CHAPTER III
EXPERIMENTAL

3.1 Materials

Polycaprolactone (PCL; 80,000 g mol^{-1}), poly(3-hydroxybutyric acid) (PHB; M_W = 300.000 g mol^{-1}) and poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV; M_W = 680,000 g mol^{-1}) was purchased from Sigma-Aldrich, USA. The HV content in PHBV was 5 mol%. Poly(1,4-butylene succinate), extended with 1,6-diisocyanatohexane (PBSu-DCH) was purchased from Aldrich chemical company Inc. (St. Louis, MO) and poly(lactic acid) (PLA) was purchased from NatureWorks company. Chloroform (Labscan, Asia, Thailand) was used as solvent for these polyesters. Sodium chloride (Ajax Finechem, Australia) (Fluka Chemika, Switzerland) was used as porogen. Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented by 10% fetal bovine serum (FBS, BIOCHROM AG, Germany), 1% L-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp., USA)] were obtained for cell culture. *Pseudomonas sp.* lipase (500 units/g) was purchased from Sigma-Aldrich, USA. Sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4) were purchased from Ajax Finechem, Australia for biodegradable tests.

3.2 Equipment

The samples were characterized for its density, morphology, mechanical properties and thermal properties using a density measurement kit (Sarotrius YKD01, Germany), JEOL/JSM-5200 scanning electron microscope (SEM), Lloyd LRX universal testing machine and NETZSCH DSC 204 F1 differential scanning calorimeter (DSC), respectively.

3.3 Methodology

3.3.1 Preparation of Polyester Scaffolds

A solvent casting and salt particulate leaching was used to prepare the scaffold. Briefly, the polymer solution was prepared by mixing the polymer powder or pellet (depends on the type of polymer) and chloroform in the concentration of 14 percentages of polymer weight by volume of the solution (w/v), then the solution was stirred at 50^0C for 2-3 h in a glass bottle with the cover (for preventing the evaporation of the solvent) after that NaCl salt particles with size of 400-500 μm (polymer/NaCl = 1/30) (w/w) were added into the glass bottle and they are mixed together. Then the mixture was packed into the cylindrical mold with the dimension of 1.2 mm in diameter and 0.8 mm in thickness. The mold was then left in the hood for 24 h and immersed in the water for 1-2 h for taking the mold out. The materials that come out were immersed in reverse osmosis water (RO water) for 48 h, during with time the water was changed approximately every 8 h under the room temperature for leaching out the salt particles. Then the materials were air-dried for 24 h and vacuum-dried overnight to obtain porous scaffolds.

3.3.2 Degradation Experiment

The porous scaffolds which produced from PCL, PBSu-DCH, PLA, PHB and PHBV were examined the degradation in the absence and presence of enzyme lipase from *Pseudomonas sp.* in phosphate buffer saline (PBS) solution. Briefly, 5 scaffolds from each type of material had been separately immersed in 5 ml of 0.1M PBS, pH 7.4 with or without lipase (*Pseudomonas sp.*, 45 units/l) for 1, 3, 5, 7, 9, 11, and 13 weeks. The buffer solution containing lipase was replaced every 84 h so that the enzyme activities were maintained at a constant level throughout the experiment. The samples were taken out at the different time intervals, and washed thoroughly with distilled water and dried in room temperature for 24 h. and then were dried in vacuum for 48 h. The weight remaining, morphological, mechanical properties and thermal properties of the scaffolds were monitored to determine the degradation of the porous scaffolds.

3.3.3 Characterization of Porous Scaffolds

3.3.3.1 Porosity Pore Volume and Pore Size

The density of the scaffolds ($\rho_{scaffold}$) is determined by using a Sartorius YDK01, Germany density measurement kit (Buoyancy method) which can be calculated using the following equation

$$\rho_{scaffold} = \frac{W_a \times \rho_{fl}}{W_a \times W_{fl}}$$

where W_a is the weight of the scaffold in air, W_{fl} is the weight of the scaffold in water and ρ_{fl} is the density of the water (at 25°C, $\rho_{fl} \approx 1$ g/cm^3).

The porosity and pore volume of the scaffolds were calculated using the following equation. (Hou *et al.*, 2003)

$$Porosity(\%) = \left(1 - \frac{\rho_{scaffold}}{\rho_{polymer}}\right) \times 100$$

$$Pore\ volume = \left(\frac{1}{\rho_{scaffold}} - \frac{1}{\rho_{polymer}}\right) \times 100$$

where $\rho_{scaffold}$ is the apparent density of the porous scaffolds and $\rho_{polymer}$ is the density of the non-porous polymer, compression moulded in the same manner.

Pore size of the scaffold was measured on the SEM micrograph with the UTHSCSA Image Tool version 3.0 software. The average values were calculated from the total 25 pores and accepted as the mean pore sizes.

3.3.3.2 Water Absorption Capability

The dry scaffolds were weighted and then were immersed in 5 ml of 0.1M PBS solution at room temperature for 10 days. At the predetermined time point, scaffolds were removed from the solution and carefully placed on the glass for 5 seconds to remove the excessive water and weighed immediately. The water absorption was calculated by use the following equation. (Kothapalli *et al.*, 2005)

$$Water\ absorption\ (\%) = \frac{(M_{wet} - M_{dry})}{M_{wet}} \times 100$$

where M_{dry} and M_{wet} are the weight of the scaffold before and after immersion in water respectively. Five measurements were performed for the calculation of an average water absorption value.

3.3.3.3 Remaining Weight of Porous Scaffolds

The scaffold remaining weight was measured and calculated by the following equation.

$$Remaining\ weight\ (\%) = \frac{W_t}{W_0} \times 100$$

Where W_0 is the initial weight and W_t is the weight of the scaffold at a single degradation time point. An average remaining weight was calculated from those of five samples in each group.

3.3.3.4 Morphology of Porous Scaffolds

The morphology of the pores, their size, distribution and also the interconnectivity between these pores of the porous scaffolds before and after degraded in the different period of time were observed by a JEOL JSM-5200 scanning electron microscopy (SEM). The scaffolds were cut with razor blade at the middle of the scaffold and mounted onto SEM stub. Cross sections of the scaffolds were coated with thin film of gold using JEOL JFC-1100E sputtering devices for 5 min prior to observation under SEM.

3.3.3.5 Compressive Modulus of Porous Scaffolds

Compressive Modulus of the scaffolds were determined with a universal testing machine (Lloyd LRX, UK) using 500 N loaded cell in the dry state at room temperature, the both before and after degraded scaffolds at the different time interval were vertically compressed at the crosshead speed of 3 mm/min. The load was applied until the scaffolds were compressed to approximately 70% of their original thickness. The initial compressive modulus were determined as the slope of the linear portion of the stress strain curve at a compressive strain of 20%

3.3.3.6 Thermal Properties of Porous Scaffolds

NETZSCH DSC 204 F1 differential scanning calorimeter (DSC) was used to investigate the crystallization behavior of the 5 types of porous scaffolds before and after degradations. Each sample of about 3 mg was first heated from room temperature to above melting temperature of those scaffolds about 50°C (the melting temperature of PCL, PBSu-DCH, PLA, PHB and PHBV are 57°C, 114°C, 150°C, 174°C and 164°C, respectively) at a rate of 10°C min^{-1}(HEAT1). After that the sample was cooled down to 20°C at a rate of -10°C min^{-1}(COOL) and then reheated to the same temperature at the same rate (HEAT2). The apparent degree of crystallinity of the each porous scaffold was assessed from the enthalpy of fusion as obtain from HEAT1.

3.3.4 Cell Culture

A human osteoblast cell line (SaOS-2) were cultured in α- minimum essential medium, supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic and antimycotic formulation (containing penicillin G sodium, streptomycin sulfate, and amphopericin B). Cells were cultivated in 5% CO_2 at 37°C in 95% relative atmospheric humidity and trypsinized every 3-4 days.

3.3.4.1 Indirect Cytotoxic Study

An indirect cytotoxic test was conducted on the 5 types of porous scaffolds by use mouse fibroblasts (L929). First, extraction media was prepared by immersing circular shape of the 5 types of porous scaffold specimens (about 15 mm in diameter and 5 mm in thickness) in wells of a 24-well culture plate in a serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) for 24 h. Each of these extraction media was used to evaluate the cytotoxicity of the scaffolds. L929 were separately cultured in wells of a 24-well culture plate in serum-containing DMEM for 16 h to allow cell attachment on the plate. The cells were then starved with SFM for 24 h, after which time the medium was replaced with an extraction medium. After 24 h of cell culturing in the extraction medium, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was carried out to quantify the amount of viable cells.

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 culture medium are aspirated and replaced with 250 µl per well of MTT solution at 5 mg/ml for a 24-well culture plate. Secondly, the plate was incubated for 1 h at 37°C. The solution is then aspirated and 900 ml per well of dimethylsulfoxide (DMSO) containing 100 ml per well of glycine buffer (pH=10) is added to dissolve the formazan crystals. Finally, after 10 min of rotary agitation, the absorbance of the DMSO solution at 570 nm is measured using a Thermospectronic Genesis10 UV/Visible spectrophotometer.

3.3.4.2 Cell Attachment and Cell Proliferation Study

The human osteoblastic cells (SaSO-2) were allowed to attach on the 5 types of porous scaffold specimens and empty wells of a TCPS for 1, 4, and 22 h. At each time point, the number of the attached cells was quantified by MTT assay. Each specimen was rinsed with phosphate buffered saline to remove unattached cells prior to the MTT assay. For the proliferation study, the cells were allowed to attach on the 5 types of specimens and empty wells of a TCPS and the number of the proliferated cells was determined by MTT assay on days 1, 2, and 3 after cell culturing.

3.3.4.3 Alkaline Phosphate Analysis (ALP)

SaOS-2 was cultured on the 5 types of porous scaffolds and empty wells of a TCPS for 5 day to observe ALP activity. Each specimen was rinsed with PBS after removal of the culture medium. Alkaline lysis buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% Triton-X100, pH 10) (1000 µl /well) was added, and the specimen was scrapped and then frozen at -20 °C for at least 30 min prior to the next step. An aqueous solution of 2 mg mL-1 *p*-nitrophenyl phosphate (PNPP; Zymed Laboratories) mixed with 0.1 M aminopropanol (10 µl/well) in 2 mM $MgCl_2$ (100 µl/well) having a pH of 10.5 was prepared and added into the specimen. It was then incubated at 37°C for 2 min. The reaction was stopped by the addition of 0.9 mL/well of 50 mM NaOH, and the extracted solution was transferred to a cuvette and placed in the UV-VIS spectrophotometer, from which the absorbance at 410 nm was measured. The amount of ALP was then calculated against a standard curve. To

determine the ALP activity, the amount of ALP had to be normalized by the amount of total proteins synthesized. In the protein assay, each specimen was treated in the same manner as in the ALP assay up to the point where it was frozen. After freezing, a bicinchoninic acid (BCA; Pierce Biotechnology) solution was added into the specimen. It was subsequently incubated at 37°C for 5 min. The absorbance of the medium solution was then measured at 562 nm by the UV-VIS spectrophotometer, and the amount of the total proteins was calculated against a standard curve.

3.3.4.4 A Morphological Observation of Cultured Cells

The human osteoblastic cells (SaSO-2) were seeded on the 5 types of porous scaffold specimens. Cells were also seeded on TCP and served as a positive control. After 1 and 7 days, the culture medium was removed and then the cell-cultured scaffold specimens were rinsed with PBS twice, the cells were then fixed with 3% glutaraldehyde solution, which was diluted from 50% glutaraldehyde solution with PBS, at 500 µl/well. After 30 min, they were rinsed again with 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%, respectively) for about 2 min at each concentration. The specimens were then dried in air. After completely dried, the specimens were mounted on an SEM stub, coated with gold and observed by SEM.

3.3.5 Statistic Analysis

Data were analyzed using the SPSS software version 15.0 for window (SPSS Inc., USA). Initially, the normal distribution was assessed by the Shapiro-Wilk test. The normal distributed data which presenting homogeneity of the variances, shown by the Levene's test, were then investigated by the One-way analysis of variance (ANOVA) with the Tukey HSD post hoc multiple comparisons. Otherwise the Dunnett T3 would be applied if the data did not exhibit the homogeneity of the variances. To compare mean between 2 groups, the students' unpaired t-test was used. The significant level was indicated at $p < 0.05$ in any case.