CHAPTER IV

EXPERIMENTAL WORK

The experimental work can be divided into three main parts:

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

4.1 Materials and reagents

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

4.1.2 Type A gelatin powder (116g bloom, pH 4.5, pI 9, lab grade, Ajax Finechem, Australia)

4.1.3 Type B gelatin powder (150g bloom, pH 5.6, pI 4.9, food grade, Erawan Chemical Co., Ltd., Thailand)

4.1.4 Sodium carbonate (Na_2CO_3, Ajax Finechem, Australia)

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

4.1.6 Mined salt (particle size 600-710μm, Thai refined salt Co., Ltd., Nakhonratchasima, Thailand)

4.1.7 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Nacalai Tesque, Inc., Japan)

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

4.1.9 Calcium chloride ($CaCl_2$, Ajax Finechem, Australia)

4.1.10 Sodium dihydrogen phosphate monohydrate($NaH_2PO_4 \cdot H_2O$, Sigma-Aldrich, Germany)

4.1.11 Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$, Sigma-Aldrich, Germany)

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

4.1.13 Modified eagle medium (α-MEM, Hyclone, USA)

4.1.14 Phosphate buffer saline without calcium, and magnesium (PBS, Hyclone, USA)

4.1.15 Trypsin-EDTA (0.25% trypsin with EDTA·Na, Gibco BRL, Canada)

4.1.16 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, USB corporation, USA)

4.1.17 Dimethyl sulfoxide (DMSO, Fisher Scientific, UK)

4.1.18 Glycine (NH_2CH_2COOH, Ajax Finechem, Australia)

4.1.19 Sodium chloride (NaCl, Analar, England)

4.1.20 Sodium hydroxide (NaOH, Analar, England)

4.2 Equipments

4.2.1 Seamless cellulose tubing (Molecular weight cut off 12000-16000, Viskase Companies, Inc., Japan)

4.2.2 -40°C freezer (Heto, PowerDry LL3000, USA)

4.2.3 Lyophilizer (Heto, PowerDry LL3000, USA)

4.2.4 Vacuum drying oven and pump (VD23, Binder, Germany)

4.2.5 Fourier transform infrared (FTIR) spectrophotometer from Perkin Elmer (Spectrum GX, UK)

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

4.2.7 Scanning Electron Microscopy (JSM-5400, JEOL Ltd., Japan)

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

4.2.9 Laminar Flow (HWS Series 254473, Australia)

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

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

4.2.12 24-well polystyrene tissue culture plates (NUNC, Denmark)

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

4.3 Experimental procedures

All experimental procedures are summarized in Figure 4.1. In brief, there are three main steps comprised in this work; preparation of silk fibroin and gelatin solutions, preparation of silk and silk-based scaffolds, and characterization of scaffolds.

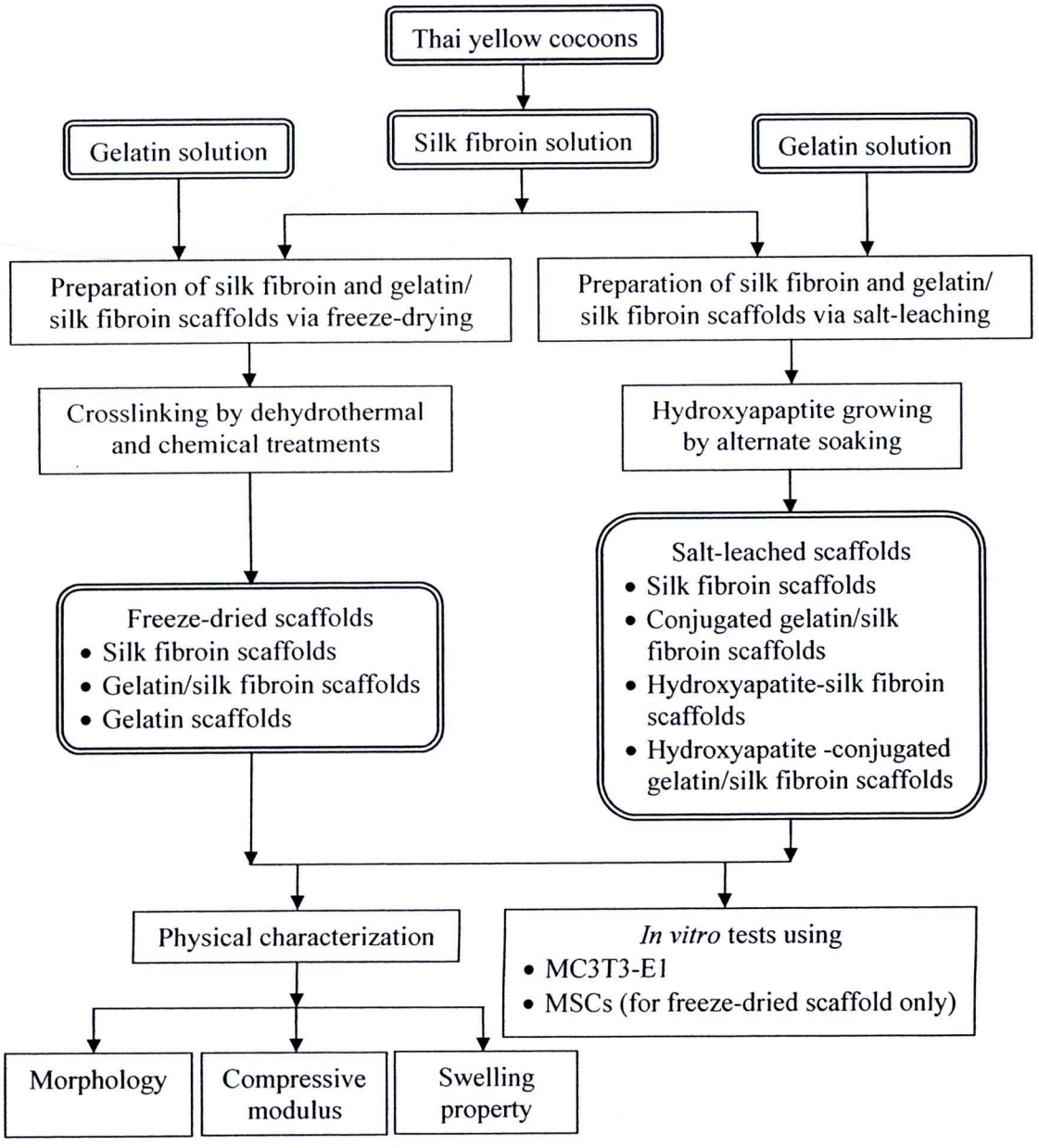

Figure 4.1 Diagram of experimental procedures.

4.3.1 Preparation of silk fibroin and gelatin solutions

4.3.1.1 Preparation of silk fibroin solution

Silk fibroin solution was prepared as described by Kim *et.al.* [1]. Cocoons were boiled in 0.02M Na_2CO_3 aqueous solutions and then rinsed thoroughly. This process was repeated until cocoon became colorless and the solution color remained the same to ensure complete removal of sericin or silk gum. The degummed silk fibroin was dissolved in 9.3M LiBr solutions at 60°C for 4 h to form 25wt% solution. The solution was dialyzed against deionized water at room temperature for 2 days and the conductivity of dialyzed water was the same as that of deionized water. After dialysis, 6.5wt% of aqueous silk fibroin solution, determined by weighing the remaining solid after drying, was obtained and further diluted to form 2wt% solution.

4.3.1.2 Preparation of gelatin solution

Type A and type B gelatin was suspended at the concentration of 2wt% in deionized water. The suspension was subsequently stirred at 40°C for 60 min to obtain gelatin solution.

4.3.2 Preparation of silk fibroin and silk fibroin-based scaffolds

4.3.2.1 Preparation of silk fibroin and gelatin/silk fibroin scaffolds via freeze-drying

Gelatin and silk fibroin solutions were blended under agitation at room temperature for 4 h. The blending weight ratios of gelatin/silk fibroin were 0/100, 20/80, 40/60, 60/40, 80/20 and 100/0. After mixing, 1ml of the 2wt% blended solution was poured into each well of 24-well plates and frozen at -50°C overnight prior lyophilized at -50°C for 48 h. The freeze dried scaffolds were treated by dehydrothermal (DHT) treatment at 140°C for 24 and 48 h in a vacuum oven. After that, the scaffolds were treated with carbodiimide solution (14mM 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 5.5mM N-hydroxy-succinimide (NHS)) at room temperature for 2 h [59]. Treatment scaffolds were rinsed thoroughly with deionized water and freeze dried.

4.3.2.2 Preparation of silk fibroin and conjugated gelatin/silk fibroin scaffolds via salt-leaching

After dialysis, 3ml of 6.5wt% silk fibroin solution was added in a cylindrical container and 9 g of mined salt (particle size: 600-710μm) was added. The container was covered and left at room temperature for 24 h to allow the gelation of silk fibroin solution. Then, the container was immersed in water to leach out salt for 2 h. The scaffold was taken out from the container and washed under stirring for 4 h. The washing water was changed every 30 min. After that, the scaffold was left to be dried overnight. The silk fibroin scaffold was punched into 11mm in diameter, 2mm in height and immersed in 0.5wt% gelatin solution under vacuum for 2 h to allow gelatin coating on silk fibroin scaffolds. Gelatin was further conjugated via DHT and EDC treatments as previously described.

4.3.2.3 Preparation of hydroxyapatite/silk fibroin and hydroxyapatite-conjugated gelatin/silk fibroin scaffolds

Hydroxyapatite was deposited on the salt-leached scaffolds by an alternate soaking process [56]. The scaffold was immersed in 0.2M $CaCl_2$ at room temperature under vacuum for 30 min. After that, the scaffold was removed from $CaCl_2$ solution and rinsed with deionized water. The scaffold was then immersed in 0.12M Na_2HPO_4 at room temperature under vacuum for 30 min. After removing the scaffold from Na_2HPO_4 solution, it was rinsed with deionized water again. This was considered as one cycle of alternate soaking. Soaking process was performed for 2, 4, and 6 cycles. Fresh solutions, $CaCl_2$ and Na_2HPO_4 were used for each soaking cycle. After desired cycles of soaking process, the hydroxyapatite/silk fibroin and hydroxyapatite-conjugated gelatin/silk fibroin scaffolds were air-dried at room temperature for 24 h.

4.3.3 Characterization of scaffolds

4.3.3.1 Chemical characterization

The conformational structure of pure silk fibroin scaffolds before and after crosslinking was investigated using fourier transform infrared spectroscopic (FTIR) and X-ray diffraction (XRD) techniques.

4.3.3.1.1 Attenuated total reflection fourier transform infrared (ATR-FTIR) spectrophotometric measurements

The silk fibroin scaffolds were analyzed by ATR-FTIR technique using Perkin Elmer Spectrum GX (FTIR system). All spectra were recorded in the wavenumber range of 1800-900cm^{-1} at the resolution of 4cm^{-1}.

4.3.3.1.2 X-ray diffraction (XRD) measurements

X-ray diffraction patterns of silk fibroin scaffolds were recorded using a Brucker D8 X-ray diffractometer at 30kV and 30mA, with Cu Kα radiation. The measurement was scanned at $2\theta = 10°$-$50°$. The scan speed used was 2.0 sec/step with the step size of $0.04°$.

4.3.3.2 Physical characterization

4.3.3.2.1 Morphology

The morphology of scaffolds was investigated by scanning electron microscopy (SEM). In order to observe the inner structure of scaffolds, the scaffolds were cut vertically and/or horizontally with razor blades. The cut scaffolds were placed on the Cu mount and coated with gold prior to SEM observation.

4.3.3.2.2 Compressive modulus

The compression tests in a dry condition were performed on all scaffolds using a universal testing machine (Instron, No. 5567) at the constant compression rate of 0.5 mm/min. The compressive modulus of the scaffolds (dimension: d=12mm, h=3mm for freeze-drying and d = 11mm, h = 2mm for salt-leaching method) 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=5).

4.3.3.2.3 Swelling property

Scaffolds were immersed in phosphate buffered saline (PBS) at 37°C, pH 7.4 for 24 h. After excess water was removed, the wet weight of the scaffold was determined. The swelling ratio of the scaffold (W_{sw}) was calculated as follow:

$$W_{sw} = \frac{(W_t - W_o)}{W_o}$$

W_t represented the weight of the wet scaffolds, and W_o was the initial weight of the scaffolds. The values were expressed as the mean±standard deviation (n=5).

4.3.3.3 Biological characterization

In this work, primary bone-marrow derived mesenchymal stem cells (MSCs) and mouse osteoblast-like cells (MC3T3-E1) were used in *in vitro* tests. Cell proliferation on all scaffolds was studied using MC3T3-E1. However, due to a limit in MSCs isolation, the proliferation of MSCs was performed only on freeze-dried silk fibroin scaffolds.

4.3.3.3.1 MSCs isolation and culture

MSCs were isolated and cultivated according to the methods of Takahashi [59]. MSCs derived from the bone shaft of femurs of 3 week old wistar rats. 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 phosphate buffer saline without calcium, and magnesium. The cell suspension was placed into tissue culture plates containing modified eagle medium (α-MEM) supplemented with 15% fetal bovine serum 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 first passage of MSCs was used in this study.

4.3.3.3.2 MC3T3-E1 culture

MC3T3-E1 were cultured in growth medium, α-MEM containing 10% FBS. They were incubated at 37°C in an atmospheric condition containing 5% CO_2. The culture medium was changed every 3 days. At confluence, MC3T3-E1 was harvested using 0.25% trypsin-EDTA and subcultivated in the same medium with 3 dilutions. The 23^{rd} passage of MC3T3-E1 was used in this work.

4.3.3.3.3 *In vitro* cell proliferation tests

The scaffolds were placed into 24-well tissue culture plates and were sterilized in 70% (v/v) ethanol for 30 min. To remove ethanol, the scaffolds were rinsed 3 times with phosphate-buffered saline (PBS).

MC3T3-E1 were seeded at a density of 2×10^4 cells per scaffold onto each scaffold placed in medium and incubated at 37°C in 5%CO_2 incubator. After cultured for a desired day, the cells were then quantified by the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT solution (0.5mg/ml in DMEM without phenol red) was added into each scaffold and incubated for 30 min to

establish cell viability. DMSO was used to elute complex crystals and the absorbance of the solution was measured at 570nm using a spectrophotometer. The same treatment of the scaffolds without cells was used as a control. The culture medium was changed every 3 days. The cells viability was determined after 3^{rd} and 7^{th} days of seeding for MSCs and 7^{th} and 14^{th} days for MC3T3-E1. All data were expressed as mean±standard deviation (n = 3).

4.3.3.3.4 MC3T3-E1 migration and morphological observation

To study the cell migration into scaffolds and the morphology of cultured cells, freeze-dried gelatin/silk fibroin scaffolds with the blending composition of 20/80, and 0/100 were selected to observe its interaction with MC3T3-E1 because they did not collapse after dehydration. For salt-leached scaffolds, silk fibroin (control) and conjugated-gelatin/silk fibroin scaffolds were observed.

Scaffolds on which cells were cultured for 14 days were fixed with 2.5% glutaraldehyde solution in PBS for 1 h. Scaffolds were then serially dehydrated by a series of ethanol, which were 30%, 50%, 70%, 80%, 90%, 95% and 100%, for 5 min at each concentration. 200 µl of hexamethyldisilazane (HMDS) was added to dry the dehydrated scaffolds at room temperature. Dried scaffolds were cross-sectional cut and cell migration, including all morphology, was observed by SEM as schematically shown in Figure 4.2. The cell-seeding side was labeled as position 1 while position 2 and 3 represented further depth inside the scaffolds to the plate-exposed side (position 4).

4.3.4 Statistical analysis

Significant levels 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.

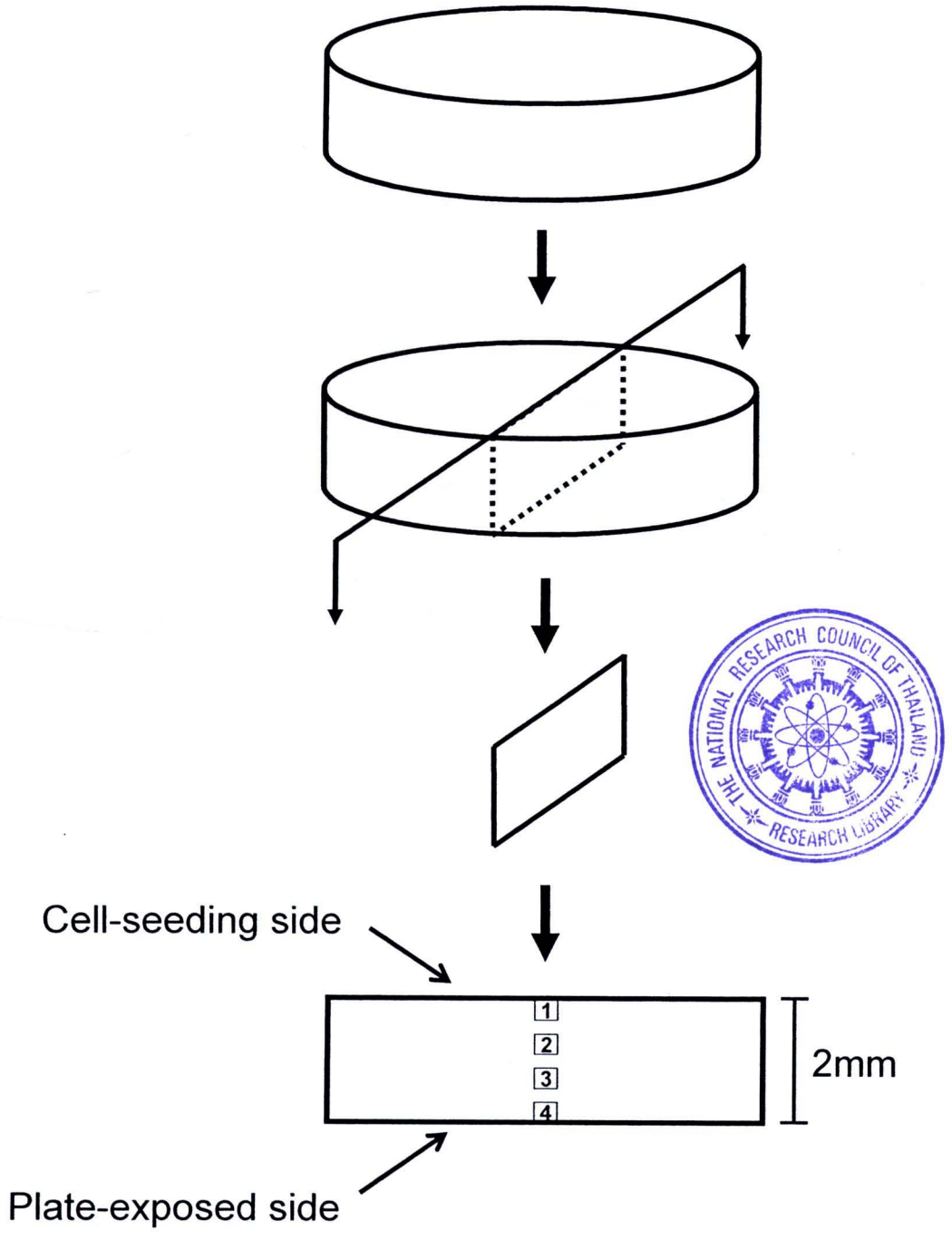

Figure 4.2 Schematic diagram of cross-sectional plane prior to the observation of cell migration and morphology.