CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials for Fabrication of Electrospun (e-spun) Fibers

3.1.1.1 Polycaprolactone

Polycaprolactone (PCL) with number-average molecular weight of 80,000 g/mol was purchased from Aldrich (USA). It was used as a base polymer material in electrospinning process.

3.1.1.2 Solvents

Dichloromethane (Carlo Erba, Italy) and *N,N*-dimethylformamide (DMF; Lab-Scan (Asia), Thailand) were used as solvents for PCL to fabricate e-spun fibers.

3.1.1.3 Fillers

- Calcium carbonate nanoparticles ($CaCO_3$; cubic form; average particle size = 40 nm) were obtained from NanoMaterials Technology, Singapore.
- Hydroxyapatite powder (HAp) nanoparticles were synthesized following the method reported by Shih *et al.* (2004).

3.1.2 Materials for Cell Culture

3.1.2.1 Model Cells

Model cells in this study were human osteosarcoma cells (SaOS2), mouse calvaria-derived, pre-osteoblastic cells (MC3T3-E1), and human fetal osteoblasts (hFOB).

3.1.2.2 Medium for SaOS2 Culture

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 amphotericin B (Invitrogen Corp., USA)] was used as culture media for SaOS2.

3.1.2.3 Medium for MC3T3-E1 Culture

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 5 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, USA).

3.1.2.4 Medium for hFOB Culture

Minimum Essential Medium Alpha Medium (with L-glutamine, ribonucleosides and deoxyribonucleosides, without sodium bicarbonate) (GIBCO, USA) supplemented by 10% fetal bovine serum (FetalPlex, animal serum complex, GEMINI, BIO-PRODUCTS, USA) and 1% Penicillin-Streptomycin (GIBCO, USA), was used as culture media for hFOB. For differentiation and mineralization studies, cells were cultured in the same medium above for the first 5 days, after that the cells were cultured in the same medium with the presence of 5 mM β-glycerophosphate and 50 $\mu g \cdot ml^{-1}$ of L-ascorbic acid.

3.2 Equipment

3.2.1 Equipment 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 Equipment for Characterization of Materials

- A Rigaku Rint2000 wide-angle X-ray diffractometer (WAXD) was used to characterize HAp nanoparticles.
- A JEOL JSM 5410LV scanning electron microscope (SEM) was used to observe the morphology of electrospun fibers and HAp nanoparticles
- A MALVERN MastersizerX particle size analyzer was used to examine the size of $CaCO_3$ and HAp powders
- A Brookfield DV-III programmable viscometer was used to examine the viscosity of spinning dopes.
- An Orion 160 conductivity meter was used to examine the conductivity of spinning dopes.
- A KRÜSS DSA10-Mk2 drop-shape analyzer was used to examine the surface tension of spinning dopes.
- A Sartorius YDK01 density measurement kit was used to determine the density of the scaffolds.
- A Lloyd LRX universal testing machine was used to examine the mechanical integrity of the scaffolds.

3.2.3 Equipment 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.
- An energy dispersive spectroscopy machine (EDS, Link ISIS series 300) was used to observe the calcium element on cell cultured-scaffolds.
- An electrophoresis machine (Power Pac Junior, Bio-Rad, USA) was used in the reverse transcription-polymerase chain reaction (RT-PCR) analysis and western blot analysis.

3.3 Methodology

3.3.1 Synthesis and Characterization of Hydroxyapatite (HAp)

Nano-sized hydroxyapatite (HAp) powder was synthesized from dicalcium phosphate dihydrate ($CaHPO_4.2H_2O$; Fluka Chemika, Switzerland) by hydrolysis method following the procedure reported by Shih *et al.* (2004). The obtained HAp powder was characterized by a Rigaku Rint2000 wide-angle X-ray diffractometer (WAXD). Both morphology and size of the as-synthesized HAp particles were characterized by a JEOL JSM 5410LV scanning electron microscope (SEM) and a MALVERN MastersizerX particle size analyzer, respectively, while size of the as-received $CaCO_3$ nanoparticulate powder was examined with the same particle size analyzer.

3.3.2 Effect of Solution and Process Parameters on e-spun PCL Fibers and Its Composite with Either $CaCO_3$ or HAp Nanoparticles

The effect of filler content, base PCL concentration and applied potential on morphology and diameter of e-spun fibers were studied. For the all mentioned experiments, weighed amount of PCL pellets was dissolved in a mixed solvent of 50:50 v/v dichloromethane and DMF. Prior to electrospinning, each of the spinning dopes was characterized for its viscosity, conductivity, and surface tension

using a Brookfield DV-III programmable viscometer, an Orion 160 conductivity meter, and a KRÜSS DSA10-Mk2 drop-shape analyzer, respectively. All measurements were carried out at room temperature (i.e. 25 °C). Each of the spinning dope was contained in a glass syringe, the opening end of which was connected to a gauge 20 stainless steel needle [outside diameter (OD) = 0.91 mm] used as the nozzle. A rotating drum (width and OD of the drum = 14 and 15 cm, respectively; rotational speed = 50 rpm) was used as a collector. The outer surface of the rotating drum was set 10 cm from the tip of the needle. A Kd Scientific syringe pump was used to control the feed rate of the polymer solution at about 1 ml/h. A collection time was 10 min. As-spun fibers were dried in vacuo at 40 °C overnight to remove as much solvent out as possible. Morphological appearance of the as-spun fibers was examined by SEM. Each sample was coated with a thin layer of gold using a JEOL JFC-1100E ion sputtering device prior to SEM observation. Diameters of the as-spun fibers were determined from SEM images using a SemAphore 4.0 software.

3.3.2.1 Effect of Filler Content

To investigate the effect of filler content, either 0.5 or 1.0% w/v of $CaCO_3$ or HAp powder was mixed in 12% w/v PCL solution. These solutions were electrospun under the applied electrostatic field strength of 21 kV/10 cm.

3.3.2.2 Effect of Base PCL Content

Weighed amount of HAp powder with the weight ratio between PCL and HAp being12:1 was mixed in 8, 10, or 12% w/v PCL solution. These solutions were electrospun under the applied electrostatic field strength of 21 kV/10 cm.

3.3.2.3 Effect of Applied Potential

A mixture of 12% w/v PCL and 1.0% w/v HAp was electrospun under an applied potential of 10, 15, or 21 kV over a collection distance of 10 cm.

3.3.3 Preparation and Characterization of Spinning Dopes and e-spun Fibrous Scaffolds Used in Cell Culture

E-spun fibrous scaffolds from section 3.3.2.1 were used in osteoblasts culture. Moreover, the corresponding solution-cast films were also used in cell culture in order to investigate how the topography of the scaffolds affects the cell response. These following are 10 types of scaffolds. Their symbols are denoted in bracket whereas E and CF stand for e-spun and cast film scaffolds, respectively.

- Neat 12% w/v PCL solution (E-PCL, CF-PCL),
- 12% w/v PCL solution with 0.5% w/v $CaCO_3$ (E-PCL/$CaCO_3$0.5, CF-PCL/$CaCO_3$0.5),
- 12% w/v PCL solution with 1.0% w/v $CaCO_3$ (E-PCL/$CaCO_3$1.0, CF-PCL/$CaCO_3$1.0),
- 12% w/v PCL solution with 0.5% w/v HAp (E-PCL/HAp0.5, CF-PCL/HAp0.5),
- 12% w/v PCL solution with 1.0% w/v HAp (E-PCL/HAp1.0, CF-PCL/HAp1.0)

A collection time of electrospinning was 10 h. Both e-spun and cast film scaffolds were dried in vacuo at 40°C overnight to remove as much solvent out as possible.

3.3.3.1 Pore Size and Porosity of the e-spun Scaffolds

Morphological appearance of the e-spun scaffolds was examined by SEM. Average fiber diameter and average pore size of the fiber mats were also examined by SEM. The average pore size was taken as an average of both the vertical and the horizontal dimensions of the pores. Lastly, porosity (ε) of the e-spun scaffolds was estimated based on the difference between the density of PCL (ρ_{PCL}) (i.e. about 1.145 g/cm^3) and the density of the scaffolds (ρ_{sc}), according to the following equation:

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

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

3.3.3.2 Mechanical Characteristics of the e-spun Scaffolds

Mechanical integrity in terms of the tensile strength and yield stress of the e-spun scaffolds was investigated using a Lloyd LRX universal testing machine (gauge length = 50 mm and crosshead speed = 10 mm/min). Mats of e-spun scaffolds about 130 μm in thickness were cut into a rectangular shape (10 mm x 70 mm).

3.3.4 The Study of *in vitro* SaOS2 Cell Response to the e-spun and Cast Film Scaffolds

3.3.4.1 Cell Culture

Human osteosarcoma cells (SaOS2) were cultured as monolayer in medium mentioned in section 3.1.2.2. The medium was replaced once in every 3 days and the cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

3.3.4.2 Cytotoxicity of the e-spun Scaffolds

Two types of cells were used: 1) human osteosarcoma cells (SaOS2) and 2) mouse fibroblasts (L929). Indirect cytotoxicity test was conducted on E-PCL, E-PCL/$CaCO_3$1.0 and E-PCL/HAp1.0. First, extraction media were prepared by immersing samples (about 15 mm in diameter and 130 μm in thickness) 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. SaOS2 and 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. Then, the cells were 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, MTT assay was carried out to quantify the amount of viable cells.

3.3.4.3 Quantification of Viable Cells (MTT Assay)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) 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 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 dimethylsulfoxide (DMSO; Carlo Erba, Italy) (900 µl/well) and glycine buffer (pH = 10) (125 µl/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 Genesis10 UV-visible spectrophotometer, from which the absorbance at 540 nm representing the number of viable cells was measured.

3.3.4.4 Cell Seeding

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), which were later sterilized in 70% ethanol for 30 min. The specimens were then washed with autoclaved de-ionized water and subsequently immersed in DMEM 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). SaOS2 from the culture 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 TCPS that were used as control. The culture was maintained in an incubator.

3.3.4.5 Cell Attachment and Proliferation

Cell attachment and proliferation were conducted on TCPS and all types of the e-spun and cast film scaffolds. For attachment study, SaOS2 were allowed to attach to the scaffold specimens and TCPS for 30 min, 1, 4, and 16 h, respectively. At each specified seeding time, the number of the attached cells was quantified by MTT assay. Each sample was rinsed with phosphate buffer saline (PBS; Sigma-Aldrich, USA) to remove unattached cells prior to MTT assay. For proliferation study, the cells were first allowed to attach on the specimens for 16 h. The proliferation of cells on the specimens was determined after 24, 48, and 72 h, respectively. After attachment for 16 h, the cells were starved by serum-free medium

(SFM). The number of cells was, again, quantified by MTT assay. Morphological appearance of the cells during attachment and proliferation period was observed by SEM.

3.3.4.6 Morphological Observation of Cultured Cells

After removal of the culture medium, the cell-cultured scaffold specimens were rinsed with PBS twice and the cells were then fixed with 3% glutaraldehyde solution, which was diluted from 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS, at 500 µl/well. After 30 min, they were rinsed again with PBS. 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 (HMDS; Sigma, USA) 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.

3.3.4.7 Production of Alkaline Phosphatase of Cultured Cells

Cells were cultured on scaffold specimens for 5 and 10 days to observe the production of alkaline phosphatase (ALP). The specimens were rinsed with PBS after removal of culture medium. Alkaline lysis buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% Triton-X100, pH 10) (100 µl/well) was added and the samples were 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 was prepared and added into the specimens. The specimens were incubated at 37°C for 25 min. The reaction was stopped by adding 0.9 ml/well of 50 mM NaOH and the extracted solution was transferred to a cuvette and placed in the UV-visible spectrophotometer, from which the absorbance at 410 nm was measured. The amount of ALP was 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 were treated in the same manner as the ALP assay up to the point was the specimens were frozen. After freezing, bicinchoninic acid (BCA; Pierce Biotechnology, USA) solution was

added into the specimens. The specimens were incubated at 37°C for 25 min. The absorbance of the medium solution was then measured at 562 nm by the UV-visible spectrophotometer and the amount of the total protein was calculated against a standard curve.

3.3.5 The Study of *in vitro* MC3T3-E1 Cell Response to the e-spun Scaffolds

The study of *in vitro* MC3T3-E1 cell response to scaffolds was conducted on TCPS, E-PCL and E-PCL/HAp1.0.

3.3.5.1 Cell Culture

Mouse osteoblasts (MC3T3-E1) were cultured as monolayer in medium mentioned in section 3.1.2.3. The medium was replaced once in every 3 days and the cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

3.3.5.2 Cell Seeding

MC3T3-E1 cell seeding procedure was similar to that of SaOS2 in section 3.3.4.4 except using MEM medium instead of DMEM medium.

3.3.5.3 Cell Attachment and Proliferation and Morphological Observation of Cultured Cells

For attachment study, MC3T3-E1 was allowed to attach to TCPS, E-PCL and E-PCL/HAp1.0 for 1, 4 and 16 h, respectively. At each specified seeding time, the number of the attached cells was quantified by MTT assay. Each sample was rinsed with PBS to remove unattached cells prior to MTT assay. For proliferation study, the cells were first allowed to attach on the specimens for 16 h. The proliferation of cells on the specimens was determined after 24, 48, and 72 h, respectively. After attachment for 16 h, the cells were starved by MEM supplemented by 2% FBS, 1% L-Glutamine and 1% antibiotic /antimycotic. The number of cells was, again, quantified by MTT assay. Morphological appearance of the cells during attachment and proliferation period was observed by SEM. The fixation and drying methods were same as that of SaOS2 in section 3.3.4.6.

3.3.5.4 Production of Alkaline Phosphatase of Cultured Cells

Cells were cultured on scaffold specimens for 1, 2, 3, 5, and 10 days to observe the production of alkaline phosphatase (ALP). The alkaline phosphatase assay was same as in section 3.3.4.7.

3.3.5.5 RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Cells were cultured on scaffold specimens for 14 days to observe gene expression level of osteocalcin (OC). The RNA was extracted by Tri Reagent (Molecular Research Center, Inc. MRC, USA). Chloroform (200 μl) was added to the homogenized samples to extract RNA, followed by precipitation using 500 μl of isopropanol (Sigma, USA). RNA pellets were washed with 70% ethanol and were dissolved in 15 μl of nuclease-free water (Promega, USA). RNA yields were evaluated by a spectrophotometer based on absorbance at 260 nm. First strand DNA was reverse transcribed from 1 μg of total RNA using RT kit (ImProm-II Reserve Transcription System, Promega, USA). For the amplification in PCR, the PCR mixture consisted of 1 μl of cDNA, sense primer, antisense primer, and reagent of PCR kit (Tag DNA Polymerase, Qiagen, USA). Twenty-eight cycles was used to amplify both genes. The PCR products were analyzed by separation with 1.8% agarose (Usb, USA) gel on electrophoresis (Power Pac Junior, Bio-Rad, USA) and visualized with ethidium bromide (EtBr; Bio-Rad, USA) staining. The stained bands were photographed under UV light and quantified intensity with Scion Image Software. Each band was normalized to that of GAPDH.

3.3.5.6 Western Blot Analysis

MC3T3-E1 were cultured on the e-spun scaffolds and empty wells of a TCPS for 21 days. The cultured medium for each type of specimens was collected and lyophilized. Lyophilized medium was dissolved with sample buffer (2% SDS, 10% glycerol, 250 mM Tris-HCl, and 0.005% bromophenol blue). Supernatant was collected and was subjected to SDS-PAGE under non-reducing condition, followed by transferring to PVDF membrane (Millipore, USA). The membrane was blocked by incubating in a blocking solution containing 5% skim milk (Difco, BD, USA) and 0.1% TWEEN 20 (Fluka Chemika, Switzerland) for 3 h. The membrane was then incubated and shaken in a primary antibody solution (mouse

osteocalcin antibody, SC-18322; Santa Cruz Biotechnology, USA) which was diluted in a blocking solution at the antibody to the blocking solution ratio of 1:200 v/v for 1 h. The membrane was washed with PBS before being incubated and shaken in a secondary antibody solution (anti-goat IgG, B7014; Biotin Conjugate, Sigma, USA) at the antibody to the blocking solution ratio of 1:2000 v/v for 20 min. The membrane was again washed with PBS. The membrane was incubated and shaken in streptavidin horseradish peroxidase (HRP) conjugate (Zymed, USA) solution at the streptavidin HRP to the blocking solution ratio of 1:500 v/v for 30 min and washed with PBS. All incubations were carried out at room temperature. The membrane was then immersed in 1 ml of SuperSignal West Pico chemiluminescent reagent (Pierce, USA). The signal was captured with a CL-XPosure film (Pierce, USA).

3.3.5.7 Alizarin Red Staining

Calcium deposition was investigated by alizarin red staining. Cells were cultured for 14 and 21 days on scaffold specimens. Cells were fixed with cold methanol for 10 min and washed with deionized water before immersing for 3 min in 370 μl of 1% alizarin red S (Sigma, USA) solution dissolved in 1:100 v/v ammonium hydroxide:water. The stained tissue was washed several times with de-ionized water and air dry at room temperature. Calcium forms an alizarin red S-calcium complex by a chelation process. The stained tissue was photographed and quantified intensity by Scion Image Software. Then the intensity of each sample was subtracted with intensity of its blank before comparing intensity.

3.3.5.8 Nodule Formation and EDS Characterization of Calcium Element

Nodule formation of MC3T3-E1 culturing on scaffold specimens were observed with a SEM at 21 days of cultured. Moreover, bone-like nodule on scaffold was characterized with EDS (Link ISIS series 300) mapping to investigate calcium deposition around nodule areas.

3.3.6 The Study of *in vitro* hFOB Cell Response to the e-spun Scaffolds

The study of *in vitro* hFOB cell response to scaffolds was conducted on TCPS, E-PCL, E-PCL/HAp1.0, and porous poly(DL lactic-*co*-glycolic acid) (PLGA).

3.3.6.1 Preparation of Porous Poly(DL lactic-co-glycolic acid)

Porous poly(DL lactic-co-glycolic acid) (PLGA) scaffold was prepared by salt leaching technique. In order to lubricate the vessel being as a template for fabricating porous PLGA scaffold, 3 ml of SIGMACOAT solution (Sigma, USA) was added into a beaker with diameter of 30 mm. The beaker was covered with parafilm and stored at 4°C overnight. A SIGMACOAT solution was removed and the beaker was let dried in air and then 1.45 g of NaCl (Mallinckrodt,USA) (size of particles was 250-425 μm collected by sieving mesh) was added into the beaker followed by 960 μl of 5% w/v PLGA (Sigma,USA) solution in chloroform. The mixture was solidified by evaporation of solvent. After solidification, a piece of scaffold was soaked overnight and washed several time with de-ionized water in order to dissolve NaCl out.

3.3.6.2 Cell Culture

Human osteoblasts (hFOB) were cultured as monolayer in medium mentioned in section 3.1.2.4. The medium was replaced once in every 3 days and the cultures were maintained at 34°C in a humidified atmosphere containing 5% CO_2.

3.3.6.3 Cell Seeding

Each scaffold was cut into circular discs (about 35 mm in diameter) and the disc specimens were placed in wells of a 6-well tissue-culture polystyrene plate (TCPS; Corning Incorporated, costar, USA), which were later sterilized in 70% ethanol for 30 min except PLGA. The specimens were then washed with autoclaved de-ionized water and exposed to UV light for 30 min. To ensure a complete contact between the specimens and the wells, the specimens were pressed with a metal ring (about 32 mm in diameter) except PLGA. hFOB cells from the culture were trypsinized with trypsin-EDTA, (GIBCO, USA), counted by a BECKMAN COULTER (Z1 coulter particle counter) and seeded at a density of

about $5x10^4$ cells/well on the scaffold specimens and empty wells of TCPS that were used as control. The volume of seeding was 4 ml/well. The culture were maintained in an incubator.

3.3.6.4 Proliferation and Morphological Observation of Cultured Cells

Cell proliferation was determined on day 7 of culture. Cells were washed with phosphate buffer saline (PBS) to remove non-adherent cells and trypsinized with 0.05% trypsin-EDTA and then counted by a Z1 Coulter Particle Counter. Morphological appearance of the cells during attachment and proliferation period was observed by SEM. The fixation and drying methods were same as that of SaOS2 in section 3.3.4.6.

3.3.6.5 Production of Alkaline Phosphatase of Cultured Cells

On day 7 of culture, ALP was harvested by scrapping cells with 500 µl of harvesting buffer per well consisting of 10 mM Tris Cl (Fisher Biotech, NJ, USA), 0.2% NonidetP40 (Roche Diagnostics Corporation, IN, USA), and 2 mM phenylmethylsulfonyl fluoride (MP Biomedicals, OH, USA). The extracted solution was sonicated and centrifuged at 2,000 rpm, 10 min, and 4° C. The 8 µl of supernatant was mixed with 192 µl of reagent of Alkaline Phosphatase kit (STANBIO LABORATORY, 0900-151, TX, USA). The OD at 405 nm was measured with a spectrophotometer (BECKMAN COULTER, AD340). The obtained ALP value was normalized with DNA content.

3.3.6.6 Calcium Content Produced by Cultured Cells

Calcium deposition from cultured cells was evaluated at 4 weeks of culture. Cells on each scaffold were incubated twice for 30 min in 5% trichloroacetic acid (TCA) (LabChem Inc., PA). Calcium extract was mixed with reagents of a calcium kit (Calcium Liquicolor: Arsenazo, STANBIO Laboratory, TX, USA) and measured colorimetrically at 550 nm using a spectrophotometer (BECKMAN COULTER, AD340).