CHAPTER III

EXPERIMENTAL WORK

The experimental work can be divided into three main parts:

1. Materials and reagents
2. Equipments
3. Experimental procedures

3.1 Materials and reagents

3.1.1 *Bombyx mori* cocoon (Nangnoi Srisaket from Nakhonratchasima province, Thailand)

3.1.2 Type A gelatin powder (pI 9, Nitta gelatin Inc., Japan)

3.1.3 Sodium carbonate (Na_2CO_3, Ajax Finechem, Australia)

3.1.4 Lithuim bromide (LiBr, Sigma-Aldrich, Germany)

3.1.5 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Nacalai Tesque, Inc., Japan)

3.1.6 N-hydroxysuccinimide (NHS, Nacalai Tesque, Inc., Japan)

3.1.7 Hydroxyapatite (HA, particle size 100-212 μm, Sigma-Aldrich, Germany)

3.1.8 Sigmacote (Sigma-Aldrich, Germany)

3.1.9 Chloroform (Analar, England)

3.1.10 Dulbecco's phosphate buffer saline without calcium and magnesium (PBS(-) powder, Nissui Pharmaceutical Co. Ltd)

3.1.11 Sodium azide (Labchem, APS, Austraria)

3.1.12 Collagenase from clostridium histolyticum (2.69 units/ml, Fluka, Biochemika, USA)

3.1.13 Ethanol (99.7-100%, VWR International Ltd., UK)

3.1.14 70% ethanol (RCM, store at RT)

3.1.15 Alpha-modified eagle powder medium (α-MEM(s), Hyclone, USA)

3.1.16 Fetal bovine serum (FBS, Hyclone or Biochrom or ICP)

3.1.17 Penicillin-Streptomycin solution (10,000 units/ml, Hyclone, USA)

3.1.18 L-glutamine (200 mM, Hyclone, USA)

3.1.19 Sodium hydrogen carbonate (Fluka)

3.1.20 Trypsin/EDTA (0.25% Trypsin in EDTA.4Na, Hyclone, USA)

3.1.21 Trypan blue solution (0.4%, Sigma-Aldrich, Germany)

3.1.22 Dimethyl sulfoxide for cell freezing (Cell culture tested DMSO, Sigma-Aldrich, Germany)

3.1.23 Glutaraldehyde solution (50% GTA, Fluka)

3.1.24 Hexamethyldisilazane (HMDS, Fluka)

3.1.25 SDS lysis buffer

3.1.26 Hoechst 33258 solution (1 mg/ml DMSO)

3.1.27 p-Nitrophenol standard solution (10 mM, Sigma-Aldrich, Germany)

3.1.28 p-Nitrophenyl phosphate liquid substrate (pNPP, Sigma-Aldrich, Germany)

3.1.29 Sodium hydroxide (0.02 N NaOH, Analar, England)

3.1.30 1M HCl

3.1.31 $CaCO_3$ standard

3.1.32 O-cresolphythalein complex substrate (OCPC, MW 636.62)

3.1.33 Ethanolamine buffer (0.88 mol/l, pH 11)

3.2 Equipments

3.2.1 Centrifuge (Kubota corporation 6500, Tokyo, Japan)

3.2.2 -50°C freezer (Heto, PowerDry LL3000, USA)

3.2.3 Lyophilizer (Christ Loc-1m, Alpha 1-4, Germany)

3.2.4 Homogenizer (T 25 digital, Ultra-turrex, Ika Co., Germany)

3.2.5 Fine coat (JFC-1100E, JEOL Ltd., Japan)

3.2.6 Scanning Electron Microscopy (JSM-5410LV, JEOL Ltd., Japan)

3.2.7 Universal Testing Machine (Instron, No. 5567, USA)

3.2.8 X-ray Diffractomerter (Bruker AXS, D8 discover, Germany)

3.2.9 Laminar Flow (HWS Series 254473, Australia)

3.2.10 CO_2 incubator (Series II 3110 Water Jacketed Incubator, Thermo Forma, USA)

3.2.11 Fluorescence microplate reader (Perkin elmer, 1420 multilabel counter, USA)

3.2.12 UV-VIS spectrophotometer (Thermo Spectronic, Genesys 10UV scanning, USA)

3.2.13 Micropipette (Pipetman P20, P200, P1000 and P5000, USA)

3.2.14 Autopipettes 10-100 μl, 100-1000 μl and 1000-5000 μl with tips (Eppendorf)

3.2.15 10 cm polystyrene tissue culture discs (Corning430167)

3.2.16 24-well polystyrene tissue culture plates (Corning3542)

3.2.17 48-well polystyrene tissue culture plates (Corning3548)

3.2.18 Black plate (96-well, Corning3550)

3.2.19 T25 tissue culture flask (Cellstar690-160)

3.2.20 T75 tissue culture flask (Cellstar658-175)

3.2.21 5 ml sterilized pipette (Costar4486)

3.2.22 10 ml sterilized pipette (Costar4488)

3.2.23 25 ml sterilized pipette (Costar4489)

3.2.24 50 ml sterilized centrifugal tubes (Corning430828)

3.2.25 1.5 ml sterilized vials (RCM, 3307-130380)

3.2.26 Hemacytometer (Counting chamber, Boeco, Germany)

3.2.27 Sterilized filter system (0.22 μm)

3.2.28 Paraffin film

3.3 Experimental procedures

All experimental procedures are summarized in Figure 3.1. In brief, there are four main steps comprised in this work; preparation of Thai silk fibroin and gelatin solutions, preparation of Thai silk fibroin/gelatin scaffolds, preparation of composited hydroxyapatite/Thai silk fibroin/gelatin scaffolds and characterization of scaffolds including morphology, weight loss (%), compressive modulus (dry and wet condition), *in vitro* biodegradability, and *in vitro* biocompatibility using bone marrow-derived stem cells (MSCs).

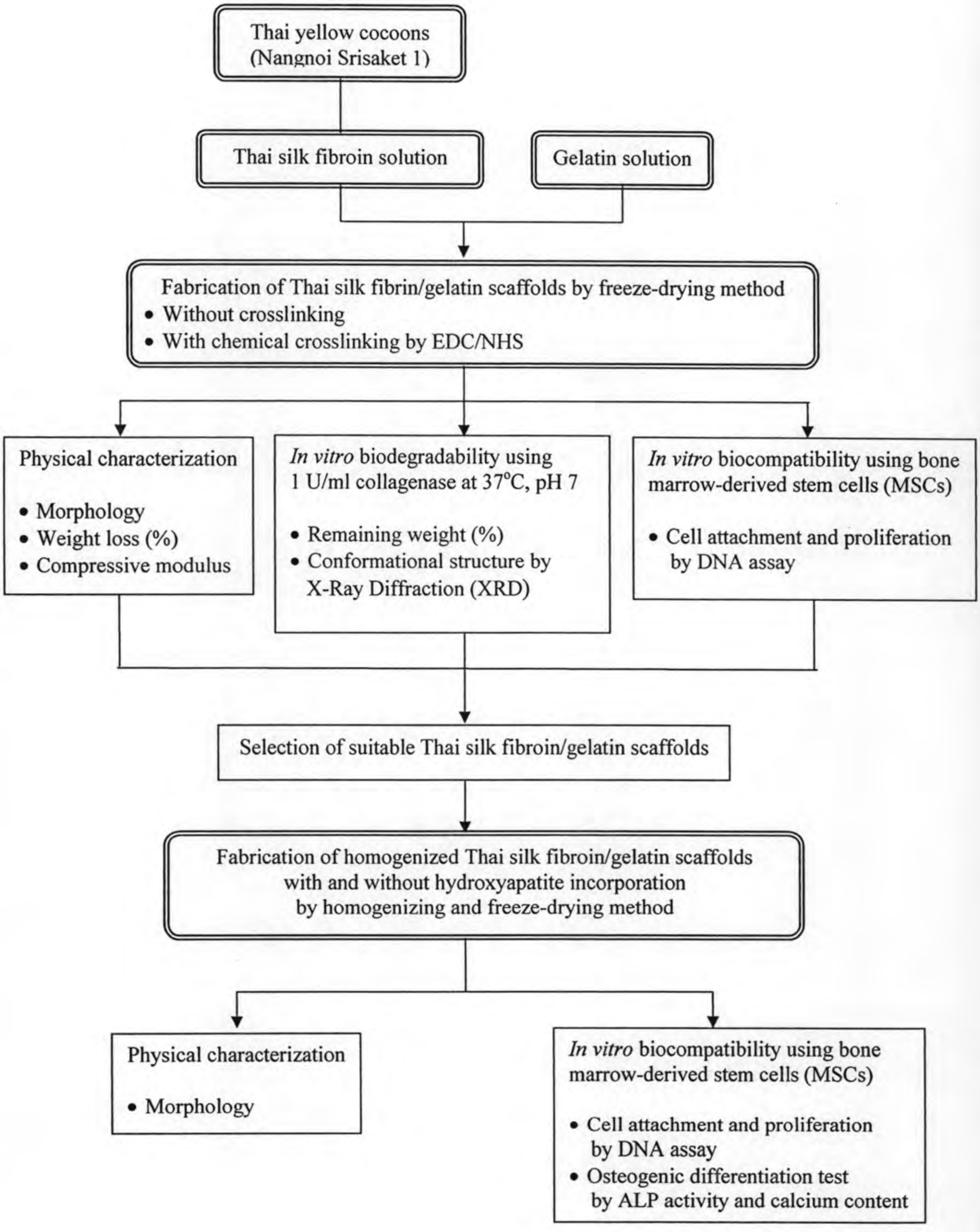

Figure 3.1 Diagram of experimental procedures.

3.3.1 Preparation of Thai silk fibroin and gelatin solutions

3.3.1.1 Preparation of Thai silk fibroin solution

Thai silk fibroin solution was prepared as described by Kim *et.al.* [9]. Cocoons were boiled for 20 min in an aqueous solution of 0.02 M Na_2CO_3 and then rinsed thoroughly with deionized water to extract the sericin. The degummed Thai silk fibroin was dissolved in 9.3 M LiBr solution at 60°C for 4 h to form 25wt% solution. The solution was dialyzed against deionized water using seamless cellulose tubing (MWCO 12000-16000, Viskase Companies, Inc., Japan) at room temperature for 2 days until the conductivity of dialyzed water was the same as that of deionized water. The final concentration of Thai silk fibroin aqueous solution was about 6-6.5wt%, determined by weighing the remaining solid after drying.

3.3.1.2 Preparation of gelatin solution

To prepare gelatin solution, type A gelatin was suspended in deionized water for 30 min. The concentration of gelatin solution was varied depending upon the desired weight blending ratio of Thai silk fibroin/gelatin scaffolds, as shown in Table 3.1. The suspension was subsequently stirred at 40°C for 60 min to obtain gelatin solution.

3.3.2 Preparation of Thai silk fibroin/gelatin scaffolds

To prepare the crosslinked Thai silk fibroin/gelatin scaffolds, 6.4wt% Thai silk fibroin solution was reacted with EDC/NHS (1.2 mg/ml) for 15 min at room temperature under gentle stirring to activate carboxylic groups of Thai silk fibroin [42]. To quench the EDC, 70 μl/ml β-mercaptoethanol was added. Then gelatin solution was blended into Thai silk fibroin solution under agitation for 30 min at room temperature (volume of each protein solution used was summarized in Table 3.1). Then blended solution was left at the room temperature for 1.5 h. In this research, the total solid weight of Thai silk fibroin/gelatin was fixed as 4wt% and the weight blending ratios of Thai silk fibroin/gelatin, (SF/G) were 0/100, 20/80, 40/60, 50/50, 60/40, 80/20, and 100/0. After that, 2.5 ml crosslinked solution was added in a cylindrical container and frozen at -50°C overnight prior to lyophilization at -55°C for 48 h. Finally, the freeze-dried Thai silk fibroin/gelatin scaffolds were washed several times with deionized water for 7 h and dehydrated again by freeze-drying. To prepare the non-crosslinked Thai silk fibroin/gelatin scaffolds, 6.4wt% Thai silk fibroin solution was directly blended with gelatin solution for 30 min at room temperature under gentle stirring. Then blended solution was left at the room temperature for 1.5 h After that, the non-crosslinked scaffold was fabricated by freeze-drying method as described previously.

Table 3.1 The concentration and volume of Thai silk fibroin and gelatin solution at each weight blending ratio of Thai silk fibroin/gelatin scaffolds.

Weight blending ratio of Thai silk fibroin/gelatin	Thai silk fibroin solution		Gelatin solution	
	Concentration (wt%)	Volume (ml)	Concentration (wt%)	Volume (ml)
0/100	0	0	4.00	100
20/80	6.40	10	3.66	70
40/60	6.40	10	3.20	30
50/50	6.40	20	2.91	44
60/40	6.40	10	2.56	16.67
80/20	6.40	10	1.60	10
100/0	4.00	100	0	0

3.3.3 Preparation of homogenized Thai silk fibroin/gelatin scaffold with hydroxyapatite incorporation

To prepare the homogenized Thai silk fibroin/gelatin scaffolds with hydroxyapatite incorporation, the non-crosslinked Thai silk fibroin/gelatin solution at the weight blending ratio of 50/50 was selected as a protein-based solution to mix with sieved hydroxyapatite particles (HA, particle size 100-212μm). The desired weight ratio of organic (Thai silk fibroin and gelatin) to inorganic (hydroxyapatite) parts in composited scaffolds was 30:70 which is the ratio reported for natural bone.

Briefly, 4 wt% aqueous solution of blended Thai silk fibroin/gelatin was mixed with HA particles and homogenized at 10000 rpm for 1.75 min. After that 5 ml chloroform solution was slowly added into the solution. The solution was further homogenized for 15 s. The resulting foamy solution was quickly poured into a prefreezed stainless steel tray and frozen at -80°C overnight prior to lyophilization at -55°C for 48 h.

3.3.4 Characterization of scaffolds

3.3.4.1 Morphology

The morphology of scaffolds was investigated by scanning electron microscopy (SEM, JSM-5410LV, JEOL Ltd., Japan). In order to observe the inner structure of scaffolds, the scaffolds were cut vertically with razor blades. The cut scaffolds were placed on the Cu mount and coated with gold prior to SEM observation.

3.3.4.2 Weight loss (%)

To observe the weight loss of the scaffolds during washing with deionized water, the freeze-dried scaffolds before and after washing step were weighed and the weight loss (%) was calculated as followed.

$$\text{Weight loss (\%)} = \frac{w_1 - w_2}{w_1} \times 100$$

Where w_1 and w_2 correspond to the weight of the dried scaffold before and after washing, respectively. The reported values were the mean±standard deviation (n=6).

3.3.4.3 Compressive modulus (dry and wet condition)

To determine the mechanical strength of the scaffolds, the compression tests in dry and wet conditions were performed using a universal testing machine (Instron, No. 5567) at the constant compression rate of 0.5 mm/min. The scaffolds used for this test was 12 mm in diameter and 3 mm in height. For the wet condition of the test, sample was immersed in PBS (-) under vacuum for 24 h at room temperature before the test. The compressive modulus of the scaffolds was determined from the slope of the compressive stress-stain curves during the strain range of 5%-30%. The reported values were the mean±standard deviation (n=6).

3.3.4.4 *In vitro* biodegradability

To investigate the *in vitro* biodegradation behavior of scaffolds, the scaffolds were punched into 12 mm in diameter and 3 mm in height. These scaffolds were incubated at 37°C, pH 7.4 in 1.5 ml solution of 1 U/ml collagenase [52] and 0.01%w/v sodium azide as an antibiotic [53] for 15 min, 30 min, 1 h, 6 h, 12 h, 1, 3, 5, and 7 days. The solution was changed every 2 days to ensure continuous enzyme activity. After each interval of time, the degraded scaffolds were taken out from the solution, rinsed with deionized water, centrifuged at 5,000 rpm for 15 min and freeze dried. The number of scaffolds used for each experimental group was three (n=3). All degraded scaffolds were characterized as follows.

3.3.4.4.1 Remaining weight (%)

To observe the remaining weight of the degraded scaffolds, the remaining samples were weighed and the percentage of remaining weight was calculated as followed.

$$\text{Remaining weight (\%)} = \frac{w_{re}}{w_{\text{int}}} \times 100$$

Where w_{int} and w_{re} correspond to the initial weight of sample before degradation and the weight of sample after degradation at different time intervals, respectively. The reported values were the mean±standard deviation (n=3).

3.3.4.4.2 Conformational structure by X-Ray Diffraction (XRD)

After enzymatic degradation, the X-ray diffraction patterns of degraded scaffolds were determined using an X-ray diffractometer (Bruker AXS, D8 discover) with Cu Kα radiation. The voltage of the X-ray source was 40 kV at a current of 40 mA. The measurement was scanned at $2\theta = 15°\text{-}40°$. The scan speed was 0.2 sec/step with the step size of 0.03°.

3.3.4.5 *In vitro* biocompatibility using bone marrow-derived stem cells (MSCs)

3.3.4.5.1 MSCs isolation and culture

MSCs were isolated from the bone shaft of femurs of 3 week old female wistar rats according to the technique reported by Takahashi et al. [46]. Briefly, both ends of rat femurs were cut away from the epiphysis and the bone marrow was flushed out by a 26-gauge needle with 1 ml of alpha-modified eagle medium (α-MEM) supplemented with 15% fetal bovine serum (FBS). The cell suspension was placed into tissue culture plates containing α-MEM supplemented with 15% FBS at 37°C in 5%CO_2 incubator. The medium was changed on the 4^{th} day of culture and every 3 days thereafter. When the cells proliferated became subconfluent, after 10 days, the cells were detached with 0.25wt% of trypsin and 0.02wt% of ethylenediaminetetraacetic acid (EDTA). The second and third passages of MSCs were used in this study.

3.3.4.5.2 Cell cuture

Before seeding cells into scaffolds (dimension: d=12mm, h=2mm), scaffolds were placed into 24-well tissue culture plates and sterilized with 70% (v/v) ethanol for 1 h. To remove ethanol, the scaffolds were rinsed 2 times with autoclaved PBS (-) and immersed in the culture medium overnight prior to cell seeding.

MSCs were seeded into the scaffolds by an agitated seeding method [46]. Briefly, the scaffolds were placed into 48-well tissue culture plates. Then MSCs were seeded (5×10^5 and 1×10^6 cells per scaffold for proliferation and osteogenic differentiation tests, respectively) into each scaffold and agitated on an orbital shaker at 250 rpm for 6 h at 37°C in 5%CO_2 incubator. After that, the scaffolds with seeded MSCs were placed into 24-well tissue culture plates and incubated in α-MEM supplemented with 15% FBS at 37°C in 5%CO_2 incubator. In case of osteogenic differentiation test, the medium was changed into osteogenic medium (α-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 50 μg/ml L-ascorbic acid, and 10 nM dexamethasone [54]) at 1 day after seeding. Along the cell culture test, the medium was changed every 2 days.

3.3.4.5.3 MSCs initial attachment and proliferation tests by DNA assay

To monitor cell adhesion and proliferation on scaffolds, the number of cells was determined by fluorometric DNA assay [46]. After the cells were cultured for a desired period of time (6 h, 1, 3, and 5 days), the cell-seeded scaffolds were washed with PBS (-). Then the samples were lysed in SDS lysis buffer at 37°C overnight to prepare cell lysate and stored at -20°C until assay. When performing the assay, standard cells and samples were thawed at room temperature. After thawing, standard cells were lysed in SDS lysis buffer at 37°C for 1 h and diluted with SDS for calibration curve (SDS was used as a blank test). Then 20 μl Hoechst solution (1 mg/ml DMSO) was diluted at room temperature with 19 ml deionized water and 1 ml sodium citrate-buffered saline solution (SSC) 20X. After that, 100 ml diluted Hoechst solution was added into standard cells and samples. The fluorescent intensity of mixed solution was measured in a fluorescence microplate reader (Perkin elmer, 1420 multilabel counter) at the excitation and emission wavelengths of 355 and 460 nm, respectively. All data were expressed as mean±standard deviation (n = 4).

3.3.4.5.4 Osteogenic differentiation test by ALP activity and calcium content

To assess the osteogenic differentiation of MSCs cultured on the scaffolds under osteogenic induction at 7, 14, 21, and 28 days after the culture, the alkaline phosphatase (ALP) activity and calcium content were used as the early and late marker for osteogenic differentiation test, respectively [54, 55].

To determine ALP activity, 10 mM p-nitrophenol solution was diluted with deionized water for calibration curve (deionized water was used as a blank test). Then 20 μl of each deionized water, p-nitrophenol standard solution, and supernatant of cell lysate prepared using the procedure described in section 3.3.4.5.3 was reacted with 100 μl p-nitrophenyl phosphate liquid substrate (pNPP) at 37°C for 15 min to converse p-nitrophenyl phosphate to p-nitrophenol. To stop reaction, 80 μl of 0.02 N NaOH solution was added. Finally, the solution was measured spectrophotometrically at 405 nm using a UV-VIS spectrophotometer (Thermo Spectronic, Genesys 10UV scanning).

To measure the calcium content, 1 M HCl was added into the cell lysate samples with equal volume and incubated at 4 °C overnight. For standard solution preparation, 20 mg/ml $CaCO_3$ was diluted with 1 M HCl for calibration curve (1 M HCl was used as a blank test). Then 1 ml of 0.88 M ethanolamine buffer and 100 μl of 0.63 M o-cresolphythalein complex substrate (OCPC) was added into 10 μl of each 1 M HCl, $CaCO_3$ standard solution, and the samples. Thereafter, calcium contents were determined spectrophotometrically at 570 nm using a UV-VIS spectrophotometer.

It was noticed that, for the case of scaffolds containing hydroxyapatite, the original amount of calcium in each of scaffolds was determined and used to substract from the amount of calcium of that scaffolds obtained after cell culture.

At each time interval, the number of cells were also determined by DNA assay and used to normalize the ALP activities and calcium content. All data were expressed as mean±standard deviation (n = 4).

3.3.4.5.5 The observation of cultured cells

After 5 and 28 days for proliferation and osteogenic differentiation tests, respectively, the scaffolds seeded with cells were washed with PBS (-) to remove non-adherent cells and then fixed in 2.5wt% glutaraldehyde solution in PBS (-) at 4°C for 1 h to fix cells. Subsequently, the scaffolds were serially dehydrated by series of ethanol, which were 30%, 50%, 70%, 80%, 90%, 95%, and 100%, for 6 min at each concentration. The scaffolds were then dried in hexamethyldisilazane (HMDS) at room temperature. Dried scaffolds were cross-sectional cut and observed under SEM [46].

3.3.5 Statistical analysis

Significant levels of each result were determined by an independent two-sample t-test. All statistical calculations were performed on the Minitab system for Windows (version 14, USA). P-values of <0.05 was significantly considered.