CHAPTER III

EXPERIMENTAL WORKS

3.1 Materials and reagents

3.1.1 *Bombyx mori* cocoon (Nangnoi Srisaket 1 from Nakhonratchasima province, Thailand)

3.1.2 Type A gelatin powder (100kDa, pI=9, Nitta Gelatin, Japan)

3.1.3 Type B gelatin powder (100kDa, pI=5, Nitta Gelatin, Japan)

3.1.4 Sodium carbonate (Na_2CO_3, Ajax Finechem, Australia)

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

3.1.6 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Nacalai Tesque, Inc., Japan)

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

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

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

3.1.10 Formic acid (98%,CARLO ERBA, Reagent grade)

3.1.11 Azo-casein (Sigma-Aldrich, USA)

3.1.12 Methylene Blue (Labpro, UK)

3.1.13 Nerve Growth Factor (NGF,CHEMICON)

3.1.14 Nerve Growth Factor Sandwich ELISA Kit (CHEMICON)

3.1.15 Phosphate Buffer Saline (PBS)

3.1.16 Collagenase powder (Sigma-Aldrich, St. Louis, USA)

3.2 Equipments

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

3.2.2 Aluminum foil

3.2.3 Syringe (5 ml)

3.2.4 Needle (20G with 0.55mm inner diameter)

3.2.5 Magnetic bar

3.2.6 Magnetic stirrer

3.2.7 Rolling collector

3.2.8 -20°C freezer (Heto, PowerDry LL3000, USA)

3.2.9 Lyophilizer (Heto, PowerDry LL3000, USA)

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

3.2.11 High – voltage power supply , Model : ES30 –5W)

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

3.2.13 Scanning Electron Microscope (JSM-5410, JEOL Ltd., Japan)

3.2.14 UV-Vis spectrophotometer

3.2.15 Viscometer (DV-II+, Brookfield)

3.2.16 Conductivity meter (Tetracon 325)

3.2.17 Universal Testing Machine (LF Plus, LLOYD Instrument)

3.3 Experimental procedures

All experimental procedures are summarized in Figure 3.1. In brief, there are four main steps comprised in this work; preparation of silk fibroin/gelatin blended solutions, electrospinning of the blended solution, characterization of electrospun fiber mats and controlled release of model compounds.

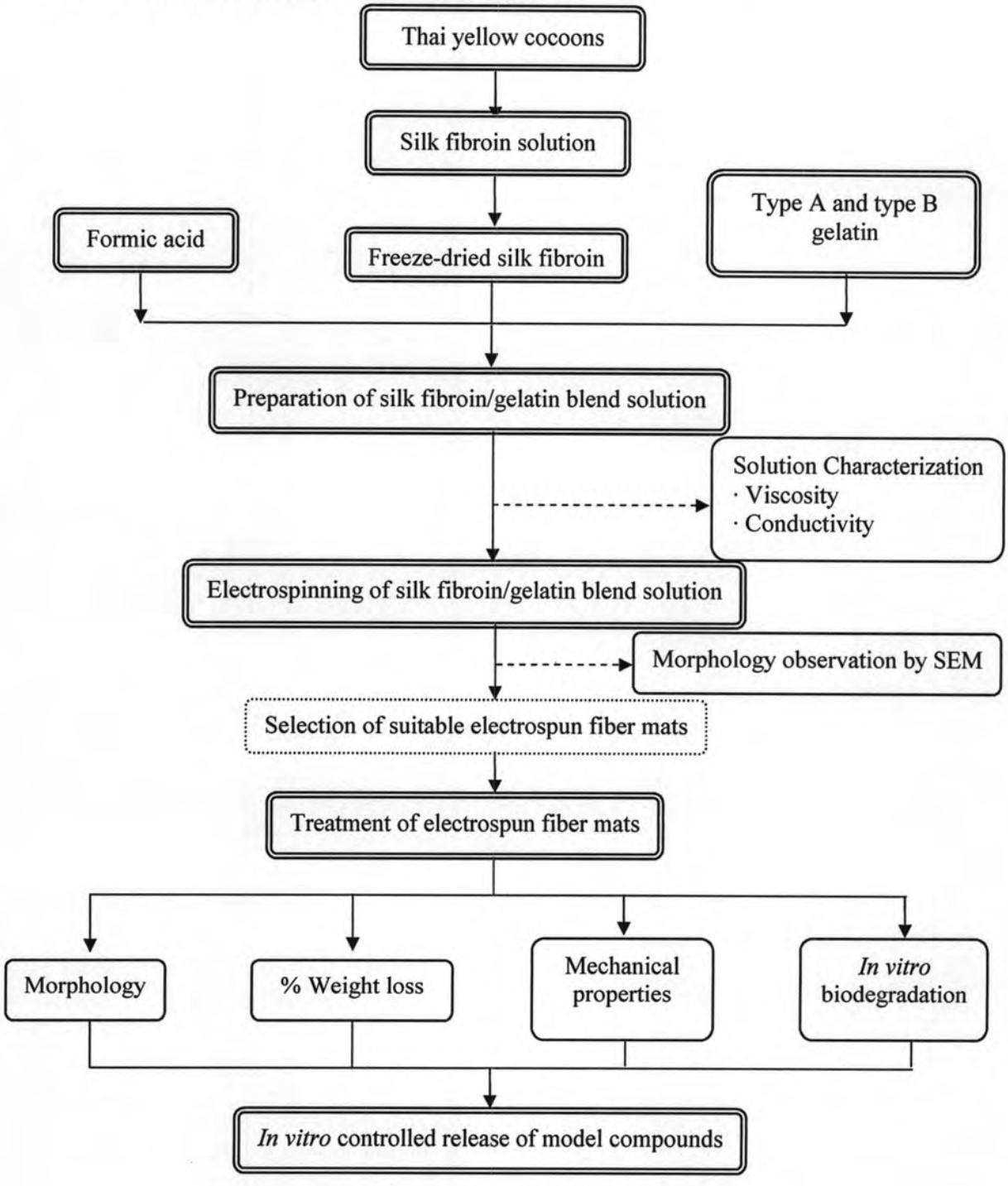

Figure 3.1 Diagram of experimental procedures

3.3.1 Preparation of regenerated Thai silk fibroin

Silk fibroin solution was prepared as described by Kim *et.al.* Cocoons of mulberry silkworm *B.mori* were boiled for 20 min in an aqueous solution of 0.02M Na_2CO_3, and then rinsed thoroughly with water. This process was repeated 2 times to remove the glue-like sericin proteins and dried overnight at 37°C. The degummed silk fibroin was then dissolved in 9.3M LiBr solutions at 60°C for 4 h. The solution was then dialyzed against deionized water with dialysis membrane at room temperature for 2 days and the conductivity of dialyzed water was the same as that of deionized water. The dialysate was centrifuged at 4,000 rpm, 4°C for 20 min to remove impurities. The final concentration of silk fibroin solution was calculated to be 6.5wt%, determined by weighing the remaining solid after drying at 60°C. The solution was further freeze-dried to obtain regenerated silk fibroin.

3.3.2 Preparation of the blended solution

The blending weight ratios of Thai silk fibroin and type B gelatin were studied. The total solid weight of silk fibroin and gelatin was maintained at 20 %. First, the calculated amount of regenerated silk fibroin was dissolved in 99% formic acid and stirred over night at room temperature then the desired amount of gelatin was added and further stirred for 3 h to obtain homogeneous solution. The silk fibroin/gelatin weight blending ratios are 10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, and 80/20. The viscosity and conductivity of blended solution were examined.

3.3.3 Electrospinning of Thai silk fibroin/gelatin blended solution

The homogeneous solution of silk fibroin/gelatin was filled up in 5 ml of syringe connected with 0.55 mm. inner diameter of needle. A high voltage in the range from 0 kV to 30 kV was applied to the droplet of solution at the tip. A grounded aluminum foil was placed at a distance 20 cm from the needle tip. Solution flow rate was fixed at 0.2 ml/hr. The fiber was collected for 10 minutes. The suitable blending

weight ratios were chosen and fabricated into fiber mats for 30 h. The collected fiber mats were dried in dessicator for over night to completely removal of solvent.

3.3.4 Treatment of Thai silk fibroin/gelatin electrospun fiber mats

Thai silk fibroin/type B gelatin electrospun fiber mats were cut into pieces (5*5 cm) and divided into three groups of different treatments.

3.3.4.1 Soaking in ethanol

Thai silk fibroin/type B gelatin electrospun fiber mats were soaked in absolute ethanol for 30 minutes (Zhang X., 2008) then rinsed with distilled water and dried at room temperature.

3.3.4.2 Soaking in EDC/NHS dissolved in ethanol

Thai silk fibroin/type B gelatin electrospun fiber mats were crosslinked as described by Jeeratawatchai H. (2008). The fiber mats were soaked in 15 ml of 14mM 1- ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 55mM N-hydroxysuccinimide (NHS) dissolve in absolute ethanol for 2 h. After that, EDC/NHS solution was removed and 15 ml of phosphate buffer saline (PBS) pH 7.4 was added. After 2 h, the fiber mats were rinsed with deionized water for 5 times.

3.3.4.3. Spraying and soaking in EDC/NHS dissolved in ethanol

Thai silk fibroin/type B gelatin electrospun fiber mats were sprayed with EDC/NHS dissolved in absolute ethanol and then left for 15 minutes. The spraying was repeated for 5 times. Then the fiber mats were soaked in the same solution following the procedure described in section 3.3.4.2

3.3.5 Characterization of Thai silk fibroin/gelatin solutions and fiber mats

3.3.5.1 Viscosity and conductivity

The viscosity and conductivity of the solutions were measured using Brookshield viscometer at spindle speed of 50 rpm and Tetracon 325 conductivity meter. The temperature was controlled at 27±1°C.

3.3.5.2 Morphology

Morphology and size of Thai silk fibroin/type B gelatin electrospun fibers were observed using JEOL JSM-6400 scanning electron microscope (SEM). Prior to observation, Thai silk fibroin/type A and B gelatin electrospun fiber mats were carefully fixed on stubs and were gold-coated using a JEOL JFC-1100 sputtering device to SEM observation. The diameter of the electrospun fibers were determined from the SEM micrographs using SemAfore 4.0 software. A measurement of 100 random fibers was used to average fiber diameter.

3.3.5.3 Mechanical properties

The mechanical properties of Thai silk fibroin/type B gelatin electrospun fiber mats were measured using the universal testing machine (LLYOD, LF Plus) with the crosshead speed of 20 mm/min under ambient condition. All samples were prepared with the dimensions of 10*50 mm^2. The thickness of Thai silk fibroin/gelatin fiber mats at 50/50, 30/70 and 10/90 were 31.66±5.77, 113.33±20.27 and 55.41±10.57 mm, respectively and gauge length was set at 20 mm (Guibo, 2008). All electrospun fiber mats were immersed in PBS, pH 7.4 for 15 minutes. The excess solution was removed immediately before test. The reported data was averaged from five repeated measurement.

3.3.5.4 *In vitro* biodegradation

3.3.5.4.1 *In vitro* biodegradation in phosphate buffer saline

The biodegradation of electrospun fiber mats was investigated in 1.5 ml of PBS (without any protease) at pH 7.4, 37°C. Briefly, the crosslinked electrospun fiber mats were cut into 1.5*1.5 cm^2 and weighed. The fiber mats were exposed in PBS solution. At each time period, the fiber mats were removed and then washed with deionized water. The degraded fiber mats were dried at 37°C in an oven and then weighed.

3.3.5.4.2 *In vitro* biodegradation in collagenase

The biodegradation of electrospun fiber mats was investigated in collagenase solution at pH 7.4, 37°C containing 0.01 wt% sodium azide to prevent bacterial growth. Briefly, the crosslinked electrospun fiber mats were cut into 2*2 cm^2 and weighed. The fiber mats were exposed in 2 ml of collagenase solution (concentration of 0.01 U/ml). At each time period, the sample was centrifuged at 2,000 rpm for 5 minutes. The supernatant was rinsed off, then washed with deionized water. The sample was dried at 37°C in an oven and weighed.

The result of in vitro biodegradation was presented as the percentage of remaining weight of dried electrospun fiber mats. The percentage of remaining weight was calculated as follows :

$$\text{Remaining weight}\,(\%) = \frac{W_f}{W_i} \times 100$$

W_i represented the initial weight of the electrospun fiber mats (g), and W_f was the final weight of the electrospun fiber mats after degraded. The values were expressed as the mean±standard deviation (n=3).

3.3.6 *In vitro* controlled release of the blended electrospun fiber mats

Three types of compounds; methylene blue, azo-casein and nerve growth factor (NGF), were used as models to investigate the release profile from Thai silk fibroin/type B gelatin electrospun fiber mats.

3.3.6.1 Loading method and controlled release of methylene blue and azo-casein

The compound was loaded into fiber mats by dropping 20 µl of compound solution onto fiber mats (1.5*1.5 cm^2). The blended fiber mats were centrifuged (10,000 rpm, 2-3 min) to obtain homogeneous distribution of solution and left at room temperature overnight to allow full absorption. The electrospun fiber mats were then moved to suspend in 1.5 ml of PBS (pH 7.4) containing 0.01%wt sodium azide and incubated at 37°C. At each time period (0h, 2h, 12h, 24h and 72h), 200 µl of buffer solution was removed and replaced with the equal volume of fresh buffer. The fiber mats were digested with 1.5 ml of papain solution at 70°C to obtain complete release of compound at the final stage. The amount of methylene blue and azo-casein released in PBS was determined using a spectrophotometer at its maximum adsorption wavelength of 600 nm and 405 nm, respectively. The effect of loading amount (5 and 10 mg of compound per g of fiber mats) was investigated.

3.3.6.2 Loading method and controlled release of nerve growth factor

Nerve growth factor (NGF, loading amount of 3.57 µg of NGF per g of fiber mats) was loaded into fiber mats as similar to the case of methylene blue and azo-casein. The blended fiber mats were left in refrigerator at 4°C. The electrospun fiber mats were then moved and suspended in 1.5 ml of PBS (pH 7.4) containing 0.01%wt sodium azide and incubated at 37°C. At each time interval (0d, 1d, 3d, 7d and 14d), the 120 µl of PBS solution was removed and replaced with the equal volume of fresh PBS buffer. The fiber mats were digested with high concentration of collagenase solution at

37°C to obtain complete release of NGF at the final stage. The amount of NGF released was determined by Enzyme-linked immunosorbent assay kit (ELISA).

The percentage of cumulative release was calculated as follows :

$$C_i = \sum_{i=0}^{t} M_i$$

$$\text{Cumulative release (\%)} = \frac{C_i}{C_T}$$

M_i represented the amount of model compound released at time i, C_i was the cumulative release at time i, C_T was the total cumulative release after electrospun fiber mats were digested.

3.3.7 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.