CHAPTER 3

Research Methodology

The research methodology of this study can be mainly separated into nine parts which are scaffolds fabrication, physical characterization, and mechanical characterization, etc.

3.1. Research Methodology

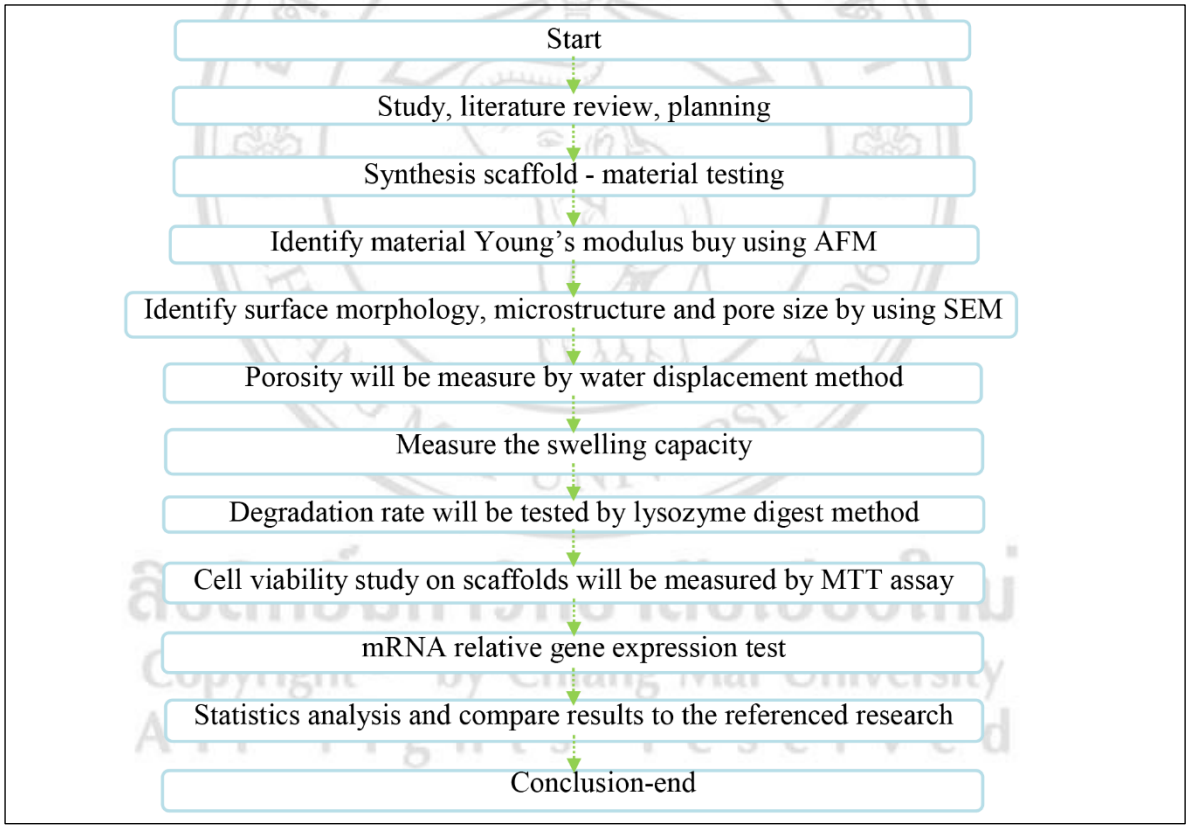

Figure 3.1 Flow chart of research methodology.

In figure 3.1 in the page, show the flow chart of this research methodology. The literature reviews and the theories use have been studied through as mentioned in the previous chapter. In this chapter in traduces the result and analysis phase, along with the fabrication method and material characterization.

3.2. Scaffold fabrication

The 10wt% gelatin solution was prepared by the following method: gelatin powder was weighed and soaked with sterilized deionized water at 50 °C in the water bath and was stirred until the solution became homogeneous.

Then the 5wt% PVA solution was prepared: PVA powder was weighted and soaked with sterilized deionized water at 80 °C in the water bath; then the solution was stirred until powder was totally dissolved and the solution was homogeneous.

And the 0.5wt% chitosan solution was prepared by weighting and soaking chitosan flake with 1% acetic acid solution and kept in room temperature for one week until solution is homogeneous. After that, 70 ml gelatin solution, 10 ml PVA solution and 20 ml chitosan solution were took with a cylinder and mixed together to make up the final concentration of 7% gelatin, 0.5% PVA and 0.1% chitosan. The following equation was used to calculate the final concentration of each material:

$$\text{final concentration} = \text{Vs} \times \text{Co}$$

The Vs means volume of solution and Co means the original concentration of solution.

The mixed solution was stirred with glass rod in the water bath at 50 °C until the solution became homogeneous. The homogeneous solution was transferred into a blender and blended for 15 seconds. After that 3 ml of blended solution was took in centrifugal tube with pipette and mixed with 7.5 μl glutaraldehyde by using blender in order to initiate crosslink before setting in each plate and all plates were kept at room temperature for 24 h.

All specimens were rinsed with sterilized deionized water 15 min for 3 times. All crosslinked materials were left in water at room temperature overnight again. Water

was discarded and all plates were kept in the freezer at -20 °C for pre-freezing. All specimens were dried in the lyophilizer at 662780 MTORR, shelf temperature 21.1 °C and condenser temperature -84.4 °C for 26 h. To analyze the Young's moduli of the specimens, all samples were cut into pieces with the size of 1 cm2. Then Young's moduli of dry specimens were analyzed using Park XE7 atomic force microscopy (AFM).

3.3. Porosity

The volumes of the dry scaffolds were measured to get Vs (both blended scaffolds and gelatin scaffolds). Dry scaffolds were weighed and recorded as Wd (dry weight). The average weight of scaffolds were calculated and the method of water displacement was applied in this study (Nazarov, Jin et al. 2004).

All scaffolds were soaked in sterilized distilled water and kept at room temperature overnight (to make sure that the scaffolds were completely equilibrated with water). Water was discarded, and the residual water was removed with tissue papers again. Wet samples were weighed again to get Ww (wet weight); The volumes of water inside scaffolds were calculated with the following equation to get Vw:

$$(\mathrm{Ww} - \mathrm{Wd}) \div \rho = \mathrm{Vw}$$

$$\rho = 1 \times 10^3\ \mathrm{kg/m^3}$$

The volumes of the dry scaffolds were measured with the following equation to get Vs:

$$Vs = \pi r^2 h$$

And then the porosity of scaffolds was calculated with the following equation:

$$\text{Porosity} = \text{Vw} \div \text{Vs}$$

Three replicates of gelatin and blended scaffolds were studied for the statistical analysis.

3.4. Swelling ratio

Preparation of 10×PBS: 80g NaCl, 2g KCl, 14.4g Na2HPO4 and 2.4g KH2PO4 were dissolved with sterilized distilled water to 1L; then the solution was put into auto-clave to sterile. 100ml of 10×PBS was mixed with sterilized distilled water to total volume 1L; the 1×PBS was sterilized with auto-clave.

Dry scaffolds were weighed and recorded as Wd. After that scaffolds were immersed in 70% ethanol for 30 min, and then washed with sterilized distilled water for 15 min, 3 times.

1×PBS was added into all plates and all of the specimens were kept in the incubator at 37 °C. All specimens were weighed again after 3 h, 7 h, 24 h incubation to get Ws. The swelling ratios of scaffolds were calculated with the following equation (Kakkar, Verma et al. 2014):

$$\text{Swelling ratio}\% = (Ws - Wd) \times 100 \div Wd$$

Here in this equation, Ws is average wet weight of scaffolds, Wd is average initial dry weight of scaffolds

3.5. Degradation rate

Dry scaffolds were weighed and recorded as Wo. After that scaffolds were immersed in 7 % alcohol for 30 min, and then washed with sterilized distilled water for 30 min, 3 times.

Sterile water was added into all plates from previous step and all of specimens were left at 4 °C overnight in the cold room. Water was discarded and removed with tissue papers again. 1 ml of lysozyme solution (1.6 μg/ml) was added to each scaffold followed by addition of PBS with 1% Pen--Strep to cover the surface of the scaffolds. All scaffolds were incubated at 37 °C. PBS with 1% Pen--Strep was added every two days to make sure that all samples were soaked in the lysozyme solution all the time.

After the desired periods of incubation, scaffolds were washed with distilled water 15 min, 3 times. All water was removed from the scaffolds. All scaffolds were frozen at -20 °C before lyophilization. After the lyophilization process dry scaffolds were weighed again for calculating the degradation rate.

$$\text{Degradation rate} = (W0 - Wt) \times 100/W0$$

3.6. Fibroblast culture

Preparation of scaffolds: scaffolds were immersed in 70% alcohol for 30 min, and then were washed with sterilized distilled water for 15 min, 3 times.

Preparation of complete culture medium (10% FBS, 1% Pen/Strep--containing DMEM): 13.5g DMEM medium was dissolved with 3.7g NaHCO3 in sterilized distilled water and the total volume was adjust to 1L (with 10 ml Penicillin Streptomycin); then 50ml fetal bovine was mixed with the resulting medium from last step to the volume 500ml. Then the complete medium was added into all plates and all of specimens were kept in the incubator at 37 °C for 24 h. Culture medium was discarded and NIH/3T3

fibroblasts were seeded onto the scaffolds 1.8×10^5 cells and cultured for 24 h. Medium was discarded and scaffolds were washed and rinsed with PBS. NIH/3T3 fibroblasts (in scaffolds) were fixed with 4% paraformaldehyde for 5-10 min and stained with methylene blue for 5 min.

NIH/3T3 fibroblasts (in scaffolds) were rinsed with lab water until cells could be observed under microscope (Nikon Eclipse TS100).

3.7. Characterization of scaffold Morphology

Scaffolds were immersed in 70% alcohol for 30 min, and then washed with sterilized distilled water for 15 min, 3 times. Complete culture medium was added into all plates and all of specimens were kept in the incubator at 37 °C for 24 h. Culture medium was discarded and NIH/3T3 fibroblasts were seeded onto the scaffolds 6.5×10^4 cells. NIH/3T3 cells were cultured on the scaffolds for 4 days. Medium was discarded, and samples were rinsed with phosphate buffer saline (PBS) once before fixing with 2.5% glutaraldehyde in PBS at 4 °C for one week. Glutaraldehyde was discarded, and the scaffolds were washed with PBS 10 min for 3 times. The scaffolds were post-fixed with 2% osmium in PBS for 2 h; then the solution was discarded, and the scaffolds were washed with PBS for 5 min, 2 times. Samples were sequentially dehydrated in 50% alcohol for 5 min, 2 times; 70% alcohol for 15 min; 85% alcohol for 15 min; 95% alcohol for 15 min and 100% alcohol for 15 min. Samples were dried using critical point drying (CPD) procedure and samples were coated with Au before observing under SEM (JSM-6610LV Scanning Electron Microscope from JEOL USA).

3.8. In vitro cell relative viability test

Scaffolds were immersed in 70% alcohol for 30 min, and then washed with sterilized distilled water for 15 min, 3 times. Complete culture medium was added into all plates and all of specimens were kept in the incubator at 37 °C for 24 h. Culture medium was discarded and NIH/3T3 fibroblasts were seeded onto the scaffolds 6.5 ×

10^4 cells/well and cultured for 4 days. Then MTT assay was applied to test the relative cell viability (Li, Dou et al. 2009). Culture medium was discarded and 2 ml of culture medium (contained MTT dye 0.5 mg/ml) was added before incubation at 37 °C for 2 h. MTT solution was discarded and samples were rinsed with PBS once. DMSO (7 ml) was added to each sample before shaking on the rotator for 15 min. The absorbance of DMSO was read at 570 nm and 630 nm. The absorbance of DMSO at 630 nm was used as background correction (OD570 nm - OD630 nm). Four replicates of gelatin and blended scaffolds were used for statistical analysis. Percent cell viability was calculated compared to the control group cultured on tissue culture plates using the following equation;

$$\%viability = \frac{\text{test (OD570 nm} - \text{OD630nm)}}{\text{control(OD570 nm} - \text{OD630nm)}} \times 100$$

3.9. Gene expression

Scaffolds were immersed in 70% alcohol for 30 min, and then washed with sterilized distilled water for 15 min, 3 times. Complete culture medium was added into all plates and all of specimens were kept in the incubator at 37 °C for 24 h. Culture medium was discarded and NIH/3T3 fibroblasts were seeded onto the scaffolds 3×10^4 cells/well and cultured for 10 days. Medium was discarded and rinsed with sterilized PBS. The RNA isolation was performed using NucleoSpin RNA - MACHEREY-NAGEL. 350 µl RA1 was mixed with 3.5 µl β-mercaptoethanol before adding into each sample to lyse cells. The lysis solution was filtered for separation of cell lysate by spinning at 11000 × g for 1 min. 350 µl of 70% ethanol was added to the filtrate before loading into silica membrane and spinning at 11000 × g for 1 min. 350 µl MDB was added into silica membrane and spun at 11000 × g, 1 min to desalt silica membrane. 95 µl DNase reaction mixture was added to silica membrane and incubated at room temperature for 15 min to digest DNA. Silica membrane was washed with 200 µl RAW2 and spun at 11000 × g for 30s followed by second washing by 600 µl RA3 and spinning at 11000 × g for 30s and 250 µl RA3 was added for the final washing before spinning at 11000 × g for 2 min. Total RNA was eluted with 60 µl RNase-free H_20 and

spun at 11000 × g for 1 min. RNA samples were kept at -20 °C for the next step. Then RNA was converted to cDNA using ReverTra Ace -α-® (Toyobo) in an Eppendorf Mastercycler®. cDNA was synthesized using the following program: priming at 25 °C for 5 min; then transcription at 42 °C for 30 min and inactivation at 85 °C for 5 min. NO-RT reaction was prepared as a negative control. SYBR Green Mastermix (SensiFAST SYBR® No-ROX Kit-Bioline) was applied for real-time PCR. The cDNA was diluted at the dilution 1:2, the 10 µl reaction was composed of 5 µl of SYBR Green RT-PCR master mix, 4 µl of cDNA and 0.25 µM of target-specific primer. LightCycler480 (Roche) was applied for RT-PCR. The polymerase chain reaction protocol consisted of 95 °C pre-incubation for 2 min, followed by 40 cycles of 95 °C for 5 sec, then 60 °C for 10 sec, and 72 °C for 20 sec. The melting peak analysis was performed at 95 °C for 5 sec, 65 °C for 1 min, and 97 °C continuous. The cooling step was performed at 40 °C for 30 sec. 18sRNA and GAPDH were used as reference genes. Relative quantification was performed with LightCycler® 480 software 1.5. Primers used in this study were shown in Table 3.1.

Table 3.1: List of primers used in this study.

Target genes	**Primer sequences (5'->3')**
1. 18s rRNA	F: GGCCCTGTAATTGGAATGAGTC
	R: CCAAGATCCAACTACGAGCTT
2. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	F: AAATCCCATCACCATCTTCCAGGAGC
	R: CATGGTTCACACCCATGACGAACA
	R: GATGCAGAACTTGGAACTAT
3. COL4A1 (*Type IV collagen*)	F: CGCTTACAGCTTTTGGCTCG
	R: GACGGCGTAGGCTTCTTGAA