CHAPTER III

EXPERIMENTAL

This work was divided into 2 parts. The first part was to study experimental parameters affecting the morphology and size of pores formed in the non-crosslinked chitosan scaffold. The second part was to prepare a mixture of chitosan-crosslinker solution that could be photo-cured to make a photo-crosslinked matrix.

In the first part, the non-crosslinked chitosan scaffolds were fabricated via freeze-drying technique. Concentration of chitosan solution, type of chitosan, and freezing temperature were evaluated. The scaffold with rectangular dimension was prepared in a glass mold. The pore size and morphology were studied by SEM. Percentage of porosity was measured by conventional weighting method avoiding an error occurring from the compressible texture of the scaffold found in mechanical method, mercury porosimetry [37]. The mechanical property of the scaffolds were analyzed in compression mode to investigate their anti-compression force.

In the second part, 1, 3-Diazido-2-propanol (DAZ), used as a crosslinker, was synthesized by applying the method reported by Fringuelli [61] and Spelberg [62]. The photo-crosslinked was then made by following a freeze-drying condition obtained from the study in the first part. Two parameters including mole ratios of DAZ:chitosan and UV irradiation time were studied. The crosslinking reaction (degree of crosslinking) was followed by determining the amount of insoluble part, and analyzed by FT-IR. The shape retention and compressive modulus of photo-crosslinked chitosan were measured and compared to the original one. Finally, cell cytotoxicity test by using L929, mouse connective tissue, was also included.

3.1 Materials

Chitosan

Shrimp and squid chitosan were purchased from Seafresh Chitosan (Lab) Co., Ltd. and Aqua Premier Co., Ltd., respectively. Their physical properties are shown in Table 1.

Table 3.1: Physical properties of chitosan

Chitosan type	%Deacetylation[(a)]	Average molecular weight (g/mol)[(b)]
Shrimp chitosan	96.9	1.0×10^6
Squid chitosan	96.9	1.1×10^6

a) analyzed by solid state ^{13}C-NMR

b) analyzed by GPC

Other Chemicals

Sodium azide, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate, and sodium sulfate were analytical grade and purchased from Fluka Co, Ltd. Epichlorohydrin and tetrabutyl ammonium bromide were from Merck Co., Inc. Glacial acetic acid (99.8%w/w), diethyl ether and ethanol were analytical grade, and purchased from Labscan Asia Co., Ltd., Thailand.

3.2 Instruments

3.2.1 Freeze Dryer

The freeze dryer (model- E-C Apparatus Modulyo Freeze Dryer) was used in the process of making the porous scaffolds.

3.2.2 Scanning Electron Microscopy (SEM)

SEM micrographs of scaffolds were taken on a JEOL JSM-5410 Scanning Electron Microscope.

3.2.3 Universal Testing Machine

The compressive modulus of the scaffolds was measured by using a static-type universal testing machine, model Instron 4502.

3.2.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton (^1H) nuclear magnetic resonance analysis of 1,3-diazido-2-propanol was carried out by using Varian Mercury-400 spectrometer operating at 400 MHz (^1H) in deuterated chloroform ($CDCl_3$). Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) by using the residual protonated solvent signal as a reference.

3.2.5 Elemental Analysis

Elemental analysis was carried out using a Perkin Elmer, CHNS/O analyzer (Series II, Model PE2400).

3.2.6 FTIR Spectroscopy

The FT-IR spectra of chitosan and photo-crosslinked chitosan were recorded with a FT-IR spectrometer (Perkin Elmer), model system 2000, with 16 scans at a resolution of 4 cm^{-1}. A frequency of 400-4000 cm^{-1} (middle IR) was observed by using TGS detector.

3.2.7 Gel Permeation Chromatography (GPC)

The effect of UV irradiation time on molecular weight of chitosan was obtained from Waters GPC systems using pump model 600E and refractive index detector model 2410. One column of Ultrahydrogel Linear was used with guard column. The eluent was 0.5M acetate buffer (pH~3-4) with the flow rate of 0.6 mL/min at constant temperature 30°C. The sample injection volume was 20 μL. Pullulans (MW = 5,800-788,000) were used as standards for calibration.

3.2.8 UV Lamp

Irradiation of the scaffold was carried out using a 8W, 330-390 nm, UV lamp (Narva, Berlin) at a distance of 15 cm from the center of the lamp. The samples were irradiated one at a time in order to control the amount of light intensity of each sample.

3.3 Methodology

3.3.1 Chitosan Scaffold Formation

Porous chitosan scaffolds, both shrimp and squid chitosan, were fabricated using freezing and lyophilization processes. Scaffolds were fabricated by dissolving 1, 2 and 3 wt% of chitosan powder in 2% (v/v) acetic acid for an overnight period, then the insoluble part was filtered out. The chitosan solution was poured into a rectangular glass mold with dimension of 5 cm ×5 cm ×0.3 cm (width×height×thickness). Pre-cooling was performed at approximately 1-2°C for overnight before immersing the mold in freezing solutions having selected temperatures; –10°C (ice:acetone, 1:1), -80°C (dry ice:acetone, 1:1) and -196 °C (liquid nitrogen). After freezing, the scaffolds were removed from the molds and then re-hydrated. For the rehydration process, the scaffolds were soaked in absolute ethanol for 2 h and then sequentially in the 60% (v/v) and 40% ethanol solutions added with 1M NaOH to neutralize the pH for 2 h each. The scaffolds were finally washed with DI water several times and then re-lyophilized before characterization.

3.3.2 Preparation of Photo-Crosslinked Chitosan Scaffolds

3.3.2.1 Synthesis of 1,3-Diazido-2-Propanol

The epichlorohydrin (13 mmol) was added to an aqueous solution of sodium azide (65 mmol in 20 ml) containing tetrabutyl ammonium bromide as the phase-transfer catalyst (13 mmol). The solution was stirred for 24 h at room temperature. 1,3-Diazido-2-propanol or DAZ was extracted from the solution with diethyl ether (3×20 ml). The combined organic phases were washed five times with sodium

phosphate buffer (pH 7.0, 25 ml). The organic phase was dried with sodium sulfate. Diethyl ether was removed by using a rotary evaporator (yield 70%).

3.3.2.2 Formation of Photo-Crosslinked Chitosan Scaffolds

Chitosan either from shrimp or squid (2%w/v) was dissolved in 2% acetic acid for overnight, then filtered to eliminate the insoluble part. The chitosan solution (SH) was then mixed with DAZ having a mole ratio of SH:DAZ equaled 1:0.05, 1:0.1, 1:0.5 and 1:1 at room temperature. Subsequently, the obtained mixture was poured into the rectangular glass molds (ca. 8g/mold) and UV-irradiated for 10-200 min. After that, they were pre-cooled at approximately 1-2°C for an overnight period before immersing into a –10°C (ice:acetone, 1:1) bath, and then freeze dried for overnight. Before characterization, the scaffolds were re-hydrated in ethanol series as the same procedure mentioned in 3.3.1.

3.3.3 Characterization of Chitosan Scaffolds

3.3.3.1 Microstructural Characterization

The scaffolds were sliced under liquid N_2 in two directions (X and Y axis, Fig 3.1). Surface morphology and pore size of the scaffolds were evaluated by SEM. Imaging was conducted at an accelerating voltage of 15 kV. Mean pore diameter of each scaffold obtained from 5 points were determined by counting the average-number of pores passing test line applied from "Quantitative Metallography" [63].

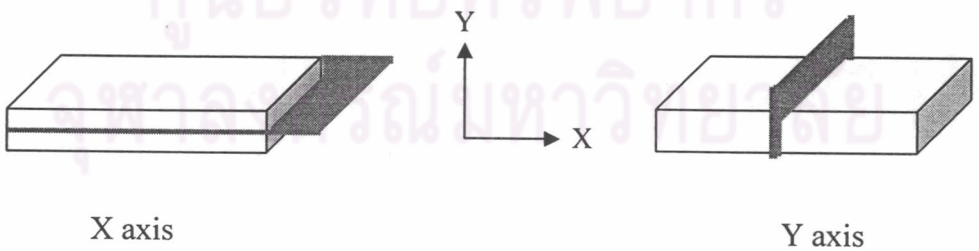

Figure 3.1 Cross-section axis for analysis of chitosan scaffold by SEM.

3.3.3.2 %Porosity

The scaffold and film were prepared from 2%wt chitosan solution. Then, the specimens (1×1 cm^2) of scaffold and film were weighed. Density was calculated from the weight and dimension of the specimens. The density measurement was performed in triplicate. Porosity was calculated using the following equation.

$$\%Porosity = \left[1 - \frac{D_s}{D_f}\right] \times 100$$

where D_s = Density of the scaffolds

D_f = Density of the films

3.3.3.3 Mechanical Testing

The compressive mechanical property of the scaffolds with dimension of 10×10×2 mm^3 was tested using a Universal Testing Machine. The scaffolds were compressed in Y axis with a 100 N load cell and 5 mm/min crosshead speed. The compressive modulus was defined as the initial linear modulus. Ten specimens were tested for each sample. To reduce an error during the compression testing, the tested scaffolds were not rehydrated since the rehydration causes surface of the scaffold to distort.

3.3.4 Characterization of Photo-Crosslinked Chitosan Scaffolds

3.3.4.1 Functional Group Analysis by FT-IR

One mg of ground sample immersed in liq. nitrogen was mechanically mixed with 100 mg of potassium bromide powder to prepare a KBr disk. A frequency ranging from 4000-400 cm^{-1} was observed.

3.3.4.2 Determination of Degree of Crosslinking

The photo-crosslinked scaffold was dissolved in 2%v/v acetic acid for overnight. To collect any insoluble materials, the sample was filtered through a pre-weighed filter paper no.1 (Whatman). The insoluble materials were washed with

acetic acid and dried. Degree of crosslinking was calculated using the following formula.

$$\%Degree\,of\,crosslinking = \left[\frac{W_i}{W_d}\right] \times 100$$

where W_d = scaffold dry weight

W_i = insoluble material weight

3.3.4.3 Shape Retention

The scaffolds were cut into square shape with size 2×2 cm^2 and precisely measured with a digital vernier. Shape retention was determined in two media; one was de-ionized water (pH 7) and the other was acetic acid solution (pH 5). The samples were immersed in either medium for 3 h. The percentage of shape retention was calculated according to the following equation.

$$\%Shape\ retention = \left[\frac{W_s\ L_s}{W_d\ L_d} - 1\right] \times 100$$

where W_d = Width of dry scaffold

W_s = Width of swelling scaffold

L_d = Length of dry scaffold

L_s = Length of swelling scaffold

3.3.4.4 Cytotoxicity

Cell culture- A cell line used in the assay was L929, mouse connective tissue, fibroblast-like cells. The growth medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), together with penicilin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 atmosphere. Once confluence was reached, the cells were subcultured for cytotoxicity study. The samples tested were sterilized with ethylene oxide gas.

Cytotoxicity test- The scaffold samples were cut into square pieces and saturated with growth medium. The sample pieces were typically placed in the middle of a 35-mm dish and belt tightly with nontoxic dental wax. L929 cells were

seeded onto the dish at a density of $6x10^4$ cells/dish and incubated for 48 h. Cell morphology and the toxic zone were evaluated by phase contrast light microscopy after a 48 h exposure to the cells. The cells were stained with 0.01 % neutral red in phosphate buffer saline (PBS) for membrane integrity. High-density polyethylene (HDPE) and natural rubber containing carbon black were used as negative and positive controls, respectively. Each sample was tested in triplicate.