The 4th International Conference on Agriculture and Agro-Industry
ICAAI2022
Transforming Value Chains for Global Security
Mae Fah Luang University, Chiang Rai, Thailand
November 10 -11, 2022

Journal of Food Science and Agricultural Technology

International peer-reviewed scientific online journal Published online: http://rs.mfu.ac.th/ojs/index.php/jfat

Original Research Article

Bombyx mori (*B. mori*) silk fibroin blend scaffold as biomaterial for corneal epithelial cell

***Pattravee Thong-on*[1]*, *Kanyanat Kaewiad*[1], *Pratthana Chomchalao*[2,3], *Pattaranapa Nimtrakul*[1], *Waree Tiyaboonchai*[3]**

[1] *Expert centre of Innovative Herbal Products, Thailand Institute of Scientific and Technological Research (TISTR), Phathum Thani, 10120, Thailand*
[2] *College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani, 34190, Thailand*
[3] *Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand*

ARTICLE INFO

Article history:
Received 7 November 2022
Received in revised form 16 October 2022
Accepted 10 November 2022
Published 7 December 2022

Keywords:
Bombyx mori (B. mori) silk fibroin
Scaffold
Human corneal epithelium cell line

ABSTRACT

Bombyx mori (*B. mori*) silk fibroin (SF) is a fibrous protein that can be readily isolated from the domesticated silkworm cocoons. It has received considerable attention over the last decades as a three-dimensional (3D) biomaterial due to its outstanding mechanical performance, tunable degradability, fabrication versatility, and biocompatibility. Here, the blending of Bombyx mori (*B. mori*) SF and collagen (C) was fabricated as scaffolds using a freeze-drying technique. The effects of collagen concentration (1 and 2 %w/w) on physical properties and cell proliferation were observed. The prepared scaffolds were sponge-like structure with highly interconnected pores. Average pore size of these scaffolds ranged from 124-197 µm. The in vitro cell viability and proliferation of human corneal cell were determined by MTT assay. The scaffolds prepared from SF blend with 2% of collagen (SF/2%C) exhibited higher cell proliferation than the SF/1%C scaffold. Cell morphology was investigated by scanning electron microscopy (SEM). SEM micrographs illustrated higher cell density in the SF/2%C scaffolds than that in the SF/1%C scaffolds. These results suggested that the blended SF scaffold with a high concentration of collagen showed a high potential for biomaterial application.

INTRODUCTION

Silk fibroin (SF) is a nature polymer which is the main component of silkworm cocoon. It can be readily purified in large quantities from the cocoons of the cultivated silkworm (Bombyx mori) (Gregory et al., 2003). 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 (Waree et al., 2011). SF is a

* *Corresponding author.* Tel.: +66 2 577 9091.
E-mail address: pattravee@tistr.or.th

ISSN: 2408-1736

suitable biomaterial for both in vivo and in vitro in different formats, such as fibers, membranes, particles, hydrogels and three-dimensional (3-D) scaffolds, due to its outstanding tunable degradability, mechanical performance, fabrication versatility, and biocompatibility (Gregory et al., 2003; Chris et al., 2018). SF biomaterials can apply for tissue engineering applications aimed to deliver and recruit (endogenous) cells. Therefore, these silk constructs must be able to provide cells with the necessary physical and biological cues to achieve the desired function (Chris et al., 2018).

During the past decade, many cornea tissue engineering researches has been focused on the scaffolds with good biocompatibility, high safety and good biomechanical properties. However, the limitation of SF on the scaffold component is its bioinertness that can delay an early adhesion of cultured cells and reduce the number of growing cells (Waree et al., 2011). An addition of inductive materials which can be recognized by cells, such as gelatin (G) or collagen (C), can improve the bioactivities of the final products. Gelatin (G) is a partially hydrolyzed product of collagen which its bioactivities act as a recognitive binding site for cell-matrix interactions (Anongnart et al., 2021). Collagen (C), one of the major components of the extracellular matrix, has been reported that it could produce scaffolds with high porosity and enhance the cell attachment (Amanda R et al., 2009; Linan et al., 2013; Eun Young et al., 2015). It is a biodegradable natural protein with low antigenicity (Waree et al., 2011).

In our previous study, we successfully developed 3-D scaffolds prepared from SF, SF/C and SF/G using a freeze-drying technique. The results showed that the SF/C scaffold provided more favorable environment of corneal epithelium cell which promoted cell proliferation than SF and SF/G scaffolds. Here, the effects of collagen concentration (1 and 2 %w/w) on physical properties and human corneal cell proliferation were observed.

MATERIALS AND METHODS

Material

Degummed Bombyx mori silk yarns were purchased from Chul Thai-Silk Co., LTD, Phetchabun, Thailand. Bovine collagen was purchased from Sigma Chamical (St. Louis, MO, USA). Keratinocyte serum-free medium (K-SFM), bovine pituitary extract, recombinant human epidermal growth factor (EGF), and Gibco antibiotic-antimycotic (100X) were purchased from Invitrogen (California, USA). Hydrocortisone solution, human insulin solution, bovine serum albumin (BSA), bovine collagen type I, and human fibronectin were obtained from Sigma-Aldrich (Steinheim, Germany).

Silk fibroin blend scaffolds fabrication

Bombyx mori silk fibroin was prepared as described by Tiyaboonchai et al., but with some modifications (Waree et al., 2011). Degummed *Bombyx mori* silk yarns was heated at 85-90 °C in a mixed solution of $CaCl_2$:H_2O:EtOH at 1:8:2 mole ration until the solution became gel-like. Then, the silk fibroin solution was dialyzed in DI water (10L) for 73-120 h within the snakeskin dialysis tube (10,000 MWCO, Thermo Fisher Scientific, USA) to remove the salts. Then, the silk fibroin gel was kept at −20°C overnight prior to freeze-dried (FreeZone Plus6, Labconco, USA) and kept in sealed plastic bag at controlled humidity until used.

Three dimensional scaffolds of 2%SF/1%C and 2%SF/2%C were prepared using a freeze-drying technique as described by Tiyaboonchai et al., but with some modifications (Waree et al., 2011). SF solutions were prepared from 2% w/v SF aqueous solution. The 1 and 2 % w/v C solution was prepared by dissolving collagen in 5% v/v acetic acid at 4°C and left overnight before use. Then, the SF and C solutions were mixed at the volume ratio of 75:25 with mild stirring for 20 min. Finally, the resulting solutions, 2%SF/1%C and 2%SF/2%C, were transferred into 12 well plate and kept at −20°C overnight prior to lyophilization. Scaffolds were punched by skin biopsy punch to attain circular- shaped scaffolds with 6 mm diameter and 2 mm thickness. Then the scaffolds were treated with methanol for 30 min and evaporate at room temperature. The sterilization of the scaffolds was conducted by autoclaving method, the scaffolds were heated to 120 ◦C (at a pressure of 0.11 MPa for 20 min) in ion-exchanged water using an autoclave (TOMY, Tokyo, Japan).

Physical characterization of the silk fibroin blend scaffolds

The morphology of porous 3-D scaffolds was investigated using a scanning electron microscopy and energy dispersive X-ray spectrometer (SEM-EDS, JSM-IT300LV, JEOL Ltd., Tokyo, Japan). The mean pore diameter of the scaffolds was determined by randomly measuring at least 30 pores from the SEM-EDS micrographs using an image analysis program called "ImageJ" (Java image processing program, downloaded from http://rsb.info.nih.gov/ij/index.html). The porosity of the prepared scaffolds was determined using liquid displacement method (Waree et al., 2011). To determine the swelling property, the scaffolds were immersed in distilled water for 24 h and the percentage water uptake was calculated from wet and dry weight of these scaffolds (Anongnart et al., 2021). Degradability of the scaffolds was conducted in cell culture medium to mimic cell culture conditions. The samples (average weight = 5.0 ± 0.1 mg, n = 3) were incubated at 37 ◦C in humidified air with 5% CO_2, before collecting, washing with DI water and freeze-drying. Subsequently, the weight of the remaining samples was measured and compared to their initial weight to calculate the weight loss percentage.

Corneal epithelium cell culture in the silk fibroin blend scaffolds

Human corneal epithelial cells (HCE-2) were purchased from ATCC (VA, USA). Until seeding, HCEs were cultured in keratinocyte serum-free medium (KSFM, Gibco, USA) with 50 µg/mL bovine pituitary extract (BPE), 5 ng/mL EGF, 0.005 mg/mL insulin and 1% penicillin/streptomycin, according to manufacturer's specifications. The coating solution was prepared from 0.01 mg/mL fibronectin, 0.03 mg/mL collagen and 0.01 mg/mL bovine serum albumin (BSA) for coating 96-well plate. All cells were cultured at 37°C with 5% CO_2 in humid conditions. A single cell suspension at a density of 2×10^4 cells/well were seeded on 2%SF/1%C and 2%SF/2%C scaffolds and cultured for 7 days. Culture media was changed every 2 days.

Cell viability and proliferation studies

The proliferation activity of the cells was quantitatively determined at 0, 3, 5, and 7 days by using a MTT assay. Briefly,

each well contained the cells to be tested with a cultured medium or rinsing solution removed. Then, 100 µL MTT solution (1 mg/mL in serum-free medium) was added to each well and the plates were incubated at 37 °C for 3 h. During the incubation, the active enzymes of the viable cells transformed the yellow MTT into purple formazan crystals. The top medium was then removed, and isopropanol was added to each well to dissolve the formazan crystals. The optical density (OD) value of absorbance at 570 nm was measured by a microplate reader (Tecan, Switzerland).

SEM examination of cell seeded scaffolds

After 7 days in culture, the scaffolds with attached cells were rinsed twice with 0.01 PBS (pH 7.4), fixed with 3% glutaraldehyde in PBS for 3 h and then rinsed twice with PBS for 10 min. The samples were dehydrated through graded ethanol solutions and air-dried overnight at room temperature. The scaffolds were then mounted on aluminum stubs, vacuum sputter-coated with gold-palladium and examined under a scanning electron microscopy and energy dispersive X-ray spectrometer (SEM-EDS, JSM-IT300LV, JEOL Ltd., Tokyo, Japan).

Statistical analysis

All data were expressed as mean ± standard error (Mean ± SE) of triplicated determination. The significance of difference was used to compare mean ($p<0.05$). One-way ANOVA followed by Tukey's Honestly Significant Difference were tested in all experiments.

RESULTS AND DISCUSSION

Physical characterization of the silk fibroin blend scaffolds

The 3-D scaffolds prepared from 2%SF/1%C and 2%SF/2%C provided a sponge-like structure with highly interconnected pores as shown in Figure 1. Both scaffolds, exhibited a thicker pore wall than SF scaffold. Mean pore size, porosity and water uptake of different scaffolds are presented in Table 1. Mean pore size of 2%SF/1%C and 2%SF/2%C were 124.37 ± 20.74 and 100.87 ± 7.71 µm, respectively. Both scaffolds showed high water uptake of ~ 90 %. Degradability of the scaffolds was conducted in the cell culture medium using the same conditions as for cell culture. The scaffolds retained their weight for 14 days which suggested that the scaffolds were stable in cell culture medium up to 14 days (data not shown). The sterilization technique is one of critical effect on the physical and biological properties of the scaffold (Tomoko et al., 2020). In this study, the scaffolds were autoclaved at 120 ◦C for 20 min. Uniform porous structures were observed in after autoclaving, indicating that sterilization by autoclaving technique did not result in a morphological.

In vitro cell viability and proliferation studies

Cell proliferation of SF/1%C and SF/2%C scaffolds in co-culture with human corneal epithelial cells (HCE-2) was measured using MTT assay, Figure 2. The obtained percentage of proliferation indicates the metabolic activity of proliferating cells. The percentage of proliferation in SF/1%C was lower than SF/2%C scaffolds. For SF/1%C scaffold, the percentage of HCE-2 proliferation was 113%, 148% and 179% at 3, 5 and 7 days, respectively. And there were significant differences in the proliferation of HCE-2 at each day activity was measured. The HCE-2 proliferation (%) of SF/2%C scaffold showed 131%, 165% and 204% at 3, 5 and 7 days. The SF/2%C scaffold showed high proliferation activity (204%) at day 7. Our previous study reported that the blended scaffolds with collagen or gelatin provided a more favorable environment for HEC-2 attachment and proliferation than that of SF scaffold and tissue culture plastic. As suggested by Harkin et al., attachment of human corneal epithelial cell line to silk fibroin film in the presence of serum is comparable to that seen on denuded amniotic membrane, a popular choice of substrate for transplanting cultured epithelial cells to the ocular surface (Damien G et al., 2011). There were significant differences in the proliferation of HCE-2 between the SF/1%C and SF/2%C scaffolds. The SF/2%C scaffold showed higher proliferation activity than SF/1%C scaffold in HCE-2. Results from MTT assay indicated that the scaffolds containing 2% w/v collagen (SF/2%C) provided a more favorable environment for HECs attachment and proliferation than that of lower percentage collagen blended within scaffold (scaffolds containing 1% w/v collagen, SF/1%C). Previous study reported that autoclaved scaffolds promoted faster proliferation than other sterilized scaffolds such as exposure to gamma or UV radiation (Jelena et al., 2015). These can be suggested that the autoclaving method did not result in a biological property, this could be promoted cell proliferation of HEC-2 for 1 to 7 days after seeding (Tomoko et al., 2020; Jelena et al., 2015).

Table 1. Mean pore size, porosity and water uptake of silk fibroin and silk fibroin blend scaffolds

Formulation	Mean pore size (µm) ± SD	Porosity (%) ± SD	Water uptake (%) ± SD
2%SF/1%C	124.37 ± 20.74	71.95 ± 0.72	86.81 ± 0.31
2%SF/2%C	100.87 ± 7.71	74.15 ± 1.64	88.45 ± 0.97

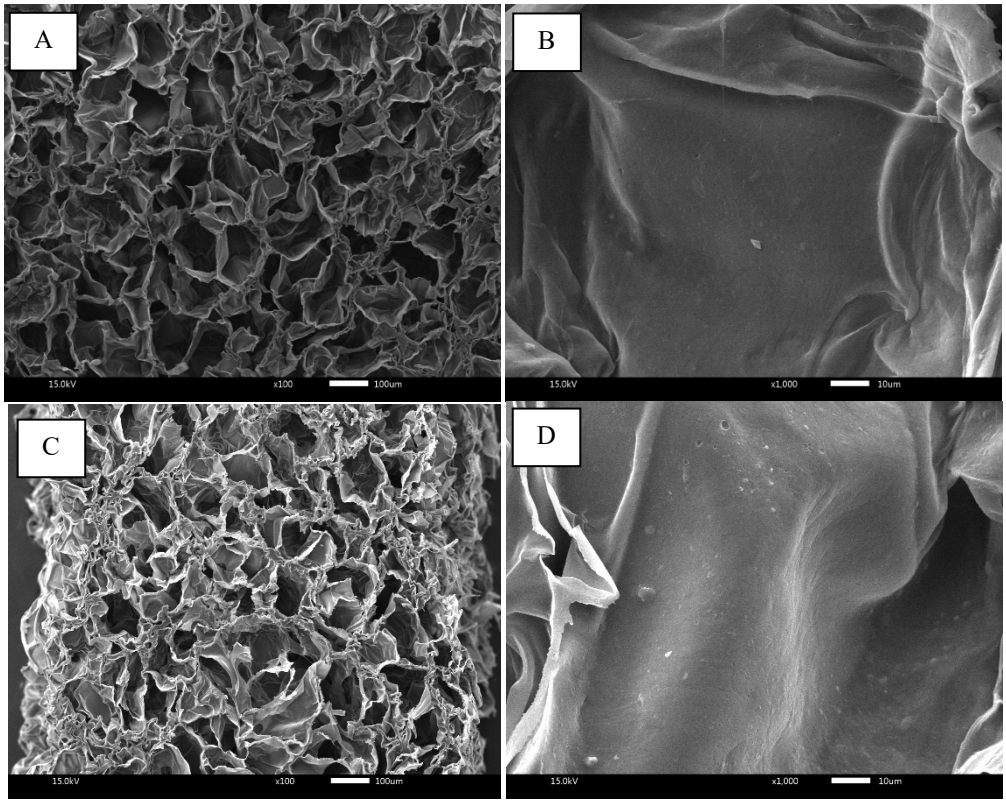

Figure 1. SEM-EDS micrographs of scaffolds after freeze-drying, methanol treatment, and autoclaved; (A) 2%SF/1%C, (B) 2%SF/2%C at a magnification of 100X; (C) 2%SF/1%C, (D) 2%SF/2%C at a magnification of 1000X. Scale bar = 100 µm.

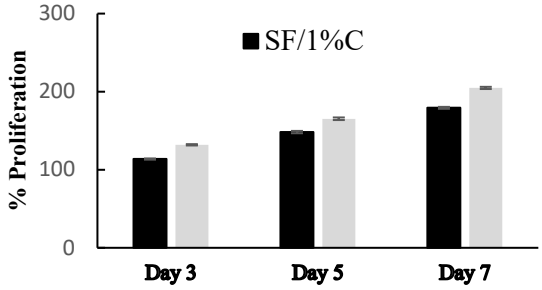

Figure 2. Proliferation activity (%) of SF/1%C and SF/2%C scaffolds in co-culture with HCE-2 at 3, 5 and 7 days.

Cell morphology and growth in the silk fibroin blend scaffolds

The morphology and distribution of HEC-2 on the surface and in the inner zone of the scaffolds after cultivation for 7 days were illustrated in Figure 3. Similarly with the proliferation study, the 2%SF/2%C scaffold was more highly populated with HEC-2 cells than the 2%SF/1%C scaffold in both of surface and inner zone. As suggested with previous study, collagen is the main structural protein of most hard and soft tissues in animals and the human body, which plays an important role in maintaining the biological and structural integrity of the ECM and provides physical support to tissues (Tomoko et al., 2020; Chanjuan et al., 2016). Moreover, cell migration is known to heavily depend on the structure and properties of the extracellular matrix (ECM) (Tomoko et al., 2020). These results suggested that high concentration of collagen type I in the blended scaffold induced higher cellular distributing and growth.

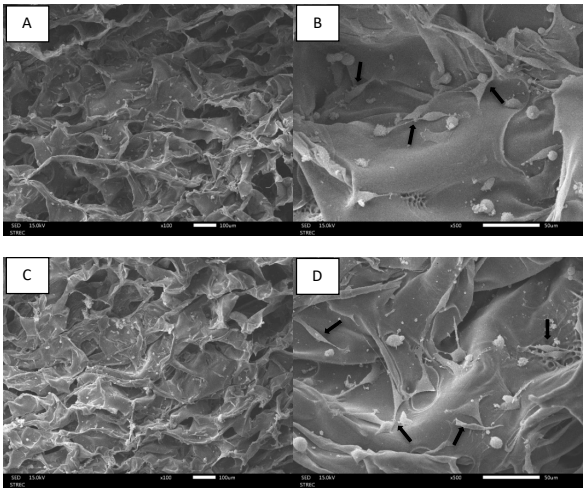

Figure 3. SEM-EDS micrographs of scaffolds cultured with HEC-2 cells for 7 days; (A) 2%SF/1%C, (C) 2%SF/2%C at a magnification of 100X; (B) 2%SF/1%C, (D) 2%SF/2%C at a magnification of 500X. Black arrows indicate human corneal epithelial cells.

CONCLUSIONS

The influences of collagen concentration (1 and 2 %w/w) on physical properties and cell proliferation of the silk fibroin blend scaffolds were investigated. There was no significant difference

between formulations in the physical properties. However, there were significant differences in the proliferation of HCE-2 between the SF/1%C and SF/2%C scaffolds. These results suggested that the scaffold with a high concentration of collagen showed higher HEC-2 proliferation activity than low concentration of collagen. In addition, high concentration of collagen in the blended scaffold induced higher cellular distributing and growth on the surface and inner zone. This study demonstrated that the optimal formulation of scaffolds exhibits a higher potential for enhancing cell mobility and the production of extracellular matrix than do fibroin or collagen alone, resulting in more favorable environment for corneal epithelial cell proliferation.

ACKNOWLEDGEMENTS

This study was a part of the research project funded by the Ministry of Higher Education, Science, Research and Innovation (MHESI) through the Thailand Institute of Scientific and Technological Research (TISTR).

REFERENCES

Altman G, Diaz F, Jakuba CM, Calabro T, Horan R, Chen J, et al. 2003. Silk-base biomaterials. Biomaterials 24: 401-416.

Dong C, Lv Y. 2016. Application of Collagen Scaffold in Tissue Engineering: Recent Advances and New Perspectives. Polymers (Basel) 8(2):42: doi: 10.3390/polym8020042

Duangpakdee, A., Laomeephol, C., Jindatip, D., Thongnuek, P., Ratanavaraporn, J.& Damrongsakkul, S. 2021. Crosslinked Silk Fibroin/Gelatin/Hyaluronan Blends as Scaffolds for Cell-Based Tissue Engineering. Molecules 26: 3191.

Guan L, Ge HY, Tang XL. 2013. Use of a Silk Fibroin-Chitosan Scaffold to Construct a Tissue-Engineered Corneal Stroma. Cells Tissues Organs 198: 190-197.

Harkin DG, George KA, Madden PW, et al. 2011. Silk fibroin in ocular tissue reconstruction. Biomaterials 32: 2445-2458: doi:10.1016/j.biomaterials.2010.12.041

Hashimoto T, Nakamura Y, Tamada Y, et al. 2020. The influence of thermal treatments on the secondary structure of silk fibroin scaffolds and their interaction with fibroblast. PeerJ Materials Science 2:e8 http://doi.org/10.7717/peerjmatsci.8

Holland C, Numata K, Rnjak-Kovacina J, Seib FP. 2018. The Biomedical Use of Silk: Past, Present, Future. Advanced Healthcare Materials. Material 8(1):1800465.

Kim EY, Tripathy N, Cho SA. 2015. Bioengineered neo-corneal endothelium using collagen type-I coated silk fibroin film. Colloids and Surfaces B: Biointerfaces 136: 394- 401.

Murphy AR, Kaplan DL. 2009. Biomedical applications of chemically-modified silk fibroin. Journal of Materials Chemistry 19: 6443-6450.

Rnjak-Kovacina J, DesRochers TM, Burke KA, Kaplan DL. 2015. The effect of sterilization on silk fibroin biomaterial properties. Macromolecular Bioscience 15:861–87: DOI 10.1002/mabi.201500013.

Tiyaboonchai W, Chomchalao P, Pongcharoen S, Sutheerawattananonda M, Sobhon P. 2011. Preparation and characterization of blended Bombyx mori silk fibroin scaffolds. Fibers and Polymer 12(3): 324-333.