CHARPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 For Electrospinning Process

3.1.1.1 Poly(vinyl acetate) solution as an adhesive from Henkel (Thailand) Ltd.

3.1.1.2 Ciprofloxacin Hydrochloride for biochemistry and microbiology 25 g CAS 86393-32-0 Assay min 98% from Sisco Research Laboratories Pvt. Ltd., India.

3.1.1.3 Coconut Oil 100% from Thai Pure Coconut Co., Ltd.

3.1.1.4 Diethyl ether as solvent

3.1.1.5 PU film from Thai Adhesive Tapes Industry Co., Ltd.

3.1.2 For Bacterial Culture

3.1.2.1 *E. coli* (*Escherichia coli*)

3.1.2.2 *S. aureus* (*Staphylococcus aureus*)

3.1.2.3 Deionized water (pH 6.5)

3.1.2.4 NaCl 0.8%w/v

3.1.2.5 Peptone broth

3.1.2.6 Agar powder

3.1.2.7 Tryptic soy broth (TSB)

3.1.3 For Cytotoxicity Test

3.1.3.1 Human fibroblast cells

3.1.3.2 Mouse fibroblast cells (L929)

3.1.3.3 DMEM (Dulbecco's MEM) or MEM (Minimum Essential Medium)

3.1.3.4 Simulated body fluid (SBF) buffer solution (pH 7.4)

3.1.3.5 L-glutamine 100x

3.1.3.6 Lactabumin

3.1.3.7 Ab/Am 100x

3.1.3.8 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazoliun bromide (MTT)

3.1.3.9 Phosphate Buffer Saline or PBS (pH 7.4)

3.1.3.10 Dimethyl sulfoxide (DMSO)

3.1.3.11 0.1 M Glycine Buffer solution

3.1.4 For *In vitro* Drug Release Study

3.1.4.1 Acetate buffer solution (pH 5.5)

3.1.4.2 Phosphate Buffer Saline (pH 7.4)

3.2 Equipment

3.2.1 Petri Dish Plastic 15 x 90 mm and a 24-well plate

3.2.2 Microscope

3.2.3 Shaking bath for *in vitro* drug release study

3.2.4 Electrospinning machine for produce electrospun fibers

3.2.5 Fourier transform infrared spectrometer (FTIR; Thermo Nicolet Nexus 670) was used to determine functional groups on the surfaces.

3.2.6 UV-Vis spectrophotometer (UV-2500) was used to characterize the formation of drug releasing

3.2.7 Field Emission Scanning electron microscope (FESEM; JSM-7001F) was used to observe the surface morphology of electrospun fibers.

3.2.8 Contact angle was used to measure indication of the chemical bonding of the uppermost surface layers of electrospun fibers

3.3 Methodology

3.3.1 Evaluating MIC and MBC value by Dilution Test and Agar Test

3.3.1.1 Dilution Test

Both bacteria from model cells were activated or incubated in different flasks containing the 3 g/mL of tryptic soy broth (TSB) as the bacterial

growing supporter and then kept them at 37°C in an incubator for 18 hours. The activating bacteria in flask will be added into the test tube containing 0.85%w/v NaCl and then adjusted the turbidity of bacterial suspensions or McFarland Standard No. into 0.5 (approximately cell density $1.5x10^8$ CFU/mL). The amount of Ciprofloxacin was varied in different quantities by considering the optimal amount from the previous MBC study, 0.016 µg/mL for *E.coli* and 2.0 µg/mL for *S.aureus*. (Chalkley *et al.*, 1985) In terms of dilution test, the amount of drug should be 15 times as much as the previous MIC value study for giving clear results. The MIC (Minimum Inhibiting Concentration) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. On the other hands, the MBC (Minimum Bactericidal Concentration) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. Both values can be the same number.

3.3.1.2 Agar Test

Each dilution will be placed on the agar plates in order to evaluate the MIC and MBC value. The MIC value will be considered as the least dilution which is no visible growth of bacteria. In contrast, the MBC value will be the least concentration that is no bacterial growth on the plate. In this study, the concentration of MBC will be decided to be the minimum concentration blended into the poly(vinyl acetate) so as to develop the electrospun fibers in further step.

3.3.2 Blending of Coconut Oil and CPF with Poly(Vinyl Acetate) Solution for Electrospinning

3.3.2.1 Blending of Coconut Oil and Ciprofloxacin (CPF) with Poly (Vinyl Acetate)

The optimum concentration of CPF from the MBC value, which was evaluated from previous result will be dissolved in 45%w/v of PVAc with 0.5 mL and 1.0 mL of coconut oil and stir them for dispersing uniformly of CPF in solution.

3.3.2.2 Electrospinning Process

The conventional electrospinning process will be set up, where the syringe has been kept inclined to flow the spinning solution. The tip-to-collector

distance was kept at 15 cm, whereas a ground aluminum foil drum covered by polyurethane film served as a counter electrode. The prepared polymer solution blended already with CPF and coconut oil will be fed into the 1 mL syringe and then the voltage will be supplied a high voltage power of 18 kV to electrospin the fibers. Finally, the sample will be dried in oven at 30°C for 24 hours to remove the residual solvent. Then, the samples will be tested in antibacterial test and cytotoxicity.

3.3.3 Bacterial Culture Evaluations

3.3.3.1 Zone Inhibition

An inhibitory test of neat PVAc, PVAc/CPF/Oil in all conditions was tested by the disc diffusion method (Bauer *et al.*, 1966). The mats were cut into small circular discs of diameter around 1.2 mm. These discs were put on the surface of the Petri dishes. The inhibition zones were estimated after 18 hours. Loaded discs and PVAc/ nanofibers only discs were placed as control discs on tested organism seeded plates. The antibacterial activity plates were incubated at 37°C. The diameters of the inhibition zones were measured in diameter with transparent ruler.

The turbidity of bacterial suspensions or McFarland Standard No. 0.5 (approximately cell density $1.5x10^8$ CFU/mL) was smeared onto the prepared agar plate thoroughly and then placed the materials or samples, which are cut into circular discs, onto the plate orderly and label each samples as well. The zone inhibition or clear zone will be appeared after the plates were kept in an incubator at 37°C for 18 hours. The diameters of the inhibition zones were measured in diameter with transparent ruler.

3.3.3.2 The Bacterial Reduction Studies

The incubated bacteria have been incubated in peptone broth or NaCl 0.8%w/v at 37°C for 1 hour and 24 hours while they were being shaken all the time with the sample materials for studying the percent reduction of bacteria in varied time. First of all, microbes are placed on the broth flask as a carrier which a sample is inside that some of the microbe-bearing carriers are treated with the disinfectant like the sample materials and others serve as untreated carriers as a control(Arora *et al.*, 2011). After the expected time, at 1 hour and 24 hours, the incubated bacteria will be placed thoroughly on the agar disc before cells will be

investigated then. The count is scaled-up to indicate the total number of colony forming units (CFUs) in the suspension. The treated carriers should hole fewer viable microbes than the untreated carriers or control. Thus, efficacy is measured by comparing the density of viable microbes on treated carriers to the density on untreated carriers. In mathematics calculation, CFUs/mL should be primarily calculated for finding the percent reduction of bacteria as following equation (3.1) and (3.2)

$$\text{CFUs/mL} = \frac{\text{Colony count (CFUs) on an agar plate}}{\text{total dilution factor of tube (used to make plate for colony count) X Volume plated}} \quad (3.1)$$

$$\text{Total dilution factor} = \text{Previous dilution factor of tube X Dilution of next tube} \quad (3.2)$$

Volume plated is the volume of the dilution that was plated on the agar plate and dilution factor is amount of specimen transferred divided by the total volume after transfer as following equation (3.3)

$$\text{Dilution factor (DF)} = \frac{\text{Amount of sample}}{\text{Volume of specimen transferred} + \text{Volume of diluent in tube}} \quad (3.3)$$

Then, the percentage of bacterial reduction in 1 hour and 24 hours can be calculated by following equation (3.4)

$$\%\ \text{Bacterial Reduction} = \frac{\text{Colony form units(CFUs) per mL of each sample}}{\text{Colony form units(CFUs) per mL of control in same dilution}} \quad (3.4)$$

3.3.4 Cytotoxicity Test *(Tova. et al. 1992)*

3.3.4.1 Materials Preparation for Cell Seeding and Cell Culturing

Both mouse fibroblast cells L929 and human fibroblast in cell lines are used in this test. They were cultured in MEM medium supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic formulation. The medium was changed in every 2 days and the cultured cells were always kept in a humidified atmosphere containing 5% CO_2 at 37◦C. Each sample materials were cut into a circular disc (about 1.2 cm in diameter) and then they were placed in a 24-well tissue-culture polystyrene plate. The specimens were washed with autoclaved deionized water,

PBS and immersed in SFM overnight. Then, culturing cells were trypsinized and counted by a hemacytometer. The density of cells about 40,000 cells/cm^2 were seeded on the specimens and empty wells of 24-well tissue-culture polystyrene plates which were used as control. The culture was maintained in a humidified atmosphere containing 5% CO_2 at 37◦C.

3.3.4.2 Indirect Cytotoxicity Evaluation

This test was conducted on a 24-well tissue-culture polystyrene plate, the neat adhesive glue, and the film dressing in all conditions. First, the extraction media was prepared by soaking samples in serum free medium (SFM; containing MEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic). They were placed under 5% CO_2 at 37◦C in 24-well plate for 1 day and 3 days. Then, the density of about 40,000 cells per well were separately cultured in a 24-well plate to allow cell attaching on the plate for one day. After that, the cells were starved with SFM for 1 day while the culture medium was replaced with the extraction medium. Finally, MTT assay will be carried out to quantify the amount of the viable cells after 24 hour cell cultured in extraction medium.

3.3.4.3 MTT Assay

Cell proliferation was measured by the colorimetric assay based on the capacity of mitochondrial enzymes of viable cells to transform the MTT tetrazolium salt into MTT formazan *(Mosmann, 1983).* MTT assay was typically used to evaluate the number of viable cells, based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystal formed is proportional to the number of viable cells. First of all, the cultured medium was moved out in each well and incubated in 400 µL MTT solution at 37°C for 30 minutes. Then, MTT was replaced with a buffer solution containing 900 µL/well DMSO and 125 µL/well glycine buffer (pH 10) was added in each well to dissolve the formal crystal. The solution was shaken for 10 min and then transferred into a spectrophotometer to measure the number of viable cells at absorbance 540 nm.

3.3.5 Characterization and Testing

3.3.5.1 Scanning Electron Microscope (SEM)

The surface morphology of the electrospun of both PVAc fibers and PVAc with CPF/Oil fibers on PU film was examined using a scanning electron microscope after silver coating using a sputter coater.

3.3.5.2 UV-Vis Spectrophotometer

The content of ciprofloxacin, which was released out from dressing, was analyzed directly at 277 nm for acetate buffer (pH 7.7) and at 272 nm for PBS (pH 5.5) using an UV-vis spectrophotometer. The concentration of ciprofloxacin in all formulated fibers on PU films was calculated using a calibration curve. The *in vitro* release experiments were performed in triplicate in an identical manner.

3.3.5.3 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of pristine PVAc, the electrospun of PVAc /CPF/Oil, were recorded using FTIR spectrophotometer (Perkin-Elmer-Spectrum RX-I, Lambda, USA) to investigate any interaction between CPF, PVAc and coconut oil in formulated fibers, and the band position of each substance. The CPF for FTIR spectroscopy were grounded with KBr to make the pellets by means of hydraulic pellet press by applying pressure of 600 kg/cm^2. The prepared pellets were scanned over the wave number range from 4,000 to 400 cm^{-1} with resolution of cm^{-1}.

3.3.5.4 In Vitro Drug Release Study

Drug release study is investigating in vitro for a human blood's and skin's pH at 7.4 and 5.5, respectively. Released drug from electrospun mats on PU film was measured by placing a known mass and approximate dimensions (1.2 × 1.2 cm) of material into 25 mL of PBS (pH 7.4) and acetate buffer (pH 5.5) under constant stirring at 37°C. At certain time points, 20 µL the buffer was taken out of the buffer and kept into a microtube for measuring the absorbance further. The amount of drug released was determined using a UV-vis spectrophotometer at λ_{max} = 277 nm for acetate buffer (pH 5.5) and λ_{max} = 272 nm for PBS (pH 7.4). The release experiments of each sample were performed in triplicate, and average values are reported.

3.3.5.5 Contact Angle

Advancing and receding air-water contact angles were measured at room temperature by an image analyzing system, using the air bubble technique. The advancing angle was obtained by placing a needle in deionized water and creating a bubble on the sample surface (1–1.5 mL) and carefully expanding the bubble volume until the part of carefully expanding the bubble volume until the part of the bubble adjacent to the sample surface stopped changing. Similarly, the receding angle was obtained when the air was sucked out of the bubble.