CHAPTER III

MATERIALS AND METHODS

3.1 Materials

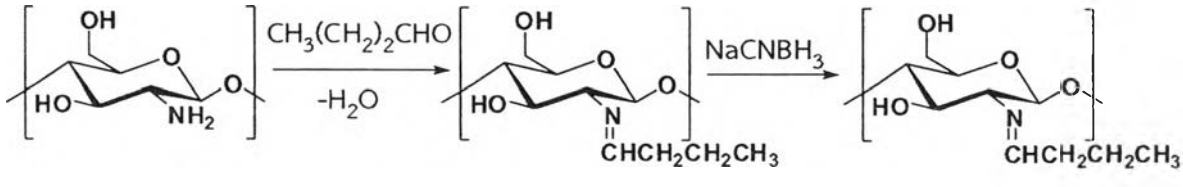
1. Chitosan, $\overline{M_w}$ =100 kDa; 95%DD (Seafresh Chitosan, Co., Ltd. Thailand)
2. Sodium alginate, $\overline{M_w}$ =120 kDa; 0.39 F_G (Sigma-Aldrich, USA)
3. Glucosamine hydrochloride, GH (Sigma-Aldrich, USA)
4. Butyraldehyde (Merck, Germany)
5. Sodium cyanoborohydride, $NaCNBH_3$ (Merck, Germany)
6. Ehanol, EtOH (Merck, Germany)
7. Sodium hydroxide (Ajax Finechem, Australia)
8. Glacial acetic acid (Merck, Germany)
9. Phenylisothiocyanate, PITC (Acros Organics, USA)
10. Calcium chloride, $CaCl_2$ (Carlo Erba, France)
11. Sodium acetate, CH_3COONa (Carlo Erba, France)
12. Tri-sodium citrate, $C_6H_5Na_3O_7*2H_2O$ (Ajax Finechem, Australia)
13. Acetonitrie HPLC grade, ACN (Carlo Erba, France)
14. Methanol HPLC grade, MeOH (Carlo Erba, France)
15. Ortho-phospholic acid, H_3PO_4 (Carlo Erba, France)
16. Tri-sodium citrate, $C_6H_5Na_3O_7*2H_2O$ (Ajax Finechem, Australia)
17. Deuterated oxide, D_2O (Merck, Germany)
18. Trifluoroacetic acid, TFA (Acros Organics, USA)
19. Phosphate buffer saline powder pH7.4 (Sigma-Aldrich, USA)
20. Triethanolamine 99% (Hong huat, Co., Ltd. Thailand)
21. Carbopol 940 (Hong huat, Co., Ltd. Thailand)
22. Dialysis membrane, MWCO 12,400 Da (Sigma-Aldrich, USA)
23. Cellulose acetate membrane filter, 0.2 µm (Merck Millipore, Ireland)

24. Centrifugal filter devices (Amicon Ultra-15), MWCO 100K (Merck Millipore, Ireland

3.2 Methods

3.2.1 Synthesis of *N*-butyl chitosan (NBC)

Chitosan (1 g, Mw = 100kDa, %DD = 95) was dissolved in acetic acid (1% v/v, 70 ml). When dissolution was complete, ethanol (50 ml) was added. Then the solution of butyraldehyde (0.1, 0.5, 1.0 or 2.0 eq/NH_2) in ethanol was added to the chitosan solution. After 1 h stirring, the pH of the solution was adjusted to 5.0 with sodium hydroxide (1M). In this solution, sodium cyanoborohydride (4eq/NH_2) was added and stirred for 24 h. The *N*-butyl chitosan derivative was precipitated by adjusting the pH of solution to 10. The precipitate was washed with 30% ethanol (v/v) and increased water content to 100%. The unreacted aldehyde and inorganic products were removed from the precipitate by refluxing with ethanol at 60 °C for 4 h and fresh ethanol was replaced every 2 h. The precipitate was washed again by DI water and dried to give the product.



Chitosan — Reductive alkylation — *N*-butyl chitosan

Figure 3.1 Synthesis of *N*-butyl chitosan

The structure of *N*-butyl chitosan was confirmed by a ^{1}H NMR spectrometer (400 MHz, Varian Mercury 400 Spectrometer, USA). The ^{1}H NMR spectra of chitosan were obtained from 1% chitosan dissolved in 1% TFA in D_2O. The degree of substitution (DS) was calculated from integration of the peaks of the NMR spectrum. All measurements were performed at 300K, using pulse accumulations of 128 scans. The calculation was as follow equation (1).

$$Degree\ of\ substitution\ (\%DS) = \left\{\frac{\int N-CH_2\boldsymbol{CH_2}R}{2 \times \int -\boldsymbol{H_2}}\right\} \times 100 \qquad (1)$$

Where: $\int N - CH_2\boldsymbol{CH_2}R$ is an integral peak of H-signal for C-2 (CH_2) in butyl group of chitosan (δ=1.5).

$\int -\boldsymbol{H_2}$ is an integral peak corresponding to proton at position 2 of the glucopyranose ring (δ=3.0).

3.2.2 Particle preparation of GH-loaded particles

Ca^{2+}-alginate-chitosan particles were prepared by ionotropic gelation method modified from Rajaonarivory et al., 1994 [7].The optimum mass ratio of sodium alginate:$CaCl_2$:chitosan to obtain the smallest particle size was 10:2.33:1.00, following the work reported by De and Robison in 2003 [9].

Sodium alginate solution (0.6 mg/ml) was mixed with GH with 2.5:1 of GH:alginate mass ratio in 30 ml of sodium alginate solution. Six ml of calcium chloride solution (0.67mg/ml) was added dropwise in this mixed solution while stirring at ~1,000 rpm. The mixed solution was sonicated at 20 min. Ultrasonic bath was used to breakdown the pre-gel aggregates to reduce a particles size. Then 6 ml of chitosan solution or *N*-butyl chitosan solution that was dissolved in 1%v/v of acetic solution (0.3 mg/ml), was added dropwise at a controlled rate to obtain particle suspension. The pH of solution was adjusted to 4.5-5.0 using 1*M* NaOH solution to prevent the alginate that will precipitate in pH solution lower than 3.3 before alginate interacted with chitosan [13]. This mixed solution was stirred for 30 min. GH-loaded particle with chitosan and *N*-butyl chitosan was labeled as GH-ALG-CTS and GH-ALG-NBC respectively. All of polymer solution was filtered through 0.45 μm filter paper to remove particulates before use.

The particle suspension was then centrifuged at 25 °C in Amicon Ultra-15 (Ultracel-100k) centrifuge tube with 100KDa cut off at 5,000 rpm for 20 min to separate free polymers from particles and stored at 4°C [14, 21].

3.2.3 Particles analysis

3.2.3.1 Determination of particle size and zeta potential

The particle size, size distribution and zeta potential were evaluated using Malvern Zetasizer Nano ZS system (Malvern Co., UK, facilitated by facilitated by Faculty of Pharmaceutical Sciences Chulalongkorn University). Size calculation was based on dynamic light scattering (DLS) method as a software protocol. Particle size was determined using He-Ne laser with wavelength at 532 nm by zeta sizer. The scattered light was collected at an angle of 270° (back scattering) through fiber optics and converted to an electrical signal by an avalanche photodiode array (APDs). All samples were run in triplicate with the number of runs set to 11 and run duration set to 20 seconds. The zeta potential of dispersion is measured by applying an electric field across the dispersion. Particles within the dispersion with a zeta potential will migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. All samples were run in triplicate with the number of runs set to 5 and run duration set to 10 seconds.

3.2.3.2 Morphology analysis

Morphology of the particles was characterized by transmission electron microscopy (TEM). It was carried out using TEM (model JSM-2100, Japan). Samples of the nanoparticles suspension (5–10 µl) were dropped onto copper grids. After drying, the samples were stained using 1% w/v phosphotungstic acid (PA, $[PW_{12}O_{40}]^{3-}$) and 1% w/v uranyl acetate (UA, $UO_2(CH_3COO)_2{\cdot}2H_2O$). Digital Micrograph and Soft Imaging Viewer software were used to perform the image capture and analysis.

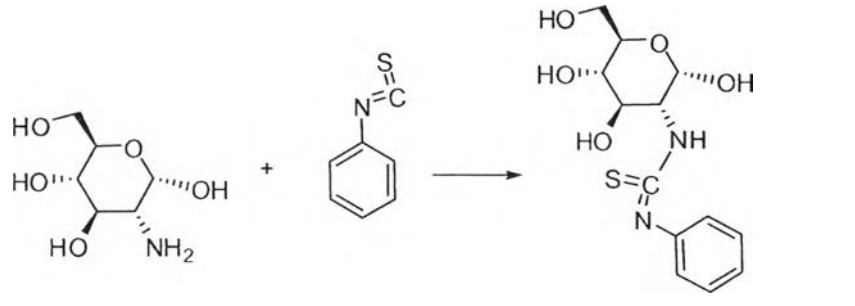
The morphology and surface appearance of particles were examined by scanning electron microscope (Model JSM-6480 LV JEOL, Japan), SEM.

3.2.3.3 Evaluation of loading efficiency

The suspensions were centrifuged by centrifuge tube with membrane (Amicon Ultra-15) at 5,000 rpm for 25 min at 4 °C. The particles on the top of membrane and filtrate solution on the bottom were collected for analysis. All fractions were analyzed by HPLC technique with suitable condition.

The particles were digested by addition 5 mL of 0.5 M sodium citrate, followed by vortex mixing for 5 min and placed in sonication bath for 15 min. The suspensions were filtered through 0.45 μm PTFE syringe filter and prepared for HPLC.

In order to detect GH by UV-visible detector, the maximum wavelength of GH is relatively low at 190 nm. GH was derivatized by reacting with phenylisothiocyanate (PITC). The derived product of GH and PITC is pkynylthiocarbonyl-glucosamine which has maximum wavelength at 245 nm (Fig. 3.2) [22].



Glucosamine PITC phynylthiocarbonyl-glucosamine

Figure 3.2 Derivatization of glucosamine

Two methods for determination of GH loading efficiency (%LE) and GH loading capacity (%LC) were performed.

Method I : Determination of GH from dried particles

The freeze-dried particles were digested by addition 5 ml of 0.5 M sodium citrate, followed by vortex mixing for 5 min and placed in sonication bath for 15 min. The suspensions were filtered through 0.45 μm PTFE syringe filter and prepared for HPLC. Then the dissolved GH from digested particles was derivatized with PITC (see 3.2.3.4).

The GH loading efficiency or the amount of GH that was contained in the particles was calculated from the following equation:

$$\%LE = \frac{weight\ of\ GH\ in\ dried\ particles}{weight\ of\ GH\ used\ in\ formulation} \times 100 \quad (2)$$

The GH loading capacity or actual loading (%w/w) was calculated from the following equation:

$$\%LC = \frac{weight\ of\ GH\ in\ dried\ particles}{dried\ weight\ of\ recovered\ particles} \times 100 \quad (3)$$

Method II : Determination of GH from the filtrate

The filtrate, aqueous solution separated from the suspension by centrifugal filter tube, was derivatized with PITC before HPLC analysis. The GH loading efficiency was calculated using reverse method applying following equation:

$$\%LE = \frac{weight\ of\ GH\ used\ in\ formulation - weight\ of\ GH\ in\ filtrate}{weight\ of\ GH\ used\ in\ formulation} \times 100 \quad (4)$$

The GH recovery was defined as percentage of the total amount of GH found in the prepared suspension (of particles) compared to the total added amount of GH in the preparation. The total amount of GH found in the prepared suspension was determined from sum of GH weight in the dried particles and in the filtrate. The percentage of GH recovery was calculated from the following equation:

$$\%Recovery = \frac{weight\ of\ GH\ in\ dried\ particles + weight\ of\ GH\ in\ filtrate}{weight\ of\ GH\ used\ in\ formulation} \times 100 \quad (5)$$

3.2.3.4 Analysis of glucosamine by PITC derivatization and HPLC

GH Standard solutions were prepared from aqueous solution in 1, 5, 10, 50, 100, 500, and 1,000 µg/ml

Four hundred µl of GH solution was transferred into a glass vial. Then, 250 µl of 0.1M sodium acetate buffer (pH 8.8) and 200 µl of methanol were added, shaken and left for 15 min before 250 µl of 1%v/v of PITC in methanol was added. The solutions were vortexed for 30 s, and then heated in water bath at 80 °C for 30 min. The sample solutions were cooled down to room temperature. The sample

solutions were filtered through a PTFE syringe filter (0.45μm) before HPLC analysis. The HPLC condition was used as follows

Column : Reversed phase C18 column (Vertical, 250 × 4.6 mm)

Mobile phase : Acetronitrile : water : phosphoric acid (10:90:0.01 by vol.)

Flow rate : 1.5 ml/min

Detector : UV-visible detector at wavelength 245 nm

Injection volume: 100 μl

Run time : 10 min

Retention time: 5.5 min

The concentration of glucosamine hydrochloride, GH was determined based on a calibration curve of GH derivative in ultra-pure water (see Appendix B).

3.2.4 Release study of GH from particles

GH release study from particles was performed using the dialysis bag diffusion technique. Five grams of GH-loaded particles, used in hydrated form, was suspended in 10 ml phosphate buffer saline (pH 7.4) and put in a dialysis bag with a molecular weight cut off of 12,400 Da. The bag was sealed and immersed in 200 ml of phosphate buffer pH 7.4 at 37±1°C under stirred condition at 200 rpm. Two milliliters of samples was withdrawn at predetermined time intervals (up to 24 h). An equal volume of fresh buffer was replaced immediately after each sampling in order to keep a constant volume of the buffer in the vessel throughout the experiment.

Each sampling solution was reacted with PITC and filtered through a PTFE syringe filter (0.45μm) before HPLC analysis. The amount of GH released at each time interval was calculated based on the calibration curve. Each sampling of released solution was performed in triplicate.

3.2.5 Formulation of gel containing GH-loaded particles

Three types of gel were prepared by using carbopol 940 as a gel base. There were 1%GH-gel and gel containing GH-loaded particle of GH-ALG-CTS and GH-ALG-46%NBC. The gel containing GH-loaded particle was prepared by dispersing carbopol 940 in water while undergoing continuous stirring. GH-loaded particle was slowly

dispersed to carbopol solution. The mixture was stirred until thicker gel was obtained. After that triethanolamine was added to neutralize until pH reached to 7. The clear gel was obtained. 1%GH-gel was similarly prepared by replacing GH-loaded particle with 1% w/w of GH. The formulations of gel are shown in Table 3.1.

Table 3.1 Formulation of gel preparation

Composition (%w/w)	Formulation		
	1% GH-gel	GH-ALG-CTS gel	GH-ALG-46%NBC gel
Carbopol 940	1	1	1
Triethanolamine	0.7	0.7	0.7
Glucosamine	1	-	-
GH-loaded particles	-	56	58
Water to	100	100	100

3.2.5.1 permeation study of drug through cellulose acetate membrane

Gel containing GH-loaded particle was prepared to study GH content that permeate across artificial membrane for transdermal drug. Franz diffusion cell was used for in vitro drug permeation study (Fig. 3.3a and 3.3b). The cellulose acetate membrane with pore size 0.2 μm, diameter 25 mm, was soaked in buffer medium (pH 7.4) for 12 h before use. The membrane was placed between the donor and receptor chambers. The upper chamber was filled with 1g of gel containing GH-loaded particle. The permeable area of Franz cell is 1.77 cm^2. The receptor chamber contained 14 ml of phosphate buffer (pH7.4) as the receptor fluid. The temperature was controlled at 37±1 ^{o}C by circulation water through a jacket surround the Franz cell body and stirring at 200 rpm. One milliliter of solution in receptor chamber was withdrawn at predetermined time intervals (up to 24 h). Fresh buffer was replaced after each sampling. Each sampling solution was reacted with PITC and filtered through a PTFE syringe filter (0.45μm) before HPLC analysis. The amount of GH released at each time interval was calculated based on the calibration curve. Each sampling of released solution was performed in triplicate.

The cumulative amount of GH permeated across a unit area of the membrane was calculated from the follow equations (6):

$$Q_p = \frac{C_{GH} \times V}{A} \quad (6)$$

where Q_c is the cumulative amount of drug permeated through a unit area of membrane (μg/cm^2), C_{GH} is the concentration of glucosamine hydrochloride permeated in receptor (g/ml), V is the volume of buffer in the receptor chamber (ml). A is the area of cellulose acetate membrane (cm^2).

The cumulative amounts of drug permeated through a unit area of membrane are present in Appendix D.

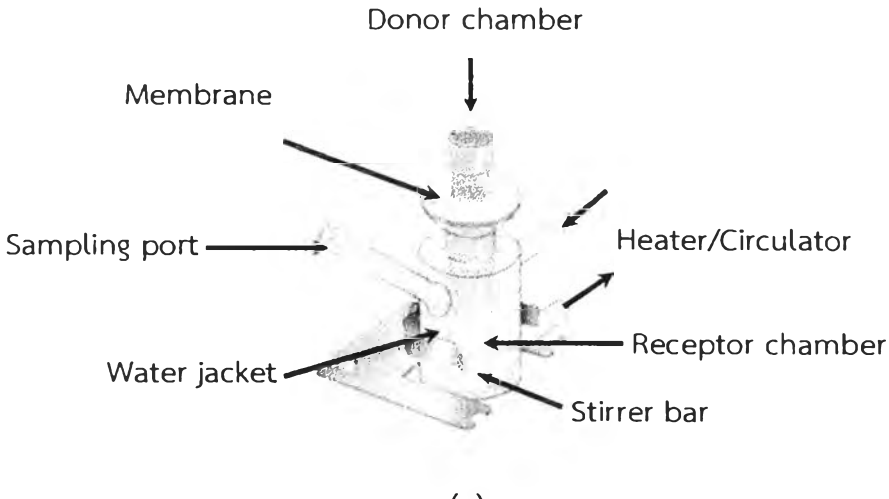
The flux of GH from gel formulation in the receptor compartment obtained from slope of the linear correlation between cumulative amount of drug (Q_p) and time.

3.2.6 Stability test

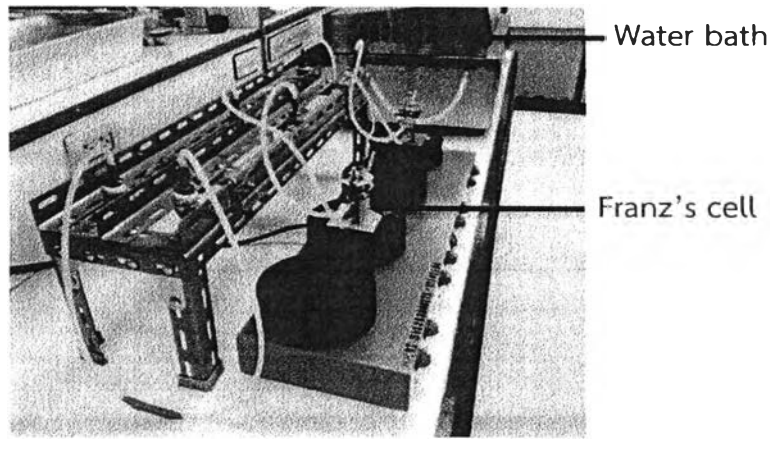
The stability of GH loaded particles was studied in suspension form. The suspension of particles was stored at room temperature for 60 days. The physical properties of suspension was investigated include pH, particle size and zeta potential. In addition, gel formula containing GH-loaded particle was evaluated for stability. Appearance of gel was observed with naked eyes. The pH of gel was measured and GH content in gel was analyzed by HPLC technique after preparation for 30 days.

3.2.7 Statistical analysis

All size and zeta measurements were performed in triplicate. Results are presented as means ± SD. Statistical analysis was performed by one-way ANOVA using Microsoft Excel (Microsoft Corporation) with $P < 0.05$ to indicate statistical significance.



(a)



(b)

Figure 3.3 The components of Franz diffusion cell cell [23] (a) and instrument setup for permeation study (b)