CHAPTER III
EXPERIMENTAL

3.1 Materials

PHB (Mw=300,000) and PHBV (Mw=680,000) were purchased from Sigma-Aldrich (SM chemaical, Thailand). The content of hydroxyvalerate in PHBV was 5% mol. Chloroform ($CHCl_3$) was purchased from Lab-scan Asia, Thailand. To carry out the *in vitro* experiments, Dulbecco's Modified Eagle Medium (DMEM) and L-glutamine were purchased from JRH Bioscience, Inc. Trypsin (0.25% trypsin/0.02% EDTA, Penicilin-streptomycin (antibiotic), HEPES and Fetal bovine Serum (FBS) form Gibco. Dimethyl Sulphoxide (DMSO) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma-Aldrich. Tissue culture dishes were purchased from Corning.

3.2 Material Preparation

3.2.1 Electrospining

The experimental setup of electrospinning was shown in Figure 2.1 The setup utilized in this study consisted of a syringe and needle (20-G), a ground electrode, stainless steel sheet on a drum whose rotation speed can be varied, and a high voltage supply. The syringe was clamped at 45 degree from a horizontal baseline to a PVC stand and holed with heater band at 50°C. The needle was connected to the high voltage supply, which can generate positive DC voltages up to 40 kV. For the electrospinning of PHB and PHBV fibers, the solutions in chloroform were prepared in the concentration range of 10–16 %wt. The distance between the needle tip and the ground electrode was fixed in 20 cm and positive voltage applied to polymer solutions were in a range of 8–14 kV. The collected as-spun fibers were dried under vacuums at room temperature overnight. Before cell culture, the scaffolds were placed in 70% ethanol for 30 min to obtain sterility. The scaffolds were washed three times in phosphate buffered saline. The scaffolds were placed in culture medium overnight.

3.2.2 Film Casting

Glass Petri dishes were used to prepare polymer Films. To prepare the PHB and PHBV film, 5g of polymer powders were dissolved in 100ml chloroform under gentle stir and heat at 60°C, and then poured into petri dishes. The dishes were kept at room temperature to allow complete evaporation of the chloroform. The evaporation of the solvent resulted in the formation of PHA films on the petri dishes. Vacuum drying was used to completely remove any possible solvent remaining in the films as the solvent is toxic to the cell lines and may influence the results. Before cell culture, the scaffolds were placed in 70% ethanol for 30 min to obtain sterility. The scaffolds were washed three times in phosphate buffered saline. The scaffolds were placed in culture medium overnight.

3.3 Characterizations

The viscosity of the polymer solution in the range of 10–16 %wt was determined by using a Brookfield Digital Viscometer (model LVTDCP) at room temperature.

Conductivity of each polymer solution were determined five times and averaged by using a conductivity meter.

The morphological appearance of the as-spun fibers was observed by a JEOL JSM-5200 scanning electron microscope (SEM). The specimens for SEM observation were prepared by cutting an Al sheet covered with the as-spun fiber mat and the cut section was carefully affixed on a copper stub. Each specimen was gold-coated using a JEOL JFC-1100E sputtering device before being observed under SEM. Diameters of the as-spun fibers were measured directly form the SEM images, with the average value being calculated from at least 100 measurements. The average number of beads per unit area (i.e. the bead density) on as-spun beaded fibers was calculated from measurements on SEM images of 500x magnification, while the average number of fibers per unit area (i.e. the fiber density) of the obtained fibers was calculated from measurements on SEM images of 500x magnification.

Tensile tests were carried out at room temperature using a Lloyd universal testing machine according to the ASTM standard 1708. The machine was operated at a crosshead speed of 20 mm/min and an initial grip-to-grip separation of 50 mm

Contact angle were measured for the films and electrospun fiber mat with a sessile drop Method using a CAM-PPLUS TANTEC contact Angle Meter. Distilled water of approximately 40 µl was gently plated on the surface of the specimens. At least five reading on different parts of the specimens were averaged for data collecting.

3.4 Cell Culture and Seeding

PHB and PHBV fibers were cut into circular disc suitably sized for tissue culture plat well. The scaffolds were sterilized with 70% alcohol, 30 min and then rinse with PBS and sterile deionised water. The samples were kept in a culture medium prior to further use

Schwann cell line RT4-D6P2T (passage 14) were culture in Dulbecco's Modified Medium supplemented with 10% fetal bovine serum (FBS) and antibiotic (penicillin-streptomycin solution), incubated in a 37°C humidified incubator with 5%CO_2. After the cells reached 80% confluence, the cells were detached by trypsin-EDTA and viable cells were count by Trypan blue assay. Then the cells were seeded on scaffolds, plated in 96-well.

3.5 Morphology of Schwann Cells

The morphology of cells was examined with SEM (JEOL JSM-5200). The cell-seeded scaffolds were rinsed twice with PBS (pH 7.4), and fixed with 3% glutaraldehyde in 1% calcium chloride for 30 min at 5 °C. after wash in PBS, the cells seeded membrane were dehydrated consecutively in 30%, 50%, 70%, 90%, and 100% ethanol for 2 min. Further, substitution to hexamethyl disyloxane was done through the cells seeded membrane. Samples were then critically point dried and covered with a thin layer of gold–palladium through sputtering under an argon atmosphere prior to SEM observation.

3.6 Cytotoxicity Tests

The indirect evaluation of cytotoxicity of the culture scaffolds were conducted in adaptation from the ISO10993-5 standard test method. The indirect evaluation of cytotoxicity of culture scaffolds were conducted in 24-well culture plate (96-well culture plate), using two types of cells: 1) mouse connective tissue, fibroblast-like L929 cells and 2) Schwann cells, RT4-D6P2T as reference cells. Cells were seeded at 5×10^4 ($1x10^4$) cells per well and incubated at 37°C. After 24 hours. The old culture medium was replaced by a serum-free medium (SFM). The number of living cells and the total amount of proteins produced were determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). Bare culture wells were used as controls. The measurements were conducted after 24h.

3.7 Cell Adhesion Tests

The assay for cell adhesion tests was modified from the method of Chatelet *et al.* Briefly, Small disks (0.32 cm^2) of PHB and PHBV mat were punched out and placed into 96-well plates. Schwann cells were seeded on the culture samples at 10^4 cells per well and incubated for 2, 4, 8 and 16 h at 37°C. A culture plate was used as control. After the removal of the culture medium, the seeded culture samples were washed twice with a sterile phosphate buffer saline (PBS) solution to eliminate free cells. Adherent cells were quantity by measurements of the MTT assay.

3.8 Cell Proliferation Assay

Cell proliferation assays were performed as described by method of Chatelet *et al.*. Small disks of material ($0.32cm^2$) were cut and placed into 96-well plates. Schwann cells were plated on the material with a concentration of 10^4 cells per well. Cell growth was quantified at 1, 3 and 5 days by measuring the cell metabolic activity (MTT assays). The absorbance level was directly proportional to cell

proliferation, assuming Schwann cell metabolic activities were similar on the different materials.

3.9 MTT Assays

The3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTΓ) 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. First, the culture medium was aspirated and replaced with 10 μl of MTT solution at 5 $mg \cdot ml^{-1}$ for a 96-well culture plate. Secondly, the plate was incubated for 1 hour at 37°C. The solution was then aspirated and 100 μl of DMSO was added to dissolve the formazan crystals. Finally, after 1 hour of rotary agitation, the absorbance of the DMSO solution at 570 nm was measured using an ELISA plate reader (ThermoLabsystems, Multiskan). A one-way ANOVA was used to compare the means of different groups, and statistical significance was accepted at 0.05 confidence level.