CHAPTER III
EXPERIMENTAL

3.1 Materials and Preparation and Characterization of Spinning Solutions

Poly(3-hydroxybutyrate) (PHB; M_w = 300,000 g·mol^{-1}) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV; M_w = 680,000 g·mol^{-1}) were purchased from Sigma-Aldrich, USA. The content of hydroxyvalerate in PHBV was 5% by mole. Chloroform, used as the solvent, was purchased from Lab-scan (Asia), Thailand. PHB and PHBV solutions with a concentration ranging between 10 and 16% w/v and PHB/PHBV blend solutions at a fixed concentration of 14% w/v were prepared in chloroform at 60°C. The weight compositional ratios between PHB and PHBV in the blend solutions were 100/0, 75/25, 50/50, 25/75, and 0/100, respectively. Prior to electrospinning, each of the spinning solutions was characterized for its viscosity and conductivity using a Brookfield DV-III programmable viscometer and an Orion 160 conductivity meter, respectively.

3.2 Electrospinning and Characterization of as-Spun Fiber Mats

In electrospinning, each of the as-prepared spinning solutions was contained in a 50-ml glass syringe, the open end of which was connected to a gauge 20 stainless steel needle (OD = 0.91 mm) used as the nozzle. The temperature of the spinning solution was maintained at 50°C using a home-made programmable heater band which was wrapped around the glass syringe. A Gamma High Voltage Research D-ES30PN/M692 power supply was used to general a DC electrical potential in a range of 8 to 14 kV. The electrical potential of positive polarity was supplied to the solution across a collection distance ranging between 15 and 25 cm. A rotating drum (i.e. width and OD of the drum = 14 and 15 cm, respectively; rotational speed = 1000 rpm) was used as a collector.

The as-spun fibers were dried *in vacuo* at room temperature overnight to remove as much solvent as possible. 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 prior to observation under SEM. Diameters of the as-spun fibers were measured directly from the SEM images using a SemAphore 4.0 software, with the average value being calculated from at least 100 measurements. The average number of beads per unit area (i.e. the bead density) of the as-spun beaded fibers and the average number of fibers per unit area (i.e. the fiber density) of the as-spun fibers were calculated from measurements on SEM images of 500x magnification. For all of the above-mentioned experiments, the collection time was about 5 min.

For further characterization, each of the spinning solutions was continuously electrospun under an applied electrostatic field strength of 12 kV/20 cm for 8 or 12 h which resulted in the formation of the as-spun fiber mats of about 85 ± 5 or 105 ± 5 μm thick, respectively. A Mettler-Toledo DSC822^e differential scanning calorimeter (DSC) was used to investigate the crystallization behavior of the as-spun pure and blend fibers. Each sample of about 2 mg was first heated from -15 to 195°C at a rate of 10°C·min^{-1} (HEAT1). After melt-annealing at 195°C for 2 min, the sample was cooled down to -15°C at a rate of -10°C·min^{-1} (COOL) and then reheated to 195°C at a rate of 10°C·min^{-1} (HEAT2). The apparent degree of crystallinity of the as-spun fibers was assessed from the enthalpy of fusion as obtained from HEAT1. Thermal degradation of the as-spun fibers was studied by a Perkin-Elmer Pyris Diamond thermogravimetric/differential thermal analyzer (TGA). Each sample of about 10 mg was heated from room temperature to 250°C at a rate of 10°C·min^{-1} in nitrogen atmosphere. For these measurements, the fiber mats of about 85 ± 5 μm thick were used.

Wettability of the fiber mat (about 85 ± 5 μm thick) and corresponding film surfaces was assessed by water contact angle measurements. The static water contact angles were measured by a sessile drop method using a Krüss contact angle measurement system. A droplet of distilled water (about 40 μl) was gently plated on the surface of each specimen. At least 10 readings on different parts of the specimen

were averaged to attain a data point. Lastly, mechanical integrity in terms of the tensile strength, Young's modulus, and elongation at break of the as-spun fiber mats was investigated using a Lloyd LRX universal testing machine (gauge length = 50 mm and crosshead speed = 20 mm·min^{-1}). Fiber mats of about 105 ± 5 μm thick were cut into a rectangular shape (10 mm x 100 mm).

3.3 Biological Compatibility Evaluation

The applicability of the as-spun fiber mats of PHB, PHBV, and their blends as tissue scaffolding materials was assessed by indirect cytotoxicity evaluation according to a procedure adapted from the ISO10993-5 standard test method using L929 mouse fibroblasts as reference. The test was conducted in a 24-well tissue-culture polystyrene plate (TCPS). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented by 10% fetal bovine serum, 1% L-glutamine and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp., USA)]. Cells were seeded at 4×10^4 cells per well and incubated at 37°C. After 24 h, the cells were re-plated once a week and cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The fiber mats of about 85 ± 5 μm thick were used in the test. Extraction media were prepared by immersing samples cut from the as-prepared fibrous scaffolds in wells of TCPS in a serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) for 24 h. L929 were separately cultured in wells of TCPS in serum-containing DMEM for 16 h to allow cell attachment. The cells were then starved with SFM for 24 h. After that, the medium was replaced with an extraction medium and cells were re-incubated for 24 h. The number of cells that were cultured on bare wells was used as control. Finally, the number of living cells in the as-prepared extraction medium was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

The assay for cell adhesion tests was carried out as following. Briefly; Small disks of PHB, PHBV and PHB/PHBV blend mats were punched out and placed in

wells of 24-well plates. SaOS2 cells were seeded on the culture samples at $4x10^4$ cells per well and incubated for 4, 16 and 24 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 quantified by MTT assay. In order to study cell proliferation, SaOS2 cells were seeded on the culture samples at $4x10^4$ cells per well. Cell growth was quantified at 24, 72 and 120 days by measuring the cell metabolic activity (MTT assays).

MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form purple formazan crystals which are largely impermeable to cell membranes, thus resulting in accumulation of the crystals within healthy cells. Solubilization of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created and was quantified by a Thermospectronic Genesis10 UV-visible spectrophotometer at 540 nm.