# CHAPTER IV

# EXPERIMENTAL WORK

The experimental work can be divided into three main parts: (1) Materials and reagents, (2) Equipments, and (3) Experimental procedures. The experimental procedures are subdivided into twelve parts: (i) Low molecular weight chitosan preparation, (ii) Determination of viscosity-average molecular weight and weight-average molecular weight, (iii) Chitosan solution preparation, (iv) Collagen solution preparation, (v) Fabrication of collagen/chitosan scaffolds, (vi) Fourier transform infrared (FT-IR) spectroscopic analysis, (vii) Differential scanning calorimetric (DSC) analysis (viii) Compressive modulus determination, (ix) Swelling ratio determination, (x) Morphology, (xi) biodegradation, (xii) cell adhesion, and (xiii) cell proliferation. All experimental procedures are summarized in Figure 4.1.

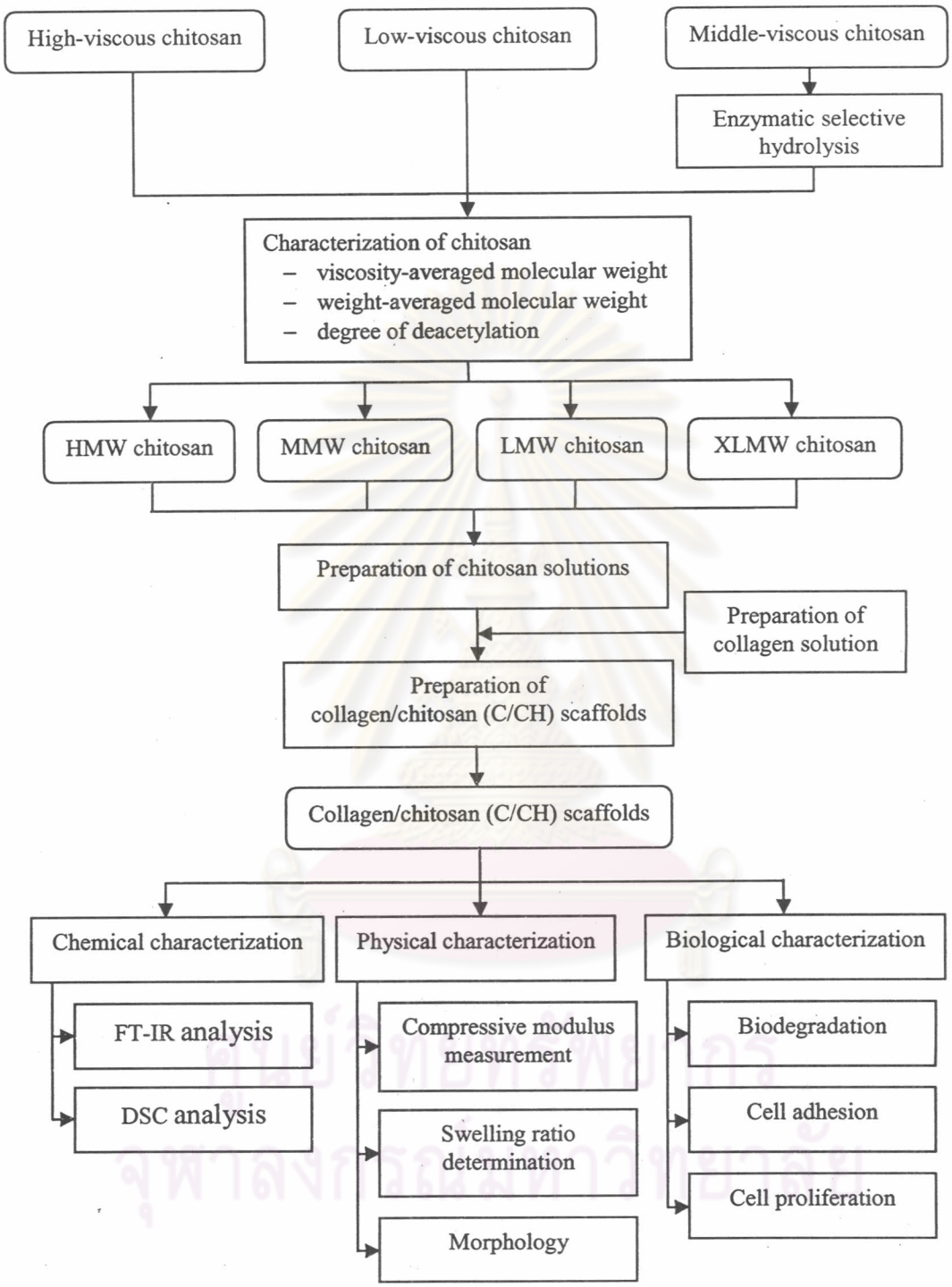



Figure 4.1 The schematic diagram of experimental procedures.

## 4.1 Materials and Reagents

1. Type I collagen

Type I collagen used in this work was derived from porcine skin by pepsin-solubilized process, purchased from Nitta Gelatin Inc., Osaka, Japan. Type I collagen was preserved in diluted hydrochloric solution pH 3 with the concentration of 6.06 mg/g.

2. Chitosan

Low-, middle-, and high-viscous chitosans from crab shell were supplied by Fluka (Germany). The viscosities of low-, middle-, and high-viscous chitosan were reported at 70, 332, and 411 mPa·s, respectively. However, before uses, the viscosity-averaged molecular weights and weight-averaged molecular weights of three different viscosity chitosans were determined according to the method detailed in section 4.3.3 and 4.3.4, respectively. The results of the molecular weight determination were reported in section 5.1.

3. Glacial acetic acid was supplied by BDH (UK).
4. Dihydrogen phosphate monohydrate and Ethyl alcohol were supplied by Merck (Germany).
5. Disodium hydrogen phosphate heptahydrate and Lysozyme from hen-egg white with 70,000 U/mg were purchased from Fluka (Germany).
6. Dulbecco's modified eagle medium (DMEM) and Trypsin-EDTA solution (0.25% Trypsin in EDTA.4Na) were purchased from Gibco (Canada).
7. Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (USA).
8. Dimethyl sulfoxide (DMSO) was supplied by Riedel-deHaën (Germany).
9. 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (USA).

**4.2 Equipments**

1. Ubbelohde viscometer from Cannon Instrument Company (BS/IP/SL 50, USA)
2. Film-casting apparatus
3. Fourier transform infrared (FT-IR) spectroscopy from Perkin Elmer (Spectrum GX, UK)
4. Differential scanning calorimeter (DSC) from Perkin Elmer (Diamond DSC, UK)
5. Lyophilizer and -40°C freezer from Thermo Electron Corporation (Heto PowerDry LL3000, USA)
6. Vacuum oven and pump from Binder (VD23, Germany)
7. High performance balance from Mettler Toledo (AG204, Switzerland)
8. pH meter from Sartorius (Professional Meter PP-50, Germany)
9. Polystyrene tissue culture discs and 24-well flat bottom polystyrene tissue culture plates from NUNC (Denmark)
10. Universal mechanical testing machine form Instron (Instron 5567, USA)
11. Scanning electron microscopy (SEM) from Joel (JSM 5400, Japan)
12. Magnetic stirrer from Eyela (RNC-3, Germany)
13. Micropipette from Gilson (Pipetman P20; P200; P1000; and P5000, USA)
14. Spectrophotometer from Genesis (10 UV Scanning, USA)
15. Phase contrast microscope from Olympus (Olympus CK2, USA)
16. $CO_2$ incubator from Thermo Electron Corporation (Series II 3110 Water Jacketed Incubator, USA)
17. Laminar flow hood from Clyde Apac (HWS-120, Australia)

## 4.3 Experimental procedures

### 4.3.1 Preparation of XLMW and LMW chitosan

XLMW chitosan was prepared according to the method of selective hydrolysis of middle-viscous chitosan usingwith *Bacillus* sp. PP8 crude chitosanase [47]. *Bacillus* sp. PP8 was classified as *Bacillus circulans* and *Bacillus coagulans*. The molecular weight of crude chitosanase from *Bacillus* sp. PP8 is 47.5 kDa. Briefly, middle-viscous chitosan from crab shell was dissolved in 1% (v/v) acetic acid to form 1% (w/w) final concentration. The pH of solution was adjusted to 5 by 1 M NaOH. The ratio of enzyme to chitosan solution at 1:5 was used. Two hundreds milliliters of crude chitosanase were added into chitosan solution and the solution was incubated and stirred in a water bath at 50°C. After 24 h, the reactions were stopped and XLMW chitosan was pricipitated by adjusting the pH of chitosan solution to approximately 10 using 1 M NaOH. The precipitate was centrifuged and washed several times with deionized water for neutralization. The neutralized chitosan precipitate was then lyophilized.

LMW chitosan was prepared according to the method of selective hydrolysis of middle-viscous chitosan with *Bacillus licheniformis* SK1 crude chitinase [48]. Briefly, middle-viscous chitosan from crab shell was dissolved in acetic acid pH 5.0 to obtain 1% (w/w) solution. A typical reaction contained 100 mU/mL of the enzyme (1 unit is equivalent to the amount of enzyme that produces 1 μmol of *N*-acetyl-D-glucosamine (GlcNAc) per min from colloidal chitin) and 10–40 mg/mL of the substrate. For this system, 25 ml of crude chitinase (100 mU/ml) was added into the chitosan solution and the solution was incubated and stirred in a water bath at 50°C. The reactions were stopped after 24 h and LMW chitosan was separated and dried using the same methods as for the case of XLMW chitosan.

### 4.3.2 Characterization of chitosans

#### 4.3.2.1 Degree of deacetylation determination

The degree of acetylation (DA) of different molecular weight chitosas was determined by using the following equation [49]:

$$DA\ (\%) = 31.92(A_{1320}/A_{1420}) - 12.20 \qquad (4.1)$$

where $A_{1320}$ and $A_{1420}$ are the absorbance peak from Perkin Elmer Spectrum GX (FT-IR system) of C-N stretching and C-H deformations, respectively. The degree of deacetylation (DD) can be calculated from 100 – DA (%).

#### 4.3.2.2 Measurement of intrinsic viscosity

The intrinsic viscosity, [η], of chitosan solutions was measured using Ubbelohde viscometer at 25°C. The solvent used was 0.3 M $CH_3COOH$/0.2 M $CH_3COONa$ aqueous solution [50-52]. Intrinsic viscosity of chitosan was determined following the ASTM standard practice for dilute solution viscosity of polymers (American Society for Testing and Materials, 2001). Viscosity of a series of diluted chitosan solutions in acetic acid was measured and the reduced viscosity, $\eta_{red}$, was calculated by:

$$\eta_{red} = \frac{(\eta/\eta_0 - 1)}{c} \approx \frac{(t/t_0 - 1)}{c} = \frac{(\eta_r - 1)}{c} \qquad (4.2)$$

where η is the viscosity of the chitosan solution at the polymer concentration c, and $\eta_0$ is the solvent viscosity while $\eta_r$ is denoted as relative viscosity. Secondly, the inherent viscosity, $\eta_i$, was calculated as:

$$\eta_i = \frac{\ln \eta_r}{c} \qquad (4.3)$$

Intrinsic viscosity, [η], of chitosan in aqueous acetic acid solutions was determined from the intercept of both inherent viscosity, $\eta_i$, and reduced viscosity, $\eta_{red}$, plots where c was near zero [53, 54].

#### 4.3.2.3 Viscosity-averaged molecular weight determination

The viscosity-averaged molecular weight was calculated based on the Mark-Houwink equation as follows:

$$[\eta] = KM_v^a \qquad (4.4)$$

where K and a are Mark-Houwink parameters usually depending on the experimental temperature, type of solvent, and degree of deacetylation of chitosan. In this work, the values of $K = 3.5 \times 10^{-2}$ (ml/g) and a = 0.76 were used based on the report of Terbojevich and coworkers [50, 51]. They have proposed these specific parameters for chitosan having degree of deacetylation around 85%. The degree of deacetylation of four chitosan samples used in this work is in the range of 84-87% (see results in section 5.1) which is approximately the same as in the work of Terbojevich and coworkers.

#### 4.3.2.4 Gel permeation chromatography (GPC)

Weight-averaged molecular weight and the polydispersity of four chitosan samples were characterized using gel permeation chromatography (GPC) technique (PL-GPC 110, Polymer labolatories Ltd., Shropshire SY6 6AX, UK). The system consists of two columns; one Ultrahydrogel linear column and one guard column (both 300 x 7.8 mm, in series), with a refractive index detector. The measurement was performed at 30°C. Chitosan aqueous solution (2.0 g/l) at pH 3 was prepared using 0.5 M acetic acid and 0.5 M sodium acetate. Eluent and chitosan solution (mixture of 0.5 M acetic acid and 0.5 M sodium acetate) were filtered through 0.45 μm Millipore filters (HAWPO 1300) before injected into the column at the flow rate of 0.6 ml/min. The injected volume of chitosan solution was 20 μl. Calibration was performed using the standard sample of pullulans.

### 4.3.3 Scaffold preparation

#### 4.3.3.1 Chitosan solution preparation

Chitosan solutions were prepared by dissolving chitosan in 0.5 M acetic acid to form 0.5% (w/w) concentration. Chitosan suspensions were vigorously stirred overnight at room temperature to ensure homogeneous and uniform solution.

#### 4.3.3.2 Collagen solution preparation

Type I collagen supplied by Nitta Gelatin Inc. is the solution of collagen dissolved in hydrochloric acid (pH 3). Before uses, stock collagen solution was lyophilized to get rid of the solvent. To prepare 0.5% (w/w) of collagen solution, lyophilized type I collagen was redissolved in 0.5 M acetic acid. The suspension of collagen in 0.5 M acetic acid was allowed to swell at 4°C overnight and then stirred at room temperature for 1 h.

#### 4.3.3.3 Fabrication of collagen/chitosan scaffolds

0.5% (w/w) collagen solution and 0.5% (w/w) chitosan solution were mixed at different proportions to form collagen/chitosan solutions at the weight ratios of 100/0; 90/10; 70/30; 50/50; 30/70; 10/90; and 0/100. Collagen/chitosan blending solutions were stirred at room temperature for 1 h and degassed under vacuum pressure. The degassed solutions were pipetted into 24-well cell culture plates with 1 ml volume of solution per well and then frozen at -40°C refrigerator for 24 h prior to lyophilize under vacuum pressure (<100 mTorr) at the condenser temperature of -40°C for 24 h. The lyophilized scaffolds were then crosslinked via dehydrothermal treatment (DHT) or vacuum heating process at 105°C for 48 h.

### 4.3.4 Chemical characterization of scaffolds

#### 4.3.4.1 Fourier transform infrared (FT-IR) spectroscopic analysis

Blending solution of collagen and chitosan was prepared as mentioned in section 4.3.3.3. To obtain the films of polymer blends for FT-IR analysis, the solution was cast on teflon plate. After solvent evaporation for 24 h at room temperature, the films with the thickness of 104 μm were further crosslinked by dehydrothermal treatment at 105°C for 48 h. The information on structural contribution was collected in the FT-IR analysis using Perkin Elmer Spectrum GX model (FT-IR system). All spectra were recorded in the wavenumber range from 4000 to 400 $cm^{-1}$ at the resolution of 4 $cm^{-1}$. The FT-IR analysis was based on the identification of absorption bands concerned with the vibrations of functional groups presented in the samples.

#### 4.3.4.2 Differential scanning calorimetric (DSC) analysis

Thermal characteristics of scaffolds containing various proportion of collagen and chitosan were carried out by differential scanning calorimeter (DSC) using Perkin Elmer Diamond DSC model. All experiments were performed in the scanning mode from 60 to 300°C at the heating rate of 20°C/min. The sample weight was about 5 mg. Dry nitrogen gas was introduced into the DSC cell as the purging gas.

### 4.3.5 Physical characterization of scaffolds

#### 4.3.5.1 Compressive modulus

A universal testing machine (INSTRON 5567, NY, USA) was used to determine the compressive modulus of scaffolds by compressing the samples of which the dimension was 13 mm in diameter and 3 mm in thickness at the constant deformation rate of 0.5 mm/min. The slopes of compressive stress-strain curves at 5 to 35% deformation were used to calculate the compressive modulus. The reported values are the mean of seven specimens.

#### 4.3.5.2 Equilibrium swelling ratio determination

Equilibrium swelling ratio ($E_s$) was measured by the conventional gravimetric method. The collagen, collagen/chitosan, and chitosan scaffolds were preweighted and then immersed in 0.05 M phosphate buffer saline (PBS) pH 7.4 at temperature of 37°C and excess surface phosphate buffer saline was blotted out with absorbent paper. The wet weight ($W_s$) of the scaffold was determined after incubating for 24 h. The equilibrium swelling ratio of the scaffolds was defined as the ratio of weight increase ($W_s$-$W_d$) with respect to the initial weight ($W_d$) of dry samples. Each value was averaged from three parallel measurements. $E_s$ was calculated using the following equation:

$$E_s = \frac{W_s - W_d}{W_d} \tag{4.5}$$

where $W_s$ and $W_d$ denote the weights of swollen and dry samples, respectively.

#### 4.3.5.3 Morphology

The morphology of collagen, collagen/chitosan, and chitosan scaffolds was analyzed using scanning electron microscope (SEM, Joel JSM 5400) at an accelerating voltage of 12–15 kV. Dry scaffolds were sputter-coated with gold (Ion sputtering device, JFC 1100) at 40 mA prior to observing under SEM.

### 4.3.6 Biological characterization of scaffolds

#### 4.3.6.1 Biodegradation

The collagen, collagen/chitosan, and chitosan scaffolds of known dried weights were sterilized by immersing in 70% ethanol for 5 min and digested in 2 ml of 0.05 M phosphate buffer saline (PBS, pH 7.4) at 37°C containing 1.6 μg/ml (112 U/ml) lysozyme (hen egg-white). The concentration of lysozyme used corresponded

to the concentration in human serum [55]. The lysozyme solution was refreshed daily to ensure continuous enzyme activity. After 7, 14, 21 and 28 days, samples were removed from the medium, rinsed with distilled water, frozen, lyophilized and weighed. The experiment was done tripicate for each scaffold. The extent of degradation was expressed as percentage of weight remained of the dried scaffold after lysozyme digestion. To separate between enzymatic degradation and dissolution, control samples were stored for 28 days under the same conditions as described above, but without the addition of lysozyme. Percentage of weight remained was calculated using the following equation:

$$\text{Weight remained} = \frac{W_i - W_f}{W_i} \times 100 \qquad (4.6)$$

where $W_i$ represents the initial weight of scaffolds and $W_f$ represents the weight of digested scaffolds.

#### 4.3.6.2 L929 and Detroit 551 cell cultivation

L929 cells, mouse connective tissue fibroblasts, and Detroit 551 cells, human dermal fibroblasts, were selected to evaluate adhesion and proliferation as a direct contact test. Cells were cultured in 6 mm diameter culture dish (NUNC, Roskilde, Denmark) using growth medium composed of Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin-B. They were incubated in a humidified incubator in an atmosphere of 5% $CO_2$ and 95% air. At confluence, L929 and Detroit 551 cells were harvested using a suspension of 0.25% trypsin and subcultivated in the same medium with 12 and 4 dilutions, respectively.

#### 4.3.6.3 L929 and Detroit 551 cell adhesion and proliferation tests

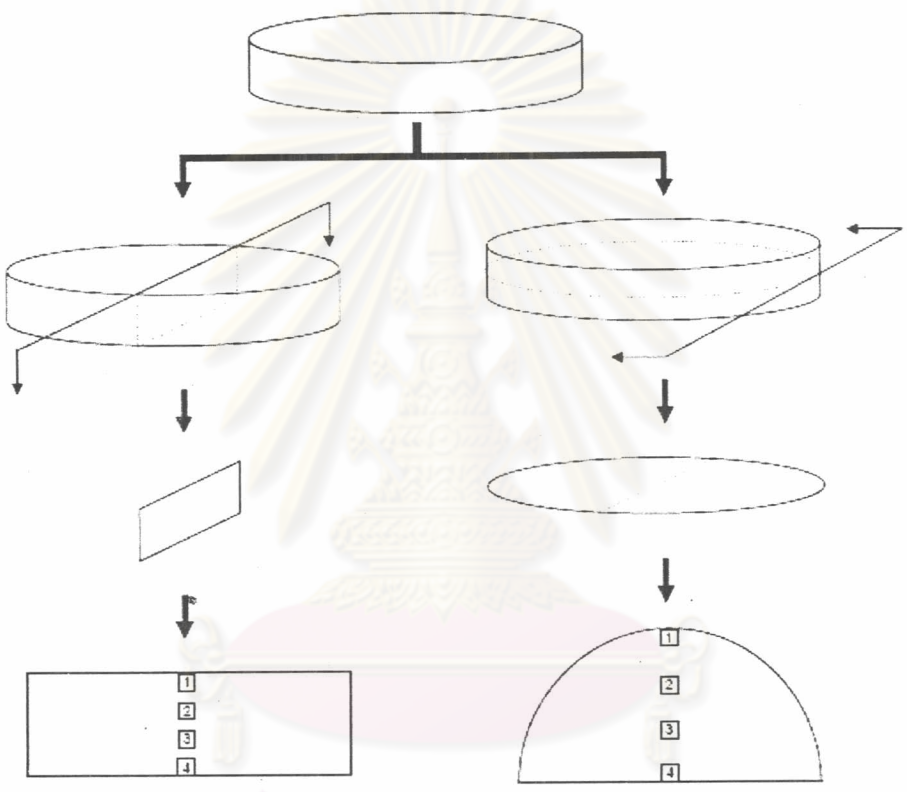
The scaffolds (13 mm in diameter and 2 mm in thickness) were immersed in 70% ethanol for 5 min for sterilization, followed with 4 times of solvent exchange by deionized water. The scaffolds were then placed on a 24-well polystyrene plate and

350 µl of culture medium was added to each well before cell seeding, $6\times10^4$ cells/well. The number of cells seeded onto scaffolds was similar in case of either L929 or Detroit 551 cells. Cells were allowed to initially attach for 5 h. For proliferation test, $2\times10^4$ cells were seeded onto each of the matrices and cultures were harvested after 5, 24, and 72 h. The similar procedure was performed in case of either L929 or Detroit 551 cells. The attached or proliferated cells were then quantified by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay [39]. Three hundreds and fifty microliters of MTT solution (0.5 mg/ml in DMEM without phenol red, filter-sterilized) were added to each culture well. After incubation for 5 h, the MTT reaction medium was removed, and 900 µl of dimethylsulfoxide and 100 µl of glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH) were added. Optical densities were determined by spectrophotometer (Genesis 10 UV scanning, NY, USA) at the wavelength of 570 nm.

#### 4.3.6.3 Detroit 551 cell spreading area observation

To study the cell spreading on scaffolds, collagen/XLMW chitosan scaffolds with blending composition of 70/30 (collagen/chitosan) was selected to observe its interaction with Detroit 551 cell since it significantly expressed cell proliferation. Collagen and XLMW chitosan scaffolds were also selected to investigate their interaction with Detroit 551 cell because they served as positive and negative controls, respectively. Scaffolds were immersed in 70% ethanol for 5 min for sterilization, followed with solvent exchange by deionized water for 4 times. The scaffolds were then placed on a 24-well polystyrene plate and 350 µl of culture medium was added to each well before cell seeding ($2\times10^4$ cells/well). Cells were allowed to proliferate for 72 h. Scaffolds containing Detroit 551 cells were washed twice with phosphate buffer saline (pH 7.4) and then fixed together with 2.5% glutaraldehyde solution in phosphate buffer saline (pH 7.4) for 24 h. Scaffolds were serially dehydrated by series of ethanol composed of 30%, 50%, 70%, 80%, 90%, and 95% for 5 min at each concentration and 15 min for 100% ethanol. Dehydrated scaffolds were dried by adding 100 µl of hexamethyldisilazane (HMDS) and allowed solvent evaporation at room temperature. Dried scaffolds were cut to observe cell

penetration in both cross-sectional and horizontal plane (see Figure 4.2) by scanning electron microscope (SEM). For cross-sectional plane, position 1 represented the cell seeding side while position 4 represented the bottom (container-exposed) side. Position 1 and position 4 in horizontal plane were located at the edge and center of scaffolds, respectively.




(a) (b)

Figure 4.2 Schematic diagram of sectional direction prior to observe cell-scaffold interaction: (a) cross-sectional plane and (b) horizontal plane.

### 4.3.7 Statistical analysis

Significant levels were determined by paired t-test. All statistical calculations were performed on the SPSS system for Windows (version 13.0, Statistical Package for Social Sciences (SPSS), Chicago, IL, USA). P-values of $<0.05$ and $<0.01$ were significantly considered.