CHAPTER III
PREPARATION AND CHARACTERIZATION OF ELECTROSPUN SILK FIBROIN FOR TISSUE REGENERATION

3.1 ABSTRACT

Thai domesticated *Bombyx mori* silk fibroin (SF) was used to fabricate as ultra-fine fibers by electrospinning process by using formic acid as a solvent. The appropriate condition of electrospinning process that could generate the electrospun fibers without the presence of beads were 25 kV and 40% (w/v). The average fiber diameter is 243.05 ± 37.6 after methanol treatment to water-insoluble structure. In addition, the electrospun SF fiber mat was fabricated for the human foreskin fibroblast (HFF) cells culture. Biological response of the cells towards the scaffolds was tested by observing the cytotoxicity, the attachment, and the proliferation of HFF cells on the scaffolds. The MTT assay was used to quantify the viability of the cells. The HFF cell morphology on the scaffolds was examined by SEM. The obtained results indicated the electrospun SF fiber mat promoted better growth of HFF cells than the film did in term of their attachment and proliferation. These data suggested that the electrospun silk fibroin fibers could be further studied and developed to be a preferable scaffold for tissue regeneration.

(**Keywords**: Electrospinning; Fiber; Silk fibroin; Tissue; Scaffold)

3.2 Introduction

Mimicking of fibrous structure of the extracellular matrix (ECM) is the first important gold to fabricate scaffold for tissue engineering. In an ideal manner, the scaffolding material should mimic the structural and biological function that found in the natural ECM, supporting the cellular activities without harm to the cells [1, 2].

Many of techniques have been developed to fabricate ultra-fine fibrous structure to mimic the native ECM. Electrospinning is the effective technique that has recently established for the fabrication of nanoscale fibers which is depending on the types of the polymer, solvent, and the processing conditions [2, 3]. Polymer solution in the syringe is subjected to an electric field generated by high voltage. A polymer jet is ejected from the capillary when the electric field overcomes the surface tension of the polymer solution. As the polymer jets travel to the collector, it suddenly dried out and forming into non-woven fibrous mat on the collector. These nano-fibers mat are of considerable interest for tissue scaffolding because of their unique properties such as high specific surface. Electrospun fibers can also create their porous nature which could be excellent functional tissue scaffolds in term of architecture. On the other hand, the biocompatibility of the polymer is also an important property that significantly plays a role on biological function [2, 4, 5].

Silk fibroin (SF) is a promising natural proteins produced by silkworm. Generally, silk fibroin is the continuous fibrous protein coated with siricin, a glue-like protein that can be removed by degumming process [6-8]. Fibroin protein contains a variety of amino acids such as glycine, alanine, and serine leading to significant homogeneity β-sheet conformation, high degree of crystallinity and importantly mechanical properties [6, 9]. With various characteristics and properties, including good biocompatibility, Silk fibroin protein provide a opportunity to produce functional devices for biomedical applications such as wound dressing, tissue engineered scaffold that has been used to culture many types of cells such as fibroblasts, bone cells, nerve cells, and Schwann cell, etc [10-14]. Electrospun of *Bombyx mori* silk fibroin were successfully prepared by using hexafluoroacetone as a solvent. The fibers diameters were in the range of 100-1,000 nm in form of thin rod-liked shape. In addition, a fibrous mat made of silk fibroin provides a remarkable of

combination of porosity and biocompatibility to support attachment and growth of bone cells [7, 8, 15-17].

Aim of this work was to fabricate electrospun fibers of Thai domesticated *Bombyx mori* silk fibroin. The electrospinning solution of SF was prepared by using formic acid as a solvent. The concentration of SF protein, voltage and distance from the collector were studied to achieve effective condition for electrospinning. Structural characteristics and morphology of the scaffolding materials were investigated through Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) respectively. The cytotoxicity of electrospun SF fiber mats was evaluated by indirect cytotoxicity procedure using mouse fibroblasts (L929). In addition, electrospun SF fiber mats were fabricated for the human foreskin fibroblast (HFF) cell culture. The attachment and the proliferation of the HFF cells were investigated by the MTT assay. The Morphological of HFF cell on the electrospun SF fiber mat was also investigated by SEM microscope.

3.3 Materials and methods

3.3.1 Materials

Cocoon of fresh Thai *Bombyx mori* silkworms (Chul 1/1) were kindly provided by Chul Thai Silk (Phetchabun, Thailand). The chemicals used for the preparation of SF and its spinning solutions were sodium carbonate (Na2CO3), lithium bromide (LiBr), were purchased from Riedel-de Haën (Germany). Formic acid was purchased from Fisher Scientific (USA).Others chemicals used for cell study were purchased from Invitrogen Corp., (USA). All chemicals were of analytical grade and used without further purification.

3.3.2 Preparation of Regenerated SF Solution

Thai *Bombyx mori* silk cocoons were degummed by three times boiling in an aqueous solution of 0.02 M Na_2CO_3 for 30 min, and then rinsed thoroughly with warm distilled water to get rid of glue-like sericin proteins. The extracted silk was then dissolved in 9.3 M LiBr (55 °C) for 30 min yielding at a final concentration of approximately 10 % (w/v) solution. This solution was dialyzed in distilled water using dialysis tubing cellulose membrane (Sigma-Aldrich, USA) for 3

days. The dialysate SF was centrifuged at 5 °C for 20 min. After filtration, the solution was lyophilized by using LABCONCO: FreezeZone 6 to obtain the regenerated SF sponges. To prepare electrospinning solution, The SF sponges was dissolved at various concentrations in 98% formic acid and stirred for 3 h at room temperature.

3.3.3 Preparation of Electrospun SF Fibers via Electrospinning

In the electrospinning process, a high electric potential in the range of 12-14 kV was applied to a droplet of the solution at a tip of a gauge 20 syringe needle. A homogeneous solution of SF was contained in a 20 ml glass syringe. A charged jet of the SF solutions was ejected to a grounded rotating drum covered with aluminum foil. The distance between the tip of the needle and the collector was fixed at 10 cm. The needle was tilted ~ 45° to maintain the flow rate of the electrospinning solution. The concentrations of SF solution were varied in the range of 10 % to 40 % by weight. The suitable condition was chosen for electrospinning to obtain electrospun fiber mats. Then, as-electrospun SF fiber mats were immerse in 98 % methanol for 10 minute and air dried to induce the conformation transition from amorphous (silk I) to β-sheet transition (silk II) which was water-insoluble. Treated fiber mats were washed in distilled water at 37 °C for 48 h.

3.3.4 Scanning Electron Microscopy

JSM-5410LV model of Scanning Electron Microscope (SEM) was used to determine the diameter and morphology of electrospun fibers by operating at 10 kV. The surface texture of fiber mats was examined after treatment with methanol. The samples were attached carefully on the stub, and then sputter coated with gold prior to evaluation. The fibers diameters were determined by measuring randomly from SEM images by using SemAphore 4.0 program.

3.3.5 Fourier Transform Infrared Spectroscopy (FTIR)

Thermo Nicolet Nexus 670 Spectrophotometer was used to record all Infrared spectra of electrospun fibers. Each spectrum was acquired by accumulation of 32 scans with a resolution of 4 cm-1 and a spectral range of 400 to 4,000 cm-1. The measurement was accomplished by using ZnSe crystal cell with an attenuated total reflectance fourier transform (ATR-FTIR) mode.

3.3.6 Indirect Cytotoxicity Evaluation

To study the cytoxicity of the electrospun SF fiber mat, the indirect method which is adapted from the ISO 10993-5 standard test was used to evaluate by using L929 cell lines. For preparing the extraction media, the specimens were sterilized by UV radiation for about 2 h. They were then immersed in a serum-free medium (SFM; DMEM containing 1 % l-glutamine, 1 % lactalbumin and 1 % antibiotic and antimycotic formulation) for 24 h at the extraction ration of 5, 10 and 20 mg/ml, and then the fiber mats were remove from the medium which were used for cell culture study. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) by adding a requisite amount of 10 % fetal bovine serum (FBS), 1 % l- glutamine, 1 % antibiotic and antimycotic formulation which contained penicillin G sodium, streptomycin sulfate, and amphotericin B. For the cell culture, the cells of L929 were seeded separately at density4×10^4cells/well in DMEM in 24-well tissue-culture polystyrene plates (TCPS) for 24 h. The cultured medium was replaced with each of extraction medium and placed in incubator for 24 h. The cell viability was obtained by using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All tests were performed in triplicate. The cell viability of L929 cells in fresh SFM was used as control.

3.3.7 Cell Attachment and Proliferation Test

Electrospun SF fiber mats were cut to 1.4 cm in diameters and sterilized by UV radiation about 1 h for each side. The fiber mats were then placed in 24-well TCPS. The human foreskin fibroblasts (HFF) cells were then cultured on the surface of both fiber mats for 2, 4 and 8 h for the cell attachment and 1; 2, and 3 days for the cell proliferation study. The plates were then placed in 5 % CO2 at 37 °C in incubator. The viabilities of the attached and proliferated cells in each time point of the experiment were determined by MTT assay. In all cases, the viability of HFF cells in the well without scaffolds was used as the controls.

3.3.8 Morphological Observation of Cultured Cells

SEM was used to observe the cell morphology that changed with time on the surface of electrospun SF fiber mats. After the culture medium had been removed, the seeded fiber mats were rinsed twice with PBS and the cells were then fixed with 3 % glutaraldehyde/PBS solution (Electron Microscopy Science, USA) for

30 min. After fixation of cells, the seeded fiber mats were rinsed again with PBS, prior to being dehydrated in increasing of concentrations of ethanol aqueous solution at 30, 50, 70, 90 % v/v and finally with pure ethanol for 2 min each. The fiber mats were then immerse in 100% hexamethyldisilazane (HMDS; Sigma-Aldrich, USA) for 5 min and later dried in air after the removal of HMDS. After being completely dried, the specimens were carefully placed on stubs and sputter coated with gold before observing by SEM. The morphology of the HFF cells that had been seeded on a glass substrate (covered glass slide, 1 mm in diameter; Menzel, Germany) was used as positive control.

3.3.9 Statistics and Data Analysis

All of the quantitative data were shown as means±standard deviations. Statistical analysis was performed by the one-way analysis of variance (one-way ANOVA) with SPSS 13.0 for Windows software (SPSS, USA). $p < 0.05$ was considered statistically significant.

3.4 Results and Discussion

3.4.1 Morphological and Physiochemical Characterization

In order to study the effect of solution concentration on the shape and size of fibers, the morphology of electrospun SF fibers mats were investigated under different concentration of SF solution while other parameters were kept constant (i.e. flow rate and electric field strength (EFS) = 20 kV/10cm). The representative SEM images of electrospun SF fibers are shown in Figure 3.1. At 10 % (w/v) of SF solution, the drops were observed and forming to cluster of fused fibers (Figure 3.1a). At 20 and 30 % (w/v) of SF concentration, the fiber structure was obtained with a mixture of bead structure (Figure 3.1(b,c)) which was decreased by increasing of SF solution concentration. The surface tension of polymer solution was the dominant factor controlling the morphology that resulted in decreasing of as-sprayed droplets. Evidently, at 40 % (w/v), the beads were disappeared. The uniform fibers were obtained.

From this condition, 40 % of SF solution was used for further investigate the effect of applied voltage on the morphology of electrospun SF fibers.

The applied voltage was varied from 15 to 30 kV, while the collector distant was kept constant at 10 cm. The selected SEM micrographs of the electrospun SF fibers obtained under different applied voltages are shown in Figure 3.2. Initially, the silk fibroin solution is held by its surface tension in the form of a droplet at a tip of the needle. As the applied voltage increased, charges were induced on the fluid surface, creating forces directly opposite to the surface tension, causing the distortion of the shape of the droplet [8].

At the applied voltages lower than 15 kV, electrospinning of the solution generated beads and beaded fibers due to the relatively low Coulombic repulsion force that is lower than the surface tension of silk fibroin solution. At the applied voltage of 15, the formation of continuous fibers without the presence of beads was obtained with average diameter of 307.20 ± 54.5 nm and decreasing to 285.08 ± 63.0 nm at 20 kV. At even higher applied voltage of 25 kV (Figure 3.2c), the ultra-fine fibers was observed with average diameter of 243.05 ± 37.6 nm, due to increasing of repulsion force in the flow of the material. In other words, at such a high applied voltage, the force exerting on the jet due to the electric field was high, causing the jet to neck down or even broke off. Both the necking and the breaking off of the jet could result in the formation of droplet on the fiber mat. From the results obtained, the optimal applied voltage among those investigated that resulted in formation of the finest electrospun SF fibers without the presence of beads was 25 kV

After methanol treatment, The FT-IR with ATR spectroscopy was used to indicate secondary structure of electrospun SF fibers which was changed to water-unsoluble. The spectra were shown in Figure 3.3. The electrospun SF fiber mats showed the obvious absorption bands at 1657 cm^{-1} (amide I, C=O stretching), 1536 cm^{-1} (amide II, N–H deformation and C–N stretching) which were shifted to 1632 cm^{-1} and 1525 cm^{-1}, respectively after methanol treatment due to the transformation of secondary structure from α-helix to β-sheet conformation [18, 19].

3.4.2 Indirect Cytotoxicity Evaluation

For indirect cytotoxicity evaluation of electrospun SF fiber mat, the viable cells were measured by MTT assay via the UV absorbance which the high UV

absorbance was also represented the high viable cells. To effortless elucidate, the data was converted to bar graphs of the percentages of the cell viability. According to Figure 3.4, the results of indirect cytotoxicity showed the viability of L929 cells that were cultured in three extraction ratios of the extraction media (i.e., 5, 10, 20 mg/ml) from silk fibroin fibrous mats and films when compared with the cells that were cultured in fresh SFM (i.e. controls). As indicated in this figure, the viabilities of L929 cells of three extraction ratios were in the range of 90 % to 98 % based on the viability of the cells that cultured in the control. There is no significant difference of absorbance values in comparison with the control. All results clearly showed that as-prepared materials were nontoxic to L929 cells, suggesting that these materials can be a good candidate to be used as tissue engineering material. Consequently, the attachment and proliferation of normal human fibroblast cells on the scaffolds at different times in culture should be further investigated to clarify the cells behavior.

3.4.3 Cell Attachment and Proliferation

With the absent of cytotoxicity, To observe and compare the biological properties of substrates, Attachment of HFF cell on control, SF film and electrospun SF fiber mat at 2, 4, 6 hours are shown in Figure 3.5, which shows the viabilities of HFF cells attached on the substrates. The results were compared with the cells that were cultured on TCPS culture plate (i.e., control). Clearly, for any given substrate, the cell viability increased with increasing time in culture. As can be seen at 2 hours, the viability of HFF cells on electrospun SF fiber mat and SF film was significantly greater than control. In addition, the cell viability of electrospun SF fiber mats was slightly higher than films. Further, trend of number of viable HFF cells on electrospun SF fiber mat was significantly greater than films and control. These results may indicate that electrospun SF fiber mats are functionally support cell attachment due to the biocompatibility of SF protein and the biomimetic fiber construction which provided large bio-functional surface area for support cellular responses[8, 17].

Nevertheless, we further investigate the spreading of HFF cells on the substrates. Proliferation of HFF cell on control, SF film and electrospun SF fiber mat at 1, 2, 3 days are shown in Figure 3.6. After day 1, the absorbance on controls and films were significantly greater than SF film. Interestingly, the cell viability of

electrospun SF fiber mat was higher than control and significantly higher than SF film. At day 2 and 3, the viability of HFF cells on the surface of control tended to increase with increasing time in culture, while film and electrospun SF fiber mat tended to increase in lower proliferation rate than control. Obviously, for all culturing time point, It was also found that the proliferation of HFF cells on the surface of control was significantly better than on the surface of electrospun SF fiber mat and film. However, comparing at all time in culture, it was found that the percentage of viable proliferated HFF cells on the surface of electrospun SF fiber mat were greater than the surface of SF films. These results may suggest that the electrospun SF fiber mat has an outstanding potential for support cell attachment and also spreading of HFF cells, which would be particularly useful for wound healing or tissue regeneration

3.4.4 Morphology of HFF Cells

To support the above results, SEM micrographs were used to elucidate the attachment and proliferation of HFF cells on each substrate. Table 2 shows selected SEM images of HFF cells cultured on bare wells (i.e., control) including SF film and electrospun SF fiber mat at various times. According to these images, at 2 h, the majority of the cells seeded on the control stretched their cytoplasm along the surface, an indication of the normal phenotype of the cells. On the contrary, the cells seeded on among the SF films, electrospun SF fiber mat were also extended their cytoplasm but almost still in their round shape. After longer times in culture (e.g., 4 and 6 h), the cells that were seeded on three types of substrates became more elongated, indicating their normal phenotype. Obviously, HFF cells were fully extend their cytoplasm on the surface of electrospun SF fiber mat. Table 3 shows selected SEM images of HFF cells for longer culture periods (i.e., 1 – 3 days), the cells that were cultured on both control appeared to spread well over the surface, while those on the SF film tended to aggregate, despite the much increase in the number of cells with increasing time in culture. Additionally, electrospun SF fiber mat show better spreading of HFF cells on their surface. From the obtained results, these suggested that HFF cells had a biocompatibility with the film and electrospun fiber mat in equally which were promote better of cell growth in vitro. In addition,

three-dimensional structure similar to ECM that played a role in supporting cell growth and cell differentiation

3.5 Conclusion

In order to mimic the natural ECM structure, electrospun SF fiber mat were obtained by electrospinning of 40% (w/v) SF solution at 25 kV/10 cm with the average fiber diameter being in the range of 243.05 ± 37.6 nm without the present of beats. ATR-FTIR data confirmed the β-sheet conformation which was water insoluble after methanol treatment. The potential use of electrospun SF fiber mats for support cell activities of HFF cell was evaluated in vitro comparing with SF films and TCPS cultured plate. Indirect cytotoxic evaluation discovered that the electrospun SF fiber mats were not toxic to HFF cells. The obtained results indicated the electrospun fiber mats promoted better growth of HFF cells than the film did significantly in term of proliferation. The attachment of HFF cells was excellent supported by electrospun SF fibers mats due to the mimic structure of ECM. These data suggested that the electrospun SF fiber mats could be further studied and developed to be a preferable scaffold for tissue regeneration.

3.6 Acknowledgments

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3.7 References

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16. Meechaisue, C., et al., *Preparation of electrospun silk fibroin fiber mats as bone scaffolds: a preliminary study.* Biomed Mater, 2007. **2**(3): p. 181-8.

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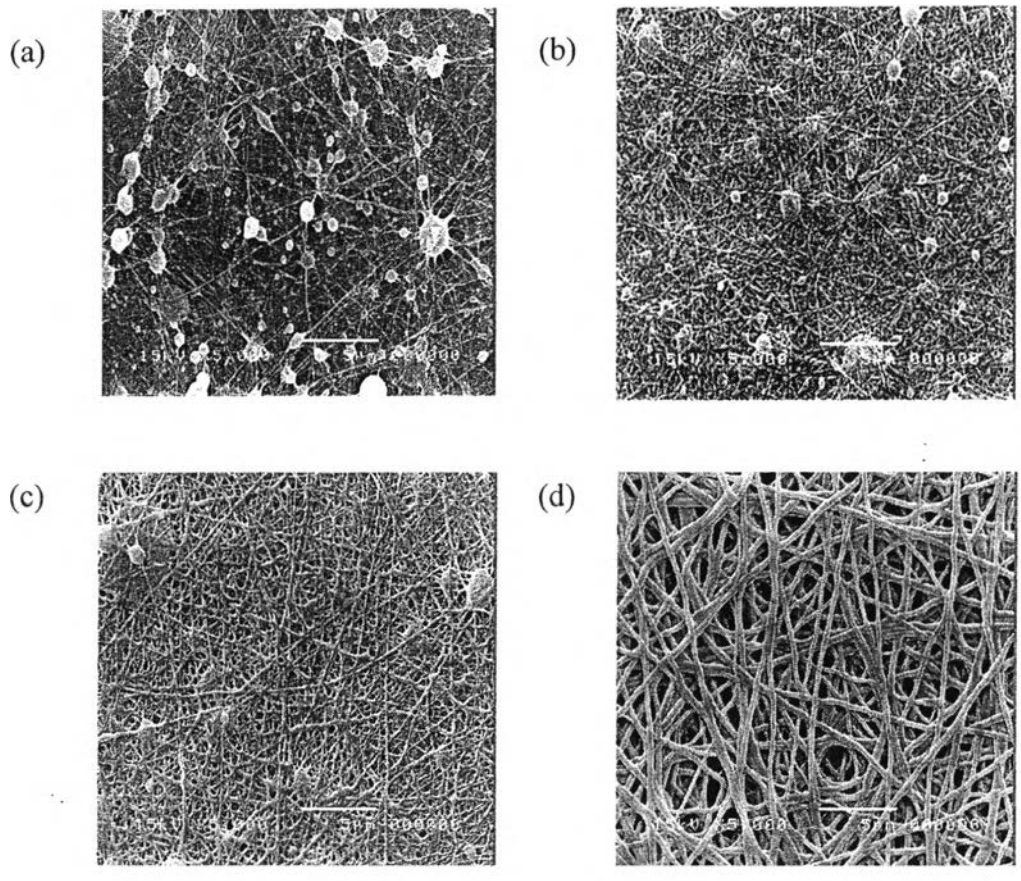


Figure 3.1 SEM images of electrospun SF fibers at different concentrations: a) 10%, b) 20%, c) 30%, and d) 40% (w/v). The EFS was applied at 20 kV/10 cm. (magnification = 5000x, scale bar = 5 μm)

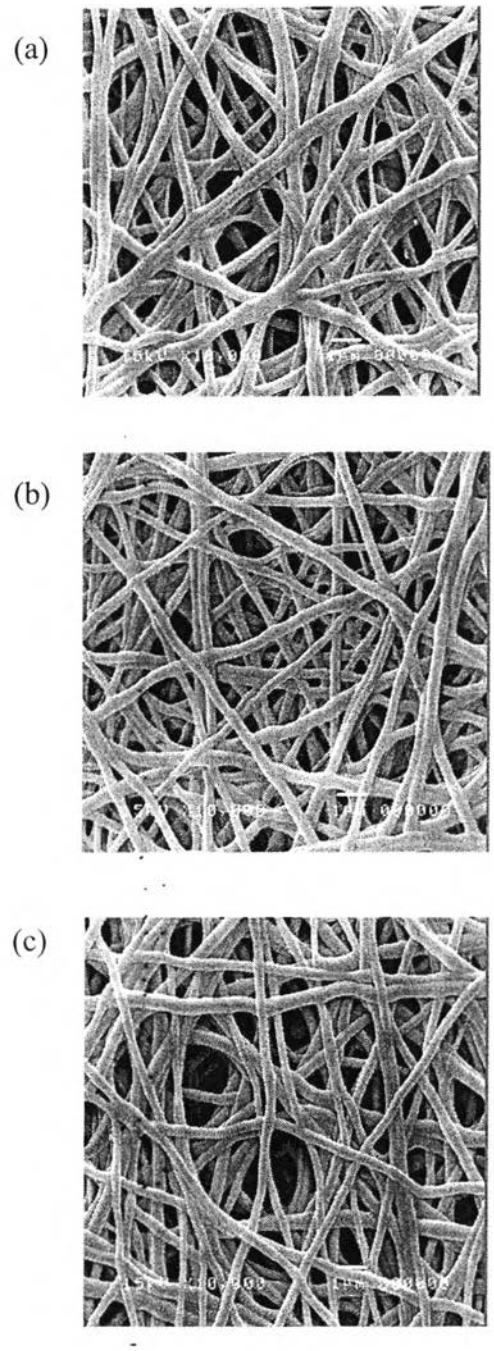


Figure 3.2 SEM images of electrospun SF fibers from 40% (w/v) SF solution after methanol treatment at different applied EFS: a) 15 kV, b) 20 kV, and c) 25 kV/10 cm. (magnification = 10000x, scale bar = 1 μm)

Table 3.1 Electrospun SF fibers diameters

Applied Voltage (kV)	Average fiber diameter (nm)	Minimum fiber diameter (nm)	Maximum fiber diameter (nm)
15	307.20 ± 54.5	188	450
20	285.08 ± 63.0	176	410
25	243.05 ± 37.6	151	378

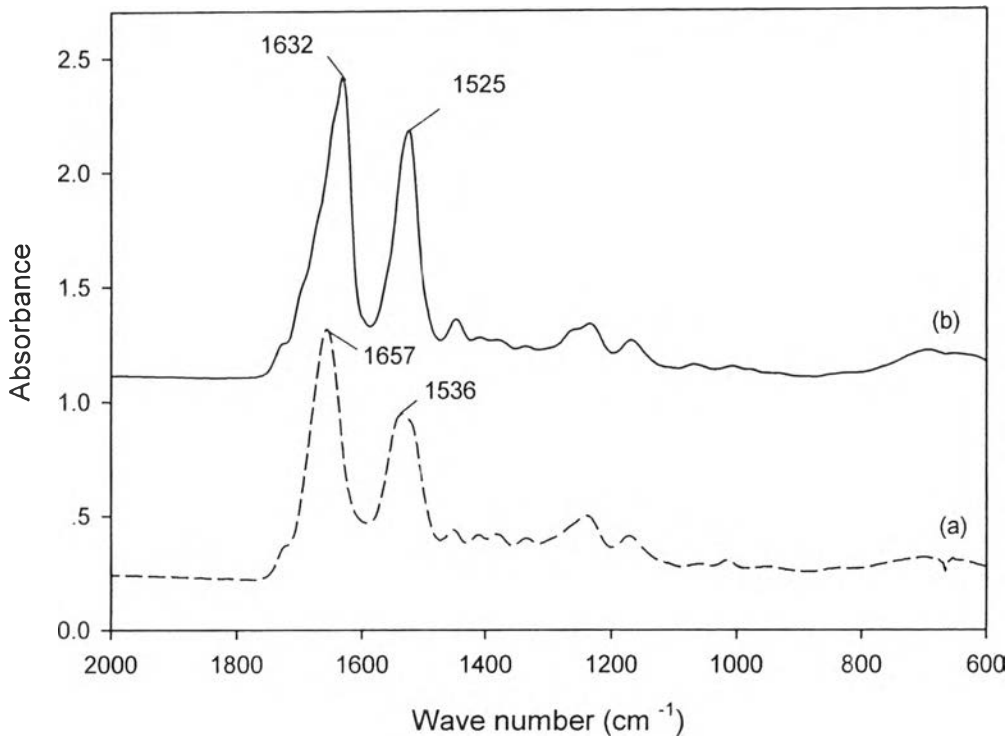


Figure 3.3 ATR-FTIR spectra of electrospun SF fiber mat: (a) electrospun SF fiber mat (b) electrospun SF fiber mat after methanol treatment.

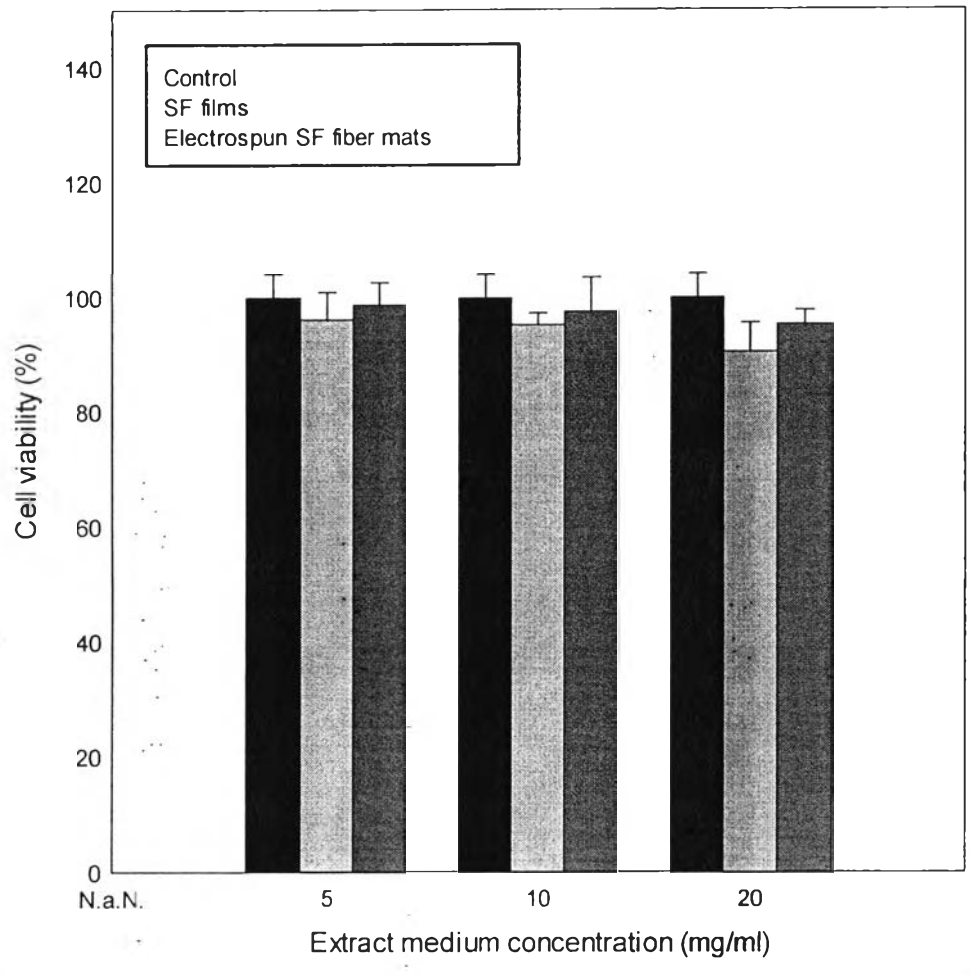


Figure 3.4 The viability of L929 cells that were cultured on electrospun SF fiber mats for indirect cytotoxicity evaluation with various extract media concentration for 24 hr.

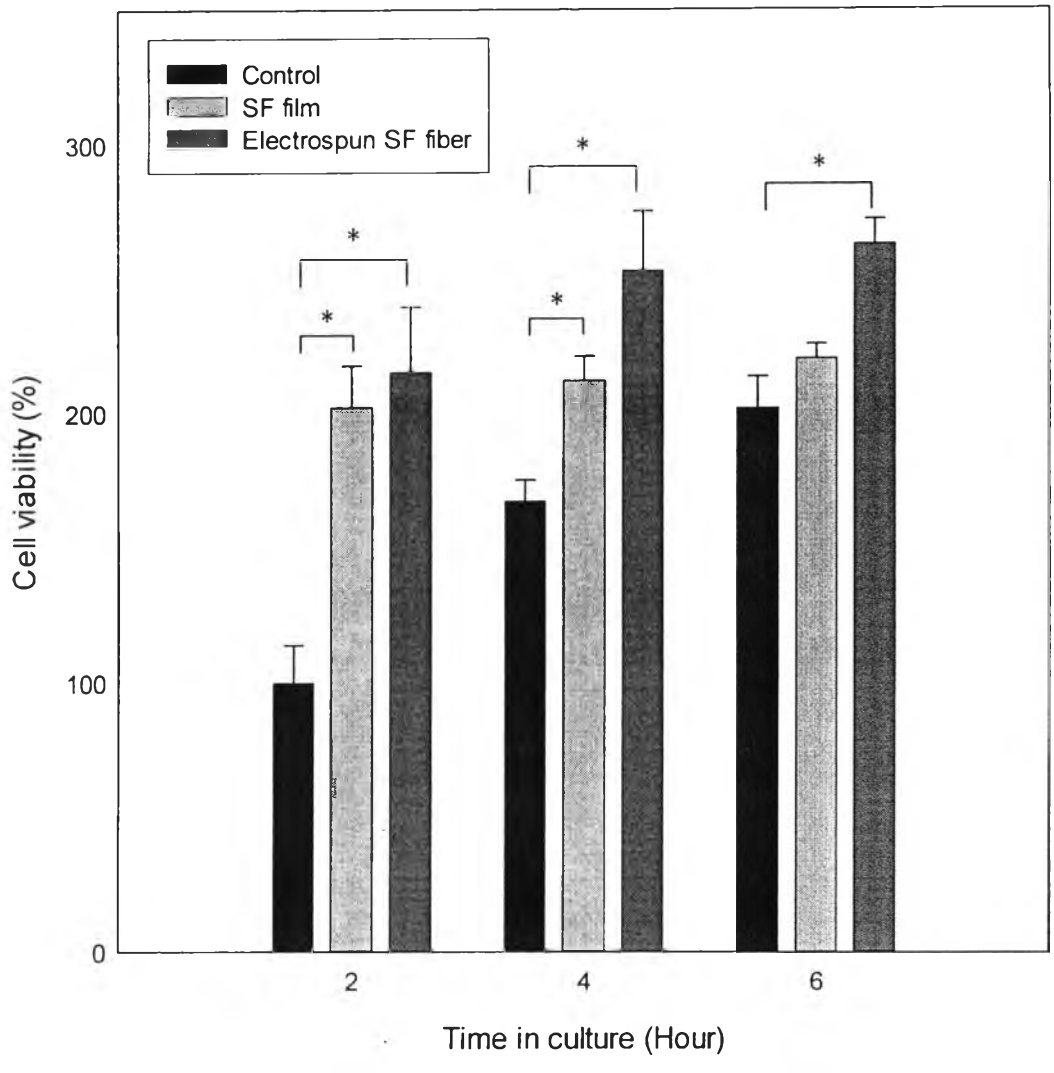


Figure 3.5 Attachment of HFF cell on control, SF film and electrospun SF fiber mat as a function of time in culture ($p < 0.05$, n = 3)

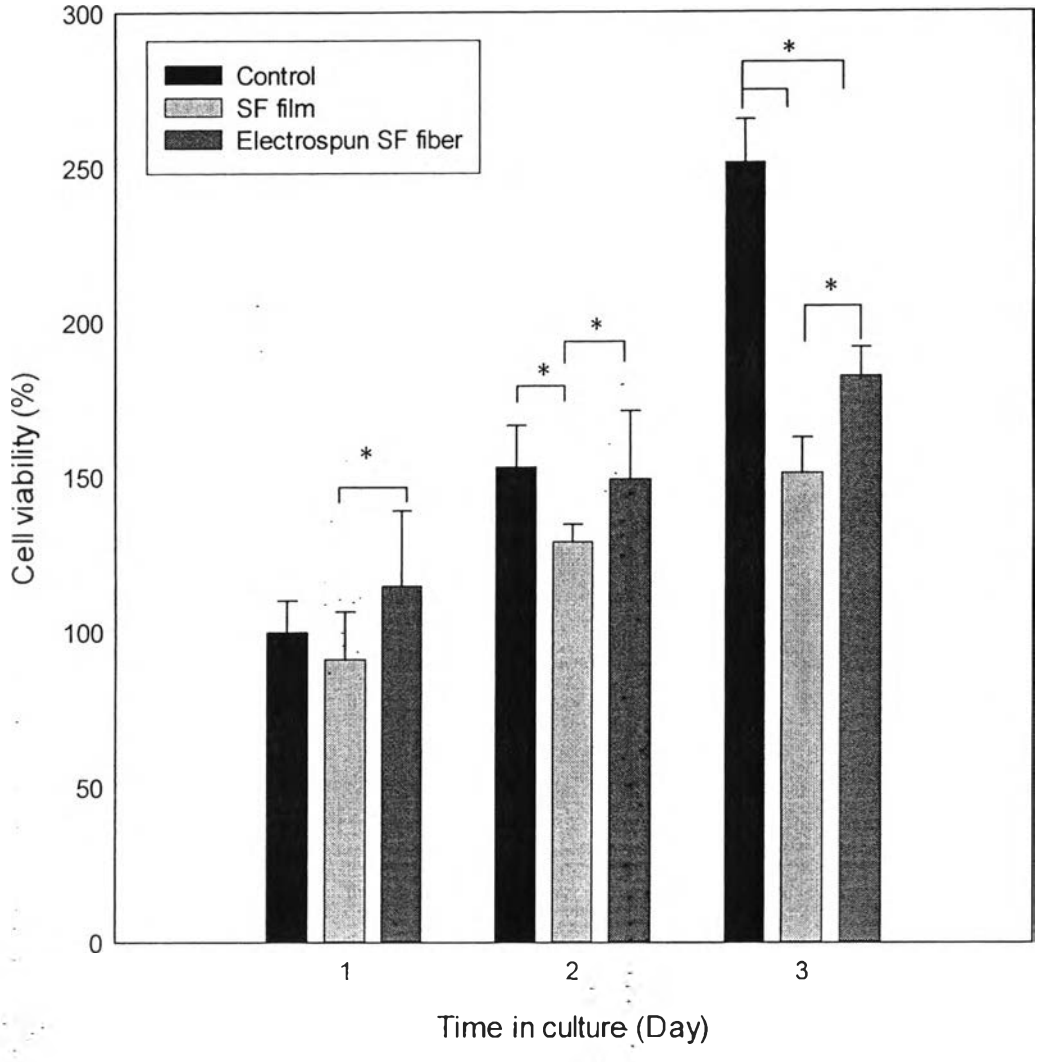


Figure 3.6 Proliferation of HFF cell on control, SF film and electrospun SF fiber mat as a function of time in culture ($p < 0.05$, n = 3)

Table 3.2 SEM micrographs of HFF cells attached on the surface of SF film and electrospun SF fiber mat comparison with control.

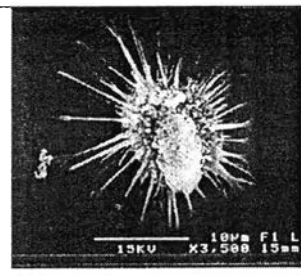
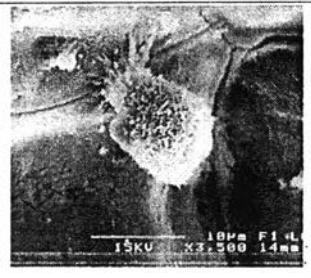
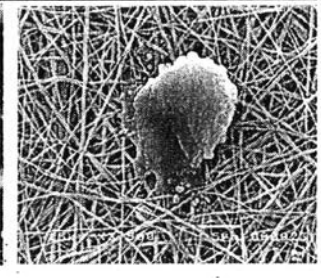
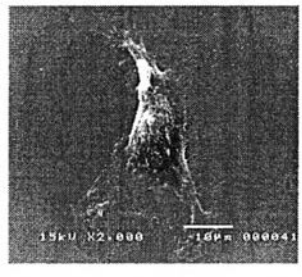
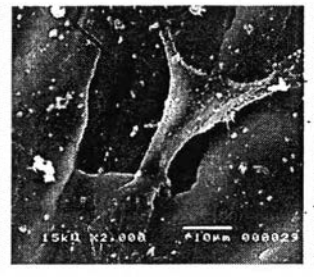
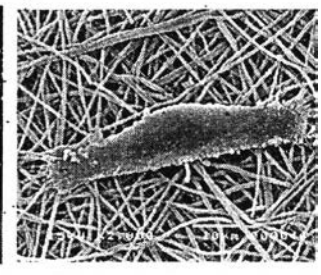
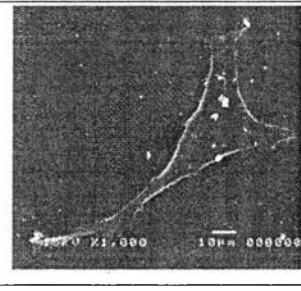
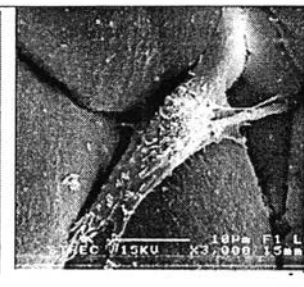
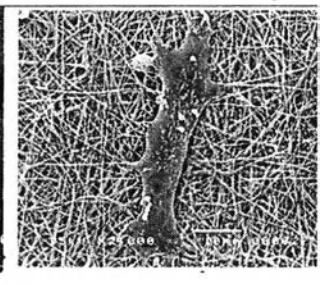
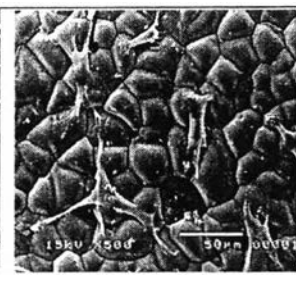
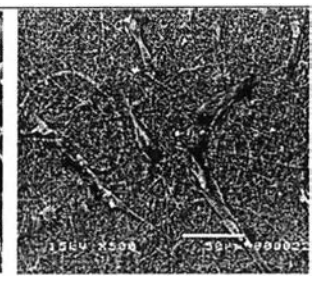
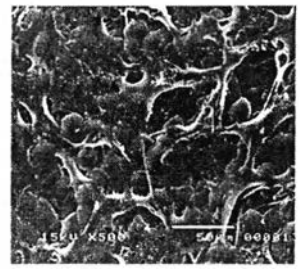
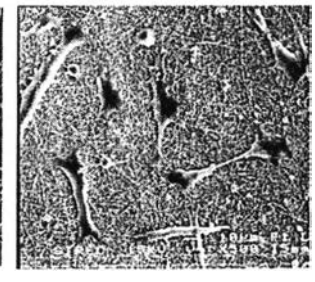
Culturing time	Type of substrates		
	Control	SF Film	SF fiber
2 h			
4 h			
6 h			

Table 3.3 SEM micrographs of HFF cells proliferated on the surface of SF film and electrospun SF fiber mat comparison with control.

Culturing time	Type of substrates		
	Control	SF Film	SF fiber
1 day			
2 days			
3 days