Preparation and characterization of silk fibroin from four different species of Thai-local silk cocoon for Bone implanted applications

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Abstract. The metal interlocking nail is normally used in diaphyseal fractures. The bio-composite interlocking nail will be fabricated in the near future by using local silk fibroin reinforce Polylactic acid filament for 3D printing. Four species of local Thai Bombyx mori silk cocoons were selected to extract silk fibroin. The silk cocoon species consist of 1) Nangnoi Srisaket-I (NN), 2) Nanglai (NL), 3) Luang Saraburi (LS), and 4) J108. After the extraction, fibroins of each silk cocoon species were characterized and compared the physical property by using Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDS) and Fourier Transform Infrared Spectroscopy (FT-IR). Then, the biological test was performed on cell viability and cytotoxicity with human fetal osteoblast cell line. The result presents that all of local silk cocoons species presented non-cytotoxicity ability which can be used in human or animal body without endangerment.

1. Introduction

Diaphyseal fractures are common orthopedic injuries which can be treat by using interlocking nails. The developments of interlocking nails were performed to increase the treatment abilities especially in femur, tibia, and humerus fractures \cite{1}. However, the removal of interlocking nails is still required due to the complications in long-term implantation \cite{2}. According to the materials of interlocking nails, titanium and stainless steel did not provided biological environment to the living cells. This research looking for the biomaterials which can eliminated the limitations of metal interlocking nails. Currently, the use of three-dimensional printing (3DP) in biomedical technology is dramatically increased \cite{3}. According to the rapidly process of 3DP, there were several studies of bone implant applications using 3DP for emergency cases \cite{4, 5}. Fused filament fabrication (FFF) technique is the most satisfied 3DP technique. Thermoplastic filament will be feed through the heating nozzle. The melted filament will be printed layer by layer until the end. Material costs, production speed, and design flexibility are the advantage of FFF technique. There were several types of biomaterials which can be printed by using
FFF technique. Thus, biopolymer such as Polylactic acid (PLA) will be used as a main structure for 3DP. In bone tissue engineering (TE), the choice of appropriate biomaterials is one of particular challenge to mimic mechanical and biological characteristics of the native tissue [6]. Several types of biomaterials were tested and implanted to the animal models to confirm the materials abilities for bone TE [7]. Silk products have widely been used as an implantation biomaterial such as silk sutured, silk proteins for wound healing or TE application [8, 9]. Fibroin is a well-known biomaterial which could be extracted from silk cocoon, spider web and red ant nests [10]. It could be fabricated into a structure or combined with other biomaterials to increase the biological and mechanical properties [11]. Previous studies have proved that fibroin has good biological and mechanical properties such as biocompatibility, biodegradability, water permeability, non-cytotoxicity and the strength and resiliency of silk fibers [12-14]. Silk fibers has an ultimate tensile strength 740 MPa while collagen and polylactic acid has an ultimate tensile strength only 0.9 to 7.4 and 28 to 50 MPa, respectively [15]. Therefore, the silk fibroin will be used as a reinforcing material for PLA to create bio-composite filament for interlocking nail 3DP.

In this study, four species of local silk cocoon were provided by the Queen Sirikit Department of Sericulture (QSDS) in Chiang Mai. After silk fibroin was extracted from each species, the characterization will be performed and compared the biological ability by using cell viability test of osteoblast-like cells. The most effective silk cocoon specie will be used to reinforce PLA for 3DP in the future study.

2. Materials and methods

2.1. Silk Fibroin Preparation

Four species of local silk cocoons consist of Nangnoi Srisaket-I (NN), Nanglai (NL), Luang Saraburi (LS) and J108 (Figure 1) were selected for silk fibroin extraction. The silk cocoons were cut into small pieces. Then, the cut silk cocoons were degummed in 100 ºC of Na₂CO₃ solution for 30 minutes and rinsed the degummed silks by 80 -100 ºC of DI water and repeated this process. The degummed silks were dried in 37 ºC hot air oven for 24 hours. Dried degummed silk was dissolved in 70 ºC of ternary solvent (CaCl₂/CH₃CH₂OH/H₂O, in mole ratio 1:2:8) for 6 hours. The silk solution was packed in dialysis tube and soaked in 4 ºC of DI water for 3 days (change the DI water every day). The dialyzed silk solution was filtered and frozen at -80 ºC freezer. Finally, the frozen silk solution was lyophilized to generated silk fibroin sponges as shown in Figure 2 [16-18].

![Figure 1. Different species of 4th silk cocoons in Chiang Mai, Thailand (A) NN (B) NL (C) LS and (D) J108.](image)

![Figure 2. Schematic of the silk fibroin extraction procedure.](image)
2.2. Fourier-transform infrared spectroscopy (FTIR)
FTIR spectra of lyophilized fibroin sample from each silk species were obtained from a Thermo Fisher FTIR spectrometer equipped with an ATR (attenuated total reflectance) accessory. Sixty-four repetitive scans from 800-2500 centimetre$^{-1}$ were averaged and presented.

2.3. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectrometer (EDS)
The structure of lyophilized fibroin from each silk species was observed under a JSM -IT300 scanning electron microscope. The surface picture will be captured and Energy Dispersive X-ray Spectrometer (EDS) was performed.

2.4. Fibroin solution preparation
Prior cell viability and cytotoxicity testing, lyophilized fibroin from each silk species were dissolved in DMEM/Ham’s F-12 medium (0.5 g/10 ml) and mixed well.

2.5. Cell viability and cytotoxicity
Human fetal osteoblast cell line (hFOB1.19, CRL NO.11372) was purchased from ATCC and expanded in a 1:1 mixture of phenol red-free DMEM/Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg /ml streptomycin (basal media). The culture cells were incubated in 37 º C with 5% CO$_2$ until reach the requirement number. The hFOB1.19 cells were seeded in 96-well plates at a density of 5 x 10$^3$ cells per well. After 24 hours incubation, the cells were cultured with 100 μl of each silk species fibroin solution for another 24 hours. At indicated time of treatment [19], Alam blue dye (10% v/v in PBS) was added to each well and incubated at 37 degree Celsius for 4 hours before the fluorescence measured at excitation 530 nanometres and emission 590 nanometres. The percentage of cell viability was calculated by the following equation 1.

\[
\text{% Cell viability} = \frac{OD \text{ of treated cell}}{OD \text{ of control cell}} \times 100
\]  

2.6. Statistical Analysis
All data were showed mean ±SD. P-value < 0.05 was measured as statistically significant. A paired t-test with two-tailed distribution was used to determine the different between control group and each silk species group.

3. Results and Discussion

3.1. Silk fibroin characterization
FTIR spectroscopy was used to investigate the conformations of fibroin protein in regenerated each silk scaffolds. The FTIR spectra of the regenerated each silk fibroin species shown at Figure 3. For C=O stretching (amide I) at 1630 to 1650 centimetres$^{-1}$, For secondary NH bending (amide II) at 1540 to 1520 centimetres$^{-1}$, and for C-N stretching (amide III) at 1270 to 1230 centimetres$^{-1}$ in their FTIR spectra show characteristic vibrational bands of protein materials [15].Furthermore, the conformations of the protein materials were indicated by the positions of these bands; Beta-sheet at 1630 centimetres$^{-1}$ and random coil at 1650 centimetres$^{-1}$ for amide I, Beta-sheet at 1520 centimetres$^{-1}$ and random coil at 1540 centimetres$^{-1}$ for amide II, Beta-sheet at 1270 centimetres$^{-1}$ and random coil at 1230 centimetres$^{-1}$ for amide III. The four species of silk scaffolds show typical Beta-sheet structures and random coil structures respectively. These band shifts occur because of the distinct hydrogen bonding states produced by different conformations adopted by the protein chains.

3.2. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectrometer (EDS)
The silk fibroin structures were observed under SEM. Structure of all silk fibroin species provided interconnect pore. The pore size of each silk species was not significantly different as shown in Figure 2A-2D. The average of each silk scaffolds pore size was about 10 to 60 microns. Each silk species scaffolds were indicated by the area EDS taken at the centre, and its consistent EDS graph is presented in the figure. As shown in Figure 4, silk fibroin weight percentage consist of Carbon (C), Nitrogen (N)
and Oxygen (O) element only which symbolize the proteinaceous compounds originating from Silk Bombyx mori.

![FTIR spectra of four types local Thai silk fibroin scaffolds.](image)

**Figure 3.** FTIR spectra of four types local Thai silk fibroin scaffolds.

### 3.3. Cell viability and cytotoxicity test using Alamar blue assay.

After 24 hours of cell culture in 100 µl of each silk species fibroin solution, the Alamar blue assay was performed on the set to observe percent cell viability and confirm non-toxicity ability of silk fibroin solution. The comparison of each silk species with control presented that cell viability percentage all scaffolds were not significantly the control (p-value > 0.05) as shown in Figure 5 and Table 1. Thus, all species of silk fibroin were non-cytotoxic materials.

|           | Mean (%) | SD    | P-value (vs. control) |
|-----------|----------|-------|-----------------------|
| Control   | 100.00   | 1.11  | -                     |
| NS        | 97.69    | 14.83 | 0.730                 |
| NL        | 106.73   | 8.26  | 0.168                 |
| LS        | 111.12   | 7.26  | 0.111                 |
| J108      | 110.41   | 3.62  | 0.051                 |

**Table 1:** Cell viability percentage
Figure 4. SEM images and Field emission scanning microscopy equipped with EDS results. (A) NN (B) NL (C) LS and (D) J108 silk fibroin.

Figure 5. Cell viability percentage of each silk species in 24 hours.
4. Conclusion
According to the purpose of the end study, silk fibroin will be used to reinforce PLA interlocking nails which will be fabricated by 3DP process [15]. Silk fibroin should provide good biological environment to the fracture area to accelerate bone healing process by providing good surface for cell adhesion, migration, and proliferation [20]. Furthermore, silk fibroin and PLA can be degraded by biologically enzyme [18]. So, the 3DP silk fibroin reinforcing PLA interlocking nails will eliminate the removal process and provided good biological environment for bone regeneration. Therefore, four species of local silk cocoon which provided by the QSDS were used as raw materials to extract silk fibroin and performed biological test to compared and confirmed the biological property of each silk species. The best silk species from biological performance will be used to reinforce PLA interlocking nails using 3DP process in the future study. From the result, all of local silk cocoons species present non-cytotoxicity ability which can be used in human or animal body without endangerment. For future work, bio-composite filament for 3DP from silk fibroin reinforcing PLA will be tested and observed the other abilities such as cell proliferation ability, mechanical properties and printing morphology.

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6. References
[1] Brumback, R. J. (1996). The rationales of interlocking nailing of the femur, tibia, and humerus: an overview. Clinical Orthopaedics and Related Research®, 324, 292-320.
[2] Seyhan, M., Guler, O., Mahirogullari, M., Donmez, F., Gereli, A., & Mutlu, S. (2018). Complications during removal of stainless steel versus titanium nails used for intramedullary nailing of diaphyseal fractures of the tibia. Annals of medicine and surgery, 26, 38-42.
[3] Ventola, C. L. (2014). Medical applications for 3D printing: current and projected uses. Pharmacy and Therapeutics, 39(10), 704.
[4] Tai, B. L., Kao, Y. T., Payne, N., Zheng, Y., Chen, L., & Shih, A. J. (2018). 3D Printed composite for simulating thermal and mechanical responses of the cortical bone in orthopaedic surgery. Medical engineering & physics.
[5] Barba, A., Maazouz, Y., Diez-Escudero, A., Rappe, K., Espanol, M., Montufar, E. B., ... & Franch, J. (2018). Osteogenesis by foamed and 3D-printed nanostructured calcium phosphate scaffolds: effect of pore architecture. Acta Biomaterialia.
[6] Hutmacher, D. W. (2006). Scaffolds in tissue engineering bone and cartilage. In the Biomaterials: Silver Jubilee Compendium (pp. 175-189).
[7] Pearce, A. I., Richards, R. G., Milz, S., Schneider, E., & Pearce, S. G. (2007). Animal models for implant biomaterial research in bone: a review. Eur Cell Mater, 13(1), 1-10.
[8] Nazarov, R., Jin, H. J., & Kaplan, D. L. (2004). Porous 3-D scaffolds from regenerated silk fibroin. Biomacromolecules, 5(3), 718-726.
[9] Kundu, B., Rajkhowa, R., Kundu, S. C., & Wang, X. (2013). Silk fibroin biomaterials for tissue regenerations. Advanced drug delivery reviews, 65(4), 457-470.
[10] Kamhaengpol, A., Maensiri, S., & Siri, S. (2010). Fibroin protein extract from red ant nests for a production of electrospun nanofibers. Asia-Pacific Journal of Science and Technology, 15(10), 919-929.
[11] Tamada, Y. (2005). New process to form a silk fibroin porous 3-D structure. Biomacromolecules, 6(6), 3100-3106.
[12] Xie, M. B., Li, Y., Li, J. S., Chen, A. Z., Zhao, Z., & Li, G. (2014). Biomedical applications of silk fibroin.
[13] Murphy, A. R., & Kaplan, D. L. (2009). Biomedical applications of chemically-modified silk fibroin. Journal of materials chemistry, 19(36), 6443-6450.
[14] Liu, T. L., Miao, J. C., Sheng, W. H., Xie, Y. F., Huang, Q., Shan, Y. B., & Yang, J. C. (2010). Cytocompatibility of regenerated silk fibroin film: a medical biomaterial applicable to wound healing. Journal of Zhejiang University SCIENCE B, 11(1), 10-16.
[15] Koh, L. D., Cheng, Y., Teng, C. P., Khin, Y. W., Loh, X. J., Tee, S. Y., ... & Han, M. Y. (2015). Structures, mechanical properties and applications of silk fibroin materials. Progress in Polymer Science, 46, 86-110.
[16] Rockwood, D. N., Preda, R. C., Yücel, T., Wang, X., Lovett, M. L., & Kaplan, D. L. (2011). Materials fabrication from Bombyx mori silk fibroin. Nature protocols, 6(10), 1612.
[17] Wattanutchariya, W., & Thunsiri, K. (2015). Effects of Fibroin Treatments on Physical and Biological Properties of Chitosan/Hydroxyapatite/Fibroin Bone's Scaffold. In Applied Mechanics and Materials (Vol. 799, pp. 488-492). Trans Tech Publications.
[18] Thunsiri, K., Oonjai, A., & Wattanutchariya, W. (2018). Characterization of Hydroxyapatite/Silk Fibroin/Chitosan Scaffold for Cartilage Tissue Engineering. In Key Engineering Materials (Vol. 775, pp. 120-126). Trans Tech Publications.
[19] Keogh, M. B., O’Brien, F. J., & Daly, J. S. (2010). A novel collagen scaffold supports human osteogenesis—applications for bone tissue engineering. Cell and tissue research, 340(1), 169-177.
[20] Qi, Y., Wang, H., Wei, K., Yang, Y., Zheng, R. Y., Kim, I. S., & Zhang, K. Q. (2017). A review of structure construction of silk fibroin biomaterials from single structures to multi-level structures. International journal of molecular sciences, 18(3), 237.