Chapter 2

Materials and methods

Methods

1 Scope of study

This study is aimed to investigate the growth and differentiation of mouse osteoblasts, MC3T3-E1 cell line on 1:1 and 1:2 chitosan-collagen sponges and compare them with growth and differentiation of cells on chitosan and collagen sponges. MC3T3-E1 cells were seeded on the sponges and cultivated for 27 days in a mineralized culture medium. Growth and differentiation of osteoblasts on each type of sponge were monitored and compared. The investigations at each time point were performed in 5 consecutive samples.

Chitosan, collagen and chitosan-collagen composite sponges were fabricated using a freeze-drying process. The microstructures of the sponges before cell seeding and the attachment of cells on the sponges at different time points were examined using a scanning electron microscope (SEM).

Cell viability was detected using MTT assay to determine the numbers of cells present. ALP activity and calcium content in an extracellular matrix were measured to determine osteoblastic differentiation of MC3T3-E1 cells in early and late osteoblastic differentiation stages, respectively. It was hypothesized that growth and differentiation of osteoblats on chitosan-collagen composite sponges would be enhanced compared to the activities of cells on chitosan and collagen scaffolds.

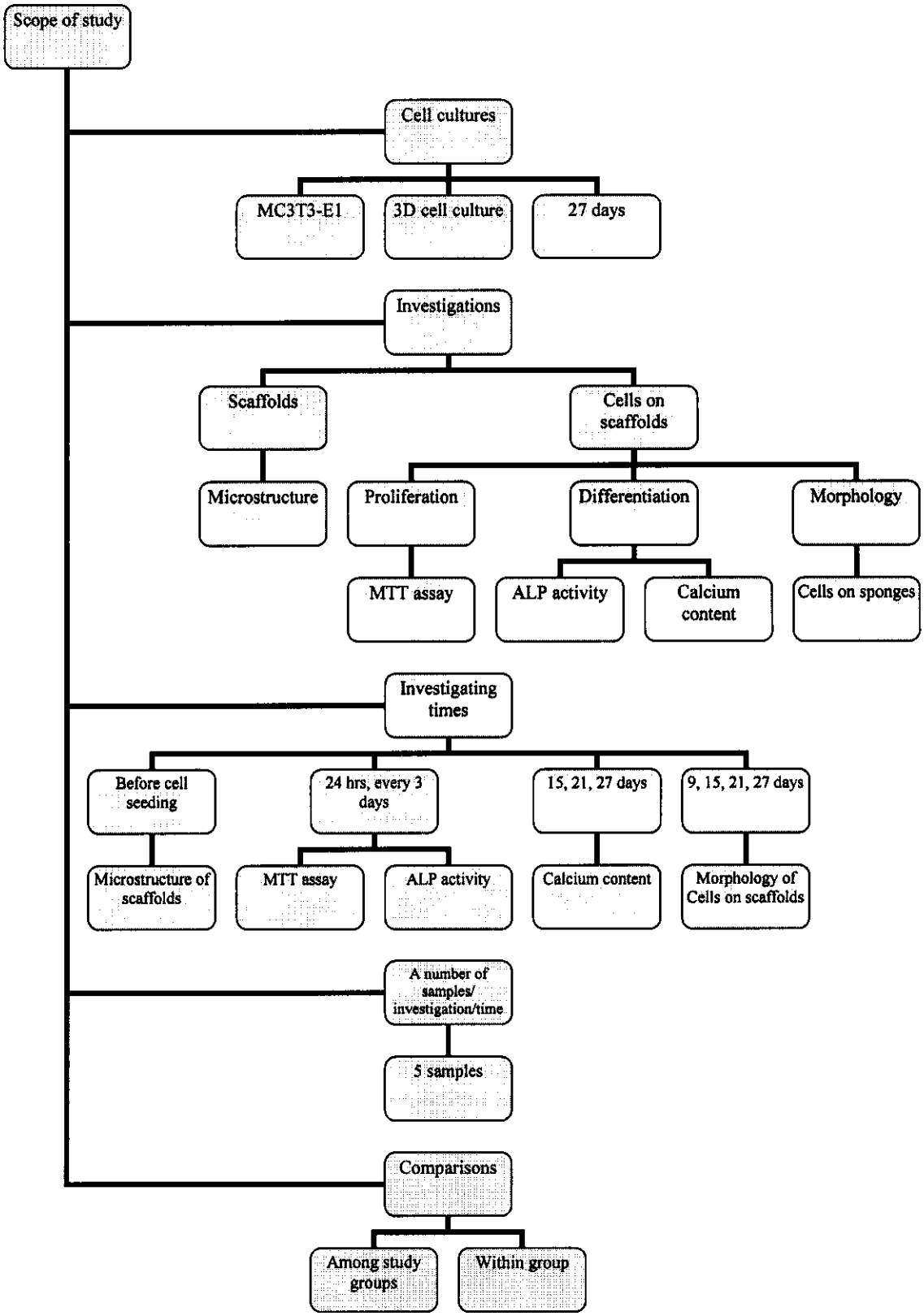

Figure 3 Scope of the study

2 Study designs

2.1 Groups of study

The study was divided into 4 categories, Group A: chitosan sponges; Group B: collagen sponges; Group C: 1:1 chitosan-collagen sponges; and Group D: 1:2 chitosan-collagen sponges (Table 1).

Table 1 Groups of study

Groups	Characteristics
A	Chitosan sponges
B	Collagen sponges
C	1:1 Chitosan-collagen sponges
D	1:2 Chitosan-collagen sponges

2.2 Osteogenic three-dimensional cell culture

Fetal mouse calvarial osteoblast cell line MC3T3-E1 was obtained from ATCC (USA). The cell line was subcultivated into passage 5-6 (Franceschi and Iyer, 1992). MC3T3-E1 cells, $1x10^5$ cells in 20 µl culture medium, were each seeded on moistened sponges of groups A-D and cultivated for 27 days in a mineralized culture medium. The medium was changed every 3 days.

2.3 Investigated parameters

Microstructure of scaffolds and morphology and attachment of cells on the sponges were examined using a scanning electron microscope. Morphology and growth of cells on sponges were investigated on culture-days 9, 15, 21, and 27. The investigations were performed in 5 samples at each time point.

Cell viability assay (MTT assay) was performed to determine the numbers of viable cells present. ALP activity and calcium content were measured as parameters of osteoblastic cell differentiation in early and late stages, respectively. MTT assay and ALP activity were determined in 3 day-intervals following cell seeding. Calcium content was measured on culture-days 15, 21, and 27. The investigations were performed in 5 samples at each time point (Table 2).

Table 2 Summary of investigation methods and times

Investigations	Methods	Investigation times
Microstructure of sponges	SEM	Before cell seeding
Morphology and growth of cells on sponges	SEM	Every 6 days (9,15, 21, 27 days)
Cell growth (numbers of cells)	MTT assay	24 hrs and every 3 days after cell seeding (1, 3, 6,....., 27 days)
Osteoblastic differentiation	ALP activity	24 hrs and every 3 days (1, 3, 6,....., 27 days)
	Calcium content	15, 21, 27 days

3 Procedures

3.1 Preparation of chitosan –collagen composite sponges

3.1.1 Preparation of 1% chitosan solution in 1% acetic acid solution (w/v)

To prepare 500 ml of 1% chitosan in 1% acetic acid solution (w/v), 5 g of chitosan powder (from crab shell, medium molecular weight, Fluka, Switzerland) was dispersed in 250 ml distilled water under continuous stirring, and then 250 ml of 2% acetic acid solution was added. The solution was stirred continuously at room temperature until a complete solubility was obtained. The solution was kept at $4^{\circ}C$ until it was used.

3.1.2 Preparation of 1:1 and 1:2 chitosan-collagen solutions

Chitosan-collagen composite solutions in ratios of 1:1 and 1:2 were prepared by adding 75 g and 50 g of 1% chitosan in 1% acetic acid solution into 75 g and 100 g of 1% atelocollagen solution (Succinyl atelocollagen from calves, Koken, Japan), respectively. The mixture was gently stirred at room temperature until a homogenous solution was obtained.

3.1.3 Crosslinking procedure

The solutions of 1% chitosan, 1% atelocollagen, and chitosan-collagen solutions were crosslinked with 0.05% glutaraldehyde (Merck, Germany). One milliliter of 0.5% glutaraldehyde was gradually added into 10 ml of the solution with a continuous stirring to generate a foam-like consistency composite. The crosslinked mixtures were then poured into 3ml-polystyrene syringes (NIPRO, Japan) and stored at 4°C for 24 hours and –70°C for 12 hours. Then the frozen solutions underwent a freeze-drying process (Flexi-Dry™, USA).

3.1.4 Freeze-drying process and preparation of sponges

The evaporation under freezing conditions changed the physical properties of the solutions into sponge-like scaffolds with different porosities. The freeze-drying process was performed at –50°C for 48 hours using a freeze-drying machine (Flexi-Dry™) (Figure 3-4) (Oungbho, 1997). After the freeze-drying process was finished, the cylindrical sponges were cut into 3x5 mm sponges (Figure 5). The sponges were sterilized by ethylene dioxide gas and stored in sterile containers at room temperature for 14 days to allow for the evaporation of the residual ethylene dioxide before being used in the cell cultures.

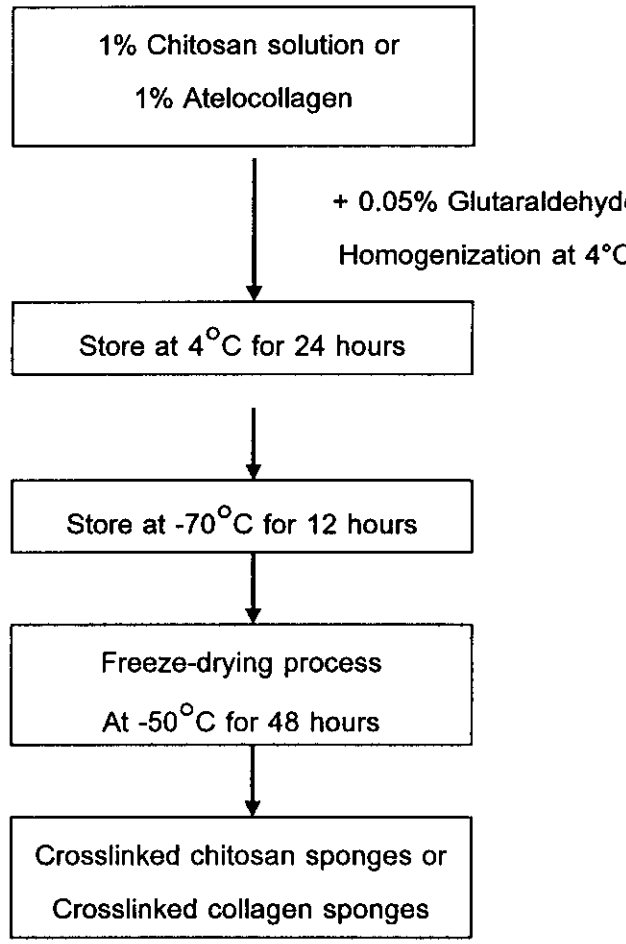

Figure 4 Prepartion of chitosan and collagen sponges (Oungbho, 1997)

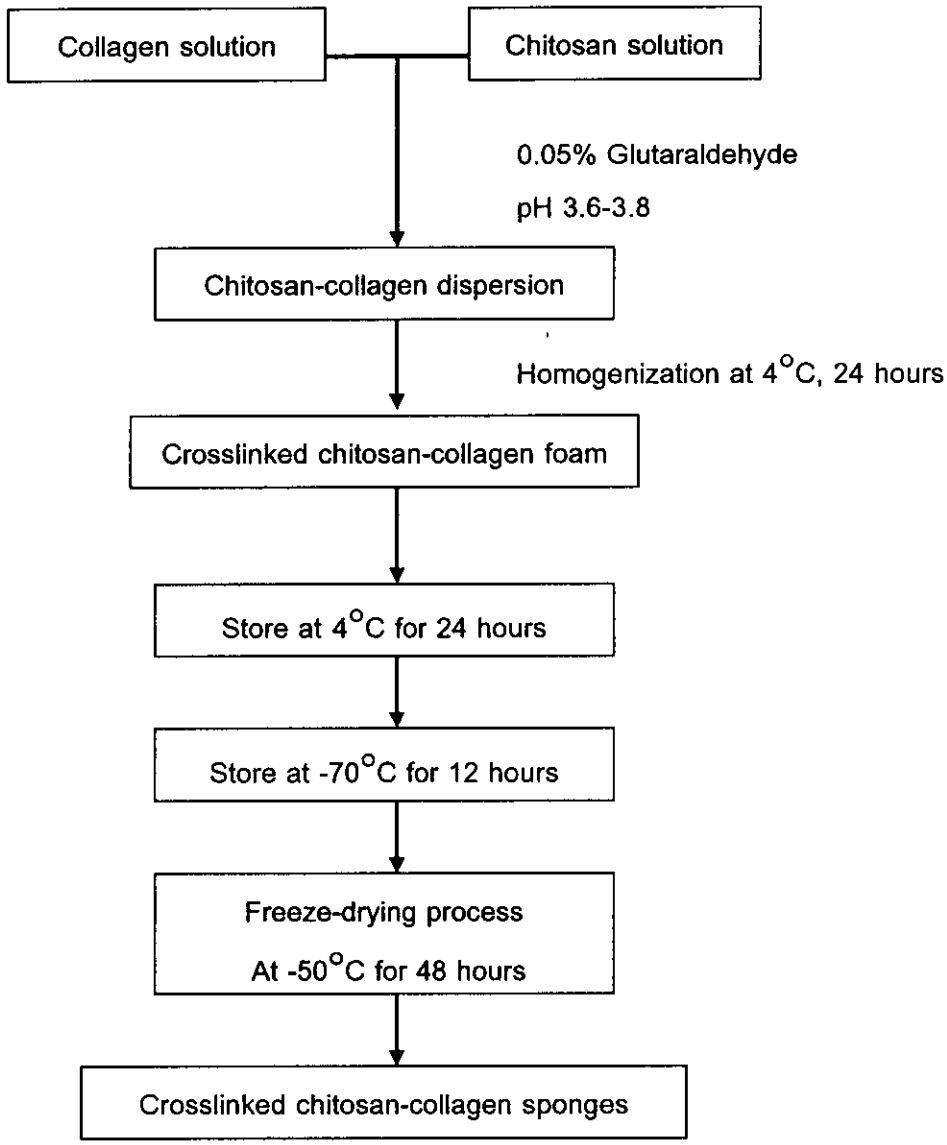

Figure 5 Preparation of 1:1 and 1:2 chitosan-collagen composite sponges (Oungbho, 1997)

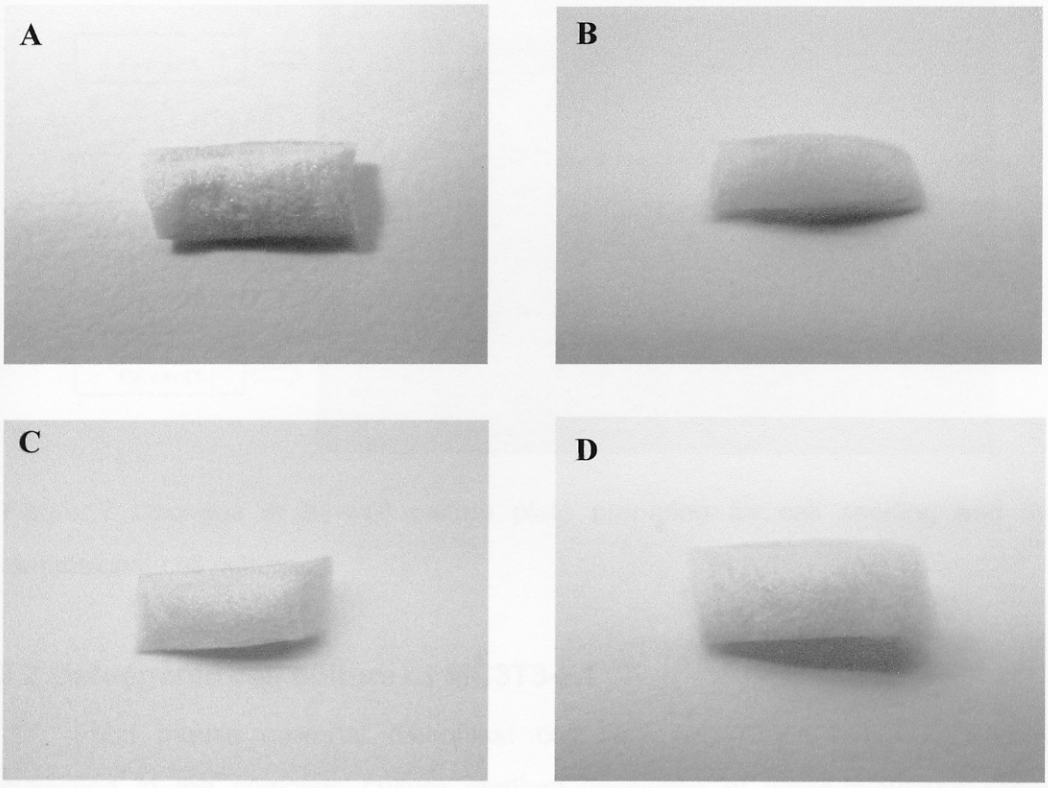

Figure 6 The sponges size 3x5 mm before immerging in culture medium, Group A: chitosan sponge (A), Group B: collagen sponge (B), Group C: 1:1 chitosan-collagen sponge (C) and Group D: 1:2 chitosan-collagen sponge (D)

3.1.5 Preparation of the sponges for cell seeding

Five days before cell seeding, the sponges were neutralized in a complete culture medium, α-MEM medium supplemented with 10% fetal bovine serum and antibiotics. In brief, sponges were washed by centrifugation two times at 100g (700 rpm) for 10 minutes. The sponges were then incubated in a complete culture medium in a humidified incubator with 5% CO_2 at 37°C for 5 days. Just before cell seeding, excess fluid was removed. The sponges were placed into 24-well culture plates for cell seeding, one sponge per well, 5 samples per group (Figure 6).

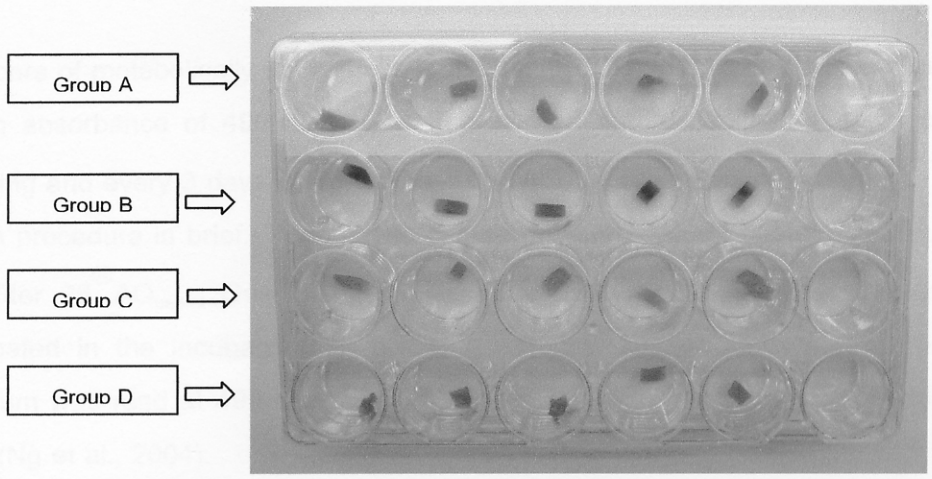

Figure 7 Sponges in 24-well culture plate preparing for cell seeding and three-dimensional cell culture

3.2 Osteogenic cell culture of MC3T3-E1

Fetal mouse calvarial osteoblast cell line, MC3T3-E1 (ATCC, USA) were cultivated in the complete culture medium consisting of alpha-minumum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 2% penicillin-streptomycin, 0.2% Fungizone, 50 μg/ml ascorbic acid and incubated in 5% CO_2 at 37°C in 95% relative atmospheric humidity. Cells were passaged every 3-4 days into passages 5–6 (Franceschi and Iyer, 1992). The culture medium was changed every 3 days.

MC3T3-E1 cells in passages 5-6, $1x10^5$ cells in 20 μl of culture medium, were statically seeded onto each sponge of groups A-D. Cells were allowed to attach on the scaffolds in a minimal culture medium for 3 hours in 5% CO_2 at 37°C in 95% relative atmospheric humidity. Subsequently, 1.5 ml of a complete culture medium supplemented with 5 mM β-glycerophosphate was added into each well. Cells were cultivated for 27 days and the culture medium was changed every 3 days.

3.3 Characterization of growth and differentiation of MC3T3-E1

3.3.1 Cell viability assay

The MTT assay is a calorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The quantity of the formazan product as measured by the amount of formazan dye formed directly correlated to the

numbers of metabolically active cells in the culture. The formazan dye was quantified at an absorbance of 490 nm. Cell viability was determined at 24 hours after cell seeding and every 3 days using CellTiter 96® AQ_{ueous} One Solution (Promega, USA).

A procedure in brief: A complete culture medium, 500 µl, containing 50 µl of the CellTiter 96® AQ_{ueous} One Solution was added into each well. The sponges were incubated in the incubator with 5% CO_2 at 37°C for 1 hour. The absorbance of medium was read at 490 nm using microplate reader (Biotrak™ II microplate reader, UK) (Ng et al., 2004).

3.3.2 Alkaline phosphatase activity (ALP)

3.3.2.1 Cell lysis

Nonionic detergent, Triton X-100, a mild and nondenaturing detergent, was used to solubilize membrane proteins for enzyme assays or immunoassays. Triton X-100 in a phosphate buffer saline (PBS) (2% Triton X-100 in PBS) was used in the cell lysis procedure to obtain protein solutions for ALP activity analysis.

A procedure in brief: At each time point, the sponges and cells in culture plates underwent repeated freezing and thawing processes. In one cycle, frozen sponges and monolayer cells were thawed in RT for 30 minutes and refrozen at -20°C for 30 minutes. This cycle was repeated three times. Next, 200 µl of 2% Triton X-100 in PBS was added into each well then the sponges were minced into small pieces. The mixtures were transferred into microcentrifuge tubes, vortexed vigorously for 30 seconds, incubated on ice for 1 hour and 30 minutes and re-vortexed vigorously for 30 seconds. All of the tubes were taken for centrifugation at 2000 g (4500 rpm) at 4°C for 10 minutes (Rozycki and Edelstein, 1996). The supernatants were transferred into new microcentrifuge tubes and stored at -20°C until the measurement of ALP activity was performed.

3.3.2.2 Measurement of ALP activity

An assay for ALP activity is based on the hydrolysis of p-nitrophenyl phosphate by ALP yielding p-nitrophenol and inorganic phosphate. p-Nitrophenol is converted into a yellow complex readily measured at 400-420 nm. The intensity of

color formed is proportional to phosphatase activity which is comparable to μM of nitrophenol. ALP activity was measured at 24 hours after cell seeding and every 3 days using Micro-plate reader (Biotrak™ II microplate reader, UK).

A procedure in brief: Cell lysis solutions were thawed on ice. Four hundred microliters of ALP lysis solution (2 mg p-Nitrophenyl phosphate in 1 ml 2-amino-2-methyl-1-propanolol) was pipetted into each tube containing 100 μl of cell lysis solution. The solution was thoroughly mixed on a vortexer and incubated at 37°C for 1 hour. Then 400 μl 0.05 N NaOH was added into each tube to stop the reaction and followed by 100 μl of distilled water. The mixtures were vortexed for a thorough mixing of the solutions.

The mixtures were added into each well of 96-well plates, 200 μl/well, 3 wells per sample. Series of p-nitrophenol standard concentrations ranging from 0.1-1 μM (0.1, 0.2, 0.4, 0.6, 0.8, and 1 μM) were prepared. Levels of p-Nitrophenol in the solutions were measured by monitoring light absorbance at 405 nm (Biotrak™ II microplate reader, UK). The levels of intensity of yellow are directly proportional to concentrations of p-Nitrophenol and levels of ALP in the standard solutions. The slope of absorbance used to calculate the ALP activity was based on values of a series of p-Nitrophenol standards (Wada et al., 1998).

3.3.3 Calcium content

Calcium reagents (Calcium, Sigma, USA) are for the quantitative, colorimetric determination of calcium. The principle of an assay is that calcium in a test solution is combined with o-cresolphthalein complexone in an alkaline medium. The reaction yields calcium-cresolphthalein complexone complex which produces a purple colored complex at pH 10-12 with a maximum absorbance at 575 nm. The intensity of the color is directly proportional to concentrations of calcium in the samples.

$$\text{Calcium} + \text{o-Cresolphthalein Complexone} \xrightarrow[\text{Medium}]{\text{Alkaline}} \text{Calcium-Cresolphthalein Complexone Complex}$$

(Hanada et al., 1997)

3.3.3.1 Demineralization procedure

Sponges used in MTT assay which were kept at -20°C were thawed in RT and washed with PBS. The sponges were minced into small pieces using a sharp surgical blade number 15 and incubated in 300 µl of 0.5 N hydrochloric acid (HCl) for 18 hours at RT. After that the mixtures were pipetted into microcentrifuge tubes and centrifuged at 4500 rpm, 25°C for 5 minutes. The supernantant was transferred into new microcentrifuge tubes and stored at –20°C until use.

3.3.3.2 Measurement of calcium content

Calcium kit and Calcium/Phosphate standard sets (Sigma Diagnostics, USA) were used in the analysis. The measurement was performed following the manufacturer's instructions. In brief, series of calcium standard concentrations ranging from 6.25-150 mg/l (6.25, 2.5, 25, 50, 75, 100, 125, and 150 mg/l) were prepared. The frozen supernatants were thawed at room temperature. One milliliter of working solution of the reagent was prepared by adding 0.5 ml of calcium binding agent into 0.5 ml of a calcium buffer and mixed well. Then 10 µl of samples, solutions of demineralized extracellular matrix or calcium standard solutions were added into each 1 ml of working solution and mixed well. Two hundred microliters of the reacted solutions were pipetted into each well of 96-well plates, 3 wells per sample. The absorbances were read at 570 nm (Biotrak™ II microplate reader, UK). The slope of absorbance was used to calculate level of calcium content based on the value of a series of calcium standards (Wada et al., 1998).

3.4 Scanning electron microscope (SEM)

Sponges were washed in PBS and fixed with 2.5% glutaraldehyde in PBS for 2 hours at RT. Then they were washed twice with PBS for 10 minutes each time and were fixed with 1% osmium tetroxide for 1 hour at RT. Next, the sponges were washed with distilled water three times and dehydrated with graded ethanol series of 50%, 70%, 80% and 90% twice for 30 minutes during each step. Finally they were dehydrated twice in 100% pure ethanol for 30 minutes each time and then the sponges were put into baskets and fixed with absolute alcohol. They were critically point dried by a critical point dryer machine (Polaron, UK). The sponges were

sputtered and coated with gold-palladium size 20 nm using a SPI-Module™ Sputter coater (SPI, USA). The sponges were examined by a SEM JEOL JSM-5800LV model (JOEL, Japan). Fields of analysis were examined at both high and low magnifications to give a qualitative confirmation of the cell proliferation on to the sponges (Attawia et al., 1999).

Pore sizes of all sponges were measured from SEM images by random selection of the 3-4 pores from a SEM image of each sponge group and 3 SEM images were selected from each type of the sponges.

4 Data analysis

Microstructure of sponges and morphologies of cells on sponges were descriptively described. The pore sizes of each sponge were analyzed by mean of descriptive statistics. Parametric data of MTT, ALP activity, and calcium content of 5 consecutive samples were presented as average values of 5 consecutive samples with standard a deviation (Mean±SD).

The data was tested for normal distribution and homogeneity of variances. Differences among groups at each time point were analyzed using one-way analysis of variance (ANOVA). When a difference was statistically significant at $p < 0.05$, a multiple comparison test was then performed. If the variances of the data were equal, the Scheffe method was selected. If the variances of the data were not equal, the Dunnette T3 method was used. Significant differences were set at 95% confidence interval ($p<0.05$).

The analyses were performed on a personal computer using Commercial SPSS software (Version 11.0, Standard Software Package Inc., USA) for data analysis and Stat View for Windows (Version 4.5, USA) for graphic demonstrations.