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Porous silk fibroin/alpha tricalcium phosphate composite scaffolds for bone tissue engineering: A preliminary study

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Abstract. Scaffolds with mechanical properties that mimic the tissue to be restored are critical to maintain the morphology and function of a scaffold after implantation and during tissue regeneration. Silk fibroin (SF), a protein from the *Bombyx mori* silk worm cocoon, is currently employed in the biomedical field and tissue engineering. The objective of this study was to construct three-dimensional porous silk fibroin/alpha tricalcium phosphate scaffolds for bone tissue engineering application. The scaffolds were fabricated using a solvent casting and salt leaching technique. The hybrid strain of degummed Thai silk fibroin, Nangnoi Srisaket 1 x Mor, was dissolved in hexafluoroisopropanol at 16% (w/v). Alpha tricalcium phosphate (α-TCP) was incorporated to produce 4, 8, 12, and 16 wt% solution and sucrose (particle size 250-450 μm; sucrose/silk fibroin = 8.5/1 w/w) was used as a porogen. The microstructure and pore size, calcium and phosphorus contents, and compressive modulus were evaluated. The scanning electron microscope images revealed the microstructure of scaffolds to be square shaped with continuous interconnected pores. The average pore size of the scaffolds was 265.70 $\pm$ 67.45 μm. The scaffolds containing 8% (w/w) α-TCP exhibited the highest compressive modulus (64.84 $\pm$ 16.65 kPa) and the highest calcium content. The results suggested that the scaffolds containing α-TCP may be a potential candidate for application in bone tissue engineering applications.

Introduction

Bone grafting is a treatment used to repair bony defects or fractures. An autograft is the gold standard for bone graft; however, it burdens the patient with an additional surgical site. Allografts and xenografts have the risk of disease transmission, foreign body reaction, and graft rejection. Arising from the limitations of such grafts, the novel and promising strategies such as tissue engineering have been developed to improve the treatment and quality of life of patients. Tissue engineering is one promising strategy to stimulate tissue formation through the use of cells, growth factors, and biomaterials.

Biomaterials for use in three - dimensional porous scaffolds for bone tissue engineering have been investigated. Natural polymers have more advantages than synthetic biodegradable polymers and ceramics; for instance, biodegradability, non-toxicity, and cellular function support. Silk fibroin (SF) derived from *Bombyx mori* cocoon demonstrates good mechanical properties [1], biodegradability [2], less immunological response [2], and is easily shaped in several architectures. Furthermore, SF scaffolds present versatility in processing for tissue engineering, for example, in nerves [3], cartilage [4], and bone [5,6]. In addition to improving osteoconductivity and osteoinductivity, ceramics were added to the SF scaffolds.

Alpha tricalcium phosphate (α-TCP) is a bioresorbable ceramic and is the major component of the powder of commercial bone cements. According to high solubility and biodegradability, α-TCP is expected to be useful for bone substitution and scaffolds for bone tissue engineering either alone or blended with other calcium phosphate. Oh et al. (2010) revealed that the differentiation of

MC3T3-E1 cells was highly stimulated by α-TCP based experimental cement [7]. de Moraes Machado et al. (2011) demonstrated the potential use of 3-D porous scaffolds based on α-TCP for tissue engineering application [8]. Qu et al. (2011) presented a mixture of SF fiber and α-TCP, $CaCO_3$, and dicalcium phosphate dehydrate can support the attachment, proliferation, and differentiation of mesenchymal stem cells [9].

Solvent casting in combination with particulate leaching is one procedure to fabricate 3-D porous scaffolds. To begin with, the polymer is dissolved in the solvent at an appropriate concentration. The porogen, sodium chloride or sucrose, is sieved into the same particle size and added to the polymer suspension. Later, the mixture is poured into a mold and allowed to evaporate the solvent. After that, the scaffolds are treated in methanol at an appropriate concentration to induce the formation of the β-sheet structure. Finally, the scaffolds are immersed in water to dissolve the porogen and dried before to use [10-13]. The advantage of this technique is the pore size can be controllable, it is easy to perform and inexpensive.

However, a few previous studies have attempted to construct a 3-D porous SF with α-TCP scaffolds. Thus, the main purpose of the experiment here was to fabricate the porous SF/ α-TCP composite scaffolds through solvent casting with particulate leaching approaches. The morphology and microstructure were investigated by scanning electron microscopy (SEM). The calcium and phosphate contents were determined by scanning electron microscopy/ dispersive X-ray spectroscopy (SEM-EDX). Finally, the mechanical properties of the SF/ α-TCP scaffolds were evaluated using a universal testing machine.

Materials and Methods

The cocoons of *B. mori* - a Thai silk hybrid strain of Nangnoi Srisaket 1 x Mor - were purchased from Queen Sirikit Sericulture Center (Khon Kaen). In this study, sucrose particles (Ajax, Australia) of chemical grade and 1,1,1,3,3,3-Hexafluoro-2-propanol (Merck, Germany) were used. α-TCP was prepared by the thermal transformation of crystalline β-TCP. The other reagents and materials were obtained from Sigma-Aldrich, USA; Merck, Germany; and Ajax, Australia.

SF was extracted from the cocoons as previously reported by Rockwood et al. (2011) with slight modifications [14]. Briefly, the cocoons were boiled for 30 minutes in 0.02 M Na_2CO_3solution and rinsed thoroughly with ultrapure water to extract sericin. The degummed SF was dissolved in $CaCl_2$2(H_2O): H_2O: C_2H_5OH (1:8:2 in molar ratio) solution to a concentration of 10% (w/v) at 65^oC for 1 h and dialyzed against deionized water by using the cellulose dialysis tubing (MWCO: 12,000; Sigma-Aldrich, USA). The dialyzed aqueous SF solution was centrifuged (Avanti J-E; Beckman Coulter Inc, USA) at 9,000 rpm, 4^oC for 20 min. The concentration of aqueous SF solution was ca. 4% (w/v) which was determined by weighing the remaining solid after drying. The aqueous SF solution was frozen at -80^oC (Premium U570; Eppendorf Inc, Germany) and lyophilized (Christ Gamma 2-16 LSC; SciQuip, UK).

The lyophilized SF was dissolved in hexafluoro-2-propanol (HFIP) to obtain a 16% (w/v) SF solution [12]. α-TCP powder was prepared as reported by Kitamura et al. (2004) [15] and was incorporated to produce 4, 8, 12, and 16 wt% solutions. According to the preliminary study, sucrose with particle diameter ranges of 250-450 μm was added at a ratio of 1:8.5 (SF: sucrose). The suspension was poured in a polystyrene mold 16 mm in diameter. The molds were covered overnight to reduce evaporation of HFIP and to provide sufficient time for the homogeneous distribution of the solution. HFIP was allowed to evaporate at room temperature for 2 days and sucrose/SF blocks were immersed in 50% (v/v) methanol for 24 h to induce the formation of the β-sheet structure. The blocks were removed, dried and sucrose was leached by immersion in deionized water at room temperature for 3 days. All scaffolds were cut into cylindrical shape with a height of 5 mm. The SF scaffolds and the SF scaffolds containing α-TCP 4, 8, 12 and 16% (w/w) were designated as SF, SF/TCP-4, SF/TCP-8, SF/TCP-12, and SF/TCP-16, respectively.

The scaffolds were visualized through SEM for their surface and pore geometries. The scaffolds were cut into half at the center, dried in a critical point dryer, coated with gold in an evaporator coater, and observed by SEM with an accelerating voltage of 15 keV and a magnification of 35 x

(Quanta 250; FEI, USA). The pore size of scaffolds was measured by SemAfore version 5.21 program (JEOL GmbH, Germany).

The Ca and P contents in the scaffolds were measured by SEM-EDX. The scaffolds were cut into half at the center, dried in a critical point dryer, and coated with carbon in an evaporator coater, and studied by SEM-EDX with an accelerating voltage of 15 keV and a magnification of 100 x (JSM-6610LV; JEOL Ltd, Japan). The Ca and P contents were presented in weight% and atomic%.

The compressive modulus of scaffolds was evaluated with a universal testing machine (EZ Test; Shimadzu, Japan) using a 0.1 kN load cell and a crosshead speed of 0.5 mm/min. The load was applied on each wetted scaffold. The compressive modulus was shown as the slope of the stress-strain curve over the compressive strain 10% [10].

Pore size, Ca and P contents, and compressive modulus were presented as averages and with standard deviations. To assess the data obtained from the SEM and compressive test, one-way analysis of variance (ANOVA) was used. Comparison between two average values was assessed with Scheffe's test at $p < 0.05$.

Results and Discussions

Silk is a natural polymer which has been utilized in medical applications and the textile industry for many decades. SF is a protein from the *Bombyx mori* cocoon currently employed in the biomedical field and tissue engineering. α-TCP is a bioresorbable ceramic, and is expected to be useful for bone substitution and as a scaffold for bone tissue engineering. Solvent casting in combination with particulate leaching is one procedure to fabricate 3-D porous scaffolds for cartilage [13] and bone tissue engineering [10,12].

In this study, the author developed α-TCP incorporated into SF scaffolds via solvent casting in combination with particulate leaching for bone tissue engineering application.

Effect of SF to sucrose ratio on the morphology of the scaffolds

The effect of the SF – to - sucrose ratio on the morphological appearance of the SF scaffolds is illustrated in Fig. 1. The particle size of sucrose was in the range of 250-450 µm. The porosity of scaffolds increased with an increase in the SF – to - sucrose ratio with the average value of 61.58 ± 3.43 to 89.05 ± 0.90 %. On the other hand, the density of scaffolds showed a reverse trend with the average value of 0.07 ± 0.00 to 0.23 ± 0.01 g/cm^3. The decrease in density was agreeable with the compressive modulus which decreased from 0.56 ± 0.16 MPa at the ratio of 1:5 to 0.02 ± 0.00 MPa at the ratio of 1:20 (the results not shown). Based on the data between the porosity and density of the SF scaffolds prepared using various SF – to - sucrose ratios, the ratio of 1:8.5 was selected for subsequent studies.

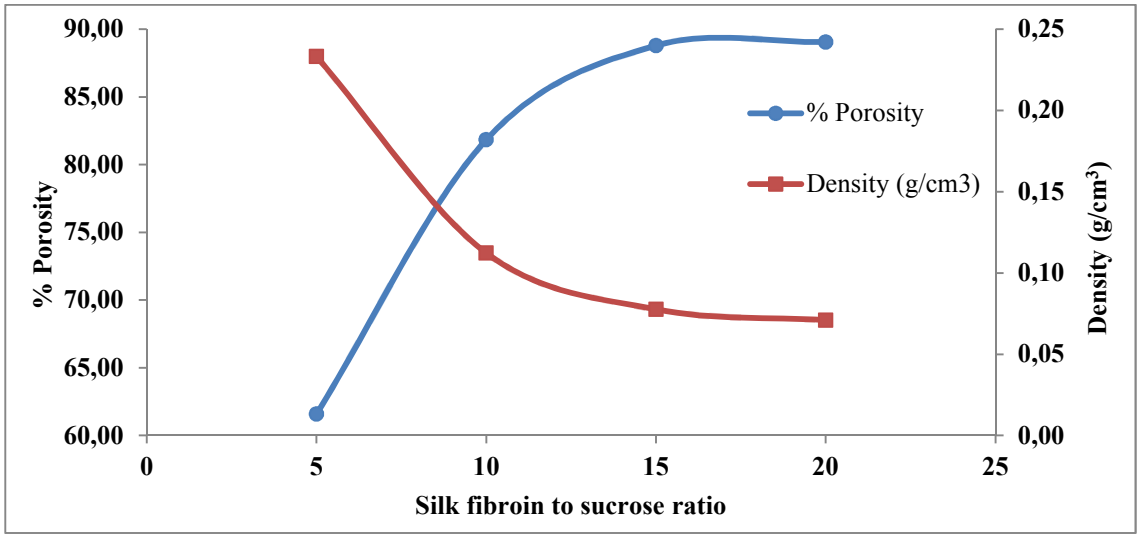

Figure 1. *The porosity and the density of the SF scaffolds obtained from the various SF – to - sucrose ratios.*

Morphology and microstructure

The morphology of all scaffolds presented highly porous structures. Fig. 2 shows SEM images of the different SF/TCP scaffolds. The microstructure of scaffolds was square shaped with continuous interconnected pores. The pore sizes were in the range 240.93 - 300.71 μm and the average pore sizes presented in Table 1. The data indicated that the pore size of SF/TCP-12 was significantly larger than that SF/TCP-4, SF/TCP-8, and SF/TCP-16 ($p < 0.05$).

There are many studies have reported the optimal pore size for cell growth and tissue formation. Karageorgiou and Kaplan (2005) [16] showed that a pore size larger than 100 μm was good for cell migration, while one higher than 300 μm had great potential for new bone and capillary formation [16] and bone formation [17]. The result of this study implies that SF/TCP scaffolds have potential for bone tissue engineering.

Calcium and phosphorus contents

Table 1 show the average Ca and P contents in the scaffolds. As can be seen, most of the Ca and P were retained in the SF/TCP-8 as compared to SF/TCP-4, SF/TCP-12, and SF/TCP-16. However, the amount of Ca and P in the composite scaffolds did not increase when increasing the amount of α-TCP. This might be because the method of adding α-TCP does not distribute it homogeneously in the scaffolds. Although many studies have demonstrated the procedure to construct silk CaP scaffolds [17, 18, 19], this study showed the simplest method to fabricate the composite scaffolds.

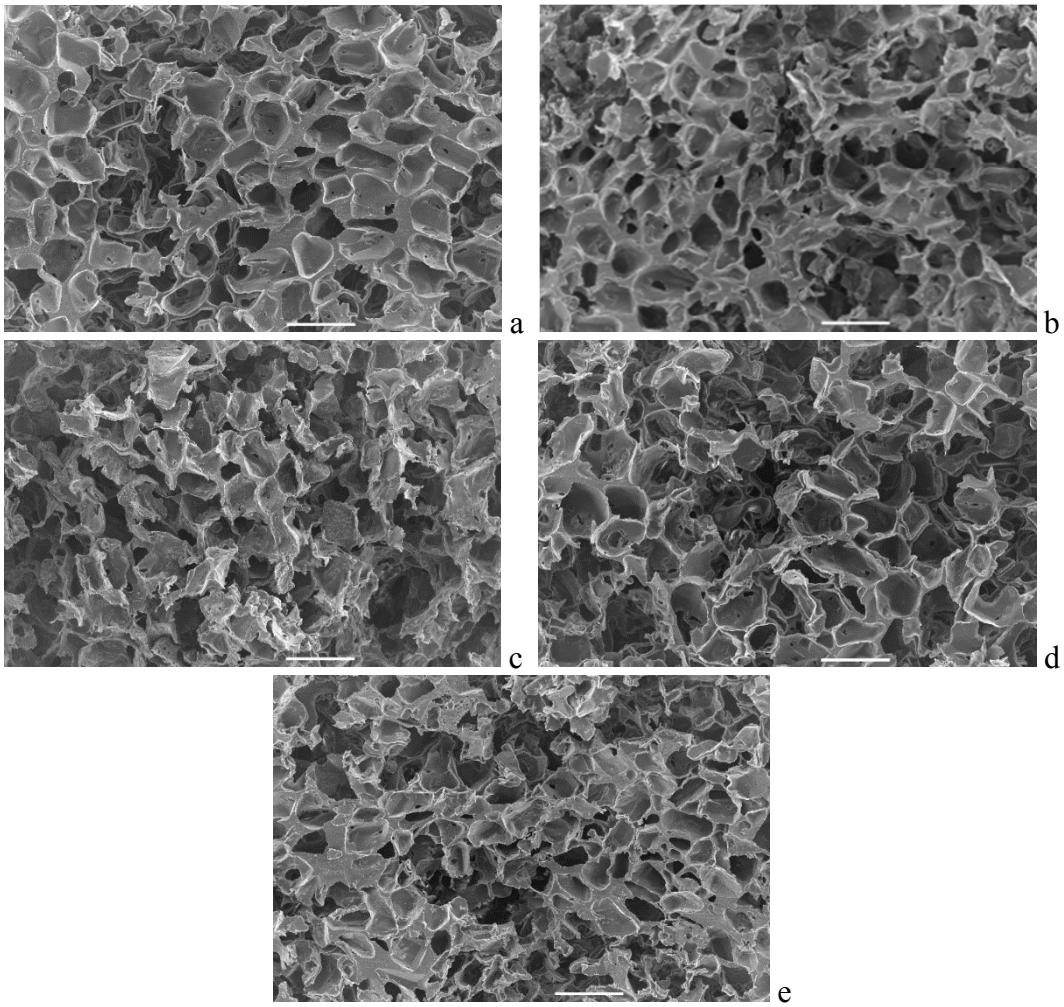

Figure 2. *SEM images of SF/TCP scaffolds: (a) SF, (b) SF/TCP-4, (c) SF/TCP-8, (d) SF/TCP-12, and (e) SF/TCP-16. Magnification: x 35 and scale bar: 500 μm.*

Mechanical properties

The compressive modulus of all scaffolds was determined in wet conditions. As shown in Table 1, the SF/TCP-8 showed the highest value, while the SF/TCP-16 presented the lowest value.

Table 1. *The pore size, calcium and phosphorus contents, and compressive modulus of SF and SF/TCP scaffolds (average ± SD).*

	Pore size (μm)	Ca (weight%)	P (weight%)	Compressive modulus (kPa)
SF	273.44 ± 58.77	0	0	57.00 ± 9.15*
SF/TCP-4	255.84 ± 54.89^{a}	0.47 ± 0.05	0.03 ± 0.06	38.21 ± 11.57
SF/TCP-8	240.93 ± 61.18^{b}	0.87 ± 0.47	0.41 ± 0.29	64.84± 16.65**
SF/TCP-12	300.71 ± 85.25^{a,b,c}	0.66 ± 0.15	0.37 ± 0.15	41.25 ± 13.77
SF/TCP-16	245.06 ± 48.69^{c}	0.79 ± 0.34	0.38 ± 0.21	14.13 ± 3.61*,**

$^{a, b, c, *, **, ***}$ Significance at $p < 0.05$ between groups.

The data demonstrated that the compressive modulus of SF/TCP-16 was significantly lower than that of SF and SF/TCP-8 ($p < 0.05$). As compared with other studies, the composite scaffolds prepared in this study showed lower compressive modulus than the study reported by Yan et al (2014) [17], Zhang et al (2010) [18], and Yan et al (2013) [19]. Moreover, the compressive modulus of the composite scaffolds prepared in this study is lower than that of natural bone which is around 20 GPa [1].

The findings suggest that the further studies should be performed to improve the mechanical properties of the SF/ α-TCP composite scaffolds. Moreover, the potential of bone formation in the critical bone defect size should be examined both in vitro and in vivo before clinical modifications for bone tissue engineering.

Conclusions

From the result of calcium and phosphorus contents, this study demonstrated that α-TCP powder can be incorporated into the silk fibroin scaffolds via the solvent casting with particulate leaching technique. The morphology and microstructure of SF/TCP scaffolds have potential for bone tissue engineering. However, an in vitro and in vivo study should be performed in further studies to evaluate the biocompatibility of the SF/TCP.

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