CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials for Fabrication of Electrospun Fibrous Scaffolds

3.1.1.1 Polymers

Poly(ε-caprolactone) (PCL) with number-average molecular weight of 80,000 g/mol and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) with number-average molecular weight of 680,000 g/mol were purchased from Sigma-Aldrich (USA).

3.1.1.2 Solvents

Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), and chloroform were purchased from Labscan (Thailand). DCM and DMF were used as solvents for PCL, while chloroform was used as a solvent for PHBV to fabricate electrospun fibrous scaffolds. Methanol and Phosphate buffered saline (PBS) (10× solution) were purchased from Fisher Scientific (USA). Nitric acid (HNO_3) and phosphoric acid (H_3PO_4) were purchased from Sigma-Aldrich (USA).

3.1.1.3 Proteins and Drug

Ovalbumin (OVA), type I collagen (COL), fibronectin (FN), and doxycycline hyclate (DOXY) were purchased from Sigma-Aldrich (USA). Crude bone protein (CBP) was extracted from pork bone.

3.1.1.4 Hydroxyapatite (HAp)

HAp was synthesized using calcium oxide (CaO) from discarded egg shells, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris-base; Sigma-Aldrich, USA), calcium carbonate ($CaCO_3$; Carlo Erba, Italy), and calcium hydrogen phosphate dehydrate ($CaHPO_4.2H_2O$; Fluka Chemika, Switzerland).

3.1.2 Materials for Cell Culture

3.1.2.1 Cells

Mouse-calvaria-derived preosteoblastic cells (MC3T3-E1) were used in these studies.

3.1.2.2 Medium for MC3T3-E1Culture

Minimum Essential Medium (with Earle's Balanced Salts) (MEM; Hyclone, 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 amphotericin B (Invitrogen Corp., USA)] was used as culture media for MC3T3-E1. For differentiation and mineralization studies, cells were cultured in the same medium above for the first 3 days, after that the cells were cultured in the same medium with the presence of 5 mM glycerol-2-phosphate disodium salt hydrate (β-glycerophosphate; Sigma, USA) and 50 $\mu g \cdot ml^{-1}$ of L-ascorbic acid (Sigma-Aldrich, USA).

3.2 Equipments

3.2.1 Equipments for Electrospinning Process

- High voltage power supply from Gamma High Voltage Research Inc. (Ormond Beach, Florida), model D-ES30PN/M692 DC was used to generate positive DC voltage.
- Syringe with volume size of 50 ml was served as a container for polymer solutions.
- Stainless steel needle with gauge number 20 (or the inner diameter of 0.91 mm) was used as the electrode to conduct the electrical from power supply to the solutions.
- The rotating drum which covered with aluminum sheet was used as a collector. Width and OD of the rotating drum were 14 and 15 cm, respectively.
- A Kd Scientific syringe pump was used to control the feed rate of the polymer solution at about 1 ml/h.

3.2.2 Equipments for Characterization of Materials

- A Rigaku D/MAX 2000 wide-angle X-ray diffractometer (WAXD) was used to characterize HAp and HAp/protein particles.
- A JEOL JSM 5410LV scanning electron microscope (SEM) was used to observe the morphology of electrospun fibrous scaffolds, HAp, and HAp/protein particles
- A JEOL JEM 2100 transmission electron microscope (TEM) was used to observe the microscopic morphology of HAp and HAp/protein particles
- A MALVERN Mastersizer 2000 particle size analyzer was used to examine the size of HAp and HAp/protein powders
- A Nicolet NEXUS 670 Fourier-transform infrared spectroscope (FT-IR) was used to examine the chemical functionalities of the specimens.
- A Brookfield DV-III programmable viscometer was used to examine the viscosity of polymer solutions.
- A KRÜSS DSA 100 drop-shape analyzer was used to examine the static water contact angles of the scaffolds.
- A Quantachrome Ultrapycnometer-1000 gas pycnometer was used to determine the true density of the scaffolds.
- A Lloyd LRX universal testing machine was used to examine the mechanical integrity of the scaffolds.
- A Perkin-Elmer TGA-7 thermogravimetric analyzer (TGA) was used to investigate the thermal behavior of the specimens.
- A Quantachrome AS-1 autosorb-1 instrument (BET) was used to analyze the pore characteristics and surface area of HAp and HAp/protein particles.
- A TA instruments DSC Q2000 (DSC) was used to examine the formation of crystalline structure of the scaffolds.

3.2.3 Equipments for Study of Cell Culture

- A Thermospectronic Genesis10 UV-visible spectrophotometer was used to measure the absorbance of the solution from the MTT and ALP assay.

3.3 Methodology

3.3.1 Synthesis and Characterization of Hydroxyapatite/Protein Particles

$CaCO_3$ and $CaHPO_4.2H_2O$ were used as precursors to prepare HAp and HAp/protein particles. The starting materials were dissolved in 1 M HNO_3 and the proteins (e.g., OVA, COL, FN, and CBP) were then added in to the solution. 1 M Tris-base solution was subsequently poured out into the mixture generating the precipitation of HAp/protein at pH 7. The product was then filtered off and washed several times with deionized water. After centrifugation, the resulting material was freeze-dried to obtain the fine powder of HAp or HAp/protein. The obtained HAp and HAp/protein particles were characterized by XRD, SEM, TEM, PSA, BET, FTIR, and TGA.

3.3.2 Preparation and Characterization of Electrospun Fibrous Scaffolds

Blend solution of 50/50 w/w PCL/PHBV of varying concentrations ranging from 4 to 14 wt% were prepared in 80/20 v/v chloroform/DMF at room temperature. PCL solution at 12 wt% was prepared in 50/50 v/v DCM/DMF at room temperature and PHBV solution at 14 wt% was prepared in chloroform at 50 °C. 1% w/v HAp or HAp/protein was added into 10 wt% PCL/PHBV solution to generate the composite fibrous scaffolds. Additionally, 4 wt% DOXY was solubilized in a small amount of methanol and added to the 10 wt% PCL/PHBV solutions to generate drug-loaded fibrous scaffolds. All fibrous scaffolds were fabricated by electrospinning method. Each of the solutions was contained in a glass syringe, the open end of which was connected to a gauge-20 stainless steel needle (o.d. = 0.91 mm), used as the nozzle. An aluminum (Al) sheet wrapped around a rotating drum (width and o.d. of the drum ~ 15 cm; rotational speed ~ 50 rpm) was employed as a collector. The distance from the tip of the needle to the outer surface of the Al sheet was set at 10 cm. A Gamma High-Voltage Research D-ES30PN/M692 power supply

was used to generate a high DC potential (i.e., 21 kV for the blend and PCL solutions and 12 kV for the PHBV solution). The emitting electrode (+) of the power supply was attached to the needle, while the grounding one was to the collector. A Kd Scientific syringe pump was used to maintain the feed rate of the solution at ~1 $mL \cdot h^{-1}$. The solutions were electrospun consecutively for ~10 h. The obtained fibrous scaffolds were characterized by SEM, ATR-FTIR, TGA, DSC, viscometer, universal testing machine, contact angle analyzer, and gas pycnometer.

3.3.3 Release Characteristics of Proteins

For the release of OVA from HAp/OVA particles, the HAp/OVA particles were immersed in PBS solution pH 7.4 at 37 °C. The amount of OVA released in the supernatant was measured at various time points by UV-VIS spectrophotometer (UV-1800) at the wavelength of 280 nm through the use of a pre-determined standard calibration curve. The gravimetric analysis was also used to confirm the amounts of OVA lost after 21 days.

For the release of COL, FN, and CBP from both HAp/protein particles and PCL/PHBV-HAp/protein fibrous scaffolds, the amounts of the proteins released were measured by using a bicinchoninic acid (BCA; Pierce Biotechnology, USA) assay. The specimens were immersed in minimum essential medium (MEM: Hyclone, Thermoscientific, USA) for various time intervals and then incubated at 37°C. The amounts of the proteins released in the withdrawn medium were determined by a Thermospectronic Genesis10 UV-visible spectrophotometer at 562 nm. The observed UV absorbance values were converted to the protein concentrations using predetermined standard calibration curves.

3.3.4 In Vitro Cell Culture Studies

3.3.4.1 Cell Culturing

Mouse calvaria-derived, preosteoblastic cells (MC3T3-E1; ATCC CRL-2593) were cultured as monolayers in minimum essential medium with Earle's Balanced Salts (MEM; Hyclone, USA), supplemented by 10% fetal bovine serum (FBS; Biochrom, UK), 1% L- glutamine (Invitrogen, USA), and a 1% antibiotic and antimycotic formulation (containing penicillin G sodium, streptomycin

sulfate, and amphopericin B (Invitrogen, USA)). The medium was changed every other day, and the cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Each scaffold was cut into circular discs (~ 15 mm in diameter), and the disc specimens were placed in wells of a 24-well TCPS (Corning, USA), which was later sterilized in 70% ethanol for 90 min. The specimens were then washed with autoclaved deionized water and subsequently immersed in MEM overnight. To ensure a complete contact between each specimen and the bottom of each well, a metal ring (~12 mm in diameter) was placed on top of the specimen. MC3T3-E1 from the cultures were trypsinized (0.25% trypsin containing 1 mM ethylenediaminetetraacetic acid (EDTA); Invitrogen, USA), counted by a hemacytometer (Hausser Scientific), and seeded at a density of ~40 000 cells cm^{-2} on each of the specimens and the empty wells of a TCPS (i.e., positive control).

3.3.4.2 Cell Attachment and Cell Proliferation

For the attachment study, the cells were allowed to attach to the fibrous substrate specimens and empty wells of a TCPS for 2, 4, and 6 h. At each time point, the number of the attached cells was quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, USA) assay. For the proliferation study, the cells were first allowed to attach on the specimens and empty wells of a TCPS for 16 h. The number of the proliferated cells was also determined by MTT assay on days 1, 2, and 3 after cell culturing. The morphology of the cells during the attachment and proliferation periods was observed by SEM.

3.3.4.3 Quantification of Viable of 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. Each cell-cultured specimen was incubated at 37 °C for 30 min with MTT solution, and then a buffer solution containing dimethylsulfoxide (DMSO; Carlo Erba, Italy) and glycine buffer (pH = 10) was added into each well to dissolve the formazan crystals. Each sample solution was then transferred into a cuvette and placed in a Thermospectronic Genesis10 UV-vis spectrophotometer, from which the absorbance at 570 nm

representing the number of viable cells was measured. The observed UV absorbance values were converted to the number of cells using predetermined standard calibration curves.

3.3.4.4 Morphological Observation of Cultured Cells

After removal of the culture medium, each cell-cultured fibrous scaffold was rinsed with PBS twice, and the cells were then fixed with 3% glutaraldehyde solution (Electron Microscopy Science, USA) at for 30 min. The specimen was rinsed again with PBS and dehydrated in an ethanol solution of varying concentrations (i.e., 30, 50, 70, 90, and 100%, respectively) for ~2 min at each concentration. It was then dried in 100% hexamethyldisilazane (HMDS; Sigma-Aldrich, USA) for 5 min and later dried in air. The specimens were then observed by SEM, and the morphology of the cells that had been cultured on glass substrates (12 mm in diameter; Menzel, Germany) was used as a positive control.

3.3.4.5 Production of Alkaline Phosphatase (ALP) of Cultured Cells

MC3T3-E1 was cultured on the fibrous substrate specimens and the empty wells of a TCPS for 3 and 7 days to observe the 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) was added, and the specimen was scrapped and then frozen at –20 °C for ~30 min. An aqueous solution of 2 mg · mL^{-1} *p*-nitrophenyl phosphate (PNPP; Invitrogen, USA) mixed with 0.1 M aminopropanol in 2 mM $MgCl_2$ was prepared and added into the specimen, which was then incubated at 37 °C for 30 min. The reaction was stopped by the addition of 50 mM NaOH, and the extracted solution was measured spectrophotometrically at 410 nm. The amount of ALP was then calculated against a predetermined standard curve and then normalized by the total protein content. 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, the BCA solution was added into the specimen and incubated at 37 °C for 30 min. The absorbance of the medium solution was eventually measured at 562 nm by the UV-vis spectrophotometer, and the amount of the total proteins was calculated against a predetermined standard curve.

3.3.4.6 Mineralization

Calcium deposition was quantified by Alizarin Red-S (Sigma-Aldrich) staining. MC3T3-E1 had been cultured on the fibrous substrate specimens and the empty wells of a TCPS for 14 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 (Sigma-Aldrich, USA) 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. The stained specimen was photographed and the redness, signifying the amount of calcium deposition, was quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich, USA) in 10 mM sodium phosphate at room temperature for 15 min and spectrophotometrically read at 570 nm.