CHAPTER III
EXPERIMENTAL

3.1 Materials

Polycaprolactone (PCL; Aldrich, USA) has a number average molecular weight of 80,000 g mol^{-1}. Chloroform (Labscan; Asia, Thailand) was used as solvent for PCL. Ipriflavone (IP; Sigma-Aldrich, USA) acts as herbal extract. Sucrose (Fluka Chemika, Switzerland) was used as porogen. Hydroxyapatite powder (HAp) was synthesized by using the hydrolysis of dicalcium phosphate dihydrate (DCPD; Fluka Chemika, Switzerland) with sodium hydroxide (NaOH; Ajax Finechem, Australia). α-Minimum essential medium (α-MEM; Sigma, USA), hemacytometer (Hausser Scientific,USA), glutaraldehyde solution (Electron Microscopy Science, USA) and 100% hexamethyldisilazane (HMDS; Sigma, USA) were obtained for cell culture tests. *Pseudomonas* lipase (500units/g) was purchased from Sigma-Aldrich (USA). Sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4) and sodium chloride (NaCl) were purchased from Ajax Finechem, Australia for biodegradable tests.

3.2 Equipment

Hydroxyapatite powder was synthesized by using high speed agitator and then dried in oven at 60°C. Solvent and moisture in the sample were evaporated using laminar flow hood and vacuum oven, respectively. The samples were characterized for its morphology, mechanical properties, thermal properties and chemical structure using a JEOL/JSM-5200 scanning electron microscope (SEM), Lloyd LRX universal testing machine, Mettler Toledo DSC 822^e differential scanning calorimeter (DSC), Perkin-Elmer Pyris Diamond thermogravimetric /differential thermal analyzer (TGA) and Thermo Nicolet Nexus 671 FT-IR Spectrophotometer (FT-IR).

3.3 Methodology

3.3.1 Preparation of Hydroxyapatite (HAp)

Hydroxyapatite was prepared by a hydrolysis reaction. Briefly, calcium hydrogen phosphate dehydrate ($CaHPO_4.2H_2O$; DCPD) was poured into a 2.5M $NaOH_{(aq)}$ (pH=13) and mixed in a high speed agitator at 75°C for 1h. After hydrolysis, the reaction was terminated by cooling in ice water. The aggregates were filtered and then rinse in the distilled water. The synthetic product were dried at 60°C and then annealed at 800°C for 4 h, at a heating rate of 1°C min^{-1} in air (Shin *et al.*, 2004).

3.3.2 Preparation of PCL Scaffolds Containing HAp and IP

PCL scaffolds containing HAp and IP were prepared using solvent casting and particulate leaching technique. Polycaprolactone (PCL) and ipriflavone (IP) were dissolving in chloroform at PCL concentration of 28 %w/v. Varying amounts of HAp (0-50 wt% of PCL) were sonicated separately in chloroform for 10 min. Then they were added to the PCL/IP solution. The mixture was stirred until the good dispersion of the HAp particles within the PCL/IP solution and it was subsequently sonicated prior to casting. Sucrose was sieved to the range of 400-500μm and added to the PCL/HAp/IP solution. The mixture was poured into a cylindrical mould (diameter = 14 mm and height = 14 mm) and allowed to dry 24 h. The scaffolds were immersed in distilled water for a period of 2-3 days, during which time the water was changed approximately every 6 h under room temperature to leach the sucrose. The porous scaffolds were dried in a vacuum over for 48 h to remove traces of water.

3.3.3 Hydrolysis of PCL Scaffolds Containing HAp and IP

The porous scaffolds with high surface hydrophilicities were prepared by immersion of the porous scaffolds in 0.1, 1.0 and 4.0 M NaOH solution at 37°C. The alkaline treated porous scaffolds were washed thoroughly distilled water .Then the porous scaffolds were dried in vacuum oven for 48 h. The morphology, compressive modulus and weight loss were analyzed.

3.3.4 Characterization of PCL Scaffolds Containing HAp and IP

3.3.4.1 Morphology of Porous Scaffolds

The morphology of the pores, their size, distribution and also the interconnectivity between these pores of the porous scaffolds were characterized by a JEOL JSM-5200 SEM. The porous scaffold was cut with a razor blade at the center of the porous scaffold and mounted onto SEM stub. Cross sections of the scaffolds were coated with gold using JEOL JFC-1100E sputtering devices prior to observation under SEM.

3.3.4.2 Mechanical Properties of Porous Scaffolds

The compressive modulus of the porous scaffolds was obtained at room temperature using a Lloyd LRX universal testing machine with a 450N load cell at a crosshead speed of 2 mm/min. The specimens were cylindrical shaped with a height of 14 mm. and diameter of 14 mm. The load was applied until the scaffold was compressed to approximately 75% of its 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 10-20%

3.3.4.3 Porosity

The bulk density of the scaffolds was determined using a liquid displacement method. Ethanol was used as the displacing liquid because it penetrated easily into the pores of the scaffolds, but not into the composite itself. The scaffolds of measured weight (W) were immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The cylinder was placed in vacuum to force the ethanol into the pores of the scaffolds until no air bubble emerged from the scaffolds. The total volume of the remaining ethanol and the ethanol-impregnated scaffolds were then recorded as V_2 by simply reading the level in graduated cylinder. The ethanol impregnated scaffolds were then removed from the graduated cylinder and the residual ethanol volume was recorded as V_3. Hence the total volume of the scaffolds were $V = (V_2-V_3)$ and the bulk density of the scaffolds were expressed as $q = W/(V_2-V_3)$. Then the porosity can be determined using the following equation (Kothapalli *et al.*, 2005):

$$\text{Porosity (\%)} = \frac{(V_1 - V_3)}{(V_2 - V_3)} \times 100\%$$

3.3.4.4 Thermal Properties of Porous Scaffolds

Small particles with large surface area were known to influence the morphology and crystallinity of PCL scaffolds containing HAp. Differential scanning calorimeter (DSC) was used to determine the above characteristics of the scaffolds. Melting temperature(T_m) was measured according to the following procedure: heating from 30°C to 100°C, isothermal for 2 min, cooling to -20°C, isothermal for 2 min and heating to 100°C. All these procedure steps were made using a rate of 10°C/min.

3.3.4.5 FT-IR Analysis

The PCL scaffolds containing HAp and IP were placed in holder to collect the FT-IR spectra in the rang 4000-500 cm^{-1}.In the case of pure HAp powder, 1 mg of sample was mixed with 60 mg of KBr powder and pressed into a pellet.

3.3.4.6 XRD Analysis

The crystalline phases and lattice parameters of the obtained HAp powder, with Si powders as a standard for calibration, were examined by WAXD (Rigaku Rint2000 wide-angle X-ray diffractometer), with a monochromatic Cu K_α radiation (λ=1.54A°). The operation voltage and current were 30 kV and 20 mA, respectively, at a scan speed of 5° min^{-1}.

3.3.4.7 TGA/DTA Analysis

The actual HAp contents in the scaffolds were determined by TGA. And DTA analyses were used to determine the melting and decomposition temperature of the scaffolds. The analyses were measured using a heating rate of 20 °C/min from 30 to 900 °C .

3.3.4.8 Water Absorption of Porous Scaffolds

The dry scaffolds were weighed and then placed in a glass bottle filled with 25 ml water for 1, 3 and 5 min. On removal, the scaffolds were carefully wiped with filter paper to remove surface water followed by measurement of the wet weight of the samples. The water absorption was calculated using the following equation (Kothapalli *et al.*, 2005):

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

where M_{wet} and M_{dry} are the wet and dry weights of the sample, respectively. Note that the water absorption measured here includes the water absorbed by both the scaffolds and the pores.

3.3.5 Cell Culture

Mouse osteoblasts (MC3T3-E1) were cultured in α- minimum essential medium, supplemented with 10% FBS, 1% L-glutamine and antibiotic as mentioned above. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Trypsinized MC3T3-E1 cells were seeded at a density of about 100,000 cells/cm^2 on the polycaprolactone scaffold specimens, counted by a hemacytometer. Cells were also seeded on TCP and served as a positive control. Each scaffold was cut into circular discs (about 15 mm in diameter) and the disc specimens were placed in wells of a 24-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland) and later sterilized in 70% ethanol for 30 min. The specimens were then washed with autoclaved de-ionized water and subsequently immersed in α-MEM overnight. To ensure a complete contact between the specimens and the wells, the specimens were pressed with a metal ring (about 12 mm. in diameter). After 48 hours, the culture medium was removed and then the cell-cultured scaffold specimens were rinsed with PBS twice, the cells were then fixed with 3% gluteraldehyde 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 100% hexamethyldisilazane for 5 min and later let dry in air after removal of HMDS. After completely dried, the specimens were mounted on an SEM stub, coated with gold, and observed by SEM.

3.3.6 Biodegradation of PCL Scaffolds Containing HAp and IP in *Vitro*

The porous scaffolds were examined the degradation in the absence and presence of *Pseudomonas* lipase in PBS kept at 37°C. Briefly, the porous scaffolds were placed in the bottle with 25 ml lipase (30units/l) in PBS or PBS alone. Samples were taken out at different time intervals, and washed thoroughly with distilled water and dried in vacuum for 48 h. The weight loss, morphological, mechanical, and thermal properties of the scaffolds were monitored to provide a measure of in *vitro* degradation versus incubation times. The serum lipase concentration in healthy adults is rang 30-190units/l. (Chawla *et al.*, 2002).