CHAPTER 3

Methodology

3.1 RESEARCH METHODOLOGY

This part will be divided into three sub parts: (1) Materials and Chemical, (2) Apparatus and Instruments, (3) Experimental procedures.

The experimental procedures are subdivided into nine parts as follows:

(1) Hydroxyapatite Preparation

(2) Fibroin Powder Preparation

(3) Fabrication of Fibroin/Chitosan/Hydroxyapatite Scaffolds

(4) Pore size and Porosity Test

(5) Swelling ratio Evaluation

(6) Mechanical Properties

(7) Biodegradation Test

(8) Biocompatibility Test

3.1.1 Materials and Chemical substances

1. Squid Chitosan was purchased from Ta Ming Enterprises Co. Ltd.
2. Lactic acid was purchased from Union Science (Chiang Mai) Co. Ltd.
3. Mollusk shell was obtained from restaurant in Chiang Mai, Thailand
4. Ammonium dihydrogen phosphate ($NH_4H_2PO_4$) was purchased from Merck KGaA Ltd.
5. Hydrogen peroxide (H_2O_2) was purchased from Union Science (Chiang Mai) Co. Ltd.
6. Glutaraldehyde was supported by Biomedical Engineering Laboratory.
7. Lysozyme from hen-egg white was purchased from Union Science (Chiang Mai) Co.Ltd.
8. Sodium Carbonate (Na_2CO_3) was purchased from Union Science (Chiang Mai) Co.Ltd.
9. Silk cocoon

3.2.2 Apparatus and Instruments

1. Freeze Drying Machine LABCONCO FreeZone 2.5litre Model 70705631
2. Freezer SANDEN INTERCOOL Model SCM 270SBD
3. X-ray Diffraction Analysis (XRD), Bruker D8 Advance
4. Universal Testing Machine, Instron 5566
5. Microplate Reader, SUNRISE TACAN
6. Scanning Electron Microscope, JBM-410LV
7. Hotplate
8. Autoclave
9. Chemical hood

3.2.3 Experimental Procedures

Hydroxyapatite Preparation

Hydroxyapatite (HA) was prepared by Chemical Precipitation Method [25]. Using Mollusk Shell as raw material. Mollusk shell were gathered and washed in Hydrogen peroxide (H_2O_2) for 72 hrs. After that cleaned mollusk shell were put into oven for calcinations at 650°C for 5 hrs. After that the mollusk shell were ground in ball mills for 2 hrs to obtain CaO powders. Then CaO were mixed with $NH_4H_2PO_4$ with the mol ratio Ca/P = 1/0.5 and dissolved in DI water 150 ml. while control the pH in range of 8-9 and stirred continuously for 2 hrs. After that leave the solution at the room temperature for 12 hrs for precipitation. The solution was filtrate by filter paper (Whatman No.45) and wash before dried in oven at 110°C for 12 hrs. Afterwards, the powder in oven at 800°C for 12 hrs. Finally, the powder were ground in ball mills and HA powder were obtained.

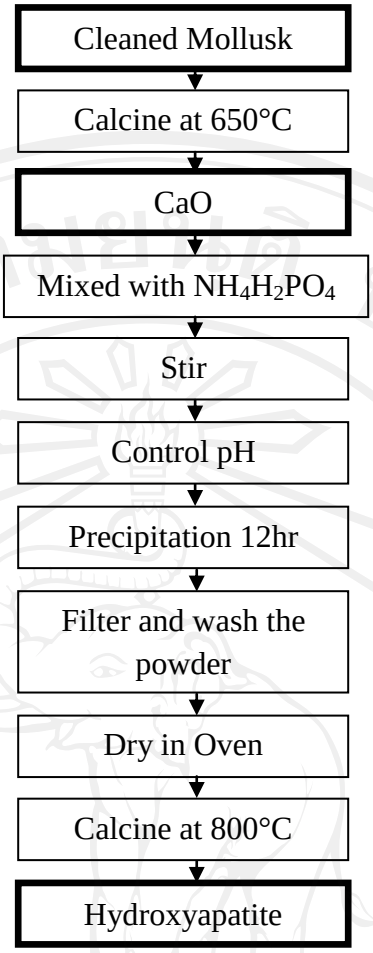

Figure 3.1 Flow chart process of HA synthesis

Fibroin Powder Preparation

Silk Cocoons were cut into small pieces and peeled the inner membrane away before degumming by boiling in 0.5% Na_2CO_3 solution at 100º C for 30 minutes. The degummed fiber were filtrated and washed with water before left and dried at room temperature. The degummed fibers were put into Ajisawa's solution and boiled at 75ºC, prior to filtration and dialyzation for 5-6 days. Next, the solution is solidified by placing in a freezer overnight. Afterward, the solid - mixture was cleaned in methanol and put in lyophilizer for 12 hours to freeze dry the solution into fibroin powders.

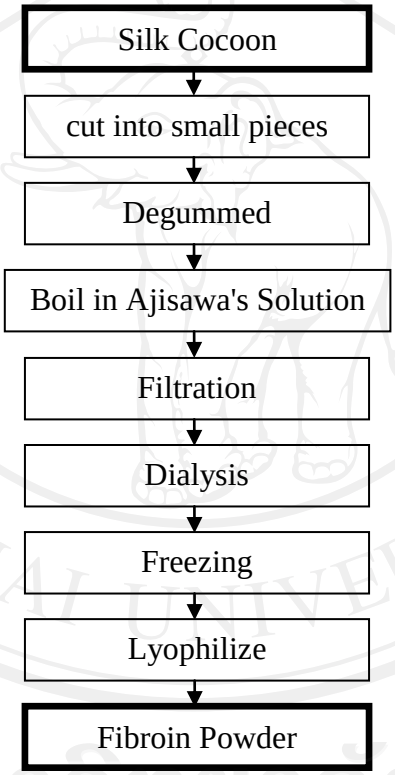

Figure 3.2 Flow chart process of fibroin extraction

X-ray Diffraction Analysis (XRD)

XRD analysis was used to investigate synthetic HA powders' phase. Data were collected from a Bruker D8 Advance in Figure 3.3 using Cu Kα radiation. Voltage and current were set at 40 kV and 40 mA, respectively. The data were analyzed from 10° - 100° 2θ with a 0.04°/s scanning rate. After the HA powder were synthesized and characterized, they were used in scaffold fabrication process.

Figure 3.3 Bruker D8 Advance Diffractometer

Phosphate buffer saline (PBS) Preparation

PBS used in swelling and biodegradation test was prepared by dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml of DI water and stirred until completely dissolve. After that DI water were added to the solution until the volume become 1000 ml. Then, the solution was put in autoclave for sterilization and the PBS was ready to used.

Fabrication of Fibroin/Chitosan/Hydroxyapatite Scaffolds

Chitosan flakes (92% deacetylation degree) were soaked in 2% (w/v) acetic acid until completely dissolved.

Figure 3.4 Chitosan flakes (left) and Chitosan soaked and dissolved in acetic acid (right)

After that HA and fibroin powder were put into the solution and mixed together until they become homogeneous. And glutaraldehyde was added into the slurry for cross-linking. Then the slurries were put into mould and moved to the freezer (-20 ^{o}C) for overnight for pre-freezing. Next the mould was transferred to lyophilizer for 48 hrs for freeze drying.

Figure 3.5 Porous scaffold fabricated by freeze drying method

Scaffold Characterization

Pore Morphology and Porosity

Pore morphology of the scaffold was examined by SEM analysis. The scaffold were coated with gold before being examined with a SEM (JBM-410LV).

Porosity of the scaffold was defined by liquid displacement method. Hexane was selected to use as the displacement liquid as it permeate through the scaffold without make the scaffold shrinking or swelling. Firstly, the size of the scaffold was measured then the volume of the scaffold were calculated and record as V_s. After that immersed the scaffold in a known volume of hexane (v_1) for 5 minutes then the scaffold was took out and the volume of the remaining hexane was measured and recorded as v_2. The porosity of the scaffold was calculated by the following equation.

$$Porosity(\%) = \frac{V_1 - V_2}{V_s} \times 100 \quad (1)$$

Swelling ratio

Swelling ratio was determined by the percentage of water absorption. Firstly, the scaffold size was measured and dry weight of the scaffold were recorded as W_s. After that scaffold was immersed in PBS for 7days in 12-well plate. After 7 days the surface water on swelled scaffold was remove by filter paper and wet weight of the scaffold were not as W_e and the swelling ratio of the scaffold were calculated by the following equation.

$$Swelling\ Ratio = \frac{W_e - W_s}{W_s} \times 100 \quad (2)$$

Mechanical Properties

A compression test was used to evaluate the mechanical properties of the scaffolds following the guidelines from ASTM D5024-95a. The specimens were placed in Instron 5566 Universal Testing Machine. The scaffolds with initial length 12mm and diameter 14mm were pressed until the thickness become 1 mm. The test was performed with 1mm/min crosshead speed and compressive strength of the samples were collected by software.

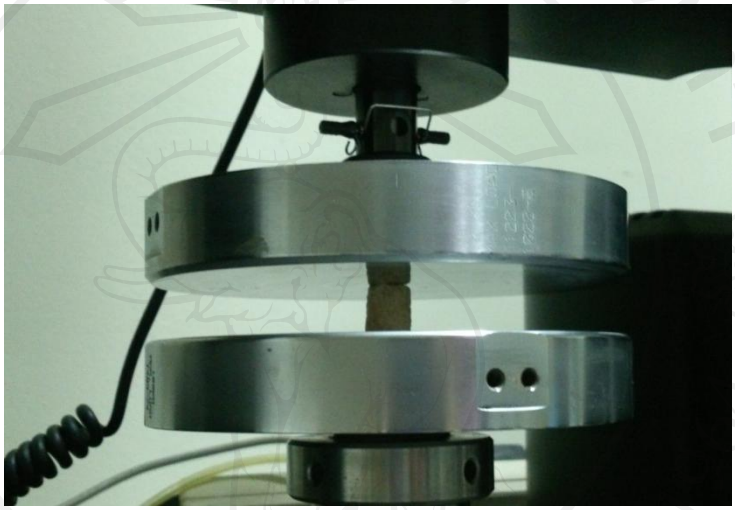

Figure 3.6 Compression test by Universal Testing Machine

Biodegradation

Biodegradability of the porous scaffold was determined by weight loss of scaffold after incubated in 1 mL PBS containing 1.6μg/mL of lysozyme from egg white at 37°C. Initial weight of the scaffold were measured. After incubated for 7days and 15days, the scaffold were washed by deionized (DI) water and freeze-dried. Finally, the weight different between the dry scaffold before and after incubated were compared and evaluated.

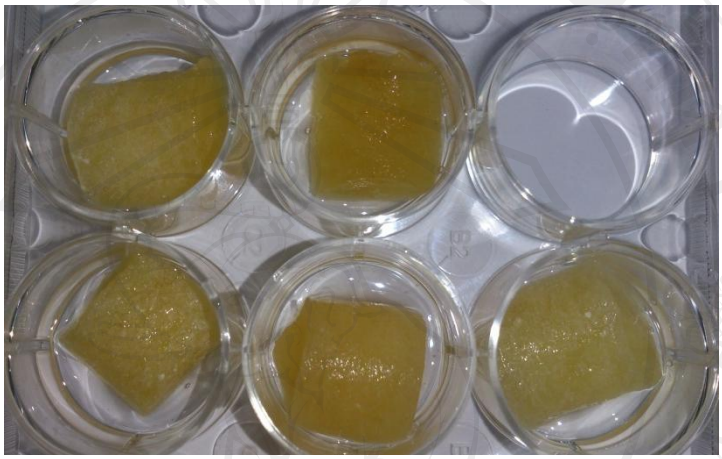

Figure 3.7 Porous scaffold soaking in PBS containing Lysozyme

Biocompatibility

Biocompatibility was tested in order to prove that prepared scaffold were non cytotoxicity. This procedure was performed by investigate cell living percentage of peripheral blood mononuclear cells (PBMC).

To assess the biocompatibility of porous scaffolds, XTT assay was performed. Equal numbers of human lymphocyte cells were seeded on scaffolds (50:50 HA:Fibroin ratio) and culture in DMEM then incubated at 37°C for 24 hours.

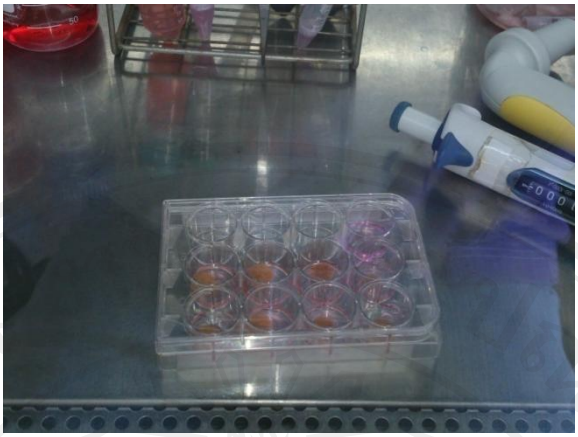

Figure 3.8 Scaffold seeded with cells and medium

After that the Activated-XTT solution were added to each wells and the plate were moved to incubator for 2 hours. Next, the optical density (OD) value of the wells were measured at 450 nm by Micro Plate Reader (SUNRISE TACAN) and the result were recorded.

Figure 3.9 XTT assay was performed to evaluate cells viability