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PREPARATION OF GLUCOSAMINE-LOADED ALGINATE-CHITOSAN NANOPARTICLES

Miss Pornphrom Peng-im

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Petrochemistry and Polymer Science
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พรพรหม เพ็งอิ่ม: การเตรียมอนุภาคไมโครแอลจิเนต-ไคโทซานที่บรรจุกลูโคซามีน PREPARATION OF GLUCOSAMINE-LOADED ALGINATE-CHITOSAN NANOPARTICLES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.วราวุฒิ ตั้งพสุธาดล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร.ปราณี เลิศสุทธิวงค์, 66 หน้า.

ในงานวิจัยนี้เป็นการเตรียมอนุภาคขนาดนาโนที่บรรจุกลูโคซามีนจากการเกิดไอโอโนโทรปิคเจลเลชั่นของแคลเซียมคลอไรด์ แอลจิเนต และไคโทซานหรืออนุพันธ์ที่มีหมู่แอมโมเนียม ซึ่งมีประจุบวกถาวร 2 ชนิดคือ เอ็น,เอ็น,เอ็น-ไทรเมทิลแอมโมเนียมไคโทซานคลอไรด์ (TMC, %DQ=35) และเอ็น-[(2-ไฮดรอกซิล-3-ไทรเมทิลแอมโมเนียม)โพรพิล]ไคโทซานคลอไรด์ (HTCC, %DQ=11, 33) การใช้โซนิเคต 20 นาที เป็นผลให้ขนาดอนุภาคเล็กลง 36.8% อย่างไรก็ตามในงานวิจัยนี้ พบว่าไม่มีความสัมพันธ์ โดยตรงระหว่างขนาดอนุภาคกับการเปลี่ยนน้ำหนักโมเลกุลของไคโทซาน การใช้ HTCC จะให้อนุภาคที่มีขนาดเล็กกว่า การใช้ไคโทซานและ TMC ที่มีความเข้มข้นของสารละลายเท่ากัน เมื่อใช้ HTCC ที่มี DQ สูง (33%) จะให้อนุภาคที่มีขนาดเล็กกว่า HTCC ที่มี DQ ต่ำ(11%) วิเคราะห์หาปริมาณกลูโคซามีนที่ปลดปล่อยจากอนุภาคด้วยเทคนิค HPLC ซึ่งพบว่าปริมาณของกลูโคซามีนที่ถูกปลดปล่อยออกมามีความสัมพันธ์เชิงเส้นกับเวลาในช่วง 360 นาที โดยไม่พบลักษณะการปลดปล่อยอย่างกระทันหันจากอนุภาคที่เตรียมขึ้นนี้ และลักษณะของอนุภาคเป็นแบบมีชั้นหุ้มแกนกลางดังผลวิเคราะห์โดยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5072376223: MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
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QUATERNARYAMMONIUM CHITOSAN/IONOTROPIC GELATION

PORNPHROM PENG-IM: PREPARATION OF GLUCOSAMINE-LOADED ALGINATE-CHITOSAN NANOPARTICLES. ADVISOR: ASST. PROF.VARAWUT TANGPASUTHADOL, Ph.D, CO-ADVISOR: PRANEE LERTSUTTHIWONG, Ph.D, 66 pp.

Glucosamine-loaded nanoparticles were prepared based on ionotropic gelation method using calcium ion, alginate, and chitosan or two chitosan derivatives that carried permanent positively charged ammonium groups. The two derivatives synthesized in this work were *N,N,N*-trimethylammonium chitosan chloride (TMC) with degree of quaternization (DQ) of 35% and *N*-[(2-hydroxyl-3-trimethylammo-nium)propyl]chitosan chloride (HTCC) with DQ of 11 and 33%. Appling the sonication time up to 20 min caused the reduction of particle size by 36.8%. In this work however no direct relationship between the molecular weight of chitosan and the particles size was observed. At the same concentration of cationic polymers, the use of HTCC tended to give smaller particles size than did the chitosan or TMC. The HTCC with high DQ (33%) when incorporated with alginate gave smaller particle size than the one with lower DQ (11%). The amount of glucosamine released was analyzed using HPLC technique. The release profiles of all particle types with show linear relationship between the cumulative amount of GH release and time in the course of 360 min. No 'burst' effect was also observed in these particle systems. The particle had a core-shell feature as characterized by TEM

Field of Study : Petrochemistry and Polymer Science Student's Signature : .....
Academic Year : 2010 Advisor's Signature : .....
Co-Advisor's Signature : .....

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LIST OF ABBREVIATIONS

ACP	: Ca^{2+}-alginate-chitosan particle
ATP	: Ca^{2+}-alginate-TMC particle
AHP	: Ca^{2+}-alginate-HTCC particle
CTS	: chitosan
ALG	: alginate
GH	: glucosamine hydrochloride
DD	: degree of deacetylation
DQ	: degree of quaternization
EE	: encapsulation efficiency
LC	: loading capacity
GTMAC	: glycidyltrimethylammonium chloride
CH_3I	: Iodomethane
TMC	: N,N,N-trimethylammonium chitosan chloride
HTCC	: N-[(2-hydroxyl-3-trimethylammonium)propyl] chitosan chloride
NMR	: Nuclear magnetic resonance spectroscopy
conc.	: concentration
kDa	: kilo dalton
h	: hour
min	: minute
µg	: microgram
mg	: milligram
mg/mL	: milligram/milliliter
mL/h	: milliliter per hour
mL	: milliliter
MW	: molecular weight
nm	: nanometer
%	: percentage

pH	:	power of hydrogen ion or the negative logarithm (base ten)
rpm	:	round per minute
SEM	:	scanning electron microscopy
TEM	:	transmission electron microscopy
SD	:	standard deviation
t	:	time
v/v	:	volume/volume
w/w	:	weight/weight
w/v	:	weight/volume

CHAPTER I

INTRODUCTION

1.1 Statement of problem

Glucosamine is a natural component of glycoproteins found in connective tissues derived from chitin. Glucosamine is in fact the monomer of chitosan. Recently, glucosamine has been used for the treatment of osteoarthritis, the most common type of joint disease in humans. It can be injected, applied on skin, or eaten as dietary supplement at a regular interval [1].

A novel technique in delivering drug that has been used utilizing micro- or nano-particles as a delivery vehicle. It is quite surprising that no specific report on the controlled release of glucosamine from particles was found. Thus the focus of this work was to investigate a controlled release system for glucosamine from particles with sizes in the range of nano to micro scales.

The major components for preparing the particles in this work were a negatively-charged polymer, alginate, and a positively-charged counterpart, chitosan. The particle formation was based on a principle of ionic complexation of the opposite charges polymers. This method is known as polyelectrolyte complexes or ionotropic gelation. Many reports showed successful particle preparation in the range of 500-1,500 nm in diameter [2-4]. In fact various methods for preparing particles have been reported, such as coacervation/precipitation, spray-drying and solvent emulsification/internal gelation [5]. But these usually resulted in the range of 1,000-5,000 nm with broad size distribution.

In this research, ionotropic gelation technique was used to prepare Ca^{2+}-alginate-chitosan particles. Two other chitosan derivatives carrying 'permanent' positive charges; *N,N,N*-trimethylammonium chitosan chloride (TMC) and *N,N,N*-[(2-hydroxyl-3-trimethylammonium)propyl]-chitosan chloride (HTCC), were also included in the study. The sizes of Ca^{2+}-alginate-chitosan or its derivatives were optimized by varying sonication time and the characteristics of chitosan (molecular weight, degree of deacetylation and

concentration of polymer solution) with the goal to achieve the smallest particles size. Loading of glucosamine into the particles was then investigated with the aim to obtain highest loading content. Finally the release of glucosamine for the prepared particles was evaluated in phosphate buffer pH 7.4 in order to find a suitable particle system for future medical application.

1.2 Objectives

The aim of this work was to create slow glucosamine delivery system from nanoparticles generated by ionic pre-gelation of Ca^{2+}, alginate, and chitosan or its derivatives.

1.3 Scope of work

1.3.1 Literature review of related works

1.3.2 Characterization of the chitosan including molecular weight, degree of moisture, and degree of deacetylation

1.3.3 Synthesis and characterization of two positively-charged derivatives of chitosan having different degrees of quaternization; TMC and HTCC

1.3.4 Preparation of blank and glucosamine-loaded particles from alginate-chitosan, alginate-TMC, and alginate-HTCC, with parameters including time of sonication, types of chitosan (molecular weight and degree of deacetylation), and concentration of chitosan or its derivative

1.3.5 Characterization of particles in terms of particle size, zeta potential, morphology, and chemical analysis

1.3.6 Evaluation of drug encapsulation efficiency and loading capacity

1.3.7 *In vitro* glucosamine release study from the prepared particles in phosphate buffer

1.3.8 Report writing

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Alginate

Sodium alginate, an anionic polymer carrying carboxylate groups, was extracted from marine brown algae. It is composed of linear chains of α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues joined by 1,4-glycosidic linkages (Figure 2.1). Water-soluble alginate can become insoluble gel with divalent ions such as calcium, strontium, or barium. However, calcium is mostly used because calcium is not harmful to humans.

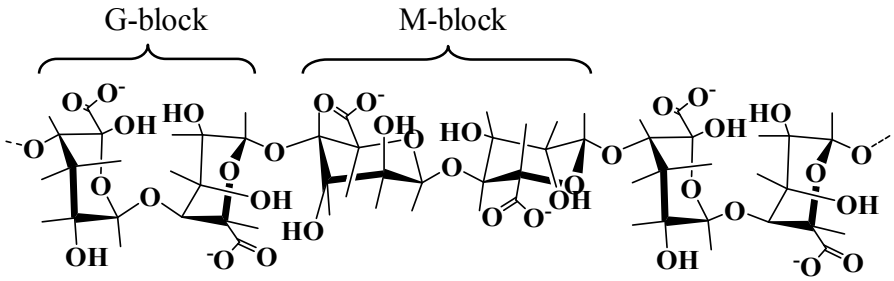


Figure 2.1 Copolymer of α-L-guluronic acid and β-D-mannuronic acid in alginate

2.2 Chitosan

Chitosan is a cationic polymer, formed by the *N*-deacetylation of chitin, which is a polymer found in crustracean shells. Chitosan is a linear copolymer that consists of D-glucosamine and *N*-acetyl-D-glucosamine units joined by β-(1-4)-glycosidic linkages. The main commercial sources of chitin are the shell wastes of shrimp, crab, lobster, krill, and squid. Molecular structure of chitin and chitosan, similar to cellulose, are long linear chain molecules of (1-4) linked glycans as shown in Figure 2.2. The amount of glucosamine unit in chitosan is generally referred to the percentage degree of deacetylation or %DD, which influencing its physical, chemical properties as well as biological activities. Various techniques can be used for determination of %*DD* such as IR, [6-7] NMR, [8] colloidal titration method, [9] UV Spectrophotometry, [10] and pH-potentiometric titration [11].

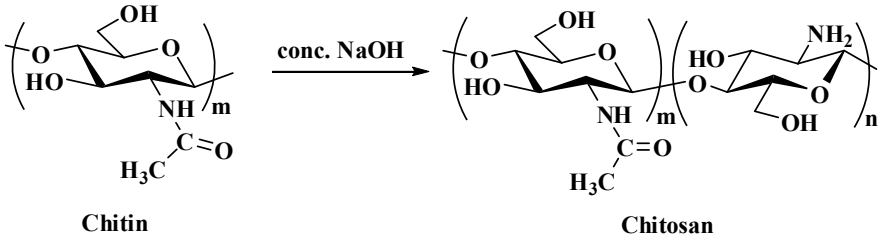


Figure 2.2 Structure of chitin and chitosan

Chitosan has a number of unique properties including antimicrobial activity, non-toxicity, and biodegradability. These attract scientific and industrial interest in such fields as cosmetics, biotechnology, pharmaceutics, wastewater treatment, agriculture, food science, and textiles. Other properties include adsorption properties and anti-microbial properties. It is, therefore, considered to be suitable for application in pharmaceutical technology [12].

Chitosan dissolves in only dilute organic acids. Organic acids such as acetic, formic and lactic acids are used for dissolving chitosan. Stability of chitosan solution is poor above pH 7 or basic condition cause gelation or precipitation. Hence the application of chitosan is limited owing to the insolubility in neutral or high pH region. Due to its reactive amino and hydroxyl groups, chemical modification of chitosan to achieve its derivatives is used to expand its application.

2.2.1 *N,N,N*-trimethylammonium chitosan chloride (TMC)

Chitosan has both reactive amino and hydroxyl groups, which can react as versatile functional groups for chemical modification under mild reaction conditions. The following related research publications have been reported on chemical modification of chitosan especially at the amino and/or hydroxyl groups to produce charged derivatives. Methylation at the amino groups was explored by many researchers [13-15]. The resulting product is *N,N,N*-trimethylammonium chitosan chloride (TMC) in Figure 2.3.

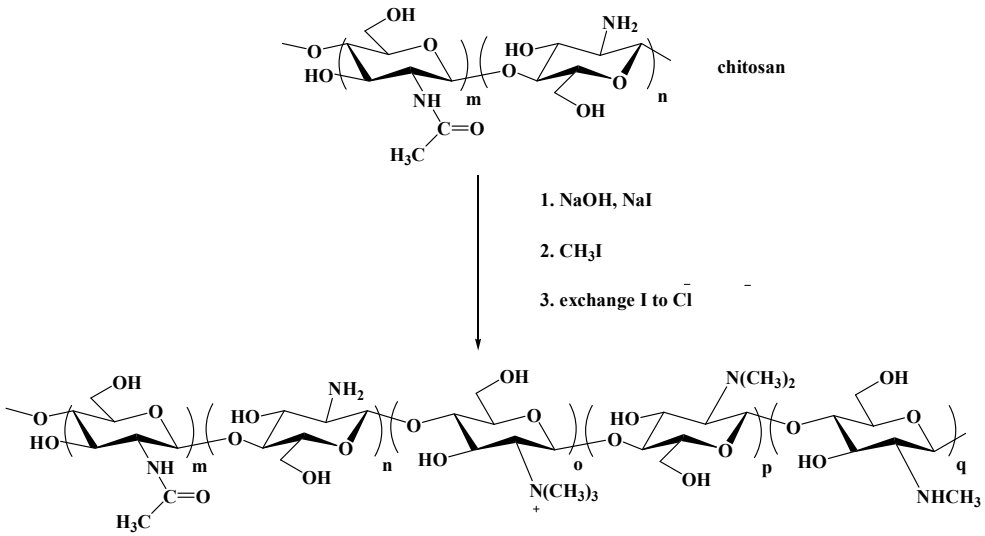


Figure 2.3 Synthesis of *N,N,N*-trimethylammonium chitosan chloride (TMC) from chitosan.

In 1998, Sieval [11] and co-workers synthesized TMC and studied their solubility comparing to the native chitosan. The product yield and degree of quaternization (%DQ) could be controlled by means of the number of methylation steps, the duration of each reaction step and the amount of methyl iodide. A two-step reaction gave products with high degrees of quaternization (40-80%). A three-step reaction procedure yielded products with a higher degree of quaternization > 80%, but with substantially decreased water solubility. This was because it also resulted in methylation at the hydroxyl group (O-methylation), which decreased the number of −OH groups along the chitosan chain, resulting in the decrease of solubility.

2.2.2 *N*-[(2-hydroxyl-3-trimethylammonium)propyl]-chitosan chloride (HTCC)

The HTCC, positively charged chitosan, was prepared by grafting glycidyltrimethyl ammonium chloride (GTMAC), a molecule carrying an ammonium group, on the chitosan chain via epoxide ring opening by the amino groups of chitosan.

Seong [16] and co-workers synthesized N-(2-hydroxyl)propyl-3-trimethyl ammonium chitosan chloride (HTCC), using a reaction of GTMAC (Figure 2.4) and chitosan. The complete substitution of the amino group in chitosan with GTMAC was

achieved when the reaction was performed at 80°C for 18 h with a 4:1 mole ratio of GTMAC to $-NH_2$ in the presence of acetic acid. HTCC showed superior antimicrobial activity to chitosan due to the quaternary ammonium group in the polymer structure. They were applied to cotton fabrics.

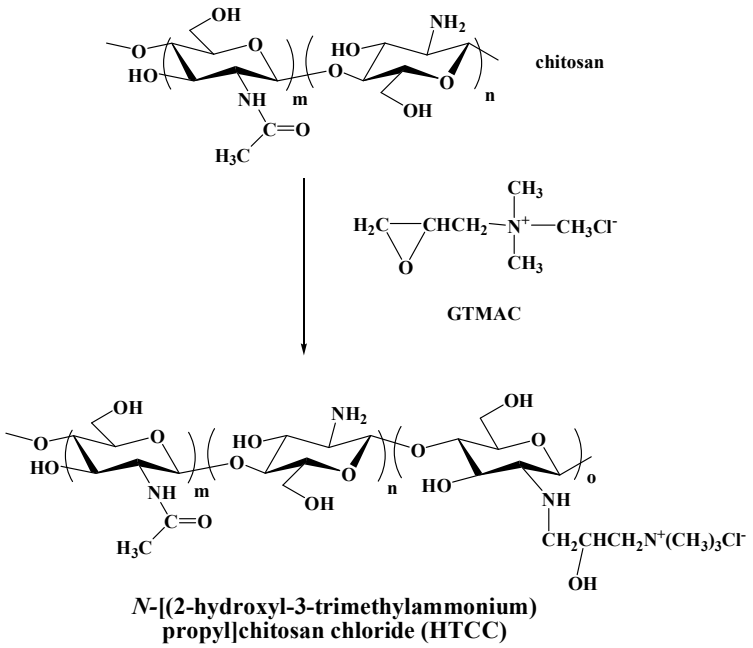


Figure 2.4 Synthesis of *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTCC) from chitosan.

2.2.3 Glucosamine hydrochloride

Glucosamine, an amino monosaccharide, is a natural component of glycoproteins found in connective tissues derived from chitin. It is a precursor of glycosaminoglycans (GAGs), disaccharide unit, which are the building blocks of the articular cartilage [17]. Recently, glucosamine was used for the treatment of osteoarthritis in humans. It can be injected or eaten as dietary supplement at a regular interval. Accordingly, glucosamine is commercially available as a nutritional supplement in the form of glucosamine hydrochloride (GH), glucosamine sulphate, and *N*-acetyl-glucosamine [18]. Most of the clinical studies examined the effect of glucosamine in the forms of hydrochloride or sulfate on osteoarthritis.

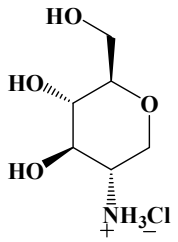


Figure 2.5 Structure of glucosamine hydrochloride ($C_6H_{13}NO_5$)

Specification of glucosamine hydrochloride is as follows:

- IUPAC name: (3R,4R,5S,6R)-3-Amino-6-(hydroxymethyl)oxane-2,4,5-triol
- Other name: 2-Amino-2deoxy-D-glucose chitosamine
- Formula: $C_6H_{13}NO_5$
- Physical state and appearance: Solid (Powdered solid)
- Odor: Odorless
- Molecular Weight: 179.17 g/mol
- Color: White
- Solubility: very soluble in water, insoluble in alcohol
- Melting Point: 105°C, 423 K, 302°F

Pharmacokinetic data:

- Bioavailability: 1,500 mg/day
- Route: Oral, topical
- Side Effects: heartburn, drowsiness, skin rashes, headache, insomnia, and mild and temporary digestive complaints such as abdominal pain, poor appetite, nausea, heartburn, constipation, diarrhea, and vomiting

2.3 Controlled release system

The means by which a drug is introduced into the body is almost as important as the drug itself. Drug concentration at the site of action must be maintained at a level that provides maximum therapeutic benefit and minimum toxicity. The pharmaceutical developer must also consider how to transport the drug to the appropriate part of the body and, once there, make it available for use [19].

Controlled drug delivery occurs when a polymer is combined with the drug or other active agents in such a way that the active agent is released from the material in a predesigned manner. The drug can be released from the system by 3 mechanisms.

2.3.1 Diffusion Controlled Release

Diffusion occurs when drug molecules pass from the polymer matrix to the external environment. As the release continues, its rate normally decreases with this type of system, since drug has progressively longer distance to travel and therefore requires a longer diffusion time to release.

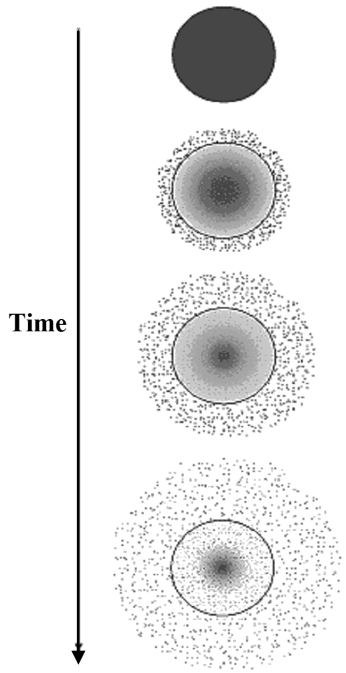


Figure 2.6 Presentation of diffusion controlled release [20]

2.3.2 Swelling Controlled Release

The swelling of the carrier increases the aqueous solvent content within the polymer matrix, enabling the drug to diffuse through the swollen network into the external environment. Most of materials used are based on hydrogel. The swelling can be triggered by a change in the environment surrounding such as pH, temperature, ionic strength, etc.

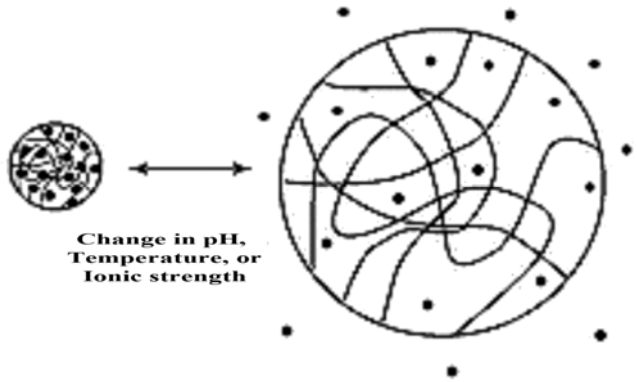


Figure 2.7 Presentation of swelling controlled release [20]

2.3.3 Erosion Controlled Release

The drug can be released from the matrix due to erosion of polymers, which can be classified into 2 types.

Bulk erosion: The polymer degrades in a fairly uniform manner throughout the polymer matrix.

Surface erosion: The degradation occurs only at the surface of the polymer device.

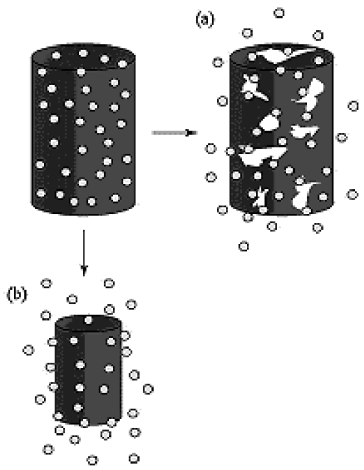


Figure 2.8 Presentation of erosion controlled release-(a) bulk erosion and (b) surface erosion [20]

2.4 Literature review on micro/nanoparticle preparation for controlled drug release

De and Robinson [21] described the preparation of chitosan–alginate nanospheres and made some comparison to poly-L-lysine–alginate system. The mass ratio range of sodium alginate:$CaCl_2$:cationic polymer (poly-L-lysine or chitosan) to prepare the nanospheres was 100:17:10. This mass ratio ensured that the calcium alginate was maintained in the pre-gel phase and sufficient cationic polymer was present to form nanospheres. At low cationic polymer concentrations, nanospheres were not formed, whereas microspheres were formed at higher concentrations. The release of entrapped methylene blue from the 2 nanospheres was directly proportional to the sodium chloride concentration in the dissolution medium.

Pan et al. [22] prepared the insulin-loaded chitosan nanoparticles by ionotropic gelation of chitosan with tripolyphosphate anions. Particle size distribution and zeta potential were determined by photon correlation spectroscopy. The ability of chitosan nanoparticles to enhance the intestinal absorption of insulin and the relative pharmacological bioavailability of insulin was investigated after the oral administration of various doses of insulin-loaded chitosan nanoparticles to rats. The positively charged, stable chitosan nanoparticles showed particle size in the range of 250–400 nm. Insulin association was up to 80%. The *in vitro* release experiments indicated initial burst effect, which is pH-sensitive. The chitosan nanoparticles enhanced the intestinal

absorption of insulin to a greater extent than the aqueous solution of CS in vivo. After administration of 21 I.U./kg insulin in the chitosan nanoparticles, hypoglycemia was prolonged over 15h.

Grenha et al. [23] prepared insulin loaded chitosan nanoparticles by ionotropic gelation method using tripolyphosphate as crosslink agent. Chitosan with degree of deacetylation of 86 was used in this study. The results indicated that particle size was ranging from 300 to 390 nm and lidocaine was released upto 75-80% within 15 min.

Lertsutthiwong et al. [24] prepared turmeric oil encaped with an alginate biopolymer coating. The alginate nanocapsules were prepared in three-step procedure using emulsification, crosslinking with calcium chloride, and solvent removal The type of solvent, concentration of turmeric oil, sonication, and oil/alginate mass ratio affected the characteristics of the nanocapsules in terms of average size, zeta potential, morphology, loading capacity, and stability at 4 ºC and 25 ºC. An increase in the oil concentration or oil/alginate mass ratio resulted in an increase in the average size of the nanocapsules.

Zhang et al. [25] prepared alginate–chitosan microspheres with narrow size distribution by membrane emulsification technique, a relatively novel technique producing all types of single and multiple emulsions, in combination with ion (Ca^{2+}) and polymer (chitosan) solidification. The preparation procedure was observed, and the physical properties (particle size distribution, surface morphology, chitosan distribution, zeta potential) of the microspheres were characterized. Subsequently, the microspheres were employed to load insulin, a model peptide. The effect of loading methods on loading efficiency and immunological activity of insulin were investigated. It was shown that the higher loading efficiency (56.7%) and remarkable activity maintenance (99.4%) were obtained when the insulin was loaded during the chitosan solidification process. Afterward, the release profile *in vitro* for the optimal insulin-loaded microspheres was investigated. Under the pH conditions of gastrointestinal environment, only 32% of insulin released during the simulated transit time of drug (2 h in the stomach and 4 h in the intestinal). While under the pH condition of blood environment, insulin release was stable and sustained for a long time (14 days). Furthermore, the chemical stability of insulin released from the microspheres was well preserved after they were treated with the simulated gastric fluid containing pepsin for 2

h. Therefore, the alginate–chitosan microspheres were found to be promising vectors showing a good efficiency in oral administration of protein or peptide drugs.

Gazori et al. [26] prepared nanoparticles comprising alginate/chitosan polymers by pregel preparation method through drop-wise addition of various concentrations of $CaCl_2$ to a defined concentration of sodium alginate. Then, chitosan/antisense solution with a certain N/P ratio was added to the pregel to make the nanoparticles. The effect of such parameters as polymer ratio, $CaCl_2$/alginate ratio and N/P ratio on the particle size distribution and loading efficacy was studied. The optimum conditions were 1:1 (w/w) Alginate to Chitosan ratio, 0.2% $CaCl_2$/Alginate ratio and N/P ratio of 5 at pH 5.3. The resulting nanoparticles had a loading efficacy of 95.6% and average size of 194 nm as confirmed by Photon correlation spectroscopy method. SEM images showed spherical and smooth particles. The zeta potential of optimized nanoparticles prepared by this method was about +30 mV which could result in good stability of nanoparticles during manipulation and storage.

Sæther et al. [4] formed polyelectrolyte complexes (PECs) of alginate and chitosan by addition of 0.1% alginate solution (pH 6.5) to 0.1% chitosan solution (pH 4.0), and by adding the chitosan solution to the alginate solution under high shearing conditions. Variations in the properties of the polymers and the preparation procedure were studied, and the resultant PEC size, zeta potential (Zp), and pH were determined using dynamic light scattering, electrophoresis and by measuring turbidity and pH. The particle size was decreased as the speed and diameter of the dispersing element of the homogenizer was increased. The net charge ratio between chitosan and alginate, and the molecular weights (MW) of both the alginate and chitosan samples were the most significant parameters that influenced the particle size, Zp, and pH. The properties of the PEC could be affected according to the molecular properties of the polyelectrolytes selected and the preparation procedures used. The resultant PEC sizes and properties of the complex were rationalized using a core-shell model for the structure of the complexes

Mao et al. [27] prepared chitosan–DNA nanoparticles using the complex coacervation technique. Important parameters such as concentrations of DNA, chitosan, sodium sulfate, temperature, pH of the buffer and molecular weights of chitosan and DNA have been investigated. At the amino to phosphate group ratio between 3 and 8 and chitosan concentration of 100 μg/mL, the particle size was optimized to100–250

nm with a narrow distribution. Surface charge of these particles was slightly positive with a zeta potential of 112 to 118 mV at pH lower than 6.0, and became nearly neutral at pH 7.2. The chitosan–DNA nanoparticles could partially protect the encapsulated plasmid DNA from nuclease degradation.

Chen et al. [28] prepared *N*-Trimethyl chitosan chloride (TMC) nanoparticles by ionic crosslinking of TMC with tripolyphosphate (TPP). Two model proteins with different pI values, bovine serum albumin (BSA, pI = 4.8) and bovine hemoglobin (BHb, pI = 6.8), were used to investigate the loading and release features of the TMC nanoparticles. TMC samples with different degrees of quaternization were synthesized to evaluate its influence on the physicochemical properties and release profiles of the nanoparticles. Sodium alginate was used to modify the TMC nanoparticles to reduce burst release. As for the alginate-modified nanoparticles, a smaller size and lower zeta potential were observed and the burst release of BSA was reduced. These studies demonstrated that TMC nanoparticles are potential protein carriers, and that their physicochemical properties and release profile could be optimized by means of various modifications.

Cafaggi et al. [29] prepared the nanoparticles with a negative or positive surface charge through electrostatic interaction of an anionic cisplatin–alginate complex with a cationic polyelectrolyte, namely chitosan or *N*-trimethyl chitosan (substitution degree of 85%). Statistical experimental design allowed the study of the influence of component amounts on the characteristics of nanoparticles. Mean particle diameter ranged from 180 nm to 350 nm. Drug loading of nanoparticles with a positive zeta potential (43mV–60mV) ranged from 13% w/w to 21% w/w and particle yield, referred to total polymers, was about 15% w/w (50% w/w if referred to cisplatin–alginate complex). Nanoparticles with a negative zeta potential (−34mV) were obtained with a yield of 40%w/w and a drug loading of 18%w/w. These results indicate that cisplatin complexes with polycarboxylate polymers can be transformed into cisplatin particulate carriers of high potential interest.

Xu et al. [30] synthesized *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC), a water-soluble derivative of chitosan (CS), by the reaction between GTMAC and CS. HTCC nanoparticles have been formed based on ionic gelation process of HTCC and sodium tripolyphosphate (TPP). Bovine serum albumin (BSA), as a model protein drug, was incorporated into the HTCC nanoparticles. HTCC

nanoparticles were 110–180 nm in size, and their encapsulation efficiency was up to 90%. In vitro release studies showed a burst effect and a slow and continuous release followed. Encapsulation efficiency was obviously increased with increase of initial BSA concentration. Increasing TPP concentration from 0.5 to 0.7 mg/ml promoted encapsulation efficiency from 46.7% to 90%, and delayed release for up to 6 days.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental Design

This work was divided into three parts. The first part was to prepare the nanoparticles of Ca^{2+}-alginate chitosan, loaded with GH by ionotropic pregelation method. Beside chitosan, two other positively charged chitosan derivatives were also investigated for their particles formation. The second part was to characterize the prepared particles for their size, zeta potential, and glucosamine loading content. The last part was to study the release profile of glucosamine hydrochloride from the nanoparticles in pH 7.4 buffer solution.

3.2 Polymers and chemicals

Glucosamine hudrochloride (GH) and sodium alginate with molecular weight of 120 kDa and guluronic acid fraction (F_G) of 0.39 were purchased from Sigma Chemicals, St Louis, MO, USA. Chitosan with molecular weight ($\overline{M_w}$) of 180 kDa and 300 kDa and % degree of deacetylation (DD) of 90% were bought from Seafresh Chitosan, Co., Ltd Thailand. Chitosans with $\overline{M_w}$ of 71, 220 and 583 kDa and DD of 85, 87, and 85%, respectively were donated from Center of Chitin-Chitosan Biomaterial Chulalongkorn University. Cellulose dialysis membrane with molecular weight cut-off at 12,400 Da (avg. diameter 21 mm; avg. flat width 33 mm) purchased from Sigma Chemicals, St Louis, MO, USA, was used to in the release study, and cellulose membrane dialysis tubing with molecular weight cut-off at 12,400 Da (avg. diameter 49 mm; avg. flat width 76 mm) purchased from Sigma Chemicals, St Louis, MO, USA was used to purify all modified chitosans.

Calcium chloride, $CaCl_2$ (Carlo); phenylisothiocyanate, PITC (Acros Organics); glucosamine hydrochloride, GH (Aldrich); sodium dihydrogen phosphate, NaH_2PO_4 (Merck); sodium phosphate, Na_2HPO_4 (Merck); paracetamol (Aldrich); iodomethane, CH_3I (Riedel-de Haen); glycidyltrimethylammonium chloride, GTMAC (Fluka); *N*-methyl-2-pyroridone, NMP (Merck); acetic acid, glacial (Merck); acetone, analytical

grade (Merck); sodium chloride, NaCl (Merck); sodium hydroxide, NaOH (Merck); sodium iodide, NaI (Aldrich); sodium sulfide, Na_2S (Merck); sulfuric acid, conc. (Merck) were used as received.

Methanol, MeOH (Carlo); ortho-phospholic acid, H_3PO_4 (Carlo); phosphotungstic acid, PITC (Acros Organic); tri-sodium citrate, $C_6H_5Na_3O_7*2H_2O$ and sodium acetate, CH_3COONa (Carlo) were used in HPLC technique.

Deuterated oxide, D_2O (Merck); trifluoroacetic acid, TFA (Fluka); deuterated methanol, CD_3OD (Merck) were used in NMR technique.

3.3 Characterization of chitosan samples

3.3.1 Molecular weight of chitosan

The molecular weight of chitosan was determined by using GPC (Gel Permeation Chromatography, Water 600E) at National Metal and Materials Technology Center. Ultrahydrogel Column (molecular weight resolving range = 1,000-20,000,000 Da) was used. Solvent was consisted of 0.5 M sodium acetate and 0.5 M acetic acid in water (acetate buffer). This solvent was filtered through nylon 66 membrane with a pore size of 0.45 μm before use. Pullulans (molecular weight, 5,900-788,000 Da) were used as standards. The analyzed sample was prepared by dissolving 0.2% w/v of chitosan in the solvent and shaken at 100 rpm for 24 h. It was then filtered with a 0.45 μm nylon 66 membrane before injection (20 μL). The flow rate of system set was at 0.6 mL per min and temperature of the column was 30°C.

3.3.2 Degree of moisture

Chitosans about 1 g were incubated at 105 ºC for 24 h in incubated crucible. The samples were left at room temperature in a desicator. The degree of moisture was determined using the following equation :

$$Degree\ of\ moisture = \frac{(B-C)}{(B-A)} \times 100$$

where :

A is weight of incubated crucible at 105°C for 2 h for removed the moisture

B is weight of chitosan and crucible before incubation

C is weight of chitosan and crucible after incubation at 105ºC for 24 h

3.3.3 Degree of deacetylation

Degree of deacetylation is defined as the percentage of glucosamine residues in the chitosan chain. The DD was calculated based on UV absorption data obtained from UV-spectrophotometer SPECORD S100. First derivative UV spectrophotometric method reported by Muzzarelli et al., 1985 [31] was used determination of the DD of chitosan. The DD was determined as follows :

$$\%DD = \left[1 - \left[\frac{A}{\frac{((10\times W)-204A)+161A}{161}}\right]\right] \times 100$$

where :

A is the concentration of N-acetyl-D-glucosamine (g/L); calculated from relative equation of a predetermined calibration curve

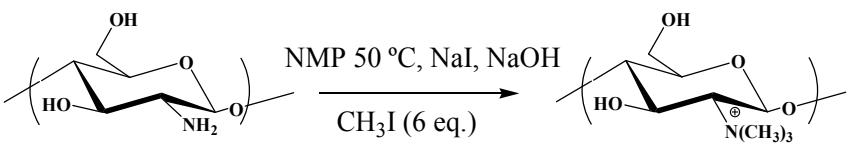
204 is the molecular weight of N-acetyl-D-glucosamine unit

161 is the molecular weight of D-glucosamine

W is the sample weight (g) in 100 mL of 0.01 M of acetic acid.

3.4 Synthesis of chitosan derivatives

3.4.1 N,N,N-trimethylammonium chitosan chloride (TMC)

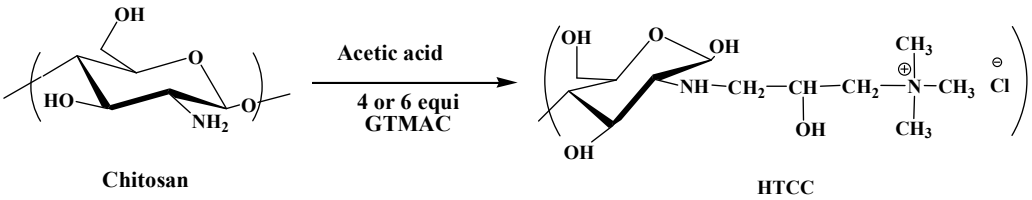


Chitosan TMC

Figure 3.1 Synthesis of TMC

TMCs were synthesized by methylation of chitosan with iodomethane in the presence of sodium hydroxide as described by Sieval et al., 2003. About two grams of chitosan ($\overline{M_w}$ =220 kDa, 87%DD) was swollen by NMP at 50 ºC for 18 h. Then a mixed solution of sodium hydroxide (2 equivalents, ~0.89 g) and sodium iodide (1 equivalent, ~ 1.7 g) were added into the swollen chitosan. After that an excess amount (6 quivalent, ~4.0 mL) of methyl iodide was added in two equal portions (2.0 mL each) at 6 h interval at 50°C. The mixture was stirred at 50°C for 18 h. After methylation, the product was precipitated in acetone and centrifuged at 3000 rpm for 30 min. The resulting crude product was then dissolved in sodium chloride solution about 5 h. Finally, the product was purified using dialysis bag (avg. flat width 32 mm, MWCO 12400) in deionized water for 3 days and freeze-dried before use.

3.4.2 N-[(2-hydroxyl-3-trimethyl-ammonium)propyl]-chitosan chloride (HTCC)



Chitosan HTCC

Figure 3.2 Synthesis of HTCC

Two percent weight by volume of chitosan solutions (M_w=220 kDa 90%DD) in 1%v/v acetic acid were prepared. Then 4 or 6 equivalent of glycidyl-trimethyl-ammonium chloride (GTMAC) was added into chitosan solution. Product was purified by dialysis method using a dialysis bag in deionized water for 3 days and freeze-dried before use.

3.4.3 Characterization of chitosan derivatives

3.4.3.1 Degree of quaternization

The Degree of quaternization (%DQ) or the percentage of quaternary ammonium group of chitosan derivatives were determined by ^{1}H nuclear magnetic resonance (NMR; Varian, USA) spectrometer. Results were reported by chemical shift (δ) comparing with tetramethylsilane (TMS). The chitosan, TMC, or HTCC sample (~10 mg) was dissolved in D_2O/trifluoro acetic acid 1 mL. All measurements were performed at 300K, using pulse accumulations of 64 scans. The calculation was as follows :

$$Degree\ of\ quaternization\ (\%DQ) \quad = \quad \left[\frac{\int N^{+}(CH_3)_3 / 9}{\left(\int H-2',3,4,5,6,6' / 6 \right) \times DD} \right] \times 100$$

where :

$\int N^{+}(CH_3)_3$ is the total peak area of the 9 H′s on the three methyl groups attached to the quaternary ammonium atom (δ=3.00).

$\int H-2',3,4,5,6,6'$ is the total peak area corresponding to the 6 protons at position 2′,3,4,5,6 and 6′ of the glucopyranose ring from 3.50 to 4.40 ppm (for TMC in the D_2O/THF solvent) and from 3.30 to 4.40 ppm (for HTCC in the D_2O solvent).

The DD of chitosan determined by UV-spectrophotometer was 0.87.

3.4.3.2 Solubility tests of chitosan derivatives

Solubility was monitored visually. Solid samples of TMC or HTCCs (100 mg) were dispersed in H_2O (20 mL) according to a method of Sashiwa et al. [32] The pH of the solution was adjusted with 0.5%w/v aqueous HCl and NaOH.

3.5 Particles preparation

3.5.1 Preparation of solution in use

Milli-Q water used in this work was filtered with glass microfiber filters with pore size 0.2 μm. Calcium chloride was dissolved in filtered water. Sodium alginate was dissolved in filtered water at 50ºC. Chitosan solution was prepared by dissolution in 1%v/v acetic acid overnight. TMC was dissolved in 1%v/v acetic acid solution. HTCCs were dissolved in filtered water at neutral pH. All polymeric solution was filtered through filter paper to remove impurities and insoluble polymer before use. No precipitate remained on the filter paper.

3.5.2 Particles preparation

Three methods of particles preparation were investigated. Each one in the order of addition. This was designed so that the effect of Ca^{2+} ion on the particle formation could be investigated.

A. Chitosan-alginate nanoparticles: twenty milliliters of sodium alginate solution (0.6 mg/mL) was added dropwise into 4 mL of chitosan solution (0.3 mg/mL) while stirring at ~1,000 rpm. Then the resultant suspension was left stand overnight at 4ºC to allow the small particles to form and reach an equilibrium.

B. Chitosan-Ca^{2+}-alginate nanoparticles: First Ca^{2+} ions were mixed into sodium alginate solution as follows. Four milliliters of calcium chloride solution was added dropwise into 20 mL of sodium alginate solution (0.6 mg/mL) while stirring at ~1,000 rpm. Then the resulting Ca^{2+}-alginate pre-gel suspension was added dropwise into 4 mL of chitosan solution (0.3 mg/mL) with stirring. The resultant suspension was left stand overnight at 4ºC to allow the small particles to form and reach an equilibrium.

C. Ca^{2+}-alginate-chitosan nanoparticles (ACP) were prepared by ionotropic pre-gelation method modified from Rajaonarivony et al., 1994 [33]. Four milliliter of calcium chloride solution was added dropwise into 20 mL of sodium alginate solution (0.6 mg/mL) while stirring at ~1,000 rpm. The mixed solution was sonicated at various lengths of time in a sonication bath (0-60 min). Then 4 mL of chitosan solution (0.15-0.60 mg/mL of 1%v/v of acetic acid solution) was added dropwise and with stirring for an additional time of 30 min. The resultant suspension was left stand overnight at 4ºC to allow the small particles to form and reach an equilibrium.

Table 3.1 Relationship between concentration of chitosan/derivatives and mass ratio of alginate:$CaCl_2$:cationic polymer

Concentration of chitosan/derivatives (mg/ml)	Mass ratio of alginate:$CaCl_2$:cationic polymer
0.15	10:2.33:0.5
0.30	10:2.33:1.0
0.45	10:2.33:1.5
0.60	10:2.33:2.0

The preparation of Ca^{2+}-alginate-TMC (ATP) and Ca^{2+}-alginate-HTCCs particles (AHP) were carried out by following method C, but chitosan solution was replaced by TMC solution (in 1% acetic acid), and HTCCs solution (in deionized water), respectively.

3.5.3 Preparation of glucosamine-loaded nanoparticles

Two methods for preparing glucosamine (GH)-loaded particles were studied.

In method A, GH (~12 mg) was dissolved in 4 mL of calcium chloride solution. After that the solution of GH/calcium chloride was added dropwise into 20 mL of sodium alginate solution (0.6 mg/mL) with stirring at ~1,000 rpm. The mixed solution was sonicated for 20 min. To the solution, 4 mL of chitosan solution or its derivatives (0.15 mg/mL) was added dropwise with stirring for 30 min. The resultant suspension was left stand overnight at 4ºC to allow small particles to form.

In method B, GH (~12 or ~120 mg) was dissolved in 20 mL of sodium alginate solution. Then 4 mL of calcium chloride solution was added dropwise into the solution of GH/sodium alginate while stirring at ~1,000 rpm. Then the ionic complexation with chitosan was carried out using the same method mention above.

3.6 Characterization of nanoparticles

3.6.1 Particle size analysis

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 (model Nano-ZS, Malvern Instruments, England). The scattered light was collected at an angle of 270º (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.

3.6.2 Zeta potential analysis

Zeta potentials were determined by using zeta sizer (model Nano-ZS, Malvern Instruments, England). In practice, 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.

3.6.3 Morphology analysis by Transmission electron microscopy

Morphological analysis of the prepared particles were 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 $[PW_{12}O_{40}]^{3-}$. Digital Micrograph and Soft Imaging Viewer software were used to perform the image capture and analysis.

3.6.4 Determination of encapsulation efficiency (%EE) and loading capacity (%LC)

Particles were separated from the dispersion by ultracentrifugation (Optima L-100XP) at 45,000 rpm for 1 h. Then the precipitate was washed with Milli-Q water in ultracentrifuge at 45,000 rpm for 30 min after pouring the supernatant.

Two methods for determining encapsulation efficiency and loading capacity were performed.

3.6.4.1 Direct method

The EE of GH-loaded ACP, ATP, and AHP were quantitatively determined by digesting the dried nanoparticles (~1 mg) in 4.5 mL of 0.5 M sodium citrate solution overnight (~12 hr). Then the dissloved GH from digested nanoparticles was derivatized with phenylisothiocyanate (see Derivatization procedure).

Derivatization procedure

In order to detect GH by UV-visible detector, GH was derivatized by reacting the compound with phenylisothiocyanate. Four hundred microliters of *GH* solution were transferred into a glass vial. Then, 250 µl of 0.3M phosphate buffer (pH 8.99) 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, then heated in water bath at 80 ºC for 30 min. The sample solutions were cooled down to room temperature, followed by the addition of 150 µl of 100 µg/mL paracetamol in methanolic solution. The sample solutions were filtered through a PTFE syringe filter (0.45µm) before HPLC analysis. The column used was a reversed phase C18 column (Vertical, 250 × 4.6 mm). The mobile phase are methanol:water:*o*-phosphoric acid at ratio 10:90:0.01 by volume (pH = 3.30) [34]. The flow rate was 1.5 mL/min and the effluent was monitored at 254 nm. All experiments were performed in triplicates.

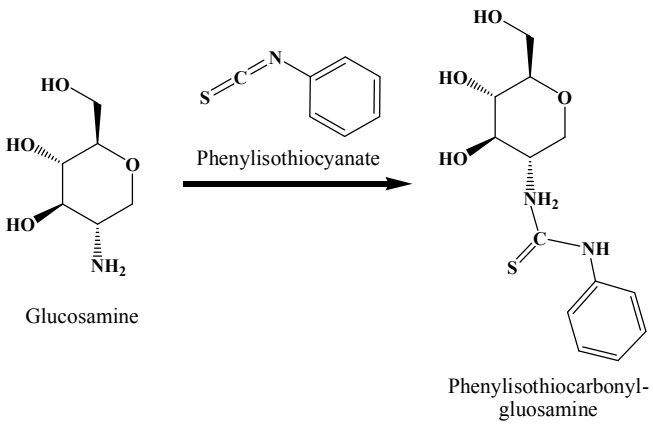


Figure 3.1 Derivatization of glucosamine

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

$$\% \text{ EE} = \frac{\text{GH content in particles as prepared}}{\text{GH content particles as planed}} \text{ x } 100$$

The *GH* loading capacity was calculated from the following equation:

$$\%\,\mathrm{LC} = \frac{\text{GH content in particles from experiment}}{\text{weight of dried particles}}\ \mathrm{x}\,100$$

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

3.6.4.2 Indirect method

The supernatant, aqueous solution separated from the suspension by ultracentrifugation, and aqueous solution from a washing steps were derivatized with phenylisothiocyanate before HPLC analysis.

The *GH* entrapment efficiency was calculated from the following equation:

$$\%\,\mathrm{EE} = \frac{\left(\text{amount of GH added} - \left[\begin{array}{l}\text{amount of GH in supernatant}\\ +\,\text{amount of GH in washed water}\end{array}\right]\right)}{\text{GH content in particles as planed}}\ \mathrm{x}\,100$$

3.6.5 *In vitro* glucosamine hydrochloride release from nanoparticles

The release of *GH* from nanoparticles was performed using the dialysis bag diffusion technique. Ten milligrams of GH-loaded particles were suspended in 10 mL phosphate buffer saline (pH 7.4) and placed in the dialysis bag with a molecular weight cut off of 3500 Da. The bag was sealed and immersed in 100 mL of phosphate buffer pH 7.4 at 37±1°C under stirred condition at 200 rpm. Two milliliter 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.

The amount of GH released was calculated following the equations:

$$\text{Amount of GH released (mg/mg of GH - loaded particles)} = \frac{\text{Concentration of GH (mg/mL)}}{\text{weight of GH - loaded particles (mg)}} \text{ x } 100 \text{ mL}$$

$$\%\,\text{Cumulative release} = \frac{\text{Amount of GH released (mg/mg of GH - loaded particles)}}{\text{Loading content of GH in particles (mg/mg of GH - loaded particles)}} \times 100$$

3.7 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 $P < 0.05$ considered to indicate statistical significance.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter was divided into three parts. The first part was the synthesis of two positively charged derivatives of chitosan; *N,N,N*-trimethylammonium chitosan chloride (TMC) and *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTCC). Both positive charged derivatives and chitosan were used the preparation of nanoparticles designed for controlled release of glucosamine in the second part. The particle preparation formation took place by ionotropic pregelation of the positive charged polymers and alginate, a negative charged polymer. Glucosamine loading methods and characterization of the particles such as size, zeta potential, morphology, and the amount of GH in the particles were investigated. Finally the release profiles of glucosamine from the GH-loaded particles were reported in the last part.

4.1 Synthesis of positive charge chitosan derivatives

4.1.1 *N,N,N*-trimethylammonium chitosan chloride (TMC)

TMC was synthesized by methylation of chitosan using iodomethane (CH_3I) as the main reagent. The theoretical equivalents of CH_3I per one amino group of chitosan is 3 in order to obtain the trimethyl derivative. From the previous study by Kerdcholpetch et al. in 2008 [35], the change in equivalent of CH_3I from 4 to 12 did not cause any change of degree of quaternization (DQ). Based on the lowest chemical costs and toxicity, TMC was synthesized in this work by using 6 equivalents of CH_3I. The %DQ of TMC was determined by ^1H NMR from relative ratios of the peak area of the signal at 3.28 ppm, assigned to three methyl groups adjacent to the quaternary nitrogen atom, and the sum of integral signal of H at the position 2′, 3, 4, 5, 6, and 6′ of pyranose ring of chitosan (δ 3.50-4.40 ppm) (Figure 4.1 and Table 4.1).

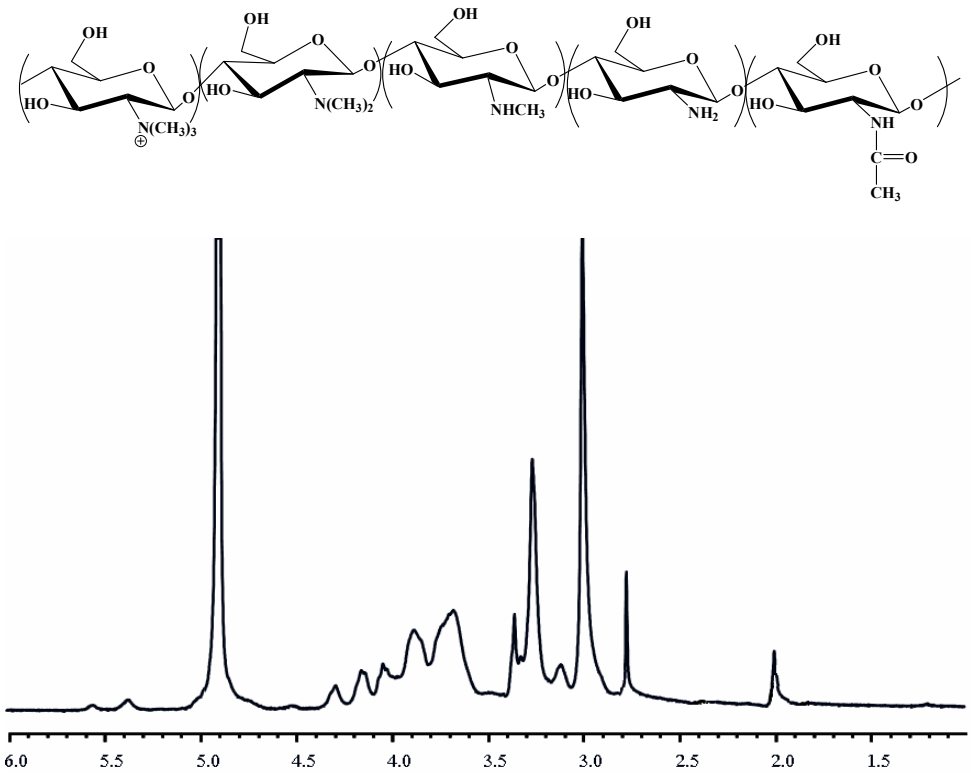


Figure 4.1 ^{1}H NMR spectrum of TMC synthesized by reacting chitosan with 6 equivalents of CH_3I (solvent: D_2O/TFA).

Table 4. 1 Chemical shift (δ) of TMC

Chemical shift (δ) (ppm)	Assignment
2.00	-COC<u>H</u>$_3$
2.80	-NHC<u>H</u>$_3$
3.0	-N(C<u>H</u>$_3$)$_2$
3.15	H-2
3.28	-N$^+$(C<u>H</u>$_3$)$_3$
3.30	6-OC<u>H</u>$_3$
3.35	3-OC<u>H</u>$_3$
3.50-4.40	H−2′,3,4,5,6,6′
4.55, 5.3-5.7	H-1
4.90	DO<u>H</u>

4.1.2 *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTCC)

HTCCs were synthesized by the epoxide ring opening reaction between glycidyltrimethyl ammonium chloride (GTMAC) and the amino groups of chitosan under acidic condition. The acidic condition caused protonation at the oxygen atom of the epoxy ring, making it more reactive towards attacks by the amino groups of chitosan. The %DQ of HTCC was determined from the relative ratio between the area of H signal from the trimethyl ammonium group of GTMAC at δ 3.20 ppm and the sum of integral signal of H−2′,3,4,5,6,6′ (δ 3.50-4.40 ppm) from all pyranose repeat units (Figure 4.2 and Table 4.2). The %DQ was increased from 11 to 33 when the amount of GTMAC used in the synthesis was increased from 4 and 6 equivalents.

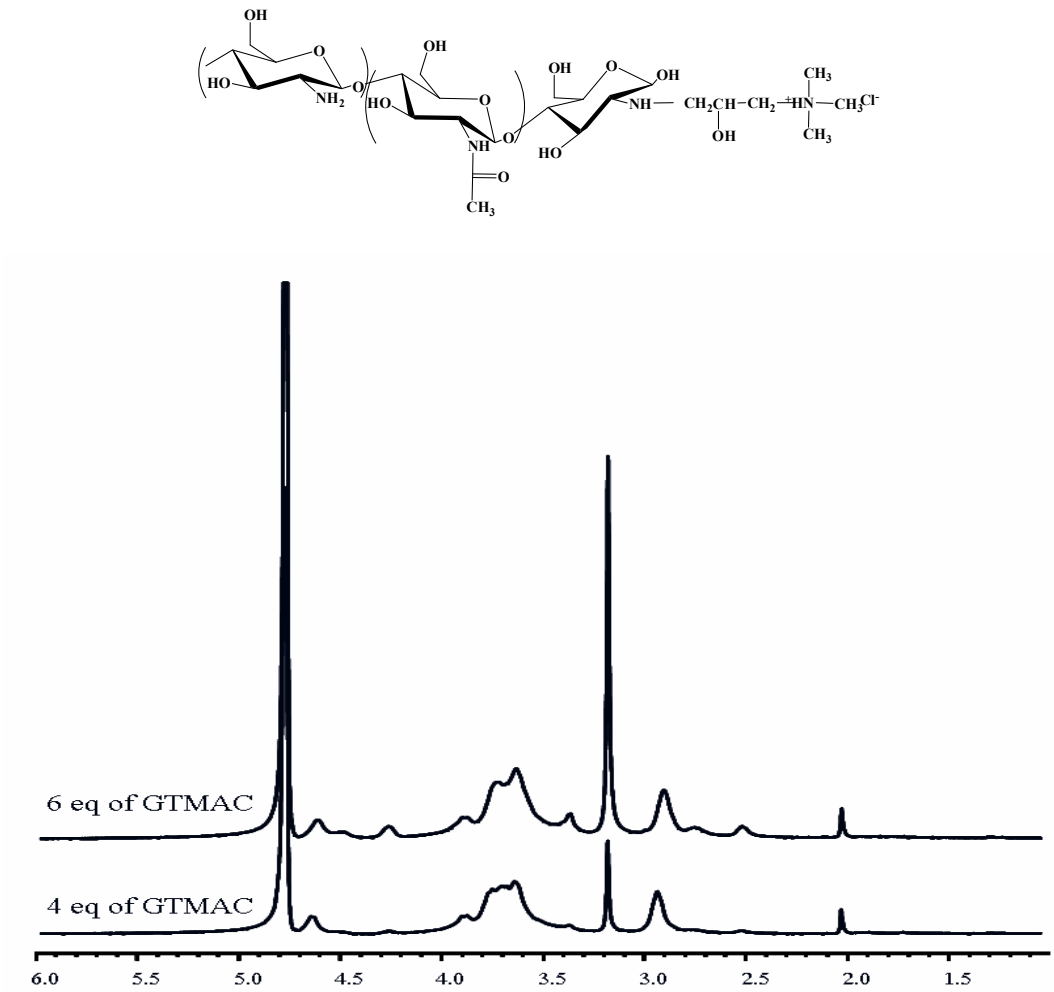


Figure 4.2 ^{1}H NMR spectra of HTCCs synthesized by reacting chitosan with 4 and 6 equivalents of GTMAC (solvent: D_2O).

Table 4.2 Chemical shift (δ) of HTCC determined by ^{1}H-NMR

Chemical shift (δ) (ppm)	Assignment
2.00	-COC<u>H</u>$_3$
2.50	-C<u>H</u>$_2$CH(OH)CH$_2$ N$^+$(CH$_3$)$_3$
2.90	H-2
3.20	-N$^+$(C<u>H</u>$_3$)$_3$
3.40	- C<u>H</u>$_2$ N$^+$(CH$_3$)$_3$
3.50-4.40	H−2′,3,4,5,6,6′
4.15	-C<u>H</u>(OH)CH$_2$ N$^+$(CH$_3$)$_3$
4.80	DO<u>H</u>

Table 4.3 Degree of quaternization and pH of the aqueous solution in which the chitosan derivative is soluble completely

Type of chitosan derivative	Degree of quaternization (%DQ)	pH range
TMC	35	1-6.5
HTCC1	11	1-14
HTCC2	33	1-14

From Table 4.3 the pH range of aqueous solution in which chitosan derivatives can dissolve is the same as reported earlier [34]. TMC can dissolve only in acidic solution. But HTCCs can dissolve in all pH range. Thus HTCC is more versatile in terms of the pH of ingredients in particle preparation step. Therefore in this work the Ca^{2+}-alginate-TMC particles were prepared by dissolving TMC first in 1% acetic acid solution while neutral deionized water was used to prepare Ca^{2+}-alginate-HTCCs particles.

4.2 Particles preparation

In this work $CaCl_2$ was first mixed with sodium alginate in order to form a "pre-gel", an ionic complex between Ca^{2+} and alginate occurred by the ion exchange between Ca^{2+} and Na^{+}. It was postulated earlier that the pre-gel state was necessary to enable the ionic interactions between alginate and chitosan to form nanospheres [32]. The mass ratio of $CaCl_2$ to alginate reportedly affected the size of particles. The mass ratio of $CaCl_2$ to alginate below 0.2 was required to maintain the critical pre-gel state essential for the preparation of nanosphere, while higher mass ratio (in the region of 0.2 to 0.6) would resulted in microspheres formation [21]. Therefore formation of nanospheres required a low concentration of calcium chloride to form the negatively charged calcium alginate pre-gel that was subsequently enveloped by the positively charged chitosan. The cationic polymers restricted further binding between calcium and alginate ions. When each drop of chitosan was mixed with rapid stirring in the Ca^{2+}-alginate pre-gel suspension, Ca^{2+}-alginate-chitosan were formed by ionic interaction between NH_3^{+} of chitosan and $-COO^{-}$ of alginate. The mass ratio of sodium alginate:$CaCl_2$:chitosan was 10:2.33:1 which followed the earlier mentioned work by De and Robison in 2003.

The ionic interactions among alginate, chitosan, and calcium ion are presented in Figure 4.3. The morphology of the particle obtained by transmission electron microscopy is shown in Figure 4.4. From this figure it seems that the particle possesses core and shell feature with size about 475 nm in diameter. The core color tone is lighter than the shell, suggesting that the core part is mainly Ca^{2+}-alginate because alginate possesses negative charge, which was not stained by phosphotungstic acid $[PW_{12}O_{40}]^{3-}$. The darker shell is most likely chitosan polymer which carries positive charges and can be stained by phosphotungstic acid.

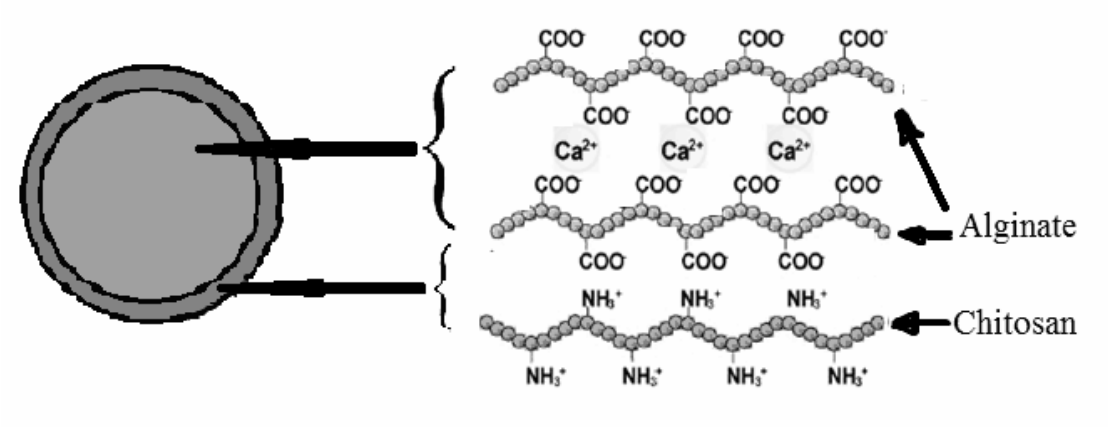


Figure 4.3 The ionic interaction among all ingredients of particle

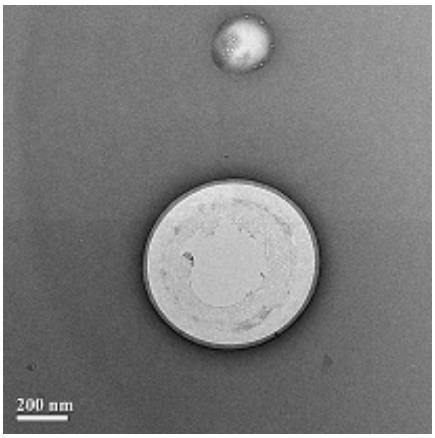


Figure 4.4 TEM micrograph of calcium alginate-chitosan particles.
(concentration of alginate= 0.60 mg/ml, time of sonication= 20 min, concentration of chitosan molecular weight 220 kDa= 0.30 mg/ml, stained by 1% phosphotungstic acid)

4.2.1 Factor affecting the preparation of blank particles

In this section detailed investigation on the particles preparation was carried out with the goal to achieve the smallest particles size. The factors being studied in this work were the addition of calcium ion, sonication time, molecular weight of chitosan, and concentration of chitosan/derivatives solution.

4.2.1.1 Effect of calcium ion and order of addition of chitosan

This part covers the study whether calcium ion is necessary for particle formation. Two formulations- one with Ca^{2+} added and the other one without-were used to prepare the particles. The particle size was found to be 392 nm in diameter for the

chitosan-Na^{+}-alginate particles and 596 nm in diameter for chitosan-Ca^{2+}-alginate particles. The morphology of particles was analyzed by TEM technique (Figure 4.5), revealing that both types of particles were quite similar with core-shell feature as already discussed and shown in Figure 4.4. Resulting the particles without Ca^{2+} show smaller size than the particles with Ca^{2+} however the particles without Ca^{2+} did not stable [29]. When the particles prepation from adding pre-gel into chitosan solution gave larger size than the particles preparing from adding chitosan solution into the pre-gel. Thus this research prepared the particles by follows the previous study [21].

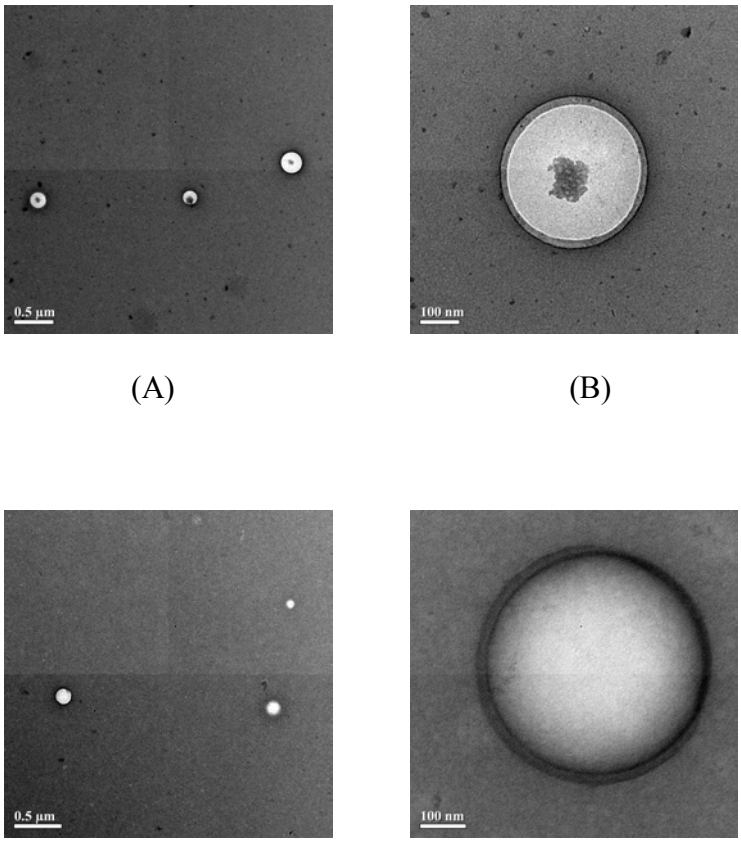


Figure 4.5 Transmission electron micrographs (TEM) of chitosan-Na^{+}-alginate (A, B), and chitosan-Ca^{2+}-alginate (C, D).

4.2.1.2 Sonication time

Sonication is the method of applying the sound energy to instigate particles. It is generally done by using an ultrasonic bath or an ultrasonic probe [36]

(Gensel et al., 1990). In this study the sonication was applied after mixing the calcium chloride solution and Na^{+}-alginate solution. The vibration of sonication brings about separation of calcium alginate molecule from the solution to form small particles. The results relationship between sonication time and the size of Ca^{2+}-alginate-chitosan particles is shown in Figure 4.6. It demonstrates that sonication time affected the size of Ca^{2+}-alginate-chitosan particles. The increase in sonication time from 0 up to 20 min caused the reduction of particle size from 751 nm (without sonication) to about 475 nm. When the time of sonication was longer than 20 min, the size of particles remained almost constant at about 460 nm and became more uniform as presented by the narrow error bars. It was also reported earlier that, the sonication was required in order to get the uniformity of particles size [37]. With only mechanical stirring, the obtained particles showed a wide range of sizes. This confirms that sonication is essential for producing the uniform-sized particles. The optimal sonication of 20 min was used however for further study in this work.

The analysis for charges on particles or zeta potential revealed that no significant difference of zeta potential of the particles was observed (Figure 4.7). Moreover the negative value of the zeta potential suggests that overall the particle was negatively charged. This in fact agreed with the amount of each polymer used to prepare the particles. The amount of alginate used in this work was about 10 folds the amount of chitosan.

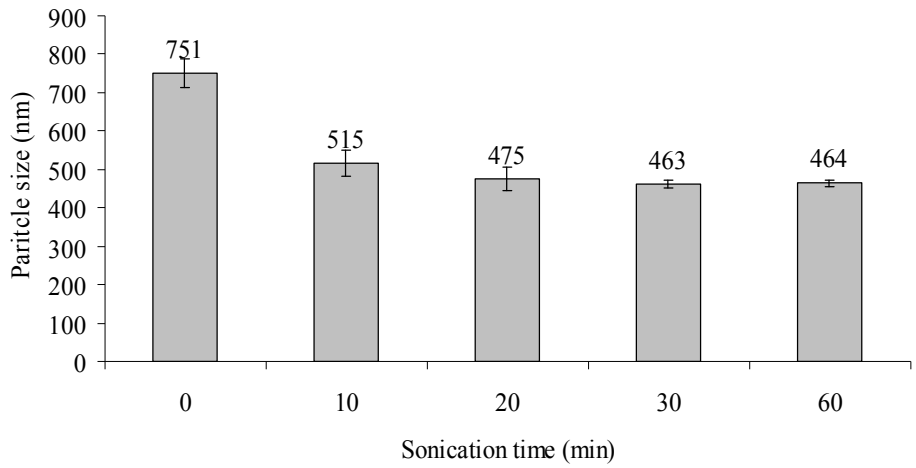


Figure 4.6 The effect of sonication time during pre-gel preparation on the size of Ca^{2+}-alginate-chitosan particles obtained from the zeta sizer (n=3).

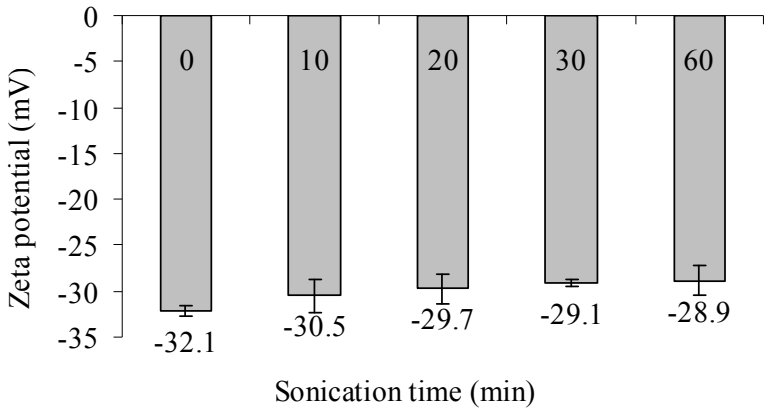


Figure 4.7 The effect of sonication time on the zeta potential of Ca^{2+}-alginate-chitosan particles obtained from the zeta sizer (n=3).

4.2.1.3 Molecular weight of chitosan

In this section the effect of molecular weight of chitosan on the particles size was studied. It was hypothesized that changing the molecular weight of chitosan could affect the particles size. In this work however no direct relation between the molecular weight of chitosan and the particles size was observed (Figure 4.8). These results were somewhat surprising since it was expected that chitosan with high molecular weight would result in large particle size. It was possible that the amount of chitosan in the particles was only 5-20% of alginate weight which was rather low relative to the amount of alginate (see also Table in section 3.5.2). Therefore it was the alginate part that dominantly designated the particles size, not chitosan. Again change in molecular weight of chitosan from 71 to 583 kDa did not cause any change in zeta potential of the particles (Figure 4.9).

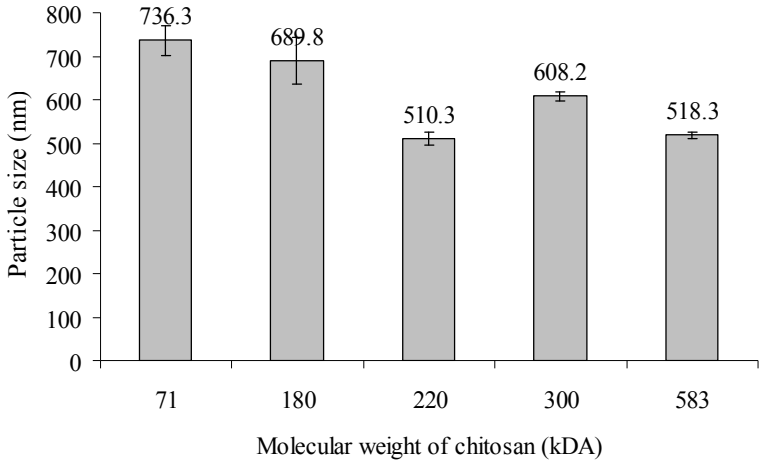


Figure 4.8 The effect of molecular weight of chitosan on the size of Ca^{2+}-alginate-chitosan particles obtained from the zeta sizer (n=3).

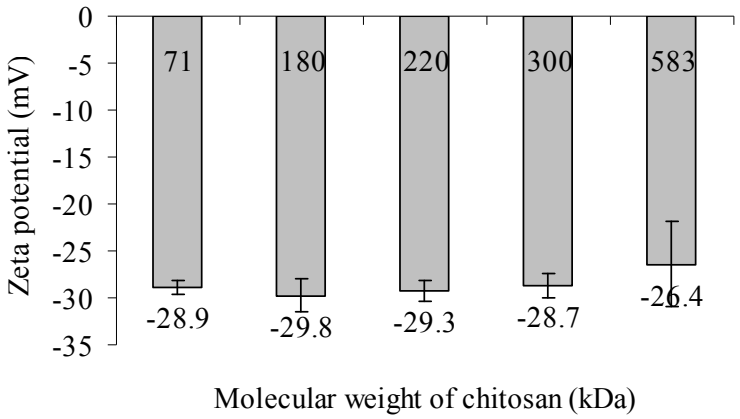
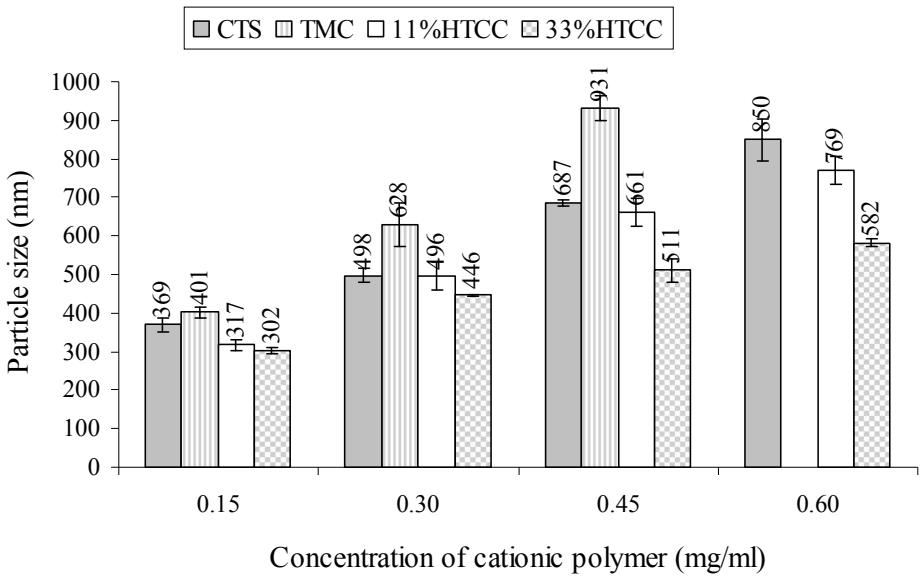


Figure 4.9 The effect molecular weight on the zeta potential of Ca^{2+}-alginate-chitosan particles obtained from the zeta sizer (n=3).

4.2.1.4 Concentration of chitosan and its derivatives

In this section the effect of concentration of cationic polymer; chitosan, TMC, HTCC (11%DQ), and HTCC (33%DQ), on particle size was investigated. It was hypothesized that increasing the concentration of the cationic polymer should lead to an increase of particles size. The results in fact agreed well with the proposed hypothesis (Figure 4.10). It could be simply explained by the fact that high polymer concentration contained a large amount of polymer in a unit volume to interact with alginate. Thus

increasing the concentration of polymer solutions from 0.15 to 0.60 mg/ml resulted in the significant increase of particle diameter. To obtain the particles size of 400 nm and lower, the concentration of chitosan, TMC, or HTCCs solution to be used in the particles formation step must be kept at 0.15 mg/ml.



**The mixture of TMC (0.60 mg/ml) and alginate precipitated completely without any measureable particles.

Figure 4.10 The effect of concentration of cationic polymer on the size of the particles having different types of cationic polymer obtained from the zeta sizer (n=3).

At the same concentration of cationic polymer, the use of HTCC tended to give smaller particle size than did the chitosan or TMC. The HTCC with higher DQ (33%) when incorporated with alginate gave a smaller particle size than the one with lower DQ (11%) did. This is most likely because HTCC with high DQ carries high percentage of ammonium group and can attract alginate chains quite effectively to form small particles with less polymer mass than the HTCC with low DQ. This is the first report about a high DQ polymer giving smaller size than the low DQ one.

As already mentioned chitosan and TMC were able to dissolve only in acidic solution, while HTCCs could dissolve in water at a neutral pH. The pHs of the suspension medium after the formulation of the particles made from chitosan and TMC

were 3.4. At this acidic pH, the carboxylate group in alginate was protonated to become a carboxylic group (-COOH), which could not interact effectively with the positive charge nitrogen in the form of ($-NH_3^+$) in chitosan and $-N^+(CH_3)_3$ in TMC. This event possibly resulted in the increase of particle size. In the case of HTCCs, the pH after formulation is 6.3. Therefore the alginate still retained its negative charge $-COO^-$ which could attract the positive charges in HTCC, resulting in close packing of polymer chains.

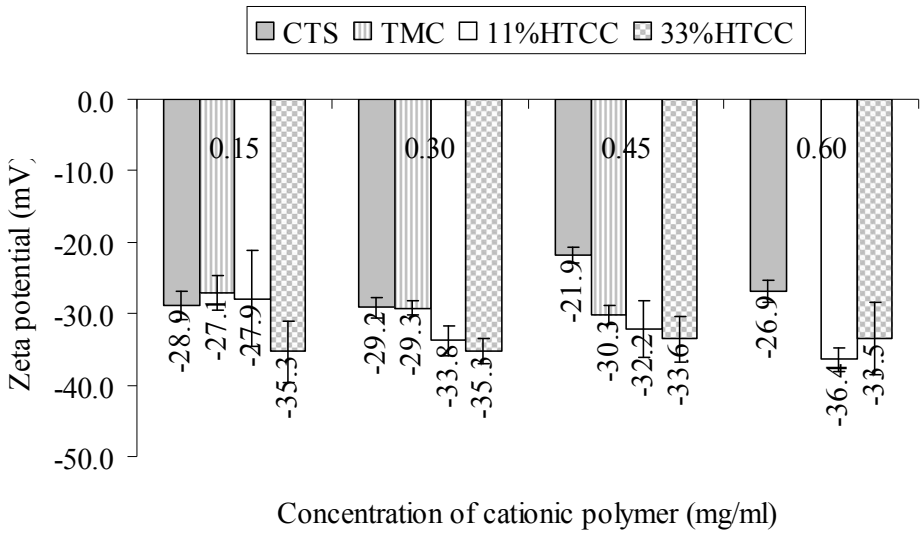


Figure 4.11 The effect of concentration of cationic polymer on the zeta potential of the particles having different types of cationic polymer obtained from the zeta sizer (n=3).

It was however noticed than the zeta potential did not changed significantly when the concentration of cationic polymers was varied from 0.15 to 0.60 mg/ml. Again this result can be attributed to the fact that the major component of the particle is alginate. Varying the amount of positively charged counterpart therefore have no effect on the net zeta potential of the particles.

The optimum formulation for the preparation of Ca^{2+}-alginate-chitosan and with two other chitosan derivatives that gave smallest particles size are listed in Table 4.5. This formulation was used to prepare GH-entrapped particles for release study.

Table 4.4 List of optimum parameters for the preparation of GH-entrapped particles for Ca^{2+}-alginate-chitosan and two other the chitosan derivatives

Preparation parameter	value
Sonication time	20 min
Molecular weight of chitosan	220 kDa
Concentration of chitosan, TMC, or HTCCs	0.15 mg/ml

4.2.2 Preparation of glucosamine-loaded particles

4.2.2.1 Order of glucosamine addition

Two methods for preparing glucosamine (GH)-loaded particles were investigated. In method A, GH was mixed into $CaCl_2$ solution before being mixed with the alginate solution. For method B, GH was mixed first into sodium alginate solution, followed by mixing with the $CaCl_2$ solution. The size and zeta potential of the particles were analyzed. The sizes of blank particles and the GH-loaded particles are shown in Figure 4.12. It turned out that the size of the blank and the GH-loaded particles were not significantly different from the others. It was possible that the amount of GH was quite low and did not affect the particle size.

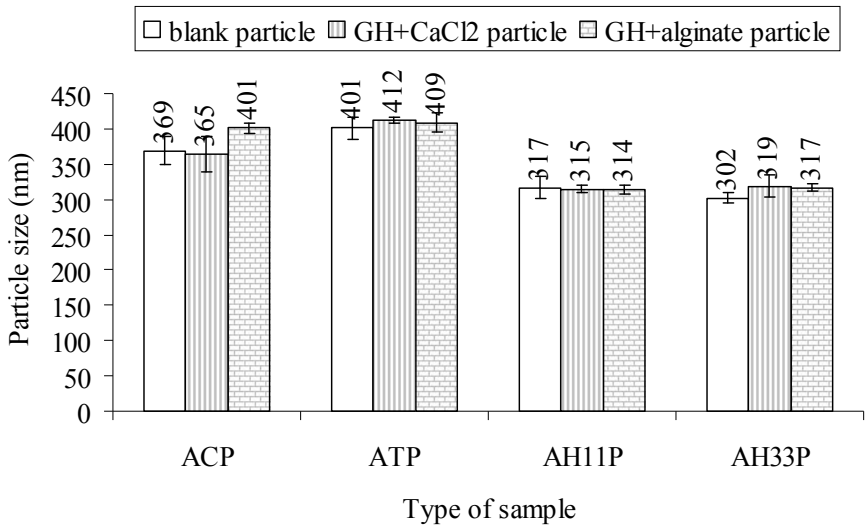


Figure 4.12 Comparison the size of blank particles and two methods of preparing GH-loaded particles obtained from a zeta sizer (n=3).

The morphology of the two preparation methods of GH-loaded particles analyzed by TEM is shown in Figure 4.13. The particle possesses core and shell feature with size of about 266 nm in diameter for the particles prepared by method A and about 218 nm in diameter for the particles prepared from method B. The core part is mainly Ca^{2+}-alginate and the shell is chitosan polymer similar to the blank particles reported earlier. In addition the loading of GH into the particles did not cause any change of zeta potential of the particles (Figure 4.14).

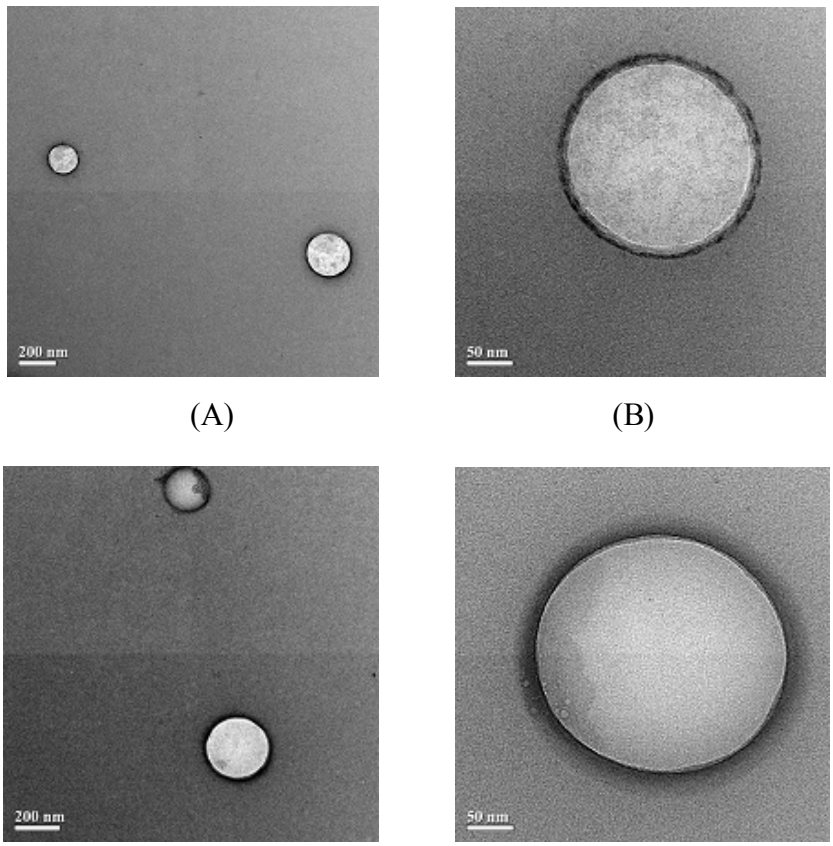


Figure 4.13 TEM micrographs of GH-loaded Ca^{2+}-alginate-chitosan particles prepared by method A (A, B) and method B of (C, D).
(concentration of alginate= 0.60 mg/ml, time of sonication= 20 min, concentration of chitosan molecular weight 220 kDa= 0.15 mg/ml, stained by 1% phosphotungstic acid)

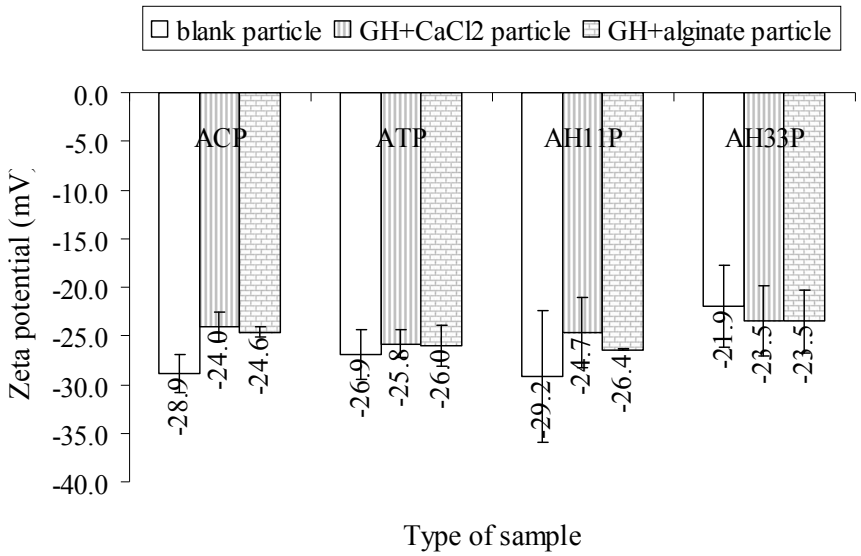


Figure 4.14 Comparison the zeta potential of blank particles and 2 methods preparing of GH-loaded particles obtained from the zeta sizer. (n=3)

4.2.2.2 Determination of encapsulation efficiency (%EE) and loading capacity (%LC)

The content of glucosamine within the particles made of Ca^{2+}-alginate-chitosan, Ca^{2+}-alginate-TMC, and Ca^{2+}-alginate-HTCCs, were analyzed by using HPLC technique. Each sample was derivatized by reacting with PITC (Figure 4.15**)** before the analysis. The mechanism of phenylthiocarbonyl-glucosamine synthesis is shown in Scheme 4.1.

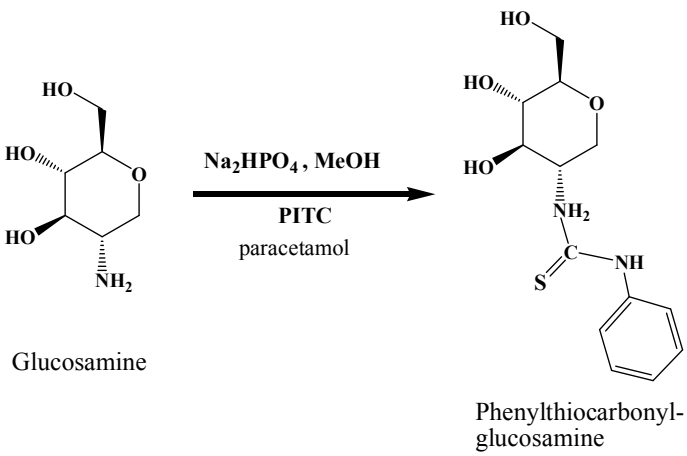
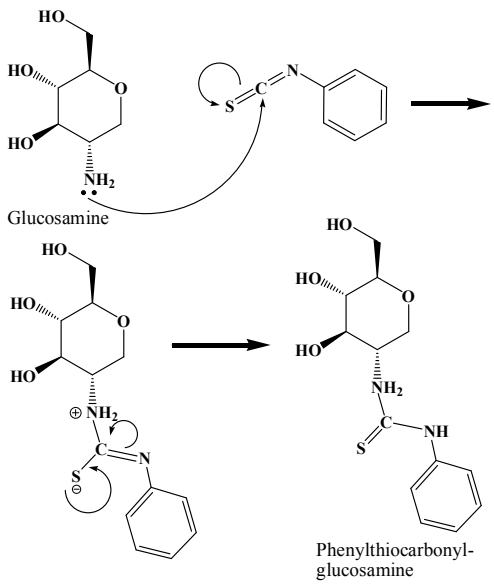


Figure 4.15 Synthesis of phenylthiocarbonyl-glucosamine from glucosamine and PITC



Scheme 4.1 Mechanism of the reaction between glucosamine and phenylisothiocyanate forming phenylthiocarbonyl-glucosamine

- Determination of loaded glucosamine by direct method

The Ca^{2+}-alginate-chitosan particles was immersed in sodium citrate (Figure 4.16) solution in order to recover all GH trapped in the particles. Since sodium citrate is smaller than alginate polymer, sodium citrate can replaced the alginate also by ionic interaction between its carboxylate group and the amino group of chitosan causing particle rupture. The results of EE and LC of two GH-loading methods are displayed in Table 4.5. From the data obtained, the amount of GH trapped in the particles prepared by loading method A was less than the one prepared by loading method B. It is possible that, as GH was dissolved in alginate solution (method B), the ionic attraction between the $-NH_3^+$ of GH and $-COO^-$ of alginate occurred instantly. Thus significant amount of GH was trapped in the alginate chains before pre-gel and the subsequent particle formation with chitosan. In the case when GH was dissolved in calcium chloride solution, the ionic repulsion between the $-NH_3^+$ of GH and Ca^{2+} in the $CaCl_2$ solution occurred. This resulted in less GH in the alginate. Thus by mixing GH with alginate first, the entrapment efficiency became high. However the net amount of loaded GH in the particle was quite low. It was possible that GH was easily dissolved in water. It tended to release out from the particles very fast during every step of particle preparation as well as during storage. The GH loading method B was used for further study in this work.

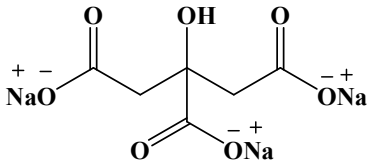


Figure 4.16 Chemical structure of sodium citrate

Table 4.5 Encapsulation efficiency and loading capacity of glucosamine in Ca^{2+}-alginate-chitosan particles (GH:alginate weight ratio 1:1), prepared by different GH loading methods. (see sample codes in section 3.5.2)

GH loading method	Sample	% Yield of dried particle	%EE[1]	%LC[1]
A	ACP	9.51	0.714±0.17	3.218±0.76
	ATP	8.53	0.581±0.15	2.995±0.77
	$AH_{11}P$	6.34	0.655±0.12	4.924±0.90
	$AH_{33}P$	5.61	0.558±0.10	4.793±0.83
B	ACP	10.66	0.941±0.14	3.914±0.59
	ATP	7.51	0.693±0.10	4.089±0.62
	$AH_{11}P$	6.06	0.802±0.14	5.868±1.00
	$AH_{33}P$	6.52	0.960±0.17	6.540±1.19

[1]Three sets of experiments were performed.

The results of encapsulation efficiency and loading capacity of GH-loaded particles from Table 4.5 demonstrate that the amount of GH trapped in the particles was rather low. Thus some modification of GH loading was carried out. The amount of GH used in the particle preparation method B was increased 10 times to obtain higher LC as shown in Table 4.6. However the EE did not change much even after increasing the amount of GH in the preparation step.

Table 4.6 Encapsulation efficiency and loading capacity of particles (GH:alginate weight ratio 10:1) by method B (n=3)

Sample	% Yield of dried particle	%EE	%LC
ACP	3.89	1.020±0.14	23.24±0.59
$AH_{11}P$	4.05	0.969±0.03	21.22±0.55
$AH_{33}P$	3.54	0.914±0.02	22.88±0.53
ATP	2.68	0.610±0.00	20.23±0.13

- Determination of loaded glucosamine by indirect method

The supernatant and washed liquid (water) obtained from the particle separation step by centrifugation were analyzed for GH (Table 4.8). It was found that the supernatant from GH-loading method A (GH+$CaCl_2$) contained higher amount of GH (96-98%) than that obtained from method B (GH+alginate). From the data obtained, a substantial amount of GH was found in the supernatant and some more in the washed liquid. This finding reflects the high solubility of GH in aqueous medium, subsequently resulted in low GH loading content in the prepared particles. Another observation will be made here that the sum of GH amount in the particle, supernatant, and washed liquid was not a little less than 100. It was possible that some GH or particles stuck to centrifuged tube or lost during sample transfer.

Table 4.7 Amount of GH from supernatant and washed water of GH-loaded particles (GH:alginate weight ratio 1:1) prepared by different methods of GH-loaded particles (n=3)

GH loading method	Sample	%GH found in			%Recovery
		gel	Supernatant	Washed water	
A	ACP	0.714	97.50	1.68	99.90
	ATP	0.581	95.75	1.95	98.36
	$AH_{11}P$	0.655	97.43	1.72	99.71
	$AH_{33}P$	0.558	96.44	1.91	98.93
B	ACP	0.941	90.61	1.86	93.40
	ATP	0.693	96.04	1.67	98.51
	$AH_{11}P$	0.802	94.22	1.67	96.84
	$AH_{33}P$	0.960	91.02	1.63	93.34

4.3 Release study

The GH loading method B was used for the GH release study from Ca^{2+}-alginate-chitosan, Ca^{2+}-alginate-TMC, and Ca^{2+}-alginate-HTCCs particles. The amount of GH within the particles was analyzed using HPLC technique. The cumulative drug release profiles from the particles having different cationic polymer were plotted as a function of time. The weight ratio between alginate:GH was fixed at 1:10 as well as the concentration of cationic polymer was constant for all types of particles. Results indicated that GH was continuously released from the Ca^{2+}-alginate-chitosan, Ca^{2+}-alginate-TMC, Ca^{2+}-alginate-HTCC (11%), and Ca^{2+}-alginate-HTCC (33%) particles to a maximum amount of 0.8164, 0.4472, 0.8373, and 0.8101, mg/mg of the particles, respectively, which were equivalent to 1.020, 0.610, 0.969, and 0.914 mg of GH (Figure 4.17). The release profiles of all particle types show linear relationship between the cumulative amount of GH release and time in the course of 360 min. The release of

GH is then discontinued after 360 min until the longest study period of 1,440 min or 24 h. No 'burst' effect was also observed in these particle systems, suggesting that there was no significant amount of GH remained on the outer surface of the particles. All raw data are shown in Appendix C.

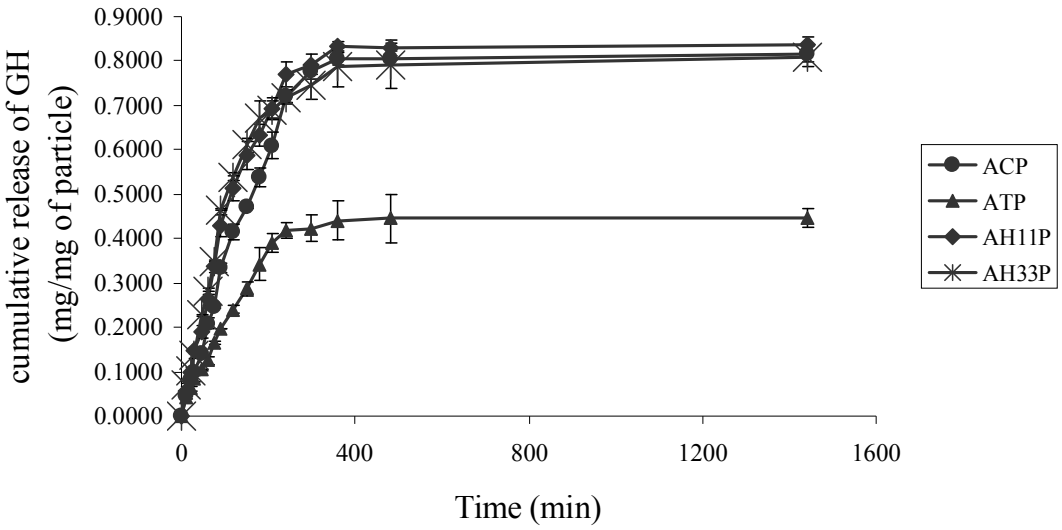


Figure 4.17 Comparison of cumulative GH release from the particles having different types of cationic polymer (GH: alginate weight ratio =10: 1).

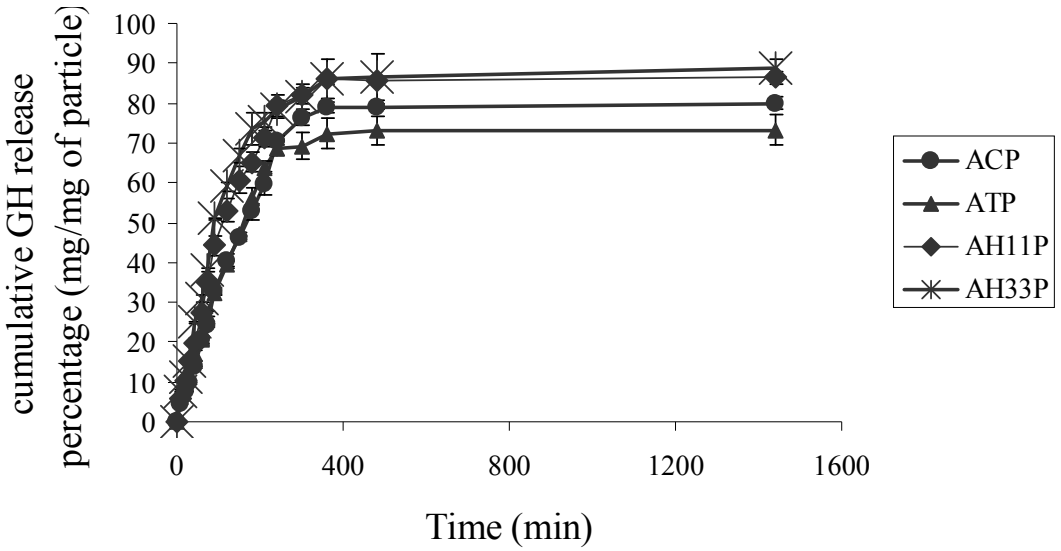


Figure 4.18 Comparison of cumulative GH release percentage from the particles having different types of cationic polymer (GH: alginate weight ratio =10: 1).

The cumulative GH release percentages from the particles made by the three types of cationic polymer were compared in Figure 4.18. The detail of data obtained from released study can be seen in Appendix C. The present results demonstrate that the GH release shows an increase of percentage of cumulative GH release with incubation time. The percentages of release from the particles reach the maximum levels after the period of 6 h; 80, 73, 86, and 89 % for Ca^{2+}-alginate-chitosan, Ca^{2+}-alginate-TMC, Ca^{2+}-alginate-HTCC (11%), and Ca^{2+}-alginate-HTCC (33%) nanoparticles respectively. It means that a small amount of GH remained in the particle, possibly by the H-bonding between the hydroxyl and amino groups of GH and those in chitosan as well as in alginate.

Based on the results obtained, the release of GH can be prolonged up to 6 hours with a constant release rate at pH 7.4. The particles made of Ca^{2+}-alginate-TMC somewhat provided less amount of GH than the others. For Ca^{2+}-alginate-chitosan and Ca^{2+}-alginate-HTCC particles, no significant difference in most aspects was observed from both particle systems, except for the slightly size difference (Figure 4.12). The author would prefer to use Ca^{2+}-alginate-chitosan particles since the chitosan part is readily available without the need to perform an extra step of chemical synthesis. In addition, all components used to fabricate the particles are biocompatible and biodegradable. Thus this system can be used for biomedical purpose in human.

CHAPTER V

CONCLUSION

Particle preparation parameters were optimized in order to achieve smallest particle sizes. In the case of Ca^{2+}-alginate-chitosan, the increase in sonication time of up to 20 min caused the reduction of particle size by 36.8%. Changing the molecular weight of chitosan in the range of 15-583 kDa did not have direct correlation with the particles size. The particle size was reduced when the concentration of chitosan or its derivatives was decreased to 0.15 mg/mL. At the same concentration of the cationic polymers used in the particle formation step, HTCC tended to give smaller particle size than chitosan and TMC did. Moreover HTCC with high DQ of 33% when incorporated with Ca^{2+}-alginate gave smaller particle size than the one with lower DQ (11%). The analysis for charges on particles or zeta potential revealed that no significant difference of zeta potential of the particles with negative value of the zeta potential (- 21.9 to -36.4) was observed.

Glucosamine-loaded particles with the size of 314-409 nm were prepared from ionotropic gelation of alginate, calcium ion, and chitosan or each of permanently positively charged TMC and HTCC. The highest GH loading content in the particles was between 20-23% w/w and not affected significantly by the types of cationic polymers used. GH was continually released from the particles with no significant burst of the drug during the initial hydrated stage in pH 7.4 aqueous solution. The release profiles of all particle types show linear relationship between the cumulative amount of GH release and time in the course of 360 min. Then the release of GH discontinued after 360 min until the longest study period of 24 h. The highest amount of GH release was found to be about 0.8 mg/mg of particles wt for the particles containing chitosan and HTCC, and was down to 0.4 mg/mg of particles wt for the one with TMC.

The key advantage of this reported system is that all components used to fabricate the particles are biocompatible and biodegradable. Thus this system is considered worth exploring more in terms of storage stability (shelf-life), formulation for consumer product, and other biological tests for its potential use in biomedical purposes.

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APPENDICES

APPENDIX A

PARTICLE SIZE AND ZETA POTENTIAL

Table 1A The particle size of Ca^{2+}-alginate chitosan particle affect of sonication time by zeta sizer

Sonication time (min)	Experiment set			mean	SD
	1	2	3		
0	764	710	780	751	37
10	511	552	483	515	34
20	449	508	467	475	30
30	469	470	450	463	11
60	466	469	456	464	7.0

Table 2A The zeta potential of Ca^{2+}-alginate chitosan particle affect of sonication time by zeta sizer

Sonication time (min)	Experiment set			mean	SD
	1	2	3		
0	-32.1	-32.3	-32.1	-32.1	0.6
10	-30.5	-31.2	-29.8	-30.5	1.8
20	-30.4	-30.1	-28.7	-29.7	1.6
30	-29.2	-28.9	-29.3	-29.1	0.4
60	-30.5	-28.1	-28.0	-28.9	1.7

Table 3A The particle size of Ca^{2+}-alginate chitosan particle affect of molecular weight by zeta sizer

Molecular weight of chitosan (kDa)	Experiment set			mean	SD
	1	2	3		
15	659	591	654	635	38
45	696	627	629	651	40
71	671	752	681	701	45
220	495	482	517	498	17
583	537	571	565	558	18

Table 4A The zeta potential of Ca^{2+}-alginate chitosan particle affect of molecular weight by zeta sizer

Molecular weight of chitosan (kDa)	Experiment set			mean	SD
	1	2	3		
15	-30.7	-30.0	-28.6	-29.8	1.7
45	-29.4	-29.3	-27.3	-28.7	1.3
71	-29.2	-29.1	-28.6	-28.9	0.7
220	-30.3	-28.4	-29.3	-29.3	1.1
583	-21.9	-27.6	-29.8	-26.4	4.6

Table 5A The particle size of Ca^{2+}-alginate chitosan particle affect of concentration of chitosan by zeta sizer

Concentration of chitosan (mg/ml)	Experiment set			mean	SD
	1	2	3		
0.15	349	371	387	369	19
0.30	495	482	517	498	18
0.45	684	680	696	687	8.4
0.60	836	804	910	850	54

Table 6A The zeta potential of Ca^{2+}-alginate chitosan particle affect of concentration of chitosan by zeta sizer

Concentration of chitosan (mg/ml)	Experiment set			mean	SD
	1	2	3		
0.15	-30.2	-28.5	-28	-28.9	2.0
0.30	-30.4	-29.15	-27.9	-29.15	1.4
0.45	-21.8	-22.9	-21.0	-21.9	1.1
0.60	-27.2	-27.1	-26.35	-26.9	1.5

Table 7A The particle size of Ca^{2+}-alginate TMC particle affect of concentration of TMC by zeta sizer

Concentration of TMC (mg/ml)	Experiment set			mean	SD
	1	2	3		
0.15	415	386	402	401	15
0.30	690	576	619	628	58
0.45	913	912	969	931	32

Table 8A The zeta potential of Ca^{2+}-alginate TMC particle affect of concentration of TMC by zeta sizer

Concentration of TMC (mg/ml)	Experiment set			mean	SD
	1	2	3		
0.15	-24.3	-27.8	-29.1	-27.1	2.5
0.30	-29.9	-29.0	-29.0	-29.3	1.0
0.45	-30.7	-30.6	-29.5	-30.3	1.3

Table 9A The particle size of Ca^{2+}-alginate 11% HTCC particle affect of concentration of 11% HTCC by zeta sizer

Concentration of HTCC (mg/ml)	Experiment set			mean	SD
	1	2	3		
0.15	332	307	313	317	14
0.30	462	503	313	496	35
0.45	665	617	596	662	35
0.60	774	768	702	769	35

Table 10A The zeta potential of Ca^{2+}-alginate 11% HTCC particle affect of concentration of 11% HTCC by zeta sizer

Concentration of HTCC (mg/ml)	Experiment set			mean	SD
	1	2	3		
0.15	-21.6	-28.5	-35.6	-32.2	6.8
0.30	-31.5	-35.2	-34.8	-33.8	2.1
0.45	-28.9	-32.2	-35.6	-32.2	1.7
0.60	-35.1	-38.5	-35.8	-36.4	4.0

Table 11A The particle size of Ca^{2+}-alginate 33% HTCC particle affect of concentration of 33% HTCC by zeta sizer

Concentration of HTCC (mg/ml)	Experiment set			mean	SD
	1	2	3		
0.15	289	311	306	302	7.6
0.30	446	445	447	446	1.4
0.45	570	480	482	511	30
0.60	591	587	569	582	9.5

Table 12A The zeta potential of Ca^{2+}-alginate 33% HTCC particle affect of concentration of 33% HTCC by zeta sizer

Concentration of HTCC (mg/ml)	Experiment set			mean	SD
	1	2	3		
0.15	-31.5	-36.3	-38.2	-35.3	4.2
0.30	-39.1	-40.4	-41.3	-40.3	1.8
0.45	-30.6	-34.3	-35.9	-33.6	3.2
0.60	-29.1	-33.4	-38.0	-33.5	5.0

APPENDIX B

CALIBRATION CURVE

The concentration versus peak area data of GH in Milli-Q water at 254 nm and are presented in Table 1. They show a linear relationship with the correlation coefficient equal to 0.9998.

Table 1B Peak area of GH in Milli-Q water determined at 254 nm

Concentration of GH in Milli-Q water (µg/mL)	Peak area of GH derivative	Peak area of paracetamol	Ratio between Peak area of GH derivative to Peak area of paracetamol
1	3457	334512	0.010
5	19073	341061	0.056
10	38616	339908	0.114
50	203805	317123	0.643
100	455785	288313	1.579
500	2533370	327624	7.733
1000	5885066	371463	15.885

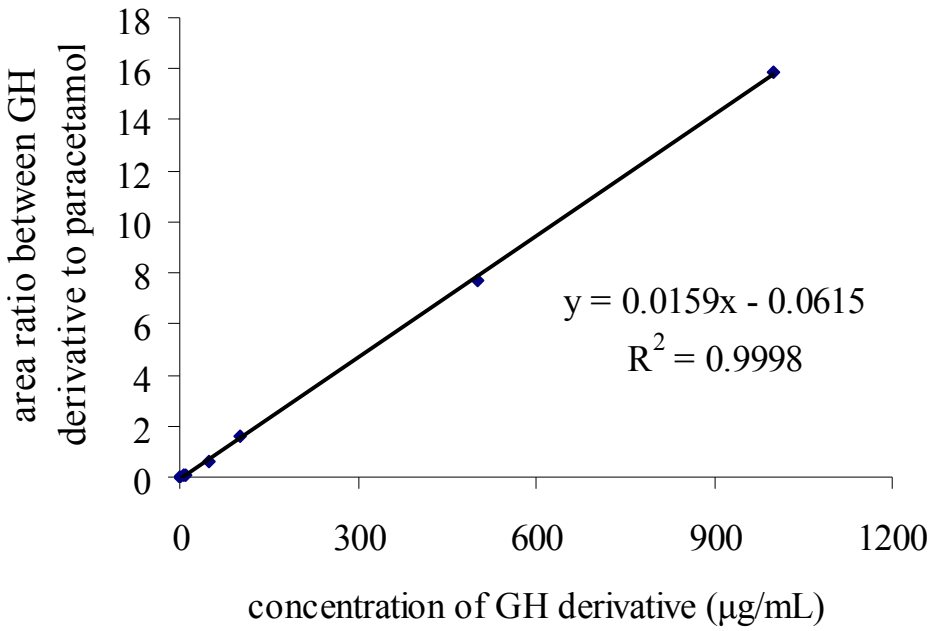

Figure 1B Standard calibration curve of area ratio between GH derivative to paracetamol in Milli-Q water

$$\text{Concentration of GH solution} = \frac{\text{Area ratio} + 0.0615}{0.0159}\ \mu\text{g/mL}$$

For the 1st method

$$\text{Amount of GH (mg)} = \frac{\text{Concentration of glucosamine (µg/mL)}}{1000\ \mu\text{g/mg}} \text{ x } 4.5\ \text{mL}$$

For the 2nd method

$$\text{Amount of GH (mg)} = \frac{\text{Concentration of glucosamine (µg/mL)}}{1000\ \mu\text{g/mg}} \text{ x volume of supernatant (mL)}$$

APPENDIX C

AMOUNT OF DRUG RELEASE

Table 1C Cumulative GH release from Ca^{2+}-alginate-chitosan particles

Time (min)	Amount of GH release (mg/mg of particles)				
	1	2	3	Mean	SD
0	0	0	0	0	0
10	0.04538	0.04401	0.04519	0.04486	0.000741
20	0.07940	0.07944	0.07913	0.07932	0.000169
30	0.09937	0.09598	0.10199	0.09911	0.003014
45	0.14478	0.13694	0.13680	0.13951	0.004566
60	0.19514	0.21745	0.21445	0.20901	0.012111
75	0.24176	0.25166	0.24770	0.24704	0.00498
90	0.34493	0.33270	0.32548	0.33437	0.009832
120	0.41968	0.42749	0.39434	0.41384	0.017333
150	0.46638	0.47075	0.47493	0.47069	0.004277
180	0.54021	0.55663	0.51733	0.53806	0.019737
210	0.62907	0.62635	0.57422	0.60988	0.030915
240	0.71809	0.70842	0.73439	0.72030	0.013125
300	0.76049	0.79358	0.77368	0.77592	0.016656
360	0.79693	0.81969	0.79578	0.80413	0.013484
480	0.80876	0.81207	0.79966	0.80683	0.006424
1440	0.82460	0.79685	0.82767	0.81637	0.016978

Table 2C Cumulative GH release from Ca^{2+}-alginate-TMC particles

Time (min)	Amount of GH release (mg/mg of particles)				
	1	2	3	Mean	SD
0	0	0	0	0	0
10	0.0438	0.0434	0.0431	0.0434	0.0004
20	0.0652	0.0586	0.0620	0.0619	0.0033
30	0.0898	0.0803	0.0867	0.0856	0.0049
45	0.1060	0.0936	0.1129	0.1041	0.0098
60	0.1306	0.1218	0.1224	0.1249	0.0049
75	0.1622	0.1706	0.1631	0.1653	0.0046
90	0.1948	0.1970	0.1979	0.1966	0.0016
120	0.2356	0.2407	0.2461	0.2408	0.0053
150	0.2848	0.2888	0.2884	0.2873	0.0022
180	0.3426	0.3253	0.3574	0.3417	0.0160
210	0.4005	0.3795	0.3889	0.3896	0.0105
240	0.4268	0.4229	0.4072	0.4190	0.0104
300	0.4387	0.4283	0.4008	0.4226	0.0196
360	0.4462	0.4608	0.4152	0.4408	0.0233
480	0.4505	0.4634	0.4207	0.4449	0.0219
1440	0.4481	0.4688	0.4246	0.4472	0.0221

Table 3C Cumulative GH release from Ca^{2+}-alginate-HTCC (11%) particles

Time (min)	Amount of GH release (mg/mg of particles)				
	1	2	3	Mean	SD
0	0	0	0	0	0
10	0.05866	0.05800	0.05542	0.05736	0.001709
20	0.09729	0.09933	0.10197	0.09953	0.002348
30	0.15519	0.14603	0.14124	0.14749	0.007087
45	0.17739	0.18440	0.20728	0.18969	0.015633
60	0.27667	0.24820	0.27023	0.26503	0.014929
75	0.33451	0.32597	0.35067	0.33705	0.012547
90	0.42726	0.40473	0.45425	0.42875	0.024796
120	0.51005	0.48902	0.54459	0.51456	0.028058
150	0.59560	0.55342	0.61492	0.58798	0.03145
180	0.60724	0.63096	0.65668	0.63163	0.024729
210	0.66447	0.70504	0.70964	0.69305	0.024853
240	0.74203	0.77210	0.79684	0.77032	0.027447
300	0.80953	0.76545	0.79873	0.79124	0.022975
360	0.82729	0.84465	0.82447	0.83213	0.010931
480	0.82645	0.83962	0.82716	0.83108	0.007407
1440	0.82713	0.85596	0.82876	0.83728	0.016194

Table 4C Cumulative GH release from Ca^{2+}-alginate-HTCC (33%) particles

Time (min)	Amount of GH release (mg/mg of particles)				
	1	2	3	Mean	SD
0	0	0	0	0	0
10	0.06794	0.07417	0.07051	0.07087	0.003132
20	0.09715	0.10256	0.10871	0.10281	0.005784
30	0.13165	0.13834	0.14566	0.13855	0.007006
45	0.22679	0.23060	0.22585	0.22775	0.002517
60	0.27444	0.28816	0.28499	0.28253	0.007187
75	0.34812	0.34987	0.34284	0.34694	0.00366
90	0.46700	0.46453	0.46569	0.46574	0.001239
120	0.53115	0.53864	0.54883	0.53954	0.008873
150	0.59601	0.60799	0.62833	0.61078	0.01634
180	0.67782	0.63196	0.70620	0.67199	0.037463
210	0.70915	0.66842	0.69391	0.69049	0.020578
240	0.73299	0.69959	0.72361	0.71873	0.017227
300	0.76280	0.71057	0.76323	0.74554	0.030281
360	0.81479	0.73509	0.80936	0.78641	0.044533
480	0.83570	0.73034	0.80724	0.79109	0.054503
1440	0.82544	0.78562	0.81931	0.81012	0.021438

VITAE

Miss Pronphrom Peng-im was born on September 22, 1985 in Nakornsawan, Thailand. She graduated with a Bachelor's Degree in Chemistry from the Faculty of Science, Kasetsart University in 2006. She has been a graduate student in the Program of Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University since 2007 and graduated in May 2010.

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2. May 21-22, 2009:	2nd Polymer Graduate Conference of Thailand, Chulalongkorn University
3. September 6-9, 2009:	8th Asia-Pacific Chitin and Chitosan Symposium or 11th International Conference on Chitin and Chitosan, National Taiwan University of Science and Technology, Taiwan
4. July 13, 2010:	25th Anniversary program of Petrochemistry and Polymer Science