CHAPTER III

EXPERIMENT

The study of electrospun silk fibroin fibers and Schwann cell culture can be divided into four steps:

1. Preparation of silk fibroin solution
2. Preparation and characterization of silk fibroin fibers via electrospinning
3. Preparation of silk fibroin scaffolds
4. Investigation of Schwann cell culture on silk fibroin scaffolds.

The details of the experiments were explained as follows.

3.1 Materials

The reagents and solvents used in this experiment were of analytical grade. Tables 3.1 through 3.3 list all the chemicals and instruments including their suppliers and models used in this research.

Table 3.1 Chemical reagents and suppliers used for preparation of electrospun silk fibroin fibers

Reagents and Solvents	Supplier
Silk fibers	Saraburee province
Sodium carbonate (Na_2CO_3)	LABSCAN ASIA CO., LTD
Ethanol (CH_3CH_2OH)	LABSCAN ASIA CO., LTD
Calcium chloride ($CaCl_2$)	LABSCAN ASIA CO., LTD
98 % Formic acid (HCOOH)	APEX CHEMICAL CO., LTD
Methanol (CH_3OH)	LABSCAN ASIA CO., LTD
Dialysis tubing cellulose membrane	ITALMAR (THAILAND) CO., LTD

Table 3.2 Chemical reagents and suppliers used for preparation of Schwann cell culture

Reagents and Solvents	Supplier
Schwann cells (Rat: RT4)	American Type Culture Collection (ATCC® US.)
Dulbecco's modified Eagle's medium (DMEM)	GIBTHAI CO., LTD
Fetal bovine serum (FBS)	GIBTHAI CO., LTD
Phosphate Buffered Saline (PBS)	GIBTHAI CO., LTD
Dimethylsulfoxide (DMSO)	GIBTHAI CO., LTD
Trypsin	GIBTHAI CO., LTD
3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) or MTT solution	GIBTHAI CO., LTD
L-glutamine	GIBTHAI CO., LTD
Penicillin streptomycin (antibiotic)	GIBTHAI CO., LTD
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid or HEPES (Buffer)	GIBTHAI CO., LTD
Hexamethyl disilazane (HMDS)	GIBTHAI CO., LTD

Table 3.3 Instruments used for characterization

Instruments	Model
Thermogravimetric Analysis (TGA)	METTLER TOLEDO (TGA/SDTA A851)
Fourier Transform Infrared Spectroscopy (FTIR)	Thermo Nicolet Nexus 670 Spectrophotometer
Scanning Electron Microscopy (SEM)	JSM-5410LV SEM scanning microscope.

3.2 Experimental Details

3.2.1 Preparation of silk fibroin solution

Bombyx mori silk cocoons were boiled in water for 4 h then degummed by boiling in an aqueous solution of 0.5 % (w/v) sodium carbonate (Na_2CO_3) for 30 min three times, and then rinsed with warm distilled water to get rid of sericin proteins. 15 g of degummed silk or fibroin fibers were dissolved in a ternary solvent system of $CaCl_2$ / CH_3CH_2OH / H_2O in molar ratio of 1:2:8, respectively, at 80 °C for 3 h. The yielding solution of 6 % (w/v) was dialyzed in distilled water using dialysis tubing cellulose membrane for 4 days. After filtration, the solution was lyophilized or freeze dried to obtain silk fibroin sponges. The silk fibroin sponges were kept in an oven at 100 °C overnight.

To prepare silk fibroin solution, the silk fibroin sponges were dissolved in 98% formic acid with the concentrations ranging from 42 to 52 % (w/v) for 3 h.

3.2.2 Preparation of electrospun silk fibroin fibers

In the electrospinning process, a high electric potential was applied to a droplet of silk fibroin solution at a tip of a gauge 20 syringe needle [outside diameter (OD) = 0.9 mm, length = 40 mm]. The concentrations of silk fibroin solutions and applied voltages were varied from 42 to 52 % (w/v) and 15 to 30 kV, respectively. A charged jet of the silk fibroin solutions was ejected and the solvent was evaporated into the air to obtain dried fibers on the ground collection screen, which was placed at a distance of 15 cm from the syringe tip. The process was carried out at room temperature in a chemical hood. Morphological appearance of the electrospun fibers were examined by SEM to find the appropriate concentration and voltage for obtaining ultrafine fibers without the presence of beads.

3.2.3 Preparation of silk fibroin scaffolds

To determine how the surface of scaffolds affect the cell response, two types of scaffolds were prepared, i.e., the electrospun fibrous mats and the solution-cast films.

The silk fibrous scaffolds were prepared by the electrospinning process, based on the optimized condition that resulted in the formation of ultrafme fibers without the presence of beads. After a high electric potential (Gamma High Voltage Research D-ES30PN/M692) was applied to the solution, the electrospun fibers were collected on a grounded rotating drum (length and diameter of the drum = 14 and 15 cm, respectively; rotational speed = 50 round per minute) for 6 h at the feed rate of the solution of about 0.5ml/h. Then, the residue solvent of electrospun fibers was completely evaporated under vacuum for 24 h. The electrospun fibers were immersed in methanol for 1 hr to convert the amorphous structure of silk into β-sheet conformational transition and then dried under vacuum. Morphological appearance of the electrospun fibers was examined by SEM.

The silk fibroin films were fabricated by solution casting technique. The silk fibroin sponges were dissolved in 98 % formic acid to obtain 2 % (w/v) silk fibroin solution concentration. The solution was cast on polypropylene plates and then left in the air for 24 h. The films were also immersed in methanol for 1 h and then dried in air.

3.2.4 Schwann cell culture on silk fibroin scaffolds

Schwann cells (RT4) were cultured onto a 25 cm^3 T-flask in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), supplemented by 2% L-glutamine, 1% penicillin streptomycin (antibiotic), and 1% HEPES (buffer). The cultures were incubated at 37 °C in a humidified atmosphere including 5% CO_2. Two types of scaffolds, mats and films, were cut out into circular discs (7 mm in diameter) with a hole puncher and sterilized in 70% ethanol for 30 min, then washed with phosphate buffered saline (PBS) and autoclaved de-ionized water, respectively. After that, four circular disc scaffolds of the mat and film samples were placed into the bare wells of the 96-wells culture plate and immersed in DMEM overnight. The circular disc scaffolds were pressed with metal rings (about 5 mm in diameter) to ensure a complete contact between the scaffolds and the wells. Schwann cells from the cultures were trypsinized, counted by a hemotometer, and seeded as a monolayer at a density of about 10,000 cells/well on the scaffolds and four empty wells of 96-well culture plate that were used for controls. Metal rings were also deposited in the empty wells. Finally, the culture plate was maintained in an incubator

at different culture time intervals. The experiment for each sample was carried in triplicate.

3.2.4.1 Quantification of viable cells (MTT assay)

The MTT assay measures the viability of cultured cells. The reduction of tetrazolium salts is now widely used as a reliable way to examine the amount of viable cells. The yellow tetrazolium salt of MTT solution or 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells through a reaction with dehydrogenase enzymes secreted from the mitocondria of the cells into the purple formazan crystals. The amount of crystals so generated is proportional to the metabolically-active cells and, under normal condition, is proportional to the number of viable cells. The amount of the crystals is conveniently quantified by spectrophotometric means.

First, 10 µl of MTT reagent (5 mg MTT dissolved in 1 ml of DMEM) was added into the wells of 96-wells culture plate which contained viable cells on the scaffolds or on the control including DMEM. The culture plate was kept in an incubator for 2 h, then the purple formazan crystals formed was visible. Secondly, the MTT solution and DMEM were clearly removed, then 100 µL of detergent reagent or dimethylsulfoxide (DMSO) was added into the wells to dissolve the formazan crystals, and left at room temperature in the dark for 2 h on a rotary shaker. Finally, the absorbance at 570 nm was recorded by a Thermo Labsystems (Multiscan Ex) spectrophotometer, which represents the proportion of the viable cells.

3.2.4.2 Cytotoxicity tests between Schwann cells and the scaffolds

There are two method, indirect and direct, for studying about the toxicity and compatibility between cells and the scaffolds.

For indirect cytotoxicity test, Schwann cells were not seed directly on the scaffolds, but they were seed onto empty wells of 96-well culture plate, after which they were cultured with extraction medium (i.e., DMEM that was used to extract any dissolvable or leachable components from the scaffolds, after the scaffolds were submerged in it for a period of 24 h). First, 12 wells of the 96-well culture plate were divided into three sections. Schwann cells were seeded on these wells with

DMEM including 10% FBS. Extraction media were then prepared by immersing the silk fibroin fibrous mat and film scaffolds in serum free medium (DMEM without FBS) for 24 h in incubation. Then, the medium in 4 of wells was replaced with the serum free medium without extraction from the scaffold (control). The medium in 8 of other wells was replaced with extraction medium from the fibrous mat scaffolds and the film scaffolds (each in 4 of wells). The cells were incubated further for 24 h, then the viability of the cells was quantified by MTT assay.

For direct cytotoxicity test, the Schwann cells were seeded directly on the scaffolds. First, after two types of the scaffolds and the control were immersed in DMEM overnight, the medium was removed and then the Schwann cells were seeded on the scaffolds by using DMEM with 10% FBS as the medium and further incubated for 24 h. Secondly, the medium was removed once more and then filled with serum free medium, incubated overnight again. Finally, the MTT assay was used to determine the viability of Schwann cells.

3.2.4.3 Schwann cell attachment and proliferation

To investigate the attachment of Schwann cells on the two types of the scaffolds, the samples and empty wells were seeded with Schwann cells in DMEM with 10% FBS and the cells were allowed to attach for 1, 2, 4, 8, and 16 h, respectively. Each sample was rinsed with fresh DMEM to remove unattached cells, prior to MTT assay. In the proliferation study, the seeded cells were allowed to proliferate for 1, 2, and 3 d, prior to MTT assay. It should be noted that, for attachment evaluation, the cells were seeded at 10,000 cells/well, while, for proliferation evaluation, the cells were seeded at 5,000 cells/well.

3.2.5 Preparation of the scaffolds coating for SEM

The scaffolds were washed twice by phosphate buffered saline (PBS ; pH 7.4) and immersed in 3% glutaraldehyde 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. Finally, the scaffolds were soaked in hexamethyl disilazane (HMDS) for 5 min followed by drying in the air.

3.3 Characterization

3.3.1 Thermogravimetric Analysis (TGA)

Thermogravimetric analyzer (TGA) was applied to determine the thermal decomposition temperature of electrospun fibers. A METTLER TOLEDO TGA / SDTA A851 was operated under nitrogen atmosphere at a heating rate of 10 ^{o}C/min from 50 to 1000 ^{o}C. The weight of electrospun fibers was about 1.8 mg.

3.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

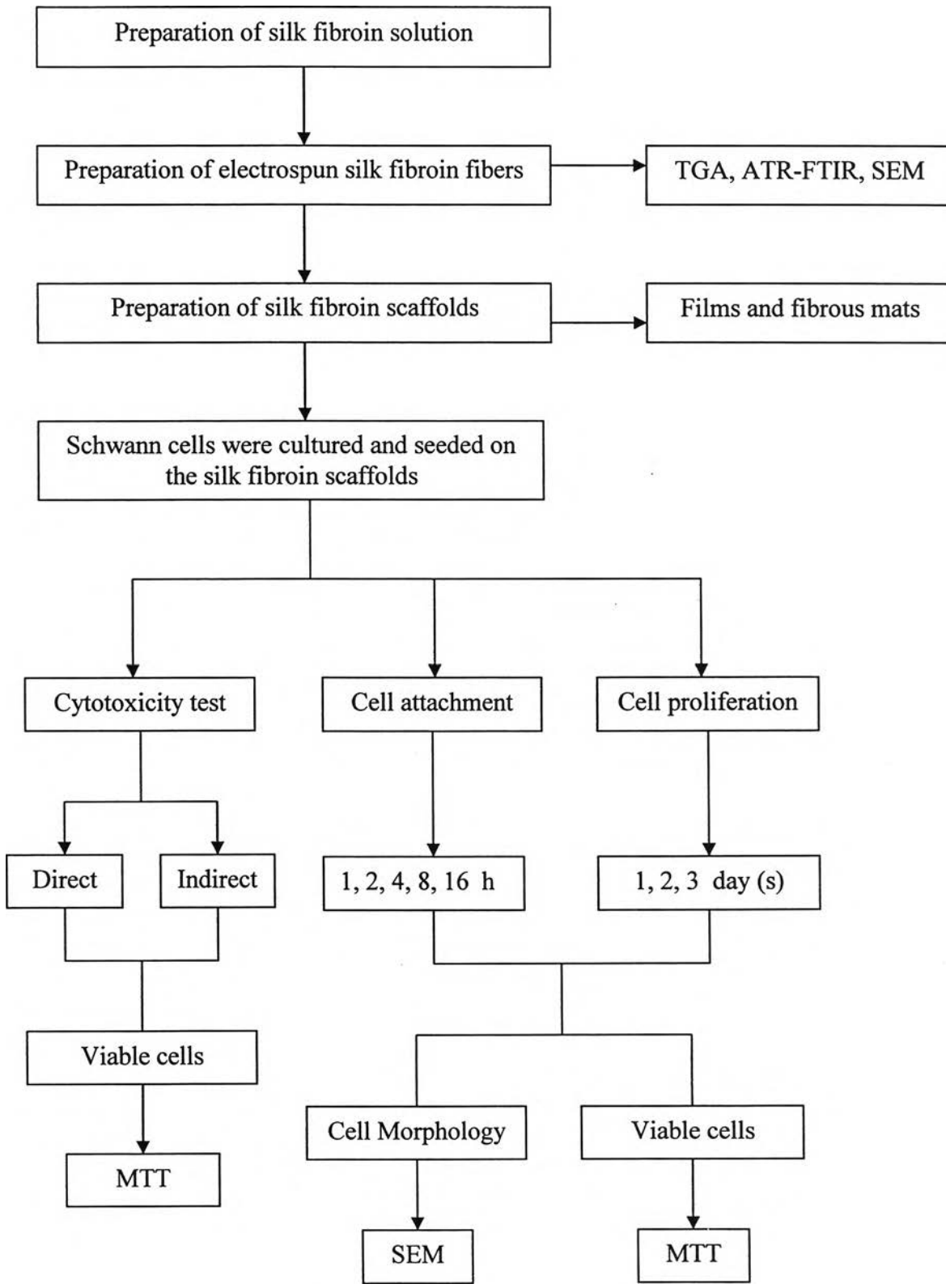
Infrared spectra of electrospun fibers were measured between 400 to 4,000 cm^{-1}, recorded with an accumulation of 32 scans and a resolution of 4 cm^{-1}. A Thermo Nicolet Nexus 670 Spectrophotometer was performed in transmittance mode by using ZnSe ATR crystal cell with an attenuated total reflectance fourier transform (ATR-FTIR).

3.3.3 Scanning Electron Microscopy (SEM)

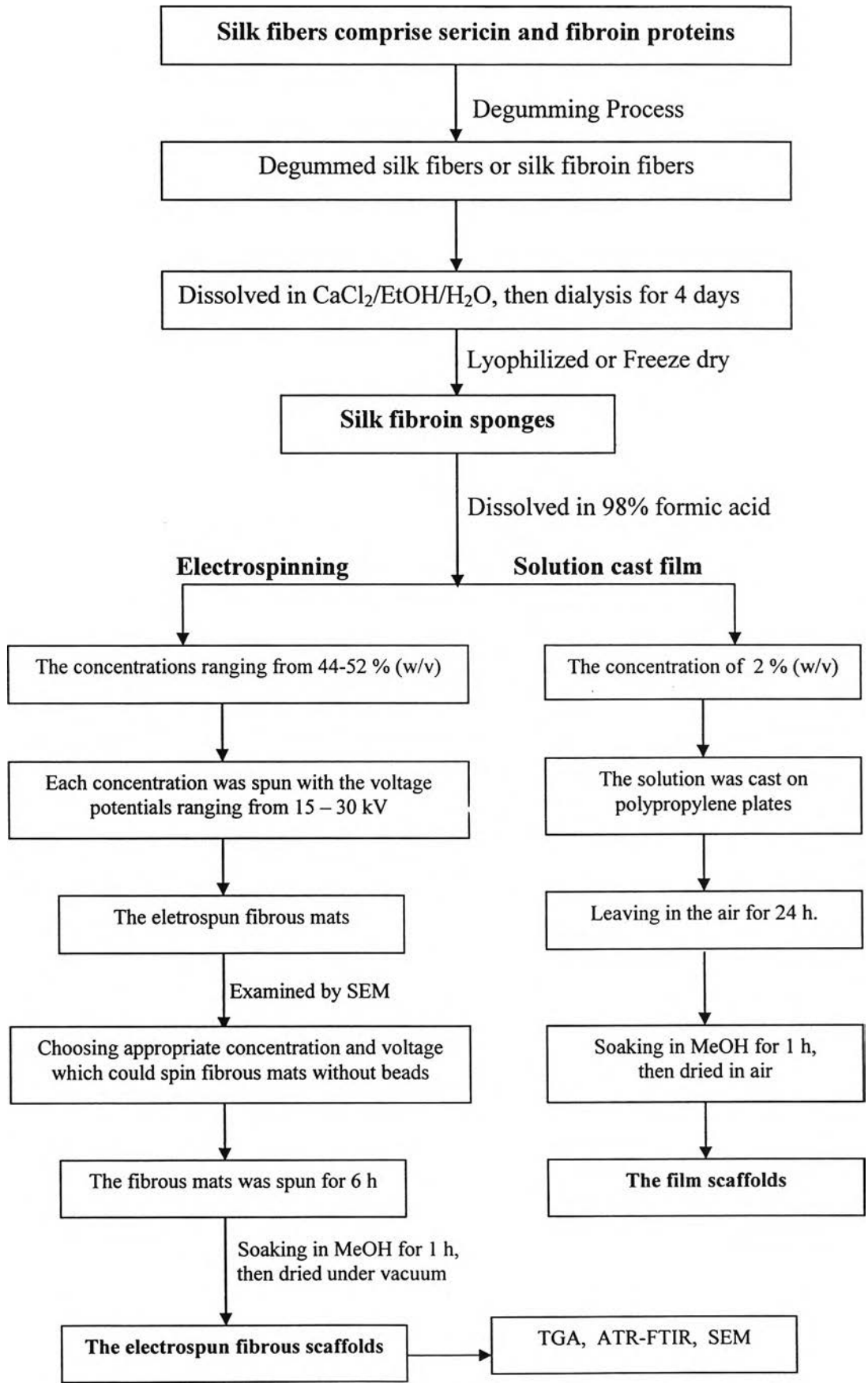
Surface morphology and diameters of electrospun fibers were investigated by using JEOLM-5410LV scanning electron microscope. The samples were sputter-coated with gold (Au) before investigating.

3.4 Flow Charts

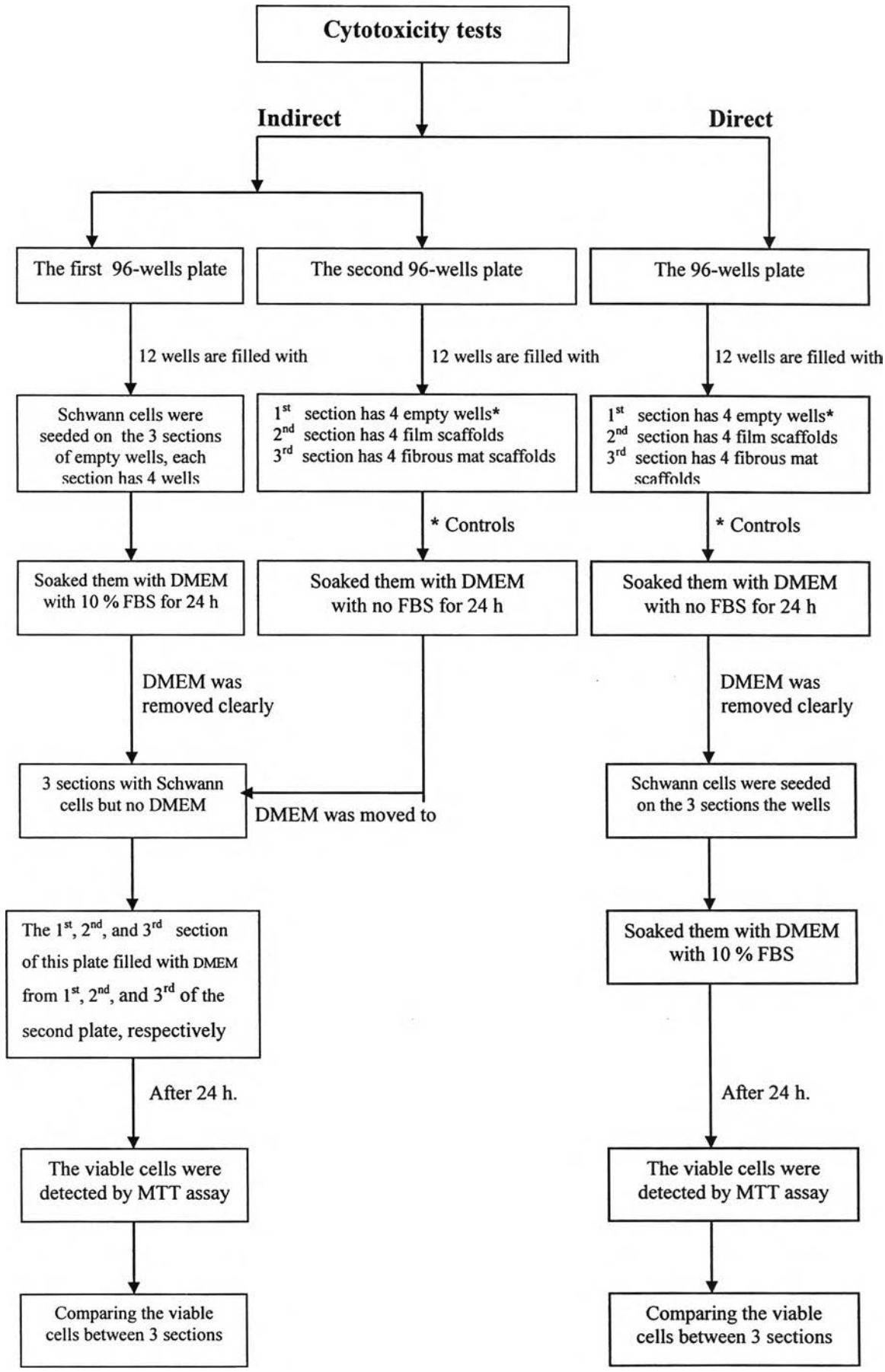
3.4.1 Experimental summary



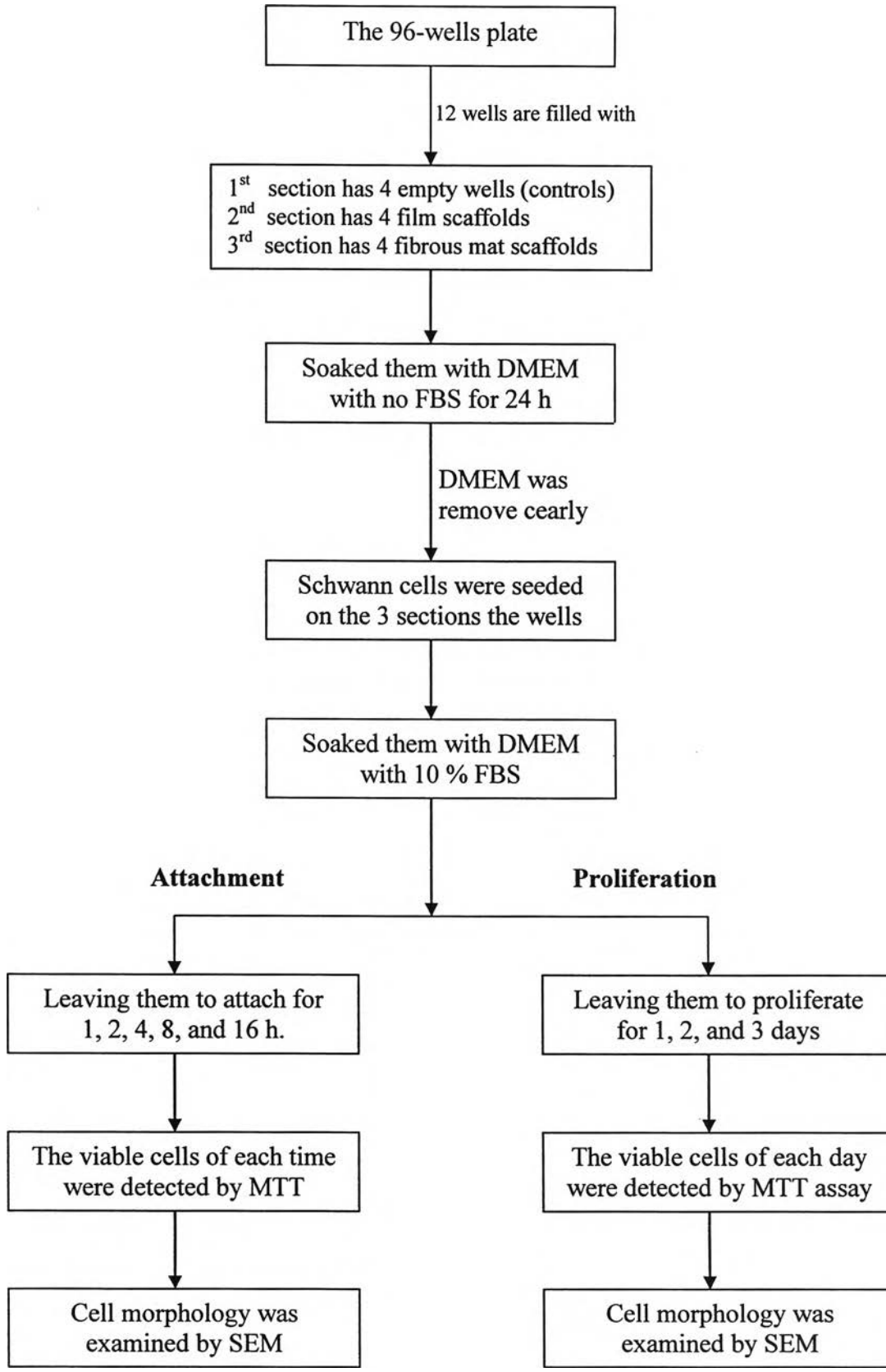
3.4.2 Scaffold fabrications



3.4.3 Cytotoxicity tests between Schwann cells and the scaffolds



3.4.4 Schwann cell attachment and proliferation



* Before MTT assay, the wells containing scaffolds including controls were rinsed with fresh DMEM to remove unattached cells.