CHAPTER III
EXPERIMENTAL

This work could be divided into two parts. The first was to investigate the effects of processing parameters including applied potential, polymer concentration, and distance between two electrodes on the morphology of electrospun chitosan fibers. The optimal condition for achieving uniformed shape and size of fibers were chosen as further investigation for in vitro nerve regeneration.

3.1 Electrospinning of Chitosan Fiber

3.1.1 Materials

Chitosan (M_n = 7.0 x 10^4; degree of deacethylation, 95%), trifluoroacetic acid (TFA, CF_3COOH), dichloromethane and glacial acetic acid (CH_3COOH) were obtained from Aldrich Chemical Co. Inc. (USA). NaOH and Na_2CO_3 were purchased from Sigma chemical Co. All chemicals and reagents were used without purification.

3.1.2 Electrospinning Setup

The experimental setup of electrospinning was shown in figure 3.1 The setup utilized in this study consisted of a 5 ml. glass syringe fitted with a needle of 0.5 mm in diameter (20-G), The needle was cut into a length of 1 cm. in advance. For controlling the feed speed by using the gravity force, the syringe was clamped at 45 degree from a horizontal baseline to a PVC stand. A high voltage supply, which can generate positive DC voltages up to 40 kV was connected between the needle tip and cylindrical collector cover with aluminum foil which could adjusted the rotating speed. The distance between the needle tip and the grounded collector was varied from 15-25 cm.

3.1.3 Preparation of Stock Solution for Electrospinning

Firstly, the effect of polymer concentration on the fiber morphology was study by dissolving chitosan in trifluoroacetic acid (TFA) at the concentration of 5, 6, 7 and 8% (w/v), stirred overnight at room temperature for in a glass bottle.

Secondly, solution of selected concentration and spinning condition were prepared in a mixture of trifluoroacetic acid (TFA) and dichloromethane (CM) at various ratios in order to increase the evaporation rate.

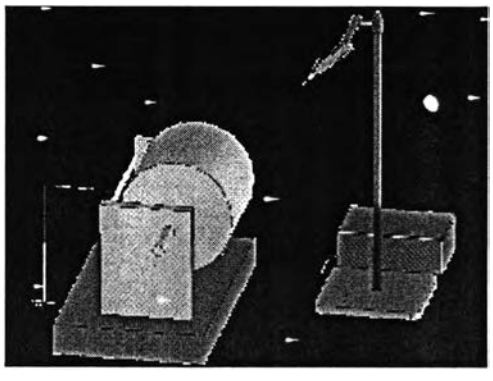

Figure 3.1 Experimental set up in electrospinning method.

3.1.4 Electrospinning Process

Five ml. of chitosan solution was placed into a 5 ml glass syringe fitted with a needle. The solution feed was driven by the gravity and the feed speed was controlled by the tilt angle of syringe to 45 degree. A high voltage of 15-25 kV was applied between the needle tip and cylindrical collector cover with aluminum foil. The distance between the needle tip and the grounded collector was varied from 15-25 cm. All the spinning experiments were performed at room temperature. The as-spun nanofibers were dried under vacuum at room temperature. The morphology of as-spun nanofiber was examined with a SEM (JOEL JSM-5200) at an accelerating voltage of 15 kV. The average diameter of electrospun nanofibers was determined by measuring the diameter of the nanofibers at 100 different points in the SEM images. The diameters were present as the average±standard deviation. The as-spun fibers were dried under vacuums at room temperature overnight.

In this work, we present an approach based on the neutralization process to improve the solubility of nanofibrous chitosan matrix by immersing chitosan fibers into in two neutralization system; 5 M of Na_2CO_3 and and 5 M NaOH solution for 3 h. After the immersion, chitosan nanofibers were repeatedly washed with distilled water until neutral pH obtained. And then, let dried at 40 °C in vacuum

oven. The as-spun chitosan mats obtained from the optimum condition with 20 μm in thickness were used in this experiment. Prior to cell culture studied, the scaffolds were sterilized by 70% alcohol for 30 min.

3.1.5 Preparation of Chitosan Membranes

For comparing the effect of surface topology on the cell culture studied, chitosan film was used. Two gram of chitosan was dissolved in 100 ml of 1% acetic acid solution and stirred until completely dissolved. Then the solution was spread on glass dishes and dried at room temperature. After the membranes had dried, the membranes were immersed in 5M NaOH solution for 3 h to neutralize the salt form in the chitosan structure and washed with distilled water until neutral pH was obtained and then, let dried at room temperature. The thickness of a dried membrane was 20 μm. Prior to cell culture studied, the scaffolds were sterilized by 70% alcohol for 30 min.

3.1.6 Characterization of as-Spun Fiber

To investigate the morphological appearance and size of as-spun fibers, a JOEL JSM-5200 scanning electron microscopy (SEM) was used. Three samples from each spinning condition were coated with gold by a JEOL JFC-1100E sputtering device for 3 min before observed under SEM at an accelerating voltage of 15 kV. The average diameter of electrospun nanofibers was determined by measuring the diameter of the nanofibers at 100 different points in the SEM images. The fiber diameters were present as the average±standard deviation.

To evaluate the effectiveness of neutralization process, FT-IR Spectroscopy was used to verify the chemical structure of as-spun chitosan nanofibers before and after the neutralization process. The measurements were carried out on a thermo Nicolet Nexus 671 FT-IR. The measurements of the packing ability were carried out at room temperature with a wide-angle X-ray diffractometer (Rigaku Rint2000). The diffraction scans were collected at 2θ = 5-90° with a speed of 5°/m

Additionally, the long term weight loss and the degree of swelling of as-spun nanofibers were investigated compared with neutralized chitosan membrane. Both chitosan film and chitosan fibers with an exact dry weight were submerged in

distilled water for 48 h at room temperature before evaluated their weight loss and swelling behavior. The weight loss (%) of each samples were calculated according to the following equation;

$$\text{Weight loss}\,(\%) = \frac{(W_b - W_d) \times 100}{W_d}$$

Where W_b is the weight of dried sample prior to submersion in distilled water and W_d is the weight of the dried sample after submersion at a given time.

The degree of swelling was evaluated by gravimetric method. Each sample was taken out from the water bath and placed between two pieces of tissue paper under 0.3 kg weight of metal for removing excess water. The degree of swelling (%) of each sample was calculated according to the following equation;

$$\text{Degree of swelling}\,(\%) = \frac{(W_s - W_d) \times 100}{W_d}$$

Where W_s and W_d are the weight of sample in swollen state and the weight of the dried sample after submersion at a given time, respectively.

3.2 Schwann Cell Culture Studies

3.2.1 Cell and Cell Culture

To carry out the *in vitro* experiments, Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA) and L-glutamine was purchased from Invitrogen Corp., USA. Trypsin (0.25% trypsin/0.02% EDTA, Penicilin-streptomycin (antibiotic), and Fetal bovine Serum (FBS) were purchased from BIOCHROM AG, Germany. Dimethyl Sulphoxide (DMSO) and MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich. Tissue culture dishes were purchased from Corning (RCM, Thailand).

Two types of cells were used in this study: 1) mouse connective tissue, fibroblast-like L929 cells line and 2) Schwann cell line RT4-D6P2T. When the cells reached 80% confluence, they were serially subcultured. Both cell types were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, USA), supplemented with 5% fetal bovine serum. The cells were incubated at 37 °C in an atmosphere containing 5% CO_2.

3.2.2 Cytotoxicity Tests

The indirect cytotoxic evaluation of the culture scaffolds were in adaptation from the ISO10993-5 standard test method, using cell line L929 and Schwann cell line RT4-D6P2T as reference cells. The culture films and fiber were submerged in fresh culture medium and were incubated for 24 hours. In the preparation of reference cells, the cells were seeded onto a 96-well plate at a density of 1.0×10^4 cells per well and incubated in 5% serum media. After incubation for 24 hours, the culture medium was removed and the as-prepared extraction media were added to the culture wells. The cells were incubated further for 24 hours, after which time the number of living cells was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays and bare culture wells were used as controls

3.2.3 Cell Adhesion Tests

The cell adhesion evaluation of culture electrospun chitosan fibers, chitosan film, and PLLA film were conducted in 96-well culture plates, using Schwann cells line RT4-D6P2T as reference cells. Cells were seeded within 96-well culture plates that covered with the substrates at 1.0×10^4 cells per well and incubated in 5% serum media at 37 °C for 2, 4, 8, and 24 h. To ensure a complete contact between the culture films and the cells, the films were pressed with a metallic ring. After which time, the culture medium was removed and the seeded culture films were washed twice with a sterile phosphate buffer saline (PBS) solution to eliminate free cells. The number of living cells was determined with MTT assay.

3.2.4 Cell Proliferation Test

The evaluation of cell proliferation on electrospun chitosan fibers, chitosan film, and PLLA film were conducted in 96-well culture plates, using Schwann cells line RT4-D6P2T as reference cells. Cells were seeded within 24-well

culture plates that covered with the scaffolds at 1.0×10^4 cells per well and incubated in 5% serum media at 37 °C for 1,3 and 5 days. After which time, the number of living cells was determined with MTT assay.

3.2.5 MTT Assays

The3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. The method was performed MTT assay included; the old culture medium in each well was removed and replaced with 10 µl. of MTT solution. Secondly, the plate was incubated for 2 hour at 37 °C until purple precipitate was visible. After 2 hour, all of solution was removed and replaced with DMSO at 100 µl per well and leave at room temperature in the dark for 2 hour. Finally, record absorbance at 570 nm microtiter plate reader. A one-way ANOVA was used to compare the means of different groups, and statistical significance was accepted at 0.05 confidence level.