CHAPTER III
EXPERIMENTAL

3.1 Materials

Alginic acid (sodium salt, Brookfield viscosity 20000-40000 cps; hereafter, sodium alginate), copper (II) sulfate ($CuSO_4.5H_2O$) and Poly(vinylpyrrolidone) , PVP were purchased from Sigma-Aldrich Corp. (St. Louis, USA). Sodium acetate (anhydrous) was purchased from Fluka (Buchs, Switzerland). Acetic acid (glacial) was purchased from Mallinckrodt Chemicals, USA. Gallic acid, MES monohydrate, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Pyocyanin from *Pseudomonas aeruginosa*, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Germany). Ethanol was purchased from Merck (Germany). All other chemicals were of analytical reagent grade and used without further purification.

DMEM media, 1% antibiotic-antimycotic, Keratinocytes SFM with EGF human recombinant, and Bovine pituitary extract were purchased from Gibco, Australia. Fetal bovine serum (FBS), Trypsin and L-Glutamine were obtained from Invitrogen, Australia. Trypan blue dye and MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma, Australia. CellTilter-BlueTM reagent was obtained from promaga, USA. NuncTM polycarbonate inserts was purchased from Nunc, Roskilde, Denmark.

3.2 Methods

3.2.1 Characterization

After the initial addition of copper (II) sulfate solution to a sodium alginate solution, the amount of free copper (II) ions in the clear solution that was left from the crosslinking of the alginate chains was quantified by a Shimadzu UV-1800 spectrophotometer. Chemical interaction between the copper (II) ions and certain chemical functional groups of alginate was examined on a copper (II) alginate film using a Nicolet Fourier transform infrared (FT-IR) 360 spectrophotometer. FT-IR spectra of sodium alginate were also recorded for comparison using the KBr pellet method. Morphologies of the copper (II) alginate films both before and after

subsequent immersion in the corresponding copper (II) sulfate solutions were observed with a JEOL JSM-5200 scanning electron microscope (SEM).

The phase structure of copper (II) stearate was characterized by an X-ray diffractometer (XRD) (Rigaku Corp., D/max-2400) equipped with graphite monochromatized Cu K radiation. Chemical interaction between the copper (II) ions and certain chemical functional groups of stearate was examined using a Nicolet Fourier transform infrared (FT-IR) 360 spectrophotometer (the KBr pellet method).

A Nicolet Fourier transform infrared (FT-110 IR) 360 spectrophotometer was used to confirm chemical structure of the obtained GA-grafted collagen scaffold with 5 different GA concentrations (5, 10, 20, 40 and 80 mM). FT-IR spectra of neat collagen scaffold were also recorded for comparison using the KBr pellet method.

3.2.2 Antibacterial Evaluation

Specimens of hydrogel (both copper (II) alginate and copper (II) stearate containing PVP hydrogel) were cut into discs (9 mm in diameter), which were further studied via the disc diffusion method [The US clinical and laboratory standards institute (CLSI) disc diffusion method]. Both gram-negative bacteria, *E. coli*, ATCC 25922 and gram-positive bacteria, *S. aureus*, ATCC 25923 were used to test for antibacterial activity of copper (II) alginate hydrogels. Methicillin resistant *S. aureus* (MRSA), DMST 20654, *S. epidermidis,* ATCC 12228 and *S. pyogenes,* DMST 17020 which caused dermal infection, were also selected to evaluate their antibacterial activity. All of the bacteria was diluted until the colonies equal to 10^8 CFU (colony forming unit)/ mL and then 200 μL bacteria solution were transferred on Difco™ Mueller Hinton agar dishes. The hydrogel specimens were placed on the agar culture dishes and incubated at 37 °C for 24 h. An inhibition zone was clearly seen around each specimen whenever there was antibacterial activity.

3.3.3 Preparation of Ca^{2+} Rich PAA Tube (Ca-PAA tube)

The PAA tube was cut into a 2 cm length for each. Then, the tubes were put in the 150 mM NaCl solution flask that being as a Na^+ rich medium and measured the length again because there was some swellings occur during immersion. The specified concentration of

calcium chloride was added to the flask in order to provide Crosslinking Bridge between the chains that led to tube contraction.

$$Length\ Contraction\ (\%) = \frac{Ls}{Lo}\ x\ 100 \qquad (1)$$

Where *Ls* is the length of the tube after shrinkage and *Lo* is the original length of the tube

The dried PAA tube, the Na-PAA tube and the obtained Ca-PAA tube were further measured on diameter as well as length change both before and after titration using microscope in order to study on anisotropic property.

3.3.4 Cell Proliferation of GA-Grafted Collagen-I Scaffolds

HDFa (from 2 patients) and MSCs (from 6 patients ; 40m-pat.357, 42m-pat.275, 49m-pat.392, 85m-pat.336, 80m-pat.355 and 80- pat.374) from the culture were trypsinized and counted by a cell counter (CASY system, AG Innovatis AG). The GA-grafted collagen scaffolds and neat collagen scaffold were dropped into the mixed cell suspension. The culture was maintained in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 for 1 hour to adhere the cells into the scaffolds. After that, the additional medium was added into the wells. The cells on the scaffold were cultured for 1, 2, 3 and 7 days for HDFa and 1, 2, 3 and 5 days for MSCs. At the different time points, the cells on the scaffolds were measured for their viability using alamar blue assay. After testing cell viability, the cells on the scaffolds were fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature. The fixed cells were washed twice with 10 % PBS buffer solutions for 5 min and once with distilled water for 5 min. The sample were stained with DNA-specified fluorochrome 4,6-diamidine-2-phenylindole dihydrochloride DAPI for 5 min, and stored at 4 °C. The staining images of the cells were examined by using fluorescence microscopy.