaffolds, the structure of the scaffolds after 1–21 days of degradation was similar to that before degradation. At the end of the incubation, the peaks exhibited only the β-sheet structure of silk fibroin. These data supported the conclusion that the conjugated gelatin on silk fibroin scaffolds was still present until 21 days. The residual of CGSF4 after 21 and 28 days of incubation indicated only the characteristics of hydroxyapatite.

3.2 In vivo biodegradation of Thai silk fibroin-based scaffolds

The physical appearance of the four types of Thai silk fibroin-based scaffolds, after 12 weeks of implantation, is shown in Fig. 5. All scaffolds remained intact in vivo after 12 weeks of implantation. The SEM micrographs of cross-sections of Thai silk fibroin-based scaffolds after 2, 4 and 12 weeks of implantation are presented in Fig. 6. After 2 weeks of implantation, some inflammatory cells invaded

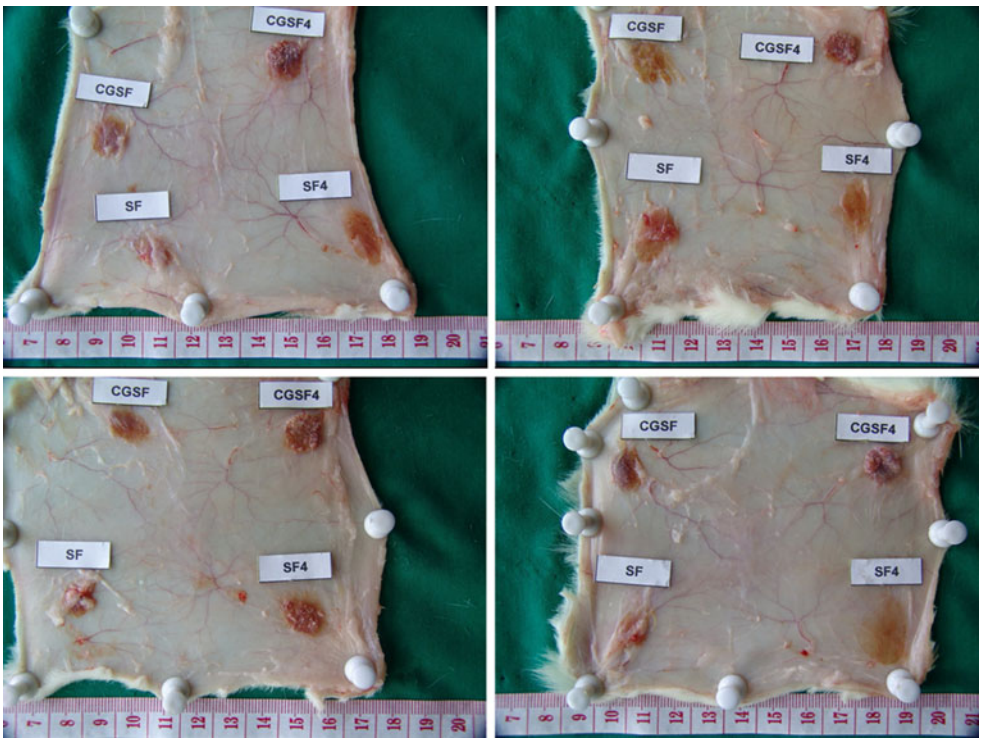


Fig. 5 Physical appearances of Thai silk fibroin scaffold (*SF*), hydroxyapatite/Thai silk fibroin scaffold (*SF4*), conjugated gelatin/Thai silk fibroin scaffold (*CGSF*) and hydroxyapatite/conjugated gelatin/Thai silk fibroin scaffold (*CGSF4*) after 12 weeks of implantation from four rats

into the SF scaffolds. The fused morphology of CGSF scaffolds was observed after 2 weeks of implantation. The deposited hydroxyapatite on SF4 scaffolds was bioresorbed whereas the morphology of the hydroxyapatite deposited on CGSF4 scaffolds was similar to the original morphology before degradation. After 4 weeks, a lot of inflammatory cells and blood cells invaded into SF scaffolds. The deposited hydroxyapatite was resorbed slightly faster on SF4 scaffolds than that on CGSF4 scaffolds. The bulk degradation of all scaffolds was observed after 12 weeks of degradation. The hydroxyapatite grown on SF4 and CGSF4 scaffolds was almost completely degraded.

Comparing in vitro and in vivo biodegradation of Thai silk fibroin-based scaffolds, the CGSF scaffolds showed the slowest in vitro degradation while the in vivo degradability of all scaffolds was similar. The double crosslinking of CGSF scaffolds promoted resistance against in vitro enzymatic degradation [22, 23]. However, in the in vivo environment, the difference in the biodegradation of four Thai silk fibroin-based scaffolds was not observed.

3.3 Evaluation of the tissue response of Thai silk fibroin-based scaffolds following ISO10993-6

The four types of Thai silk fibroin-based scaffolds were implanted into the subcutaneous tissue of Wistar rats. The Wistar rats were healthy throughout the implantation period and no signs of inflammation (i.e. redness, swelling,

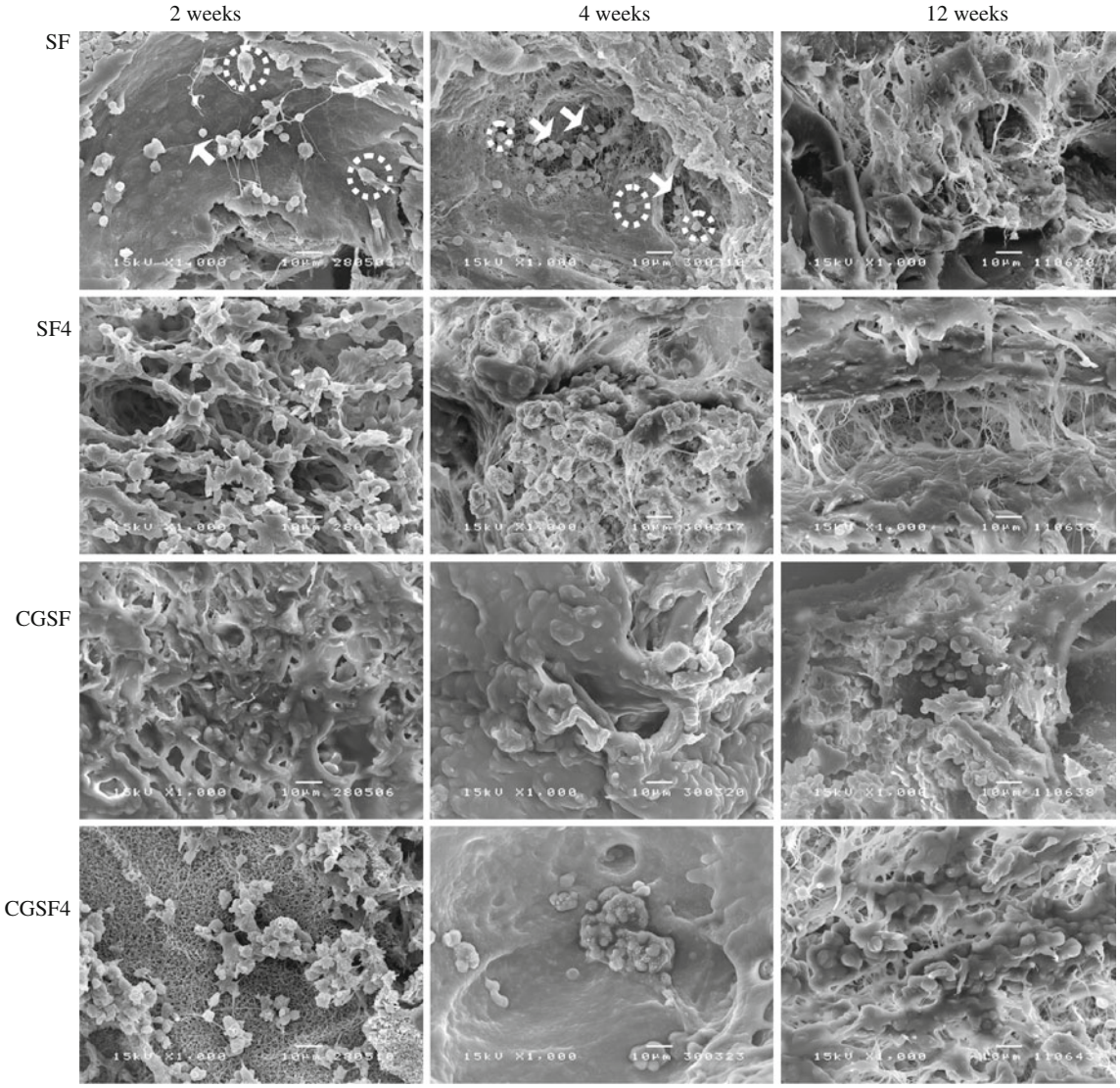


Fig. 6 SEM micrographs of SF, SF4, CGSF and CGSF4 scaffolds after 2, 4 and 12 weeks of implantation. *Circle* indicates inflammatory cells while *arrow* indicates extracellular matrix (scale bar = 10 μm)

Table 2 Level of irritation of Thai silk fibroin-based scaffolds after 2 and 4 weeks of subcutaneous implantation

Types of material	Level of irritation (2 weeks)		Level of irritation (4 weeks)	
	First evaluation	Second evaluation	First evaluation	Second evaluation
SF				
$n = 1$	Non-irritant	Non-irritant	Non-irritant	Non-irritant
n = 2	Non-irritant	Non-irritant	N/A	N/A
$n = 3$	Slight-irritant	Non-irritant	N/A	N/A
$n = 4$	Slight-irritant	Non-irritant	N/A	N/A
SF4				
$n = 1$	Non-irritant	Non-irritant	N/A	N/A
$n = 2$	Non-irritant	Non-irritant	N/A	N/A
$n = 3$	Slight-irritant	Slight-irritant	Slight-irritant	Slight-irritant
$n = 4$	Non-irritant	Non-irritant	N/A	N/A
CGSF				
$n = 1$	Non-irritant	Non-irritant	N/A	N/A
$n = 2$	Non-irritant	Slight-irritant	Slight-irritant	Slight-irritant
$n = 3$	Slight-irritant	Slight-irritant	N/A	N/A
$n = 4$	N/A	N/A	N/A	N/A
CGSF4				
$n = 1$	Non-irritant	Non-irritant	N/A	N/A
$n = 2$	Non-irritant	Non-irritant	N/A	N/A
$n = 3$	Slight-irritant	Slight-irritant	N/A	N/A
$n = 4$	Non-irritant	Non-irritant	N/A	N/A

N/A No evaluation since control sample was completely degraded

pain and heat) were observed. After 2 and 4 weeks of implantation, the cross-sections of retrieved SF and CGSF scaffolds were brownish yellow whereas that of SF4 and CGSF4 scaffolds appeared white and light yellow. There was no difference in the gross appearance of each scaffold. No abscesses were detected during the implantation period, nor was there an inflammatory reaction around the implantation sites.

The surrounding inflammatory reaction for each type of scaffolds was compared to that of the control material (Gelfoam®), after 2 and 4 weeks of implantation. Scoring of the histopathology of tissue reaction according to ISO10993-6 was performed twice by the same pathologist. The level of irritation of all scaffolds is shown in Table 2. For the first evaluation at 2 weeks after implantation, 2/4 rats implanted with Thai silk fibroin scaffold (SF) were classified as "non-irritant" and the remaining (2 rats) as "slight-irritant". In the case of the SF4 and CGSF4 scaffolds, there were more than 50% of the rats showing "non-irritant". For CGSF scaffolds, the fourth rat could not be evaluated since the control material disappeared. Result of the second-round evaluation were similar to that of the first round. The tissue surrounding the SF scaffolds, after 2 weeks of implantation, showed minimal inflammatory response, which consisted of a rare macrophage and neovascularisation (Fig. 7). In addition, giant cells were noted in SF4, CGSF and CGSF4, indicating a slight tissue response (Fig. 7). The tissue response in control samples comprised rare macrophages. Therefore, the level of irritation of all four types of scaffolds was "non-irritant" to "slight-irritant", and that, at the 2nd week of implantation, the tissue response to the four Thai silk fibroin-based scaffolds did not significantly differ from that of the control (Gelfoam®).

After 4 weeks of implantation, the level of irritation of most scaffolds could not be evaluated because more than 50% of the implanted control was degraded. The inflammatory cells and tissue response decreased around SF, SF4 and CGSF4 scaffolds at the 4th week, compared to those at the 2nd week. However, the CGSF scaffold still showed minimal macrophages, similar to the reaction at the 2nd week. There was no significant difference in inflammatory cells and responses observed in all scaffolds, compared to that of the control. Results of the second-round evaluation did not differ significantly from that of the first round. Hence, it can be concluded that the tissue response to all Thai silk fibroin-based scaffolds, after 4 weeks of implantation, was less than that observed at the 2nd week.

4 Conclusions

The biodegradation behavior of four types of scaffolds were investigated in vitro (in collagenase solution) and in vivo (in a subcutaneous rat model). Among the four types

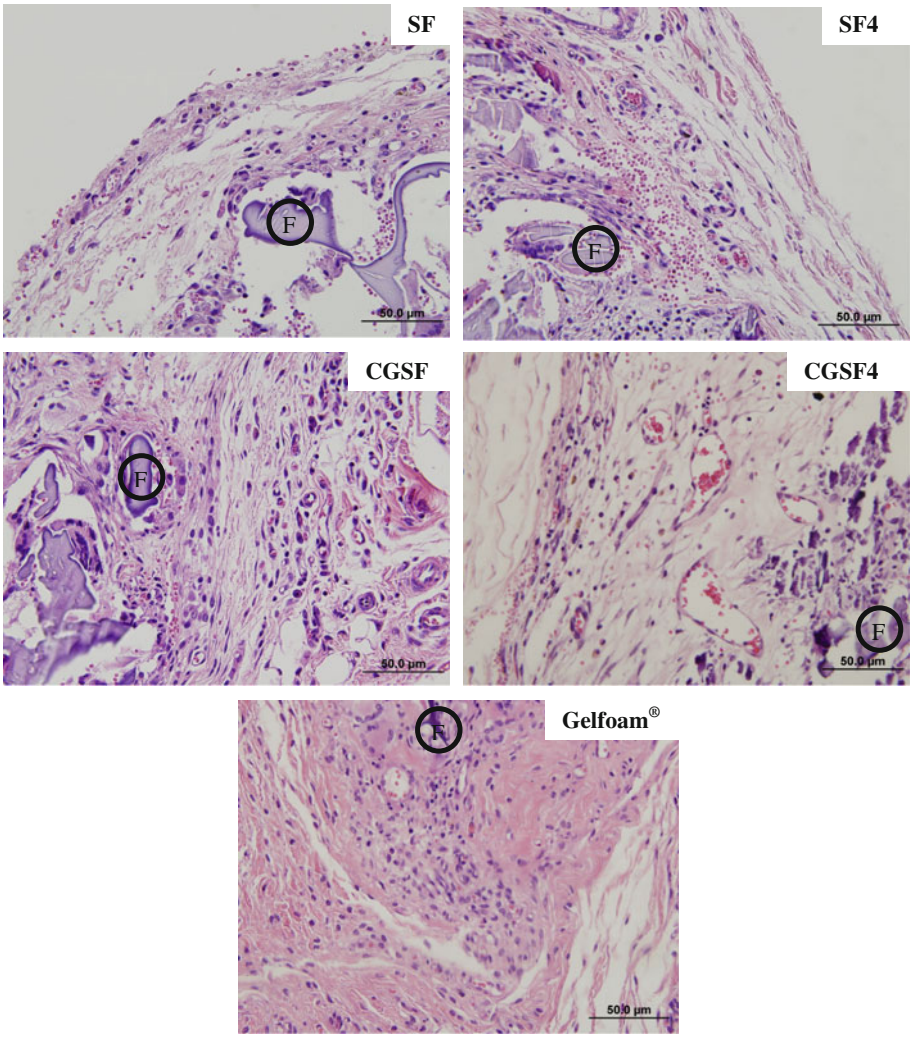


Fig. 7 Histological section of subcutaneously implanted Thai silk fibroin-based scaffold in Wistar rat after 2 weeks of implantation. Ⓕ indicates fragment of scaffold. (magnification ×400)

of scaffolds, the biodegradability of conjugated gelatin/Thai silk fibroin scaffold (CGSF) in collagenase solution appeared to be slowest. This was because gelatin conjugation by dehydrothermal and EDC/NHS treatments led to more stable structures. These differences could also be at least partly due to the conditions to which the samples were prepared, as well as the differences in composition of the various materials. Elucidation of these differences would require additional inquiry into selective components of the various scaffolds. All scaffolds remained in vivo after 12 weeks of subcutaneous implantation in Wistar rats. This is the first to report of tissue responses to Thai silk fibroin-based scaffolds, evaluated by the International Standard Organization 10993-6: Biological evaluations of medical devices. All four types of scaffolds were classified as "non-irritant" to "slight-irritant" after 2 weeks of subcutaneous implantation. After 4 weeks, the number of inflammatory cells decreased from that observed at 2 weeks after implantation. All implanted scaffolds, at 2 and 4 weeks postoperatively, showed slight irritation as evaluated by ISO10993-6. Based on the data collected in the present study, these naturally-modified materials may have future potential for clinical applications.

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