CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Chemicals Used in the Fabrication of Protein-loaded Hydroxyapatite

- Dicalcium phosphate dihydrate (DCPD; Fluka, Germany)
- Calcium carbonate ($CaCO_3$; Carlo Erba, Italy)
- Tris-base (Tris[hydroxymethyl]amino methane) (Sigma-Aldrich, USA)
- Nitric acid (ACS reagent 69%; J.T.Baker, USA)
- Ovalbumin (OVA; Sigma-Aldrich, USA)
- Gelatin type B (Sigma-Aldrich, USA)
- Bovine serum albumin (BSA; Sigma-Aldrich, USA)
- Sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4; Ajex Finechem, Australia)

3.1.2 Chemicals Used in The Fabrication of PCL-HAp Scaffold

- Polycaprolactone (PCL;80,000 g mol^{-1}; Sigma-Aldrich, USA)
- Sodium chloride (Ajax Finechem, Australia)
- Chloroform (Labscan; Asia, Thailand)

3.1.3 Chemicals Used for Cell Culture

- Modified Eagle's medium (MEM; Thermo Scientific, USA)
- Fetal bovine serum (FBS;Sorali, Campo Grande Brazil)
- 1% L-glutamine (Invitrogen Corp., USA)
- 1% Lactabumin (Invitrogen Corp., USA)
- 1% antibiotic (Invitrogen Corp., USA)
- Bicinchoninic acid (BCA; Thermo Scientific, USA)

3.2 Equipments

3.2.1 Thermogravimetric Analysis (TGA)

3.2.2 Fourier-Transformed Infrared Spectrophotometer (FT-IR)

3.2.3 Autosorb-1

3.2.4 Scanning Electron Microscope (SEM)

3.2.5 Transmission Electron Microscope (TEM)

3.2.6 Energy Dispersive Spectrophotometer (EDS)

3.2.7 X-ray Diffraction (XRD)

3.2.8 UV-Visible Spectrophotometer

3.2.9 Zeta potential (ZP)

3.3 Methodology

3.3.1 Preparation of Protein-Loaded Hydroxyapatite

Calcium hydrogen phosphate dihydrate ($CaHPO_4.H_2O$, DCPD) and calcium carbonate ($CaCO_3$) were used as precursors of Ca and P to prepare protein-loaded hydroxyapatite. The molar ratios of Ca to P were fixed at 1.67 which mixed with nitric acid 1 mol/l at 75°C for 1 h under stirring. Then, pour DI water at room temperature following protein. Ovalbumin (Sigma-Aldrich A-5253), Gelatin type B, Bovine serum albumin (BSA, Aldrich A-3912) and Crude bone protein from pork bone (CBP) were selected as candidate proteins in this study. The proteins aqueous solution of 25 mg/ml were prepared by dissolving proteins powder or pellet into distilled water for each synthesis. Regulated pH at a constant value, tris (hydroxymethyl) aminomethane. The aggregates were rinsed with the distilled water until pH=7 (distilled water was boiled and decarbonated before use) and further centrifuged at 4500 rpm for 10 mins, freezed at -45°C and lyophilized at -50°C. The samples were kept in dessicator until used.

3.3.2 Crude Bone Protein preparation

CBP was extracted from the pork leg bone on the back. In particular, bone was initially washed and cleaned thoroughly in tap water and then sectioned into small pieces with a high speed motor machine. Pieces of bones were further

crushed into powder in liquid nitrogen. Then, the as-prepared powder was immersed in 0.6 N HCl at 4°C. After three days, the bony solution was centrifuged and the supernatant was collected, dialyzed for 48 h and lyophilized. The dry CBP was kept in desiccators until use.

3.3.3 Preparation of Polycaprolactone(PCL)-Hydroxyapatite(HAp) Scaffold

A solvent casting and salt particulate leaching was used to prepare the scaffold. Brifly, PCL granules were put into a glass bottle with chloroform to make up a PCL solution in the concentration of 14 percentages of polymer weight by volume of the solution (w/v), then the solution was stirred at room temperature for 2 h after that NaCl salt particles with size of 400 μm (PCL/NaCl = 1/30) (w/w) were added into the glass bottle and they were mixed together. Then the mixture was packed into the petri-dish with the dimension of 10x10 cm. The mold was then left in the hood for 24 h and immersed in the DI water 1 day for taking the mold out. The materials that come out were immersed in DI water for 2 days, during with time the water was changed approximately every 8 h under the room temperature for the leaching out the salt particles. Then the materials were air-dried for 24 h and vacuum-dried overnight to obtain porous scaffolds (Prasansuklap *et al.*, 2008).

3.3.4 Release Test

Proteins-loaded Hydroxyapatite (20 mg) were dispersed in 10 ml phosphate buffer saline (PBS) solution at pH 7.4 with shaking rate 70 rpm at 37°C. The proteins release test was carried out by taking 1 ml of released solution. UV-visible spectroscopy was used for the characterization of absorbance peaks at 280 nm to determine the ovalbumin and BSA concentration, 215 nm to determine the gelatin type B concentration through the use of a pre-determined standard concentration—intensity calibration curve.

3.4 Characterization

3.4.1 Thermogravimetric Analysis (TGA)

The samples were analyzed by TGA using a Perkin Elmer (TG-DTA) instrument under N_2 flow of 5 ml/min. The heating process was conducted from 30-950°C at a rate of 10°C/min.

3.4.2 Fourier-Transformed Infrared Spectrophotometer (FT-IR)

A Thermo Nicolet Nexus® 670 Fourier-transformed infrared spectrophotometer (FT-IR) was used to investigate chemical functionalities of hydroxyapatite powder by the KBr disk method. Hydroxyapatite powder was randomly selected from each group of samples and detected for the FT-IR spectra over 32 scans with a resolution of 4 cm^{-1}.

3.4.3 Autosorb-1

Surface areas of the powders were analyzed by nitrogen adsorption in a Autosorb-1.

3.4.4 Scanning Electron Microscope (SEM)

For the morphological study, hydroxyapatite were mounted on brass stubs, coated with gold using a JEOL JFC-1100 sputtering device, and observed for their microscopic morphology using JEOL JSM-5200 scanning electron microscopy (SEM). For the morphology of the pores, their size, distribution and also the interconnectivity.

3.4.5 Transmission Electron Microscope (TEM)

The microstructural and morphological features of HAp powders were analysed in JEM-2100 operating voltage of 200 kV. Sample for TEM were prepared by air-drying a drop of a sonicated ethanol suspension of particles onto a carbon-coated copper grid and air-dried.

3.4.6 Energy Dispersive Spectrophotometer (EDS)

Energy dispersive X-ray spectroscopy (EDS) is an analytical technique used for the elemental analysis or chemical characterization of sample.

3.4.7 X-ray Diffraction (XRD)

The phase compositions of HAp powders were characterized by X-ray diffraction (XRD) with copper target. Data were collected over the scanning range (2θ) from 5° to 60° at a scan speed 2° /min.

3.4.8 UV-Visible Spectrophotometer

Determination of protein by UV/Vis spectrophotometry at 215 nm for Gelatin type B, 280 nm for OVA and BSA, 275 nm for CBP

3.4.9 Zeta Potential (ZP)

Zeta potentials (or electrophoretic mobility) of the hydroxyapatite were determined using Zeta-Meter 3.0+ (Zeta-Meter, Inc., USA). Briefly, the suspension of 100 mg hydroxyapatite in 20 ml of deionized water was filled in an electrophoresis cell. Two electrodes were inserted into the cell and connected to the Zeat-Meter 3.0+ unit. Once the electrodes were energized, microspheres were aroused to move toward one electrode. A microsphere was observed under a microscope for its movement along a specific distance which was indicated by a built in grid. The zeta potential value was detected at a right time point when the microsphere moved to the end. Measurement was repeated 10 times for each preparative condition and the average values were calculated.

3.4.10 Porosity, Pore Volume, and Pore Size

Porosity and pore volume of the scaffolds were measured gravimetrically according to the following equations:

$$\text{Porosity (\%)} = (1 - \rho_{scaffold}/\rho_{polymer}) \times 100 \quad (1)$$

$$\text{Pore volume (\%)} = (1/\rho_{scaffold} - 1/\rho_{polymer}) \times 100 \quad (2)$$

where $\rho_{polymer}$ is the density of the polymer from which the scaffolds were fabricated and $\rho_{scaffold}$ is the apparent density of the scaffolds which was measured by a Sartorius YDK01 density measurement kit. Here, ρ_{PCL} was taken the value of 1.145 $g \cdot cm^{-3}$. Five specimens were measured for both the porosity and the pore volume and an average value for each property was calculated. On the other hand, pore size of the scaffolds was directly measured from SEM images, using a UTHSCSA Image Tool version 3.0 software. At least 25 pores for each of the cross and the longitudinal sections (i.e., at least 50 pores in total) were measured and the average values for all of the scaffolds investigated were calculated.

3.5 Cell Culture

Mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) were cultured as monolayers in minimum essential medium with Earle's Balanced Salts (MEM; Thermo Scientific, USA), supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic and antimycotic formulation (containing penicillin G sodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp., USA). Cells were cultivated in 5% CO_2 at 37°C in a humidified atmosphere.

3.5.1 Direct and Indirect Cytotoxic Study

Direct cytotoxic test was conducted on tissue culture polystyrene (TCPS) with 10μg/ml of proteins in wells of a 24-well culture plate in 2%MEM (containing MEM, 2% FBS, 1% L-glutamine, 1% antibiotic and antimycotic formulation) by using MC3T3-E1. An indirect cytotoxic test was conducted on the proteins-loaded hydroxyapatite and HAp-PCL scaffold. First, extraction media was prepared by immersing powder of each protein-loaded hydroxyapatite about 50 mg/ml and circular shape of each HAp-PCL scaffold specimens (about 15 mm in diameter) in wells of a 24-well culture plate in 2% MEM for 24 h. Both of the protein concentration for direct cytotoxic and extraction media for indirect cytotoxic were used to evaluate the cytotoxicity of proteins, HAp powder and HAp-PCL scaffold. MC3T3-E1 was separately cultured in wells of a 24-well culture plate in serum-containing MEM for 24 h to allow cell attachment on the plate. The cells were then starved with 2% MEM for 24 h, after which time the medium was replaced with 10 μg/ml of proteins for direct cytotoxic and an extraction medium for indirect cytotoxic. 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 via 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 were aspirated and replaced with 400 μl per well of MTT solution at 0.5 mg/ml for a 24-

well culture plate. Secondly, the plate was incubated for 30 mins at 37°C. The solution was then aspired and 900 µl per well of dimethylsulfoxide (DMSO) containing 125 µl per well of glycine buffer (pH=10) was added to dissolve the formazan crystals. Finally, after 20 min of rotary agitation, the absorbance of the DMSO solution at 570 nm was measured using a Thermospectronic Genesis 10 UV-Visible spectrophotometer.

3.5.2 Cell Attachment and Cell Proliferation Study

Mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) were allowed to attach on the porous scaffold specimens and empty wells for 2, 4, and 6 h. For the proliferation study, the cells were allowed to attach on the porous scaffold specimens and empty wells for 1, 2, and 3 d. At each time point, a number of the attached and proliferated cells were quantified by MTT assay. Each specimen was rinsed with phosphate buffered saline to remove unattached cells prior to the MTT assay. The morphology of the cells during the attachment and proliferation periods was observed by SEM. At each time point, 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 dilute from 50% glutaraldehyde solution with PBS, at 500 µl per well. After cell fixation 30 min, the specimens were dehydrated in an ethanol solution of 30%, 50%, 70% and 90% concentration, respectively. The specimens were dried with hexamethyldisilaxane (HMDS) for 5 min and exposed in air. After completely dried, the specimens were mounted on an SEM blass stub, coated with gold and observed by SEM.

3.5.3 Alkali Phosphate Analysis (ALP)

Mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1) were cultured on porous scaffolds and empty wells for 3, 5, and 7 d to observe ALP activity. Each porous specimen was rinsed with PBS after removal of the culture medium. Alkaline lysis buffer (10 mM tris-HCl, 2mM $MgCl_2$, 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 (10µl/well) in 2 mM $MgCl_2$ (100µl/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 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; Thermo scientific, USA) solution was 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 BSA standard curve.

3.5.4 Mineralization Analysis

Calcium deposition was quantified by Alizarin Red S staining. MC3T3-E1 were cultured on the porous scaffolds and empty wells for 21 days, after which the cells were fixed with cold methanol for 10 min and washed with deionized water prior to immersion for 3 min in 370 μl of 1% Alizarin Red S solution dissolved in 1:100 (v/v) ammonium hydroxide/water mixture. Each stained specimen was washed several times with deionized water and air-dried at room temperature. Calcium forms an Alizarin Red S-calcium complex in a chelating process. The stained specimen was photographed.