CHAPTER III
EXPERIMENTAL

3.1 Materials

Chitin from shrimp shells was provided by Seafresh (Lab) Company Limited, Thailand. Sodium hydroxide, hydrochloric acid, methanol, and isopropanol were purchased from RCI Labscan Limited, Thailand. Epichlorohydrin (C_3H_5ClO) and chloroacetic acid ($ClCH_2COOH$) were purchased from Sigma–Aldrich. All chemicals were used as received without further purification.

3.2 Instrument and Equipment

3.2.1 Fourier Transform Infrared Spectrophotometer (FTIR)

Structural characterization related to functional groups was done by a Nicolet/Nexus 670 with 32 scan at a resolution of 4 cm^{-1}, and recorded in frequency range of 4000-650 cm^{-1}.

3.2.2 Nuclear Magnetic Resonance Spectrometer (NMR)

Structural characterization related to types of protons was done by a Bruker Avance proton-nuclear magnetic resonance spectrometer (^1H NMR) 500MHz, and recorded chemical shift from 16 to 0 ppm.

3.2.3 Thermal Gravimetric Analysis (TGA)

Thermal stability of the chitosan gel was determined by a Dupont thermo gravimetric analyzer under N_2 atmosphere at the temperature range between 50°C to 800°C and heating rate of 10°C/min.

3.2.4 Scanning Electron Microscope (SEM)

Morphology of the injectable gel was studied by a Hitachi scanning electron microscope (SEM). The gel samples were cut and coated with a platinum thin layer.

3.2.5 Transmission Electron Microscope (TEM)

Morphology of nanofiber was studied by using a Hitachi transmission electron microscope (TEM). The samples were dispersed in water and dropped on copper grids.

3.26 Rheometry

The rheological measurements were performed by using a Rheometric Scientific (TA Instruments). The samples were placed between parallel plate geometry and dropped with mineral oil in order to prevent evaporation during the measurements. The dynamic strain sweep test was applied to determine storage modulus (G′) and loss modulus (G″).

3.3 Methodology

3.3.1 Preparation of Chitosan Nanoscaffold

Chitin whiskers, CW, were prepared as described by Nair and Dufresne with some modifications. In brief, chitin, (1.00 g), was treated in 3 M HCl (100.0 ml) and stirred at 105 °C for 3 h to obtain colloidal solution. The solution was centrifuged, followed by treating with hydrochloric acid for two times and dialyzing in distilled water, before lyophilizing to obtain white fine fibrous product of CW (Scheme 3.1).

FTIR (KBr, cm^{-1}): 3485, 3310 and 3120 (-OH and -NH) 1663, 1627, and 1560 (amides I and II).

Chitosan nanoscaffold, CN, was prepared as previously described by Phongying et al. In brief, CW (20 ml, 2.45 g) was stirred in aq. NaOH (40% w/v, 100.0 ml) at 150 °C for 7 h and left at room temperature overnight. The precipitates were collected followed by treating for two more times with NaOH to accomplish the treatment time of 21 h. The crude product was dialyzed in distilled water several times until neutral, and lyophilized to obtain white fine fiber of CN (Scheme 3.1).

FTIR (KBr, cm^{-1}): 3484 (-OH), 1640 (amide I), 1595 ($-NH_2$), and 1154-896 (pyranose ring). ^{1}H-NMR (δ, ppm, 500 Hz, 2% CD_3COOD/D_2O, 338 K): 2.472 (NHAc), 3.593 (H2 of GlcN unit in chitosan), 4.412-4.305 (H2 of GlcNAc and H3-H6 of pyranose ring), 4.927 (H1 of GlcNAc), 5.274 (H1 of GlcN).

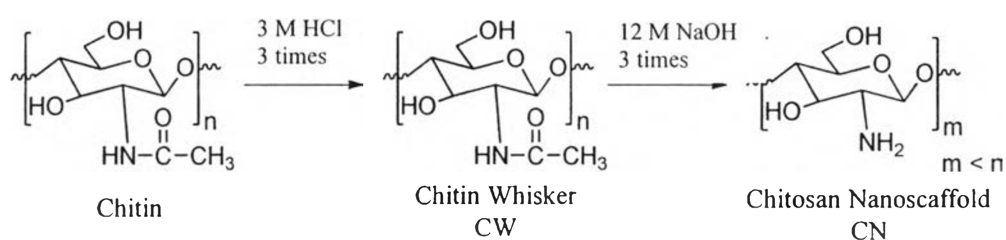
Molecular weight (Mw) of CN was determined based on pullulan standards by using a Shimadzu LC-10AD, size exclusion chromatography (SEC) equipped with a TSK-GEL WS44228 column (7.8 mm × 300 mm; Milford; Massachusetts, USA) under the conditions of an eluent flow rate at 1.0 ml/min

operating temperature at 40 °C (Lingyun *et al.*, 2006). The eluent was 0.5 mol/L CH_3COOH/ 0.5 mol/L CH_3COONa aqueous solution. Pullulans were products of Showa Denso K.K. with Mw of 6.0×10^3, 1.0×10^4, 2.2×10^4, 4.9×10^4, 1.1×10^5, and 2.1×10^5, 3.9×10^5, 8.1×10^5, as evaluated by using 0.5 mol/L CH_3COOH/ 0.5 mol/L CH_3COONa aqueous solution at 1 mg/mL. Pullulans were filtered through 0.45 mm millipore filters and the weight-average molecular weight was calculated by the following equation.

$$\text{Log (Mw)} = -0.4734Rt + 12.659\ (R^2 = 0.9996) \qquad (1)$$

whereas Rt is the retention time.

The Mw of chitosan nanoscaffold is about 103 kDa ± 6.5 kDa and polydispersity (PDI) of CN is 3.0.



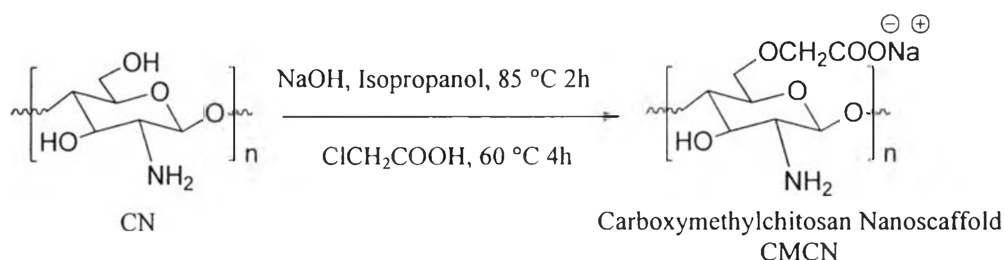
Scheme 3.1 Preparation of chitosan nanoscaffold

3.3.2 Synthesis of Carboxymethylchitosan Nanoscaffold, CMCN

Compound CN (10.0 g/ 6.2 mmol) was suspended in 50% (w/v) NaOH (100 ml) for 1 h at room temperature and kept at -40 °C overnight, then thawed at room temperature. The alkali chitosan was suspended in isopropanol (75 ml), refluxed at 85 °C for 2 h before cooling to room temperature. Chloroacetic acid, $ClCH_2COOH$ 60% (w/v) (100 ml/ 64.8 mmol), was added to the alkali chitosan solution in five equal portions every 5 min before raising to 60 °C and stirring for 4 h. The solution obtained was centrifuged and collected followed by adjusting pH to 7.0 with 1.0 M NaOH. The solution was precipitated in cold methanol and washed with methanol/ H_2O (60: 40) several times. The product was dissolved in water, filtrated out the insoluble particles and freeze-dried at -50 °C to obtain white powder of CMCN

(Scheme 3.2). The solubility of CMCN was applied to see the rang of pH and concentration for using CMCN as a liquid phase.

FTIR (KBr, cm^{-1}): 3467 (-OH), 1609 ($-COO^-$), 1418 ($-COO^-$), and 1164-893 (pyranose ring). ^{1}H-NMR (δ, ppm, 500 Hz, D_2O, 298 K): 4.7-5.0 (H1 of GlcN), 4.5-4.7 (H1 of GlcNAc), 4.3-4.4 (H3 of O-CH_2COOH), 4.0-4.3 (H6 and H3 of O-CH_2COOH), 3.3-4.0 (H3-6 of pyranose ring), 3.0-3.3 (H2 of N-CH_2COOH), 2.7-3.0 (H2 of GlcN unit in chitosan), 2.1 (H2 GlcN unit in chitin).

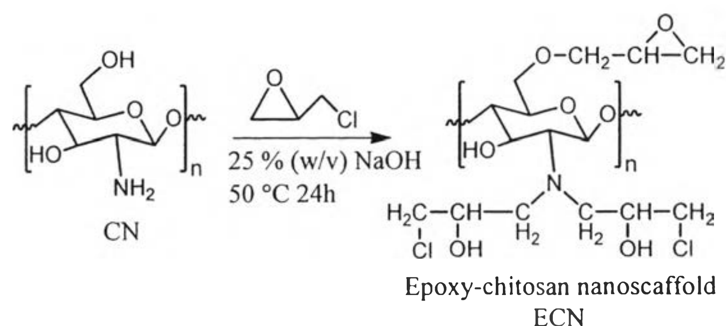


Scheme 3.2 Synthesis of carboxymethylchitosan nanoscaffold

3.3.3 <u>Synthesis of Epoxy-chitosan Nanoscaffold (ECN)</u>

Compound CN (1.00 g) was suspended in 25% (w/v) NaOH, and NaOH, and epichlorohydrin, EPC (2.0 ml) was added before stirring at 50 °C for 24 h (Scheme 3.3). The heterogeneous solution was filtrated to collect the white precipitates. The precipitates were washed with methanol several times to obtain epoxy-chitosan nanoscaffold (ECN).

FTIR (KBR, cm^{-1}): 3400 (-OH), 2929 (-CH), 1432 ($-CH_2$), 1150-1085 ($-CH_2-O-CH_2$), 928 (oxirane ring), and 729 ($-CH_2-Cl$).



Scheme 3.3 Synthesis of epoxy-chitosan nanoscaffold

3.3.4 Preparation of Carboxymethylchitosan Nanoscaffold Gel, CMCN gel

Compound CMCN, (20 mg), was added in deionized water 1.0 ml to obtain CMCN-2 (2% (w/v)). In similar, CMCN for, 40, 60, 80, 100, 150 and 200 mg, also added in deionized water 1.0 ml to obtain CMCN-4, -6, -8, -10, -15, and -20. The pH values of the solutions were in the range of 6.80-7.40. EPC was added for 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 ml to investigate appropriate amount of crosslink agent. Gelation time and gelation temperature were systematically examined by test tube inverting method. The temperature was varied from room temperature to 60 °C.

FTIR (KBr, cm^{-1}): 3426 (-OH), 1736 (-C=O ester linkage), 1598 (-NH_2), and 1151-898 (pyranose ring).

3.3.5 Preparation of CMCN Blend Gel, CMCNB Gel

In the case of CMCNB gel preparation, CN (20 mg) was added in the solution of CMCN-10 to form CMCNB-2 gel. In similar, series of CN, (50, 100, 200, 400, and 600 mg), were added into solution of CMCN-10 to form CMCNB-5, -10, -20, -40, -60 gel. All mixtures were allowed stirring and sonicating for 15 min. EPC (0.2 ml) was added into the mixture and allowed stirring and sonicating until the solution obtained became a fluid viscous gel. The fluid gel was transferred to syringe before gelation was completed. The fluid gel was injected into the mold to determine percent swelling of CMCNB gel.

FTIR (KBr, cm^{-1}): 3426 (-OH), 1737 (-C=O ester linkage), 1597 (-NH_2 high intensity), and 1150-898 (pyranose ring).

3.3.6 Preparation of Carboxymethylchitosan Nanoscaffold Epoxy-chitosan Nanoscaffold, CMCNE Gel

For preparation of CMCNE gel, ECN, (20 mg), was added in the solution of CMCN-10 to form CMCNE-2 gel. In similar, series of ECN, (50, 100, 200, 400, and 600 mg), were added into solution of CMCN-10 to form CMCNE-5, -10, -20, -40, -60 gel. All mixtures were allowed stirring and sonicating for 15 min. EPC (0.2 ml) was added into the mixture and allowed stirring and sonicating until the solution obtained became a fluid viscous gel. The fluid gel was transferred to syringe before gelation was completed. The fluid gel was injected into the mold to determine percent swelling of CMCNE gel.

FTIR (KBr, cm^{-1}): 3426 (-OH), 1736 (-C=O ester linkage), 1596 ($-NH_2$), and 1151-898 (pyranose ring).

3.3.7 Swelling Measurement

Gel was cut into a piece with size of 1.0 × 1.0 × 0.3 cm and was weighed (m_b) before placing in distilled water at room temperature. The gel was taken out of water at regular time, and then the water on hydrogel was removed thoroughly by with filter paper and weighed (m_t). The gel was weighed until constant (m_e). The gel was weighed at dry state (m_0). The percentage of swelling, %S, and equilibrium water content, EWC, were calculated as equations (2) and (3), respectively (Wang and Wu 2005).

$$\% \text{ Swelling} = \frac{(m_t - m_b)}{m_b} \times 100 \qquad (2)$$

$$EWC = \frac{(m_e - m_0)}{m_0} \qquad (3)$$

The kinetic data of swelling process were used to study water diffusion into gels via equations (4) (Thakur *et al.*, 2010), (Sunil *et al.*, 2006).

$$\frac{M_t}{M_\infty} = kt^n \qquad (4)$$

M_t and M_∞ represent the mass of water absorbed by the hydrogel at time t and at equilibrium whereas k is a gel characteristic constant, which is related to the structural characteristics of the polymer, especially the interaction with the solvent. Swelling exponent, n, describes the mechanism of water penetrated into the hydrogel. The constants n and k were calculated from slope and intercept of log M_t/M_∞ versus log t for the hydrogel.

3.3.8 Network Parameters

The number average molecular weight between crosslink, M_c is one of the basic parameters to describe the crosslink polymeric networks. M_c was calculated by using equilibrium swelling theory (Paul *et al.*, 1943), (Jordan *et al.*, 1983), as described previously by Flory and Rehner equation, Eq. (5). The volume fraction of

polymer, ϕ, in the swollen state explained about the amount of liquid which can be imbibed into a hydrogel and is defined as a ratio of the polymer volume to the swollen gel volume, Eq. (6) (Martin *et al.*, 2010).

$$M_c = \frac{-d_p V_s \phi^{1/3}}{[\ln(1-\phi) + \phi + \chi\phi^2]} \qquad (5)$$

$$\phi = \left(1 + \frac{d_p}{d_s}\left[\frac{m_a}{m_b}\right] - \frac{d_p}{d_s}\right)^{-1} \qquad (6)$$

Herein, ϕ is volume fraction of polymer in hydrogel, V_s is molar volume of solvent (18 cm^3/mol), χ is Flory-Huggins polymer-solvent interaction parameter, d_s and d_p are the density of solvent and polymer, respectively. The terms of m_a and m_b are the mass of polymer after and before swelling. The χ parameters of hydrogels can be examined from experiment by using equation (7) (Tuncer *et al.*, 2006).

$$\chi = \frac{1}{2} + \frac{\phi}{3} \qquad (7)$$

As shown in Eq. (7), χ parameter is always ≥ 0.50. The crosslink density, V_e, can be determined by using Eq. (8). Herein, N_A is Avagadro's number (6.023×10^{23} mol^{-1}) (Sunil *et al.*, 2006).

$$V_e = \frac{d_p N_A}{M_c} \qquad (8)$$

3.3.9 Morphology of Injectable Gel

Structural morphology of CMCN-10 gel, CMCNB-10 gel, and CMCNE-10 gel were investigated both optical, and scanning electron microscopes (SEM). In case of SEM, CMCN-10 gel, CMCNB-10 gel, and CMCNE-10 gel were lyophilized before placing on aluminum stub with conductive paint and were sputter-coated with platinum.

3.3.10 Rheological Analysis

Rheological measurements were performed by using a Rheometric Scientific (TA Instruments) at 25 °C with parallel plate geometry (plate diameter of 25 mm, gap of 3 mm). In order to determine storage modulus (G′) and loss modulus (G″), CMCN-10 gel, CMCNB-10 gel, and CMCNE-10 gel were placed on parallel plate and dropped mineral oil to prevent evaporation during the measurements. The dynamic strain sweep test was applied with percent strain from 0.1 to 100 at constant frequency (ω = 1 rad/s) was evaluated

3.3.11 Hydroxyapatite Cooperation (Tachaboonyakiat *et al.*, 2001)

CMCN-10 gel, CMCNB-10 gel, and CMCNE-10 gel, (1.00 g), were soaked in $CaCl_2$ (200 mM)/Tris-HCl (pH 7.4) aqueous solution (20 ml) at 37 °C for 2 h followed by washing several times with deionized water. The products were soaked in Na_2HPO_4 (120 mM) aqueous solution (20 ml) at 37 °C for 2 h and washed thoroughly with water. Five cycles of soaking were operated.

3.3.12 Cell Culture Studies

Cell culture studies were studied by using a non-transformed cell line SaOs-2 cells (sarcoma osteogenic). The SaOs-2 with 90–95% obtained from T-75 flask cultures were used to seed onto CMCN-10 gel, CMCNB-10 gel, and CMCNE-10 gel. All gels were prepared in 24-well culture plate and incubated with culture medium for 3 h at 37 °C in an incubator with 5% CO_2 and 95% air. Cells were dropwisely seeded onto the top of the gels (5×10^4 cells/50 μl of media/gel). The seeded gels were kept at 37 °C in an incubator with 5% CO_2 for 6 h, 1d, 3d, 5d, and 7d to determine the number of cells in gel by DNA assay technique. The fresh media were changed every two days. The number of cells was analyzed by fluorescent bisbenzimide (Hoechst 33258). Fluorescent measurement used to evaluate cell number by converted from calibration curve of known cell number at 355 nm (Excitation) and 460 nm (Emission) using fluorescent microplate readers.