CHEPTER III
EXPERIMENTAL

3.1 Materials

Acetobacter xylinum TISTR 975 was purchased from Microbiological Resources Centre, Thailand Institute of Scientific and Technological Research (TISTR). D-glucose anhydrous (analytical grade) was purchased from Ajax Finechem. Yeast extract powder (bacteriological grade) was purchased from Biobasic. Sodium hydroxide anhydrous pellet (analytical grade) was purchased from Ajax Finechem. Glacial acetic acid (analytical grade) was purchased from RCI lab scan. Lenin, cotton and shefong (polyester) fabrics were purchased from Kikuya shop. Muslin and filter cloth fabrics were purchased from Suksapanpanit. Nylon mesh was purchased from NBC meshtec inc. Japan.

3.2 Methodology

3.2.1 Production of Bacterial Cellulose and Bacterial Cellulose Composites

3.2.1.1 Culture Medium

The culture medium used for bacterial cellulose synthesis of *Acetobacter xylinum* contained 4.0 % w/v D-glucose and 2.0 % w/v Yeast extract powder in distilled water. Then, the culture medium was sterilized by autoclaving at 121 °C for 15 min.

3.2.1.2 Culture Condition

Pre-inoculum was prepared by adding *Acetobacter xylinum* TISTR 975 in a 100 mL Erlenmeyer flask containing 200 mL of culture medium. After a static incubation at 30 °C for 2 days, the bacterial cellulose pellicle appeared on the surface of culture medium. After that, 10 mL of stock culture medium was transferred to a 500 mL Erlenmeyer flask containing 100 mL of culture medium, followed incubation at 30 °C for 4 days. For production of bacterial cellulose composites, after 10 mL of stock culture medium was transferred to a 600 mL beaker

containing 100 mL of culture medium, immersed porous supporting fabric on the surface of culture medium, followed by incubation at 30 °C for 2 days.

3.2.1.3 Purification of Bacterial Cellulose and Bacterial Cellulose Composites

After incubation, bacterial cellulose pellicles produced on the surface of culture medium were harvested and purified by boiling them in 1.0 % w/v sodium hydroxide solution at 90 °C for 2 hrs (repeated 3 times) to remove bacterial cells and culture medium, followed by neutralized with 1.5 % w/v acetic acid solution at room temperature for 30 min and then immersed in distilled water until pH become neutral. The bacterial cellulose pellicles were kept in distilled water prior to use.

3.2.2 Dielectric Barrier Discharge (DBD) Plasma Treatment

The dielectric discharge has the thickness of 2 mm. The two parallel electrodes are stainless steel. The porous supporting fabrics were cut into square shape with the dimension of 10 x 10 cm and were put into the parallel plate dielectric barrier discharge (DBD) plasma reactor for plasma treatment before being immersed in culture medium to produce bacterial cellulose composites. The experiment was operated with the condition of voltage of 50 kV, frequency of 325 Hz and the electrode gap of 4 mm. The flowing air gas was introduced directly through the gap of electrodes.

3.3 Characterization

3.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was used to investigate the chemical functional groups of bacterial cellulose and fabrics. The FTIR spectra were measured at wavenumbers ranging from 4000 to 650 cm^{-1} with a Thermo Nicolet Nexus 670.

3.3.2 Scanning Electron Microscope (SEM)

The surface and cross sectional morphology of bacterial cellulose, bacterial cellulose composites and fabrics were observed by using scanning electron microscope (JEOL JSM 5410LV) operating at 15 kV.

3.3.3 Mechanical Properties

Bacterial cellulose and bacterial cellulose composites were cut into rectangle shape with width of 1.0 cm and height of 5.0 cm. These samples were test the tensile strength. The tensile strength of bacterial cellulose and bacterial cellulose composites were measured in both wet and dry state by using universal testing machine (UTM Lloyd) with 450 N load-cell at crosshead speed of 50 mm/min.

3.3.4 Water Absorption Capacity

To determine the WAC, the freeze dried materials were immersed in distilled water at room temperature until equilibration. After that the materials were removed from water and the excess water at the surface of materials was blotted out with tissue paper. The weights of swollen material were measured and the procedure was repeated until no further weight change was observed. The WAC was calculated with the following equation:

$$WAC = \frac{W_h - W_d}{W_d}$$

Where W_h and W_d were the hydrate and dry weight, respectively.

3.3.5 Water Vapor Transmission Rate (WVTR)

Samples were cut into disc shape with diameter of 33 mm. The bottle containing 25 ml distilled water was weighted, after that the samples were placed and sealed on a mouth as a cap of bottle and placed in incubator at 35 °C for 24 hours. The WVTR was calculated according to the following equation:

$$WVTR = \frac{W_i - W_f}{A}$$

Where W_i and W_f were the weight before and after being placed in an incubator, respectively. A was area of the bottle mouth (m^2).

3.3.6 Wicking Test

A fabric strip (10 mm x 80 mm) was suspended vertically above the colored distilled water surface in a glass beaker in a way that vertical bottom edge slightly touches the colored water. A spontaneous wicking occurs due to capillary force. The water absorption time was recorded when the height of moving liquid was 20 mm.

3.3.7 The In Vivo Experiment

3.3.7.1 Preparation of Animals

This experiment was approved by the national laboratory animal center (NLAC) Mahidol University. Twenty four male rats (Sprague Dawley), Each rat was 8-9 weeks old, weighting approximately 280-300 g, were used in this study. The rats were kept in separate cages at a temperature of 22 °C in a light dark cycle of 12 hours for 3 days prior to the experiment. They were had unlimited supply for food and drinking water.

3.3.7.2 Wound Creation

The hair on the back of each of the rats was removed. The rats were anaesthetized with isofluran 3-4 %. All surgical procedures were performed under strict aseptic protocol. Each animal were made four incision of 6 mm in diameter with biopsy puncher and distance of approximately 1 mm among them as shown in figure 3.1.

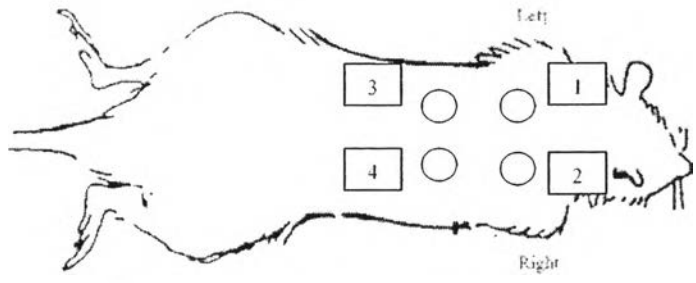

Figure 3.1 Identification of the wound created surgically for the respective treatment groups.

Position 1-3, pure BC, BC/Cotton and BC/Nylon composites were put on the wound, respectively. Position 4, 3M tegraderm film 1624W was put on the wound as the control. Subsequently, animals were accommodated in cage properly isolate and were monitored daily. Their weight, food and water uptake, healthy and wound size were measured. The percent of wound contraction was calculated according to the following equation :

$$\text{Wound contraction (\%)} = \frac{A_{t=0} - A_t}{A_t} \times 100$$

$A_{t=0}$ and A_t were the wound areas at 0 day and 5, 7,14 and 21 days, respectively.

3.3.8 The In Vitro Experiment : MTT Cytotoxicity Test

This assay was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and ISO10993-5). The MTT assay1 is a tetrazolium-dye based colorimetric microtitration assay. Metabolism-competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a plate reader. It is assumed cells that are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell viability. The cells were seeded into 6-well tissue culture dish on the sample's surface at a density of 100,000 cells/dish/3 ml, and incubated for 48 hours in fresh medium and then tested with MTT assay. Briefly, 500 µl of MTT in PBS at 5 mg/ml was added to the medium in

each dish and the cells were incubated for 4 hours. Medium and MTT were then aspirated from the dishes, and formazan solubilized with 1 ml of DMSO and 50 µl of Sorensen's Glycine buffer, pH10.5. The solution was then seeded into 96-well plate for the reading. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The experiments were done 3 times to get the values and standard deviation. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the %survival for each sample compared to controls.

3.3.8.1 Cell Culture

The target cells were human dermal skin fibroblast cell lines (HDF, neonatal, C-004-5C). The human dermal skin fibroblast cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 unit/ml penicillin and 100 ug/ml streptomycin. The cells were incubated at 37 °C in a fully humidified, 5% CO_2 : air atmosphere.