CHAPTER III
EXPERIMENTAL

This work could be divided into two parts. The first is to derive an optimum condition for preparing electrospun poly(butylene succinate) extended with 1,6-diisocyanatohexane (PBSu-DCH) fibers and to investigate the effect of processing parameters including polymer concentration, applied electrical potential, and collection distance on the morphology of electrospun PBSu-DCH fibers. The optimum condition for achieving uniformed shape and size of fibers will be chosen as further evaluation for the potential use as bone scaffolding materials.

3.1 Electrospinning of PBSu-DCH Fiber Mats

3.1.1 Materials and Preparation and Characterization of Spinning Solutions

Poly(1,4-butylene succinate) extended with 1,6-diisocyanatohexane ($C_{16}H_{32}N_2O_{10}$; Sigma-Aldrich, USA), Dichloromethane (CH_2Cl_2; Fisher Scientific, UK) and trifluoroacetic acid ($C_2HF_3O_2$; Fluka, Switzerland) are used as solvents for PBS. All chemicals and reagents were used without purification.

Poly(1,4-butylene succinate) extended with 1,6-diisocyanatohexane (PBSu-DCH) solutions with a wide range concentration were prepared in solvent mixture of dichloromethane (DCM) and trifluoroacetic (TFA) with various volume ratio between these two solvents. To ensure good homogeinity of PBSu-DCH solutions, PBSu-DCH pellets were dissolved under mechanical stirring in the solvent mixture at room temperature until the pellets were completely dissolved. Prior to electrospinning, each of spinning solutions was characterized for its viscosity and conductivity by using a Brookfield DV-III programmable viscometer and an Orion 160 conductivity meter, respectively.

3.1.2 Electrospinning and Characterization of as-Spun Fiber Mats

The electrospinning set up utilized in this study consisted of a 5 ml glass syringe fitted with a gauge 20 stainless steel needle that was cut into about 1.5 cm. The syringe was clamped at about 45 degree from horizontal baseline to a PVC

stand. A Kd scientific syringe pump was used to control the feed rate of polymer solutions at about 1 ml/hr. A rotating drum (width and OD of the drum =14 and 15 respectively) was use as a collector. A Gamma High Voltage Research D-ES30PN/M692 power supply, which can generate a high DC potential, was connected between the needle tip and a rotating cylindrical collector, the surface of which was wrapped around by an aluminum sheet.

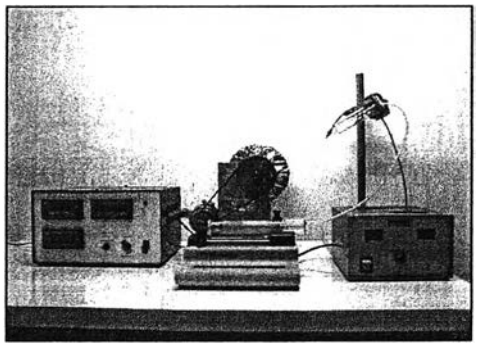

Figure 3.1 The electrospinning apparatus utilized in the production of ultrafine fibers.

The optimum condition for preparation ultrafine fiber mats based on electrospun PBSu-DCH was studied by varying polymer concentration, solvents, applied electrical potential, and collection distance. Each of spinning solutions was contained in glass syringe, the openning end of which was connected to a needle. A rotating drum was used as a collector and a syringe pump was used to control the feed rate of polymer solutions. A high voltage of 15-25 kV was applied to the solutions and the distance between the needle tip and the ground collector was varied from 10-25 cm. All of the spinning experiments were performed at room temperature. 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). 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 nanofibers at 100 different points in the SEM images with

a SemAphore 4.0 Software. The diameters were presented as the average ± standard deviation. The fabrication of the solution-cast scaffolds was conducted to investigate how the surface topography of the scaffolds affected the cell response. The film scaffolds were fabricated from 8% PBSu-DCH solutions in chloroform. In order to ensure good homogeinity, the solutions were stirred until completely dissolved. After that the solutions were spread on the glass dish and dried at room temperature.

Mechanical integrity in terms of tensile strength, Young's modulus, and % elongation at break of both types of scaffolds was investigated using a Lloyd LRX universal testing machine (gauge length = 30 mm and crosshead speed = 20 mm/min) on the samples that were cut into a rectangular shape (10 mm × 70 mm).

Pore volume and porosity (ε) of the as-spun fibrous scaffolds were investigated based on the difference between the density of the fibrous mats (ρ_{sc}) and the density of PBSu-DCH (ρ_{PBS}) (i.e. about 1.3 g/cm^3), according to the following equation:

$$\varepsilon(\%) = \left(1 - \frac{\rho_{sc}}{\rho_{PBS}}\right) \times 100$$

The density of the fibrous scaffolds was measured by a Sartorius YDK01 density measurement.

Additionally, thermal properties of the as-spun PBSu-DCH fibers in comparison with the solution-cast films and the as-received pellets of PBSu-DCH were investigated by using a Mettler-Toledo DSC822^e differential scanning calorimeter (DSC) and a perkin-Elmer Pyris Diamond thermogravimetric/differential thermal analyzer (TGA). For these measurement, the spinning solutions were continuously electrospun for about 10 hr to obtained the as-spun fiber mats of about 120 μm thick. The the melting and crystallization behavior of the as-spun PBSu-DCH fibers in comparison with the solution-cast films and the as-received pellets of PBSu-DCH were studied by a DSC and Indium was used as the calibration standard. Each sample of about 5 mg sealed in aluminum pan was first heated from 25 to 150°C at a rate of 10°C/min (HEAT 1). After melt annealing at 150°C for 5 min, the sample was cool down to 25°C at a rate of 10°C/min (COOL) and then reheated to 150°C at a rate of 10°C/min (HEAT 2). The apparent degree of crystallinity of the as-spun fibers was assessed from the enthalpy of fusion that obtained from HEAT 1. A perkin-Elmer

Pyris Diamond thermogravimetric/differential thermal analyzer (TGA) was used to investigate thermal degradation of the as-spun fibers compared with the film and the pellet one. Each sample of about 5 mg was heated from 30°C to 500°C at a rate of 10°C/min in nitrogen atmosphere.

3.2 Cell Culture Studies

3.2.1 Cell Culture and Cell Seeding

To carry out the in vitro experiments, two types of cells were used in this study : 1) Mouse fibroblasts (L929) and 2) Human osteoblasts (SaOS-2). The cells were cultured as monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented by 10% fetal bovine serum, 1% l-glutamine, and 1% antibiotic and antimycotic formulation (containing penicillin · G sodium, streptomycin sulfate, amphopericin B (Invitrogen,USA)). The medium was replaced every certain time and the culture were maitained at 37 °C in a humidified atmosphere containing 5% CO_2. For cell seeding, SaOS-2 from the cultures were trypsinized [0.25% trypsin containing 1 mM EDTA (Invitrogen Crop.,USA)], counted by a hemacytometer (Hausser Scientific,USA), and seeded at a density of about 36,000 cells/cm^2 on the scaffold specimens and empty wells of a 24-well tissue-culture polystyrene plate (TCPS) that used as controls. The cultures were maintained at 37°C in an incubator.

3.2.2 Indirect Cytotoxicity Evaluation

The biological compatibility of the as-spun fiber mats of PBSu-DCH was assessed by indirect cytotoxicity evaluation using mouse fibroblasts (L929) 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; Sigma-Aldrich, USA), supplemented by 10% fetal bovine serum, 1% l-glutamine, and 1% antibiotic and antimycotic formulation (containing penicillin G sodium, streptomycin sulfate, amphopericin B (Invitrogen,USA)). Cells were seededat 40,000 cells per well and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Extraction media were prepared by immersing samples cut from the as-prepared

fibrous and film scaffolds in wells of TCPS in serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) for 24 h. After 24 h of culturing, the culture medium was removed and 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. After which time the viability of cells in the as-prepared extraction medium was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and the viability of cells that were cultured on bare wells was used as control.

3.2.3 Cell Attachment and Proliferation

For attachment study, SaOS-2 will be allowed to attach on the scaffold specimens and TCPS for 1, 4, and 16 h, respectively. At each specified seeding time, the number of attached cells will be quantified by MTT assay. Each sample will be rinsed with phosphate buffer saline solution (PBS; Sigma- Aldrich, USA) to remove unattached cells prior to MTT assay. For proliferation study, the cells will be first allowed to attach on the specimens for 16 h. The proliferation of cells on the specimens will be determined after 1, 2, and 3 day(s), respectively. After attachment for 16 h, the cells will be starved by SFM twice and then the number of cells will be quantified by MTT assay. Morphological appearance of the cells during attachment and proliferation period will be observed by SEM.

3.2.4 Quantification of Viable Cells (MTT Assay)

The 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. First, each sample was incubated at 37°C for 1 h with 250 µl/well of MTT solution at 0.5 mg/ml without phenol red. After incubation, MTT solution was removed. A buffer solution containing dimethyl sulfoxide (DMSO; Carlo Erba, Italy) (900µl/well) and glycine buffer (pH=10) (125µl/well) was added into the wells to dissolve the formazan crystals. After 10 min of rotary agitation, the solutions were then transferred into a cuvette and placed in a

thermospectronic Genesis 10 UV-visible spectrophotometer, from which the absorbance at 570 nm.

3.2.5 Morphological Observation of Cultured Cells

After removal of the culture medium, the cell-cultured scaffold specimens will be rinsed with PBS twice and the cells were then fixed with 3% glutaraldehyde solution, which will be diluted from 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS, at 500 µl/well. After 30 min, they will be rinsed again with and kept in PBS at 4°C. After cell fixation, the specimens will be dehydrated in an ethanol solutions of varying concentration (i.e., 30, 50, 70, 90, and 100% , respectively) for about 2 min at each concentration. The specimens will be then dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min and later let dry in air after removal HMDS. After completely dried, the specimens will be mounted on an SEM stub, coated with gold, and observed by SEM.

3.2.6 Production of Characteristic Protein of Cultured Cells

SaOS-2 will be cultured on scaffold specimens for 3, 5, and 10 days to observe the production of alkaline phosphatase (ALP). The specimens will be rinsed with PBS after removal of culture medium. Alkaline lysis buffer (10 mM Tris-HCl, 2mM $MgCl_2$, 0.1% Triton-X100, pH 10) (100 µl/well) will be added and the samples will be scrapped and then frozen at –20°C for at least 30 min prior to the next step. An aqueous solution of 2 mg/ml *p*-nitrophenyl phosphate (PNPP; Zymed Laboratories, USA) mixed with 0.1 M amino propanol (10 µl/well) in 2 mM $MgCl_2$ (100 µl/well) having a pH of 10.5 will be prepared and added into the specimens. The specimens will be incubated at 37°C for 2 min. The reaction will be stopped by adding 0.9 ml/well of 50 mM NaOH and the extracted solution will be transferred to a cuvette and placed in the UV-visible spectrophotometer, from which the absorbance at 410 nm will be measured. The amount of ALP will be then calculated against a standard curve. In order to calculate for the ALP activity, the amount of ALP had to be normalized by the amount of total protein synthesized. In the protein assay, the samples will be treated in the same manner as the ALP assay up to the point that the specimens will be frozen. After freezing, bicinchoninic acid (BCA; Pierce

Biotechnology, USA) solution will be added into the specimens. The specimens will be incubated at 37°C for 2 min. The absorbance of the medium solution will be then measured at 562 nm by the UV-visible spectrophotometer and the amount of the total protein will be calculated against a standard curve.

3.3 Statistic Analysis

Statistical comparisons were performed using one-way ANOVA with SPSS software (SPSS, Germany). P values < 0.05 were considered statistically significant.