CHAPTER III
EXPERIMENTAL

3.1 Materials

Chitosan powder [degree of deacethylation (DD) = 95%], both the weight-average and the number average molar masses of chitosan were determined using a Waters 600E size exclusion chromatograph (medium = 0.5M acetate buffer, column set: Ultrahydrogel linear 1 column, detector: refractive index, temperature = 30°C, and software: PL Logical) to be about 570000 and 70000g· mol^{-1}, respectively. Sodium acetate (pH = 5.5), used as the medium for weight loss, swelling and drug release assessment, was purchased from Ajax Chemicals (Australia), Sodium carbonate (Na_2CO_3) used as neutralizing agent, were supplied from Sigma-Aldrich (USA). Tetrahydrocurcumin or THC was obtained from Ramkhamhaeng University (Thailand). Glacial acetic acid was purchased from Carlo Erba (Italy). Glutaraldehyde was purchased from Sigma-Aldrich (USA). Trifluoroacetic acid (TFA, CF_3COOH~98% purity) was purchased from Sigma-Aldrich (USA). Dichloromethane (DCM) was supplied from Sigma- Aldrich (USA).

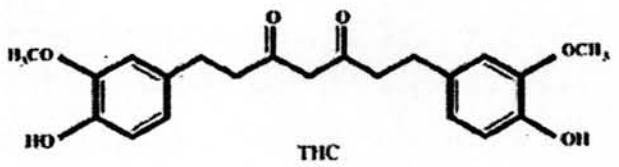

Figure 3.1 Chemical structure of tetrahydrocurcumin (THC).

3.2 Equipment

3.2.1 High Voltage Power Supply

A high voltage power supply (Gammar High Voltage Research model no.D-ES30PN/M692, Ormond Beach, Florida) was used to supply electrostatic field between a needle tip and a collection screen.

3.2.2 Scanning Electron Microscope (SEM)

Scanning electron microscope (JOEL model JSM-5200-2AE) was used to investigate the surface l morphology of fibers with the magnifications of 2000x and 5000x times at an acceleration voltage of 15 kV.

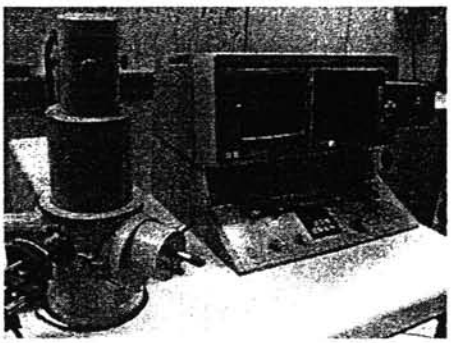

Figure 3.2 Scanning electron microscope.

3.2.3 A JEOL JFC-1100E Sputtering Device

The fiber samples were cut into small pieces and adhered on brass-stub by using adhesive tape and were coated with thin layer of gold by using a JFC-1100E ion sputtering device.

3.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of the chitosan fibers, the post-neutralized chitosan fibers, the chitosan/THC fibers, the post-neutralized chitosan /THC fibers, the crosslinked chitosan/THC fibers, the post-neutralized and crosslinked chitosan/THC fibers, and as-received THC powder were recorded with Nicolet Nexes 671 FTIR spectroscopy with 32 scans at a resolution of 4 cm^{-1}.

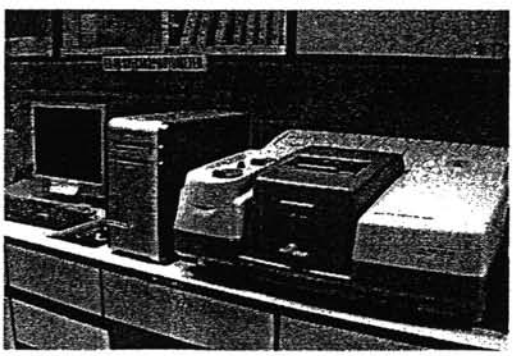

Figure 3.3 Fourier Transform Infrared (FTIR) Spectroscope.

3.2.5 UV- Spectrophotometer (Perkin Elmer, Lambda 10)

UV- spectrophotometer (Perkin Elmer, Lambda 10) was used to study the release characteristics of THC from the post-neutralized crosslinked electrospun chitosan /THC fiber mats at wavelength 280 nm that correspond to the maximum wavelength (λmax) for THC.

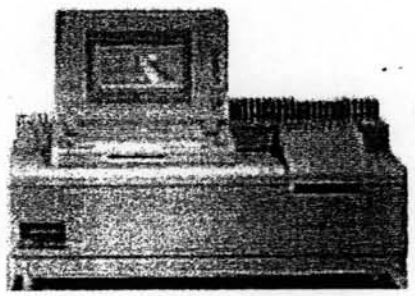

Figure 3.4 UV- spectrophotometer.

3.3 Methodology

3.3.1 Electrospinning Setup

The experimental set up for study the effects of solution parameters including polymer and tetrahydrocurcumin concentrations. Each THC loaded in chitosan solution was placed in a 5-ml glass syringe fitted with a needle and camped to PVC stand. The solution feed was driven by the gravity and syringe pump with flow rate 1ml/min and the feed speed was controlled by the tilt angle of syringe to 45 degree. A rotating drum is used at the constant speed with a flat aluminum foil for collecting electrospun fibers. The electrospinning solution was subjected to external electrical field 25 kV by attaching a positive electrode to the needle tip and a negative electrode to the rotating drum cover with aluminum foil. The constant pressure was applied into the syringe to suspend the polymer drop at the tip of

nozzle. The distance between the needle tip and the grounded collector screen was 20cm. The electrospun fibers were dried under vacuum for 24 h at ambient temperature prior to characterization to ensure a complete drying of the sample.

3.3.2 Preparation of Electrospining Solution and Film Solution

The effects of solution parameters on the fiber morphology which are tetrahydrocurcumin and polymer concentrations were studied. THC was dissolved in dichloromethane 30% (v/v) of solution at various concentrations in the range of 10 to 20wt.% (based on the weight of chitosan power) and the chitosan power was added into solution by varying chitosan concentration from 6.5 to 7.5% (w/v), and then trifluoroacetic acid (TFA) 70% (v/v) was filled and stirred 6 h at room temperature in a glass bottle. The solution of chitosan/THC films was prepared by using 4% (w/v) chitosan and 20wt.%THC (based on the weight of chitosan power) in 70:30 (v/v) TFA:DCM

3.3.3 Selection of the Optimum Condition of Electrospining Solution

Each electrospining solution was prepared in a manner similar method and electrospun fibers were collected on a collector metal screen. The collection time was about 5 min for morphological study and the electrospun fibers were further investigated by using Scanning electron microscope (SEM) to find the optimum condition that gives uniformed shape and size of bead-free fibers.

3.3.4 Crosslinking Treatment of the Electrospun Chitosan/THC Fiber Mats

This procedure used glutaraldehyde (GTA) as crosslinking agent. Firstly, the electrospun chitosan/THC fiber mats and chitosan/THC films were placed in a vapor chamber containing 15 ml of GTA liquid. The GTA liquid vaporized when it warmed to room temperature (37°C) and was allowed to crosslink the fibers for 1h prior to neutralization and characterization.

3.3.5 Neutralization Treatment of the Post-Crosslinked Electrospun Chitosan/THC Fiber Mats

The crosslinked electrospun chitosan/THC fiber mats and chitosan/THC films were neutralized by immersing in saturated Na_2CO_3, NaOH aqueous solution, respectively for 30 minute at ambient temperature. After that the post-neutralized and crosslinked fiber mats and films were repeatly washed with distilled water until neutral pH was obtained, and then the fiber mats were kept in

vacuum for 24 hr at 40 ^{o}C to ensure a complete drying of fibers prior to characterization.

3.3.6 Characterization of Electrospun Chitosan/THC Fiber Mats

a) The morphological appearance

The morphological appearance of the electrospun chitosan/THC, the crosslinked electrospun chitosan/THC, the post-neutralized and crosslinked electrospun chitosan/THC fiber mats and films were observed by a scanning electron microscope (SEM; JOEL model JSM-5200-2AE). All of electrospun chitosan /THC mats and films were sputtered with a thin layer of gold prior to SEM observation. Base on SEM images, the average diameter of the post-neutralized and crosslinked electrospun chitosan/THC fiber mats could be measured. The results were reported as average value from as least 50 measurements.

b) The degree of swelling and weight loss behavior

The post-neutralized and crosslinked chitosan/THC fiber mats were submerged and investigated in acetate buffer solution at various time intervals [i.e., for the weight loss measurement (up to 9 weeks) and the swelling measurement (up to 120 minutes)]. The post-neutralized and crosslinked electrospun chitosan/THC fiber mats were submerged in acetate buffer for 24 h at ambient temperature prior to official timing of both the weight loss and the swelling measurements. The weight loss (%) of each sample (circular disc of about 1.5cm in diameter) was calculated according to the following equation:

$$\text{Weight loss (\%)} = \frac{(W_{di} - W_{dt}) \times 100}{W_{di}} \qquad (1)$$

Where W_{di} denotes the initial weight of the sample in its dry state prior to submersion in acetate buffer and W_{dt} denotes the weight of the sample in its dry state after submersion in acetate buffer after an arbitrary submersion time. The swelling behavior of each sample (circular disc of about 1.5cm in diameter) was assessed by gravimetric method. Each sample, after submersion in acetate buffer for a required time, was taken out and placed between two pieces of tissue paper. A flat

metal sheet (300g) was placed on top of the sample to remove excess acetate buffer. The degree of swelling (%) of each sample was then calculated according to the following equation

$$\text{Degree of swelling (\%)} = \frac{(\text{Wst} - \text{Wdt}) \times 100}{\text{Wdi}} \quad (2)$$

Where Wst denotes the weight of the sample in its wet state after submersion in acetate buffer after an arbitrary submersion time.

c) Fourier Transform Infrared Spectroscope

A Nicolet Nexus 671 Fourier Transform Infrared Spectroscope (FTIR) was used to verify the chemical structure of as-received THC powder, the chitosan fibers, the post-neutralized chitosan fibers, the electrospun chitosan/THC fibers, the post-neutralized electrospun chitosan/THC fibers, the crosslinked electrospun chitosan/THC fibers, and the post-neutralized and crosslinked electrospun chitosan/THC fibers. All spectra were taken in the spectral range of 4000-400 cm^{-1} and observed by using deuterated triglycerinesulfate detector (DTGS) with specific detectivity of $1 \times 10^{9} cm \cdot Hz^{1/2} \cdot w^{-1}$.

3.3.7 Release of Model Drug From the Post-neutralized and Crosslinked Electrospun Chitosan/THC Fiber Mats and Chitosan/THC Films:

a) Preparation of acetate buffer solution

Acetate buffer was chosen to simulate human skin pH condition of 5.5. Sodium acetate 150g was dissolved in 250 ml of distilled water and then glacial acetic acid 15 ml was added into the aqueous sodium acetate solution. Finally, the distilled water was then added into the solution until the final volume up to 1000 ml.

b) Investigation of the actual THC content

The actual THC content in the electrospun chitosan/THC fiber mats was quantified by dissolving the pre-crosslinked electrospun fiber mats in 10 ml acetate buffer after that 0.5 ml of the solution was pipetted to measure the amount of THC content by using a UV spectrophotometer (Perkin Elmer, Lamda 10) at the wavelength of 280 nm which is the maximum wavelength (λ_{max}) for THC. The amount of originally present in as-spun fibers was back-calculated from the obtained

data against a predetermined calibration curve for model drug. The results were reported as averages from at least 3 measurements.

c) Drug release assay

The release characteristic of THC from electrospun mats was studied by immersing the post-neutralized and crosslinked electrospun chitosan/THC fiber mats into 10 ml acetate buffer solution at 37 ^{o}C. At specified immersion period ranging between 0 and 10 h, 0.5 ml of buffer solution is taken out and fresh buffer in equal amount must be refilled. In case of the transdermal diffusion through the pig skin technique, THC loaded as-spun chitosan mats were placed on a fresh piece of pig skin (abdomen; epidermal hair, subcutaneous fat, and underlying tissues removed; final thickness = 1-1.5mm) which is turn, was placed on top of the acetate buffer solution on a modified Franz diffusion cell. At specified immersion period ranging between 0 and 10 h, 0.3 ml of buffer solution is taken out and fresh buffer in equal amount must be refilled. The amount of THC in the withdraw solutions was determined using the UV spectrophotometer at the same wavelength previously mentioned against the predetermined calibration curve for model drug. These data are calculated to determine the accumulative amount of THC released from the samples at the each specified immersion or diffusion period. The experiments are carried out in triplicate and the results are reported as average values.

3.3.8 Cytotoxicity Evaluation

The indirect cytotoxicity evaluation of all electrospun chitosan/THC fiber mats were conducted in adaption from the ISO10993-5 standard test method in a 24 well TCPS, using cell line L929 as reference cell. Extraction media were first prepared by immersing samples cut from the as-spun fiber mats (viz. the diameter of the samples was 15mm) in wells of TCOS in a serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) for 1 day and 7 days, respectively. Cells were cultured in serum-containing DMEM for 16 h to allow cell attachment on the plate. The cells were then starved with SFM for 24 h, after which time the medium was replaced with an extraction medium and cells were re-incubated for 24 h. The viability of cells cultured with fresh SFM was used as control. Finally, the viability of the cells cultured with fresh SFM (i.e., control) and the extraction media was determined with

3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT;Sigma-Aldrich, USA) assay.