CHAPTER III
METHODOLOGY

This work could be divided into two parts. The first was to investigate the effect of processing parameters of synthesis including surfactant concentration for adsorption isotherm and pyrrole monomer concentration. The optimal condition for achieving uniform shape and fiber size was chosen to further investigation for in vitro nerve regeneration.

3.1 Procedure Method

3.1.1 Electrospinning of Aligned and Random Scaffold

3.1.1.1 Materials used in the Fabrication of the Fibrous Scaffolds

- Poly(lactic acid), or (PLA) Mn~200,000 $gmol^{-1}$
- Choloform (Lab-Scan (Asia), Thailand)

3.1.1.2 Electrospinning Setup

The polymer solution with concentration of 10% w/v was prepared by dissolving PLA in chloroform and stirred for 4 hrs. at room temperature. The solution was electrospun from a glass syringe with 20-gauge stainless steel needle. A Gamma High Voltage Research DES30PN/M692 power supply was used to generate a fixed direct current potential of 15 kV. After that it was applied to the tip of the needle attached to the syringe and fluid jet is ejected. A rotating drum width and OD of the drum ≈ 15 cm; rotational speed = 1200 and 600 rpm was used to collect both aligned and random fibers as a collector. The distance between the needle and collector was 15 cm.

3.1.2 Polypyrrole Coating by Admicellar Polymerization

3.1.2.1 Materials used in Coating the Fabrication

- Pyrrole, or Py (reagent grades, 98 % purity)
- Dodecylbenzenesulfonic acid, sodium salt or DBSA
- Iron(III) chloride or $FeCl_3$ (reagent grades, 97 % purity)

All chemical and reagents were purchased from Sigma Aldrich chemical Co. Reagent grade pyrrole was purified by distillation for 4 hrs. and stored in a refrigerator at about 4°C before using (I. Cucchi *et al.*, 2009). Iron(III) chloride was used without purification.

3.1.2.2 Determination of Surfactant Adsorption Isotherm

The adsorption of DBSA on alignment fibers were obtained by exposing 0.05 g piece of alignment fiber to 25 ml of DBSA solution with known initial concentration of pH = 4. The mixture was equilibrated at 30 °C for 15 hours in 32 ml vial. The amounts of DBSA in supernatant were measured by UV spectrometer at a wavelength of 224 nm. The initial DBSA concentrations in this experiment were varied from 10-6,000 μM which covered the region below and above the critical micelle concentration (CMC) of DBSA. A plot of equilibrium supernatant concentration versus the amount of adsorbed surfactant yielded is acquired, which is known as the surfactant adsorption isotherm.

3.1.2.3 Varies Pyrrole Monomer for Coating by Admicellar Polymerization

The admicellar polymerization of pyrrole monomer on PLA fibers were carried out using 0.8 mM (below CMC) aqueous DBSA solution at pH 4 (adjusted by using HCl) as surfactant with pyrrole monomer concentrations. DBSA:Py monomer molar ratio were varied in the range of 1:2, 1:4, 1:6, 1:8, 1:10 and 1:12 respectively. Ferric chloride : oxidant ratio was fixed at ratio 1:1. The 25 cm^2 square electrospun fibers were placed in the test tube containing both surfactant and monomer solution. Then the test tube will be placed in the shaking bath at 30 °c for 15 hours to allow admicellar formation and monomer adsolubilization into the admicelle. Total volume of the solution was 100 mL. The oxidant was added into the test tube and placed in the shaking bath at 30 °c for 4 hrs for polymerization to take

place (Lekpittaya *et al.*, 2003). The PPy-coated PLA fibers were then removed from the test tube and washed 3 times by distilled water, to remove the upper layer of DBSA and leave the polymer exposed. Finally the PPy-coated PLA fibers were placed in room temperature overnight to evaporate the remaining water on the surface as well as any non-polymerized monomer.

3.1.2.4 Immobilization of Bioactive Molecules on PPy-coated Aligened PLA Fibers

Subsequently PPy-coated aligned PLA fibers at ratio 1:8, the PPy-coated aligned PLA fibers were immersed in (N-morpholino) ethanesulfonic acid (MES) buffered solution (0.10 M, pH 5.0) contained of 5 mg/mL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 5 mg/mL of N-hydroxysuccinimide (NHS) for 1 h at 37 °C. The PPy-coated aligned PLA fibers were then rinsed with MES buffer and immersed in laminin solution (1 μg/mL) for 48 h at 4 °C. The bioactive molecules were immobilized through a condensation reaction between the amino or ester carbonyl group on the fibrous surface and functional group of bioactive molecules. Covalently bound laminin/ PPy-coated aligned PLA fibers were then rinsed thoroughly with sterile 0.10 M PBS. The samples were kept sterile for cell culture studies or dried completely for characterization.

3.1.3 Testing and Characterization

3.1.3.1 Surface Morphology of Fibers

The morphology of the PLA fibers and PPy-coated PLA fibers were observed by a JEOL JSM-5200 scanning electron microscopy (SEM). Each specimen was coated with a thin layer of gold by using a JEOL JFC-1100E ion sputtering device prior to SEM observation. The average diameters of as-spun fibers were determined by measuring the diameters of fibers at 100 different points in the SEM images at 1500x magnification with SemAphore 5.0 Software. The diameter were presented as average ± standard deviation. Moreover, The degree of fiber alignment was characterized by measuring the mean fiber angle from 5 SEM images. These values were then normalized to 90 degrees and plotted in a histogram. Closer to 90 degrees indicates more alignment.

3.1.3.2 Surface Conductivity

The surface conductivity of the PPy-coated PLA fibers and laminin coated on (PPy-coated aligned PLA fibers) were measured at a vacuum chamber. The specimens were kept in this condition for more than 24 hrs before testing. The PPy-coated PLA fiber was placed between two probes (Two-Point Probe Meter, and Keithley Model 8009). These probes were connected to a source meter (Keithley, Model 6517A) for a constant voltage source and for reading current. The applied voltage was plotted versus the current change to determine the linear Ohmic regime of each sample. The applied voltage and the current change in the linear Ohmic regime were converted to the electrical conductivity of PPy-coated PLA fiber using equation (3.1) but the elastomers and elastomer blends using equation (3.2) as follow:

$$\sigma = \frac{1}{\rho} = \frac{1}{R_s \times t} = \frac{I}{K \times V \times t} \qquad (3.1)$$

where σ is specific conductivity (S/cm), ρ is specific resistivity (Ω.cm), R_S is sheet resistivity (Ω), I is measured current (A), K is geometric correction factor, V is applied voltage (voltage drop, V), and t is fiber mat thickness (cm).

$$\sigma = \frac{1}{\rho} = \frac{t \times I}{22.9 \times V} \tag{3.2}$$

where σ is the specific conductivity (S/cm), ρ is the specific resistivity (Ω.cm), I is the measured current (A), V is the applied voltage (voltage drop) (V), t is the sheet thickness (cm).

The geometrical correction factor was taken into account for geometric effects, depending on the configuration and probe tip spacing and was determined by using standard materials where specific resistivity values were known; we used silicon wafer chips (SiO_2). In our case, the sheet resistivity was measured by using the two-point probe and then the geometric correction factor was calculated by equation (3.3) as follow:

$$K = \frac{\rho}{R \times t} = \frac{I \times \rho}{V \times t} \tag{3.3}$$

where K is the geometric correction factor, ρ is the known resistivity of standard silicon wafer (Ω.cm), t is the film thickness (cm), R is the film resistance (Ω), and I is the measured current (A).

3.1.3.3 Water Contact Angle Measurements

A contact angle goniometer (KRUSS Gmbh Germany; Model: DSA 10-Mk2 T1C) equipped with a Gilmont syringe and a 24-gauge flat-tipped needle was used to determine contact angles of a water drop on the surfaces of both the PLA fibers, Polypyrrole (PPy)-coated PLA fibers and laminin coated on (PPy-coated aligned PLA fibers). The measurements were carried out by the sessile drop method in air at room temperature in centuplicate on different areas of each sample.

3.1.3.4 Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR)

Chemical functional groups that were present on the surfaces of both the PLA aligned fibers, PPy-coated PLA alignment fibers and laminin coated on (PPy-coated aligned PLA fibers) were analyzed by attenuated total reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR; Thermo Nicolet Nexus 670) at a resolution 3 cm^{-1} and 64 scans.

3.1.3.5 X-ray Photoelectron Spectrometer (XPS)

The surface analysis of the samples was carried out using a Thermo Fisher Scientific Theta Probe XPS. Monochromatic Al K(X-ray was employed for analysis of one spot on each sample with photoelectron take-off angle of 50°(with respect to surface plane normal). The analysis area was 400 µm * 400µm on the polymer sample surfaces. The maximum analysis depth lay in the range of ~4-8nm. A special designed electron flood gun with a few eV Ar^+ ion was used for the charge compensation. Electron beam and ion beam were focused and steered towards the analysis position. Survey spectra and high resolution scans were acquired for surface composition analysis. Further correction of binding energy was made by setting C-C/C-H components in C 1s peak at 284.6 eV using the manufacturer's standard software.

3.2 Biological Characterizations

To carry out the *in vitro* experiments, neuro 2a and neural stem cell response to the e-spun scaffolds was conducted on TCPS, Alignment and random PLA fibers, PPy-coated on aligned and random PLA fibers, and laminin coated on (PPy-coated aligned PLA fibers).

3.2.1 Materials Used for Cell Culture

3.2.1.1 Model Cells

Model cells in this study were neuro 2a and neural stem cell

3.2.1.2 Medium for Neuro 2a Culture

- Dulbecco's modified Eagle Medium(MEM-EBSS; Sigma Aldrich., (USA)
- L-glutamine, Trypsin (0.25 % trypsin/0.02% EDTA), Pencilinstreptomycin (antibiotic), Sodium pyruvate were purchased from Gibco Invitrogen Crop., USA.
- Fetal bovine Serum (FBS) was purchased from Thermo Scientific.,(USA)

3.2.1.3 Medium for Neural Stem Cell Culture

- Neuralbasal media, B27 and Epidermal growth factor (EGF) were purchased from Gibco Invitrogen Crop., USA.
- Basic Fibroblast Growth Factor (bFGF) was purchased from R&D System.
- Heparin was purchased from Sigma Aldrich., (USA)

3.2.2 Cytotoxicity Tests

The indirect cytotoxic evaluation of scaffold were in adapted from the standard test method, using cell Neuro 2a as reference cells. The scaffold fiber was submerged in a serum-free medium (SFM; containing MEM-EBSS, L-glutamine, Pencilin streptomycin (antibiotic)) and was incubated for 24 hrs. In the preparation of reference cells, the cells were seed onto a 96-well plate at a density of 1.0×10^4 cells per well and incubated in SFM to allow cell attachment on the plate. After 24 h, the culture medium was removed and the as-prepared extraction media were added to the wells. The cells were incubated further for 24 h, after which time the number of viable cells was quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; was purchased from Sigma- Aldrich) assay. The viability of the cells that were cultured with fresh SFM was used as the control.

3.2.3 Quantification of Viable Cells by MTT Assay

The MTT assay is the method used to quantify the amount of viable cells on the basis of 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. First, the culture medium of each cultured specimen was removed and replaced with 200 μL/well of MTT solution (Sigma-Aldrich) at 5 $mg \cdot mL^{-1}$ for a 96-well TCPS (or 500 μL/well for a 24-well TCPS), and then the plate was incubated for 1 h. After incubation, the MTT solution was removed. Then, 200 μL/well of dimethyl sulfoxide (DMSO; Riedel-de Haën,Germany) was added to dissolve the formazan crystals (or 500 μL/ well for a 24-well TCPS), and the plate was left at room temperature in the dark for 1 h on a rotary shaker. Finally, the absorbance at 570 nm, representing the proportion of viable cells, was recorded by a Thermo Labsystems (Multiscan Ex) spectrophotometer.

3.2.3 Cell Attachment Tests

The cell attachment evaluation of culture PLA fibers and PPy-coated aligned PLA fibers were conducted in 24-well culture plates, using neural stem cells as reference cells. Cells were seeded within 24-well culture plates that covered with the substrates at 4.0×10^4 cells per well and incubated in serum media at 37 °C for 4 hrs and 3 days. To ensure a complete contact between the fibers and cells, All fibers were pressed with glass ring. After which time, the culture medium was removed and seeded PBS twice times and the cells were fixed with 3% glutaraldehyde solution, which was diluted from 25% glutaraldehyde solution (Electron Microscopy Science) with PBS, at 500 μL/well. After 30 min, they were rinsed again with PBS and kept in PBS at 4 °C. After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentration (i.e., 30%, 50%, 70%, 90%, and 100%) for about 2 min at each concentration. The specimens were then dried in 100% hexamethyldisilazane (HMDS; Sigma-Aldrich) for 5 min and later dried in air after the removal of HMDS. After being completely dried, the specimens were mounted on copper stubs, coated with gold using a JEOL JFC-1100E sputtering device for 3 min, and observed by a JEOL JSM-5200 scanning electron microscope. For comparison, the morphology of the cells that were seeded or cultured on a glass substrate (cover glass slide, 1 mm in diameter, Menzel, Germany) was also investigated.

3.2.4 *In Vitro* Electrical Stimulation

Rat hippocampal neural stem cell were plated into cell wells assembled onto the PPy-coated aligned PLA scaffolds into 24-well tissue culture plates at densities of 4.0×10^4cells per well. Autoclaved glass ring with an inner diameter of 1.4 cm and an outer diameter of 1.5 cm was fitted into the wells of the tissue culture plate to eliminate any scaffold buoyancy. It was allowed to incubate for 24 hrs to permit attachment and spreading. After this initial 24 hrs period, the neural stem cells were subjected to a steady potential of 100 mV for 2 h. For electrical stimulation, a pair of stainless steel probes were inserted into each well as the anode and cathode with a gap of 0.5 cm, The tissue culture plates were then placed in a 5% CO_2 incubator at 37 °C with potential generator connected to each pair of probes in parallel configuraion. After electrical stimulation the scaffolds were extracted to investigate differential gene expression by using RT-PCR.

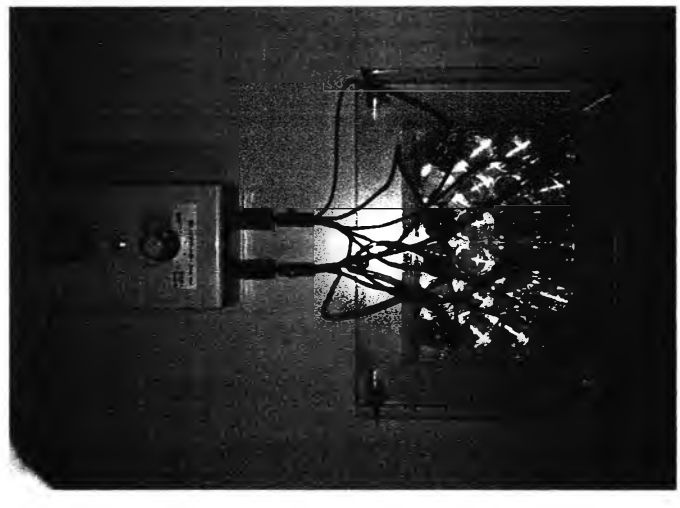

Figure 3.1 Measurement for electrical stimulation.

3.2.5 Real Time-Polymerase Chain Reaction (RT-PCR) Analysis

Rat hippocampal neural stem cell were cultured on the Laminin coated on (PPy-coated aligned PLA fiber meshes) for 1 day. RNA, extracted by Tri Reagent (Molecular Research Center), was harvested and pooled from four wells for each type of specimen. Chloroform (200 μL) was added to thehomogenized specimens to extract RNA, followed by precipitation using 500 μL of isopropyl alcohol (Sigma). RNA pellets were washed with 70% ethanol and were dissolved in 15 μL of nuclease-free water (Promega). RNA yields were evaluated by the UV-vis spectrophotometer based on absorbance at 260 nm. First strand DNA was reverse transcribed from 1 μg of total RNA using a RT kit (ImProm-II ReserveTranscription System, Promega). For the amplification in PCR, the PCR mixture consisted of 1 μL of cDNA, sense primer, antisense primer,and reagent of the PCR kit (Tag DNA Polymerase, Qiagen). The PCR oligonucleotide primers for c-Fos and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) Twenty-eight cycles were used to amplify both genes. The PCR products were analyzed by separation on 1.8% agarose (Usb) gel using electrophoresis (Power Pac Junior, Bio-Rad) and visualized with ethidium bromide (EtBr; Bio-Rad) staining. The stained bands were photographed under UV light, and the intensity was quantified with Scion Image Software.