Use of *Bombyx mori* and Eri Silk Fibroin Blended Films in Fibroblast Cell Culture

การใช้แผ่นฟิล์มที่ทำจากส่วนผสมไฟโบรอินของไหมบ้านกับไหมอีรี่ในการเลี้ยงเซลล์ไฟโบรบลาสต์

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ABSTRACT

The purpose of this study was to prepare film materials from the blend mixtures of *Bombyx mori* and Eri silk fibroin for fibroblast cell culture. Fibroin from either source was dissolved in lithium bromide, freeze-dried into powder and redissolved in water. Four concentrations of Eri silk fibroin solution, including 0.05, 0.1, 0.2 and 0.4%, were mixed with *B. mori* fibroin solution at a fixed concentration of 3 %. Each fibroin mixture was used to form film for the culture of fibroblast cells (NIH 3T3). The constituent of Eri silk fibroin caused the film surface non-smooth. Cell growth was optimal on the film with 0.1% Eri silk fibroin blend, which was also superior to the growth on pure *B. mori* fibroin film. In addition, the presence of Eri silk fibroin in films helped protection of the oxidative cytotoxicity from hydrogen peroxide.

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์ในการศึกษาการใช้แผ่นฟิล์มที่ผสมระหว่างโปรตีนไฟโบรอินจากไหมบ้านและไหมอีรี่ในการเลี้ยงเซลล์ไฟโบรบลาสต์ โดยเริ่มจากการละลายโปรตีนไฟโบรอินจากไหมทั้งสองชนิดด้วยสารละลายลิเทียมโบรไมด์แล้วทำให้เป็นผงโปรตีนด้วยวิธีการทำแห้งแบบแช่เยือกแข็ง ละลายผงโปรตีนไฟโบรอินในน้ำให้มีไหมอีรี่ความเข้มข้น 0.05, 0.1, 0.2 และ 0.4 % ตามลำดับ ผสมกับไฟโบรอินไหมบ้านที่ความเข้มข้น 3 % นำสารละลายโปรตีนผสมทำเป็นแผ่นฟิล์มแล้วใช้เลี้ยงเซลล์ไฟโบรบลาสต์ (NIH 3T3) พบว่า ไฟโบรอินไหมอีรี่ทำให้แผ่นฟิล์มผสมมีลักษณะพื้นผิวหยาบ เซลล์เจริญได้ดีที่สุดเมื่อใช้แผ่นฟิล์มผสมไฟโบรอินไหมอีรี่ 0.1% และดีกว่าการเจริญของเซลล์บนแผ่นฟิล์มไฟโบรอินไหมบ้าน ส่วมผสมของไฟโบรอินไหมอีรี่ในฟิล์มช่วยป้องกันผลกระทบออกซิเดชันจากไฮโดรเจนเปอร์ออกไซด์ต่อเซลล์

Keywords: *Bombyx mori,* Eri, Fibroin film

คำสำคัญ: ไหมบ้าน ไหมอีรี่ แผ่นฟิล์มไฟโบรอิน

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Introduction

Silk is composed of two protein components which are fibroin and sericin. The silk fibroin (SF) is natively a water insoluble fibrous protein underlying the fiber structure of silk, while sericin is a group of amorphous adhesive proteins gumming and coating around the layers of fibroin (Mondal et al., 2007). Apart from being garment fabric, SF has been explored for various applications, such as food industries (Keiko, Michiko, 2004; Hu et al., 2008), cosmetics (Jayawardane et al., 2016), and biomedicine (Deptuch, Dams-Kozlowska, 2017). SF has several advantages that support its biomedical application including good oxygen and water vapor permeability, excellent biocompatibility (non-cytotoxicity, low antigenicity and non-inflammatory), and biodegradability at slow rate (Min et al., 2004; Qi et al., 2017). Diverse biomaterials have been established from SF of household silkworm (*Bombyx mori*), dependent on different purposes (Rockwood et al., 2011). Among those biomaterials, SF film has been recently published for its success in promoting wound healing at preclinical tests in animals (rabbits and pigs) and clinical studies (Zhang et al., 2017). The concept to improve SF film is to find additives that bring about novel properties. For an example, the property of SF film in stimulating cell proliferation was enhanced by the addition of collagen (Hu et al., 2006).

Eri (*Philosamia ricini* Boisd. renamed as *Samia ricini* Donovan) is natively a wild silk moth but, nowadays, it can be reared by feeding on a wide range of food plants including castor and cassava (Singh, Benchamin, 2002). Eri fibroin derived electrospun nanofiber scaffold was superior to the *B. mori* fibroin scaffold in cell culture tests for cell attachment, cell viability and cell spreading, and it is less susceptible to microorganism (Andiappan, Sundaramoorthy, 2015). Therefore, Eri fibroin is attractive for using as an artificial substratum for cell growth in wound healing. The limitation is that Eri silk fiber exhibits poor solubility. In this study, Eri fibroin was consequently designed as an additive in the preparation of film materials from *B. mori* fibroin. The SF films thereof were investigated in the culture of mouse fibroblast NIH 3T3 cells whether the films could enhance cell proliferative activity and protect cells from oxidation by hydrogen peroxide (H_2O_2). The results would be informative for applying the films as wound dressings.

Objectives of the study

SF films would be made by the optimal constituents of Eri SF and *B. mori* SF, and evaluated for the ability to support growth of mouse fibroblast NIH 3T3cells and anti-oxidation against H_2O_2.

Materials and methods

Preparation of SF

The hot-air dried Eri or *B. mori* cocoon shells were cut into small pieces, treated with 0.25% Na_2CO_3 at 100°C for 90 min to get rid of sericin (Nuchadomrong et al., 2008), and finally rinsed 3 times with hot distilled water. The so-obtained SF fiber was made dry at 80°C and stored in a dessicator at room temperature.

The method of using 9 M lithium bromide solution was as previously described to dissolve SF fiber (Senakoon, 2009). The fibroin solution was excessively dialyzed with several changes of distilled water at room temperature for 48 h. The dialysate was freeze-dried into a powder and dissolved in water to obtain fibroin solution for preparation of SF films.

Preparation of SF film

SF films were prepared by the combination of *B. mori* fibroin and Eri fibroin. The *B. mori* fibroin powder was added to make a concentration of 3% in four different concentrations of Eri fibroin solution which were 0.05, 0.1, 0.2 and 0.4%. The 0.3-ml mixture was applied onto a cover slip fitted in the well of a 24-well cell culture plate and dried at room temperature to set up SF film. The cover slip coated with SF film was immersed in 70% ethanol for 3 h, then air-dried at room temperature and sterilized by autoclaving. The foresaid procedures were also used in preparing film from the pure *B. mori* SF (3% solution).

Cell culture

Mouse fibroblast NIH 3T3 cells were cultured in RPMI 1640 medium enriched with 10% fetal bovine serum, 100 unit/ml penicillin and 100 µg/ml streptomycin on a 25 cm^2 culture flask at 37°C in 5% CO_2 and 98% humidified cell incubator. At 80-100% confluence, the cells were trypsinized using 0.25% trypsin-EDTA. Then, the trypsin effect was diluted by adding fresh enriched medium. After brief centrifugation (1000 rpm, 90 sec), cell pellet was resuspended in the enriched medium for subculturing to obtain sufficient cells for tests.

Cell viability on SF film

A 0.3-ml aliquot of trypsinized cell suspension (5×10^4 cells/well) was cultured on the sterile SF film coated cover slip placed in the well of 24-well culture plate. Cell viability on the film was determined by thiazolyl blue tetrazolium bromide (MTT) assay, according to the manufacture's protocol, at time intervals of 24 and 48 h. In brief, the culture medium was removed and 0.3 ml of serum-free medium containing MTT was added. After 3-h incubation, the medium was discarded and 0.3 ml of dimethylsulfoxide was added to dissolve the purple formazan crystals of MTT reduced by the mitochondrial activity of viable cells. Optical density (OD) was measured at 550 nm (for the reduced MTT) and 655 nm (as reference wavelength for turbidity) using a microplate reader. The net OD was calculated as OD_{550}- OD_{655} (Khaopha et al., 2015). The control cell viability was performed by seeding the same amount of cells in the wells containing the SF film non-coated cover slips and undergoing MTT assay in parallel with the tests. Cell viability in the tests was calculated as a percentage of the control values.

Anti-oxidation assay

The lethal dose of H_2O_2 was preliminarily evaluated as followings. Cells were cultured in 0.1-ml enriched medium for 24 h in a 96-well culture plate at 1×10^4 cells/well, then, 90 µl of fresh enriched medium were replaced. Ninety microliters of filtered-sterile aqueous H_2O_2 was added to make the final concentrations of 0.25-2.00 mM. Cell viability was measured by the MTT assay after 24-h incubation comparing to the untreated experiment. The H_2O_2concentration that caused 50% death was defined as 50% lethal concentration (LC_{50}) which was evaluated at 1 mM.

In anti-oxidation study, trypsinized cells were resuspended in the enriched medium at an appropriate cell density. A 0.3-ml aliquot of the cell suspension (5×10^4 cells) was added to each well of a 24-well culture containing SF film. At specific times of 24 and 48 h, the culture medium was replaced by 0.27 ml of fresh enriched medium and 30 µl of filtered-sterile H_2O_2 to make the final concentration of LC_{50} (1 mM). After 24-h incubation with H_2O_2, cell viability was measured by MTT assay. The anti-oxidation effect was evaluated from the cell viability in the tests compared to the paralleled experiments without the H_2O_2 treatment.

Data analysis

All experiments were performed in triplicates, the data were reported as average ± S.D and statistical analysis was performed by one-way analysis of variance (ANOVA). Differences between the groups with $p < 0.05$ were considered to be statistically significant and indicated with asterisks (*) in figures.

Results

SF films were obtained by using solely *B. mori* SF (3%) or that mixed with Eri SF in the range of 0.05-0.4% (Figure 1). The characteristic of *B. mori* SF film was visibly changed when Eri SF was blended in the preparations. It became non-smooth and thicker. The maximal addition of Eri SF was 0.4% because the higher amount made the film fragile. According to the method, the film was coated on one side of the glass cover slip having the dimension fitted with the well of a 24-well culture plate.

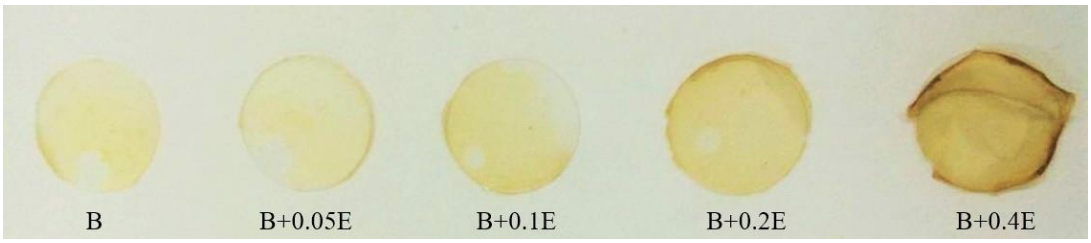

Figure 1 SF films coated cover slips. Films were made of pure *Bombyx mori* SF (B), or that blended with Eri SF including 0.05% (B+0.05E), 0.1% (B+0.1E), 0.2% (B+0.2E) and 0.4% (B+0.4E).

The proliferative activity of mouse fibroblast NIH 3T3 cells on the *B. mori* SF film and on the film of the *B. mori*-Ei SF blend was followed after 24- and 48-h culture (Figure 2 and 3, respectively). Cell growth was also detectable in the culture using film non-coated cover slip. It was thought to be from cells attached to the well beneath the cover slip and it was consequently used as the basal growth as 100% cell viability to evaluate the effect of SF films. The results from both 24-h and 48-h tests showed that proliferative activity of cells on SF film increased with statistical significance ($p < 0.05$) compared with the test using cover slip (control). Besides, the 24-h test demonstrated that the 0.1% constituent of Eri SF in 3% *B. mori* SF film was optimized for its growth supportive effect superior to *B. mori* SF. However, the culture for 48 h was found too long to observe such effect of 0.1% Eri SF meanwhile the films did not change their morphology. Cells probably became aging and weak. It was this reason that the anti-oxidation effect was studied in 24-hculture.

Oxidative cytotoxicity was generated by H_2O_2 at LD_{50} concentration to mouse fibroblast NIH 3T3 cells. As showed in Figure 4, cell viability was minimal in the H_2O_2 treated cell culture on *B. mori* SF film which was found did not have an anti-oxidation effect. Eri SF additives, in the range of 0.05-0.4%, in the *B. mori* SF film demonstrated the tendency to protect cells from H_2O_2.

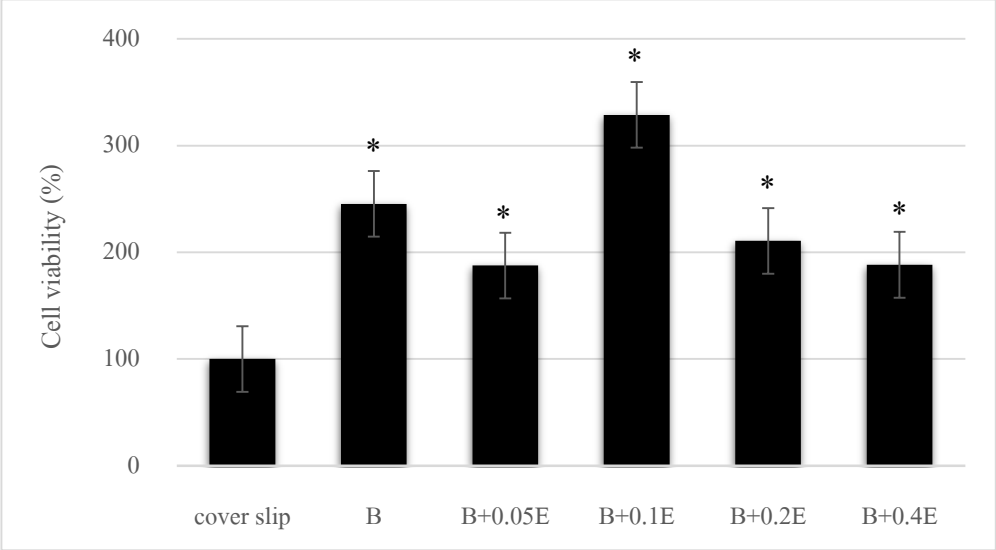

Figure 2 Cell viability after 24-h culture in the tests for the effect of SF film on proliferative activity of mouse fibroblast NIH 3T3 cells. The experiments were performed with films made of *Bombyx mori* SF (B) or blended with Eri SF 0.05% (B+0.05E), 0.1% (B+0.1E), 0.2% (B+0.2E) and 0.4% (B+0.4E). Cells growth using cover slip without film (cover slip) was refered as the base-line growth. The asterisks (*) indicate significant differences at $p < 0.05$ and the data are average ± S.D (n=3).

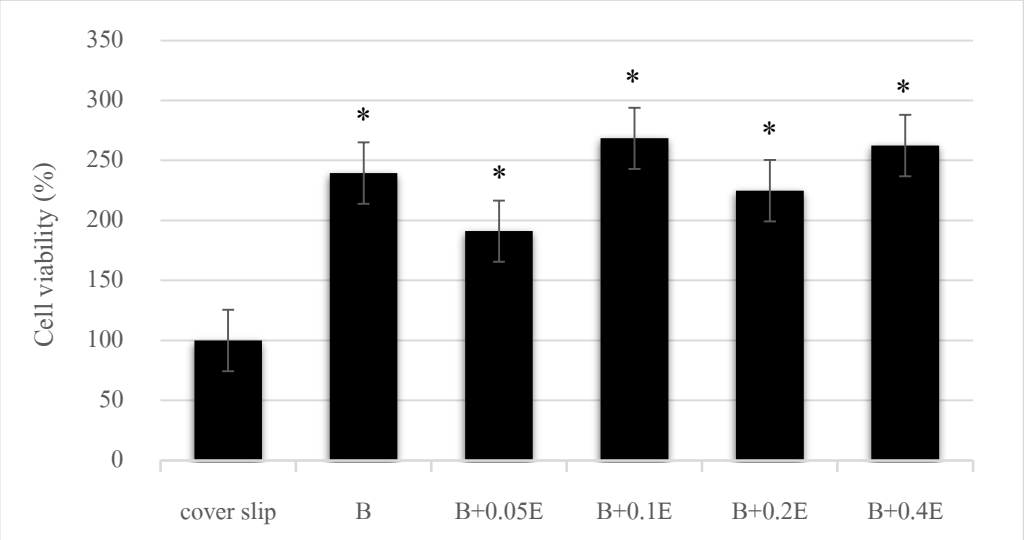

Figure 3 Cell viability after 48-h culture in the tests for the effect of SF film on proliferative activity of mouse fibroblast NIH 3T3 cells. The experiments were performed with films made of *Bombyx mori* SF (B) or blended with Eri SF 0.05% (B+0.05E), 0.1% (B+0.1E), 0.2% (B+0.2E) and 0.4% (B+0.4E). Cells growth using cover slip without film (cover slip) was refered as the base-line growth. The asterisks (*) indicate significant differences at $p < 0.05$ and the data are average ± S.D (n=3).

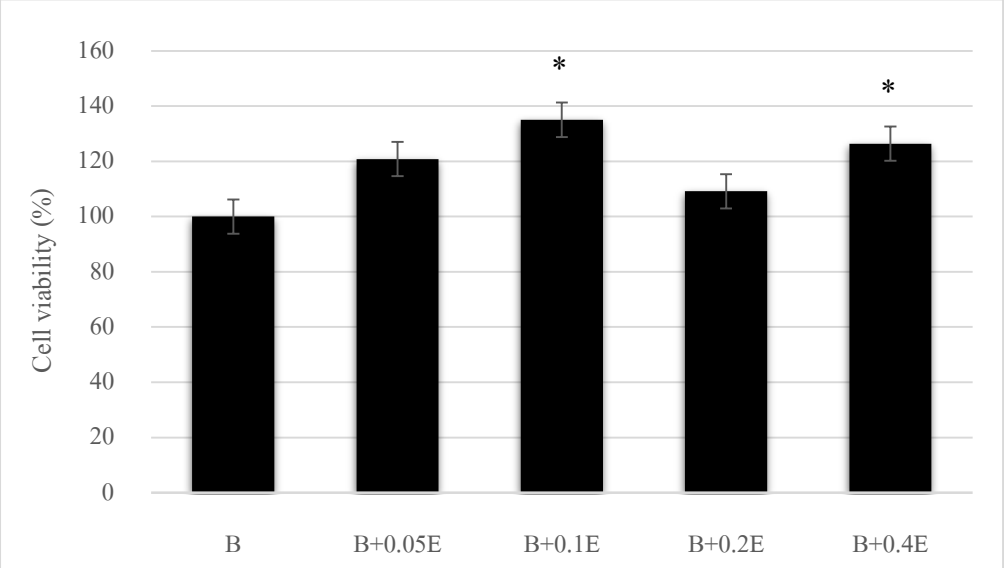

Figure 4 Cell viability representing anti-oxidation effect against H_2O_2 on mouse fibroblast NIH 3T3 cells of films containing Eri SF. The experiments were performed with film made of *Bombyx mori* SF (B) or blended with Eri SF 0.05% (B+0.05E), 0.1% (B+0.1E), 0.2% (B+0.2E) and 0.4% (B+0.4E). The asterisks (*) indicate significant differences at $p < 0.05$ and the data are average ± S.D. (n=3).

Discussion and Conclusion

The SF film was expected to be applied for tissue or skin recovery in hardly healing wound like the wound from thermal injury and diabetic wound. The healing process involves cell migration and proliferation into the reconstituting extracellular matrix (Schultz, Wysocki, 2009). Once wounded, the surrounding tissue undergoes inflammation and produces H_2O_2. It has been reviewed for the essence of H_2O_2 as a damage signal to turn on the mechanisms of healing, however, H_2O_2 leads to deleterious effect in case of chronic wounding (van der Vliet, Janssen-Heininger, 2014). Among several ideal properties, wound dressing materials need to be good gaseous exchange, non-cytotoxic to nearby healthy tissues, promote a moist wound environment and cell growth for tissue regeneration, and microbiological barrier as well as antimicrobial (Jones et al., 2006). As cited in literatures, novel wound dressings have been focused on the use of natural or artificial scaffolds to adopt the characteristics of dermis. The most importance is that they must be made from biocompatible and biodegradable materials (International Organization for Standardization 10993-5 and 10993-13; https://www.iso.org>standard). Commercially ready-to-use scaffolds are mostly formulated with collagen which is the basal extracellular matrix. However, collagen is not able to prevent microbial infection and may induce hyperimmune response (Fonder et al., 2008).

SF meets the criteria of good wound dressings, and Eri SF is superior to that of *B. mori* for its antimicrobial property (Min et al., 2004; Hu et al., 2006; Andiappan, Sundaramoorthy, 2015; Qi et al., 2017). There has been little knowledge of Eri SF film because of the limitation of SF solubility using the conventional method of dissolution. Each laboratory keeps its own tricks although the method has been published (Srisuwan et al., 2012; Andiappan, Sundaramoorthy, 2015). In this work, the lithium bromide method was used with modifications following the

previous work by Senakoon (2009). The regenerated Eri SF powder was obtained but had limitation of dissolution in water. Therefore, it was investigated to supplement the use of *B. mori* SF. The concentration of *B. mori* SF at 3% is common in film preparation as cited in literatures. The features of the SF blend films were non-smooth which were dominated by the characteristic of Eri SF, although the content of Eri SF was mixed in the low ratio. It was reported elsewhere that the surface of Eri SF film was rough and porous (Srisuwan et al., 2012). Surface roughness was important for cell adherence (Vepari, Kaplan, 2007). The 0.1% Eri SF constituent in the blend film was suggested to produce appropriate porous structure of the blend film which was attributed to the optimal growth enhancing effect. Higher Eri SF content, 0.2% and 0.4%, might result in too large pore and reduce the area for cell attachment. The *B. mori* SF has not been reported for its protective effect against H_2O_2. However, such effect was found in the *B. mori* SF hydrolysate having the contents of aromatic amino acids (Yeo et al., 2006). The anti-oxidation of Eri SF blend film was interesting and has not been previously evident. It might be associated with the higher content of phenylaline (Ahmad et al., 2004). Cell viability on cover slip was usually low even without H_2O_2 effect, therefore, it was not included as a control group in the anti-oxidation test.

In conclusions, this work was successful in the production of *B. mori* SF (3%)-Eri SF (0.1%) blend film which exhibited enhancing effect on the growth of mouse fibroblast NIH 3T3 cells and protection to H_2O_2 cytotoxicity.

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