CHAPTER IV

EXPERIMENTAL WORKS

4.1 Materials

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

4.1.2 Type B gelatin powder (152g bloom, pH 5.64, pI 4.9, pharmaceutical grade, Geltech Co., LTD., Bangkok, Thailand)

4.1.3 Collagen solution (pH 3.1, Nitta Gelatin Inc., Tokyo, Japan)

4.1.4 Chondroitin-6-sulfate (Sodium salt from shark cartilage, approx 90%, Sigma Co., St. Louis, USA)

4.1.5 Hydrochloric acid (HCl 36.5-38%, J.T. Baker, NJ, USA)

4.1.6 2,4,6-trinitrobenzene sulphonic acid (TNBS)

4.1.7 β-Alanine (Nacalai Tesque, Inc., Kyoto, Japan)

4.1.8 Sodium hydrogen carbonate ($NaHCO_3$ 99%, Fluka, Buchs, Germany)

4.1.9 Sodium dihydrogen phosphate monohydrate($NaH_2PO_4 \cdot H_2O$, Merck, Darmstadt, Germany)

4.1.10 Sodium phosphate dibasic heptahydrate (Na2HPO4·7H2O, Sigma Co., St. Louis, USA)

4.1.11 Lysozyme (70,000 U/mg, Fluka, Buchs, Germany)

4.1.12 Ethanol (99.7-100%, VWR International Ltd., Poole Dorset, United Kingdom)

4.1.13 Mouse skin fibroblasts (L929 or murine fibroblasts),

4.1.14 Dulbecco's modified eagle medium, DMEM (10%medium + L-glutamine + AB, Hyclone, Utah, 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, Cleveland, OH, USA)

4.1.17 Dimethylsulfoxide, DMSO (Sigma-Aldrich, Germany)

4.2 Equipments

4.2.1 -40°C freezer (Heto, PowerDry LL3000, USA)

4.2.2 Lyophilizer (Heto, PowerDry LL3000, USA)

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

4.2.4 UV-Vis spectrophotometer

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

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

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

4.2.8 Laminar Flow (HWS Series 254473, Australia)

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

4.2.10 Spectrophotometer (Thermo Spectronic, Genesys 10UV scanning)

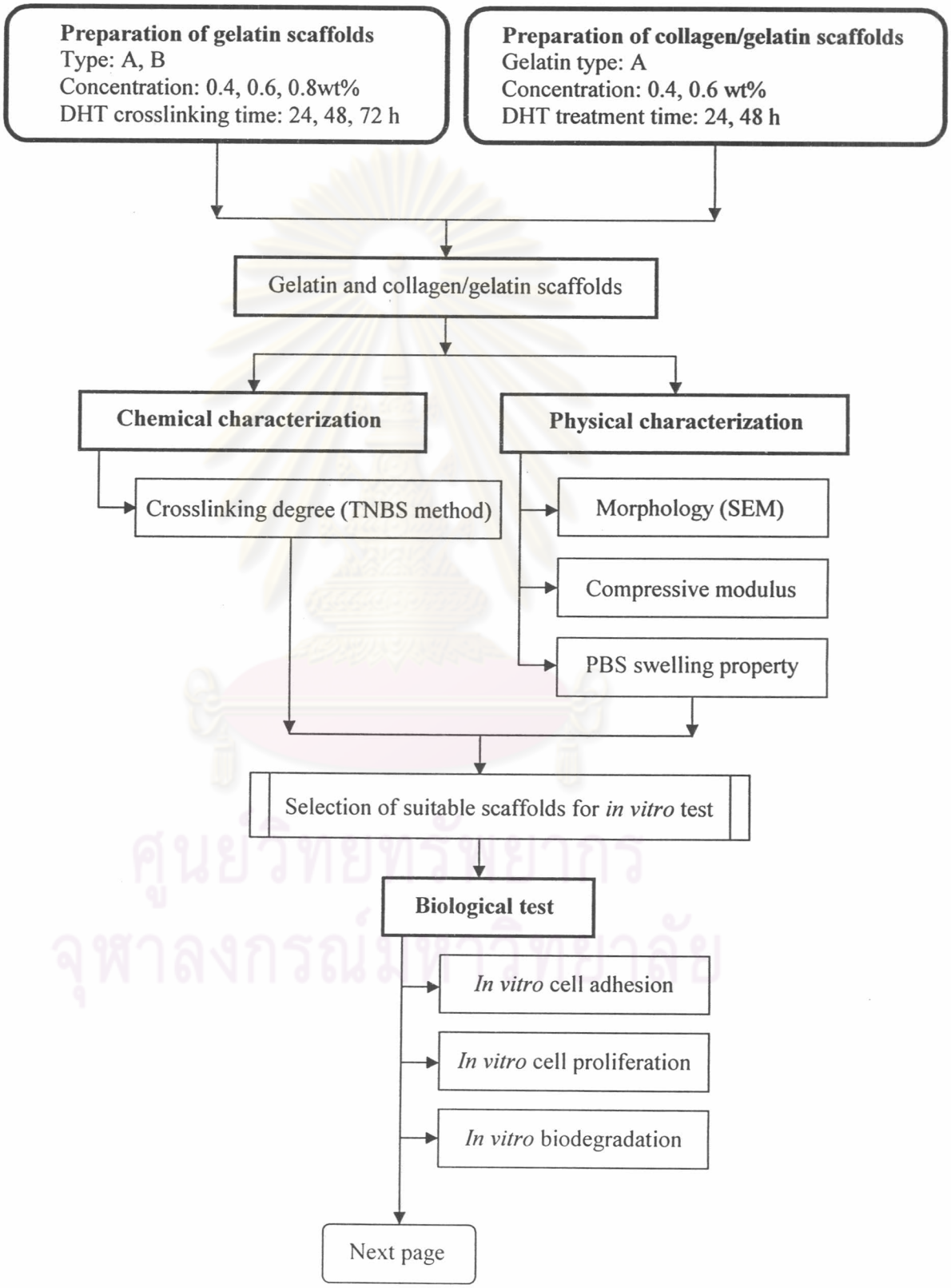
4.2.11 pH meter (Professional Meter PP-50, Germany)

4.2.12 Polystyrene tissue culture discs (NUNC, Denmark)

4.2.13 24-well and 48-well polystyrene tissue culture plates (NUNC, Denmark)

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

4.3 Methods



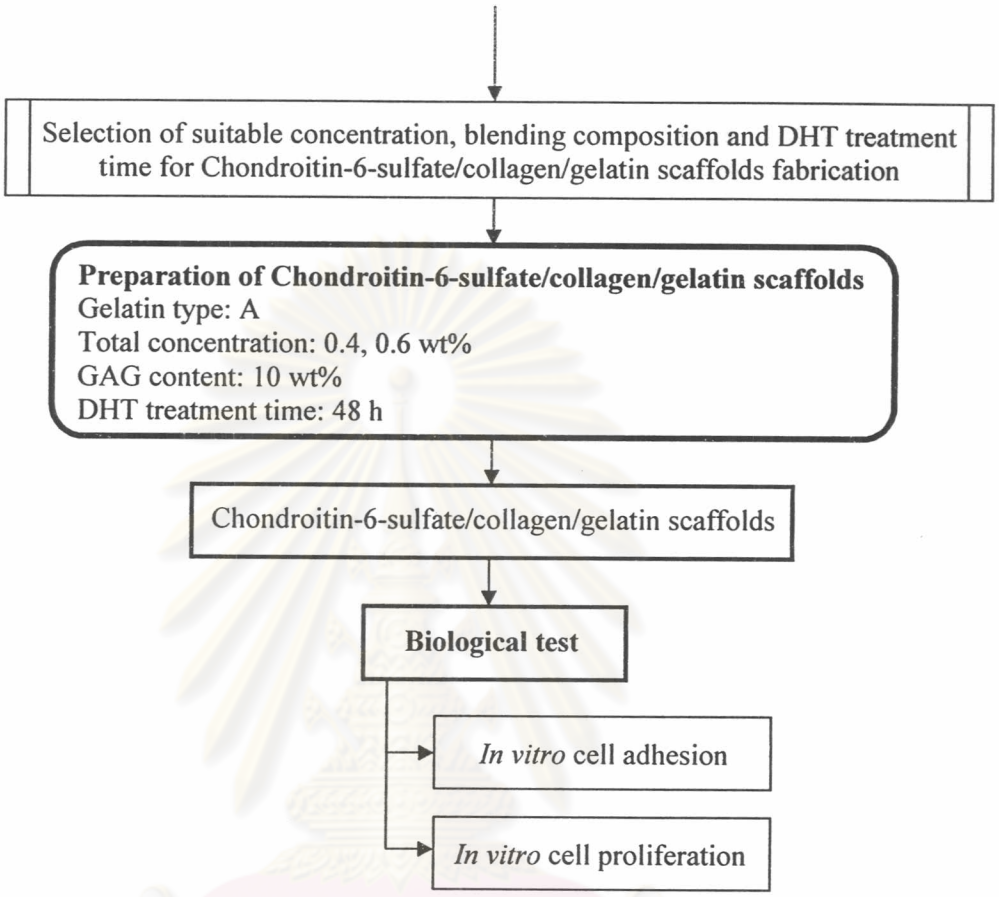


Figure 4.1: Diagram of the experimental procedure

4.3.1 Preparation of the scaffolds

Gelatin scaffolds: Type A and type B gelatin (pI 9, 116g bloom and pI 4.9, 152g bloom, respectively) were swollen in deionized water at room temperature for 1 h and then dissolved at 37°C under agitation for another 3 h to obtain 0.4wt%, 0.6wt%, and 0.8wt% (w/w) solutions. The solutions were then degassed centrifugally at 37°C. After measuring pH of the solutions, 1 ml of the solution was poured into each well of polystyrene 24-well plates and frozen at -50°C overnight prior lyophilized at -50°C for 24 h. The resulting freeze dried gelatin scaffolds were crossslinked by dehydrothermal (DHT) treatment at 140°C for 24, 48 and 72 h in a vacuum oven. Table 4.1 listed 18 experiments of gelatin scaffolds as well as the pH of their solutions.

Table 4.1: Experiments of gelatin scaffolds and pH of the solutions

Solution concentration	**DHT treatment time (h)**			**pH**
	24	**48**	**72**	
0.4wt% type A gelatin	*0.4A-24*	*0.4A-48*	*0.4A-72*	5.24
0.6wt% type A gelatin	*0.6A-24*	*0.6A-48*	*0.6A-72*	5.15
0.8wt% type A gelatin	*0.8A-24*	*0.8A-48*	*0.8A-72*	5.20
0.4wt% type B gelatin	*0.4B-24*	*0.4B-48*	*0.4B-72*	6.50
0.6wt% type B gelatin	*0.6B-24*	*0.6B-48*	*0.6B-72*	5.80
0.8wt% type B gelatin	*0.8B-24*	*0.8B-48*	*0.8B-72*	5.30

Collagen/gelatin scaffolds: Type A gelatin (pI 9, 116g bloom) was swollen in HCl solution (pH 3) at room temperature for 1 h and then dissolved at 37°C under agitation for 1 h. To obtain 0.4wt% and 0.6wt% (w/w) collagen/gelatin solutions, the gelatin solutions were mixed with the collagen solution (6.06 mg collagen in 1 g collagen solution in HCl) at the blending composition of collagen/gelatin to be 0/100, 10/90, 20/80, 30/70 and 100/0. The mixed solutions were stirred at 37°C for another 3 h, then degassed centrifugally at 37°C. The collagen/gelatin scaffolds were prepared via lyophilization and DHT crosslinking techniques as previously described for the

case of gelatin scaffolds. The 24 and 48 h DHT treatment time were employed for collagen/gelatin scaffolds. Table 4.2 listed 20 experiments of collagen/gelatin scaffolds as well as the pH of their solutions.

Table 4.2: Experiments of collagen/gelatin scaffolds and pH of the solutions.

Solution concentration	Blending composition of collagen/gelatin	DHT treatment time (h)		pH
		24	48	
0.4wt%	0/100	*0.4A-24*	*0.4A-48*	5.24
	10/90	*0.4CG10/90-24*	*0.4CG10/90-48*	3.87
	20/80	*0.4CG20/80-24*	*0.4CG20/80-48*	3.85
	30/70	*0.4CG30/70-24*	*0.4CG30/70-48*	3.84
	100/0	*0.4C100-24*	*0.4C100-48*	3.08
0.6wt%	0/100	*0.6A-24*	*0.6A-48*	5.15
	10/90	*0.6CG10/90-24*	*0.6CG10/90-48*	4.10
	20/80	*0.6CG20/80-24*	*0.6CG20/80-48*	3.97
	30/70	*0.6CG30/70-24*	*0.6CG30/70-48*	3.92
	100/0	*0.6C100-24*	*0.6C100-48*	3.22

CS/collagen/gelatin scaffolds: Type A gelatin was swollen in HCl solution (pH 3) at room temperature for 1 h and then dissolved at 37°C under agitation for 1 h. 10wt% of chondroitin-6-sulfate (CS) was dropped into the gelatin solutions. The suspension of CS/gelatin was mixed with the collagen solution (6.06 mg collagen in 1 g collagen solution in HCl) at the blending composition of collagen/gelatin to be 10/90, 20/80, and 100/0 in order to obtain 0.4 wt% and 0.6 wt% (w/w) solutions. The mixed suspensions were stirred at 37°C for another 3 h, then degassed centrifugally. The suspensions were immediately frozen in liquid nitrogen to prevent the separation of the suspensions. The lyophilization and DHT crosslinking techniques as previously mentioned were also used to prepare CS/collagen/gelatin scaffolds. Only 48 h DHT treatment time was employed for CS/collagen/gelatin scaffolds. Table 4.3 listed 6 experiments of collagen/gelatin scaffolds as well as the pH of their solutions.

Table 4.3: Experiments of CS/collagen/gelatin scaffolds and pH of the solutions.

Solution concentration	Blending composition of collagen/gelatin	48 h DHT treatment time	pH
0.4wt%	10/90	*0.4CS-CG10/90-48*	4.34
	20/80	*0.4CS-CG20/80-48*	4.11
	100/0	*0.4CS-C100-48*	3.25
0.6wt%	10/90	*0.6CS-CG10/90-48*	4.54
	20/80	*0.6CS-CG20/80-48*	4.46
	100/0	*0.6CS-C100-48*	3.41

4.3.2 Morphological observation

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

4.3.3 Determination of crosslinking degree

The determination of crosslinking degree was carried out by modifying the method of Bubins *et al.*[58]. The concept of this method was to react free amino groups of gelatin and collagen, which indicate the uncrosslinking groups, with 2,4,6-trinitrobenzene sulphonic acid (TNBS). The TNBS reaction was shown in Figure 4.2. The absorbance of uncrosslinking groups from TNBS reaction was detected by UV spectroscopy. Then, the crosslinking degree could be relatively obtained from the differences between the absorbance values of uncrosslinked and croslinked scaffolds.

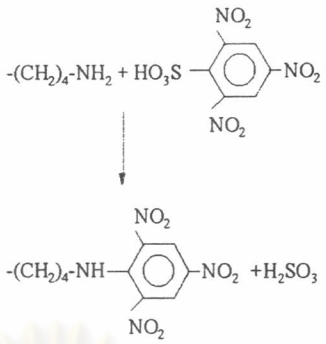


Figure 4.2: TNBS reaction

Briefly, about 5 mg of the scaffolds was weighed into a test tube where 1 ml of 0.5%TNBS solution and 1 ml of 4%sodium hydrogen carbonate ($NaHCO_3$, pH8.5) were added. It was then heated in a water bath maintained at 40°C for 2 h. The uncrosslinked primary amino groups of gelatin and collagen in the scaffolds would react with TNBS and form a soluble complex. This solution was further treated with 2 ml of 6 N HCl at 60°C for 1.5 h. The absorbance of the solutions was determined at 415 nm after suitable dilution spectrophotometrically. The crosslinking degree was then calculated by the following equation:

$$\text{Crosslinking degree (\%)} = \left(1 - \frac{\text{Absorbance of crosslinked scaffold}}{\text{Absorbance of uncrosslinked scaffold}}\right) \times 100$$

The values were expressed as the mean±standard deviation (n=2).

4.3.4 Mechanical testing

A universal testing machine was used to determine the slope from 5% to 30% strain of the stress-stain curves of the scaffolds (dimension: d = 14.5 mm, h = 5 mm) at a constant compression rate of 0.5 mm/min. The compressive modulus was determined and reported as the mean±standard deviation (n=5).

4.3.5 PBS swelling property

The water sorption capacities of gelatin and collagen/gelatin scaffolds were determined by swelling them in phosphate buffered saline (PBS) at 37°C, pH 7.4. A known weight of the scaffold was placed in the PBS solution for 5 and 24 h, which were the time for the initially cell attachment and equilibrium swelling, respectively. The wet weight of the scaffold was determined by first blotting the scaffold on a lint-freed paper (Kimwipe) to remove excess water, and then weighed immediately. The swelling ratio of the scaffold, W_{sw}, was calculated from the equation:

$$W_{SW} = \frac{(W_t - W_O)}{W_O}$$

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=3).

4.3.6 *In vitro* biodegradation

The biodegradation of scaffolds was investigated using 1 ml phosphate buffered solution (PBS, pH 7.4) at 37°C containing 1.6 μg/ml lysozyme. The concentration of lysozyme chosen corresponded to the concentration in human serum. Briefly, scaffolds of known dry weights were sterilized in 70%ethanol and incubated in the lysozyme solution. The lysozyme solution was refreshed daily to ensure continuous enzyme activity. Every other day, samples were taken from the medium, rinsed with deionized water, freeze dried and weighed. The experiment was done triplicate for each different scaffolds.

The extent of in vitro degradation was expressed as percentage of remaining weight of the dried scaffold after lysozyme treatment.

$$\text{Remaining weight (\%)} = \left(\frac{W_d}{W_O}\right) \times 100$$

W_o denoted the initial weight of the scaffolds, while W_d was the remaining weight of the scaffolds at time t. The values were expressed as the mean±standard deviation (n=3).

4.3.7 *In vitro* cell culture

Mouse skin fibroblasts (L929) were cultured in growth medium, DMEM containing 10% FBS, 2mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. They were incubated at 37°C in air containing 5% CO_2 and 95% air. The culture medium was changed every 3 days. At confluence, L929 cells were harvested using 0.25% trypsin-EDTA and subcultivated in the same medium with 12 dilutions.

The MTT assay was used to measure the relative cell viability according to the methods of Mosmann [59]. Series of L929 cells were plated onto the 24-well tissue culture plates with 10%DMEM containing serum. After 5 hours of the cell incubation, media was removed and PBS was added for washing the media. 350 μl of 5 mg/ml MTT in DMEM without phenol red was added into each well containing cells and incubated in a 5% CO_2 incubator at 37°C for 30 min. After that, MTT was removed and DMSO was employed to dissolve the dark blue formazan crystals formed by the cleaving of viable cells to the tetrazolium rings of the pale yellow MTT. The absorbance of the solution was measured at 570 nm. The result was used as a standard curve for cell adhesion and cell proliferation test.

4.3.8 *In vitro* cell adhesion and proliferation tests

Before seeding the cells for cell adhesion and proliferation tests, the scaffolds were plated in 48-well tissue culture plates and sterilized in 350 μl/well of 70% ethanol for 5 min. PBS was used for washing ethanol from the scaffolds.

For cell attachment, L929 cells (60,000 cells per scaffold) were seeded onto the selected gelatin, collagen/gelatin and CS/collagen/gelatin scaffolds placed in 48-well tissue culture plates in 10%DMEM containing serum. After 5 h, scaffolds were

rinsed with PBS and 350 µl/well of MTT (0.5 mg/ml) was added and incubated at 37°C in 5% CO_2 incubator for 30 min to establish cell viability. DMSO was used to elute the ice crystals of MTT and the absorbance of the solution was measured at 570 nm using a spectrophotometer. The treatment of the scaffolds without cells was used as the control.

For cell proliferation, L929 cells (30,000 cells per scaffold) were seeded onto the scaffolds instead. Medium was changed every other day. At each time interval, 24^{th} and 48^{th} h, scaffolds were rinsed with PBS and the MTT treatments were performed as mentioned previously.

The same procedure of proliferation step was repeated with DMEM without serum in order to remove the effect of serum on cell proliferation.

All experiments were run in duplicate. All data were expressed as mean±standard deviation (n = 3).

4.3.9 L929 cells spreading area observation

For both concentrations, scaffolds with collagen/gelatin blending composition of 20/80 were selected to observe the cell spreading because they expressed appropriate physical and biological properties. Moreover, cell response showed no significant difference from the collagen (control) scaffolds.

Scaffolds were sterilized in 70% ethanol and washed repeatedly with PBS. L929 cells (30,000 cells per scaffold) were seeded onto the scaffolds immersing in 10%DMEM containing serum. Cells were allowed to proliferate for 48 h. Scaffolds, which cells proliferated on, were washed with PBS and then fixed with 2.5% glutaraldehyde solution in PBS for 1 h. Scaffolds were then serially dehydrated by 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 cut in cross-sectional plane to observe cell penetration by SEM. From cross-sectional plane in Figure 4.3, position 1, 2, 3 and 4 represented the cell seeding side down to the plate-exposed side.

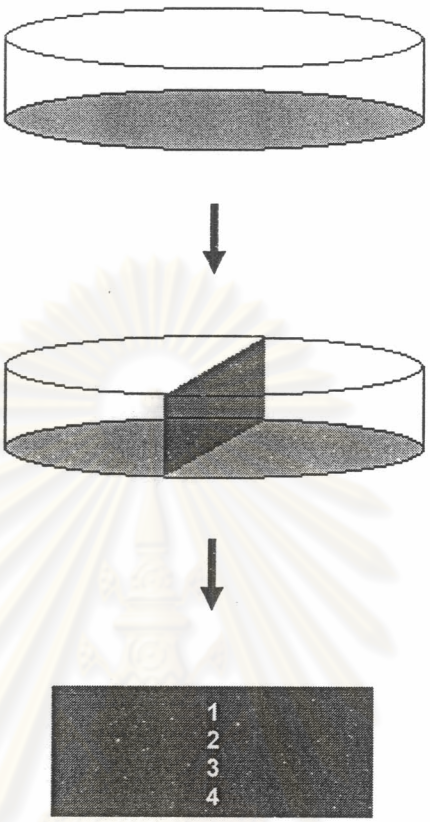


Figure 4.3: Schematic diagram of cross-sectional plane prior to cells spreading area observation.

4.3.10 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.