CHAPTER III

EXPERIMENTAL

The experiment in this research can be divided into four parts.

1. Preparation of silk fibroin spinning solution
2. Fabrication of silk fibroin nanofibers via electrospinning and characterization of as-prepared silk fibrin nanofibers
3. Preparation of silk fibroin scaffolds from as-prepared and hyaluronic acid coated silk fibroin nanofibers and silk fibroin films
4. Investigation of keratinocyte and fibroblast cell culture and testing of cell response on scaffolding materials

The details of the each part were explained as following.

3.1 Materials

Bombyx mori silk cocoons used as the starting material were brought from Saraburee province. Other chemicals and materials used in this work were listed in Table 3.1. The instruments used in this research were also listed in Table 3.2.

Table 3.1 Chemical reagents and suppliers

Chemical	Supplier
Sodium Carbonate (Na_2CO_3)	Lab Scans Co., Ltd., Thailand
Calcium Chloride ($CaCl_2$)	Lab Scans Co., Ltd., Thailand
Ethanol (C_2H_5OH)	Lab Scans Co., Ltd., Thailand
98 % Formic acid (HCOOH)	Apex CHEMICALS Co., Ltd., Thailand
Methanol (CH_3OH)	Lab Scans Co., Ltd., Thailand
Hyaluronic acid (HA)	Lab Scans Co., Ltd., Thailand

Table 3.1(continued)

Chemical	Supplier
50% Glutaraldehyde (for coating)	A.C.S. Co., Ltd., Thailand
50% Glutaraldehyde (for cell fixing)	Sigma-Aldrich,USA
37% Hydrocholic acid	Lab Scans Co., Ltd., Thailand
Dialysis tubing cellulose membrane	ITALMAR (THAILAND) CO., LTD
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich,USA
Phosphate buffered saline (PBS)	Sigma-Aldrich,USA
Fetal bovine serum (FBS)	BIOCHROM AG, GERMANY
Dimethylsulfoxide (DMSO)	Sigma-Aldrich,USA
Trypsin-EDTA (1X)	Invitrogen Corp., USA
3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl-2H-tetrazolium bromide [MTT]	USB Corp.,USA
L-glutamine	Invitrogen Corp., USA
AntiBiotic-Antimycotic	Invitrogen Corp., USA
Hexamethyldisilazone (HMDS)	Sigma-Aldrich,USA

Table 3.2 Instruments

Instrument	Model
Scanning electron microscope (SEM)	JEOL JSM-5410LV
FT-IR spectrophotometer	Thermo Nicolet NEXUS 670
Thermogravimeter	METTLER TOLEDO (TGA/SDTA A851)
UV-visible spectrophotometer	Thermospectronic Genesys 10 UV scanning

3.2 Experimental

Figure 3.1 presents the overview of this research, and the details of each steps were explained in the next page.

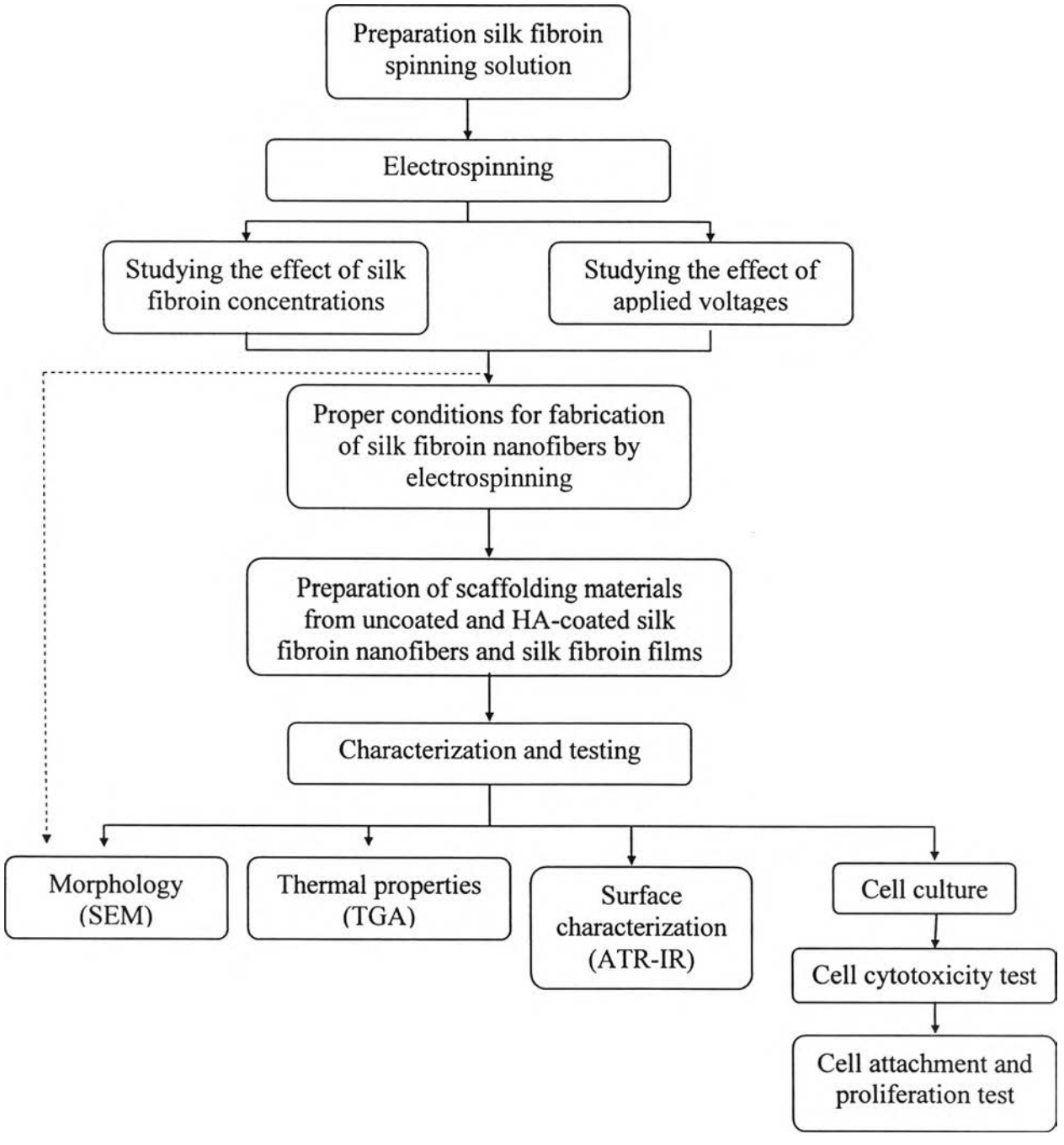

Figure 3.1 Overview of this research

3.2.1 Preparation of silk fibroin (SF) spinning solution

B.mori silk cocoons were boiled in water for 4 h. After that, they were degummed with 0.5% (w/v) Na_2CO_3 solution at 100°C for 30 min three times and then rinsed with warm distilled water. 15 g of degummed silk fibers were dissolved in a ternary solvent system of $CaCl_2/CH_3CH_2OH/H_2O$ (1:2:8 in molar ratio) at 80°C yielding a 6 % (w/v) solution for 3 h. The SF solution was dialyzed against dH_2O with cellulose tubular membrane for 4 days, the SF solution was filtered and lyophilized (freezed drying) to obtain the regenerated SF sponges. SF sponges were kept in oven at 100 ^{o} C overnight. SF solutions for electrospinning were prepared by dissolving the regenerated SF sponges in 98 % formic acid for 3 h. Concentrations of SF solutions were in the range from 44% to 52% w/v.

3.2.2 Preparation of electrospun silk fibroin fibers

For the electrospinning process, SF solution was added into a 3 ml syringe where its opening tip was connected to a nozzle made from a 1 cm long blunt end stainless-steel needle gauge 20 (0.9 × 40 mm). The syringe was tilted ca. 45° from a horizontal baseline and the positive charges were applied to SF spinning solution. A rotating drum (width and outer diameter = 14 and 15 cm, respectively) was used as a collector. The applied voltages were applied to a droplet of silk fibroin solution at a tip of a syringe needle ranging from 15 to 30 kV. A charge jet of the solution was ejected and the solvent was evaporated into the air to obtain the dried fibers on the ground collector, which was placed at a distance of 15 cm from the syringe tip. The electrospinning process was carried out under hood at room temperature. Morphology of the electrospun fibers were examined by SEM to find the appropriate concentration and voltage for obtaining the fine fibers without beads on the fibers.

3.2.3 Preparation of silk fibroin scaffolds

To investigate the ability of scaffolds on the cell responses, three forms of scaffolds, uncoated and HA-coated electrospun SF fibers were prepared.

For the electrospun SF fibers, the scaffold was prepared by the electrospinning process. When obtaining the proper conditions for producing the fine fibers without beads, the SF fibers were spun by the electrospinning process for 6 h. Then, the as-electrospun SF nonwoven was kept in vacuum oven for 24 h. The scaffolding materials were then fabricated by plunging nonwoven with punch (14 mm in diameter). After that, the scaffolding materials were immersed in methanol for 1 h to insolubilize them and then dried under vacuum. Morphology of the scaffolding materials were examined by SEM.

To prepare hyaluronic acid (HA) coated SF as scaffolding materials, 1 % HA (M.W. = 1,000,000) powder was dissolved in an 80:20 (v/v) ethanol : water mixture, with the addition of 0.01 N HCl and 250 mM glutaraldehyde. The as-electrospun SF fiber scaffolds were immersed in HA solution at 37°C for 24 h and then dried under vacuum for 24 h.

For the preparation of scaffolding materials in the film form, the 2 % (w/v) SF solution was prepared by dissolving 2 g of SF sponges in 98 % formic acid. Then this solution was poured onto polypropylene plates and maintained at room temperature to allow the complete evaporation of 98% formic acid overnight. Then, after completely drying, these films were cut out with punch(14- mm in diameter). The films were also immersed in methanol for 1 h and then dried under vacuum.

3.2.4 Keratinocyte and fibroblast cell culture and testing of cell response onto scaffolding materials

Three cell types used in this research were consisted of the mouse fibroblast cell lines (L929), human foreskin fibroblasts (HFF) primary cells, and immortalized human keratinocyte cell line HaCaT. The invidual cell types were cultured by seeding on 60-mm Petri dish and allowed to proliferate in Dulbecco's Modified Eagle Medium (DMEM) ,supplemented by 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate and amphopericin B] as culture medium. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and the 3 ml of culture medium were changed every 3 days.

3.2.4.1 Cell cytotoxicity test

In this research, to study the cytotoxicity of uncoated (as-prepared) and hyaluronic acid coated fibers and film scaffolds, indirect cytotoxicity test was used to examine these scaffolds. For indirect cytotoxicity test, the scaffolds are not direct contact with the cells. This test was used to verify the toxicity and compatibility between cells and the scaffolds before the interesting materials will be used as potential skin scaffolds for approval of medical devices. L929 cell lines were used to study cytotoxicity test of all scaffolds. This test was mandatory according to ISO 10993-5 standard method. The wells without scaffolds were used as the controls in this research. For preparing the extraction media, first, individual scaffolds(n = 3) were placed into each wells of 24-well cell culture plate and then sterilized by addition of 70% ethanol for 30 min followed by rinsing in dI-H_2O twice. After that, the individual scaffold pieces were added with 350 μL of serum-free medium (SFM;containing DMEM, 1% L-glutamine, 1% antibiotic and antimycotic formulation) to immerse the of scaffolds. On the other plate, when the cells were approximately 80% confluence on 60-mm Petri dish, they were spilt with trypsin/EDTA and counted these cells with haemocytometer. The counted cells were seeded at a density of 4×10^4 cells/ml into wells of another 24-well plate including control followed by addition of culture medium to total volume of 350 μl and then incubated for 16 h at 37 ° C, 5% CO_2 to allow cell attachment. The culture media was removed then immersed with SFM for starving cells. After 3 h, the cells were added fresh SFM instead of immersed SFM for 24 h. After 24 h, these SFM were replaced with extraction media for 24 h in incubator. After 24 h, the cytotoxicity of cells in the extraction media was determined by MTT assay.

MTT assay was applied in this study to quantitatively assess the number of viable cells attached and grown on tested scaffolds. Briefly, 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution in DMEM without phenol red were added to each scaffolds, followed by incubation at 37 °C for 10 min to 30 min (this time depends on cell type). After incubation, MTT formazan crystals were formed , and then MTT solution were removed and washed the remained solution with PBS. For dissolving the formazan crystals, DMSO-glycine buffer solution was used. The optical density (OD) or absorbance at 570 nm was determined against DMSO-glycine buffer

solution blank using UV-visible spectrophotometer. The OD represents the numbers of viable cell on scaffolds. Three parallel replicates were read for each sample.

3.2.4.2 Cell attachment and proliferation test

Cell attachment test was used in this research to investigate the ability of cell to attach on all prepared scaffolds. These scaffolds have to sterilize before testing as previously explained in 3.2.4.1. HFF and HaCaT cells were used in this test. Individual cells were seeded onto these scaffolds for 2, 4 and 6 h, respectively and then incubated at 37 °C in an atmosphere of 5% CO_2. After specific time, the cells in 24-well plate were determined the cell attachment by MTT assay. On the other hand, for cell proliferation test, the cells were seeded onto the scaffolds similar to the attachment test but incubated in an incubator for 1, 2, and 3 days, respectively. After specific day, the ability in cell proliferation was determined by MTT assay. In all cases, the wells without scaffolds were used as the controls.

3.2.5 Preparation of scaffolds for SEM

The scaffolds were washed twice by phosphate buffered saline (PBS) and immersed in PBS containing 3% glutaraldehyde (pH 7.4) for 30 min. They were then rinsed with PBS again and dehydrated in increasing concentrations of ethanol (from 30%, 50%, 70%, 90%, to 100%) for 2 min at each concentration, followed by immersing in HMDS. Then these scaffolds were dried in the air before being coated with gold for SEM analysis.

3.3 Characterization and testing

3.3.1 Scanning electron microscope (SEM)

Surface morphology and fiber diameter was investigated by scanning electron microscopy. The dried samples were mounted on aluminum stubs, sputter-coated with gold, and examined by JSM-5410LV SEM with an accelerating voltage of 15 kV.

3.3.2 Attenuated total reflection (ATR)-Fourier transform infrared spectrophotometer (FTIR)

FT-IR was performed using a Nicolet NEXUS 670 spectrophotometer in the spectral region of 400-4000 cm^{-1}. The solid samples (hyaluronic acid powder) were prepared by pressing the samples with KBr and spectra was collected by FT-IR. As-electrospun SF fibers and hyaluronic acid coated SF fibers FTIR spectra were collected in transmittance mode by using ZnSe ATR crystal cell with an attenuated total reflectance fourier transform (ATR-IR). The spectra of all samples were recorded with an accumulation of 32 scans and a resolution of 4 cm^{-1}.

3.3.3 Thermogravimetric analysis (TGA)

TGA was performed using METTLER TOLEDO (TGA/SDTA A851). The as-electrospun and HA coated SF fibers samples with approximately 3 mg. were heated at a constant rate of 10 °C/min under nitrogen atmosphere from 60 to 1000 °C. All samples were run in duplicate.