อนุภาคควอเทอไนซ์ไคโทซานเตรียมโดยการประกอบตัวเองของแอมฟิฟิลิกไคโทซาน

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QUATERNIZED CHITOSAN PARTICLES PREPARED BY SELF-ASSEMBLY OF AMPHIPHILIC CHITOSAN

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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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Field of Study	Petrochemistry and Polymer Science
Thesis Advisor	Associate Professor Voravee P. Hoven, Ph.D.

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นัดดา บูรณะบัญญัติ: อนุภาคควอเทอไนซ์ไคโทซานเตรียมโดยการประกอบตัวเองของแอมฟิฟิลิกไคโทซาน (QUATERNIZED CHITOSAN PARTICLES PREPARED BY SELF-ASSEMBLY OF AMPHIPHILIC CHITOSAN) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วรวีร์ โฮเว่น, 66 หน้า.

เตรียมอนุภาคควอเทอร์ไนซ์ไคโทซานด้วยการประกอบตัวเองของแอมฟิฟิลิกไคโทซานที่มีหมู่ทาโลอิล, เฮกซิล หรือโดเดกซิล เป็นหมู่ไม่ชอบน้ำ และหมู่พอลิ(เอทิลีนไกลคอล)เมทิลอีเทอร์ หรือ เอ็น-[(2-ไฮดรอกซิล-3-ไทรเมทิลแอมโมเนียม)]โพรพิล เป็นหมู่ชอบน้ำ ซึ่งตรึงบนไคโทซานโดยการทำปฏิกิริยาเคมีของหมู่แอมิโนและ/หรือไฮดรอกซิล การทำให้อนุภาคที่มีหมู่ พอลิ(เอทิลีนไกลคอล)เมทิลอีเทอร์ เป็นประจุบวกของหมู่ควอเทอร์นารีแอมโมเนียมจำเป็นต้องอาศัยการทำปฏิกิริยาเมทิลเลชันแบบต่างวัฏภาค ซึ่งต่างจากอนุภาคที่มีหมู่ เอ็น-[(2-ไฮดรอกซิล-3-ไทรเมทิลแอมโมเนียม)]โพรพิล พิสูจน์ยืนยันโครงสร้างทางเคมีของอนุพันธ์แอมฟิฟิลิกไคโทซานด้วยโปรตอนเอ็นเอ็มอาร์และเอฟที-ไออาร์สเปกโทรสโกปี จากการทดลองพบว่าสัดส่วนการแทนที่สัมพัทธ์ของแต่ละหมู่ฟังก์ชันแปรเปลี่ยนได้ตามสัดส่วนโดยโมลของรีเอเจนต์ที่ใช้ในแต่ละขั้น ควอเทอร์ไนซ์แอมฟิฟิลิกไคโทซานแสดงค่าความเข้มข้นวิกฤติในการเกิดไมเซลล์ในช่วง 7-17 ไมโครกรัม/มิลลิลิตร จากการวิเคราะห์ด้วยเอสอีเอ็มพบว่าอนุภาคมีลักษณะเป็นทรงกลม มีขนาดอยู่ในช่วง 100-300 นาโนเมตรซึ่งไม่ขึ้นกับสัดส่วนการแทนที่ และอนุภาคที่มีหมู่เฮกซิลและโดเดกซิลเป็นหมู่ไม่ชอบน้ำ และหมู่เอ็น-[(2-ไฮดรอกซิล-3-ไตรเมทิลแอมโมเนียม)]โพรพิล เป็นหมู่ชอบน้ำที่มีสัดส่วนของการเกิดควอเทอร์ไนเซชันมากกว่า 50 เปอร์เซนต์เท่านั้นที่แสดงประจุเป็นบวกในสัดส่วนสูง

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต.....

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5172336223 : MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
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NADDA BOORANABUNYAT: QUATERNIZED CHITOSAN PARTICLES PREPARED BY SELF-ASSEMBLY OF AMPHIPHILIC CHITOSAN. ADVISOR: ASSOC. PROF. VORAVEE P. HOVEN, Ph.D., 66 pp.

Quaternized chitosan particles were prepared by self assembly of amphiphilic chitosan having phthaloyl (Ph), hexyl (He) or dodecyl (Do) groups as hydrophobic entity and poly(ethylene glycol)methyl ether (mPEG) or *N*-[(2-hydroxyl-3-trimethylammonium)]propyl (HTAP) groups as hydrophilic entity that were immobilized on chitosan via chemical reactions of amino and/or hydroxyl groups. Unlike the chitosan particles having HTAP moieties, a subsequent heterogeneous methylation was necessary to introduce positively charged quaternary ammonium groups to the particles having mPEG moieties. Chemical structures of all amphiphilic chitosan derivatives were verified by ^{1}H NMR and FT-IR spectroscopy. The relative degree of substitution (%DS) of each functionality can be varied as a function of mole equivalent of reagent used in each step of reaction. All quaternized amphiphilic chitosan exhibited a critical micelle concentration in a range of 7-17 μg/mL. According to SEM analysis, the particles have spherical morphology and a size range of 100–300 nm which is independent on %DS variation. And only the particles having He and Do as hydrophobic entities and HTAP as hydrophilic entities having degree of quaternization more than 50% exhibited high positive charges.

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

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LIST OF ABBREVIATIONS

CS	: Chitosan
CMC	: Critical micelle concentration
DCS	: Dodecylchitosan
DgM	: Dodecylchitosan grafted poly(ethylene-glycol)methyl ether terminated with carboxylic group
DgH	: Dodecylchitosan grafted *N*-[(2-hydroxyl-3-trimethylammonium)]propyl
DD	: Degree of deacetylation
DS	: Degree of substitution
DQ	: Degree of quaternization
DMF	: *N*,*N*-dimethylformamide
EDC	: 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide
FT-IR	: Fourier transform infrared spectroscopy
GTMAC	: Glycidyltrimethylammonium chloride
HCS	: Hexylchitosan
HTAP	: *N*-[(2-hydroxyl-3- trimethylammonium)]propy
HgM	: Hexylchitosan grafted poly(ethylene-glycol)methyl ether terminated with carboxylic group
HgH	: Hexylchitosan grafted *N*-[(2-hydroxyl-3-trimethylammonium)]propyl
mPEG	: Poly(ethylene glycol)methyl ether
mPEG-COOH	: Poly(ethylene glycol)methyl ether terminated carboxylic
NMR	: Nuclear magnetic resonance spectroscopy
NHS	: *N*-Hydroxysuccinimide
NMR	: Nuclear magnetic resonance spectroscopy
$NaBH_4$	: Sodium borohydride
NaOH	: Sodium hydroxide

PhCS	: Phthaloylchitosan
PCS	: Photon correlation spectroscopy
PgM	: Phthaloylchitosan grafted poly(ethylene-glycol)methyl ether terminated carboxylic group
PgH	: Phthaloylchitosan grafted *N*-[(2-hydroxyl-3-trimethylammonium)]propyl
QPgM	: Quaternized phthaloylchitosan grafted poly(ethylene-glycol)methyl ether terminated with carboxylic group
QHgM	: Quaternized hexylchitosan grafted poly(ethylene-glycol)methyl ether terminated with carboxylic group
QDgM	: Quaternized dodecylchitosan grafted poly(ethylene-glycol)methyl ether terminated with carboxylic group
SEM	: Scanning electron microscopy
TEM	: Transmission electron microscopy
ζ-potential	: Zeta-potential

CHAPTER I

INTRODUCTION

1.1 Statement of Problem

Chitosan is a natural biopolymer derived by deacetylation of chitin, an abundantly available biopolymer found in exoskeletons of insects, the shell of crustaceans, and fungal cell walls. Chitosan has a number of unique characteristics such as nontoxic, biodegradable, biocompatible and possesses antibacterial properties Chitosan shows its antibacterial activity only in acidic medium above its pKa (pH 6.5). Introducing quaternary ammonium groups to chitosan via quaternization of amino groups has been recognized as a potential way to enhance the antibacterial activity of chitosan in a broader pH range. In addition, it has been recently demonstrated that chitosan as well as quaternized chitosan in the form of nanoparticles exerted higher antibacterial activity than chitosan solution because the greater surface area of the particles provides intimate contact with the surface of bacterial cells. Our recent work suggested that surface-quaternized chitosan particles have a great potential as alternative antibacterial fillers. However, relative low yield was obtained via ionic crosslinking route used.

In this research, we have proposed to generate chitosan particles having positive charges of quaternary ammonium groups by self-assembly of amphiphilic chitosan. It is believed that this route should be a more effective way to generate chitosan particles with high yield and well control over particle size and charge. Phthaloyl (Ph), hexyl (He) or dodecyl (Do) groups as hydrophobic entities and poly(ethylene glycol)methyl ether (mPEG) or *N*-[(2-hydroxyl-3-trimethylammonium)]propyl (HTAP) as hydrophilic entities were immobilized on chitosan backbone via chemical reactions of amino and hydroxyl groups, respectively. Formation of particle was then induced by self-assembly of the resulting amphiphilic chitosan derivatives upon dialysis in water. Unlike the chitosan particles having HTAP moieties, a subsequent heterogeneous methylation was necessary to introduce positively charged quaternary ammonium groups to the chitosan particles having mPEG moieties. Moreover, effects

of molecular weight of chitosan, type of hydrophobic/hydrophilic groups as well as relative %DS of hydrophobic/hydrophilic groups on the particle size/morphology will be determined. A series of characterization techniques, namely Fourier transformed infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (^{1}H NMR), photon correlation spectroscopy (PCS), scanning electron micrpscope (SEM) transmission electron microscopy (TEM) and critical micelle concentration (CMC) were employed to confirm the success of amphiphilic chitosan particle formation.

1.2 Objectives

To synthesize and characterize the amphiphilic chitosan particles containing quaternary ammonium groups formed by self-assembly

1.3 Scope of the Investigation

1.3.1 Attachment of hydrophobic entities to chitosan

- Synthesis of phthaloylchitosan (PhCS), hexylchitosan (HCS), and dodecylchitosan (DCS)

1.3.2 Preparation of amphiphilic chitosan particles

- Amphiphilic chitosan particles having mPEG as hydrophilic entity
- Quaternization of amphiphilic chitosan particles having mPEG as hydrophilic entity
- Amphiphilic chitosan particles having *N*-[(2-hydroxyl-3-trimethylammonium)]propyl (HTAP) as hydrophilic entity

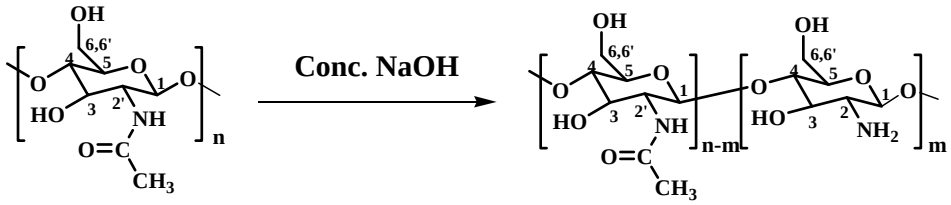
1.3.3 Characterization of amphiphilic chitosan particles

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Chitin and Chitosan

Chitosan can be prepared by alkaline *N*-deacetylation of chitin, the second most abundant polysaccharide found on earth next to cellulose. Chitin is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi. Chitosan is a linear polysaccharide composed of randomly distributed *β*-(1-4)-linked *D*-glucosamine (deacetylated unit) and *N*-acetyl-*D*-glucosamine (acetylated unit). The solubility of chitin is remarkably poorer than that of cellulose, because of the high crystallinity of chitin, supported by hydrogen bonds mainly through the acetamido group [1]. A common method for the synthesis of chitosan is the deacetylation of chitin using sodium hydroxide in excess as a reagent and water as a solvent [2]. Chitosan has abundant of hydroxyl groups (C-6 and C-3) and highly reactive amino group (at C-2). Chemical structures of chitin and chitosan are shown in Scheme 2.1. The degree of deacetylation (%DD) in commercial chitosan is in the range 60-100 %. The amino group in chitosan has a pKa value of ~6.5, which leads to a protonation in acidic to neutral solution with a charge density dependent on pH and the degree of acetylation value (%DA).



Chitin **Chitosan**

Scheme 2.1 Structures of chitin and chitosan

Chitosan is biodegradable, biocompatible, non-toxic, non allergenic, renewable biomaterials. The applications of chitosan include uses in a variety of areas, such as pharmaceutical and medical applications, cosmetics, biotechnology,

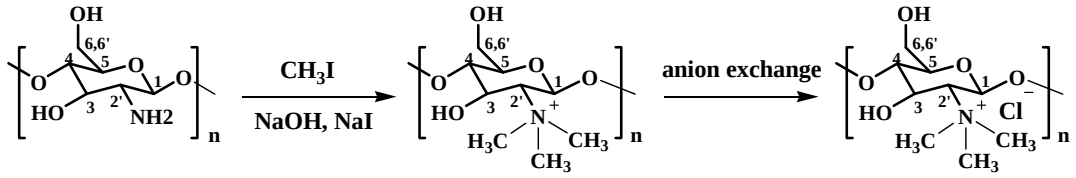
waste water treatment, textiles, paper production, food processing, and agriculture. [3-4]. However, many applications of chitosan are in neutral or basic medium that chitosan is not soluble especially those in medicine, cosmetics and food. To improve the solubility property of chitosan, the reactive amino groups of chitosan can be modified to achieve derivatives that are water soluble and can be used in various applications [1, 5-6].

2.2 Chitosan Derivatives

The applications of chitosan are limited due to its limited solubility. From this reason, many researchers have attempted to modify the functional group of chitosan to make it soluble in a wider pH range. Chitosan has three reactive groups, i.e., primary (C-6) and secondary (C-3) hydroxyl groups on each repeat unit and the amino (C-2) group on each deacetylated unit. These reactive groups are readily subject to chemical modification to alter mechanical and physical properties and solubilities of chitosan. The chemical modification of chitosan side chain at the hydroxyl and amino positions have been widely used because the modification should not change the fundamental skeleton of chitosan, and the original physicochemical and biochemical properties should be remained. In general, the nonbonding pair of electrons on the nitrogen atoms of amine groups as well as on oxygen atoms of the hydroxyl groups also make chitosan a potent nucleophile. Not only chemical functionalization improve the solubility of chitosan, most often it can also bring about new or improved properties to chitosan. The hydrophobic behavior is based on the presence of both the main polysaccharide backbone and the *N*-acetyl groups at C-2 position [7].

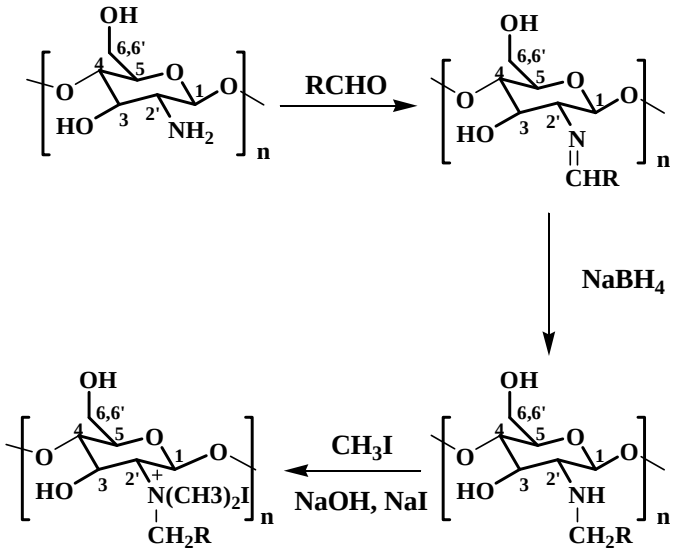
2.2.1 Quaternized Chitosan Derivatives

In 1985, Domard *et al*. [8] synthesized chitosan derivative having a quaternary ammonium salt, *N*,*N*,*N*-trimethyl chitosan chloride (TMC). The reaction was performed by reaction of a low acetyl content chitosan with methyl iodide and sodium hydroxide under controlled conditions (Scheme 2.2). The reaction yielded TMC with various degrees of quaternization. Furthermore, TMC can be soluble in water.



Scheme 2.2 Synthesis of *N*,*N*,*N*-trimethyl chitosan chloride (TMC)

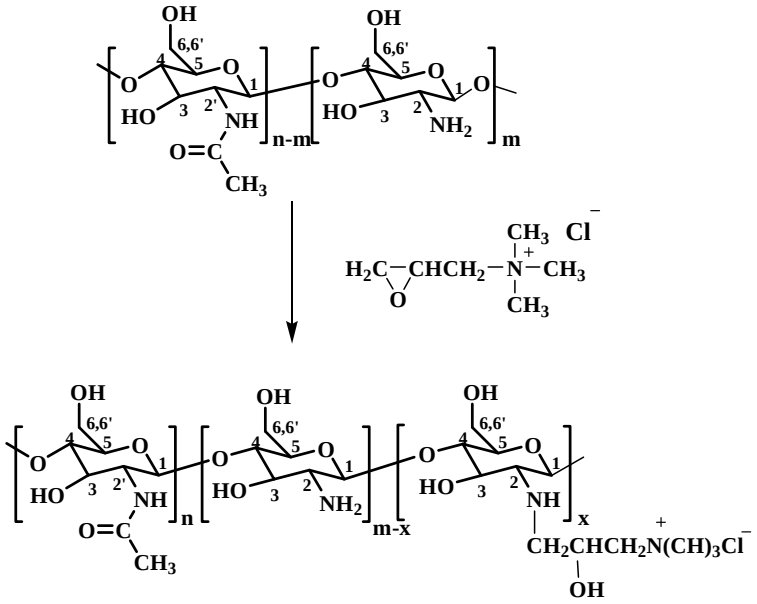
Aldehydes are generally used for protecting via Schiff's base formation on the amino groups of chitosan and to allow hydroxyl groups to be modified. In 2001, Jia *et al*. [9] prepared water soluble chitosan derivatives with quaternary ammonium salt, *N*,*N*,*N*-trimethyl chitosan, *N*-propyl-*N*,*N*-methyl chitosan and *N*-furfuryl-*N*,*N*-dimethyl chitosan (Scheme 2.3). These derivatives were prepared by reductive alkylation of amino groups of chitosan followed by methylation with methyl iodide. It was found that antibacterial activity of the quaternized chitosan against *E*. *coli* is stronger than chitosan.



Scheme 2.3 Synthesis of quaternized *N*-alkyl chitosan

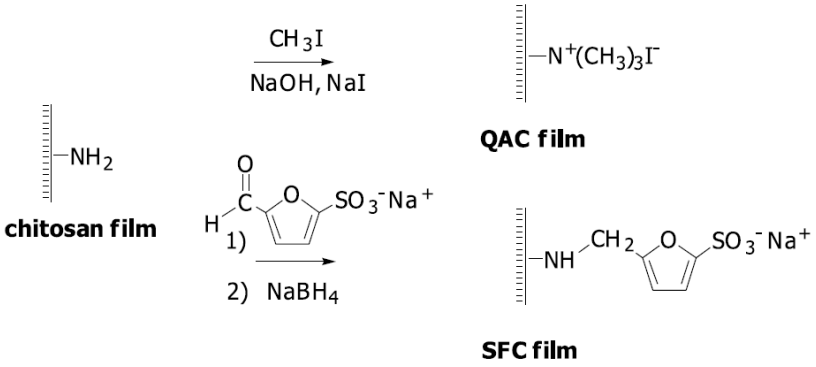
In 2000, Seong *et al.* [10] synthesized *N*-(2-hydroxyl)propyl-3-trimethyl ammonium chitosan chloride (HTACC) using a reaction of GTMAC (Scheme 2.4) and chitosan. The complete substitution of -NH_2 in chitosan with GTMAC was achieved when the reaction was performed at 80°C for 18 h with a 4:1 mole ratio of GTMAC to –NH_2 in the presence of acetic acid. HTACC showed superior

antimicrobial activity to chitosan due to the quaternary ammonium group from the substitution of NH_2 in chitosan with GTMAC.



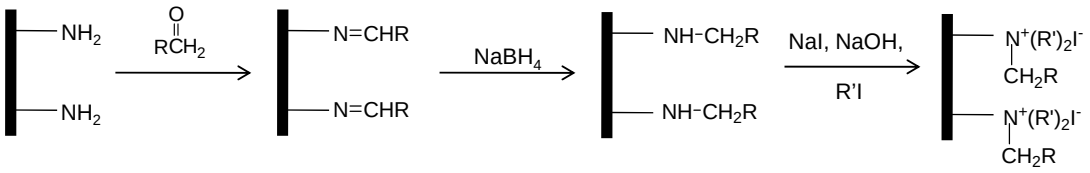
Scheme 2.4 Synthesis of *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC) from chitosan

In 2007, Hoven *et al*. [11] surface-functionalized chitosan film with positive and negative charges via methylation using methyl iodide and reductive alkylation using 5-formyl-2-furan sulfonic acid (Scheme 2.5). The chitosan film having negative charges of *N*-sulfofurfuryl groups and positive charges of quaternary ammonium groups on surface demonstrated selective protein adsorption against both negatively charged proteins (albumin and fibrinogen) and positively charged proteins (ribonuclease, lysozyme).



Scheme 2.5 Introduction of charged functional groups to the surface of chitosan

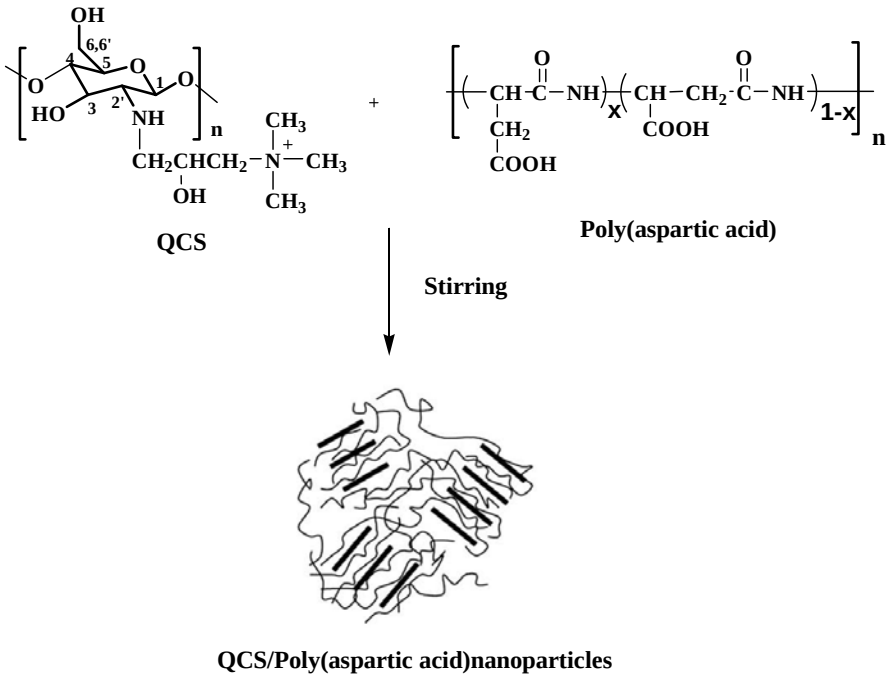
In 2006, Vallapa [12] prepared quaternary ammonium chitosan film via reductive alkylation using butyraldehyde followed by methylation with methyl iodide under heterogeneous condition (Scheme 2.6). The antibacterial activity of surface-quaternized chitosan film against *Staphylococcus aureus* and *Escherichia coli* were superior to that of the virgin chitosan film. The positive charge and hydrophobicity introduced via surface quaternization made the chitosan film more favorable substrate for interacting with the negatively charged membrane of the bacteria.



Scheme 2.6 Surface quaternization of chitosan film

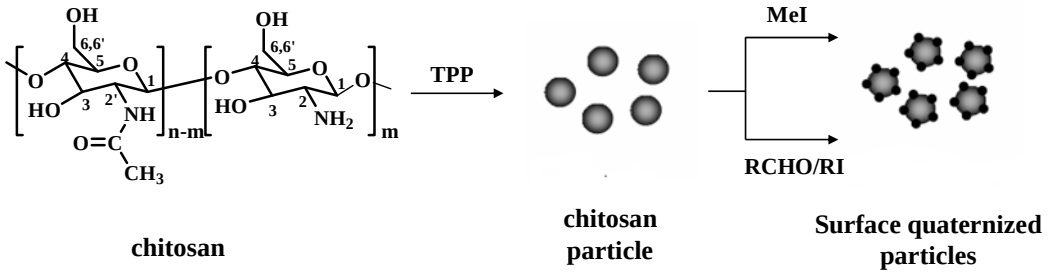
In 2009, Sajomsang *et al*. [13] synthesized quaternary ammonium chitosan containing monosaccharides or disaccharides moieties by reductive *N*-alkylation then quaternized by *N*-(3-chloro-2-hydroxypropyl) trimethyl ammonium chloride (Quat-188). It was found that the degree of *N*-substitution (DS) was in the range of 12–40% while the degree of quaternization (DQ) was in the range of 90–97%. The quaternary ammonium chitosan derivatives were water-soluble in a wide range of pH and displayed antibacterial activity against *S. aureus* and *E. coli* as determined from the minimum inhibitory concentration. The antibacterial activity decreased with increasing the DS. The low MIC values (8–32 g/mL) were obviously observed when the %DS of quaternary ammonium *N*-octyl and *N*-benzyl chitosan derivatives was lower than 18%.

In 2009, Wang *et al*. [14] prepared nanoparticles from quaternized chitosan (QCS) and poly(aspartic acid) via the ionotropic gelation technique (Scheme 2.7). The ionic gelation process was mild and involved the mixture of two aqueous phases at room temperature. The nanoparticles were spherical in shape. The nanoparticle size increased with increasing the QCS concentration. The zeta potential of QCS and QCS/poly (aspartic acid) nanoparticles had a high positive charge value.

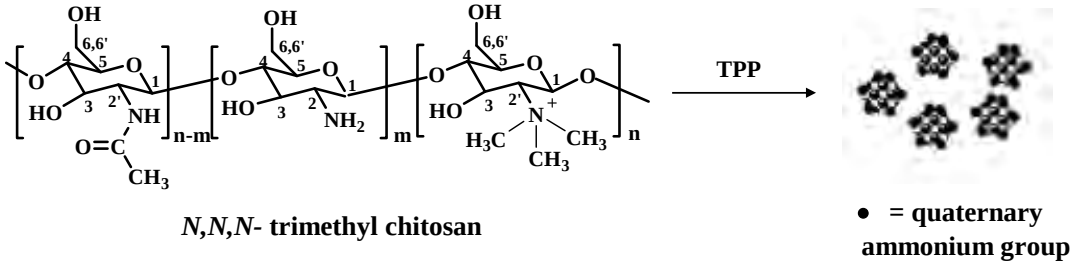


Scheme 2.7 Preparation of QCS/poly (aspartic acid) nanoparticles [14]

In 2009, Wia-rachai [15] prepared quaternary ammonium-containing chitosan particles by two methods. The first method was to prepare chitosan particles by ionic crosslinking then modified the particles by heterogeneous quaternization through *N*-reductive alkylation with aldehyde followed by alkylation with alkyl iodide (Scheme 2.8). The second method was to synthesize *N*,*N*,*N*-trimethyl chitosan by homogeneous quaternization using methyl iodide followed by particle formation by ionic crosslinking (Scheme 2.9). The particle size was in the range of 0.23-0.49 μm. The quaternized chitosan particles exhibited superior antibacterial activity against, *S. aureus*, the gram-positive bacteria, to the native chitosan particles at neutral pH.



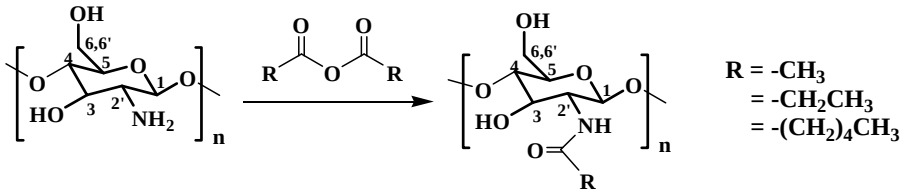
Scheme 2.8 Preparation of quaternized chitosan particles by heterogeneous quaternization of chitosan particles [15]



Scheme 2.9 Preparation of quaternized chitosan particles from TMC [15]

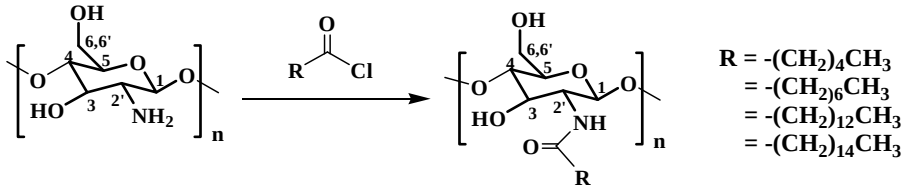
2.2.2 *N*-alkylation of Chitosan

In 2007, Hu *et al.* [16] synthesized selectively *N*-acetyl chitosan (NACS), *N*-propionic chitosan (NPCS) and *N*-hexanoyl chitosan (NHCS) under homogeneous condition (Scheme 2.10). Intramolecular aggregation of NPCS and NACS was stronger for NPCS than NACS. Hydrophobic interactions of *N*-acylated substituted chitosan with longer acyl chains was stronger. In addition, *N*-acylated chitosan with lower DD had inhibitory effect on the growth of bacteria than that with moderate DD.



Scheme 2.10 Synthesis of *N*-akylation chitosan

In 2003, Le Tien *et al*. [17] synthesized *N*-acylated chitosan with various fatty acid (C6–C16) chlorides (Scheme 2.11). Acylation with longer side chains (C8–C16) resulted in a higher degree of order. The palmitoyl chitosan which had degree of substitution in the range of 40-50% exhibied the best mechanical characteristics and drug release properties.

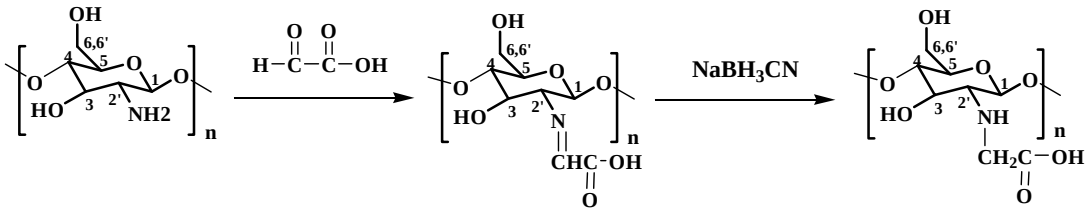


Scheme 2.11 Synthesis of chitosan derivatization with fatty acyl chlorides

In 2008, Sajomsang *et al*. [18] synthesized methylated chitosan containing different aromatic moieties by two steps, the reductive amination and the methylation. The water solubility of the methylated chitosan derivatives decreased with increasing concentration and pH. As the result, it was found that the antibacterial activity was not only dependent on the DQ but it was also dependent on the positively charged location and the molecular weight.

2.2.3 Carboxyalkylated Chitosan Derivatives

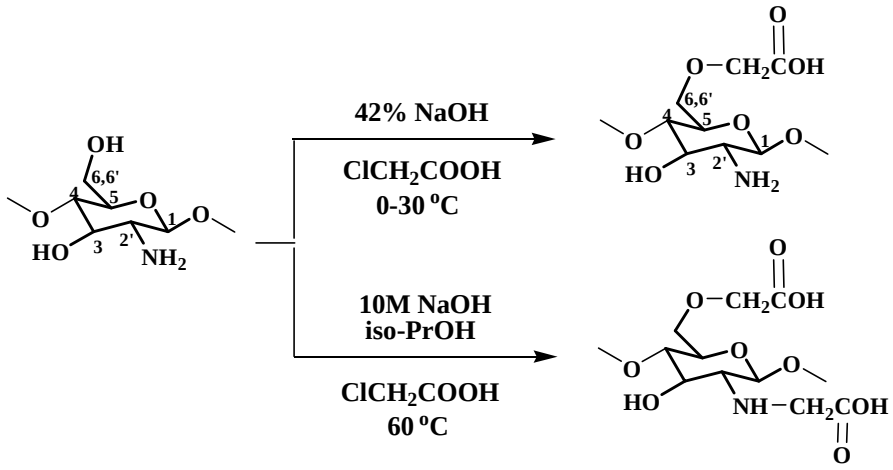
Besides, the method of synthesize chitosan derivative to improve the solubility of chitosan, is carboxyalkylation of chitosan. In 1982, Muzzarelli *et al*. [19] prepared *N*-carboxymethyl chitosan (NCMC) by treating an aqueous suspension of chitosan with glyoxylic acid followed by pH adjustment and reduction with sodium cyanoborohydride (Scheme 2.12). The NCMC was soluble in water at all pH values.



Scheme 2.12 Synthesis of *N*-carboxymethyl chitosan (NCMC)

In 2009, Tan *et al*. [20] prepared hydrogel nanoparticles using linoleic acid (LA) modified carboxymethyl chitosan (CMCS) after the sonication. Self-aggregated nanoparticles demonstrated an increased loading capacity and adriamycin (ADR) loading efficiency, decreased sustained release with an increasing ratio of the hydrophobic LA to hydrophilic CMCS. The formation of self-aggregates depends on the degree of substitution (DS), and the critical aggregation concentration (CAC) values decreased with increasing DS.

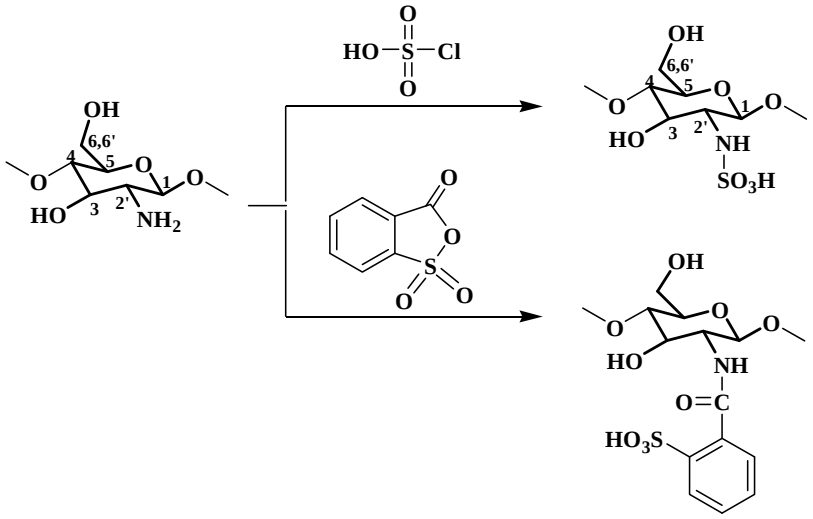
In 2001, Liu *et al*. [21] synthesized *N*,*O*-carboxymethyl chitosan (NOCMC) and *O*-carboxymethyl chitosan (OCMC) (Scheme 2.13) and tested their antibacterial activity against *E.Coli*. The NOCMC did not show antibacterial activity, whereas OCMC was more antibacterial than chitosan.



Scheme 2.13 Synthesis of *O*-carboxymethyl chitosan (OCMC) and *N*, *O*-carboxymethyl chitosan (NOCMC)

2.2.4 Chitosan Derivatives with Sulfonyl Groups

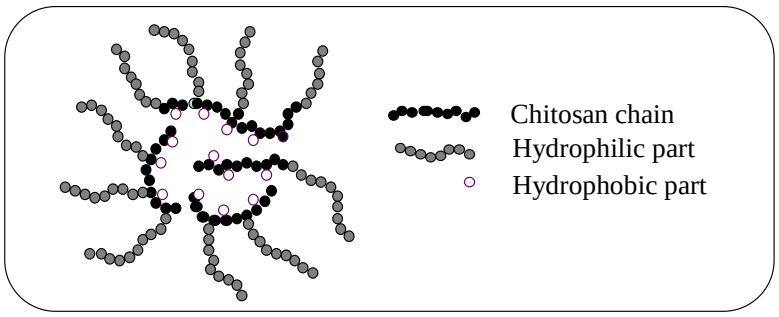
In 1998, Chen *et al*. synthesized *N*-sulfonated [22] and *N*-sulfobenzoyl [23] chitosan (Scheme 2.14). *N*-Sulfonated chitosan was prepared by a reaction between chitosan and chlorosulfonic acid in pyridine. *N*-sulfonated and *N*-sulfobenzoyl chitosan with different sulfur contents were prepared by varying the amount of chlorosulfonic acid. The antibacterial activity of both *N*-sulfonated and *N*-sulfobenzoyl was found to be superior to virgin chitosan.



Scheme 2.14 Synthesis of *N*-sulfonated and *N*-sulfobenzoyl chitosan

2.3 Self-assembly

Molecular self-assembly is a strategy for nanofabrication that involves designing molecules and supramolecular entities so that shape-complementarity causes them to aggregate into desired structures [24]. Self-assembly based on selective control of non-covalent interactions provides a powerful tool for the creation of structured systems at a molecular level [25]. These molecules automatically organize themselves into more complex and biologically useful structures, for example in cells (such as the self-assembly of the lipid bilayer membrane) and other biological systems, as well as in human engineered systems. Also, self-assembly is a manufacturing method used to construct things at the nanometer scale. Amphiphilic polymer consisting of hydrophobic entity and hydrophilic entity is known to form micelles via self-assembly. In principle, when molecule self-assemble, the hydrophobic entity would form the inner core part and the hydrophilic entities would form outer layer as corona in polar solvent. The situation would be opposite in non-polar solvent**.** Amphiphilic polymer is soluble in both organic and aqueous solvents and able to self-assemble. An example of particle formation of amphiphilic chitosan in polar solvent was shown in Scheme 2.15.



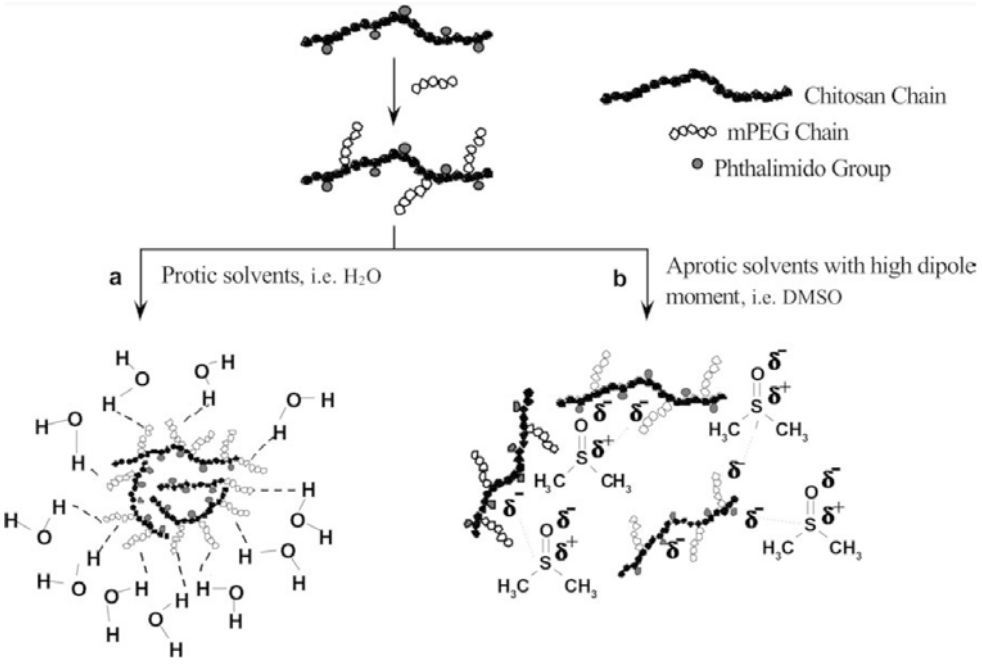
Scheme 2.15 Formation of amphiphilic chitosan [26]

At the result, self assembly is a useful and convenient method to prepare the particles for encapsulation of active ingredients such as drugs or vitamin which can be used in medical, pharmaceutical or cosmetic applications.

2.3.1 Self-assembly of Amphiphilic Chitosan

In 2004, Yoksan *et al*. [26] synthesized *N*-phthaloylchitosan grafted poly(ethylene glycol) methyl ether (mPEG) to study the colloidal phenomena and

nanosphere formation. The stability of the appearance of the milky solution was enhanced in protic solvents. The mPEG (Mn = 5,000) gave spheres with sizes about 80–100 nm, whereas mPEG (Mn = 550) provided spheres with an average size of 400–500 nm. Scheme 2.16 shows the formation of *N*-phthaloylchitosan grafted poly(ethylene glycol) methyl ether (mPEG) in protic and aprotic solvent.



Scheme 2.16 The formation of *N*-phthaloylchitosan grafted poly(ethylene glycol) methyl ether (mPEG) in protic and aprotic solvent

In 2009, Choochottiros *et al*. [27] prepared chitosan nanosphere having core–corona structure by conjugating chitosan with phthaloyl group as hydrophobic core and mPEG chain as hydrophilic corona. Degree of substitution of mPEG, having molecular weight of 2k and 5k were about 35–60% and 7–18%, respectively. The particles demonstrated negative charge in neutral and alkaline condition. The amphiphilic chitosan in various pH reflected the self-assembly phenomena which the colloidal appearances became transparent in basic condition.

In 2007, Opanasopit *et al*. [28] amphiphilic grafted copolymers, *N*-phthaloylchitosan grafted poly (ethylene glycol) methyl ether (PLC-g-mPEG), were synthesized from chitosan with different degree of deacetylation (DD = 80%, 85%, 90% and 95%). The particle sizes were in the range of 100–250 nm, and increased with increasing the %DD of chitosan. Release of camptothecin (CPT) from the

micelles was dependent on the %DD and a sustained release was obtained at high %DD.

In 2009, Liu *et al*. [29] synthesized amphiphilic chitosan grafted with polycaprolactone (PCL) and poly(ethylene glycol) methyl ether which act as hydrophobic and hydrophilic entity, respectively. The reaction was carried out by reacting phthaloyl protected chitosan with functional PCL-COOH and mPEG-COOH. The particle formation of amphiphilic chitosan was induced by dialysis in water. The particles were characterized by dynamic light scattering and transmission electron microscopy. It was found that the amphiphilic chitosan could form nano-size particles in acedic solution and stability was decreased with increasing pH of solution.

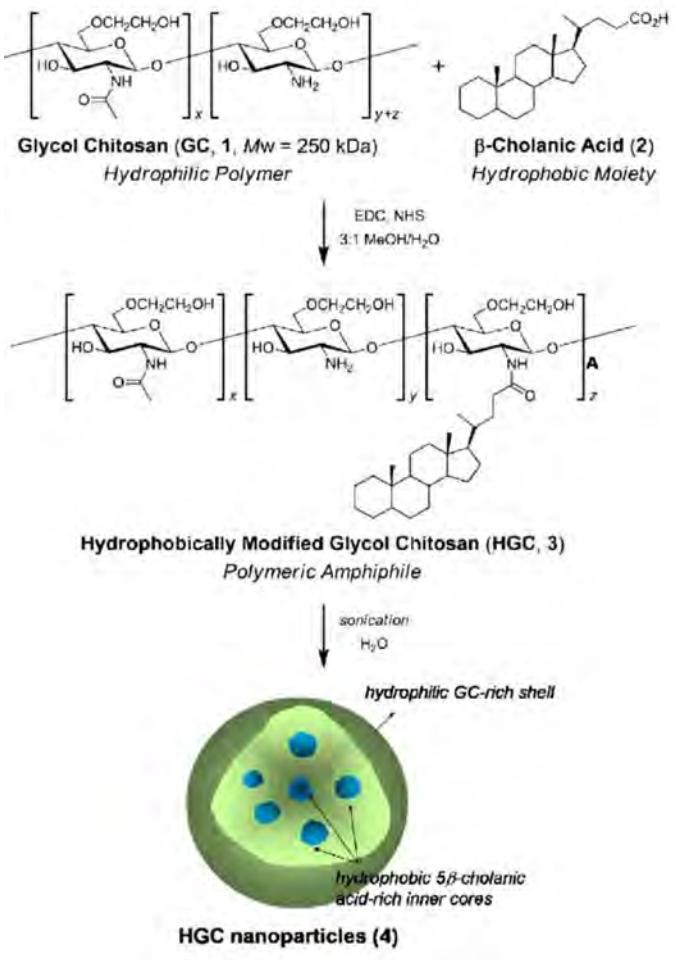
In 2008, Anumansirikul *et al*. [30] synthesized methyl ether terminated poly(ethylene) glycol)-4-methoxy cinnamoyl phthaloylchitosan (PCPLC) and methyl ether terminated poly(ethylene glycol) phthaloylchitosan (PPLC). Then the formation of particles by self-assembly and 2-ethylhexyl-4-methoxy cinnamate (EHMC) encapsulation of the particles were investigated. It was found that minimal *E* to *Z* photoisomerization was observed when EHMC was encapsulated in PCPLC particles. The results indicated that the grafted UVB absorptive chromophore, 4-methoxycinnamoyl moieties, situated at the shell of PCPLC helped protecting EHMC from photoisomerization.

In 2009, Ngawhirunpat *et al*. [31] incorporated camptothecin (CPT) in a polymeric micelle carrier system prepared from cholic acid chitosan grafted poly(ethylene glycol)methyl ether (CS-mPEG-CA). Among three incorporation methods (dialysis, emulsion and evaporation methods), an emulsion method showed the highest CPT incorporation efficiency. The mean particle sizes of bare-polymeric micelles were 93 to 120 nm whereas those loaded with CPT ranged from 150 to 390 nm.

In 2005, Kim *et al*. [32] prepared deoxycholic acid modified glycol chitosan self-aggregates by covalent attachment of deoxycholic acid to glycol chitosan (GCD). The mean sizes of self-aggregates were in the range of 245-450 nm, depending on the DS value. The critical aggregation concentration (CAC) values of the GCD conjugates decreased with the increasing the content of hydrophobic deoxycholic acid. The CAC values of the GCD conjugates were in the range of 0.038-0.260

mg/mL. The GCD of which degree of substitution was 30% maintained its self-aggregate structure in physiological conditions and its stability was maintained for 10 days.

In 2009, Nam *et al*. [33] prepared hydrophobic modified glycol chitosan (HGC) nanoparticle by a partial derivatization of the free amino groups of glycol chitosan (GC) with 5β-cholanic acid (Scheme 2.17), had a globular shape with the average diameter of 359 nm as measured by dynamic light scattering (DLS) and the zeta potential was 22 mV. Furthermore, HeLa H2B-GFP containing in HGC particles exhibited an intense fluorescence signal along the cell membrane suggesting a strong binding of HGC at the cellular surface. The cultured HeLa cells treated with HGC nanoparticles showed a full viability.



Scheme 2.17 Schematic diagram of hydrophobic modified glycol chitosan nanoparticles (HGC)

CHAPTER III

EXPERIMENTAL

3.1 Materials

Chitosan flakes (degree of deacetylation 85%, molecular weight 45 kDa and degree of deacetylation 95%, molecular weight 100 kDa) was obtained from Seafresh Chitosan (Lab) Co., Ltd. (Thailand). Phthalic anhydride (PhA), poly(ethylene glycol)methyl ether (mPEG; Mn = 5,000), succinic anhydride (SA), *N*-hydroxysuccinimide (NHS), hexanal, dodecanal and 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDC) were purchased from Aldrich (USA). Methyl iodide (MeI), sodium iodide (NaI) and glycidyltrimethylammonium chloride (GTMAC) were purchased from Fluka (Switzerland). Methanol (MeOH) and sodium hydroxide (NaOH) were purchased from Merck (Germany). *N*,*N*-Dimethyl formamide (DMF) was obtained from Carlo. All reagents and materials are analytical grade and used without further purification.

3.2 Equipments

3.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H NMR spectra were recorded in solution of CF_3COOH/D_2O, D_2O or DMSO-d_6 using a Varian, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. Chemical shifts were reported in part per million (ppm) relative to tetramethylsilane (TMS) or using the residual protonated solvent signal as a reference.

3.2.2 Fourier Transform -Infrared Spectroscopy (FT-IR)

IR spectra were collected using a Nicolet 6700 FT-IR spectrometer with 32 scans at resolution 4 cm^{-1}. A frequency of 400-4000 cm^{-1} was collected by using TGS detector. All samples were prepared as KBr pellets.

3.2.3 Scanning Electron Microscopy (SEM)

The size and the morphology of particles were examined by scanning electron microscope (SEM, Model JEOL JSM-6480LV). The average diameter was determined from measurements of 100 random particles. The size analysis was calculated using Semafore software.

3.2.4 Photon Correlation Spectroscopy (PCS)

The size and ζ-potential of chitosan particles were determined using a Nanosizer Nano-ZS (Malvern Instruments, UK). The particles (~10 mg) were first dispersed in 20 mL of Milli-Q water by sonication for 3 min prior to measurement. The analysis was performed at 25^{o}C using a scattering angle of 173^{o}**.** All data were displayed as the mean $\pm$ one standard deviation and are derived from at least three independent experiments. The data were calculated using the Helmholtz-Smoluchowski equation.

3.2.5 Critical Micelle Concentration (CMC) [34]

The CMC of amphiphilic chitosan were determined using a spectrofluorophotometer (Varian, Cary Eclipse) using pyrene as a fluorescent probe. The emission and excitation fluorescence spectra of pyrene were recorded with a micelle concentration that ranged from 0.5 to 450 µg/mL. In each experiment, pyrene in acetone solution (5µL, 6.0x10^{-7} M) was added to a 4 mL polymeric micelle solution. Then the solution was stirred for 24 h until acetone was evaporated prior to measurement. Pyrene was excited at 334 nm and its emission was recorded at 373 and 384 nm, which correspond to the first and third vibrational peaks, respectively. The ratio of fluorescence intensity at 373 and 384 nm (I_{374}/I_{384}) was calculated and plotted against the logarithm of the concentration of micelles.

3.3 Methods

3.3.1 Attachment of Hydrophobic Entities to Chitosan

3.3.1.1 Synthesis of Phthaloylchitosan (PhCS) [27]

Chitosan 1.0 g (4.8 mmol of $–NH_2$ group) was reacted with PhA (1, 3 and 5 mole equivalent to amino groups on chitosan.) in 20 mL of DMF at 110 $^{\circ}$C under nitrogen atmosphere for 6 h. The temperature was then reduced to 60°C and left overnight under nitrogen atmosphere. Dark brown solution obtained was then precipitated in cool water and vacuum dried to obtain the final product of light yellow powder.

3.3.1.2 Synthesis of Hexylchitosan (HCS) and Dodecylchitosan (DCS) [35]

Chitosan 1.0 g (4.8 mmol of $–NH_2$ group) was dissolved in 0.2 M acetic acid (70 mL). The solution was diluted with 70 mL DMF. Hexanal or dodecanal (1, 3 and 5 mole equivalent to amino groups of chitosan) was added and stirred at ambient temperature for 1 h. Then pH of the solution was adjusted to 5 by adding 0.1 M NaOH (aq). After that, $NaBH_4$ (0.38 g, 2 mole) was added to the resulting solution. The solution was stirred at ambient temperature for 24 h, followed by adjusting the pH to 7 with 0.1 M NaOH (aq). The solution was dialyzed against deionized water in a dialysis bag at ambient temperature for 4 days to remove residual small molecules. The final product was obtained after lyophilization by a freeze dryer (model Freezeone 77520 Benchtop, Labconco, USA).

3.3.2 Preparation of Amphiphilic Chitosan Particles

3.3.2.1 Synthesis of Poly(ethylene glycol)methyl ether Terminated with Carboxyl Groups (mPEG-COOH) [27]

The synthesis of mPEG (3.0 g, 0.6 mmol) was reacted with SA (1 mole equivalent to mPEG) in DMF 10 mL using pyridine as catalyst at 60°C overnight. White powder was obtained as a product after precipitation in diethyl ether and drying *in vacuo*.

3.3.2.2 Preparation of Amphiphilic Chitosan Particles having mPEG as Hydrophilic Entity

Chitosan having different hydrophobic entity obtained from section 3.3.1 (PhCS, HCS or DCS) 0.1 g was reacted with mPEG (1, 3 and 5 mole equivalent to amino groups on chitosan) in 4 mL of DMF. NHS (1 mole equivalent to amino groups of chitosan) was added and stirred until the solution became clear. Then EDC (1 mole equivalent to amino groups of chitosan) was added and the solution was stirred for 24 h. Finally, The particles were formed after dialysis the solution against deionized water for 4 days and lyophilization. The amphiphilic chitosan particles having mPEG as the hydrophilic entity and phthaloyl, hexyl and dodecyl group as the hydrophobic entity are defined as PgM, HgM, and DgM, respectively.

3.3.2.3 Quaternization of Amphiphilic Chitosan Particles having mPEG as Hydrophilic Entity

An anhydrous methanol solution (10 mL) was added into a flask containing amphiphilic chitosan particles (obtained from section 3.3.2.2) 0.05 g. NaOH (0.13 g, 3.3 mmol) and NaI 0.28 g (1.9 mmol) were added to the flask. Subsequently, the reaction mixture was stirred at 50°C for 8 h. MeI (0.4 mL, 6.4 mmol) was added twice every 4 h. The synthesized particles were isolated by centrifugation at 10,000 rpm for 30 min. Supernatant was discarded and the particles were extensively rinsed with methanol and dried *in vacuo*. The amphiphilic chitosan particles having mPEG as the hydrophilic entity and phthaloyl, hexyl and dodecyl group as the hydrophobic entity obtained after quaternization are defined as QPgM, QHgM, and QDgM, respectively.

3.3.2.4 Preparation of Amphiphilic Chitosan Particles having *N*-[(2-hydroxyl-3-trimethylammonium)]propyl (HTAP) as Hydrophilic Entity

Chitosan having different hydrophobic entity obtained from section 3.2.1 (PhCS, HCS or DCS) 0.2 g was dissolved in 4 mL DMF at ambient temperature. Then pH of the solution was adjusted to 9 and 5 by adding 0.1 M

NaOH (aq). GTMAC (1, 3 and 5 mole equivalent to amino groups on chitosan) was added and the reaction was stirred at 70°C for 24 h. The particles were formed after dialysis the solution against deionized water for 4 days and lyophilization. The amphiphilic chitosan particles having HTAP as the hydrophilic entity and phthaloyl, hexyl and dodecyl group as the hydrophobic entity are defined as PgH, HgH, and DgH, respectively.

CHAPTER IV

RESULTS AND DISCUSSION

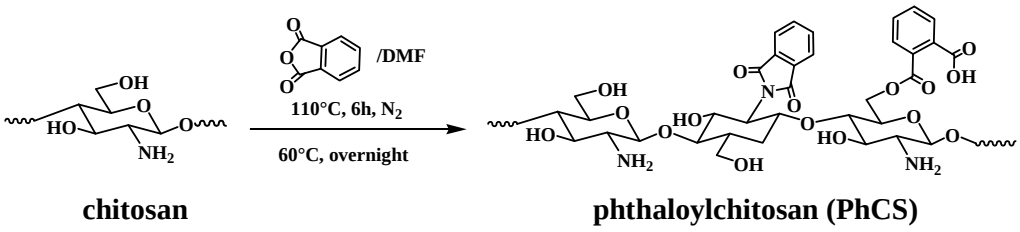
This research focused on the synthesis of amphiphilic chitosan. The synthesized method was divided into three steps. In the first step, the hydrophobic entities, phthaloyl groups (Ph), hexyl groups (He) or dodecyl groups (Do), were grafted via chemical reactions of amino groups on chitosan backbone. The hydrophilic entities, poly(ethylene glycol)methyl ether (mPEG) or *N*-[(2-hydroxyl-3-trimethylammonium)]propyl (HTAP), were subsequently immobilized to hydroxyl groups on chitosan backbone. Unlike the derivatives grafted with HTAP, the amphiphilc chitosan having mPEG as hydrophilic entity, the quaternary ammonium groups were later introduced via methylation of the unreacted amino groups. Effects of chitosan molecular weight, equivalent and type of reagent used for introducing hydrophobic and hydrophilic entities on degree of substitution and formation of amphiphilic chitosan particles were studied. The amphiphilic chitosan particles were characterized by nuclear magnetic resonance spectroscopy (NMR), scanning electron microscopy (SEM), transmission electron microscopy (TEM), fourier transform infrared spectroscopy (FTIR), and photon correlation spectroscopy (PCS).

4.1 Attachment of Hydrophobic Entities to Chitosan

Chitosan samples having molecular weight of 45 and 100 kDa were chosen for synthesis. %DS of each functional group was determined by ^{1}H NMR.

4.1.1 Phthaloylchitosan (PhCS)

Phthaloyl groups (Ph) were immobilized on chitosan backbone via ring opening of PhA by amino and hydroxyl groups of chitosan. [36] (Scheme 4.1).



Scheme 4.1 Synthesis of phthaloylchitosan (PhCS)

Figure 4.1 shows the ^{1}H NMR spectra of the synthesized PhCS in comparison with chitosan. The signals of aromatic protons at 7.2-8.2 ppm can be assigned to phthaloyl groups. Degree of substitution of phthaloyl groups (%DS_{Ph}) on chitosan was calculated from the relative ratio between the peak integration of protons from the aromatic protons at 7.2-8.2 ppm and integral intensities of H-2 from chitosan at 2.9 ppm (equation. 4.1).

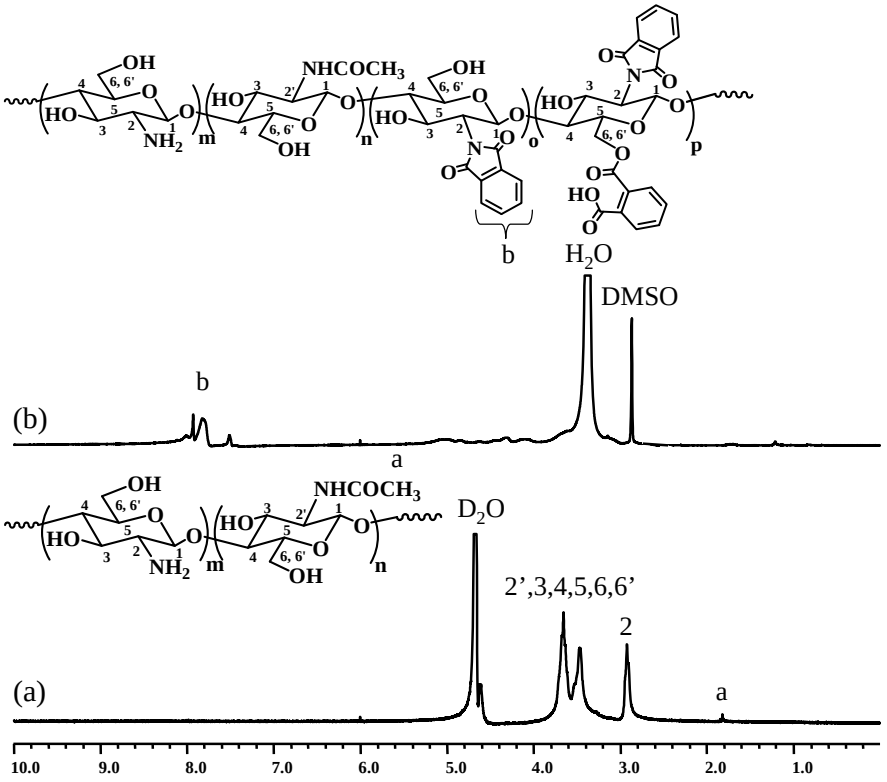


Figure 4.1 ^{1}H NMR spectra of (a) chitosan (D_2O) and (b) phthaloylchitosan (PhCS) (DMSO-d_6)

$$\%\, DS_{Ph} = \left\{ \frac{\text{intergral of the } C_6H_4 / 4}{\text{integral of the H - 2 / 1}} \right\} \times 100 \qquad (4.1)$$

The structure of PhCS could also be verified by FT-IR analysis. The spectrum is displayed in Figure 4.2. As compared with the FT-IR spectrum of chitosan, a new peak at 721 cm^{-1} which can be assigned to the –CH deformation out of plane of aromatic rings was found on the FT-IR spectrum of PhCS. In addition, a decrement of N-H bending of primary amine in chitosan at 1,598 cm^{-1} and an emergence of a peak at 1,710 cm^{-1} and 1,770 cm^{-1} assigned to C=O stretching of cyclic imide were also observed. Upon increasing the mole of PhA equivalent to amino groups on chitosan from 1 to 3 and 5, $\%DS_{Ph}$ were in the range from 80.2% to 73.4 and 72.8 and from 51.8 to 85.5 and 85.2%, for chitosan having molecular weight of 45 and 100 kDa, respectively. Presumably, a relative high $\%DS_{Ph}$ was achieved because the phthaloyl groups were capable of substituting at both amino and hydroxyl positions on chitosan backbone [27].

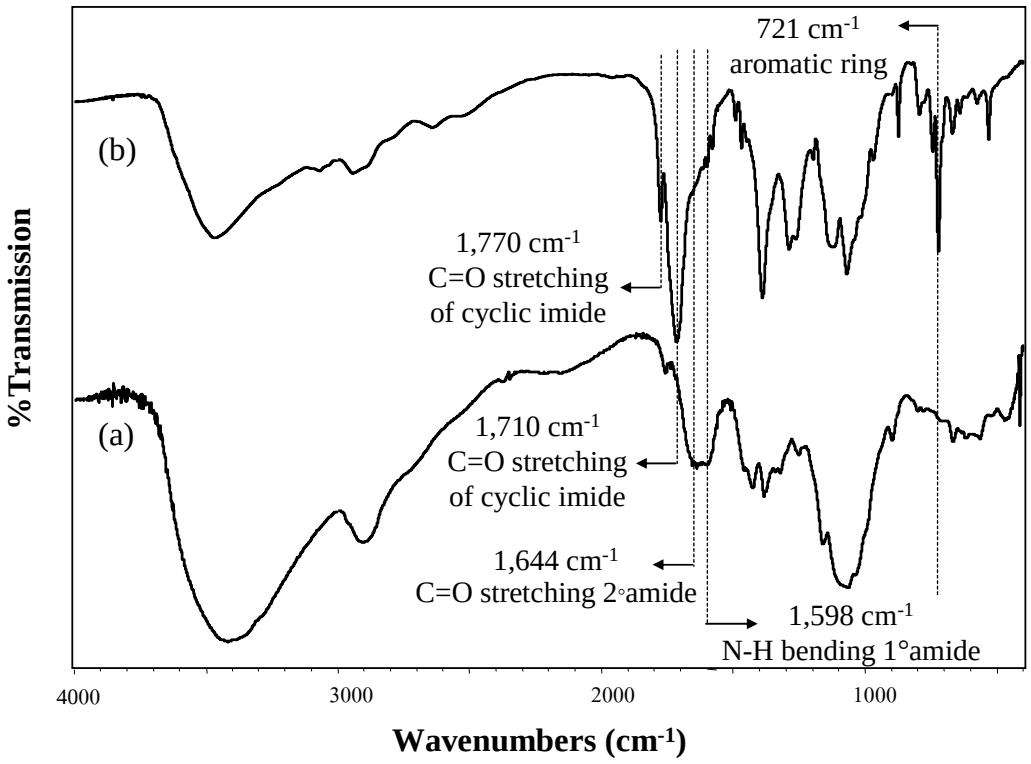
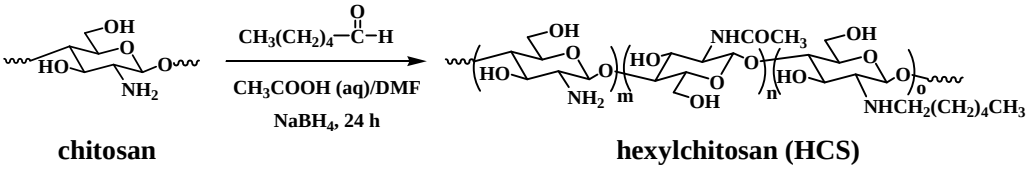


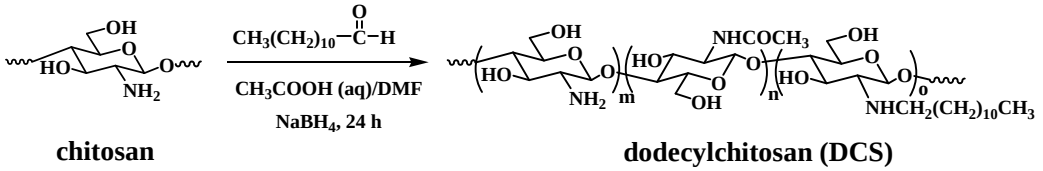
Figure 4.2 FT-IR spectra of (a) chitosan and (b) phthaloylchitosan (PhCS)

4.1.2 Hexylchitosan (HCS) and Dodecylchitosan (DCS)

Hexyl groups (He) (Scheme 4.2) and dodecyl groups (Do) (Scheme 4.3) were immobilized on chitosan backbone via *N*-reductive alkylation of *N*-hexanal and *N*-dodecanal, respectively [35].



Scheme 4.2 Synthesis of hexylchitosan (HCS)



Scheme 4.3 Synthesis of dodecylchitosan (DCS)

Figure 4.3 shows ^{1}H NMR spectra of HCS (b) and DCS (c). The signals corresponding to methylene and methyl protons appeared at 1.2 and 0.8 ppm, respectively. Degree of substitution of hexyl groups (%DS_{He}) and degree of substitution of dodecyl groups (%DS_{Do}) were calculated from the relative ratio between the peak integration of protons from the methyl protons at 0.8 ppm and integral intensities of H-2 at 2.9 ppm from chitosan (equation 4.2). Upon increasing the mole of *N*-hexanal equivalent to amino groups on chitosan from 1 to 3 and 5, %DS_{He} was increased from 38.0 to 46.3 and 65.3% and from 55.8 to 51.0 and 66.7% for chitosan having molecular weight of 45 and 100 kDa, respectively. In the case of DCS, %DS_{Do} was increased from 53.7 to 72.3 and 83.3% and from 20.3 to 41.0 and 55.2%, for chitosan having molecular weight of 45 and 100 kDa, respectively with increasing the mole of *N*-dodecanal equivalent to amino groups on chitosan from 1 to 3 and 5.

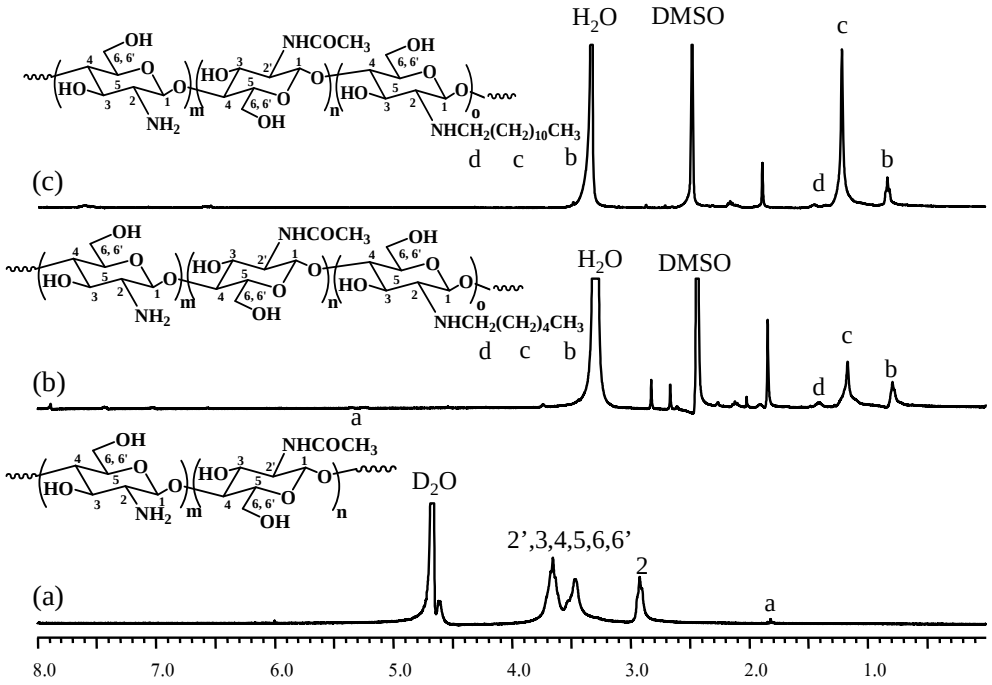


Figure 4.3 ^{1}H NMR spectra of (a) chitosan (D_2O), (b) hexylchitosan (HCS) and (c) dodecylchitosan (DCS) (DMSO-d_6)

$$\% DS_{HCS,DCS} = \left\{ \frac{\text{intergral of the } CH_3 / 3}{\text{integral of the H - 2 / 1}} \right\} \times 100 \qquad (4.2)$$

FT-IR analysis of HCS and DCS was conducted in comparison with these of chitosan. As shown in Figure 4.4, the C-H bending signals of $–CH_3$ and $–CH_2–$ groups from alkyl chains of hexyl or dodecyl groups appeared at the wavenumber of 1,380 and 1,466 cm^{-1}, respectively together with a strong C-H stretching signal at 2,950 cm^{-1} in the spectra of both HCS and DCS. Upon hexyl or dodecyl substitution, a signal from N-H bending at 1,598 cm^{-1} from chitosan disappeared whereas a signal from C=O stretching at 1,644 cm^{-1} still remained.

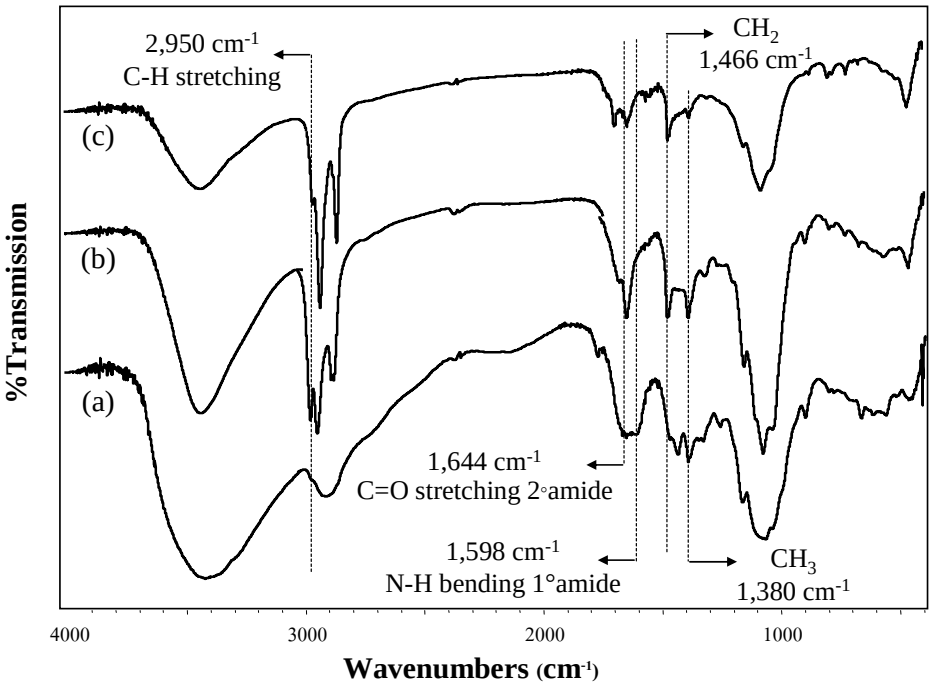


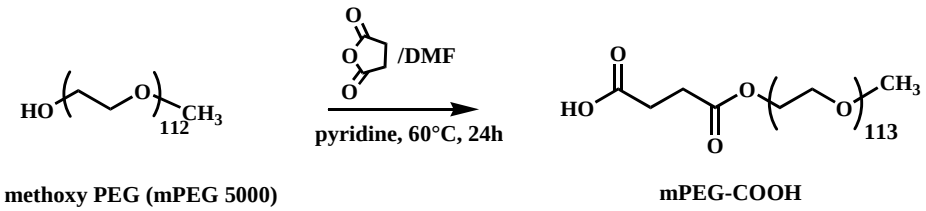
Figure 4.4 FT-IR spectra of (a) chitosan, (b) hexylchitosan (HCS) and (c) dodecylchitosan (DCS)

4.2 Preparation of Amphiphilic Chitosan Particles

Poly(ethylene glycol)methyl ether (mPEG, Mn =5000) cannot directly react with hydroxyl groups of chitosan unless being functionalized with carboxyl group.

4.2.1 Synthesis of Poly(ethylene glycol)methyl Ether Terminated with Carboxyl Group (mPEG-COOH)

Carboxyl-terminated poly(ethylene glycol)methyl ether (mPEG-COOH) was synthesized by ring opening of succinic anhydride by hydroxyl group at the chain end of mPEG-COOH (Scheme 4.4). ^{1}H NMR spectrum of mPEG-COOH is illustrated in Figure 4.5. There were peaks belonging to methylene protons of succinic anhydride and mPEG appearing at 2.5 and 3.4 ppm, respectively indicating the success of mPEG-COOH synthesis.



Scheme 4.4 Synthesis of mPEG-COOH

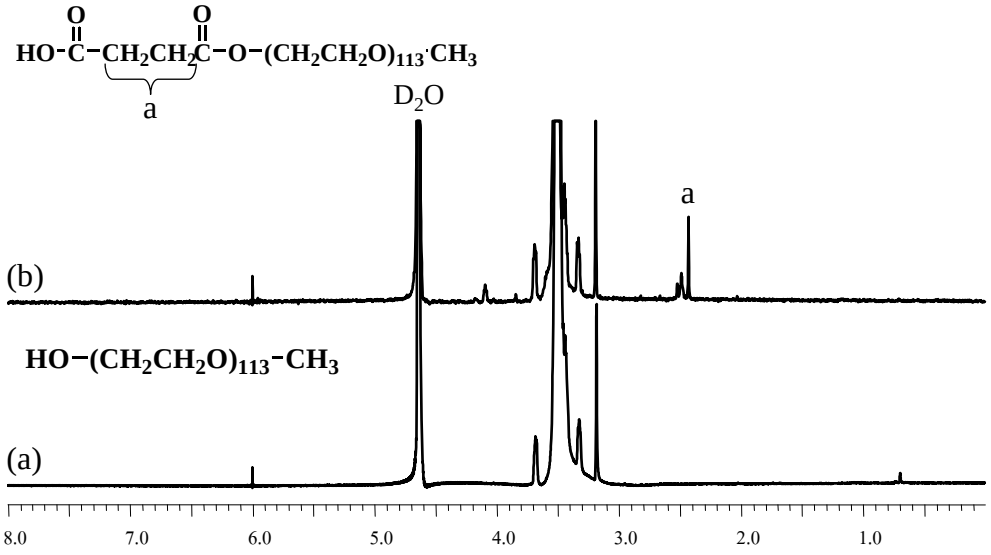


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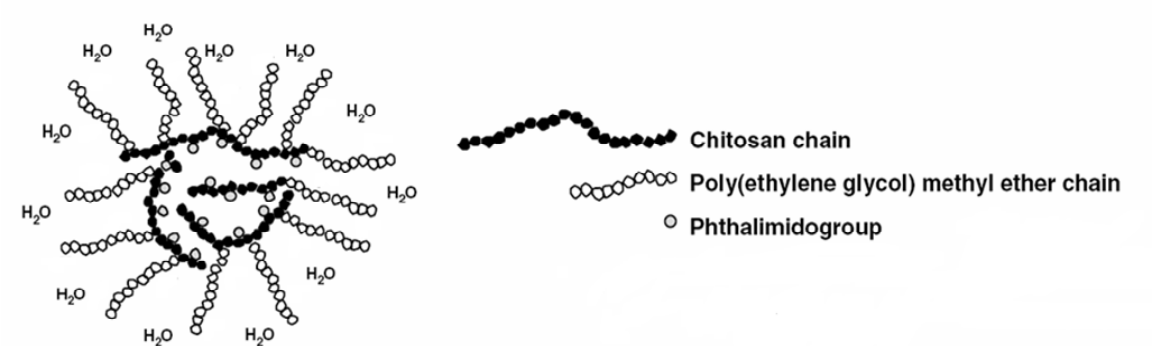
Figure 4.5 ^{1}H NMR spectra (D_2O) of (a) mPEG and (b) mPEG-COOH

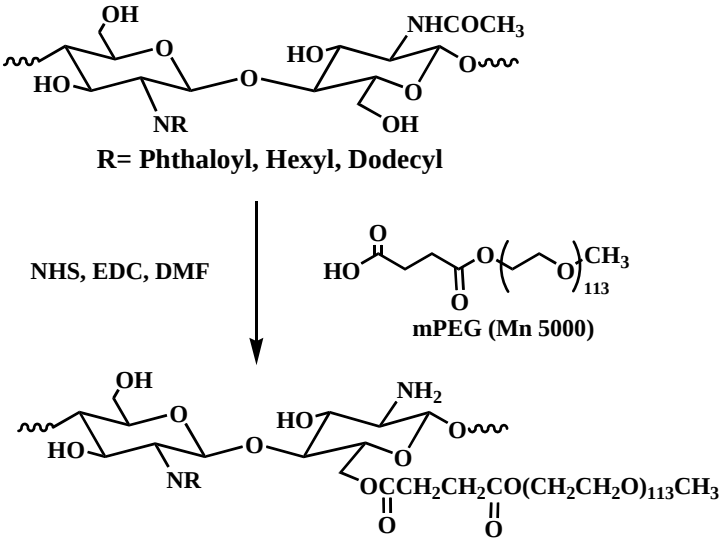
4.2.2 Preparation of Amphiphilic Chitosan Particles having mPEG as Hydrophilic Entities (PgM, HgM and DgM)

In this research, amphiphilic chitosan having mPEG as hydrophilic entity was obtained by reacting chitosan previously functionalized with hydrophobic groups, PhCS, HCS and DCS with mPEG-COOH using EDC/NHS as coupling agents to form ester linkages. The mole of mPEG-COOH equivalent of amino groups on chitosan was varied from 1 to 3 and 5 mole. Particles can be simultaneously formed during purification of the synthesized amphiphilic chitosan via dialysis in water. It is known that self-assembly of amphiphilic chitosan in polar solvent would form particles having hydrophobic entities locate inside the core of the particles whereas the hydrophilic entities would situate at the periphery of the particles as shown in Scheme

4.5. The resulting amphiphilic particles having mPEG as hydrophilic entities that were prepared from PhCS, HCS and DCS are designated as PgM, HgM and DgM, respectively (Scheme 4.5).



Scheme 4.5 Amphiphilic chitosan particles having mPEG as hydrophilic entity formed by self-assembly in polar solvent [37]



Scheme 4.6 Synthesis of PgM, HgM and DgM

Figure 4.6 shows ^{1}H NMR spectra of chitosan, PgM, HgM and DgM. All spectra exhibited a multiplet signal at 4.2-3.2 ppm belonging to H 2',3, 4, 5, 6 and 6', a signal at 2.9 and 1.9 ppm assigned to the H-2 proton and *N*-acetyl proton of glucosamine ring, respectively. They are characteristic peaks of chitosan. A signal corresponding to aromatic protons of phthaloyl groups appeared at 7.2-8.0 ppm in the

spectrum of PhCS. Signals at chemical shift of 0.8 and 1.2 ppm which belonged to -CH_3 and -CH_2- protons of hexyl and dodecyl groups were observed on the spectra of HgM and PgM. Signals at chemical shift of 3.3 and 2.6 ppm can be assigned to methylene protons of mPEG and succinic anhydride, respectively. The degree of substitution of mPEG (%DS_{mPEG}) in the structure of PgM HgM and DgM can be determined from the relative ratio between the integration of 4 protons from mPEG and peak integration of 1 proton of H-2 at 2.9 ppm from chitosan (equation 4.3).

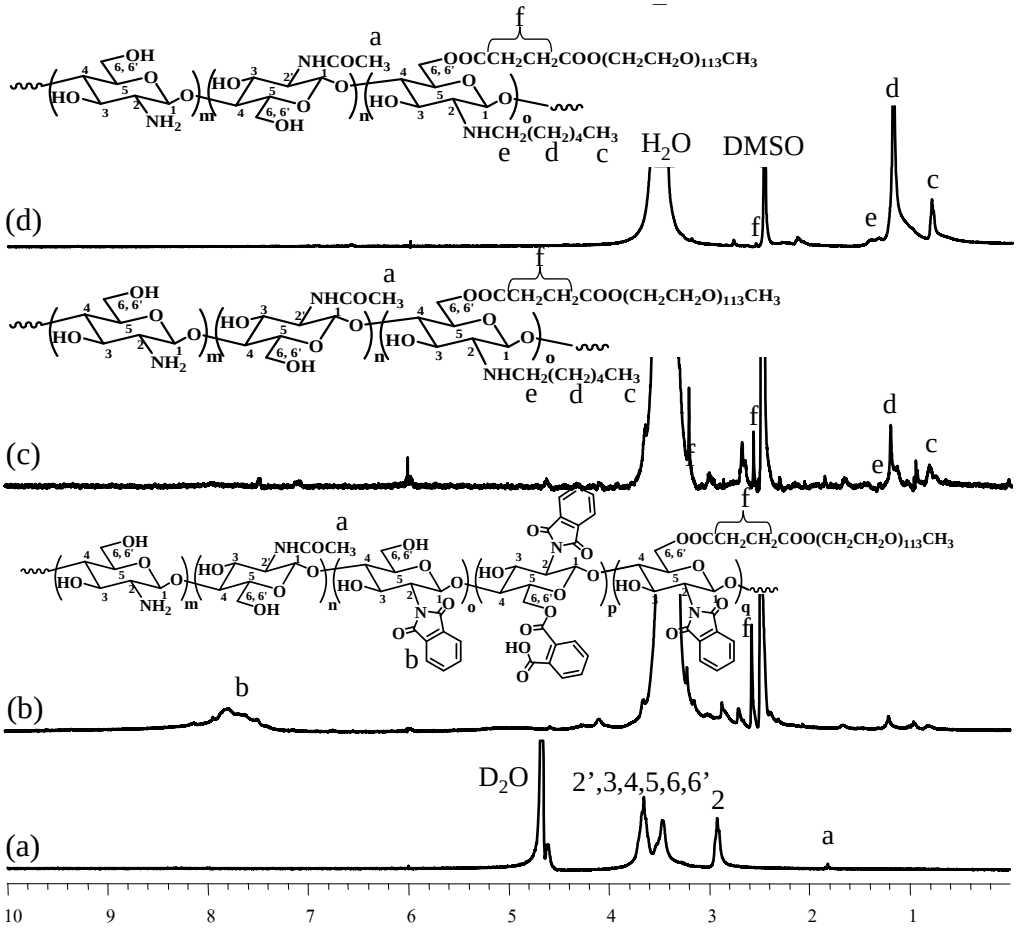
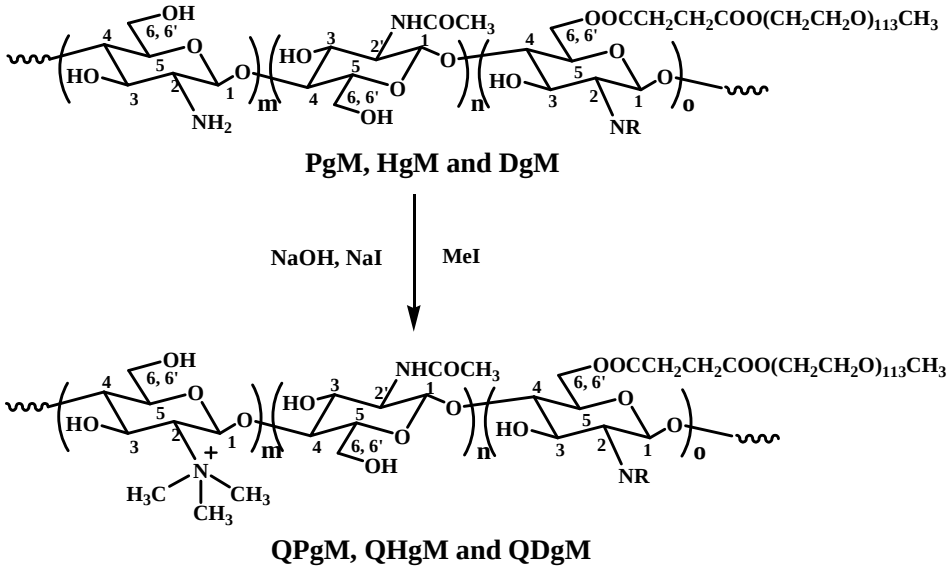


Figure 4.6 ^{1}H NMR spectra of (a) chitosan (D_2O) and amphiphilic chitosan particles having mPEG as hydrophilic entities (DMSO-d_6): (b) PgM, (c) HgM and (d) DgM

$$\%\, DS_{mPEG} = \left\{ \frac{\text{intergral of the OOCC}\underline{H}_2\text{C}\underline{H}_2\text{COO} / 4}{\text{integral of the H-2} / 1} \right\} \times 100 \qquad (4.3)$$

4.2.3 Quaternization of Amphiphilic Chitosan Particles having mPEG as Hydrophilic Entities



Scheme 4.7 Quaternization of amphiphilic chitosan having mPEG as hydrophilic entities by methylation with methyl iodide

To introduce positive charges to the structure of amphiphilic chitosan having mPEG as hydrophilic entities, PgM, HgM, and DgM were quaternized by methylation with methyl iodide (MeI) and yielded quaternized amphiphilic chitosan particles which can be assigned to QPgM, QHgM, and QDgM, respectively (Scheme 4.7). Under alkaline condition, the methylation preferentially occurs at amino groups than the hydroxyl groups. HI, the by-product from the reaction was neutralized by NaOH. The reaction could be promoted by excessive addition of NaI [38]. The success of quaternization can be confirmed by ^{1}H NMR analysis. As demonstrated in Figure 4.7, a signal at 3.2-3.4 ppm assigned to the methyl protons of the quaternary ammonium group can be found in all spectra except that of chitosan. The degree of quaternization (%DQ) of QPgM, QHgM and QDgM can be determined from the relative ratio between the integration of 9 protons from 3 methyl groups ($-N^{+}(CH_3)_3$) and the peak integration of 1 proton of H-2 at 2.9 ppm from chitosan using equation 4.4.

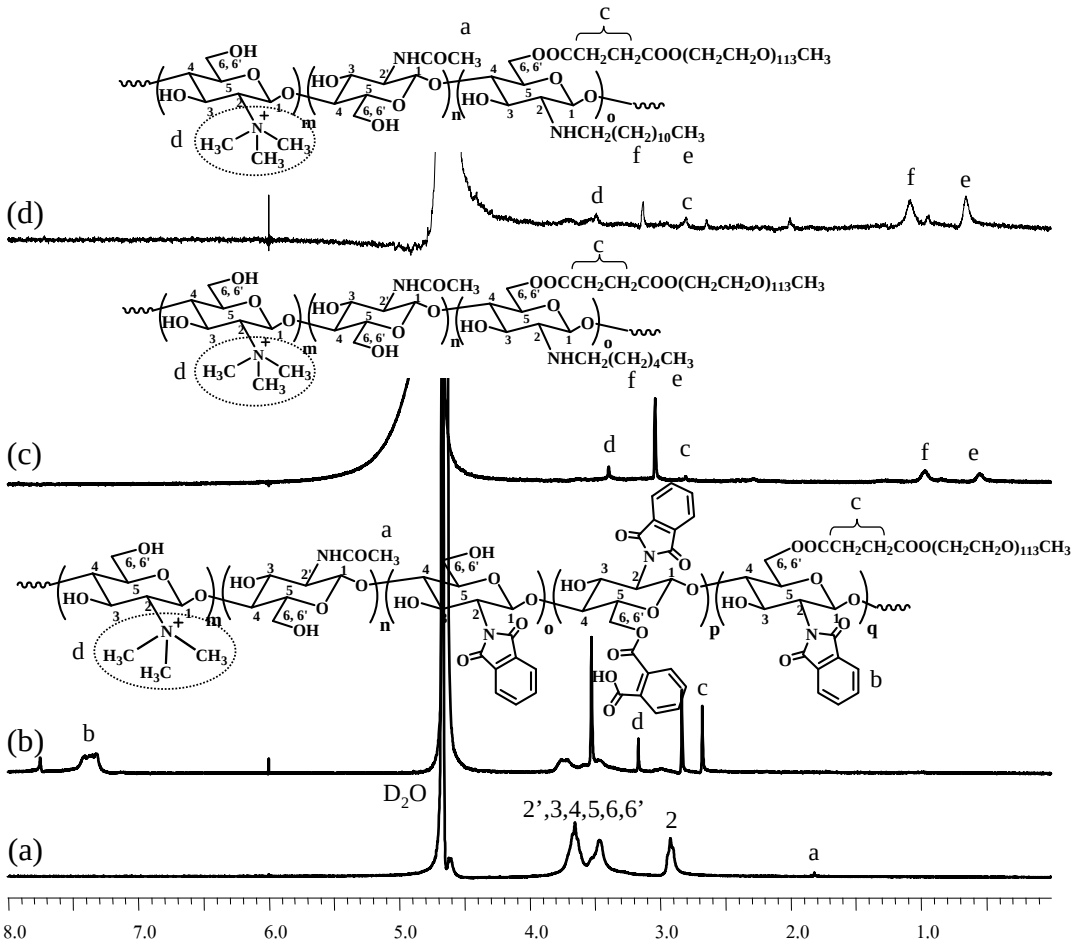
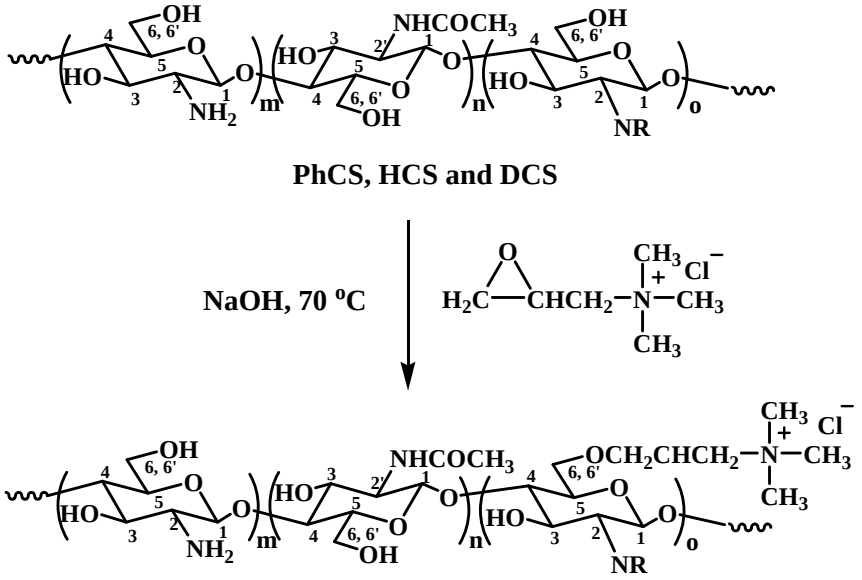


Figure 4.7 ^{1}H NMR spectra of (a) chitosan (D_2O) and quaternized amphiphilic chitosan having mPEG as hydrophilic entities (DMSO-d_6): (b) QPgM, (c) QHgM and (d) QDgM

$$\%\ DQ\ or\ \%DS_{HTAP} = \left\{ \frac{\text{intergral of the } N^{+}(CH_3)_3 / 9}{\text{integral of the H-2 / 1}} \right\} \times 100 \qquad (4.4)$$

4.2.4 Preparation of Amphiphilic Chitosan Particles having *N*-[(2-hydroxyl-3-trimethylammonium)]propyl (HTAP) as Hydrophilic Entities



Scheme 4.8 Preparation of amphiphilic chitosan having HTAP as hydrophilic entity

The positively charged *N*-[(2-hydroxyl-3-trimethylammonium)]propyl (HTAP) group can be introduced to the structure of chitosan by reacting chitosan previously functionalized with hydrophobic groups, PhCS, HCS and DCS with glycidyltrimethyl ammonium chloride (GTMAC) that yielded amphiphilic chitosan having HTAP as hydrophilic entities, PgH, HgH, and DgH, respectively (Scheme 4.8). The reaction took place via ring opening of epoxide by hydroxyl groups of chitosan under basic condition. It is known that GTMAC mainly reacts with amino groups of chitosan under acidic condition but preferably reacts with hydroxyl groups under neutral and alkaline conditions [39]. Formation of particles was then induced by self-assembly of the resulting amphiphilic chitosan derivatives upon dialysis in water.

Figure 4.8 illustrates ^{1}H NMR spectra of the synthesized PgH, HgH, and DgH. The signals corresponding to the protons of aromatic ring from phthaloyl groups appeared at chemical shift 7.2-8.0 ppm in the spectrum of PgH. Signals at chemical

shift of 0.8 and 1.2 ppm which belonged to -CH_3 and -CH_2- protons of hexyl and dodecyl groups were observed on the spectra of HgH and PgH. Peaks at chemical shift in a range of 3.00-3.05 ppm in the spectra of PgH, HgH, and DgH indicates the presence of quaternary ammonium groups in the obtained products. Degree of HTAP substitution (%DS_{HTAP}) can be calculated from the relative ratio between the integration of 9 protons from 3 methyl groups (-$N^+(CH_3)_3$) and the peak integration of 1 proton of H-2 at 2.9 ppm from chitosan also using equation 4.4.

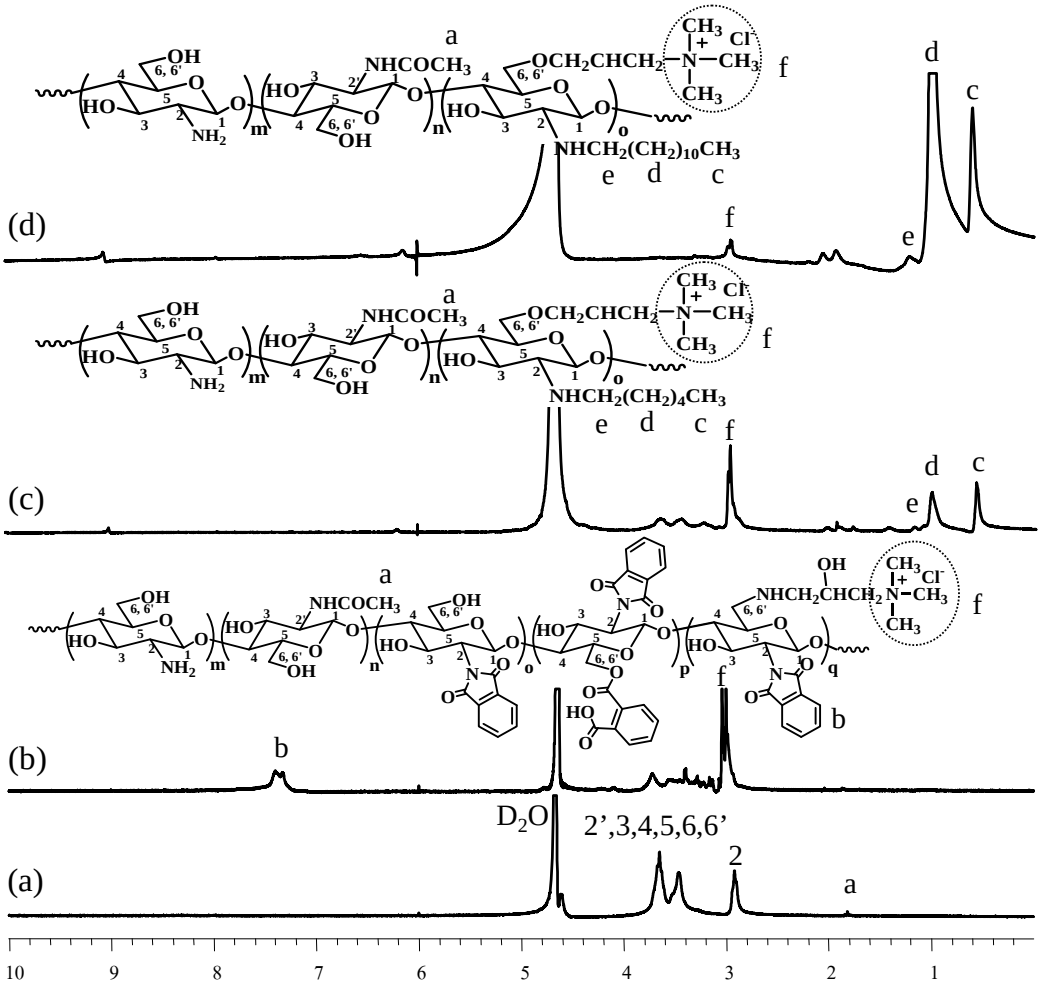


Figure 4.8 ^{1}H NMR spectra (D_2O) of (a) chitosan and amphiphilic chitosan particles having HTAP as hydrophilic entities: (b) PgH, (c) HgH, and (d) DgH

All amphiphilic chitosan particles having quaternary ammonium groups were characterized by FT-IR. As shown in Figure 4.9, the N-H bending shifts from 1,598 cm^{-1} for chitosan to 1,544 cm^{-1} for amphiphilic chitosan particles implying that there were

substitutions at amino groups, presumably by hydrophobic groups. The same signal can also be used together with the C-H deformation peak at 1,470 cm^{-1} as indications of quaternary ammonium functionality. A peak at 718 cm^{-1} in the spectra of QPgM and PgH corresponded to the aromatic ring from phthaloyl groups. In addition, the strong peaks due to C-H stretching at 2,923 cm^{-1} in the spectra of QHgM, QDgM, HgH, and DgH apparently verified the presence of alkyl chains, hexyl and dodecyl groups, in the structures of the amphiphilic derivatives. The spectra of all amphiphilic chitosan having mPEG as hydrophilic entity show the peaks at 1,078 cm^{-1} which can be assigned to C-O/C-O-C stretching of ethylene oxide units. All peak assignments are also summarized in Table 4.1.

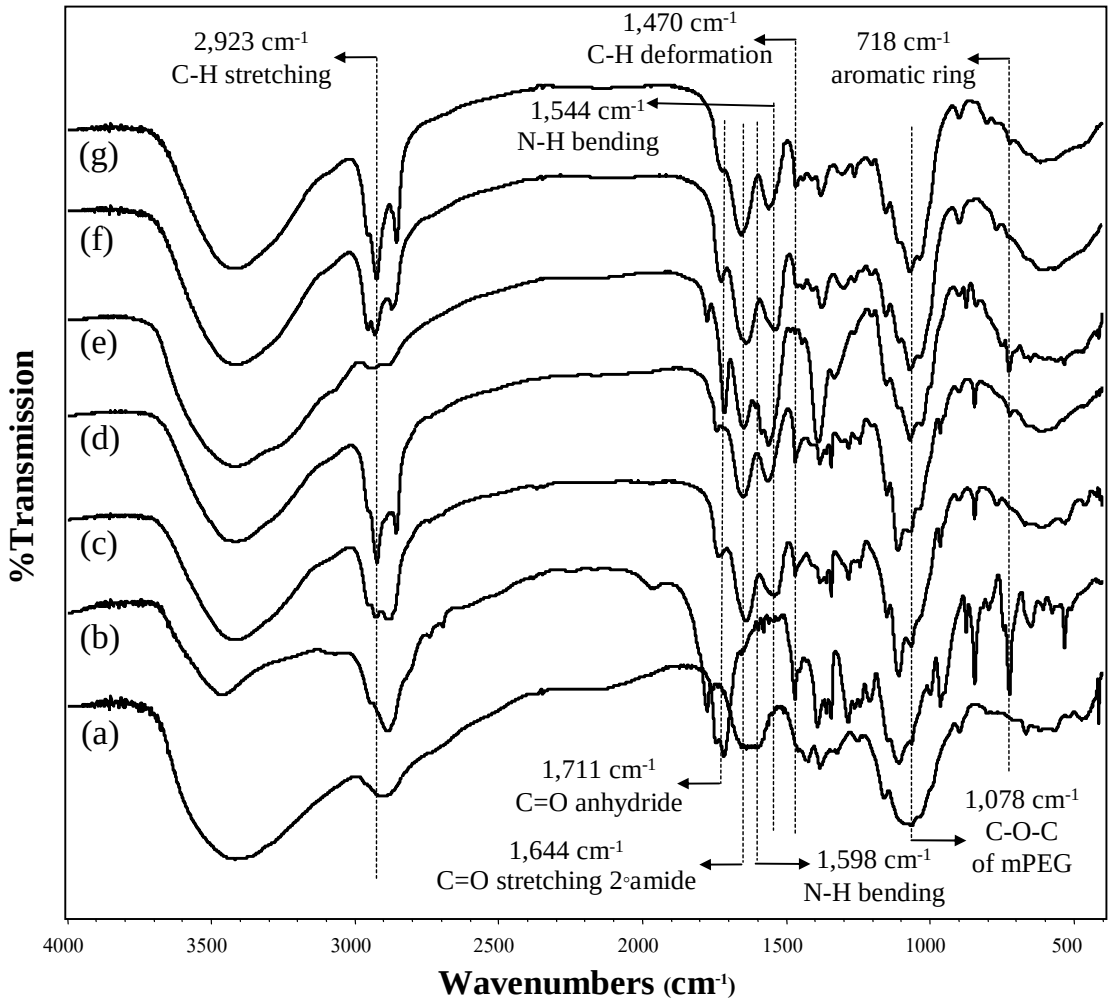


Figure 4.9 FT-IR spectra of (a) chitosan, (b) PgH, (c) HgH, (d) DgH, (e) QPgM, (f) QHgM, and (g) QDgM

Table 4.1 Peak assignment of amphiphilic chitosan

Wavenumbers (cm^{-1})	Assignments
3,000-3,700	O-H, N-H stretching
2,923	C-H stretching
1,770 and 1,711	C=O stretching of cyclic imide
1,600-1,700	N-H deformation of amino groups
1,470	C-H deformation of quaternary ammonium groups
1,417-1,377	C-N stretching and N-H deformation
1,153-897	C-O and C-O-C stretching
718	aromatic out-of plane C-H deformation

4.3 Degree of Substitution (%DS) of Functional Group in Amphiphilic Chitosan Particles

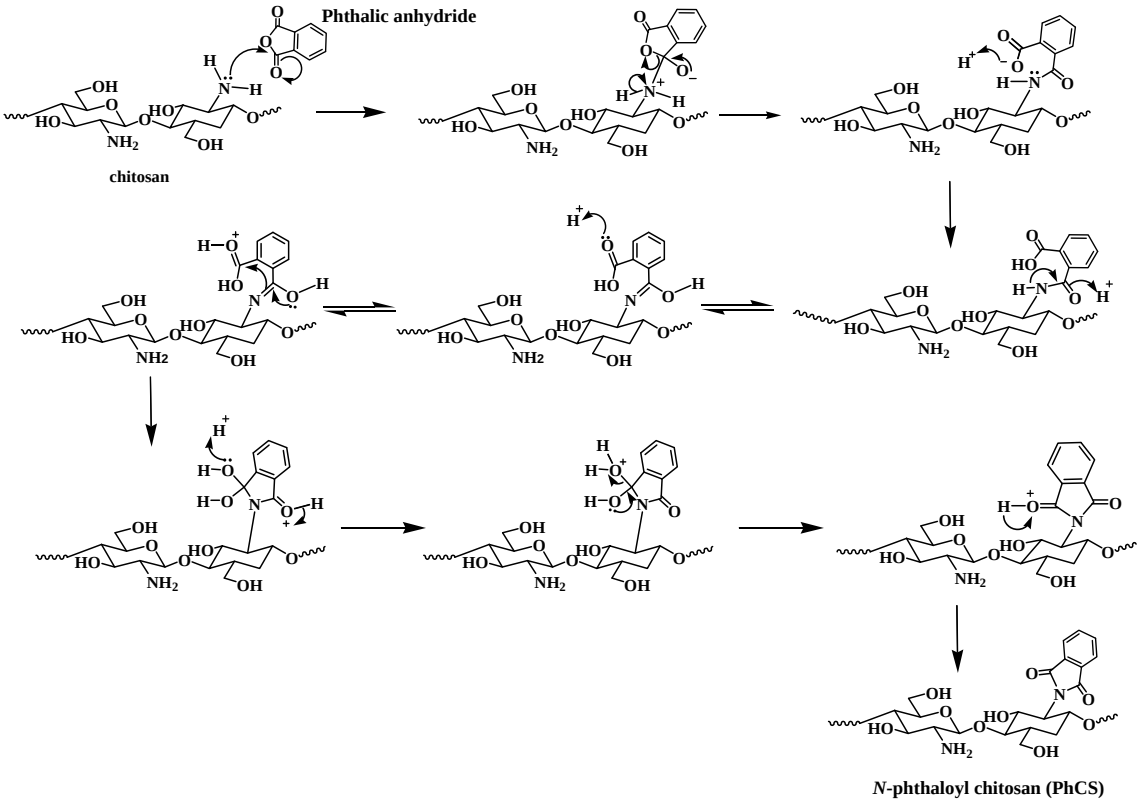
The relative ratio between the hydrophobic entities and the hydrophilic entities can be varied as a function of mole ratio between the reagents used in the step of hydrophobic group attachment (PhA, hexanal, dodecanal) and the reagents used in the step of hydrophilic group attachment (mPEG-COOH, GTMAC). The amount of each reagent was designated as equivalent to amino groups of chitosan which is the original substrate.

4.3.1 Amphiphilic Chitosan having mPEG as Hydrophilic Entity

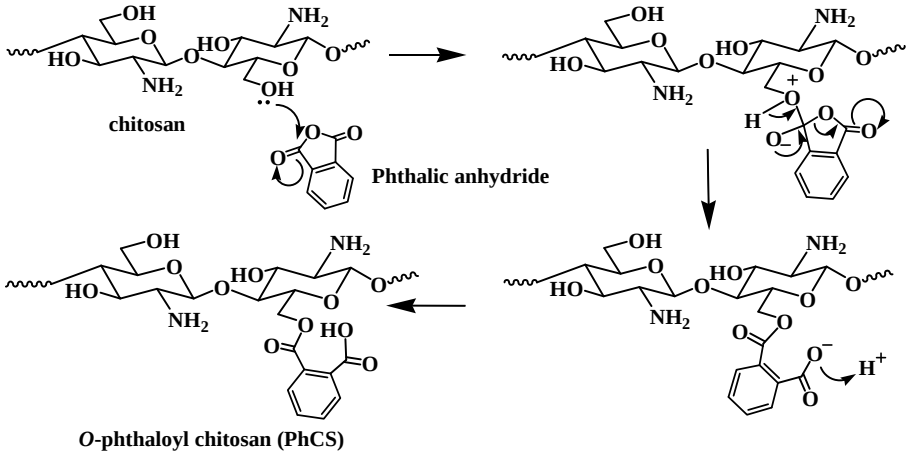
Phthaloyl groups have been widely used as hydrophobic entity in the synthesis of amphiphilic chitosan and is known to effectively induce particle formation by self-assembly of amphiphilic chitosan. [18, 40-41]. It is generally introduced to chitosan by ring opening of phthalic anhydride (PhA) by amino and hydroxyl groups of chitosan. Because nitrogen is less electronegative than oxygen, amino groups are thus more nucleophilic than hydroxyl groups, especially under neutral to alkaline conditions. The nucleophilicity of the amino groups became inferior in acidic media in which amino groups can be protonated. If amino groups act as nucleophiles, phthalimide is obtained as a product (Scheme 4.9). This stable cyclic amide (imide) would allow planar rearrangement of the aromatic ring that should promote $\pi-\pi$ stacking interactions between phthalimido groups, the main driving force for self

assembly. If hydroxyl groups act as nucleophiles, the anhydride is obtained as a product (Scheme 4.10). The cyclic ester is hydrolytically unstable and generally broken into carboxyl group.

Degree of functional group substitution of QPgM, amphiphilic chitosan having phthaloyl groups as hydrophobic entities, mPEG groups as hydrophilic entities and being quaternized by methyl iodide prepared from chitosan having molecular weight of 45 and 100 kDa is shown in Table 4.2 and 4.3, respectively. Because PhA can react with both amino and hydroxyl groups of chitosan, $\%DS_{Ph}$ was as high as 80.2% for 45kDa chitosan even only when 1 mole equivalent was used. $\%\ DS_{Ph}$ cannot be further increased upon increasing PhA equivalent. Chitosan having higher MW (100 kDa) seems to be less reactive towards the same reaction than the lower one. At least 3 equivalents of PhA were necessary to obtain > 80% DS_{Ph}.



Scheme 4.9 Mechanism of PhA ring opening by amino groups of chitosan that yields *N*-phthaloylchitosan



Scheme 4.10 Mechanism of PhA ring opening by hydroxyl groups of chitosan that yields *O*-phthaloylchitosan

Table 4.2 %DS and %DQ of QPgM prepared from chitosan having molecular weight of 45 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of PhA:mPEG-COOH	$\%DS_{Ph}$	$\%DS_{mPEG}$	%DQ
QPgM	1:1	80.2±8.4	7.8±0.4	11.7±1.3
	1:3		38.5±1.7	7.6±2.1
	1:5		18.0±0.9	13.7±1.6
	3:1	73.4±5.0	7.3±0.7	11.4±0.2
	3:3		31.5±0.5	9.0±0.9
	3:5		13.3±0.5	10.6±1.6
	5:1	72.8±10.4	8.70±0.9	8.6±0.4
	5:3		23.3±1.5	9.3±0.9
	5:5		13.4±1.2	10.6±1.0

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Table 4.3 %DS and %DQ of QPgM prepared from chitosan having molecular weight of 100 kDa as determined by ^{1}H NMR

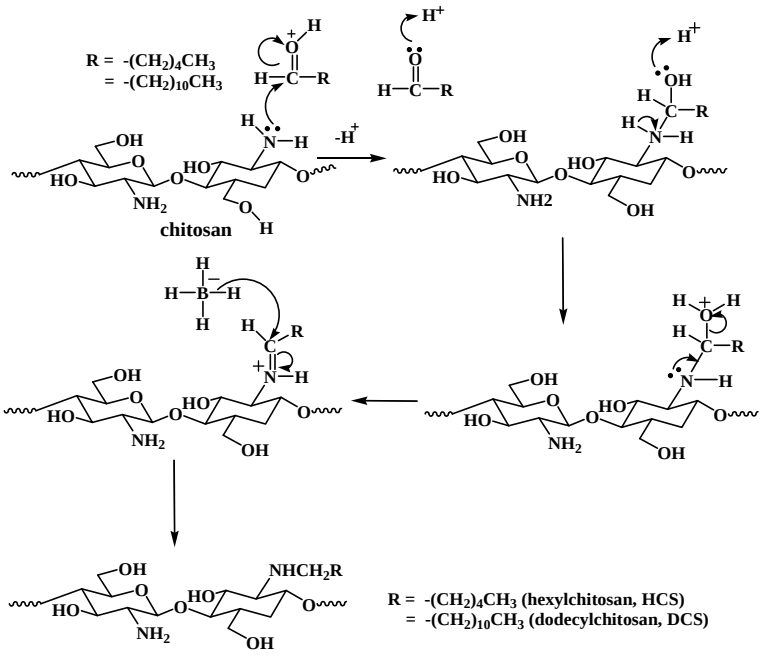
Sample	Mole equivalent of PhA:mPEG-COOH	$\%DS_{Ph}$	$\%DS_{mPEG}$	%DQ
QPgM	1:1	51.8±9.9	25.8±1.3	21.6±1.4
	1:3		20.5±0.9	10.6±1.0
	1:5		31.5±1.0	8.2±0.5
	3:1	85.5±4.0	15.0±1.2	8.8±0.6
	3:3		15.5±2.0	5.9±0.8
	3:5		25.0±1.6	13.2±0.3
	5:1	85.2±9.6	6.8±0.5	12.4±1.4
	5:3		24.0±1.0	10.7±0.9
	5:5		15.8±1.2	4.2±1.8

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

For 45 kDa chitosan, $\%DS_{mPEG}$ can significantly be increased upon increasing mole equivalent of mPEG-COOH from 1 to 3. Increasing the mole equivalent from 3 to 5, the $\%DS_{mPEG}$ was somehow lower. We suspected that it could be explained by the limited accessability of the mPEG-COOH to hydroxyl groups of chitosan at high concentration. This can be evidenced for all PhCS having $\%DS_{Ph}$ in a range of 72.8-80.2%. A similar trend was only observed for the PhCS having $\%DS_{Ph}$ of 85.2% and prepared from chitosan having molecular weight of 100 kDa. Upon using 5 mole equivalent of mPEG-COOH, $\%DS_{Ph}$ of 85.5% was unexpectedly high. Overall, $\%DS_{mPEG}$ of the PhCS grafted with mPEG-COOH (PgM) prepared from chitosan having molecular weight of 45 and 100 kDa was in a range of 3.25-38.5% and 6.8-45.0%, respectively. It was not surprising to see that these $\%DS_{mPEG}$ values were relatively low considering that a number of hydroxyl groups may react with PhA in the step of hydrophobic group introduction and only few were left to react with mPEG-COOH. Subsequent heterogeneous methylation of PgM particles yielded QPgM particles with very low %DQ, 7.6-11.7%, 4.2-23.2% for chitosan having molecular weight of 45 and 100 kDa, respectively. The low %DQ can be explained as

a result of most amino groups were substituted and only few were left to be quaternized by methyl iodide.

We were also interested to employ hexyl groups as another hydrophobic entities to the structure of amphiphilic chitosan. They are capable of inducing particle formation by self-assembly [42-44]. In this research, hexyl groups were introduced to chitosan by the product was prepared from reductive alkylation of chitosan by *N*-hexanal (Scheme 4.11). Degree of functional group substitution of QHgM, amphiphilic chitosan having hexyl groups as hydrophobic entities, mPEG groups as hydrophilic entities and being quaternized by methyl iodide prepared from chitosan having molecular weight of 45 and 100 kDa is shown in Table 4.4 and 4.5, respectively. For both molecular weight of chitosan (45 and 100 kDa), it was found that both $\%DS_{He}$ and $\%DS_{mPEG}$ increased with increasing the mole of *N*-hexanal (He) and mPEG-COOH, respectively. Unlike $\%DS_{Ph}$ in the case of QPgM, the highest $\%DS_{He}$ obtained in the case of QHgM did not exceed 67%. This can be described based on the fact that hexyl substitution only occurred at the amino groups of chitosan. Although hydroxyl groups did not take part in the step of hydrophobic group substitution, $\%DS_{mPEG}$ of QHgM was not higher than that of QPgM. The highest $\%DS_{mPEG}$ obtained in the former case was 31% suggesting that steric hindrance of mPEG-COOH having MW of 5,000 g/mol may be the major cause of the ineffective hydrophilic group substitution. Upon heterogeneous methylation, the obtained QHgM particles possessed very low %DQ, 7.4-14.6%, 6.1-14.7% for chitosan having molecular weight of 45 and 100kDa, respectively. The fact that %DQ being independent on the $\%DS_{He}$ suggested that methyl iodide may not be able to efficiently reach the unreacted free amino groups of chitosan in the HgM structure.



Scheme 4.11 Mechanism of *N*-reductive alkylation of chitosan by *N*-hexanal that yields *N*-Hexylchitosan (HCS)

Table 4.4 %DS and %DQ of QHgM prepared from chitosan having molecular weight of 45 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of He:mPEG-COOH	$\%DS_{He}$	$\%DS_{mPEG}$	%DQ
QHgM	1:1	38.02±3.1	7.80±0.4	11.33±1.7
	1:3		27.5±0.4	12.3±1.9
	1:5		31.5±1.1	13.9±1.6
	3:1	46.32±4.5	17.0±1.0	12.7±1.3
	3:3		19.3±0.3	14.0±2.1
	3:5		23.3±1.0	7.4±1.1
	5:1	65.32±6.8	13.5±0.4	14.6±2.5
	5:3		19.0±1.2	13.1±1.6
	5:5		23.5±1.1	11.6±1.7

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Table 4.5 %DS and %DQ of QHgM prepared from chitosan having molecular weight of 100 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of He:mPEG-COOH	$\%DS_{He}$	$\%DS_{mPEG}$	%DQ
QHgM	1:1	55.8±7.1	22.8±0.6	11.4±1.8
	1:3		18.3±0.5	14.7±1.3
	1:5		28.5±1.0	10.9±0.7
	3:1	51.0±5.8	17.8±0.5	9.3±0.4
	3:3		21.0±1.4	7.8±1.2
	3:5		26.5±0.5	8.4±1.9
	5:1	66.7±7.5	17.0±0.6	8.8±0.2
	5:3		24.8±0.5	6.1±1.4
	5:5		31.0±1.4	7.1±0.9

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Having changed the type of aldehyde from *N*-hexanal to *N*-dodecanal with a longer alkyl chain length, QHgM, amphiphilic chitosan having hexyl groups as hydrophobic entities, mPEG groups as hydrophilic entities and being quaternized by methyl iodide was obtained as a product. Degree of functional group substitution of the derivatives prepared from chitosan having molecular weight of 45 and 100 kDa is presented in Table 4.6 and 4.7, respectively. Similar tendencies were observed on the degree of hydrophobic group substitution ($\%DS_{Do}$) and the degree of hydrophilic group substitution ($\%DS_{mPEG}$) which can be varied as a function of the mole equivalent of reagent used in each step. It was obvious that the 100 kDa chitosan was less reactive towards the N-reductive alkylation *N*-dodecanal than the 45 kDa chitosan. That is why the highest $\%DS_{Do}$ was only 55.2%. In comparison with QHgM, a similar range of $\%DS_{mPEG}$ and %DQ were obtained.

Table 4.6 %DS and %DQ of QDgM prepared from chitosan having molecular weight of chitosan 45 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of Do:mPEG-COOH	$\%DS_{Do}$	$\%DS_{mPEG}$	%DQ
QDgM	1:1	53.7±5.3	12.3±0.7	8.1±3.2
	1:3		17.0±0.7	13.2±1.6
	1:5		18.3±1.2	9.3±2.7
	3:1	72.3±6.8	11.3±1.2	11.5±0.8
	3:3		13.0±0.4	8.3±1.3
	3:5		23.0±0.5	7.1±1.4
	5:1	83.3±6.5	28.0±1.2	8.3±0.5
	5:3		30.5±2.3	12.8±1.6
	5:5		22.3±0.5	10.7±2.0

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

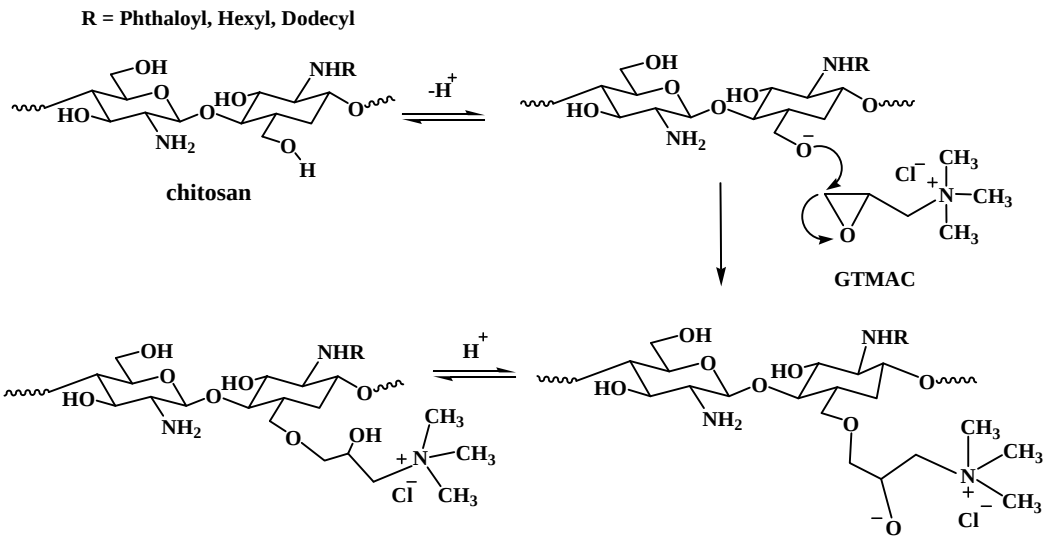
Table 4.7 %DS and %DQ of QDgM prepared from chitosan having molecular weight of chitosan 100 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of Do:mPEG-COOH	$\%DS_{Do}$	$\%DS_{mPEG}$	%DQ
QDgM	1:1	20.3±2.4	3.5±0.5	9.4±1.1
	1:3		18.8±2.1	7.7±0.2
	1:5		22.7±0.7	13.1±2.5
	3:1	41.0±3.0	20.5±1.0	13.3±2.1
	3:3		26.5±0.9	15.1±2.4
	3:5		30.8±1.8	7.9±0.5
	5:1	55.2±4.9	31.0±1.9	8.7±0.5
	5:3		32.8±0.9	10.3±0.5
	5:5		37.5±1.8	12.4±1.4

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

4.3.2 Amphiphilic Chitosan having HTAP as Hydrophilic Entity

O-(2-hydroxyl-3-trimethylammonium)propyl or HTAP group was introduced to chitosan as another hydrophilic entity via epoxide ring opening of glycidyltrimethyl ammonium chloride (GTMAC) by hydroxyl groups of chitosan under alkaline condition. The mechanism is illustrated in Scheme 4.12. The resulting amphiphilic chitosan designated as PgH, HgH and DgH were prepared from PhCS, HCS and DCS, respectively. The %DS and %DQ were determined from ^{1}H NMR data. For system, the $\%DS_{Ph}$ and $\%DQ_{HTAP}$ of PgH prepared from chitosan having molecular weight of 45 and 100 kDa are illustrated in Table 4.8 and 4.9, respectively. Apparently, the $\%DQ_{HTAP}$ increased with increasing the GTMAC equivalent. In addition, it was also found that these values are much higher than the %DQ of the derivatives having mPEG-COOH as hydrophilic entity. The former values are in a range of 21.4-51.7% whereas the latter values are in a range of 4.2-23.2% depending on the derivatives. It may be because the GTMAC is much smaller than mPEG-COOH so it can easily reach the reactive sites of chitosan. The same tendency was also observed for the HgH (Table 4.10-4.11) and DgH (Table 4.12-4.13) particles with slight lower $\%DQ_{HTAP}$. It should be emphasized that no specific correlation between the degree of hydrophobic substitution ($\%DS_{Ph}$, $\%DS_{He}$, $\%DS_{Do}$) and $\%DQ_{HTAP}$.



Scheme 4.12 Mechanism of GTMAC ring opening by amino groups of chitosan that yields amphiliclic chitosan having HTAP as hydrophilic entity

Table 4.8 %DS and %DQ of PgH prepared from chitosan having molecular weight of 45 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of PhA:GTMAC	$\%DS_{Ph}$	$\%DQ_{HTAP}$
PgH	1:1	80.2±8.4	42.0±1.3
	1:3		40.3±2.1
	1:5		51.7±3.0
	3:1	73.4±5.0	45.5±3.2
	3:3		39.1±0.9
	3:5		46.4±0.4
	5:1	72.8±10.4	36.1±3.7
	5:3		21.4±3.2
	5:5		47.6±4.2

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Table 4.9 %DS and %DQ of PgH prepared from chitosan having molecular weight of 100 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of He:GTMAC	$\%DS_{Ph}$	$\%DQ_{HTAP}$
PgH	1:1	51.8±9.9	36.4±0.6
	1:3		28.8±2.4
	1:5		40.8±1.1
	3:1	85.5±4.0	31.2±3.3
	3:3		38.6±3.3
	3:5		50.4±1.8
	5:1	85.2±9.6	28.9±2.6
	5:3		42.6±0.3
	5:5		51.1±3.5

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Table 4.10 %DS and %DQ of HgH prepared from chitosan having molecular weight of 45 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of He:GTMAC	$\%DS_{He}$	$\%DQ_{HTAP}$
HgH	1:1	38.02±3.1	24.3±2.3
	1:3		30.8±0.8
	1:5		33.6±1.3
	3:1	46.32±4.5	17.1±1.0
	3:3		29.0±3.2
	3:5		31.6±3.3
	5:1	65.32±6.8	16.3±2.3
	5:3		25.8±1.3
	5:5		29.8±3.5

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Table 4.11 %DS and %DQ of HgH prepared from chitosan having molecular weight of 100 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of Do: GTMAC	$\%DS_{He}$	$\%DQ_{HTAP}$
HgH	1:1	55.8±7.1	14.3±2.4
	1:3		31.8±0.4
	1:5		36.7±1.2
	3:1	51.0±5.8	17.7±0.5
	3:3		19.0±1.4
	3:5		26.9±0.7
	5:1	66.7±7.5	24.8±2.1
	5:3		29.5±2.1
	5:5		36.3±2.2

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Table 4.12 %DS and %DQ of DgH prepared from chitosan having molecular weight of 45 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of Do: GTMAC	$\%DS_{Do}$	$\%DQ_{HTAP}$
DgH	1:1	53.7±5.3	16.8±0.6
	1:3		19.2±1.2
	1:5		25.1±1.2
	3:1	72.3±6.8	11.9±1.9
	3:3		29.6±1.2
	3:5		22.9±1.2
	5:1	83.3±6.5	16.8±2.0
	5:3		21.4±1.4
	5:5		35.8±2.1

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Table 4.13 %DS and %DQ of DgH prepared from chitosan having molecular weight of 100 kDa as determined by ^{1}H NMR

Sample	Mole of Do: GTMAC	$\%DS_{Do}$	$\%DQ_{HTAP}$
DgH	1:1	20.3±2.4	12.3±1.5
	1:3		20.1±0.6
	1:5		23.8±0.2
	3:1	41.0±3.0	22.6±0.6
	3:3		24.5±1.0
	3:5		23.1±0.9
	5:1	55.2±4.9	16.7±1.4
	5:3		21.4±1.5
	5:5		24.7±1.5

Remark: All data were displayed as the mean ± one standard deviation and are derived from three independent experiments

Considering %DQ in all previous data, it was found that %DQ did not exceed 50%. It was suspected that the $\%DQ_{HTAP}$ may not be high enough for the particles to possess positive charges. For this reason, we tried to find a method to enhance $\%DQ_{HTAP}$. In this particular case, pH of the solvent used in the step of GTMAC ring opening was changed from alkaline condition (pH = 9) to a more acidic condition (pH = 5) by adding acetic acid solution of 1.0% (v/v) in the formerly used solvent, DMF. It was anticipated that a slightly acidic condition would minimize GTMAC consumption by hydroxide ion in the system and still allow some free amino groups to take part in the ring opening of GTMAC. The success of $\%DQ_{HTAP}$ elevation is demonstrated in Table 4.14 for the derivatives prepared from chitosan having molecular weight of 45 kDa. The introduced acid can bring $\%DQ_{HTAP}$ up above 50% in most cases.

Table 4.14 %DS of PgH, HgH and DgH in DMF+1.0%(v/v) acetic acid using chitosan having molecular weight of 45 kDa as determined by ^{1}H NMR

Sample	Mole equivalent of hydrophobic reagent: GTMAC	$\%DS_{hydrophobic}$	$\%DQ_{HTAP}$
PgH	1:5	80.2	58.2
	3:5	73.4	52.6
	5:5	72.8	42.4
HgH	1:5	38.0	55.0
	3:5	46.3	59.1
	5:5	65.3	68.1
DgH	1:5	53.7	59.4
	3:5	72.3	65.4
	5:5	83.3	51.7

4.4 Charge Characteristic of Amphiphilic Chitosan Particles

The charge characteristic of the amphiphilic chitosan particles were determined by PCS. As shown in Table 4.15, all particles having mPEG as hydrophiphilic entities that were quaternized by methyl iodide, QPgM, QHgM and QDgM, exhibited

negative charges having QPgM show the highest negative charges. There are a number parameters contributing to this outcome. First, %DQ values of all particles were less than 20%, so it was not too surprising for the particles to exhibit negatively charged ζ-potential. Second, the mPEG with ethylene oxide repeat unit carrying a large number of lone pair electrons of oxygen atoms. And this is known to introduce the negative charges to the particles [27]. And third, in the case of particles having phthaloyl groups as hydrophobic entities (QPgM and PgH), the unavoidable presence of negatively charged carboxylate groups dissociated from carboxyl groups (pKa 4.4), upon cyclic anhydride ring opening can significantly introduce negative charges. It should be noted that cyclic anhydride is the product obtained when hydroxyl groups act as nucleophiles in the step of phthaloyl group introduction via PhA ring opening. This last contribution was most obvious for the QPgM particles of which $\%DS_{Ph}$ was very high and %DQ was quite low. The positive charge introducing by methylation was not high enough to counteract the negative charges from the carboxylate groups. As a result the QPgM particles possess the highest negative ζ-potential of -44 mV. With a greater %DQ (the highest was 51.7%) introduced from HTAP groups, the PgH exhibited less magnitude of negative ζ-potential indicating the majority of the negative charges from the carboxylate groups were cancelled out. But that high content of HTAP was still not high enough to render the PgH particles positive charge even when the reaction was performed in a slight acidic condition in the presence of 1%(v/v) acetic acid, of which yielded the PgH particles with the highest $\%DQ_{HTAP}$ of 58.2%

In the case of the HgH and DgH particles, the positive magnitude of the ζ-potential was not high as expected when prepared in DMF without 1%(v/v) acetic acid added. This may stem from their $\%DQ_{HTAP}$ values of approximately 30% were not high. Upon an addition of 1%(v/v) acetic acid in DMF solution, $\%DQ_{HTAP}$ can be raised from +22.5 to +35.3 mV and from +15.5 to +48.5 mV for HgH and DgH particles, respectively. It should be noted that it may not be reasonable to compare these ζ-potential values with that of the unmodified chitosan particle which was prepared by a different method based on ionic crosslinking between chitosan and tripolyphosphate, also shown for comparison in Table 4.15.

Table 4.15 Charge characteristic of amphiphilic chitosan particles as determined by PCS

Sample	%DS of Hydrophobic:Hydrophilic	%DQ	ζ-potential (mV)*	ζ-potential (mV)**
Chitosan	-	-	+27.1	+27.1
QPgM	73:13 *	9	-44.3	-
QHgM	46:19 *	14	-10.6	-
QDgM	72:13*	8	-6.5	-
PgH	73:46*/ 73:53**	-	-13.2	-10.4
HgH	46:31*/ 46:59**	-	+22.5	+35.3
DgH	72:29*/ 72:65**	-	+15.5	+48.5

Remark : * = Particles were prepared in DMF

**** =** Particles were prepared in DMF+1.0% (v/v) acetic acid

4.5 Critical Micelle Concentration (CMC) Determination

Critical micelle concentration (CMC) is the minimum concentration required for the formation of micelles. The CMC of amphiphilic chitosan was determined by fluorescent spectroscopy method using pyrene as an environmentally dependent fluorescence dye. The CMC can be determined from the change of the ratio between the first and third vibrational peak (I_1/I_3) in pyrene fluorescence emission spectrum [34]. Below the CMC, the micelle cannot yet be formed. The ratio of I_{373}/I_{384} is high because pyrene, as a hydrophobic dye, is unable to disperse in aqueous solution. When the concentration is above the CMC, the micelle can be formed. Therefore, pyrene is encapsulated and able to disperse in apolar interiors of the micelle resulting in decreasing of I_{373}/I_{384} (Figure 4.10). Figure 4.11 shows the plot of the intensity ratio I_{373}/I_{384} of the pyrene emission spectra against the logarithm of the amphiphilic chitosan concentration. As shown in Table 4.16, the I_{373}/I_{384} ratio decreased around 7-17 µg/mL of polymer concentration suggesting that pyrene can be encapsulated in apolar interiors of the amphiphilic chitosan micelle. With the same hydrophobic entities (phthaloyl, hexyl, dodecyl), the particles having HTAP as hydrophilic entities exhibited lower CMC values than the particles having mPEG as hydrophilic entities

implying that the formation of the former particles were more effective than that of the latter. Considered at the same degree of substitution for each hydrophobic group, these results corresponded well with their hydrophilic contents: $\%DQ_{HTAP} > 50\%$ for PgH, HgH, and DgH and $\%DS_{mPEG} + \%DQ < 50\%$ for QPgM, QHgM, and QDgM.

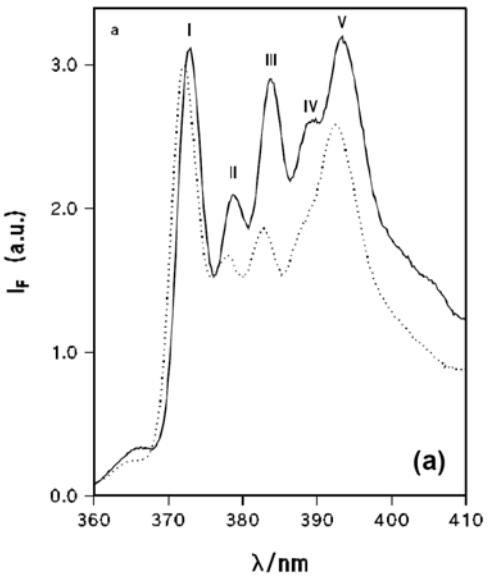


Figure 4.10 Fluorescence emission spectra of pyrene (—) above the CMC, (…) below the CMC [34]

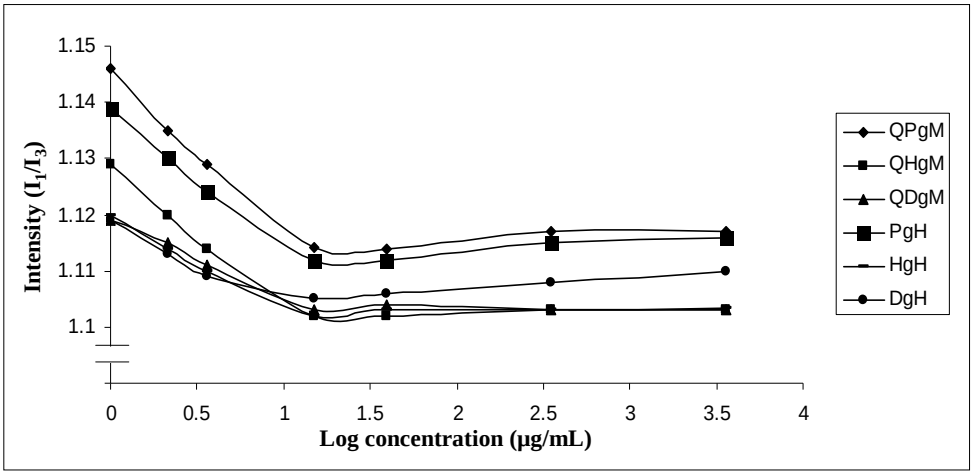


Figure 4.11 Plot of the intensity ratio (I_{373}/I_{384}) of pyrene as a function of logarithm concentration of micelles forming from a function of quaternary ammonium chitosan. Data are plotted in the mean ±S.D. of three measurements.

Table 4.16 The critical micelle concentration (CMC) of amphiphilic chitosan particles

Sample	CMC (µg/mL)
QPgM	15.9
QHgM	16.9
QDgM	12.6
PgH	13.6
HgH	10.0
DgH	7.10

4.6 Morphology and Particle Sizes of Amphiphilic Chitosan Particles

SEM images illustrated in Figure 4.12 and revealed that all particles were quite spherical in shape. As determined by SEM analysis, the particles had sub-micron diameters in a range of 0.11 to 0.29 µm (Table 4.15). In contrast, the data obtained from PCS analysis were much larger. The size ranged from 1.29 to 3.1 µm in diameter. This is mainly due to the fact that the size measured by PCS is a hydrodynamic size which is measured in solution taking into account the swelling. Also, it was found that the particles grafted with mPEG gave particles with a size smaller than those grafted with HTAP. It is believed that the hydrogen bonding between the long chains of grafted mPEG entities may make the hydrophobic groups in the core pack more efficiently and resulting in the smaller particles.

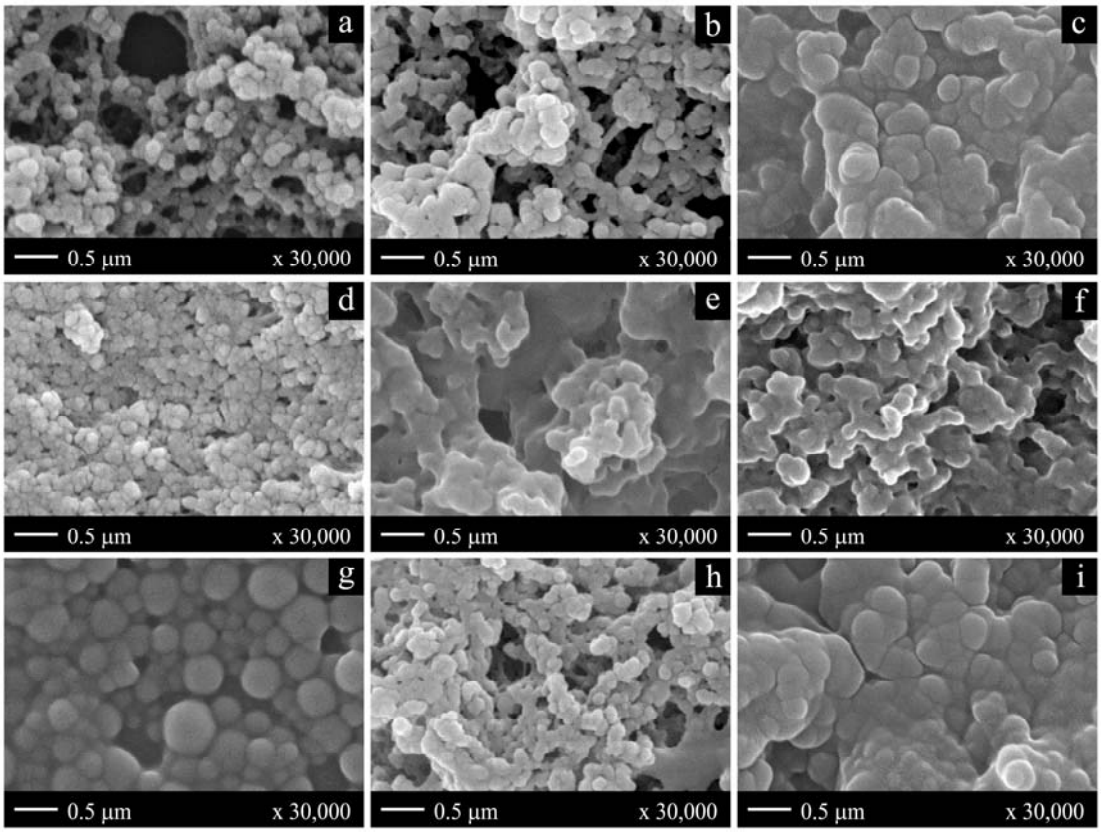


Figure 4.12 SEM images of amphiphilic chitosan particles: (a) PgM, (b) HgM, (c) DgM, (d) QPgM, (e) QHgM, (f) QDgM, (g) PgH, (h) HgH and (i) DgH

Table 4.17 Particle sizes of amphiphilic chitosan particles analyzed by SEM and PCS

Particles	Size (µm)	
	SEM	PCS
PgM	0.11±0.03	2.24
HgM	0.14±0.03	2.67
DgM	0.25±0.05	2.89
QPgM	0.13±0.03	1.29
QHgM	0.17±0.05	1.78
QDgM	0.19±0.04	1.85
PgH	0.29±0.04	3.10
HgH	0.21±0.02	2.10
DgH	0.26±0.02	2.95

CHAPTER V

CONCLUSION AND SUGGESTIONS

Quaternized chitosan particles can be successfully prepared by self-assembly of amphiphilic chitosan. Self-assembly of the resulting derivatives upon dialysis gave stable colloidal solution in water at room temperature. Phthaloyl (Ph), hexyl (He) dodecyl (Do) as hydrophobic entities, poly(ethylene glycol)methyl ether (mPEG) and *N*-[(2-hydroxyl-3-trimethylammonium)]propyl (HTAP) as hydrophilic entities were immobilized on chitosan backbone via chemical reactions of amino and hydroxyl groups, respectively. Chemical structures of the synthesized amphiphilic chitosan were verified by ^{1}H NMR and FT-IR spectroscopy. The relative degree of substitution (%DS) of each functionality as determined from ^{1}H NMR data suggested that the molar ratio of substituted hydrophobic to hydrophilic groups can be varied as a function of mole equivalent of reagent used in each step of reaction. Total degree of quaternization (%DQ) of the particles having mPEG and HTAP as hydrophilic entities calculated from ^{1}H NMR data was in a range of 7-23% and 21-51%, respectively. The %DQ of the particles grafted with HTAP can be raised to 51-82% upon an introduction of 1%(v/v) acetic acid to the reaction having DMF as a solvent

Charge characteristic of the particles determined by PCS indicated that only the particles having hexyl and dodecyl as hydrophobic entities and HTAP as hydrophilic entities that were prepared in the slight acidic condition exhibited high positive charges**.** On the other hand, the particles having mPEG as hydrophilic entities possessed negative charges even after methylation implying the large negative charge contribution from the lone pair electrons of the ethylene oxide units of mPEG. Alternatively, another source of negative charges was from the carboxyl groups of the particles having Ph groups as hydrophobic entities. Having pKa around 4, the carboxyl group can dissociate into the negatively charged carboxylate groups under neutral condition. The critical micelle concentration (CMC) of quaternized amphiphilic chitosan as determined by measuring the fluorescence intensity of a fluorescent probe, pyrene were in a range of 7-17 μg/mL. The results from SEM

analysis of representative particles suggested that the sub-micron particles can be formed with a diameter in a range of 0.11-0.29 μm.

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APPENDIX

APPENDIX A

Figure A-1 Fluorescence emission spectrum of pyrene of QPgM particle

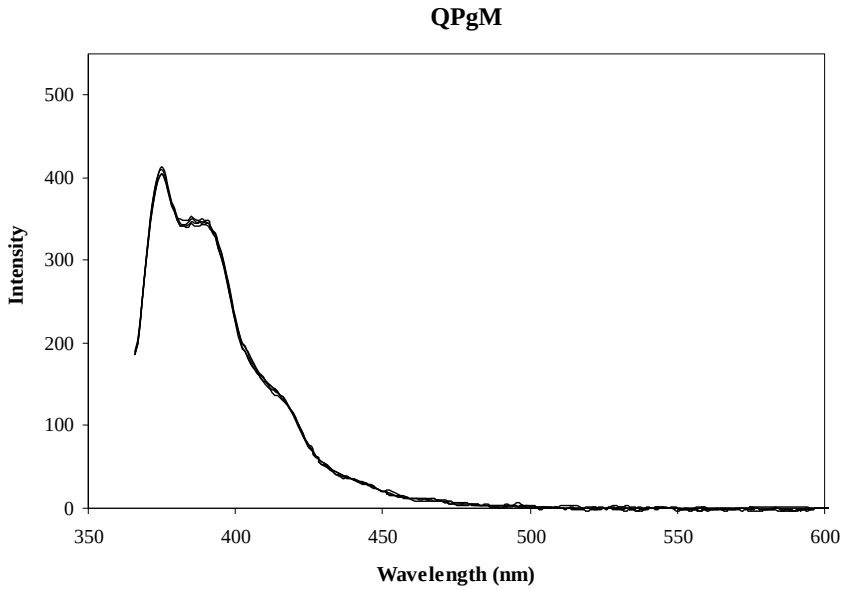

Figure A-2 Fluorescence emission spectrum of pyrene of QHgM particle

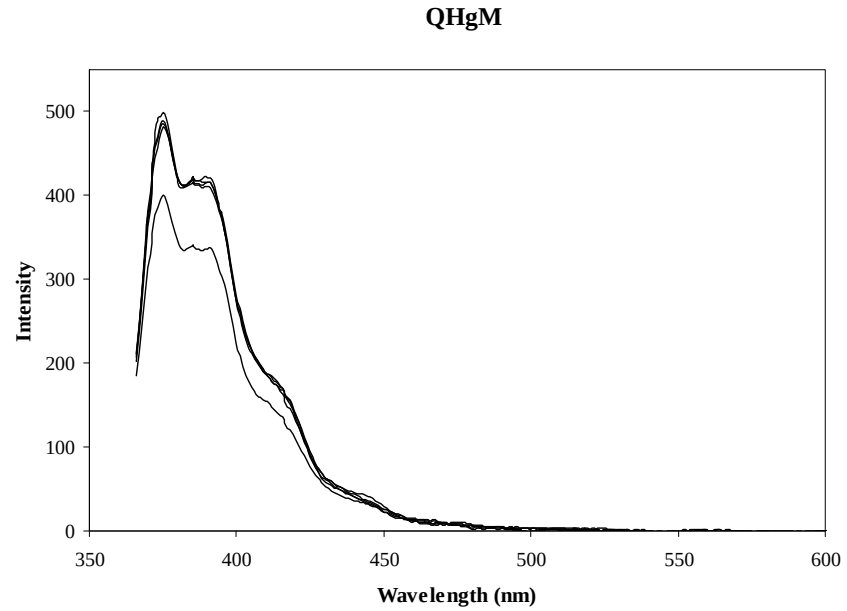

Figure A-3 Fluorescence emission spectrum of pyrene of QDgM particle

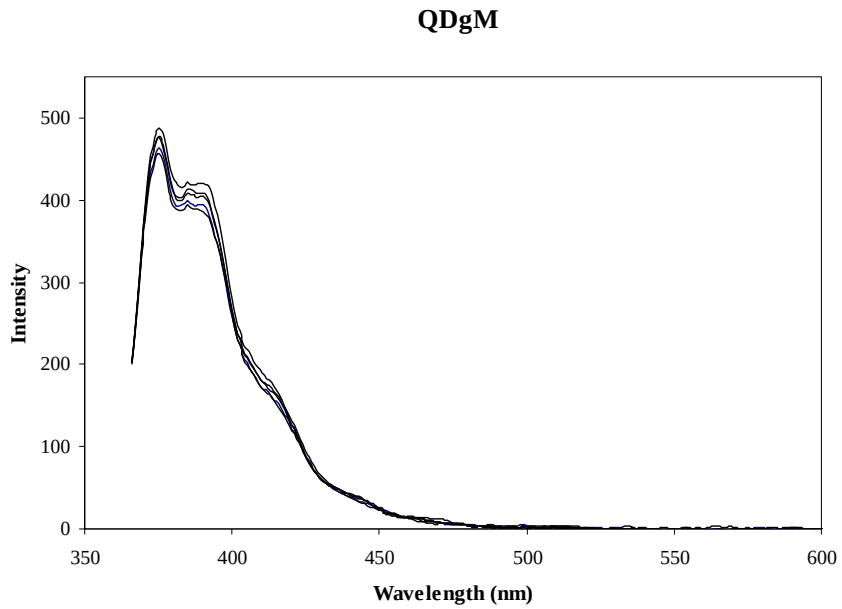

Figure A-4 Fluorescence emission spectrum of pyrene of PgH particle

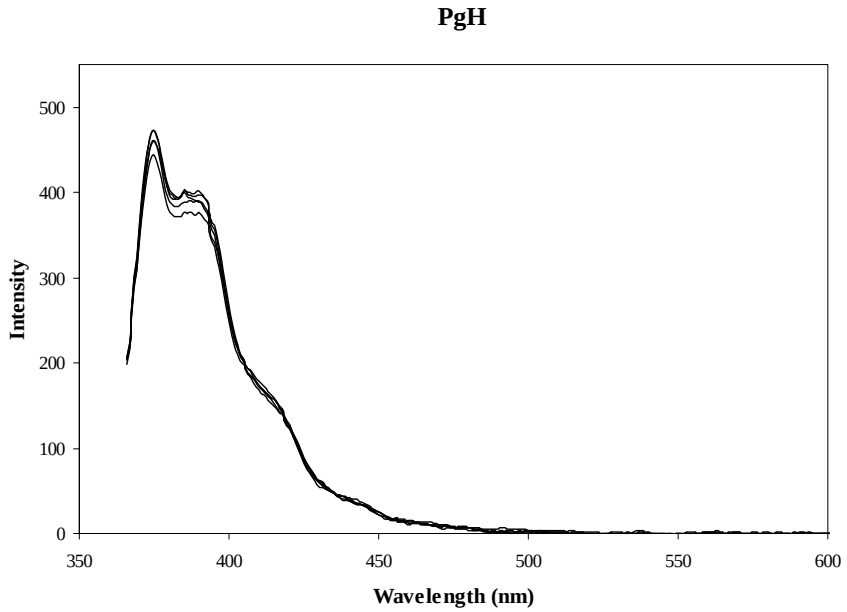

Figure A-5 Fluorescence emission spectrum of pyrene of HgH particle

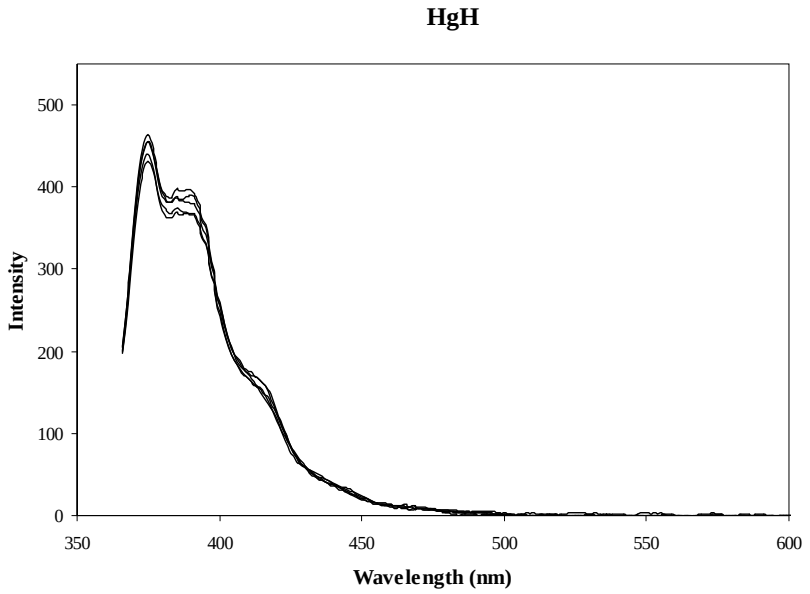

Figure A-6 Fluorescence emission spectrum of pyrene of DgH particle

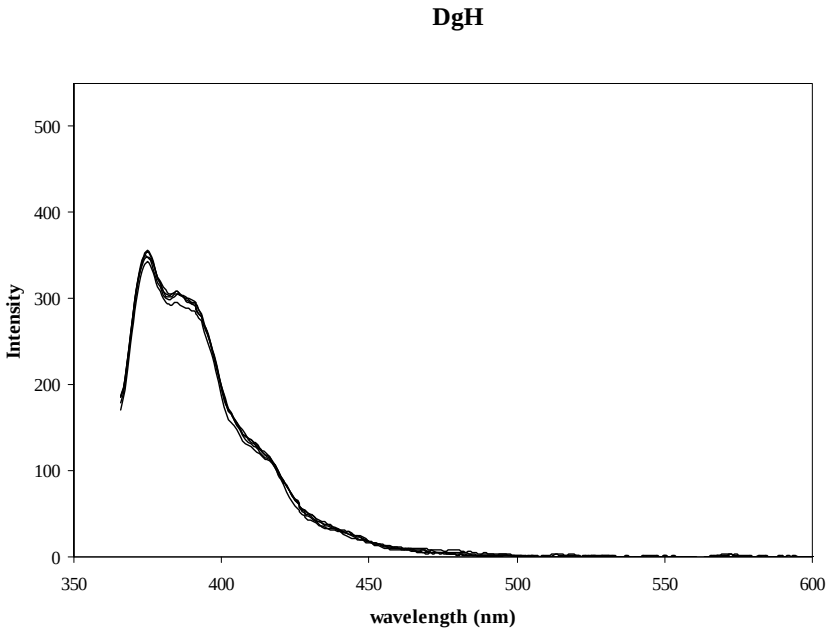

Table A-1 Data of the intensity ratio I_{373}/I_{384} of QPgM particle

Particles	Log Concentration	Intensity of 373 nm (I_{373})	Intensity of 384 nm (I_{384})	I_{373}/I_{384}
QPgM	0.00102	384.97186	335.89140	1.14612
	0.33490	387.59315	341.21518	1.13592
	0.55760	390.24726	345.54727	1.12936
	1.17609	397.37448	356.57515	1.11442
	1.59794	405.46530	363.74388	1.11470
	2.54406	408.10959	365.19220	1.11752
	3.55321	413.46159	372.79933	1.11712

Table A-2 Data of the intensity ratio I_{373}/I_{384} of QHgM particle

Particles	Log Concentration	Intensity of 373 nm (I_{373})	Intensity of 384 nm (I_{384})	I_{373}/I_{384}
QHgM	0.00102	394.36789	349.27322	1.12911
	0.33490	445.46921	396.04952	1.12434
	0.55760	455.29432	408.43828	1.11472
	1.17609	470.35641	426.73938	1.10221
	1.59794	480.99460	436.25650	1.10255
	2.54406	485.16086	439.80787	1.10312
	3.55321	498.59967	452.03549	1.10301

Table A-3 Data of the intensity ratio I_{373}/I_{384} of QDgM particle

Particles	Log Concentration	Intensity of 373 nm (I_{373})	Intensity of 384 nm (I_{384})	I_{373}/I_{384}
QDgM	0.00102	443.03045	395.84565	1.11923
	0.33490	448.47232	402.10914	1.11534
	0.55760	452.25634	406.92491	1.11141
	1.17609	451.62292	409.44961	1.10303
	1.59794	456.87835	413.60680	1.10462
	2.54406	477.27328	432.64586	1.10315
	3.55321	484.54147	439.24240	1.10313

Table A-4 Data of the intensity ratio I_{373}/I_{384} of PgH particle

Particles	Log Concentration	Intensity of 373 nm (I_{373})	Intensity of 384 nm (I_{384})	I_{373}/I_{384}
PgH	0.00102	428.40554	376.05823	1.13921
	0.33490	435.39721	384.58573	1.13212
	0.55760	439.51211	390.87547	1.12443
	1.17609	442.96389	398.34524	1.11201
	1.59794	441.31567	396.78097	1.11224
	2.54406	453.90905	406.96192	1.11536
	3.55321	455.53158	408.17145	1.11603

Table A-5 Data of the intensity ratio I_{373}/I_{384} of HgH particle

Particles	Log Concentration	Intensity of 373 nm (I_{373})	Intensity of 384 nm (I_{384})	I_{373}/I_{384}
HgH	0.00102	415.27542	370.41121	1.12112
	0.33490	417.39812	374.68077	1.11401
	0.55760	420.21123	378.04418	1.11154
	1.17609	423.74935	384.48916	1.10211
	1.59794	436.45785	395.68992	1.10303
	2.54406	438.57150	397.60974	1.10302
	3.55321	444.83148	403.10598	1.10351

Table A-6 Data of the intensity ratio I_{373}/I_{384} of DgH paticle

Particles	Log Concentration	Intensity of 373 nm (I_{373})	Intensity of 384 nm (I_{384})	I_{373}/I_{384}
DgH	0.00102	331.18960	295.85028	1.11945
	0.33490	332.32142	298.54414	1.11314
	0.55760	335.11238	302.16165	1.10905
	1.17609	338.48092	306.28154	1.10513
	1.59794	341.95581	309.10421	1.10628
	2.54406	341.88284	308.52511	1.10812
	3.55321	344.89654	310.65603	1.11022

VITAE

Miss Nadda Booranabunyat was born in Nakhonratchasima, Thailand, on August 20th, 1985. She received a Bachelor Degree of Science (Chemistry) from the Department of Applied Chemistry, Faculty of Science, King Monkut's University of Technology North Bangkok in 2007. In the same year, she started as a Master Degree student with a major in Program of Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University and completed the program in 2011.

Proceedindings:

August 2010	Poster presentation in The 6th National Chitin-Chitosan Conference (6th NCCC), Miracle Grand Convention Hotel, Bangkok, Thailand.

Presentation in Conference:

March 2011	Oral presentation in The 19th Science Forum, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.
January 2011	Poster presentation in the Yearly Meeting for Research Team Consolidation Grant, The Thailand Research Fund and Fund-Higher Education Commission (RTA5280002)
September 2010	Poster presentation in The 7th International Symposium on Advanced Materials in Asia-Pacific JAIST International Symposium on Nano Technology 2010, Ishikawa, Japan.
July 2010	Oral presentation in Graduate Conference celebrating the 25th Anniversary Program of Petrochemistry and Polymer Science, Mahamakut Building, Faculty of Science, Chulalongkorn University.