CHAPTER III
EXPERIMENTAL

3.1 Materials

Silk cocoon (*Bombyx mori*) from four species; Nang Noi, Nang Lai, Dok Bua, and Luang Pairote were purchased from local sericulture in Thailand. Sodium-bentonite was supported from Thai Nippon Co.,Ltd, Thailand. The bentonite is a commercial sodium activated bentonite (Mac-Gel© (GRADE SAC)) with cationic exchange capacity (CEC) of 49.74 meq/100 g clay and used without extra modification. Poly(vinyl alcohol) or PVA was purchased from KURARAY POVAL Co.,Ltd, Japan with average molecular weight at 9000-10000 (characterized by GPC) , hydrolyzed at 87-89 mol% and viscosity is 40-48 mPa.s (in 4% aqueous solution at 20°C). Glutaraldehyde ($C_5H_8O_2$, CAS No.111-30-8) used as chemical cross-linked agent was purchased from Sigma Aldrich Corp., USA with molecular weight 100.12 g/mol and used without further purification.

3.2 Experimental Procedures

3.2.1 Extraction of silk sericin

The method was prepared according to the method of Aramwit *et al.* (2010). Silk cocoons were rinsed with water to eliminate contaminated matter. 20 g of silk cocoons were cut in to small pieces about 5×5 mm^2 and mixed with 300 ml of purified water. Silk cocoons were autoclaved under pressure of 0.8-0.9 atm at 120°C for 60 min. The silk fiber (fibroin) was filtered out to obtain the sericin solution. This method was repeated 2 times in order to extract silk sericin from silk cocoons as much as possible. Silk sericin solution was frozen in the glass shells at -40°C for 12 hr. After freezing, the glass shells were put in to a freeze-dryer maintained at -110 °C for 48 hr under vacuum. Then, freeze-dried silk sericin was grinded in to powder.

3.2.2 Preparation of silk sericin/PVA/clay aerogels

3.2.2.1 Aerogels preparation

Silk sericin powder and PVA (5 wt%) were dissolved in purified water and heated at 90°C until the mixture was completely dissolved. The Na-bentonite was added into the mixture followed by vigorous stirring for 2 hr. After cooled down the clay gel precursor to the ambient temperature, the gel was immediately frozen in cylindrical glass shells at -40°C for 12 hr and attached to a freeze-dryer maintained at -110°C for 48 hr to sublime the ice out.

3.2.2.2 Cross-linked aerogel preparation

The silk sericin/PVA/clay gel precursor was prepared as described above. After 2 hr of vigorous stirring, glutaraldehyde was added in to the gel under constant stirring followed by continuous stirring for 1 hr. The gel was cooled down to the ambient temperature and subjected to the freeze-dried procedure mentioned above in order to create the cross-linked silk sericin/PVA/clay aerogel. After 48 hr in freeze-dryer, the aerogel was removed and post cured in the oven at 120°C to ensure the maximum curing of the aerogel and removed the residual glutaraldehyde as much as possible.

3.3 Characterizations

3.3.1 Solid contents

The solid contents of silk sericin were determined by weighing method. After freeze-dried, silk sericin powder was weighed in order to obtain the dried weight of silk sericin. This method was repeated at less 3 times in each species. The percentage of solid content of silk sericin was calculated by equation:

$$\%\ silk\ sericin = \frac{g\ of\ freeze\text{-}dried\ silk\ sericin\ powder}{g\ of\ dry\ silk\ cocoons} \times 100 \qquad \text{(eq. 3.1)}$$

3.3.2 Fourier Transform Infrared Spectroscope (FTIR)

The functional groups of freeze-dried silk sericin were analyzed by Thermo Nicolet Nexus 670 FTIR spectrometer. About 1-2 mg of silk sericin powder was ground with KBr and was pelletized into the pellet with the thickness less than 0.5 mm. The spectra were recorded over the wavenumber range from 400 to 4,000 cm^{-1} with the resolution of 4 cm^{-1} and the number of scan at 64.

3.3.3 High Performance Liquid Chromatography (HPLC)

Amino acid composition of freeze-dried silk sericin was analyzed by Waters Alliance 2695 High Performance Liquid Chromatography using Hypersil Gold column C18 (4.6*150mm, 5um). The method was according to In house method based on J. of AOAC, Vol.78 No.3,1995 (CIF T001). Silk sericin powder was hydrolyzed in 6 N of HCl to obtain sericin hydrolysate. The internal standard was added in hydrolysate and diluted with deionized water. The sample was derivatized by mixed filtrate with AccQ-fluor derivatization buffer and AccQ-fluor reagent and heated at 55 °C for 10 minutes before analyzed.

3.3.4 Density measurement

The density of the silk sericin/PVA/clay aerogels was calculated by mass and dimension measurement using Sartorius BS 224 S analytical balance and digital vernier caliper. The calculation was according to equation:

$$\rho = \frac{M}{V} \qquad \text{(eq. 3.2)}$$

where ρ is mass density (g/cm^3), M is mass of sample (g) and V is volume of sample (cm^3). The aerogel was prepared in the cylindrical shape with ~20 mm in diameter and ~10 mm in height.

3.3.5 Field Emission Scanning Electron Microscope (FE-SEM)

The morphology of silk sericin/PVA/clay aerogels was observed using Hitachi S-4800 Field Emission Scanning Electron Microscope. The samples were cut

in liquid nitrogen, fixed on stubs with carbon tape and coated with platinum under vacuum. FE-SEM micrographs were taken with low magnification range between 45- 400 and high magnification at 10.0 and 20.0 k using an accelerator voltage of 2.0 kV.

3.3.6 X-ray Diffractometer (XRD)

The d-spacing of bentonite, silk sericin, PVA and silk sericin/PVA/clay aerogels were characterized by Bruker AXS Model D8 Discover X-ray Diffractometer. The X-ray beam was Cu (lambda = 0.15406 nm) and the radiation operated at a tube voltage of 40 kV with a tube current of 30 mA. The aerogels were scanned at a scan rate of 2°/min from 2θ = 5°–30°. The interlayer spacing (d_{001}-spacing) was calculated via the Bragg equation:

$$\lambda = 2d \sin\theta \qquad \text{(eq. 3.3)}$$

where λ is the X-ray wavelength, d is the interlayer spacing and θ is the diffraction angle. The bentonite, silk sericin and PVA were used in the form of powder and the aerogel was sliced into the thin plate before measured.

3.3.7 Thermogravimetric-Differential Thermal Analyzer (TG-DTA)

Thermal stability of freeze-dried silk sericin and silk sericin/PVA/clay aerogels were examined by Perkin-Elmer Pyris Daimond thermogravimatric analysis. The weight of sample was in the range of 5-7 mg and heated at the heating rate of 10 °C/min from 30-900 °C in nitrogen atmosphere with 30 ml/min of nitrogen flow rate.

3.3.8 Universal Testing Machine

The initial modulus and young's modulus of silk sericin/PVA/clay aerogels were investigated by LLOYD Lrx Universal Testing Machine in compression mode with 500 N load cell at constant crosshead speed of 1 mm/min. The aerogels were prepared in the cylindrical shape with ~20 mm in diameter and height. Five samples of each composition were tested for reproducibility. The initial

compressive modulus was calculated from the slope of the linear portion of the stress-strain curve.

3.3.9 Sulfur analyzer

The sulfur content in silk sericin in all species was determined by LECO®Elemental Analyzer (TruSpec®S). The silk sericin powder was weighed around 0.1 g in a ceramic boat. The temperature of furnace was 1,350 °C.

3.3.10 Swelling behavior

The swelling behavior of cross-linked silk sericin/PVA/clay aerogels was studied by using conventional gravimetric procedure. The aerogels was dried in an oven over night and weighed to obtain weight of dry sample. The dried aerogels were immersed in Phosphate buffer saline pH 7.4 at room temperature. The swollen gels were withdrawn, wiped to remove the excess water out, and reweighed. The swelling ratio was calculated followed an equation:

$$SR = \frac{weight\ of\ swollen\ sample - weight\ of\ dry\ sample}{weight\ of\ dry\ sample} \times 100 \qquad \text{(eq. 3.4)}$$

3.3.11 In *vitro* biological tests

a) Direct contact test

Fibroblast cells derived from human gingival fibroblast (HGF) in the passage 5 were directly cultured on the aerogel with composition of clay 6 wt% and PVA 5 wt%. The samples in the size of 3×7 mm^2 were sterilized using UV radiation for 30 minutes. The sterilized samples (sponge) were transfer to culture plates and adhered with dental wax. The solution of cell suspension (containing 4×10^4 cells/ml) was added to the cultured plates and incubated in the incubator at 37 °C under 5% CO_2 and 100% humidity atmosphere. The cell cultured media was changed once in every two days. The reactivity of human gingival fibroblast cells was studied at 24, 48 and 72 hr. This experimental was repeated using human

gingival fibroblast from two human donors. All equipment was sterilized in alcohol before tested.

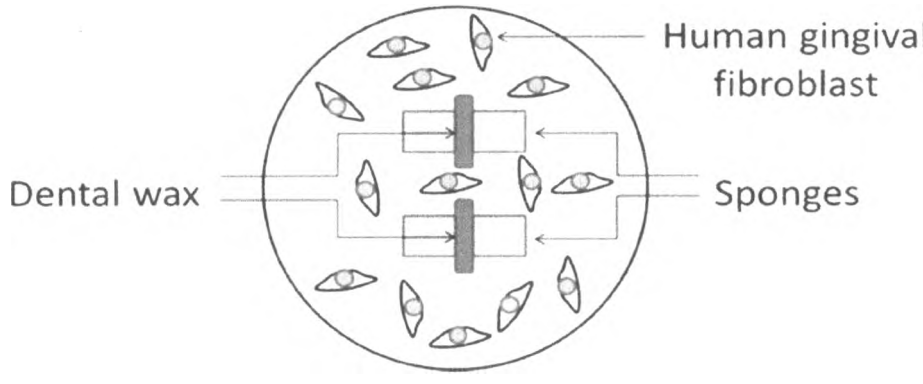

Figure 3.1 The schematic draw of cell cultured plate for direct contact test.

b) MTT assay

Cell viability and mitochondrial activities was studied using MTT assay. The aerogel with composition of 6 wt% of clay and 5 wt% of PVA was cut in to round shape with the dimension around 4 mm and sterilized using UV radiation for 30 minutes. The samples were immersed in cell cultured media at 37 °C for 24 hr. Human gingival fibroblast cells were seeded in to the 24-wellplate with the concentration of 4×10^4 cells/ml. The cultured plates were incubated in the incubator at 37 °C under 5 % CO_2 atmosphere at 100% humidity. After 24 hr of incubation, the cell cultured media was removed and replaced with the cell cultured media obtained from the immersion of samples. The cell cultured plates were incubated for a second time at 37 °C in the incubator for 48 hr. The cell cultured media was removed and replaced by 50 µl of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)** and 300 µl of DMEM without phenol red, followed by incubation for 4 hr at 37 °C. The MTT solution was removed and added 1 ml of dimethyl sulfoxide in order to dissolve the formazan crystals. The optical density (OD) was measured by spectrophotometer to compare the OD valued between cell

cultured media from samples and control. The experiment was carried twice using human gingival fibroblast cells from two human donors.

** The MTT solution was prepared by dissolved MTT powder in phosphate buffer saline with the concentration of 5 mg/ml and filtrated through the porous membrane with pore diameter around 0.22 μm.

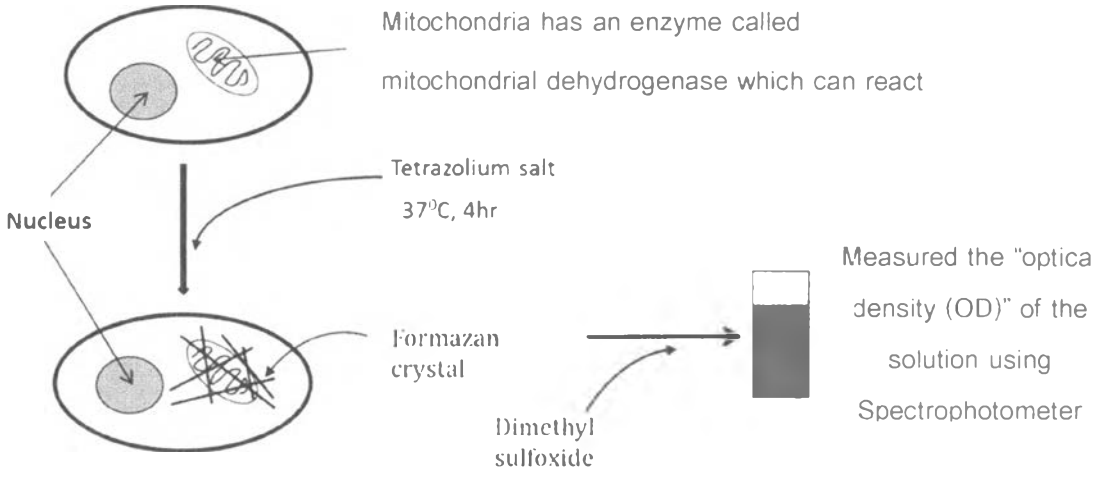

Figure 3.2 The schematic draw presented the principle of MTT assay.

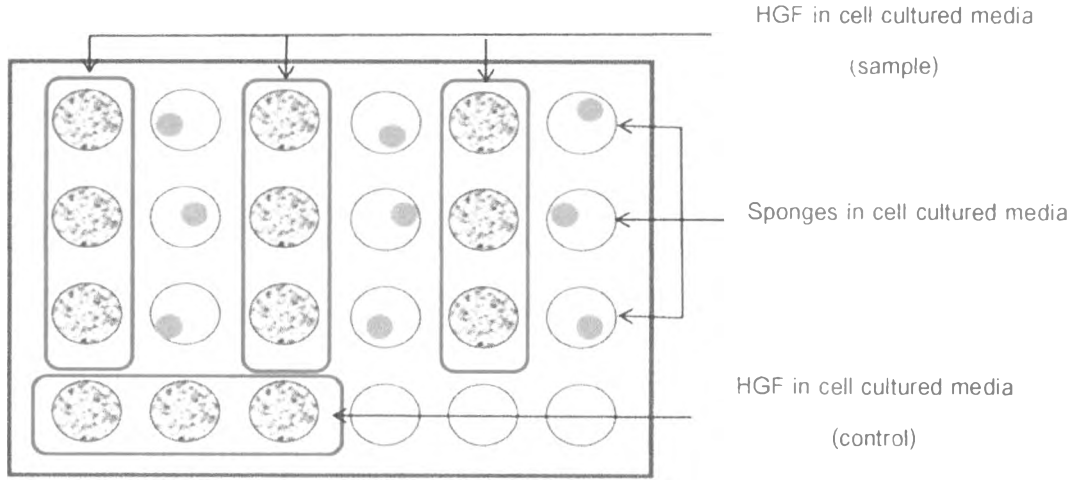

Figure 3.3 Schematic draw showed the seeding of the cell onto the 24-wellplates for MTT assay test.