สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

# CHAPTER III

# EXPERIMENTAL

## 3.1 Materials

Gelatin from porcine skin type A strength 180 g bloom, Biochemika, Fluka, Germany, hyaluronic acid (Sodium salt Mw 1,000,000) extracted from unbiblical cord of animals, glacial acetic acid,Mallinckrodt Chemicals, USA, distilled water, glutaraldehyde, sodium metabisulfite, Riedel-deHaen, Germany. All chemicals were used without further purification.

## 3.2 Equipments

The electrospinning set up requires a high voltage power supply, a 5 mL glass syringe connected to syringe pump and a rotating metal collector connected to ground, basic laboratory glassware, water bath, magnetic stirrer and hot plate.

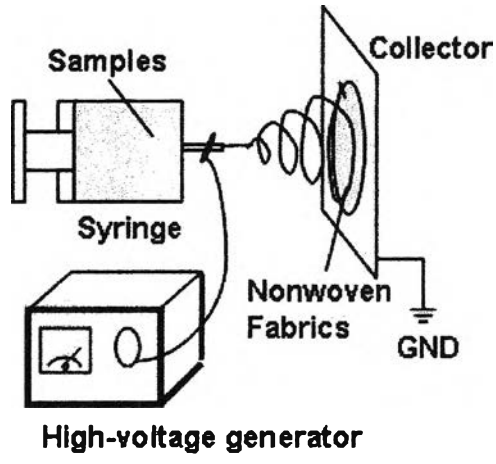
## 3.3 Methodology

### 3.3.1 Preparation of Electrospinning Solution (Gelatin/Hyaluronic acid)

Prepare 5 concentrations of electrospinning solutions by dissolving 5%, 10%, 15%, 20%, 25% (w/v) in 40% acetic acid solution with the fixed hyaluronic acid concentration at 0.5% (w/v). Stir continuously until homogenous solution is obtained.

### 3.3.2 Fabrication of Electrospun Fiber Mats

Prepare electrospinning solutions. Load the solution into 5 mL syringe fitted with a 25 gauge blunt needle. Connect the power supply, attach the anode to the needle and attach the ground to the aluminum foil rotating collector. Place the collector 20 cm away from the tip of the needle. Turn on the power supply to 20 kV for 3 days or until the electrospinning solution run out. After the electrospinning process was completed, store the fiber mats in the desiccators immediately.




3.3.3 Crosslinking by Glutaraldehyde

Prepare saturated glutaraldehyde vapor environment by pouring glutaraldehyde solution into an air-tight container and immerse it in the water bath at 37°C prior to the crosslinking process. The glutaraldehyde solution needs to be replace each time to ensure sufficient vapor. Expose the electrospun mat to glutaraldehyde vapor and continue immersing the container in water bath for 30min, 1hr, 2hr, 4hr. After the desired cross-linking time was reached, quench the reaction by immersing the electrospun mat in sodium metabisulfie solution overnight. Wash the electrospun mat 2-3times in distilled water to remove sodium metabisulfite residue. Dry the specimen in the oven at 60°C overnight to remove the residue solvent.

Because the length of time varied in crosslinking the electrospun fiber mats will results in different morphology and different mechanical properties of the fiber mats, four time periods: 30 min, 1 hr, 2 hr and, 4hr were investigated to observe the change in cross-linked fiber mats' properties.

3.3.4 Fabrication of Gelatin and Gelatin/Hyaluronic acid Films

In biological compatibility tests of the gelatin/hyaluronic acid electrospun fiber mats such as cytotoxicity test, cell attachment test and cell proliferation test, reference materials are needed for comparison. Therefore each of the components of the fiber blend must be tested for their biocompatibility. Films of Gelatin and gelatin/hyaluronic acid were prepared and direct addition of glutaraldehyde acted as the cross-linking agent. To quench the cross-linking reaction and remove glutaraldehyde prior to biocompatibility tests since glutaraldehyde is known to be toxic to living organisms, the Gelatin films and gelatin/hyaluronic acid films were immersed in Sodium bimetasulfite solution overnight and wash with distilled water before oven dry at 60°C to remove residue solvents.

## 3.4 Testing Properties

3.4.1 Scanning Electron Microscopy (SEM)

To characterize the morphology of the electrospun fiber mats, a JOEL JSM-5200 scanning electron microscopy was used. A small piece of sample was taken and coated with gold by using JEOL JFC-1100E sputtering device for 4 min, and stored in desiccators to absorb water before being observed by sing SEM at an accelerating voltage of 15 kV. The fiber diameters were measured by taking 50 points measurements of the SEM images and the morphology of the as-spun gelatin/hyaluronic acid fibers, and cross-linked gelatin/hyaluronic acid fibers were also observed.

3.4.2 Swelling Test

To investigate the level of cross-linking density, the swelling test was conducted. Samples were cut into round pieces with the diameter of 1.5 cm and immersed in stimulated body fluid (SBF) at 37°C for 30 min, 1 hr, 2 hr, and 4 hr. The samples put between two sheets of filter paper and a weight of 0.5 kg was applied for 30 seconds, then the samples were weighed. The degree of swelling (%) of the samples was calculated by the following equation;

$$\text{Degree of swelling (\%)} = \frac{(W_b - W_d) \times 100}{W_d}$$

3.4.3 Tensile Test

Loyd tensiometer were used to measure the tensile properties of the crosslinked electrospun fiber mats after cross-linking. The samples were cut into rectangular pieces with the dimension of 1x8 cm and immersed in distilled water for 4 hr to allow the fiber mats to absorb water and let them expand to the maximum limit before testing. The values of percentage strain at break and selected tensile profiles are reported.

3.4.4 Fourier Transform Infrared Spectrophotometer (FTIR)

Funtional groups of the blended fiber mats were checked by using thermo Nicolet Nexus 671 FT-IR. The comparison between each pure component, blended fibers and cross-linked fibers are shown.

3.4.5 Thermogravimetry (TGA)

The thermal degradation temperature profiles were measured by using themogravimetry analyzer. Values comparing between the pure components, blended fibers and cross-linked fibers are shown.

**3.5 Cell Culture Studies**

The *in vitro* cell culture studies were carried out to investigate the biocompatibility of the electrospun fiber mats. Thus the samples were compared with polystyrene tissue culture plate (PSTC), Gelatin electrospun fiber mats, Gelatin films, and gelatin/hyaluronic acid films.

Mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented by 10% fetal bovine serum (FBS;

BIOCHROM AG, Germany), 1% L-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphopericin B (Invitrogen Corp., USA)]. The cultures were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$.

Each scaffold was cut into circular discs (about 15 mm in diameter) and were placed in a 24-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland) and later sterilized in 70% ethanol for 30 min. T o ensure a complete contact between the specimens and the wells, the specimens were pressed with a metal ring (about 12 mm in diameter). Mouse fibroblasts from the cultures were trypsinized [0.25% trypsin containing 1 mM EDTA (Invitrogen Crop., USA)], counted by a hemacytometer (Hausser Scientific, USA), and seeded at a density of approximately 4,000 cells/well on the scaffold.

3.5.1 Cytotoxicity Tests

Indirect cytotoxicity test were carried out using mouse fibroblast (L929). The sterilized samples were immersed in fresh serum-free media (SFM) and incubated for 24 hours. Then the extraction serum-free media (SFM) media were used to culture L929 cells and incubated overnight. The number of viable cells was determined using standard MTT assay method. Fresh SFM were used as control.

3.5.1 Attachment and Proliferation Tests

Mouse fibroblast cells (L929) were cultured on the sterilized samples for 1, 2, 3, and 4 days at the cell density of 10,000 cells each well. When the required culture time was reached, the culture media was removed and the samples were dyed with fluorescence propidium iodide dye, place on to glass slide and covered with glass cover slip. Then observe the specimen under optical microscope.