ฤทธิ์ยับยั้งแบคทีเรียของอนุภาคไคโตซานที่มีหมู่ควอเทอร์นารีแอมโมเนียม

นางสาวอรพรรณ เวียรชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIBACTERIAL ACTIVITY OF QUATERNARY AMMONIUM-CONTAINING CHITOSAN PARTICLES

Miss Oraphan Wia-rachai

A Thesis Submitted in Partial Fulfillment of the Requirements
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งานวิจัยนี้สนใจที่จะพัฒนาสารตัวเติมอินทรีย์ที่มีฤทธิ์ในการยับยั้งแบคทีเรีย เป็นมิตรกับสิ่งแวดล้อม จากอนุภาคไคโตซานที่มีหมู่ควอเทอร์นารีแอมโมเนียม ซึ่งน่าจะสามารถประยุกต์ในอุตสาหกรรมที่เกี่ยวข้องกับวัสดุทางชีวการแพทย์ สุขภาพ และสิ่งทอ อนุภาคไคโตซานที่มีหมู่ควอเทอร์นารีแอมโมเนียมถูกเตรียมโดย 2 วิธี วิธีแรกเป็นการเตรียมอนุภาคไคโตซานด้วยการเชื่อมขวางทางไอออนิกตามด้วยการดัดแปลงพื้นผิวของอนุภาคด้วยควอเทอร์ไนเซชันแบบวิวิธพันธ์ผ่านเอ็น-รีดักทีฟอัลคิเลชันกับอัลดีไฮด์ตามด้วยอัลคิลเลชันด้วยอัลคิลไอโอไดด์หรือเมทิลเลชันโดยตรงกับเมทิลไอโอไดด์ วิธีที่สองเป็นการสังเคราะห์ *เอ็น,เอ็น,เอ็น*-ไทรเมทิลไคโตซานด้วยควอเทอร์ไนเซชันแบบเอกพันธ์โดยใช้เมทิลไอโอไดด์ตามด้วยการเตรียมเป็นอนุภาค จากการวิเคราะห์ด้วยเทคนิคเอฟที-ไออาร์ โปรตอนเอ็นเอ็มอาร์ พีซีเอส และทีอีเอ็มช่วยยืนยันการเกิดอนุภาคควอเทอร์ไนซ์ไคโตซานที่มีขนาดเล็กกว่า 1 ไมครอน มีลักษณะเป็นทรงกลม และมีประจุเป็นบวก จากการทดสอบฤทธิ์ในการยับยั้งแบคทีเรียด้วยการวัดความขุ่นและการนับจำนวนแบคทีเรียที่ยังคงมีชีวิต พบว่าอนุภาคควอเทอร์ไนซ์ไคโตซานทุกชนิดมีฤทธิ์ในการยับยั้ง *S. aureus* ซึ่งเป็นแบคทีเรียแกรมบวกที่เหนือกว่าอนุภาคไคโตซานในสภาวะที่มีพีเอชเป็นกลาง ทั้งนี้อาจเป็นผลเนื่องมาจากการเชื่อมด้วยพันธะไอออนิกระหว่างประจุบวกบนอนุภาคและประจุลบของกรดไทโชอิกบนผนังด้านนอกของ *S. aureus* ในขณะที่มีเพียงอนุภาคควอเทอร์ไนซ์ไคโตซานบางชนิดโดยเฉพาะอย่างยิ่งที่มีความหนาแน่นประจุสูงและมีหมู่แทนที่อัลคิลขนาดใหญ่ที่สามารถยับยั้งการเติบโตของ *E. coli* ซึ่งเป็นแบคทีเรียแกรมลบที่ผนังด้านนอกมีไลโปพอลิแซคคาไรด์ และนอกจากนี้ยังพบว่าประสิทธิภาพในการยับยั้งขึ้นกับปริมาณของอนุภาคซึ่งประเมินค่าเชิงปริมาณได้จากความเข้มข้นต่ำสุดที่ทำให้เกิดการยับยั้ง

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2551 ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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4972572423 : MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEYWORDS: CHITOSAN PARTICLES/ QUATERNIZATION/ ANTIBACTERIAL ACTIVITY

ORAPHAN WIA-RACHAI: ANTIBACTERIAL ACTIVITY OF QUATERNARY AMMONIUM-CONTAINING CHITOSAN PARTICLES. THESIS ADVISOR: ASST. PROF. VORAVEE P. HOVEN, Ph.D., THESIS CO-ADVISOR: PROF. SUDA KIATKAMJORNWONG, Ph.D., 87 pp.

This research aims to develop environmentally friendly, organic antibacterial fillers from quaternary ammonium-containing chitosan particles that may be applicable for biomedical device, health, and textile industries. Quaternary ammonium-containing chitosan particles were prepared by two methods. The first method was to prepare chitosan particles by ionic crosslinking. Their surface was then modified by heterogeneous quaternization through *N*-reductive alkylation with aldehyde followed by alkylation with alkyl iodide or direct methylation with methyl iodide. The second method was to synthesize *N,N,N*-trimethyl chitosan by homogeneous quaternization using methyl iodide followed by particle formation. As analyzed by FT-IR, ^{1}H NMR, PCS, and TEM, sub-micron, spherical, and positively charged quaternized chitosan particles were formed. Antibacterial activity tests were performed by turbidimetric method and viable cell counts. All quaternized chitosan particles exhibited superior antibacterial activity against, *S. aureus*, the gram-positive bacteria, to the native chitosan particles at neutral pH. This may be explained as a result of ionic binding between the positive charges on the particles and the negatively charged teichoic acid on the outer membrane of *S. aureus*. Only some quaternized chitosan particles, especially those having high charge density and bearing large alkyl substituents, were capable of suppressing the growth of *E. coli*, the gram negative bacteria, of which outer membrane contains lipopolysaccharide. The inhibitory efficiency quantified by minimum inhibitory concentration was also found to be dose-dependent.

Field of study: Petrochemistry and Polymer Science Student' signature .....
Academic year: 2008 Advisor's signature .....
Co-Advisor's signature .....

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LIST OF SCHEMES

LIST OF ABBREVIATION

CFU	: Colony forming unit
DS	: Degree of substitution
EtI	: Ethyl iodide
EtOH	: Ethanol
E. coli	: *Escherichia coli*
EMC	: Ethylated methyl chitosan
FT-IR	: Fourier transform infrared spectroscopy
MIC	: Minimum inhibitory concentration
MEC	: Methylated ethyl chitosan
MeOH	: Methanol
MPC	: Methylated propyl chitosan
MBzC	: Methylated benzyl chitosan
MeI	: Methyl iodide
OD	: Optical density
QAC	: Quaternary ammonium-containing chitosan
PCS	: Photon correlation spectroscopy
S. aureus	: *Staphylococcus aureus*
TEM	: Transmisssion electron microscopy
TMC	: *N*,*N*,*N*-trimethyl chitosan
TPP	: Tripolyphosphate
ζ	: Zeta

CHAPTER I

INTRODUCTION

1.1 Statement of Problem

Incorporation of antibacterial fillers is an efficient approach to introduce antibacterial activity to materials. There are many types of filler taken into the materials to induce their antibacterial activity such as antibiotic [1,2], nitric oxide [3], halogen species [4,5], biocide [6] and heavy metal [7-9]. From several types of antibacterial fillers, heavy metal in the form silver nanoparticles, are well recognized to exhibit effective antibacterial activity and widely used in many applications such as medical devices [10-12], paint [13], coating [14] and textiles [15-17]. Recently, it has been reported that the silver nanoparticles could affect human tissues and routes of exposure including respiratory system, skin, and gastrointestinal tract [18]. It has been proposed that silver nanoparticles may interact with thiol groups of proteins and enzymes within mammalian cells. These proteins and enzymes are key components of the antioxidant defense mechanism of the cell. Inhibition of this mechanism would result in an increase of reactive oxygen species that can disturb the function of the cell. However, these effects depend on the amount of silver nanoparticles. Early study has found that the silver nanoparticles can be released from commercial clothing (socks) into wash water [19]. These results suggested that the releasing of the particles to wash water might increase the risk of environmental problem. Therefore, it is highly desirable if the antibacterial fillers can be developed from natural, nontoxic and environmentally friendly material.

Chitosan is a natural biopolymer derived by deacetylation of chitin, an abundantly available biopolymer found in exoskeletons of insects, shell of crustaceans, and fungal cell walls. It is nontoxic, biodegradable, biocompatible and possesses antibacterial properties [20]. Chitosan shows its antibacterial activity only in acidic medium, which is ascribed to the poor solubility of chitosan above its pKa (pH 6.5). For this reason, a number of chitosan derivatives have been developed not only to expand the use of chitosan in a broader pH range and media but also to improve the bactericidal

actions of chitosan. Among all derivatives that exhibit superior antibacterial activity to native chitosan, quaternary ammonium-containing ones have gained the most attention.

Quaternization of amino groups to introduce quaternary ammonium groups to chitosan has been recognized as a potential way to enhance the antibacterial activity of chitosan in a broader pH range. The reaction can simultaneously introduce the permanent positive charge and hydrophobicity, the two parameters believed to affect the antibacterial activity. Many research groups have reported the synthesis of chitosan derivatives having different quaternary ammonium groups such as *N*,*N*,*N*-trimethyl chitosan, *N*-propyl-*N*,*N*-dimethyl chitosan, *N*-furfuryl-*N*,*N*-dimethyl chitosan [21,22], *N*-diethylmethyl chitosan [23], quaternized carboxymethyl chitosan [24], chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride [25,26], quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan, and quaternized *N*-(4-pyridylmethyl) chitosan [27].

Recent work reported by Qi and coworkers have demonstrated that chitosan in the form of nanoparticles exerted higher antibacterial activity against *E. coli, S. choleraesis, S. typhimurium, and S. aureus* than chitosan solution because of the greater surface area of the particles provided intimate contact with the surface of bacterial cells. The studies also indicated that the antibacterial activity of chitosan and chitosan nanoparticles in acidic media were higher than that in neutral media. Moreover, the antibacterial activity can be enhanced by incorporating Cu^{2+} into chitosan particles in order to promote ionic interactions with the negatively charged components of the cell membrane [28]. In addition, there are a few studies suggesting that chitosan particles loaded with various metal ion such as Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, exhibited higher antibacterial effect against *E. coli*, *S. choleraesuis*, and *S. aureus* than chitosan particles [29,30]. Also, Shi and co-workers fabricated particles by ionic crosslinking tripolyphosphate with chitosan that was formerly quaternized by hexyl bromide. The quaternized chitosan particles were later tested as bactericidal fillers against *S. aureus* and *S. epidermidis* in poly(methyl methacrylate) bone cement under neutral environment [31].

This research aims to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles using two methods. The first method was to prepare chitosan particles and their surface was then modified by heterogeneous quaternization through *N*-reductive alkylation with aldehyde followed by alkylation with alkyl iodide or direct methylation with methyl iodide. The second method was to synthesize *N*,*N*,*N*-trimethyl chitosan by homogeneous quaternization using methyl

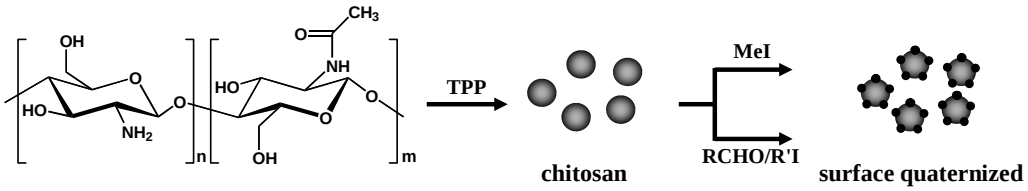
iodide followed by particle formation. A series of characterization techniques, namely Fourier transformed infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (^{1}H NMR), photon correlation spectroscopy (PCS), and transmission electron microscopy (TEM) are employed to confirm the success quaternization and particle formation. Antibacterial activity tests against both a gram-positive bacteria, *Staphylococcus aureus*, and a gram negative bacteria, *Escherichia coli*, are performed by turbidimetric method and viable cell counts**.** Minimum inhibitory concentration of some particles is also determined.

1.2 Objectives

1. To prepare quaternary ammonium-containing chitosan particles
2. To test antibacterial activity of quaternary ammonium-containing chitosan particles

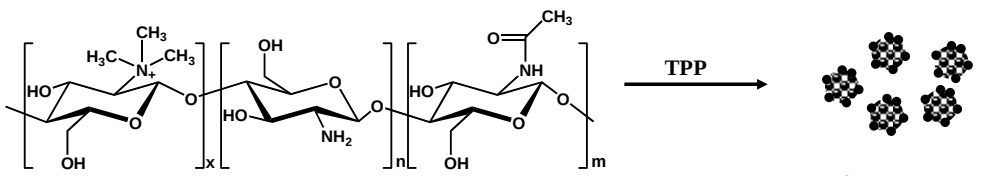
1.3 Scope of the Investigation

1. Preparation of quaternary ammonium-containing chitosan particles by heterogeneous quaternization



chitosan | **chitosan particles** | **surface quaternized particles**

2. Preparation of quaternary ammonium-containing chitosan particles by homogeneous quaternization followed by particle formation



***N, N, N* – trimethyl chitosan** | **● = quaternary ammonium group**

3. Characterization of the quaternary ammonium-containing chitosan particles

4. Determination of antibacterial activity of quaternary ammonium-containing chitosan particles against *Staphylococcus aureus* (gram positive bacteria) and *Escherichia coli* (gram negative bacteria) using turbidimetric method and viable cell counts.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Bacteria [32]

Bacteria are small and deceptively simple prokaryotes. They are most abundant microorganism and it can be found everywhere in nature. In order to explore the role and effect of bacteria, it is necessary to understand several aspects of the structure of prokaryotic cells.

2.1.1 The Bacterial Cell Wall

Bacteria can be divided into two groups by the Gram staining technique. These staining reactions involve an attraction of the cell to a charge dye, it is important to note that the terms gram positive and gram negative are not used to indicate the electrical charge of cells or dyes. The different results in Gram staining are due to difference in structure of the cell wall and how it reacts to the series of reagents applied to the cells.

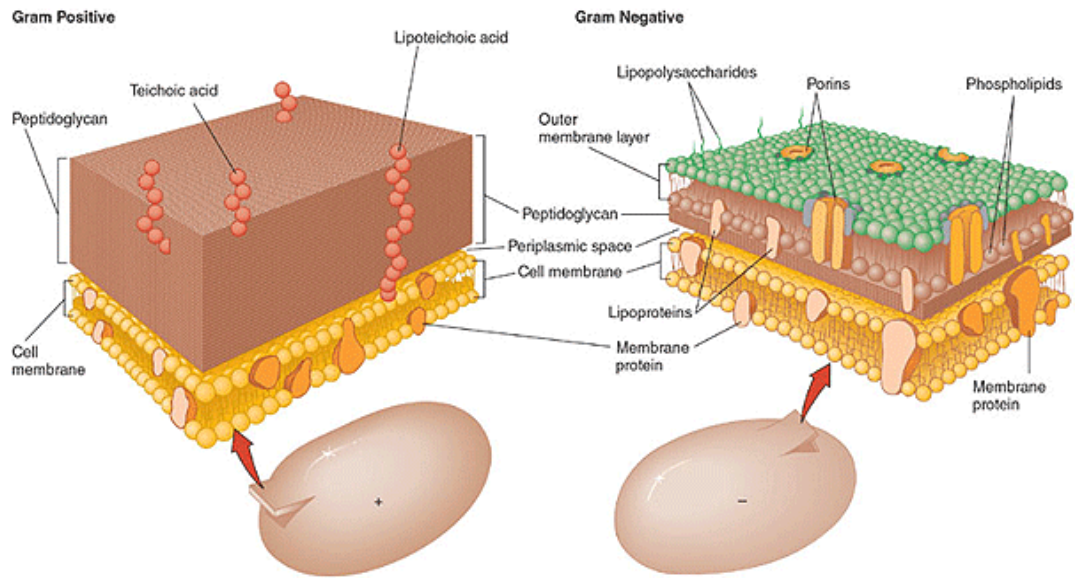


Figure 2.1 Structure of gram positive and gram negative cell walls

The Gram Positive Cell Wall

The bulk of the gram positive cell wall is a thick, homogeneous sheath of peptidoglycan ranging from 20 to 80 nm in the thickness. It also contains tightly bound acidic polysaccharides, including teichoic acid and lipoteichoic acid (Figure 2.1). Teichoic acid is a polymer of ribitol or glycerol and phosphate embedded in the peptidoglycan sheath. Lipoteichoic acid is similar in structure but is attached to the lipids in the plasma membrane. These molecules appear to function in cell wall maintenance and enlargement during cell division, and they also contribute to the acidic charge on the cell surface. In some case, the cell wall of gram positive bacteria is pressed tightly against the cell membrane with very little space between them, but in other cell, a thin periplasmic space is evident between the cell membrane and cell wall.

The Gram Negative Cell Wall

The gram negative cell wall is more complex in morphology because it contains an outer membrane, has a thinner shell of peptidoglycan, and has an extensive space surrounding the peptidoglycan (Figure 2.1). The outer membrane is somewhat similar in construction to the cell membrane, except that it contains specialized types of polysaccharides and proteins. The uppermost layer of the outer membrane contains lipopolysaccharide. The polysaccharide chains extending off the surface function as antigens and receptors. The innermost layer of the outer membrane is a phospholipid layer anchored by means of lipoproteins to the peptidoglycan layer below. The outer membrane serves as a partial chemical sieve by allowing only relatively small molecules to penetrate. Access is provided by special membrane channels formed by porin proteins that completely span the outer membrane. The size of the, porin can be altered so as to block the enhancement of harmful chemicals, making them one defend of gram negative bacteria against certain antibiotics.

The bottom layer of gram negative wall is a single, thin (1-3 nm) sheet of peptidoglycan. Although it acts as a somewhat rigid protective structure as previously describe, its thinness gives gram negative bacteria a relatively greater flexibility and sensitivity to lysis. There is a well developed periplasmic space surrounding the peptidoglycan. This space is an important reaction site for a large and varied pool of substances that enter and leave the cell.

2.1.2 Bacterial Growth [33]

Bacterial growth is the division of one bacterium into two daughter cells in a process called binary fission. Providing no mutational event occurs, the resulting daughter cells are genetically identical to the original cell. In autecological studies, bacterial growth in batch culture can be modeled with four different phases: lag phase, exponential or log phase, stationary phase, and death phase (Figure 2.2).

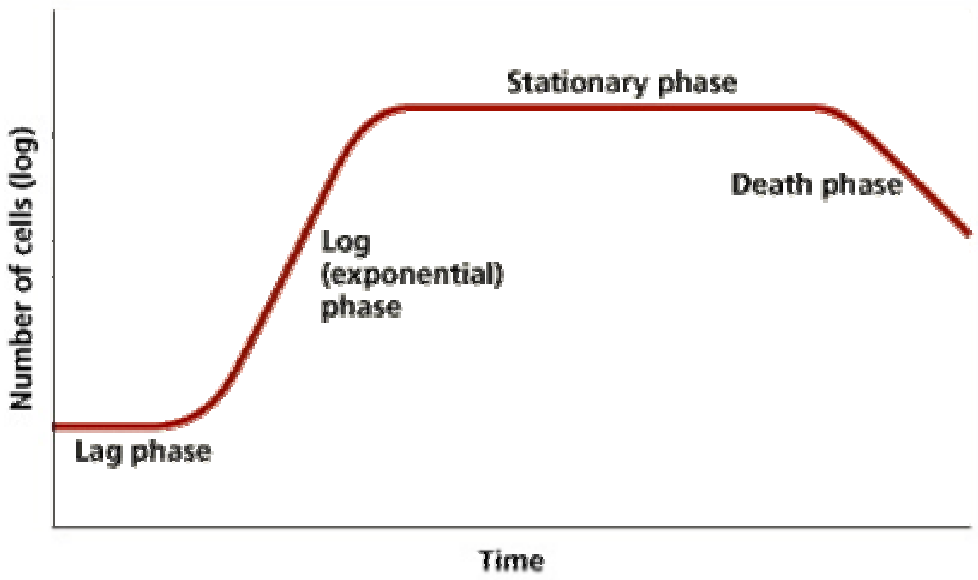


Figure 2.2 Bacterial growth curve

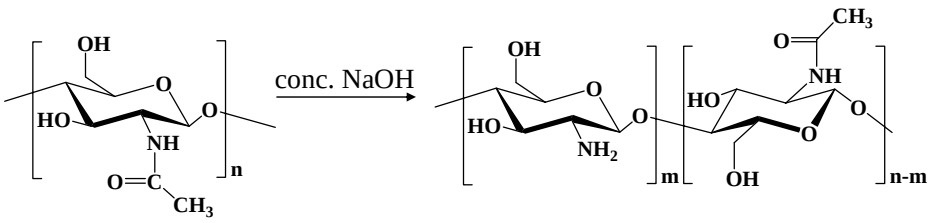
In lag phase, bacteria adapt themselves to the growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs.

At exponential phase (log phase) is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate so both the number of cells and the rate of population increase to doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time. The actual rate of this growth (slope of the line in Figure 2.2) depends upon the growth conditions, which affect the frequency of cell division events and the

probability of both daughter cells surviving. Exponential growth cannot continue indefinitely. However, because the medium is soon depleted of nutrients and enriched with wastes.

During stationary phase, the growth rate slows down as a result of nutrient depletion and accumulation of toxic products. This phase is reached as the bacteria begin to exhaust the resources that are available to them. This phase is a constant value as the rate of bacterial growth is equal to the rate of bacterial death. At death phase, bacteria run out of nutrients and die.

2.2 Chitin and Chitosan



Chitin Chitosan

Scheme 2.1 Structures of chitin and chitosan

The shells of crabs, shrimps, prawns, and lobster are used for the industrial preparation of chitin. The isolation includes two-step process: demineralization with HCl and deproteinization with aqueous NaOH. Lipids and pigments may also be extracted. Isolated chitin is a highly ordered copolymer of 2-acetamido-2-deoxy-β-D-glucose and 2-amino-2-deoxy-β-D-glucose. As a point of difference from other abundant polysaccharides, chitin contains nitrogen. 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 [20].

Chitosan are biodegradable, biocompatible, non-toxic, nonallergenic, renewable biomaterials and find applications in fields such as medicine, cosmetics, food industry and agriculture. Chitosan is prepared by deacetylation of chitin as shown in Scheme 2.1. At the elemental analysis, chitosan have nitrogen content higher than 7% and degree of acetylation lower than 0.40. Removal of the acetyl group is a harsh treatment usually performed with concentrated NaOH solution (either aqueous or alcoholic). Protection from oxygen, with a nitrogen purge or addition of sodium borohydride to the alkali

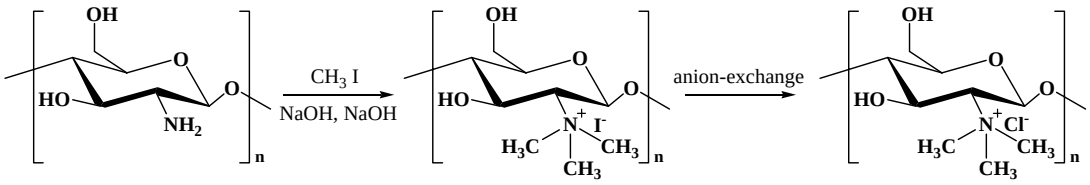
solution, is necessary in order to avoid undesirable reactions such as depolymerization and generation of reactive species. The presences of 2-amino-2-deoxyglucose units in a chitosan bring the polymer into solution by salt formation. As a result, chitosan is a primary aliphatic amine that the pKa of chitosan amine is 6.3 so chitosan can be soluble in pH values of lower than 6.0. 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 its derivatives that are water soluble and can be used in various applications [20,34,35].

2.3 Charged Derivatives of Chitosan

Applications of chitosan are limited due to its limited solubility. From this reason, many researchers have tried to modify the functional group of chitosan to make it soluble in a wider pH range. 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. 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, reacting readily with most electrophiles. Not only can chemical functionalization improve the solubility of chitosan, most often it can also bring about new or improved properties to chitosan.

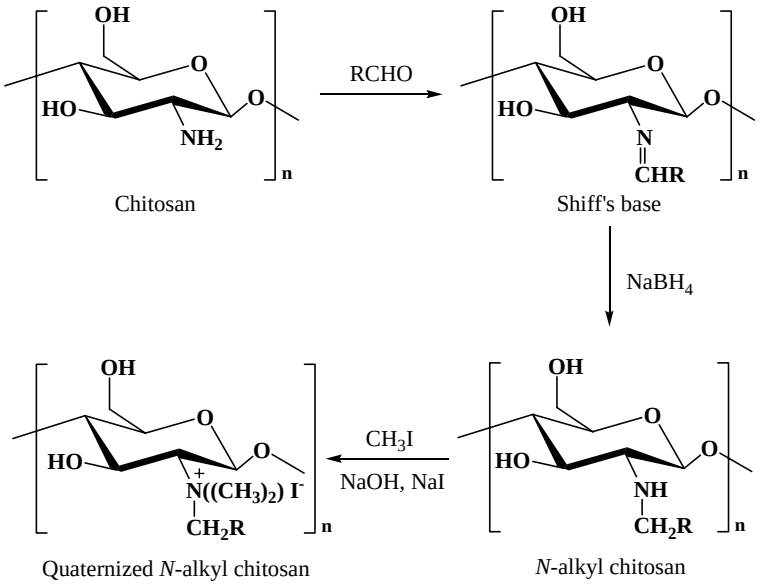
2.3.1 Quaternized Chitosan Derivatives

In 1985, Domard *et al*. [36] 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.



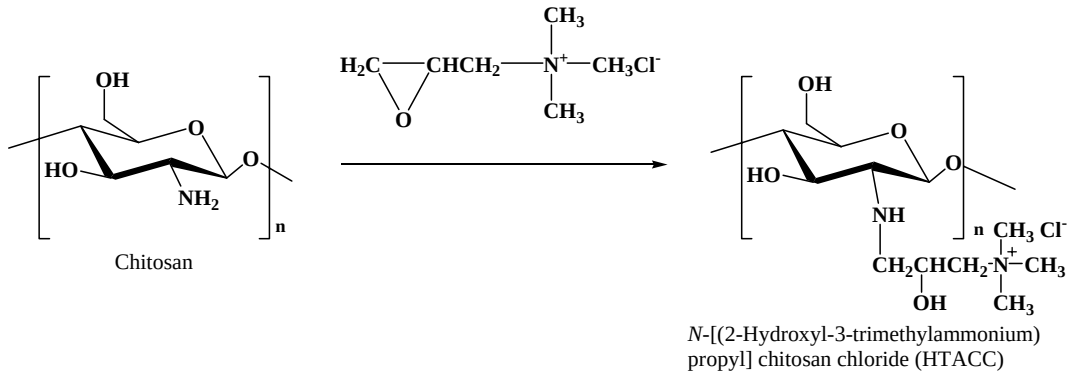
Scheme 2.2 Synthesis of *N*,*N*,*N*-trimethyl chitosan chloride (TMC)

In 2001, Jia *et al*. [37] 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. These derivatives were prepared by the reaction between amino groups of chitosan and appropriated aldehydes to form Schiff base intermediate and then quaternized with methyl iodide (Scheme 2.3).



Scheme 2.3 Synthesis of quaternized *N*-alkyl chitosan

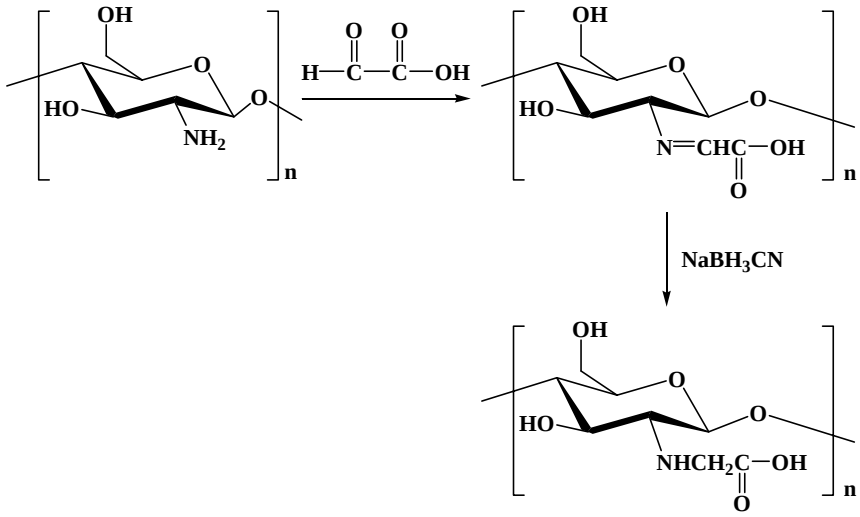
In 2000, Seong, *et al*. [38] synthesized the quaternary ammonium salt using glycidyl trimethylammonium chloride as the quaternizing agent. The product which is called *N*-[(2-hydroxyl-3-trimethylammonium)propyl] chitosan chloride (HTACC) shows excellent solubility in water (Scheme 2.4).



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

2.3.2 Carboxyalkylated Chitosan Derivatives

Another method to improve the solubility of chitosan is carboxyalkylation of chitosan. Muzzarelli *et al*. [39] prepared *N*-carboxymethyl chitosan (NCMC) (Scheme 2.5) by treating an aqueous suspension of chitosan with glyoxylic acid followed by pH adjustment and reduction with sodium cyanoborohydride. The NCMC was soluble in water at all pH values.

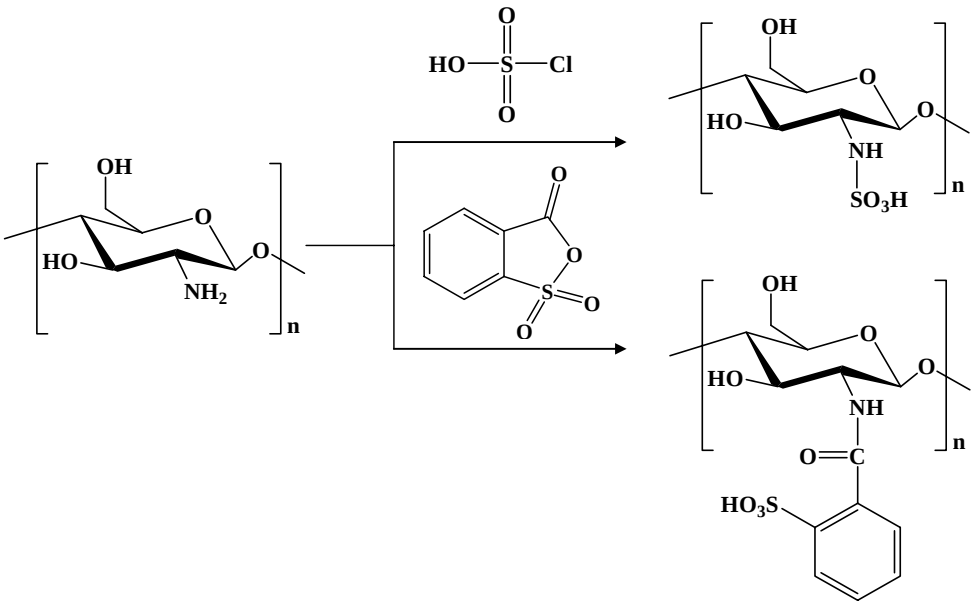


Scheme 2.5 Synthesis of *N*-carboxymethyl chitosan (NCMC)

2.3.3 Chitosan Derivatives with Sulfonyl Groups

In 1992, Muzzarelli [40] prepared *N*-sulfofurfuryl chitosan and sulfoethyl *N*-carboxymethyl chitosan by the reaction of chitosan with sodium salt of 5-formyl-2-furansulfonic acid and 2-chloroethanesulfonic acid, respectively. The ^{13}C NMR and FT-IR spectra showed typical signals of furane carbons. Circular dichroism measurements were used to indicate its polyampholyte nature. The chelating reaction with metal ion was used to prove the chelating ability.

In 1998, Chen *et al*. synthesized *N*-sulfonated [41] and *N*-sulfobenzoyl [42] chitosan (Scheme 2.6). *N*-Sulfonated chitosan (SC) 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.



Scheme 2.6 Synthesis of *N*-sulfonated and *N*-sulfobenzoyl chitosan

2.4 Antibacterial Activity of Chitosan

2.4.1 Antibacterial Mechanism

The present of primary amino groups of chitosan making them exhibit more pronounced activity than chitin. Under mildly acidic conditions (~pH 6), the amino groups display a positive charge which is usually associated with the demonstrated activity. The exact mode of interaction between chitosan, its derivatives and the microorganism is still unknown, but different mechanisms have been proposed to explain antimicrobial activity. It is believed that the polycationic nature of chitosan initiates binding with the cell membrane by means of electrostatic attraction with negatively charged microbial cell membrane.

In 1991, Cuero, *et al*. [43] proposed that chitosan can bind with DNA and inhibit mRNA and protein synthesis upon penetration into the nuclei of fungi.

In 1999, Tsai, *et al*. [44] indicated that the cytoplasmic membrane of bacteria was detached from the inner part of the cell wall after chitosan treatment by using TEM analysis. Once bound to the cell surface, chitosan is thought to affect membrane permeability which results into the leakage of proteinaceous material and other intracellular constituents of the microbial cell causing death due to the loss of essential fluids.

In 1999, Shahidi *et al*. [45] reported the different antibacterial mechanism of chitosan and their derivatives.

- Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents.
- It acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth.
- It activates several defense processes in the host tissue, acts as a water binding agent and inhibits various enzymes.
- Binding of chitosan with DNA and inhibition of mRNA synthesis occurs via chitosan penetrating the nuclei of the micro-organisms and interfering with the synthesis of mRNA and proteins.

2.4.2 Factors Affecting Antibacterial Activity

The antimicrobial activity of chitosan is controlled by many factors such as molecular weight (MW), degree of deacetylation (DD), pH and temperature. It is necessary to understand these factors for the effective application of chitosan as an antimicrobial agent.

In 2003, Lim *et al*. [46] suggested that antibacterial activity of chitosan depends on the MW and type of bacterial target. For example, medium and high MW chitosan exhibited higher activities against *Bacillus circulans* as compared to chito-oligosaccharides [47]. However, the activity was reversed when the tests were performed on *Escherichia coli*. When comparing the trend against *Escherichia coli*, the activity seems to increase with the MW, until it reaches a certain value (MW= 30,000), after which the activity starts declining. [48]

In 2006, Liu *et al.* [49] reported that the effect of antimicrobial activities of chitosan and acetic acid against *Escherichia coli*. All of the chitosan samples with MW from 5.5×10^4 to 15.5×10^4 Da had antimicrobial activities at the concentrations higher than 200 ppm. The antibacterial activity of low MW chitosan is higher than that of the high molecular weight samples. However, the chitosan sample with the middle MW (9.0×10^4 Da) could promote the growth of bacteria.

Antibacterial activities of chitosan exhibit a direct correlation with the degree of deacetylation (DD) [46]. As a result, the number of amino groups on chitosan is increased when the DD increases. This can be implied that increasing of amino groups on chitosan would increase the interaction between positively charged chitosan and negatively charged cell walls of microorganisms. On the contrary, antimicrobial activity of chitosan is increased when pH is decreased. To study effect of temperature to antibacterial activity of chitosan against *Escherichia coli* [44], it was found that at temperatures of 25˚C and 37˚C, the *Escherichia coli* cells were completely killed within 5 h and 1 h respectively. The authors suggested that the reduced antimicrobial activity resulted from the decreased rate of interaction between chitosan and cells at a lower temperature.

2.4.3 Antibacterial Applications of Chitosan

Chitosan have been widely used as an antimicrobial agent in many applications. As a result, it is a safe biopolymer that it can be used especially in food and biomedical applications.

In 2003, Rebea *et al.* [50] reported that the antimicrobial activity of chitosan was observed against a wide variety of microorganisms including fungi, algae, and some bacteria (Table 2.1). However, the antimicrobial action is influenced by intrinsic factors such as the type of chitosan, the degree of chitosan polymerization, the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates or both, and the environmental conditions. Chitosan film was successfully prepared as food wrap. The film was tough, elastic and durable; comparable to many of the medium strength commercial polymers. When applied as a coating over fruits and product, the film controls the influx of moisture and oxygen and also reduces transpiration loss and delays ripening process while preserving the food stock. Moreover, it was observed that the packaging film prepared from chitosan possesses antimicrobial activity.

Table 2.1 Antibacterial and antifungal activity of chitosan

Bacteria	**MIC(ppm)**	***Fungi***	**MIC(ppm)**
Agrobacterium tumefaciens	100	*Drechstera sorokian*	100
Escherichia coli	20	*Rhizoctonia solani*	1000
Pseudomonas fluorescens	500	*Trichophyton equinum*	2500
Staphylococcus aureus	20	*Botrytis cinerea*	10
Micrococcus luteus	20	*Fusarium oxysporum*	10

In 2004, Qi *et al*. [28] revealed that chitosan nanoparticles and copper loaded chitosan nanoparticles could inhibit the growth of various microorganisms (*E. coli, S. choleraesuis, S. typhimurium, S. aureus*). Chitosan nanoparticles exhibited higher antibacterial activity than chitosan solution. Because of the larger surface area of the chitosan nanoparticles, it could be tightly adsorbed onto the surface of the bacterial cells so as to disrupt the membrane. This would lead to the leakage of intracellular components, thus killing the bacteria cells. Moreover, copper loaded chitosan nanoparticles exhibited the highest antibacterial activity. The higher surface charge density of copper loaded nanoparticles, which enhances the affinity with the negatively charged bacteria membrane, is probably responsible for their higher antibacterial activity.

In 2005, Ye *et al.* [51] synthesized core-shell particles that consist of poly(*n*-butyl acrylate) (PBA) cores and chitosan shells as a novel antibacterial coating for cotton fabrics. The cotton fabric was coated with PBA-chitosan particles by using a conventional pad-dry-cure method. Its antibacterial efficiency was then evaluated quantitatively against *Staphylococcus aureus* with a shake flask method. The cotton treated with PBA-chitosan particles demonstrates an excellent antibacterial activity with bacterial reductions more than 99%. Later in 2006, Ye *et al.* [52] prepared nanosized particles by grafting of antibacterial chitosan shells onto polymer cores via a surfactant free emulsion copolymerization in aqueous chitosan. The core-shell particles, one with poly (*n*-butyl acrylate) soft core and another with crosslinked poly(*N*-isopropylamide) hard core, were synthesized and also applied to cotton fabric by a conventional pad-dry-cure process. Using the shake flask method in which the reduction of the number of cells was counted, the treated fabric exhibited excellent antibacterial property against *Staphylococcus aureus* with bacterial reduction higher than 99%. The antibacterial activity maintained at over 90% reduction levels even after 50 times of home laundering.

2.4.4 Antibacterial Activities of Quaternized Chitosan Derivatives

Numerous of the researches have continued to study about the antibacterial activity of chitosan derivatives. As a result, the water soluble chitosan derivatives are advantageous for its wide application. There are several derivatives of chitosan have been studied about their antibacterial activity such as the carboxyalkylated chitosan derivatives [53-55], chitosan derivative with sulfonyl groups [41,56], ethylamine hydroxyethyl chitosan [53], guanidinylated chitosan [57] and oleyl chitosan [58]. Besides, these derivatives, quaternized chitosan derivative were interested to be used in antibacterial applications because their permanent positive charges have a potential to be bound with negatively charged membrane of bacteria. Furthermore, this derivative not only displays the positive charge but their functional groups can be modified to present hydrophobic part that it might enhance the antibacterial activity of the derivative.

In 1997, Kim *et al.* [59] reported that quaternized *N*-alkyl chitosan derivatives were synthesized by introducing alkyl groups into the amine groups of chitosan via Schiff's base intermediates and then quaternized by using methyl iodide to produce water soluble cationic polyelectrolytes. The results showed that the derivative exhibited antibacterial activities against *Staphylococcus aureus*. The antibacterial activities of the chitosan derivatives with quaternary ammonium salt had a direct relationship with the chain length of the alkyl substituent, and this increased activity could be occurred due to the increasing of hydrophobic properties of the derivatives.

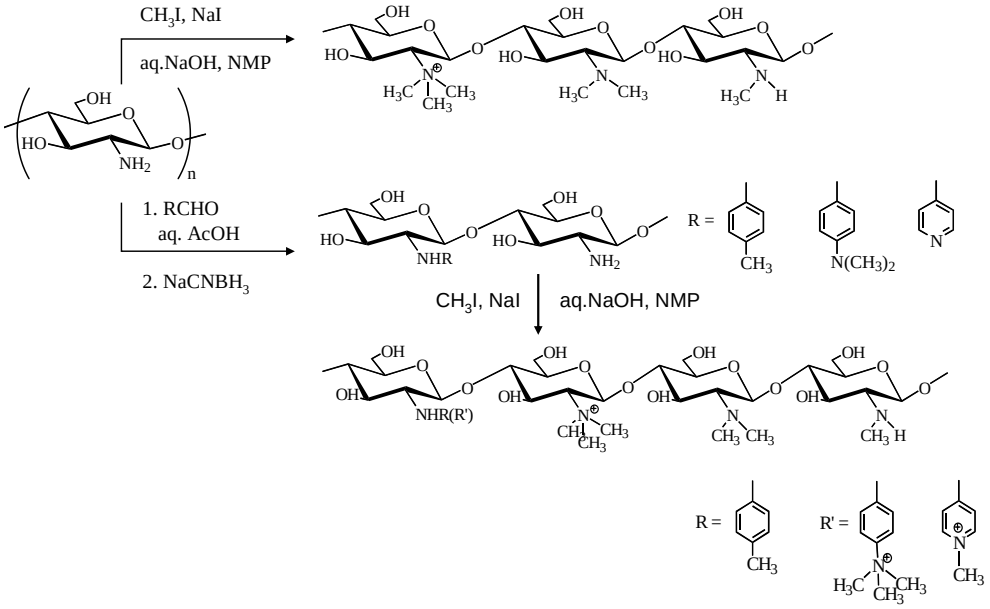
In 2001, Jia *et al*. [37] studied the antibacterial activities of quaternized chitosan; *N,N,N*-trimethyl chitosan, *N*-propyl-*N,N*-dimethyl chitosan and *N*-furfuryl-*N,N*-dimethyl chitosan against *Escherichia coli*. Results showed that the antibacterial activity of quaternized chitosan against *Escherichia coli* is related to its molecular weight. The antibacterial activity of quaternized chitosan in acetic acid medium is stronger than that in water. Furthermore, it was also found that the antibacterial activity of quaternized chitosan was stronger than that of chitosan.

In 2004, Avadi *et al*. [23] synthesized quaternized chitosan (*N*-diethylmethyl chitosan, DEMC) based on a modified two-step process: reductive alkylation using formaldehyde followed by quaternization with ethyl iodide The antimicrobial activities of chitosan and DEMC against *Escherchia coli* were compared by calculation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration

(MBC). From the results, it could be indicated that the antimicrobial activity of DEMC is higher than that of chitosan in acedic medium.

In 2006, Sun *et al*. [24] indicated that quaternized carboxymethyl chitosan (QCMC) prepared by the reaction of carboxymethyl chitosan (CMC) with 2, 3-epoxypropyl trimethylammonium. QCMC exhibited the antbacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Having the same degree of substitution (DS) of QCMC and quarternary chitosan (QC), they found that QCMC has stronger antimicrobial activity.

In 2008, Sajomsang *et al*. [27] reported the synthesis of *N*-arylated chitosan via Schiff bases formed by the reaction between the primary amino groups of chitosan with aromatic aldehydes followed by reduction of the Schiff base intermediates with sodium cyanoborohydride (Scheme 2.7). After that, *N*-arylated chitosan were treated with iodomethane under basic conditions. Finally, methylated *N*-alkylated chitosan was occurred. The total degree of quaternization of chitosan derivatives depends on the extent of *N*-substitution and the sodium hydroxide concentration in methylation step. The antibacterial activity of the derivatives was tested against *Escherichia coli* and *Staphylococcus aureus*. Minimum inhibitory concentrations (MIC) of these derivatives ranged from 32 to 128 μg/mL.



Scheme 2.7 Synthesis of methylated chitosan and *N*-aryl chitosan

2.4.5 Quaternized Chitosan Derivatives in Antibacterial Applications

The antimicrobial activity of chitosan against various bacteria and fungi is well known because of the polycationic nature of chitosan. Chitosan can be used in a variety of application, including food science, agriculture, medicine, pharmaceutics and textiles. Antibacterial activities of quaternized chitosan derivatives have been reported by many researches. They study the activity of the derivatives in various fields of applications.

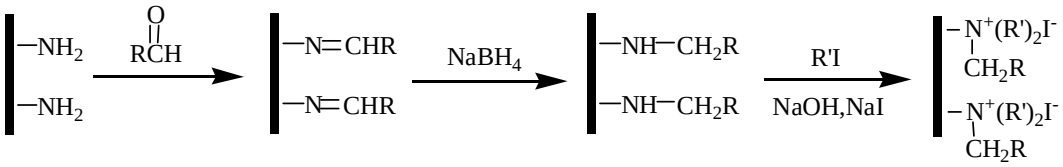
In 2006, Sun *et al*. [24] synthesized quaternized carboxymethyl chitosan (QCMC) by the reaction between *N*-quaternary ammonium groups and 2,3-epoxypropyl trimethylammonium. Antibacterial activities of QCMC were evaluated against *Escherichia coli* and *Staphylococcus aureus*. The antibacterial activities of QCMC were affected by the degree of substitution (DS) of quaternary group and the molecular weight while no clear effect of DS of carboxymethyl group on the antimicrobial activity was observed. QCMC was complexed with calcium hydroxide for used as pulp-cap in dental application. From animal experiment results, it was found that QCMC can strongly induce reparative dentine formation and showed a better ability in dentin inducing than calcium hydroxide.

In 2006, Shi *et al*. [31] prepared chitosan nanoparticles (CS NP) and quaternary ammonium chitosan derivative nanoparticles (QCS NP) as bactericidal agents in poly(methyl methacrylate) (PMMA) bone cement. The antibacterial activity was tested against *Staphylococcus aureus* and *Staphylococcus epidermidis.* The antibacterial effectiveness remained high even after the modified bone cements had been immersed for 3 weeks in an aqueous medium. Using MTT cytotoxicity assay, CS NP and QCS NP loaded cements did not exhibit cytotoxic effect against a mouse fibroblast. Mechanical tests indicated that the addition of the CS and QCS in nanoparticulate form allowed the retention of a significant degree of the bone cement's strength. These results indicate a new promising strategy for combating joint implant infection.

In 2006, Ignatava *et al.* [60] prepared nano-fibres containing quaternized chitosan (QCh) (*N*-butyl-*N*,*N*-dimethyl chitosan iodide) by electrospinning of QCh solutions mixed with poly(vinyl alcohol) (PVA). The higher the content of QCh induced the smaller diameter of the nanofibers. The photocrosslinked electrospun mats exhibited a good antibacterial activity against *Staphylococcus aureus* and *Escherichia*

coli. These results showed that the electrospun mats revealed their high potential for wound dressing applications.

In 2007, Vallapa [61] modified chitosan surface by introducing quaternary ammonium groups via a heterogeneous two-step process: reductive alkylation using selected aldehydes followed by methylation with methyl iodide. The antibacterial activity of the surface-modified chitosan film against *Staphylococcus aureus* and *Escherichia coli* were superior to that of the virgin chitosan film. The additional positive charge and hydrophobicity introduced to the chitosan film after surface quaternization made the quaternary ammonium-containing chitosan film a more favorable substrate for interacting with the negatively-charged membrane of the bacteria.



Chitosan film

Surface-quaternized *N*-alkyl chitosan

Scheme 2.8 Surface quaternization of chitosan film

In 2008, Belalia *et al.* [62] indicated that the hydroxypropylcellulose (HPC) films coatings with chitosan and *N*,*N*,*N*-trimethylchitosan (TMC) exhibited antibacterial activity against the *Listeria monocytogenes* and *Salmonella typhimurium*. The HPC-chitosan and HPC-TMC coatings exhibited a total inhibition on solid medium of both bacterial strains. From antibacterial activity and physicochemical properties of films revealed that this film has a potential to be used in food applications.

In 2008, Sadeghi *et al*. [63] prepared *N*-trimethyl chitosan (TMC) and *N*-diethylmethyl chitosan (DEMC) nanoparticles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods (PEC). The obtained nanoparticles by the PEC method had higher insulin loading efficiency and zeta potential both required for an effective permeation enhancement across the intestinal epithelium. Results from antibacterial test against *Staphylococcus aureus* revealed that the nanoparticles displayed lower inhibition effect than the polymers in free soluble form.

CHAPTER III

EXPERIMENTAL

3.1 Materials

Chitosan flake (DAC 85%, M_V = 45,000 Da) was purchased from Seafresh Chitosan (Lab) Co., Ltd (Thailand). Acetaldehyde, methyl iodide (MeI), propionaldehyde, sodium borohydride ($NaBH_4$), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium iodide (NaI) and tripolyphosphate (TPP) were purchased from Fluka (Switzerland). Acetic acid, benzaldehyde, ethanol (EtOH), formaldehyde, and methanol (MeOH) were purchased from Merck (Germany). Ethyl iodide (EtI) was purchased from Sigma (USA). All reagents and materials are analytical grade and used without further purification. *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* were provided by National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) were purchased from Difco (USA). Mueller Hinton agar plate was supplied by Department of Medical Science, Ministry of Public Health, Thailand. Ultrapure distilled water was obtained after purification using a Millipore Milli-Q system (USA) that involves reverse osmosis, ion exchange, and a filtration step.

3.2 Equipments

3.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H NMR spectra were recorded in solution of CF3COOH/D_2O or D_2O 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 Impact 410 FT-IR spectrometer. All samples were prepared as KBr pellets.

3.2.3 Transmission Electron Microscopy (TEM)

The size and the morphology of chitosan and quaternary ammonium-containing chitosan particles were examined by transmission electron microscope (TEM, Model JEM-2100, Japan). The average diameter was reported from measurement 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 are 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.

3.3 Methods

3.3.1 Preparation of Chitosan Particles

Chitosan flasks were dissolved at selected concentration with 1% (v/v) acetic acid. pH of the solution was raised to 4.6-4.8 with 10 N NaOH. Chitosan particles formed spontaneously upon an addition of 3 mL of an aqueous TPP solution to 3 mL of chitosan under magnetic stirring. The particles were isolated by centrifugation at 12,000 rpm using a high speed refrigerated centrifuge (Model GL21M, Yingtai Instrument, China) for a 30 min interval. Supernatants were discarded and the particles were centrifugally washed with deionized water (5x) and then lyophilized by freeze dryer, model Freezone 77520, Benchtop, Labconco, USA, before further use or analysis.

3.3.2 Preparation of *N*-alkyl Chitosan Particles

An anhydrous methanol solution of a selected aldehyde (10 mL) having a desired concentration was added into a flask containing chitosan particles (0.03 g). After stirring for a given time at ambient temperature, $NaBH_4$ (0.3 g, 0.8 mol) was added into the reaction mixture and the solution was stirred for 24 h. The particles were isolated by centrifugation at 12,000 rpm for 30 min. Supernatants were discarded and the particles were centrifugally washed with methanol (3x) and dried *in vacuo*.

3.3.3 Preparation of Quaternized *N*-alkyl Chitosan Particles

An anhydrous methanol solution of NaI (0.2 M) was added into a flask containing *N*-alkyl chitosan particles (0.03 g) and NaOH (0.13 g, 0.3 mol). A selected alkyl iodide was added via syringe. The total volume of the reaction mixture was 10 mL. The reaction mixture was stirred at 50°C for a given time. The particles were isolated by centrifugation at 12,000 rpm for 30 min. Supernatants were discarded and the particles were centrifugally washed with methanol (3x) and dried *in vacuo*.

3.3.4 Preparation of *N,N,N*-Trimethyl Chitosan

N,N,N-trimethyl chitosan was synthesized according to a modified method of Sieval et al [64]. Chitosan (2 g) was dispersed in 80 mL of *N*-methyl-2-pyrrolidone for 18 h, followed by an addition of NaOH (0.81 g, 0.02 mol) and NaI (1.51 g, 0.01 mol). The reaction mixture was stirred at 40°C under magnetic stirring. MeI (4.32 g, 0.03 mol) was added every 4 h. The mixture was stirred at 40°C for 8 h. The product was precipitated using acetone. The precipitate was dissolved in 6% (w/v) NaCl solution in order to replace the iodide counterion with a chloride counterion. The suspension was then transferred to the dialysis membrane (cut-off molecular weight of 12,400 g/mol, Sigma, USA) where it was dialyzed against deionized water. The final product was obtained after lyophilized.

3.3.5 Preparation of *N,N,N*-Trimethyl Chitosan Particles

N,N,N-trimethyl chitosan was dissolved at selected concentration with deionized water. The particles formed spontaneously upon an addition of 3 mL of an aqueous TPP solution to 3 mL of *N,N,N*-trimethyl chitosan solution under magnetic stirring. The particles were isolated by centrifugation at 12,000 rpm for a 30 min interval. Supernatants were discarded and the particles were centrifugally washed with deionized water (3x) and then lyophilized before further use or analysis.

3.3.6 Evaluation of Antibacterial Activity

All glasswares used for the tests were sterilized in an autoclave at 121°C for 15 min prior to use. All particles were sterilized by exposing to UV radiation for 60 min prior to the tests. Mueller-Hinton broth dissolved in deionized water was autoclaved for 15 min at 121°C. The broth was stored at 4 °C before use. Mueller-

Hinton agar dissolved in deionized water (5 mL) was transferred into each test tube before autoclaved for 15 min at 121°C. The tubes are placed in a slanted position to allow the agar to solidify. The agar slant was stored at 4 °C before use.

S. aureus and *E. coli* were used as gram positive and gram negative bacteria, respectively. A loopful of bacteria were streaked on agar slant, then incubated at 37°C in an incubator shaker (rotary incubator shaker Model G-25, New Brunswick Scientific Co., Inc., USA) for 24 h. Sterile deionized water (10 mL) was added in the tube containing agar slant to obtain bacterial suspension. The optical density of the suspended bacteria in sterile deionized water was determined by UV-visible spectrophotometer (Model MV, the Bausch ε Lomb, USA) at a wavelength of 600 nm (OD_{600}). The value was adjusted to 0.5 by sterile deionized water.

3.3.6.1 Turbidimetric Method

The test was performed according to a modified method of Qi et al. [28] Chitosan and quaternary ammonium-containing chitosan particles (2.5 mg) were added to the test tubes each containing 5 mL of Mueller–Hinton broth. The tubes were inoculated under aseptic conditions with 50 μL of the freshly prepared bacterial suspension. pH of the broth was about 7.4. The negative and positive controls were a broth solution alone and a broth solution with ampicillin (50 mg/mL), respectively. After mixing, the tubes were incubated at 37°C, at a shaking speed of 110 rpm for 24 h. The OD_{600} of the solution was measured to determine a density of bacteria in the broth.

3.3.6.2 Viable Cell Counting Method

Chitosan particles and quaternary ammonium-containing chitosan particles were (2.5 mg) were added to the test tubes each containing 5 mL of Mueller–Hinton broth. The tubes were inoculated under aseptic conditions with 50 μL of the freshly prepared bacterial suspension. pH of the broth was about 7.0. After mixing, the tubes were incubated at 37°C, at a shaking speed of 110 rpm for 24 h. Then, the mixture (100 μL) of the bacterial solution was diluted to 10^6 times. A 100 μL of the diluted bacterial solution was then spreaded onto the Mueller-Hinton agar. After incubating at 37°C for 18 h, a number of viable bacteria were then counted. The results after multiplication with the dilution factor were expressed as mean colony forming units per volume (CFU/mL).

3.3.6.3 Determination of the Minimum Inhibitory Concentration (MIC)

The chitosan particles were suspended in 5 mL of Mueller Hinton broth. To the first tube containing 5 mL of broth was added by 5 mL of the particle suspension. After mixing, 5 mL of the mixture was transfered to the second tube and similar transformations were repeated. Therefore, each tube that contains the sample solution reveals half of the concentration of the previous one. The tubes were inoculated under aseptic conditions with 50 μL of the freshly prepared bacterial suspension having OD_{600} of 0.5. After incubating at 37°C for 24 h, the solution in each tube was subjected to testing of antibacterial activity based on two methods described above. The lowest concentration of the particles that inhibited the growth of bacteria was considered as the minimum inhibitory concentration (MIC).

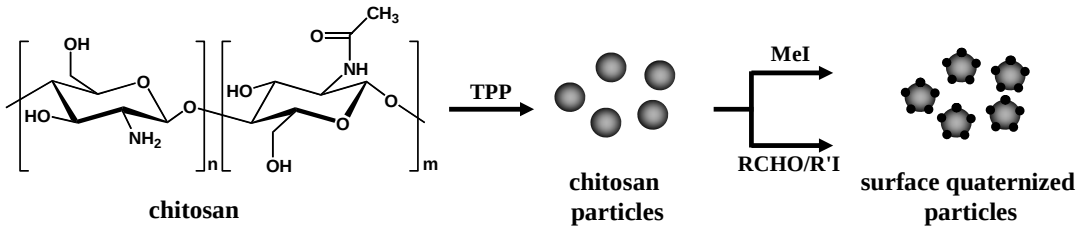
3.3.6.4 Statistical Analysis

All data obtained from the tests of antibacterial activity are expressed as mean ± one standard deviation (SD) of a representative of three similar experiments carried out in triplicate. Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) version 17.0 software. Statistical comparisons made by One-Way Analysis of Variance (ANOVA) with the Least Square Difference (LSD) tests were used for post hoc evaluations of differences between groups. Statistical significance was associated with the value of $p < 0.01$.

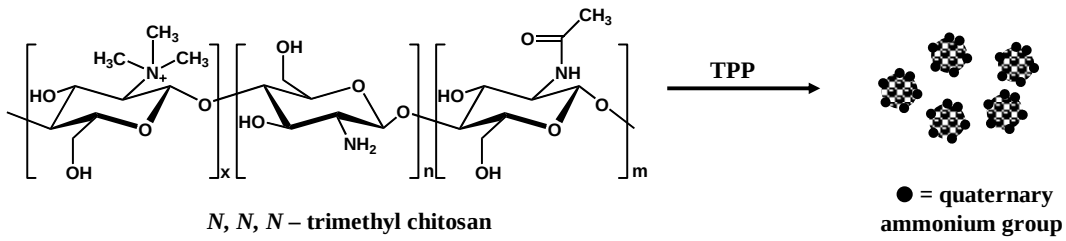
CHAPTER IV

RESULTS AND DISCUSSION

This part is divided into 2 sections. The first section reveals the preparation of quaternary ammonium-containing chitosan particles using two methods. The first method was to prepare chitosan particles and then modified their surface by heterogeneous quaternization using selected aldehydes and alkyl iodides (See Table 4.1). The second method was to synthesize *N*,*N*,*N*-trimethyl chitosan (TMC) by homogeneous quaternization using methyl iodide followed by the preparation of the particles. The second section involves determination of antibacterial activities of the quaternary ammonium-containing chitosan having different alkyl group.



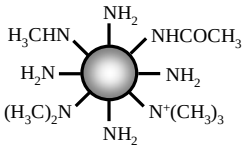
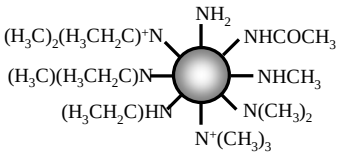
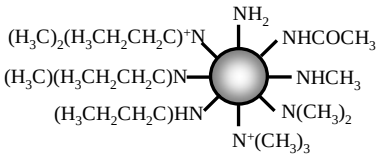
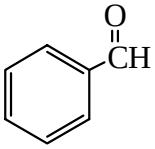
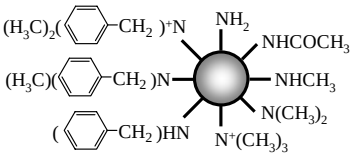
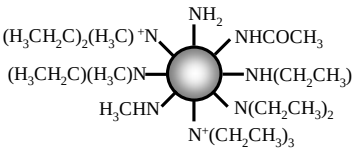
Scheme 4.1 Preparation of quaternized chitosan particles by heterogeneous quaternization of chitosan particles



***N, N, N* – trimethyl chitosan**

Scheme 4.2 Preparation of quaternized chitosan particles from TMC

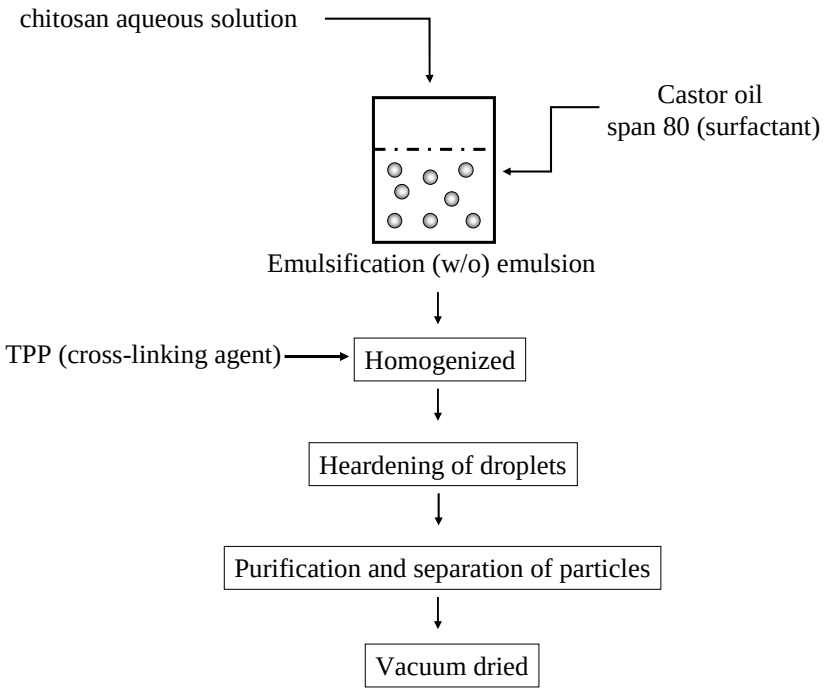
Table 4.1 Aldehydes and alkyl iodides used for surface quaternization of chitosan particles

RCH (O)	**R'I**	**Particles**
-	CH_3I	QAC (Quaternary ammonium-containing chitosan) ; heterogeneous quaternization TMC (*N*,*N*,*N*-trimethyl chitosan) ; homogeneous quaternization 
CH_3CH(O)	CH_3I	MEC (Methylated Ethyl chitosan) 
CH_3CH_2CH(O)	CH_3I	MPC (Methylated Propyl chitosan) 
	CH_3I	MBzC (Methylated Benzyl chitosan) 
HCH (O)	CH_3CH_2I	EMC (Ethylated Methyl chitosan)) 

4.1 Preparation of Quaternary Ammonium-Containing Chitosan Particles by Heterogeneous Quaternization

4.1.1 Attempt to Prepare Chitosan Particles by Emulsion Cross-linking

Different methods have been used to prepare chitosan particles such as emulsion cross-linking, precipitation, spray-drying, emulsion-droplet coalescence method, ionic gelation, reverse micellar method and sieving method [65]. At first, emulsion cross-linking was chosen for the preparation of chitosan particles. The stepwise procedure is shown in Scheme 4.3. The size and shape of the particles can theoretically be varied as a function of aqueous droplets. In the experiment, water-in-oil (w/o) emulsion was prepared by mixing the 0.2% (w/v) chitosan solution in castor oil (oil phase) by homogenizer. Aqueous droplets were stabilized using 2% (v/v) span 80 as a surfactant. The chemical structures of span 80 and TPP are displayed in Figure 4.1. The stable emulsion was crosslinked by TPP in order to harden the droplets. Chitosan particles were isolated by centrifugation. Supernatants were discarded and the particles were centrifugally washed with EtOH (5x) and then dried *in vacuo*. The chitosan particles prepared using this method appeared as white fine powder.



Scheme 4.3 Stepwise procedure for the preparation of chitosan particles by emulsion cross-linking

As shown in Figure 4.2, the spectrum of chitosan particles obtained from FT-IR analysis exhibited the shifting of the N-H bending signal from 1599 cm^{-1} for chitosan to 1539 cm^{-1} for chitosan particles. This suggested that the ammonium groups of chitosan were crosslinked with TPP molecules. Moreover, the spectra of chitosan particles showed a characteristic peak at 1741 cm^{-1} which can be assigned to C=O stretching signal of ester group in the structures of span 80 and castor oil. This result indicated that the surfactant and the oil phase used in the preparation of the chitosan particles could not be completely removed. The particles prepared by this method was not suitable to be further used as substrates for antibacterial filler because the remaining span 80 and castor oil may possibly interfere the subsequent step of quaternization or potentially interact with the bacteria via different mechanism and lead to ambiguous interpretation of antibacterial activity of the particles. For this reason, ionic gelation was then selected as an alternative method for the preparation of chitosan particles. The problem due to the interference of reagents should be overcome since the process can be done in aqueous solution in the presence of only one reagent, TPP in this particular case.

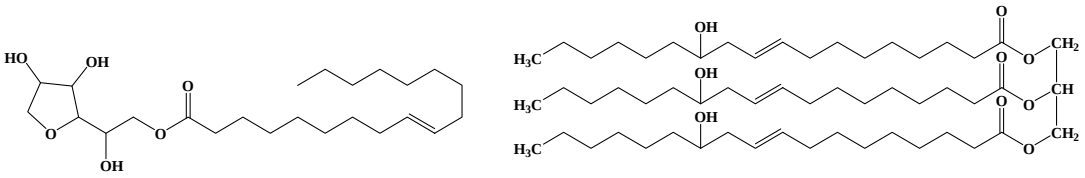


Figure 4.1 Chemical structures of span 80 and castor oil

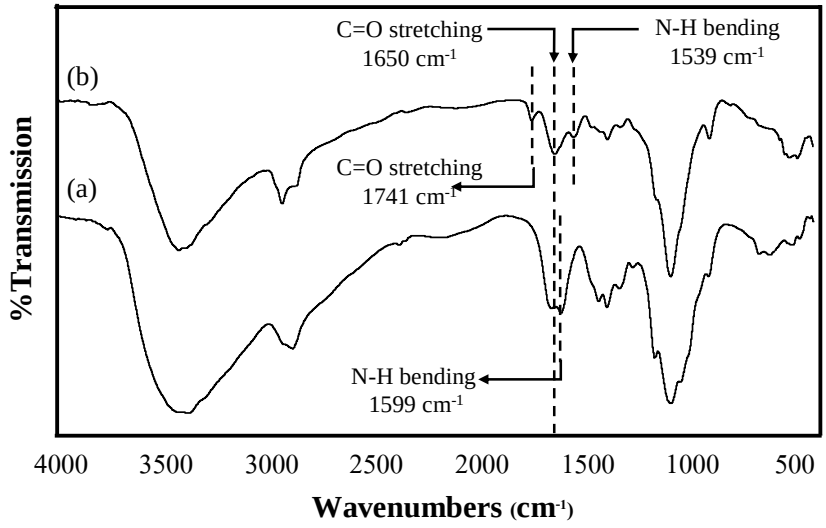
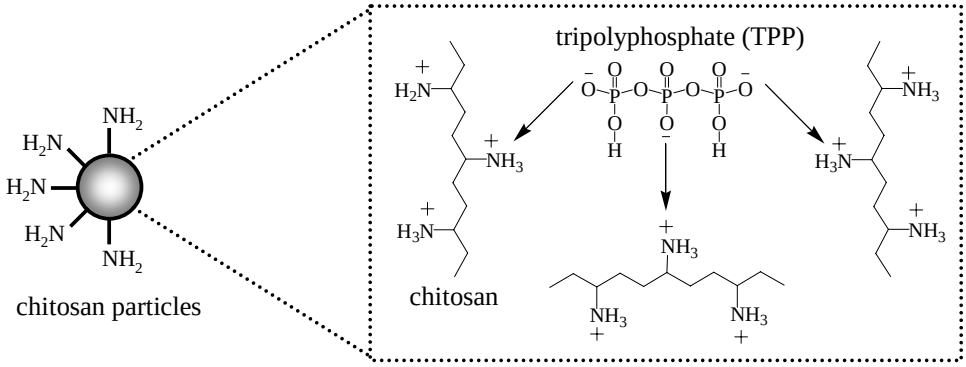


Figure 4.2 FT-IR spectra of (a) chitosan and (b) chitosan particles prepared by emulsion cross-linking

4.1.2 Preparation of Chitosan Particles by Ionic Gelation

In this particular study, ionic gelation is based on ionic interactions between positively charged chitosan and negatively charged TPP (Scheme 4.4). The process was conducted at ambient temperature in the absence of surfactant and oil phase. Although TPP consists of five acidic protons in its structure, it has been previously reported that one of them is strongly acidic so the conjugate base, oxyanion (O^-) cannot be protonated by weak acid. [66]. There are also other 2 acidic protons that can be ionized. One has a pKa of 8.8 and the other has a pKa of 6.2. It should be noted that the pH of TTP solution is about 9. Upon mixing with the chitosan solution (pH ~ 4.0), the pH would go down to ~7. Each molecule of TPP is considered to be able to provide 2-3 negative charges which can be bound with the positive charges of chitosan.



Scheme 4.4 Chitosan particles prepared by ionic gelation between chitosan and TPP

It has been reported that the size of chitosan particle should be decreased with the decreasing MW and concentration of chitosan and the ratio of chitosan to TPP [67-70]. As shown in Figure 4.3, it can be seen that upon increasing the amount of TPP relative to chitosan 1:0.25, 1:0.4, and 1:0.5, larger chitosan particles tended to be formed. This can be realized from the prompt precipitation of the particles at the bottom of the tubes even without the centrifugation. It should be noted that the smaller the particles, the better the particles disperse in the medium. That is why centrifugation at high speed is necessary for the separation of sub-micron particles. This study agrees well with previous research reported by Zhou that the diameter of the chitosan-TPP particles increased with the elevation of TPP concentration [70]. From this experiment, it can be concluded that the suitable mole ratio of amino group ($-NH_2$) of chitosan:TPP for preparing chitosan particles is 1: 0.1.

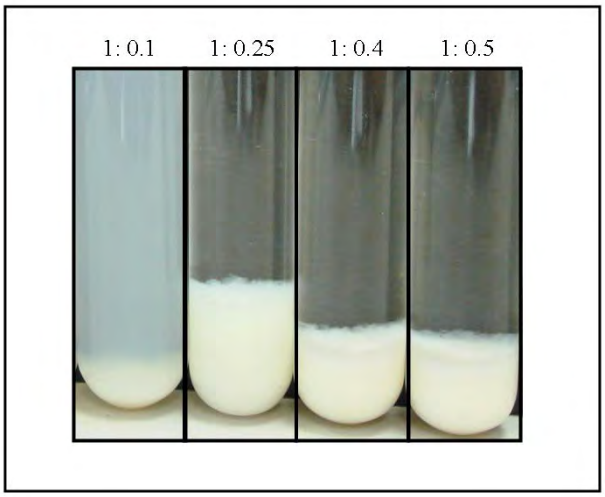


Figure 4.3 Chitosan particles prepared by ionic gelation with fixed concentration of chitosan (0.5% (w/v)) and varied mole ratio between amino groups of chitosan and TPP from 1: 0.1 to 1:0.5

To determine the effect of chitosan concentration on the particle size, the concentration of chitosan (% (w/v)) was varied from 0.2% to 0.5% and 1.0% and the mole ratio of amino groups of chitosan ($-NH_2$) to TPP was kept constant at 1:0.1. As analyzed by photon correlation spectroscopy (PCS) the size of chitosan particles apparently became larger as the increasing concentration of chitosan (Table 4.2). It should be noted that the size determination was conducted right after the addition of TPP and before the centrifugation. This result agrees quite well with what has been previously reported by Gan *et al* [69].

Table 4.2 Size of the chitosan particles prepared using different concentration of chitosan

Concentration of chitosan %(w/v)	size (µm)
0.2	0.163
0.5	0.479
1.0	0.856

Based upon the data shown above, the optimal condition that should be used for the preparation of chitosan particles from chitosan having Mv of 45,000 was to use 0.2% (w/v) of chitosan solution and the mole ratio of amino group ($-NH_2$) of chitosan: TPP of 1:0.1. The resulting chitosan particles appeared as white fine powder after drying and were insoluble in water, dilute acidic and alkali solution.

FT-IR analysis of chitosan and chitosan particles was performed to characterize the chemical structure of the particles. As shown in Figure 4.4, the appearance of a characteristic peak at 1213 cm^{-1} assigned to P=O groups of TPP and the shifting of the N-H bending signal from 1599 cm^{-1} for chitosan to 1544 cm^{-1} for chitosan particles implies that the ammonium groups of chitosan were crosslinked with TPP molecules.

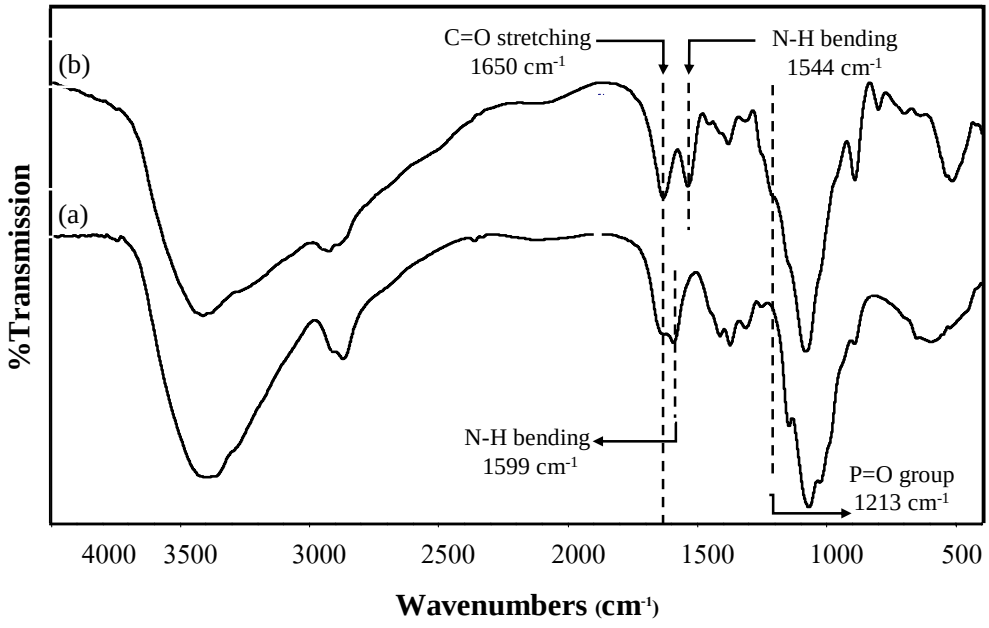
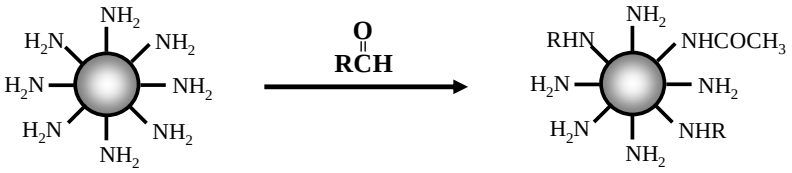


Figure 4.4 FT-IR spectra of (a) chitosan and (b) chitosan particles prepared by ionic gelation

4.1.3 Preparation of *N*-alkyl Chitosan Particles



chitosan particles *N*-alkyl chitosan particles

Scheme 4.5 Reductive *N*-alkylation of chitosan particles

Heterogeneous reductive *N*-alkylation of chitosan particles with formaldehyde, acetaldehyde, propionaldehyde and benzaldehyde yielded *N*-methyl chitosan particles, *N*-ethyl chitosan particles, *N*-propyl chitosan particles and *N*-benzyl chitosan particles, respectively. The optimized conditions for each reaction (reaction time and reagent concentration) previously identified by Vallapa [61] were used (Table 4.3).

Table 4.3 Optimized conditions used for heterogeneous reductive *N*-alkylation of chitosan particles [61]

Aldehyde	Concentration (M)	Time (h)
Formaldehyde	0.4	1.5
Acetaldehyde	0.4	1.5
Propionaldehyde	1.0	4
Benzaldehyde	1.0	4

The success of the heterogeneous reductive *N*-alkylation of chitosan particles was verified by FT-IR analysis. As shown in Figure 4.5, the appearance of a characteristic peak at 1213 cm^{-1} assigned to P=O groups of TPP and the decrement of the N-H bending signal at 1544 cm^{-1} in all spectra implied that the amino groups were functionalized by alkyl moiety introduced from the aldehyde molecule.

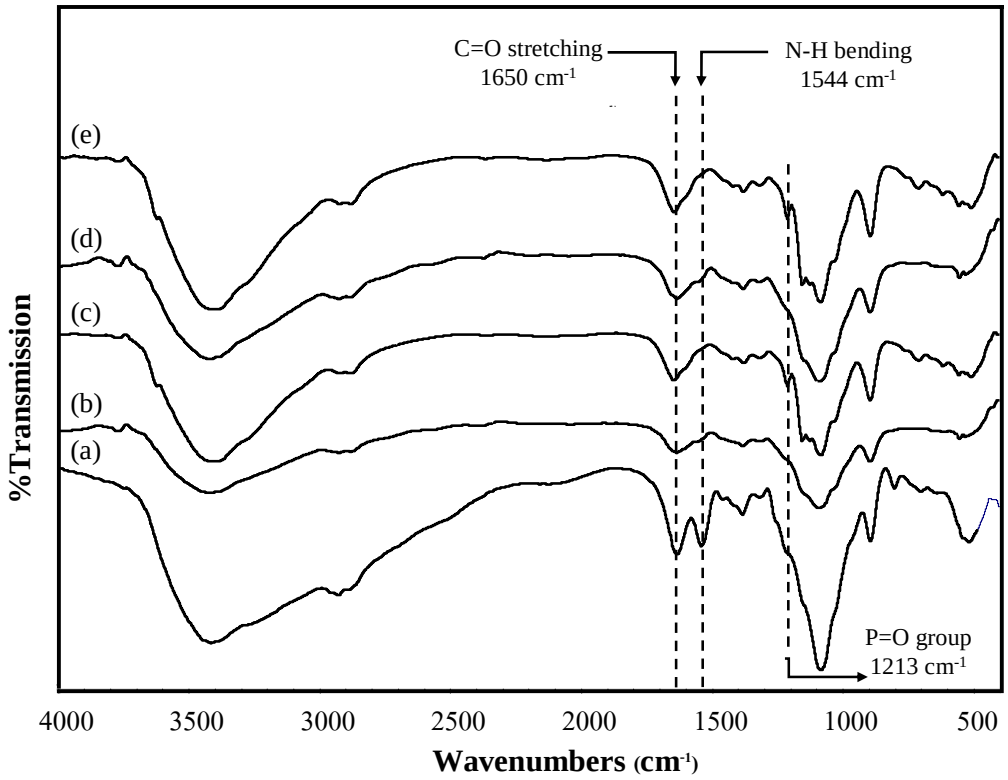
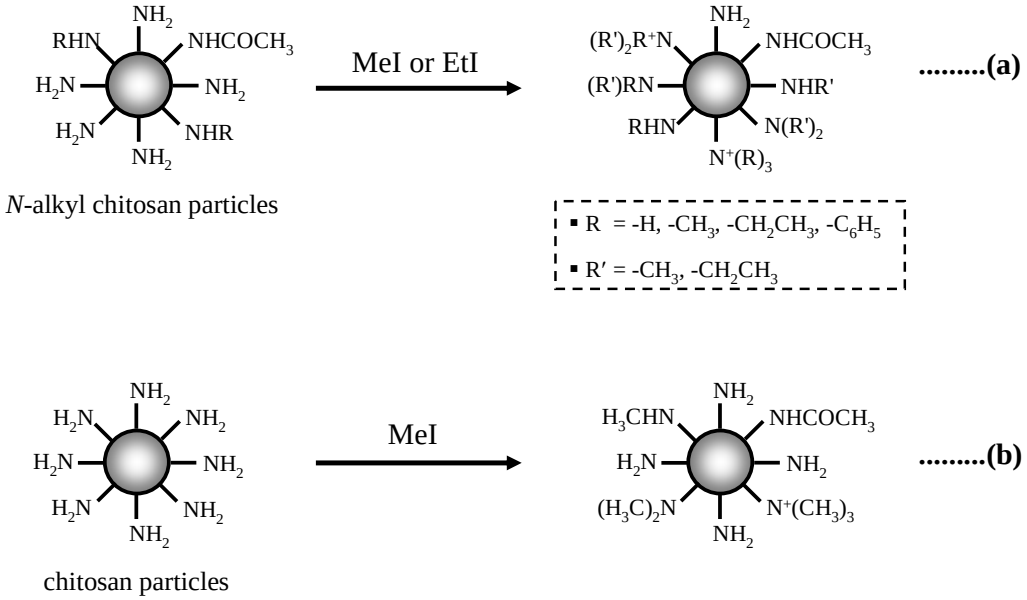


Figure 4.5 FT-IR spectra of (a) chitosan particles, (b) *N*-methyl chitosan particles, (c) *N*-ethyl chitosan particles, (d) *N*-propyl chitosan particles, and (e) *N*-benzyl chitosan particles

4.1.4 Preparation of Quaternized *N*-alkyl Chitosan Particles and Quaternized Chitosan Particles

Using the optimized conditions for quaternization of *N*-alkyl chitosan particles identified in the recent study [61] (Table 4.4), four types of quaternized *N*-alkyl chitosan particles were prepared. The quaternization of *N*-ethyl chitosan particles, *N*-propyl chitosan particles and *N*-benzyl chitosan particles with methyl iodide (MeI) yields methylated ethyl chitosan (MEC), methylated propyl chitosan (MPC) and methylated benzyl chitosan (MBzC) particles, respectively. Ethyl iodide (EtI) was used for quaternization *N*-methyl chitosan particles to produce ethylated methyl chitosan (EMC) particles. Furthermore, quaternization was also conducted directly on chitosan particles using MeI as a reagent and gave quaternary ammonium-containing chitosan (QAC) particles as products.



Scheme 4.6 Preparation of quaternary ammonium-containing chitosan particles by (a) quaternization of *N*-alkyl chitosan particles by MeI or EtI and (b) direct quaternization of chitosan particles with MeI

Table 4.4 Optimized conditions for quaternization of *N*-alkyl chitosan particles with MeI and EtI [61]

Particles	MeI (M)	EtI (M)	Time (h)
chitosan particles	1.2	-	8
N-ethyl chitosan particles	1.2	-	12
N-propyl chitosan particles	1.2	-	12
N-benzyl chitosan particles	2.0	-	12
N-methyl chitosan particles	-	2.0	12

FT-IR spectroscopy was also used to confirm the success of quaternization of *N*-alkyl chitosan by MeI and EtI. As shown in Figure 4.6, the intensity of the characteristic N-H bending peak of chitosan at 1544 cm^{-1} correspondingly decreased after the reaction implying there were fewer number of free amino groups available in the structure. The peaks in the range of 1470-1460 cm^{-1} observed on all spectra of quaternized particles are the characteristic peaks of C-N stretching of which intensity

apparently increased as a result of alkyl substitution at the amino groups in the step of quaternization.by MeI or EtI

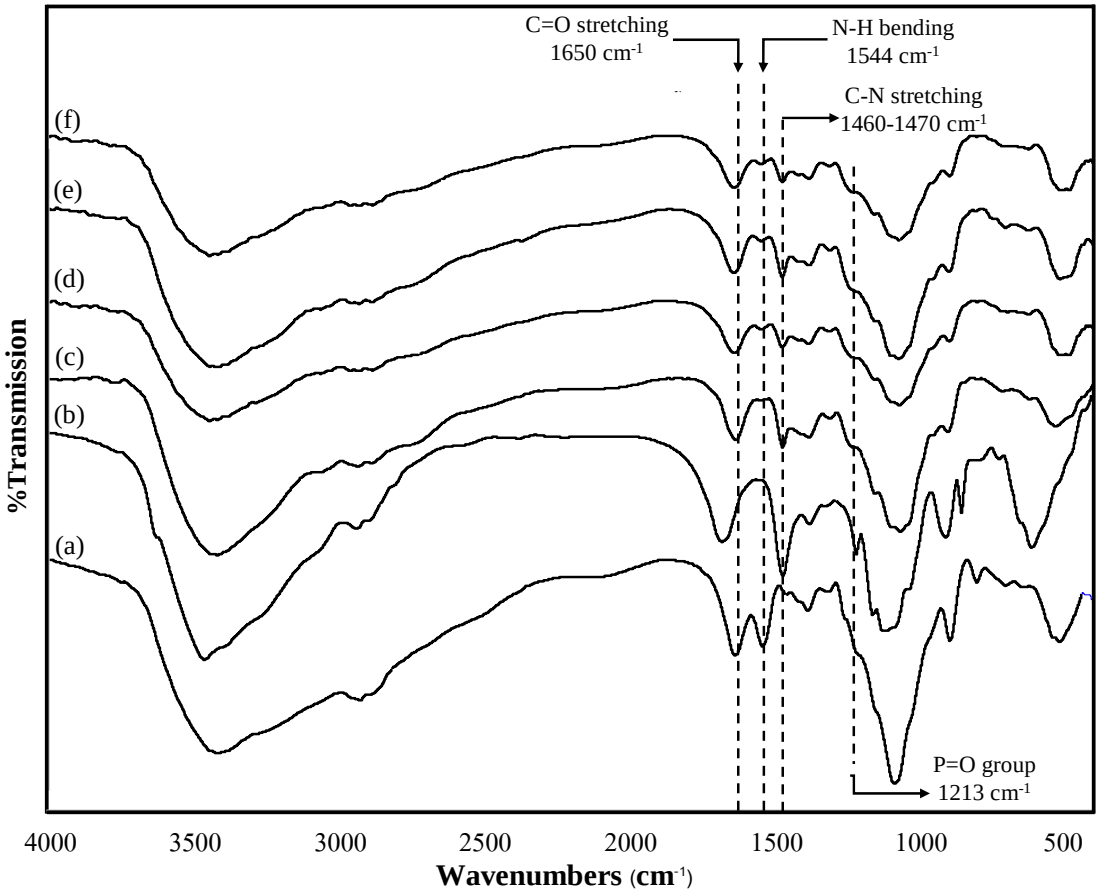


Figure 4.6 FT-IR spectra of (a) chitosan particles, (b) QAC particles, (c) MEC particles, (d) MPC particles, (e) MBC particles, (f) EMC particles

From ^{1}H NMR analysis of *N*-alkyl chitosan particles after quaternization by MeI illustrated in Figure 4.7, the signal at 3.1-3.2, 2.9, and 2.7 ppm can be assigned to the methyl protons of the trisubstituted (B), disubstituted (C) and monosubstituted (D) amino groups, respectively. In the case of MPC particles (Figure 4.7(d)), the signals of methyl protons (0.80 ppm) and methylene protons (1.0 ppm) were also observed. These evidences confirmed the attachment of propyl groups at the amino moieties of chitosan in the step of *N*-reductive alkylation. However, the spectrum of MEC particles (Figure 4.7(c)) only exhibited the signal of methylene protons with extremely low intensity at 1.0 ppm whereas the signal of methyl protons cannot be detected. For the MBzC

particles (Figure 4.7(e)), the signals of aromatic proton at 7.4 ppm could be used to verify the attachment of benzyl groups at the amino groups of chitosan.

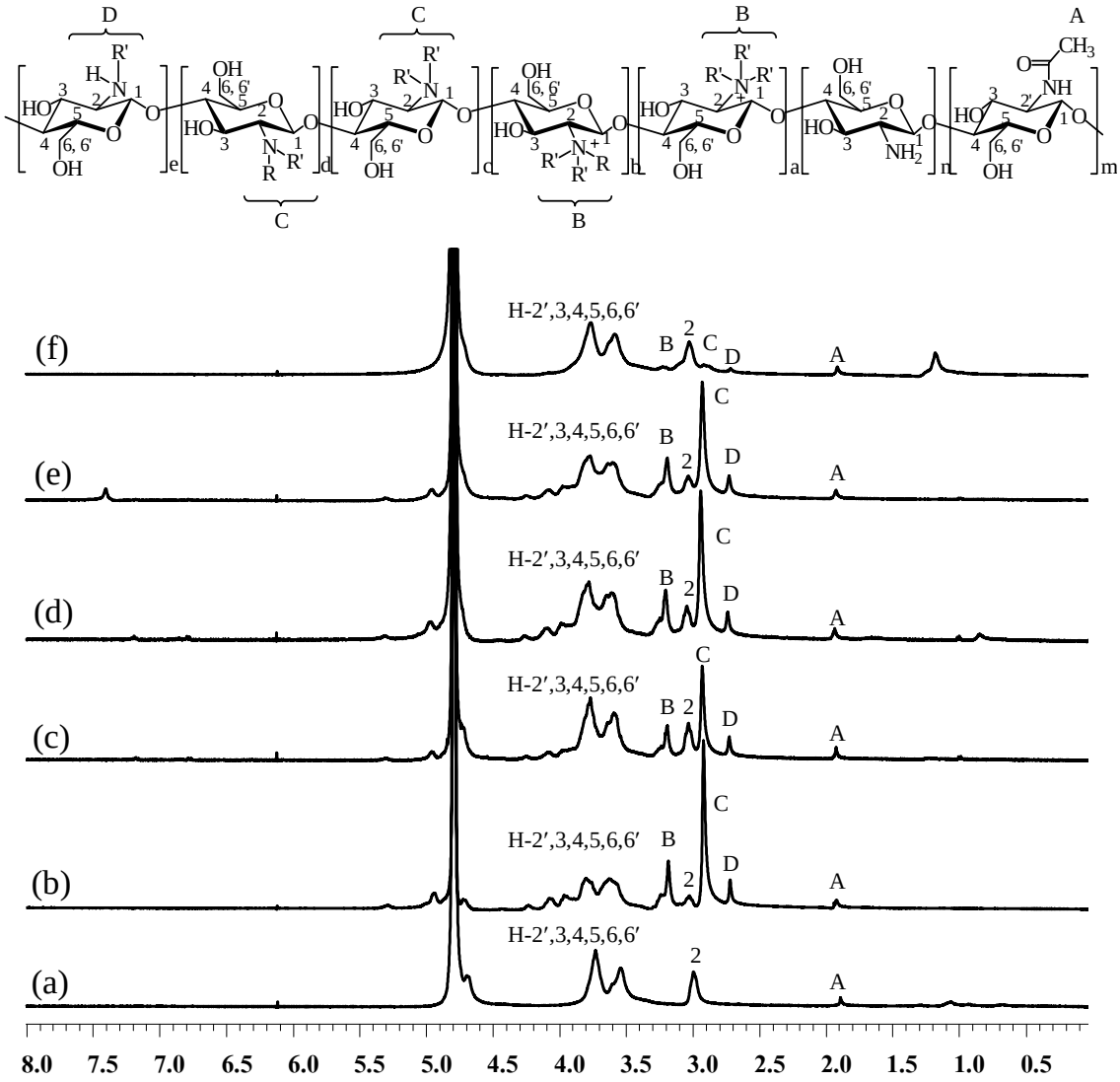


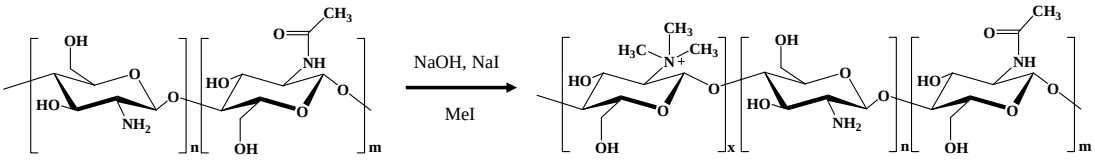
Figure 4.7 ^{1}H-NMR spectra of (a) chitosan particles, (b) QAC particles, (c) MEC particles, (d) MPC particles, (e) MBzC particles and (f) EMC particles.

Even though there was a signal of methyl protons from ethyl groups appearing at 1.25 ppm indicating the substitution of ethyl groups in the step of quaternization, the signal of trisubstituted (B) disubstituted (C) and monosubstituted (D) amino groups having one methyl substituent in the spectrum of EMC particles were rather low in intensity (Figure 4.7(f)). This strongly suggested that the alkylation by EtI at the monosubstituted amino groups (-$NHCH_3$), and disubstituted amino groups (-$NCH_3(C_2H_5)$ in order to form (-$NCH_3(C_2H_5)$) and (-$N^+CH_3(C_2H_5)_2$) was not effective.

The substitution was somewhat limited by the steric hindrance of the EtI upon the attack by substituted amino groups of bulky polymeric substance like chitosan. It is thus reasonable to assume that most of the ethyl substitution would occur at the unsubstituted free amino positions yielding ($-NH(C_2H_5)$), ($-N(C_2H_5)_2$), and ($-N^+(C_2H_5)_3$).

4.2 Preparation of Quaternary Ammonium-Containing Chitosan Particles by Homogeneous Quaternization

4.2.1 Preparation of *N,N,N*-trimethyl Chitosan (TMC) and *N,N,N*-trimethyl Chitosan (TMC) particles



Scheme 4.7 Preparation of *N,N,N*-trimethyl chitosan by homogeneous quaternization with methyl iodide

N,N,N-trimethyl chitosan (TMC) was synthesized via methylation of amino groups of chitosan by methyl iodide (MeI) (Scheme 4.7). Under basic condition, the methylation preferentially occurs at amino groups than the hydroxy groups. HI, the by-product from the reaction was neutralized by NaOH. The reaction could be promoted by NaI. From ^{1}H NMR spectrum of TMC shown in Figure 4.8(b), the signal at 3.2-3.3 ppm can be assigned to the methyl protons of the quaternary ammonium group while the signal at 2.8 and 2.6 ppm can be assigned to the methyl protons of the disubstituted and mono-substituted amino groups, respectively. TMC particles were also prepared by ionic gelation using TPP as a crosslinking agent. The same ^{1}H NMR pattern was also observed for TMC particles Figure 4.8(c).

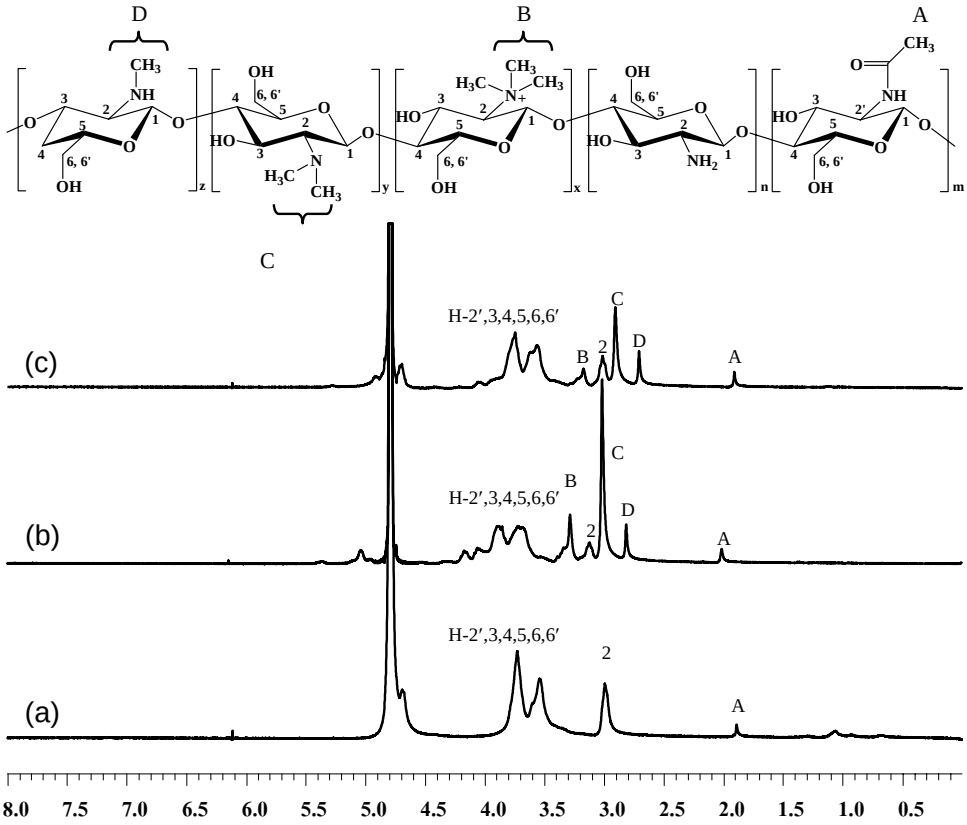


Figure 4.8 ^{1}H-NMR spectra of (a) chitosan and (b) *N*,*N*,*N*-trimethyl chitosan and (c) *N*,*N*,*N*-trimethyl chitosan particles

The degree of quaternization or degree of tri-substitution of methyl groups ($-N^{+}(CH_3)_3$) on TMC and TMC particles can be determined from the relative ratio between the integration of 9 protons from 3 methyl groups ($-N(C\underline{H}_3)_3$) of TMC and TMC particles and the peak integration of 6 protons of chitosan (δ 3.4-4.4 ppm). The relative ratio from amount of amino group on chitosan (chitosan; DAC 85%) can be calculated using the following equation.

$$\%DS_{(tri-)} = \frac{\left(\frac{\int N^{+}(CH_3)_3}{9}\right)}{\left(\frac{\int H\text{-}2',3,4,5,6,6'}{6} \times \frac{85}{100}\right)} \times 100 \qquad \text{……..(4.1)}$$

The degree of disubstitution (-N(C$\underline{H}_3$)$_2$) can be determined from the relative ratio between the integration of 6 protons from 2 methyl groups of TMC and TMC particles and 6 proton of chitosan (δ 3.4-4.4 ppm) by using the following equation.

$$\%DS_{(di-)} = \frac{\left(\frac{\int N(CH_3)_2}{6}\right)}{\left(\frac{\int H\text{-}2',3,4,5,6,6'}{6} \times \frac{85}{100}\right)} \times 100 \quad \ldots\ldots\ldots(4.2)$$

The degree of monosubstitution (-NH(C$\underline{H}_3$)) can be determined from the relative ratio between the integration of 3 protons from methyl groups of TMC and TMC particles and the peak integration of 6 protons of chitosan (δ 3.4-4.4 ppm). These can be calculated using the following equation.

$$\%DS_{(mono-)} = \frac{\left(\frac{\int N(CH_3)}{3}\right)}{\left(\frac{\int H\text{-}2',3,4,5,6,6'}{6} \times \frac{85}{100}\right)} \times 100 \quad \ldots\ldots..(4.3)$$

From the data displayed in Table 4.5, it was found that the degree of quaternization or degree of trisubstitution of methyl groups (-$N^+(CH_3)_3$) on TMC particles was lower than that of TMC. This may stem from the fact that the some positive charges of TMC were consumed for ionic crosslinking with negatively charged TPP upon the particle formation.

Table 4.5 Degree of substitution of TMC and TMC particles

Substrate	**Integration**				**%DS**			%DS $_{total}$
	H-2',3,4,5,6,6'	-N^+(C$\underline{H}_3$)$_3$ tri-substution	-N(C$\underline{H}_3$)$_2$ di-substution	-NHC$\underline{H}_3$ mono-substution	tri-	di-	mono-	
TMC	100	15.0	44.2	5.5	11.8	51.9	12.9	76.6
TMC particles	100	2.7	20.1	6.4	2.1	23.6	15.1	40.8

FT-IR spectroscopy was used to confirm the success of quaternization of chitosan by MeI. As shown in Figure 4.9, the intensity of the characteristic N-H bending peak of chitosan at 1599 cm^{-1} correspondingly decreased after the reaction. The peaks in the range of 1470-1460 cm^{-1} observed on the spectra are the characteristic peaks of C-N stretching. Furthermore, the shifting of the N-H bending signal from 1599 cm^{-1} of chitosan and TMC to 1549 cm^{-1} of TMC particles implies that the quaternary ammonium groups were crosslinked with TPP molecules.

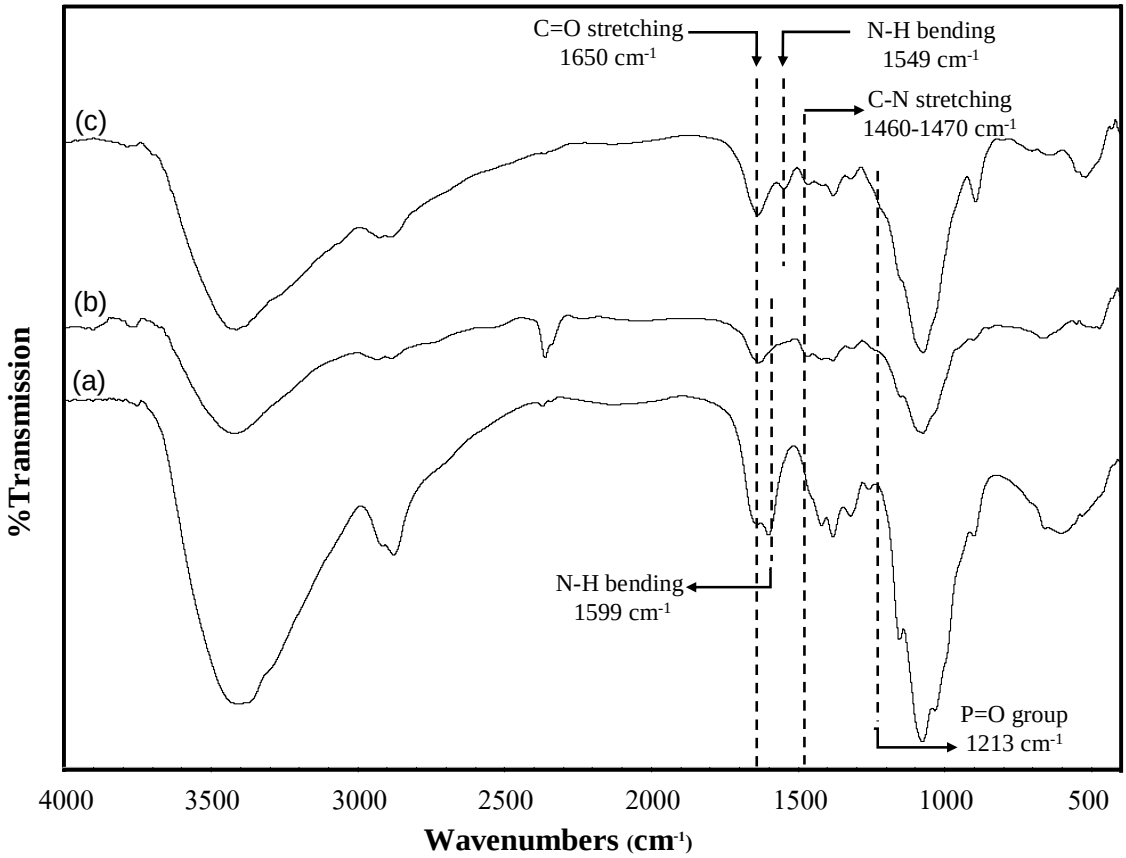


Figure 4.9 FT-IR spectra of (a) chitosan, (b) TMC, and (c) TMC particles

4.3 Degree of Substitution and Charge Characteristic of Quaternary Ammonium-Containing Chitosan Particles

Evidences from the NMR analysis presented in section 4.1 implied that the heterogeneous quaternization can be realized by a bulk technique like ^{1}H NMR. The degree of substitution of quaternized *N*-alkyl chitosan particles can thus be estimated and compared from the ^{1}H NMR integration in very much the same way as we have just done for TMC and TMC particles. To simplify the calculation, it was assumed that the majority of quaternary ammonium groups existed in the form of $-N^{+}(C\underline{H}_3)_3$ and only few were present in the form of $-NR^{+}(C\underline{H}_3)_2$. This assumption is based on the fact that the non-alkylated or free amino groups ($-NH_2$) left after the step of *N*-reductive alkylation are less bulkier, although more electronically reactive than the alkylated amino group (-NHR) towards nucleophilic substitution of MeI. Such characteristic should favor the formation of $-N^{+}(C\underline{H}_3)_3$ as opposed to that of the $-NR^{+}(C\underline{H}_3)_2$. Similar assumption was also applied for mono- and di-substitution. As a result, the degree of mono-, di- and tri-substitution of MEC, MPC, and MBzC particles can be calculated by using eqn. 4.1, 4.2, and 4.3, respectively. The estimation was not neccessary for QAC particles since the modification was done in the absence of reductive *N*-alkylation. In the case of EMC, there are 2 possible ways that can be used to calculate degree of substitution. The first way determines overall substitution of ethyl groups from the relative ratio between the integration of 3 protons from methyl moieties of the ethyl groups (δ 1.25 ppm) and the peak integration of 6 protons of chitosan (δ 3.4-4.4 ppm) using eqn. 4.4.

$$\%DS_{(ethyl)} = \frac{\left(\frac{\int N(CH_2C\underline{H}_3)}{3} \right)}{\left(\frac{\int H\text{-}2',3,4,5,6,6'}{6} \times \frac{85}{100} \right)} \times 100 \quad \text{……..(4.4)}$$

The second way is to use the signal of methyl protons that are attached to mono- ($NHCH_3$), di- ($-NCH_3(C_2H_5)$), and tri-substituted ($-N^{+}CH_3(C_2H_5)_2$) amino groups which appeared at the position D, C, and B, respectively. This calculation is based on the assumption that and each substituted amino group only bears one methyl group which

was incorporated in the step of *N*-reductive alkylation. So the degree of mono-, di-, and tri-substitution can be calculated using eqn. 4.5, 4.6, and 4.7, respectively. It should be noted that the %DS calculated from those 3 equations did not include trisubstituted amino groups in the form of ($-N^+ (C_2H_5)_3$)

$$\%DS_{(mono-)} = \frac{\left(\frac{\int NH(CH_3)}{3}\right)}{\left(\frac{\int H\text{-}2',3,4,5,6,6'}{6} \times \frac{85}{100}\right)} \times 100 \quad \text{……..(4.5)}$$

$$\%DS_{(di-)} = \frac{\left(\frac{\int NCH_3(C_2H_5)}{3}\right)}{\left(\frac{\int H\text{-}2',3,4,5,6,6'}{6} \times \frac{85}{100}\right)} \times 100 \quad \text{……..(4.6)}$$

$$\%DS_{(tri-)} = \frac{\left(\frac{\int N^+CH_3(C_2H_5)_2}{3}\right)}{\left(\frac{\int H\text{-}2',3,4,5,6,6'}{6} \times \frac{85}{100}\right)} \times 100 \quad \text{……..(4.7)}$$

From the data shown in Table 4.6, total degree of substitution ($\%DS_{total}$) was in a range of 7-82%. The degree of di-substitution possessed higher value than those of mono- and tri-substitution. Among all particles, QAC particles exhibited the highest degree of tri-substitution (quaternization) as well as $\%DS_{total}$. However, it should be emphasized that the quaternized particles were not completely dissolved in D_2O/TFA, the solvent used for ^1H NMR analysis. The data obtained from ^1H NMR analysis may not reflect the actual degree of substitution. The values can only be used as semi-quantitative measure of the extent of substitution.

The charge characteristic of the quaternary ammonium-containing chitosan particles was determined by Photon Correlation Spectroscopy (PCS). As shown in Table 4.7, all particles prepared by heterogeneous quaternization via *N*-reductive alkylation followed by methylation or ethylation (MEC, MPC, MBzC, and EMC

particles) exhibited greater zeta potential values than those of chitosan, TMC and QAC particles.

Table 4.6 Degree of substitution of quaternary ammonium-containing chitosan particles from homogeneous and heterogeneous quaternization

Particles	Integration				%DS			%DS $_{total}$
	H-2',3,4,5,6,6'	-C<u>H</u>$_3$ tri-substution	-C<u>H</u>$_3$ di-substution	-C<u>H</u>$_3$ mono-substution	tri-	di-	mono-	
TMC	100	2.7	20.1	6.4	2.1	23.6	15.1	40.8
QAC	100	9.1	53.5	5.3	7.1	62.9	12.5	82.5
MEC	100	5.1	21.4	3.4	4.0	25.2	8.0	37.2
MPC	100	5.3	33.5	3.6	4.2	39.4	8.47	52.1
MBzC	100	7.2	33.2	4.0	5.65	39.0	9.4	54.1
EMC[a]	100	1.0	1.2	0.8	2.35	2.82	1.88	7.1
EMC[b]	100	13.3			31.3			31.3

[a]the calculation is based on methyl group using eqn, 4.5-4.7

[b]the calculation is based on methyl group using eqn, 4.4

Table 4.7 ζ-potential of quaternary ammonium-containing chitosan particles

Particles	ζ-potential (mV)
Chitosan	+27.1
N, *N*, *N*-trimethyl chitosan (TMC)	+23.2
Quaternary ammonium-containing chitosan (QAC)	+29.3
Methylated Ethyl Chitosan (MEC)	+38.2
Methylated Propyl Chitosan (MPC)	+45.9
Methylated Benzyl Chitosan (MBzC)	+46.8
Ethylated Methyl Chitosan (EMC)	+44.1

4.4 Size and Morphology of Quaternary Ammonium-Containing Chitosan Particles

TEM images illustrated in Figure 4.10 revealed that all particles were quite spherical in shape. The particle size of all particles was determined by TEM and PCS analysis as presented in Table 4.8. According to TEM analysis, the particles had

diameters in a sub-micron range of 0.23-0.49 µm. In contrast, the data obtained from PCS analysis were much larger. The size ranged from 1 to 2.4 µm in diameter. This is mainly due to the fact that the size measured by PCS is a hydrodynamic size which was measured in solution takes into account the swelling. The TEM analysis, on the other hand, was done under reduced pressure atmosphere so the particles were dehydrated.

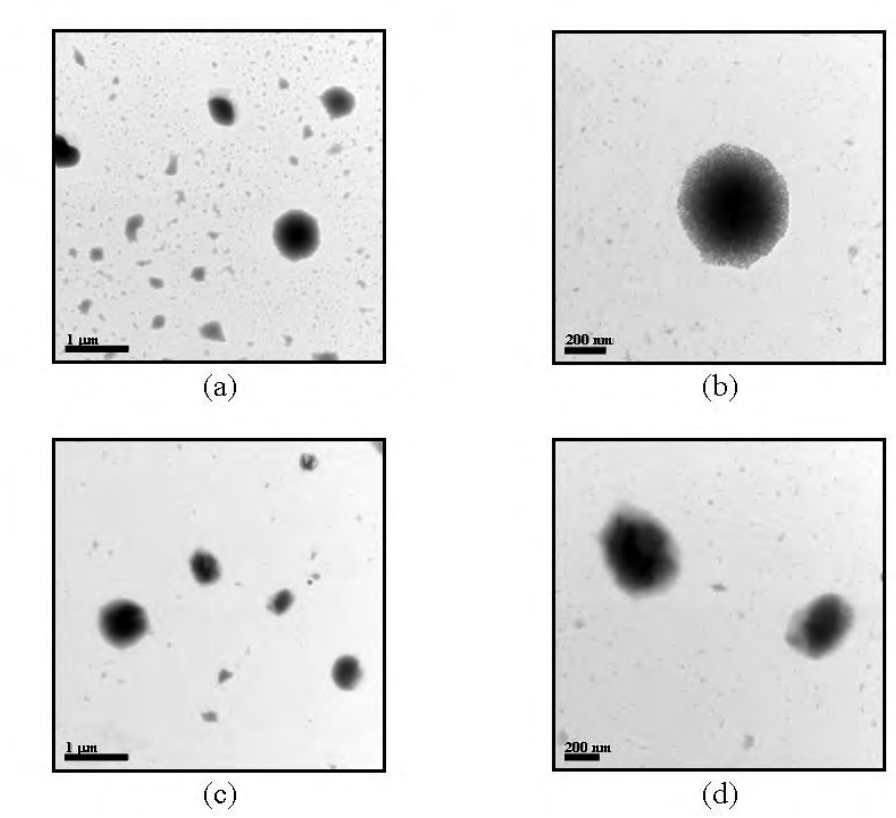


Figure 4.10 Transmission electron micrographs of (a,b) chitosan particles, (c,d) TMC particles, (e,f) QAC particles, (g,h) MEC particles, (i,j) MPC particles, (k,l) MBzC particles and (m,n) EMC particles

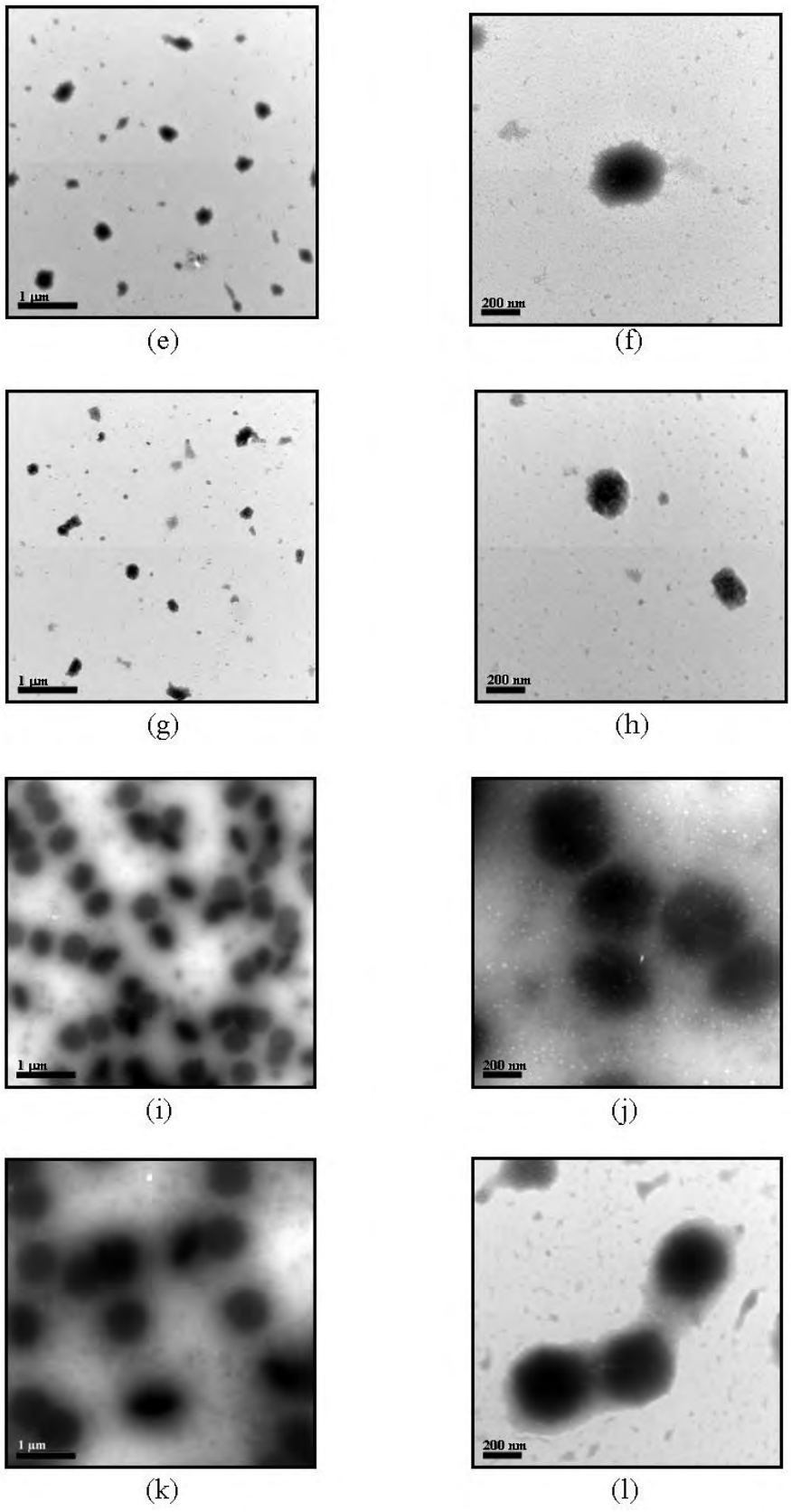
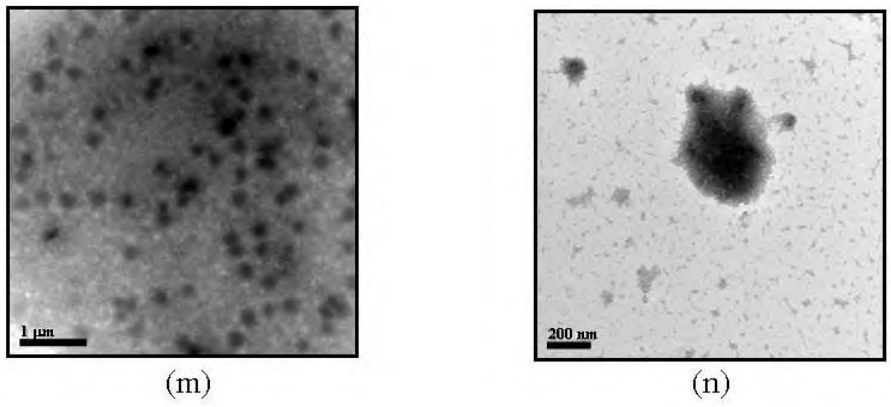


Figure 4.10 continued



(m) (n)

Figure 4.10 continued

Table 4.8 Particles size of quaternary ammonium-containing chitosan particles analyzed by TEM and PCS

Particles	Size (µm)	
	TEM	**PCS**
chitosan	0.32 ± 0.16	1.49
TMC	0.29 ± 0.21	1.33
QAC	0.26 ± 0.09	1.00
MEC	0.26 ± 0.11	2.08
MPC	0.44 ± 0.12	1.74
MBzC	0.49 ± 0.21	1.37
EMC	0.23 ± 0.04	2.35

4.5 Antibacterial Activity of Quaternary Ammonium-Containing Chitosan Particles

There are many researches studying about antibacterial mechanism of chitosan and its derivatives. Two major antibacterial mechanisms have been proposed for chitosan. The first is the absorption of polycationic of chitosan to negatively charged microbial cell membrane leading to cell membrane disruption and cell leakage [44]. The second is the penetration of chitosan into bacterial cell leading to inhibition of various enzymes to synthesize mRNA and proteins [43]. Furthermore, recent study has suggested that in the case of chitosan particles, the live cell of bacteria could be killed by the first mechanism [28]. In this study, the antibacterial activities of quaternary ammonium-containing chitosan particles were tested against gram-positive bacteria, *S. aureus* and gram-negative bacteria, *E. coli* using 2 methods: turbidimetric method and viable cell count.

4.5.1 Antibacterial Activity of Quaternary Ammonium-Containing Chitosan Particles against *S. aureus* and *E. coli*

To test the antibacterial activity of quaternary ammonium-containing chitosan particles, broth solution with ampicillin (50 mg/mL) and broth solution alone were used as a positive and negative control, respectively. The number of bacteria was determined from the OD_{600} value of the suspension after incubation with quaternary ammonium-containing chitosan particles which was prepared under the optimized condition. The higher the OD_{600} value indicate the greater the number of bacteria. Also, the higher the viable count (CFU/mL) value, the greater the survivable rate of bacteria is.

As presented in Figure 4.11 and 4.12, there was a good correlation between the data obtained from turbidimetric and viable cell counting method in case of *S. aureus*. The results indicated that quaternary ammonium-containing chitosan particles, TMC, QAC, MEC, MPC, MBzC and EMC particles exhibited significantly higher antibacterial activity against *S. aureus* (gram positive bacteria) than the chitosan particles at pH 7 indicating the introduction of positive charges via quaternization can effectively improve the antibacterial activity of chitosan particles especially in neutral media. In contrast, chitosan particles cannot at all inhibit the growth of bacteria considering that their OD_{600} and viable count are almost indistinguishable from those of the negative control. These evidences really verify that the superior antibacterial

activities of some quaternized chitosan particles (MEC, MPC, MBzC and EMC particles) to the chitosan particles are mainly due to the fact that the former possessed higher charge density in neutral condition than the latter (See Table 4.7 for ζ-potential data). This also implied that chitosan particles would exhibit its potential in suppressing the bacterial growth only when they are in acidic media in which they are positively charged. Overall, this trend is in accordance with previously observed data reported by Qi and coworkers [28]. They have proposed that the positively charged chitosan particles interacting favorably with negatively charged cell membranes, causing an increase in membrane permeability and eventually rupture and leakage of intracellular components. This proposed mechanism is particularly reasonable in the case of the gram positive, *S. aureus* since their outer membrane consists of teichoic acid, the negatively charged molecules that are believed to be responsible for the ionic binding with the positively charged particles.

Although the TMC particles exhibited slightly lower ζ-potential than the chitosan particles, they can still inhibit the bacterial growth suggesting the killing mechanism did not solely rely on the charge density, but may also influence by the charge inside the particles or the ability to dissolve in the media considering that TMC is water soluble and not all of TMC molecules were crosslinked by TPP during the particle formation. Similar explanations may also be applied in the case of QAC particles whose charge density (ζ-potential = +29.3 mV) was slightly higher than those of both chitosan particles (ζ-potential = +27.1 mV) and TMC particles (ζ-potential = +23.2 mV). Having the same type of quaternary ammonium groups (trimethyl), The QAC particles exhibited better antibacterial activity than the TMC particles implying that the heterogeneous quaternization was the more effective route to promote the antibacterial activity of chitosan particles than the homogeneous one.

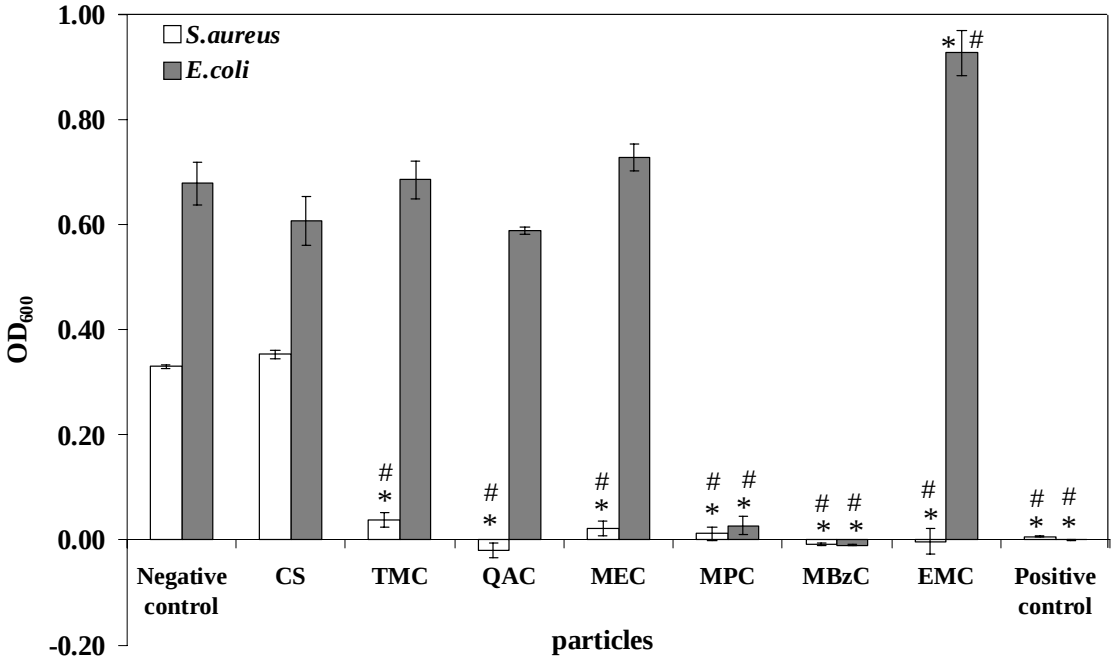


Figure 4.11 Optical density (OD_{600}) of *S. aureus* and *E. coli* in suspension incubated with different quaternary ammonium-containing chitosan particles (0.5 mg/mL) for 24 h $^{*}p < 0.01$ (compared with the negative control), $^{\#}p < 0.01$ (compared with the chitosan particles).

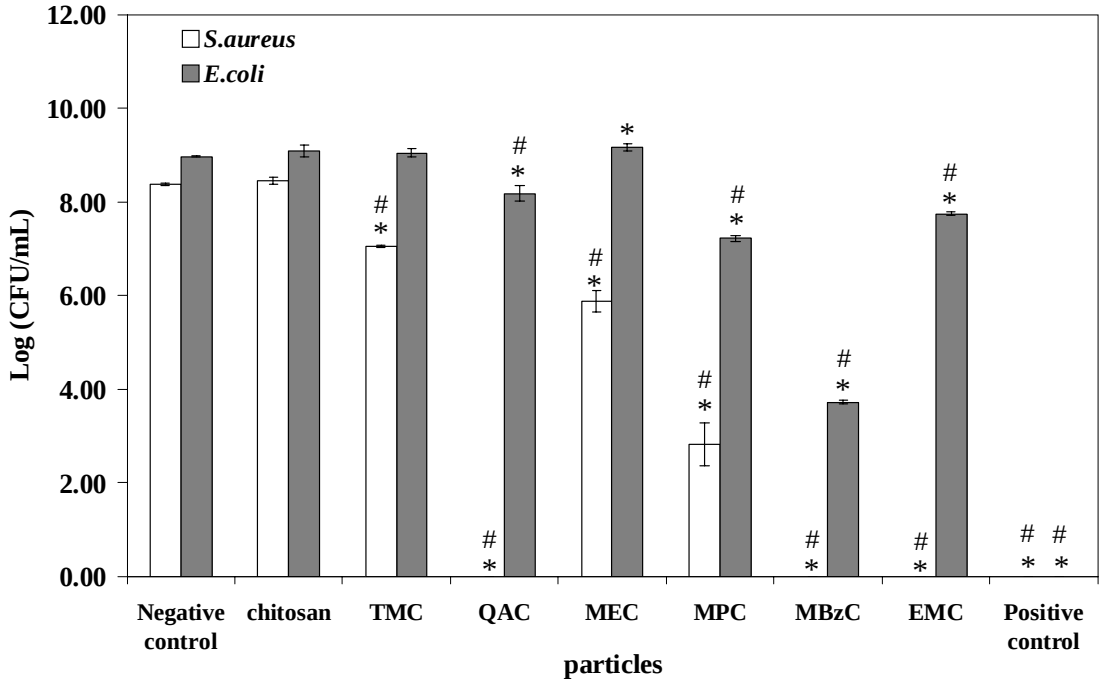


Figure 4.12 Viable cell counts of *S. aureus* and *E. coli* in suspension incubated with different quaternary ammonium-containing chitosan particles (0.5 mg/mL) for 24 h $^{*}p < 0.01$ (compared with the negative control), $^{\#}p < 0.01$ (compared with the chitosan particles).

The trend observed in the case of the gram-negative *E. coli* is quite different from that of the gram-positive *S. aureus* described above. In comparison with the negative control and chitosan particles, the quaternized particles having not too high charge density, namely TMC and QAC particles, did not possess significant antibacterial action based on the OD_{600} values shown in Figure 4.11. The particles with higher charge density, namely MPC and MBzC particles, on the other hand, exerted much higher antibacterial activity against the *E. coli.* However, that did not seem to be the case for MEC and EMC particles of which charge density were also high in magnitude.

It should be underlined at this point that the antibacterial activity determined from the viable counting method should be more reliable and accurate than those obtained from the turbidimetric method. Besides bacteria, there may be some other components in the solution, which can cause opaqueness and led to unavoidable interference to the measurement. In the case of quaternized particles whose activity was not so potent, it is also possible that the bacterial cells were not entirely destroyed, but was damaged to a slight extent so that they still maintained their overall integrity. That is the reason why the solution remained opaque with high OD_{600} value. This seems to be conceivable especially for QAC and EMC particles of which subsequent viable counts shows the opposite trend to the OD_{600} values.

The information obtained from these two methods as shown in Figure 4.11 and 4.12 can thus be used as an indication of the antibacterial effectiveness of each quaternized chitosan particles. In the case of MPC and MBzC particles, the antibacterial activity was so powerful. The results from OD_{600} and viable count confirmed that there was also low survival rate in comparison with other particles.

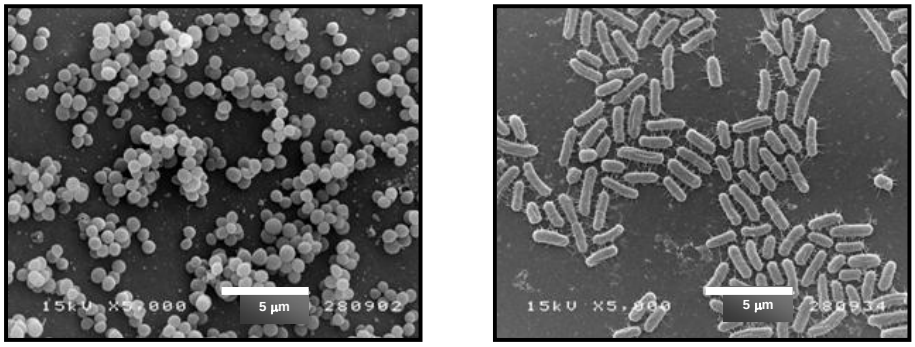
The antibacterial activity of the quaternized chitosan against the *E. coli* did not appear to have correlation with the ζ-potential of the particles, taking MEC particles as an exception. However, it seems to this point that the longer and the larger of the alkyl group introduced in the step of *N*-alkylation (benzyl > propyl > ethyl > methyl), the better the antibacterial activity. This is reasonable considering that the uppermost layer of the outer membrane of *E. coli* contains lipopolysaccharide and, unlike the *S. aureus*, the teichoic acid is not present. This may explain why the activity against *E. coli* is very much dictated by the hydrophobicity of the particles, not the charge magnitude.

Comparatively, as outlined in Table 4.9, it was found that *E. coli* had greater resistance to quaternary ammonium-containing chitosan particles than the *S. aureus*. This can be explained by three reasons: (1) the absence of outer membrane and the presence of negatively charged teichoic acid within a thick peptidoglycan layer (20-80 nm) on the surface of *S. aureus* should made them more attractive to the positively charged, quaternized chitosan particles and easier to be damaged through contact-killing mechanism than *E. coli*, (2) the presence of porins on the outer membrane of *E. coli* may help blocking the entrance of the particles into the inner bacterial cell, making them more difficult to inhibit than *S. aureus*, and (3) the smaller dimension of *S. aureus* may partly account for more intimate contact with the quaternary ammonium-containing chitosan particles, making the antibacterial activity more effective than the *E. coli.* The morphologies of both bacteria are illustrated in Figure 4.13.

Table 4.9 Viable cell counts of *S. aureus* and *E. coli* suspension in the presence of different quaternary ammonium-containing chitosan particles

Sample	**ζ-potential (mV)**	**Viable cell counts (Log(CFU/mL))**	
		S. aureus	***E. coli***
Negative control	-	8.38 ± 0.03	8.97 ± 0.01
Chitosan particles	+27.1	8.45 ± 0.09	9.09 ± 0.13
TMC particles	+23.2	7.05 ± 0.02	9.04 ± 0.08
QAC particles	+29.3	N/A	8.18 ± 0.18
MEC particles	+38.2	5.88 ± 0.23	9.16 ± 0.07
MPC particles	+45.9	2.83 ± 0.46	7.22 ± 0.07
MBzC particles	+46.8	N/A	3.73 ± 0.05
EMC particles	+44.1	N/A	7.75 ± 0.05
Positive control	-	N/A	N/A

N/A = There were no bacteria so the count was esentially zero.



(a) (b)

Figure 4.13 Morphologies of (a) *S. aureus* and (b) *E. coli*.

To determine the effectiveness of antibacterial activity, the viable counts of the *S. aureus* suspension was determined after incubation with the selected quaternized particles, QAC particles in this case, every 6 h for 24 h. The results are displayed in Figure 4.14.

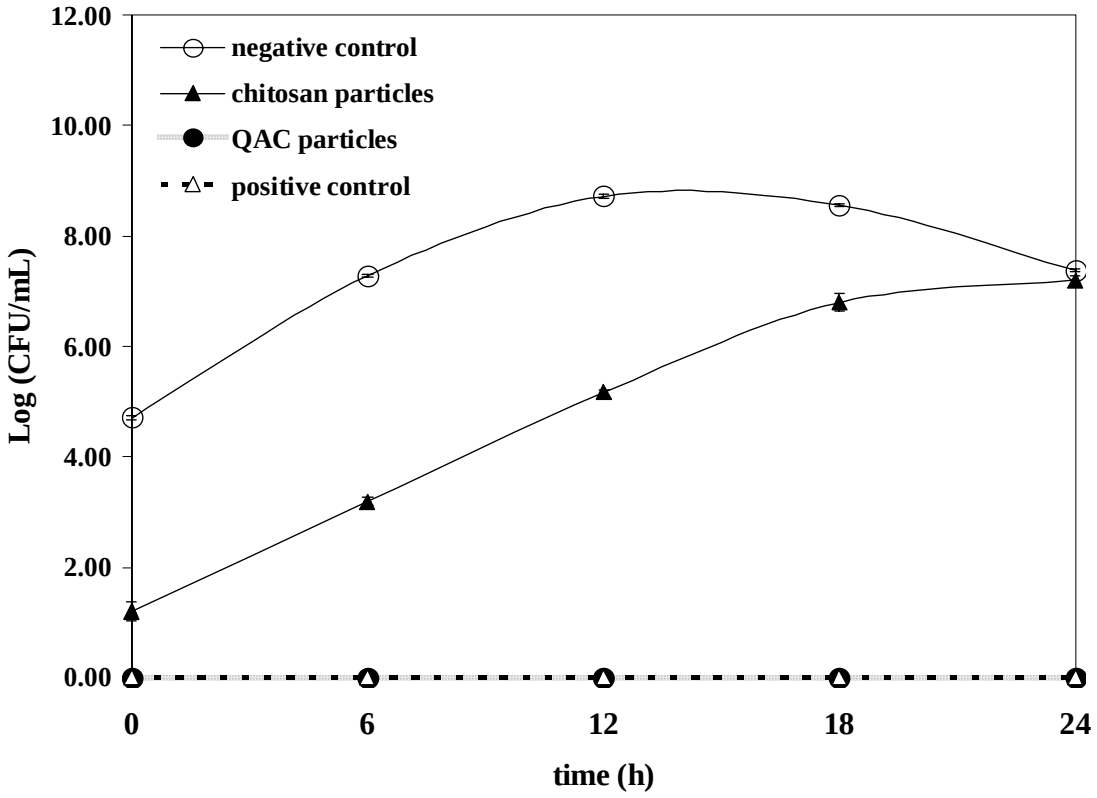


Figure 4.14 Viable cell counts of *S. aureus* in suspension incubated with chitosan and QAC particles (0.5 mg/mL) for 24 h

Considering the negative control, it was found that the bacterial reproduction initially increased and reached the maximum at 12 h of incubation, after that the growth rate of bacteria started decresing. The lag and stationary phases were not observed in this case. This can be explained by the stage of bacterial growth. When bacteria are first introduced to a new environment, they go through a lag phase, which seems to be very short in this investigation. In such phase, the bacteria do not reproduce because they have to adjust themselves to their new environment. Therefore, the number of bacteria remains constant. After a short time, the bacteria will reproduce and the production rates quickly speed up to the logarithmic growth phase. As the bacteria grow, they will use the food and oxygen in their environment and they also excrete wastes which pollute their environment. Eventually, the environment conditions degrade to a point at which the bacterial death rate exceeds the birth rate and the population of bacteria begins to fall in the accelerated death phase.

Unlike the regular growth of bacteria (negative control), the bacterial growth reached the maximum at 18 h and entered the stationary phase from that point on. There were no observable lag and death phases up to 24 h of incubation. Unlikely, the growth inhibition by QAC particles against *S. aureus* was so rapid that the stepwise bacterial evolution was not observed suggesting that the bacteria were killed at the early stage. It should be noted at this point that this investigation is preliminary and further studies on other particles and antibacterial tests against *E. coli* remained to be explored.

4.5.2 Minimum Inhibitory Concentration (MIC) of Some Particles

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that can inhibit the growth of microorganism. This value is an important parameter that indicates the capability of the substance when it is used in any applications. In this study, the MIC values of chitosan, TMC and QAC particles were analyzed by both turbidimetric and viable cell counting methods. Results for chitosan, TMC and QAC particles are displayed in Figures 4.15 - 4.16, 4.17 – 4.18, and 4.19 – 4.20, respectively. Each MIC value was identified by comparing the difference of OD_{600} and viable cell counts (Log (CFU/mL)) between the suspension having the particles and negative control. Statistical significance was associated with the value of $p < 0.01$. The lower MIC (mg/mL) value indicated the more efficiency of the particles in suppressing the antibacterial growth.

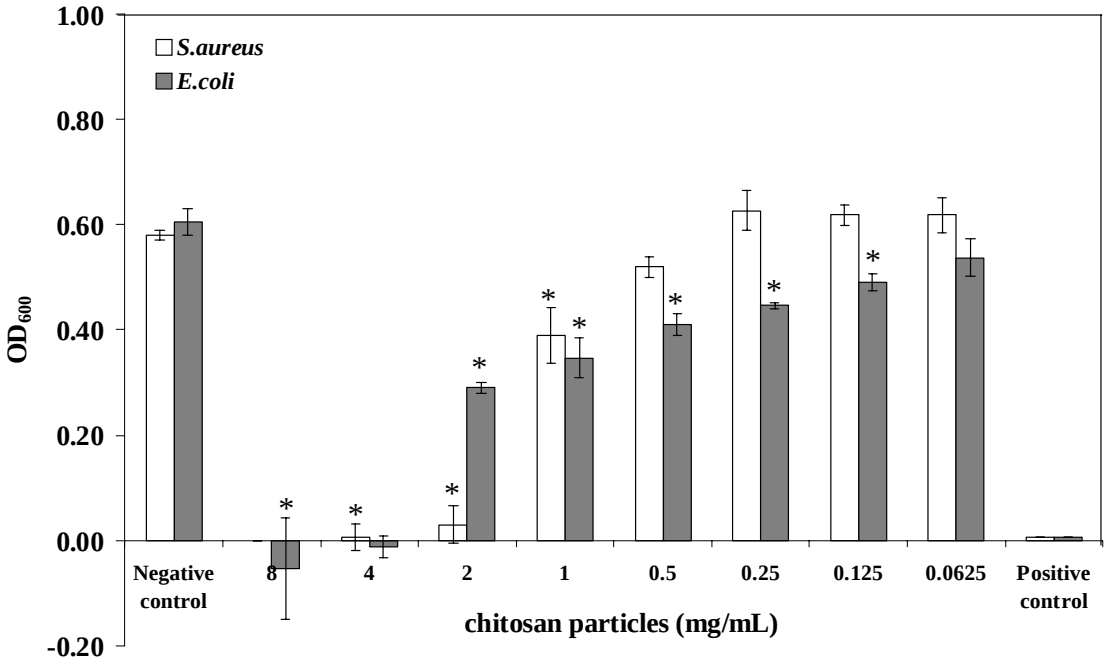


Figure 4.15 Optical density (OD_{600}) of *S. aureus* and *E. coli* in suspension incubated with different concentration of chitosan particles (mg/mL) for 24 h *$p < 0.01$ (compared with the negative control).

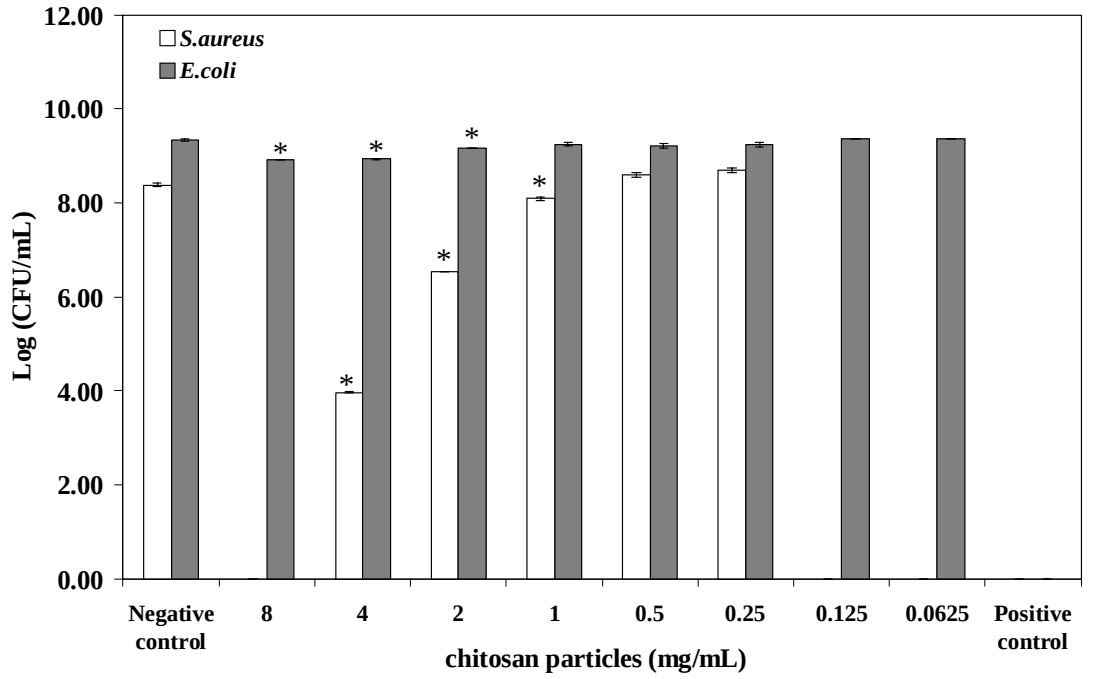


Figure 4.16 Viable cell counts of *S. aureus* and *E. coli* in suspension incubated with different concentration of chitosan particles (mg/mL) for 24 h *$p < 0.01$ (compared with the negative control).

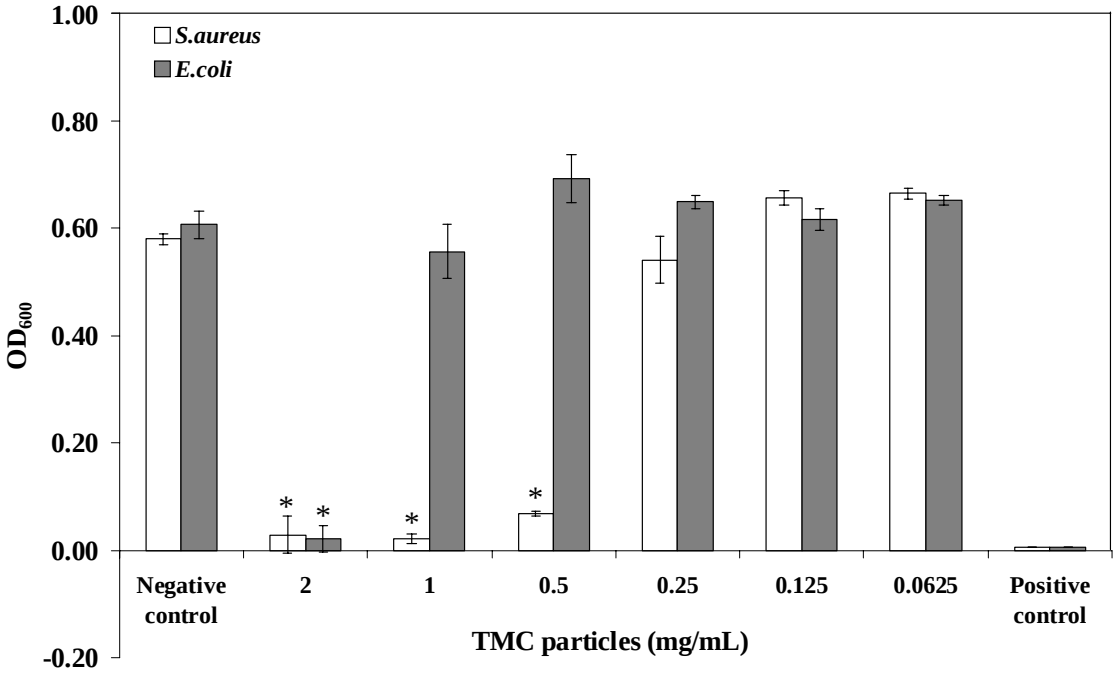


Figure 4.17 Optical density (OD_{600}) of *S. aureus* and *E. coli* in suspension incubated with different concentration of TMC particles (mg/mL) for 24 h *$p < 0.01$ (compared with the negative control).

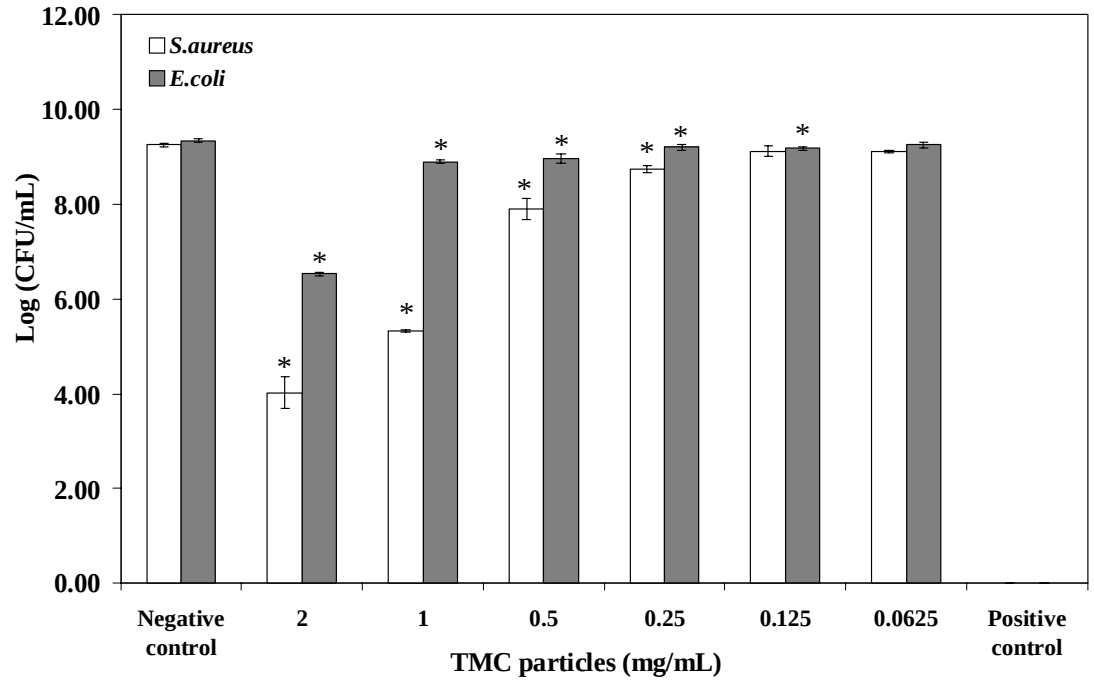


Figure 4.18 Viable cell counts of *S. aureus* and *E. coli* in suspension incubated with different concentration of TMC particles (mg/mL) for 24 h *$p < 0.01$ (compared with the negative control).

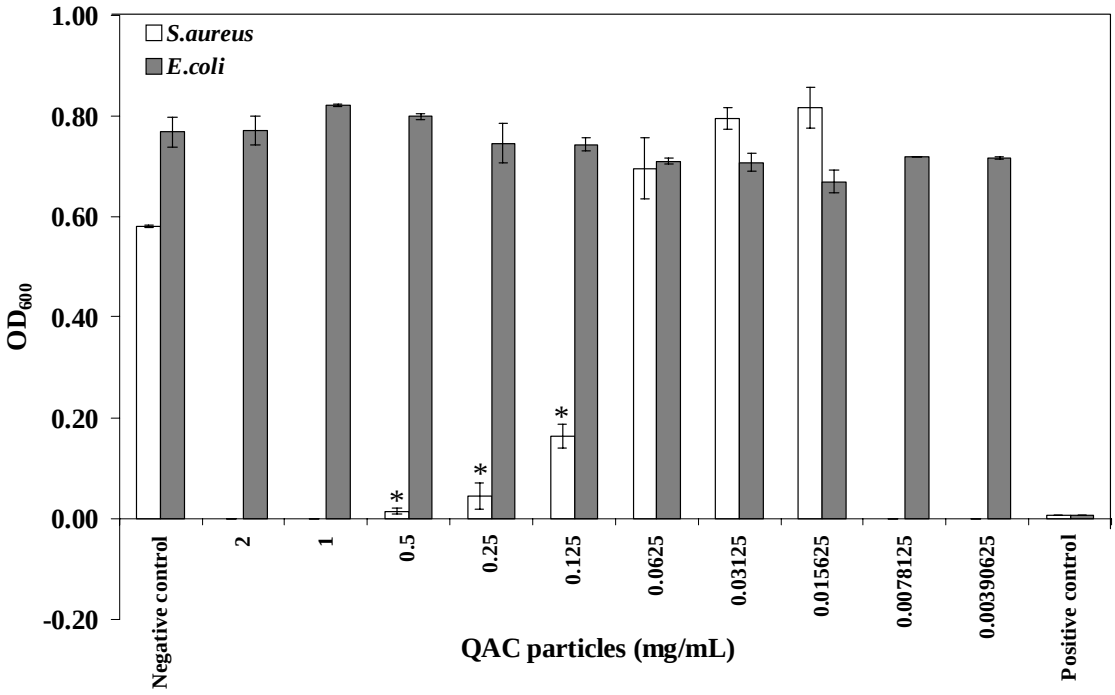


Figure 4.19 Optical density (OD_{600}) of *S. aureus* and *E. coli* in suspension incubated with different concentration of QAC particles (mg/mL) for 24 h *$p < 0.01$ (compared with the negative control).

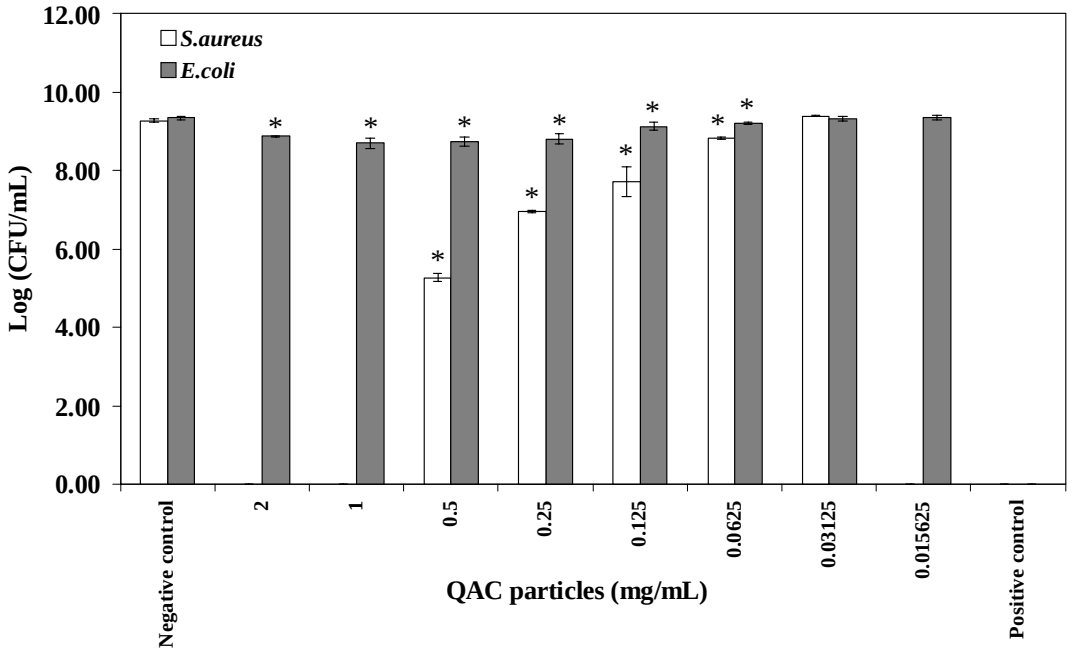


Figure 4.20 Viable cell counts of *S. aureus* and *E. coli* in suspension incubated with different concentration of QAC particles (mg/mL) for 24 h *$p < 0.01$ (compared with the negative control).

MIC values of chitosan, TMC and QAC particles determined based on the turbidimetric method and viable counts are listed in Table 4.10. In the case of *S. aureus*, it is obvious that QAC particles exhibited the highest antibacterial activity in comparison with chitosan and TMC particles. The fact that the concentration of less than or equal to 0.5 mg/mL (the concentration used for the tests shown previously) of TMC and QAC particles were required agree well with the results formerly described in section 4.5.1. The concentration of as high as 1.00 mg/mL was neccessary for chitosan particles to express their inhibitory power. This is why chitosan showed no activity when the concentration lower than that was used (See Figure 4.11). For *E. coli*, the concentration of 0.5 mg/mL for both TMC and QAC particles were not definitely enough to inhibit the bacterial growth. In particular, to start suppressing the growth of *E. coli* by QAC particles demanded the concentration higher than 2.0 mg/mL. These results confirmed our previous assumption that *E. coli* is more tolerable than *S. aureus*.

As presented in Table 4.10, Based on the MIC value, the antibacterial activity can be ranked from high to low as follows: QAC > TMC > chitosan. Determination of MIC values of other quaternized chitosan particles is currently being investigated. QAC particles exhibited the highest antibacterial activity against both bacteria because the particles not only showed positively charged in neutral region but it also exhibited the higher surface charge density than chitosan and TMC particles. This results can be used to confirm that the particles prepared form heterogeneous quaternization have higher antibacterial activity than the particles prepared from homogeneous quaternization.

Table 4.10 Minimum inhibitory concentration (MIC) of chitosan, TMC and QAC particles (mg/mL) against *S. aureus* and *E. coli*

Bacteria	**Method**	**Particles (mg/mL)**		
		Chitosan	**TMC**	**QAC**
S. aureus	Turbidimetric	1.00	0.50	0.125
	Viable count	1.00	0.25	0.0625
E. coli	Turbidimetric	0.125	2.0	> 2.0
	Viable count	2.0	0.125	0.0625

CHAPTER V

CONCLUSION AND SUGGESTIONS

Quaternary ammonium-containing chitosan particles having different alkyl substitutents were prepared by 2 methods. The first method was to prepare chitosan particles and then modified their surface by heterogeneous quaternization through *N*-reductive alkylation with aldehyde followed by alkylation with alkyl iodide or direct methylation using methyl iodide (MeI). The particles that were synthesized by the first method are methylated ethyl chitosan (MEC), methylated propyl chitosan (MPC), methylated benzyl chitosan (MBzC), ethylated methyl chitosan (EMC) particles. The second method was to synthesize *N*,*N*,*N*-trimethyl chitosan by homogeneous quaternization using MeI followed by the preparation of particles which was called TMC particles. It was found that the appropriate method for formulating particles was ionic gelation using tripolyphosphate as a crosslinking agent. The process can be done without surfactant or dispersing agent, unlike the emulsion crosslinking. It has been demonstrated from ^{1}H NMR and FT-IR analyses that all quaternary ammomium-containing chitosan particles could be successfully prepared. Total degree of substitution calculated from ^{1}H NMR data was in a range of 7-82%. Charge characteristic of the particles as determined by photon correlation spectroscopy (PCS) suggested that all particles carried positive charges with ζ-potential ranging from +23.2 to +46.8. The results from transmission electron microscopy (TEM) indicated that the sub-micron quaternized particles having diameter in a range of 0.23-0.49 μm were spherical in shape.

Antibacterial activity of the quaternary ammonium-containing chitosan particles were evaluated using turbidimetric and viable cell counting methods. Data from both tests suggested that all quaternary ammonium-containing chitosan particles, TMC, QAC, MEC, MPC, MBzC and EMC particles exhibited significantly higher antibacterial activity against *S. aureus* (gram positive bacteria) than the chitosan particles in neutral media. It is believed that the ionic binding between the positive charges on the particles and the negatively charged teichoic acid on the outer membrane of *S. aureus* was responsible for their superior antibacterial activity. In contrast, only the

MPC, MBzC particles of which charge density were high and their alkyl groups were relatively large, were capable of suppressing the growth of *E.coli* (gram negative bacteria). An evidence obtained after periodical monitoring (every 6 h, for up to 24 h) the *S. aureus* growth in the presence of chitosan particles implied that the delay in the log phase of the bacterial growth in the presence of some quaternized particles may account for such trend. The minimum inhibitory concentration (MIC) values obtained led to three important conclusive remarks: (1) the antibacterial efficiency of each particle was dose-dependent, (2) having the same alkyl substituents as TMC, QAC was a more potent antibacterial material. This fact indicated that the heterogeneous quaternization was a more effective method than the homogeneous one, and (3) *E. coli* had greater resistance to all quaternary ammonium-containing chitosan particles than the *S. aureus* which can be explained as a result of their difference in shape and outer membrane properties.

Several aspects on antibacterial activity of the quaternary ammonium-containing chitosan particles remain to be explored. The kinetic monitoring of bacterial growth and MIC values of other quaternized particles, TMC, MEC, MPC, MBzC, and EMC particles should lead to basic understanding on the efficiency of their antibacterial activities. It is anticipated that conducting the antibacterial tests against fluorescent protein-expressing bacteria in combination with fluorescence-based characterization techniques may provide useful information that can be used to explain the antibacterial mechanism of these quaternized chitosan particles.

REFERENCES

[1] Kohnen, W.; Kolbenschlag, C.; Teske-Keiser, S.; Jansen, B., Development of a long-lasting ventricular catheter impregnated with a combination of antibiotics. *Biomaterials* 24 (26) (2003): 4865-4869.

[2] Changez, M.; Koul, V.; Dinda, A. K., Efficacy of antibiotics-loaded interpenetrating network (IPNs) hydrogel based on poly(acrylic acid) and gelatin for treatment of experimental osteomyelitis: *in vivo* study. *Biomaterials* 26 (14) (2005): 2095-2104.

[3] Nablo, B. J.; Chen, T. Y.; Schoenfisch, M. H., Sol-gel derived nitric-oxide releasing materials that reduce bacterial adhesion. *Journal of the American Chemical Society* 123 (39) (2001): 9712-9713.

[4] Sun, Y.; Sun, G., Synthesis, characterization, and antibacterial activities of novel *N*-halamine polymer beads prepared by suspension copolymerization. *Macromolecules* 35 (23) (2002): 8909-8912.

[5] Su, Y.; Sun, G., Novel refreshable *N*-halamine polymeric biocides: *N*-chlorination of aromatic polyamides. *Industrial and Engineering Chemistry Research* 43 (17) (2004): 5015-5020.

[6] Iconomopoulou, S. M.; Voyiatzis, G. A., The effect of the molecular orientation on the release of antimicrobial substances from uniaxially drawn polymer matrixes. *Journal of Controlled Release* 103 (2) (2005): 451-464.

[7] Cioffi, N.; Torsi, L.; Ditaranto, N.; Tantillo, G.; Ghibelli, L.; Sabbatini, L.; Bleve-Zacheo, T.; D'Alessio, M.; Zambonin, P. G.; Traversa, E., Copper nanoparticle/polymer composites with antifungal and bacteriostatic properties. *Chemistry of Materials* 17 (21) (2005): 5255-5262.

[8] Rai, M.; Yadav, A.; Gade, A., Silver nanoparticles as a new generation of antimicrobials. *Biotechnology Advances* 27 (1) (2009): 76-83.

[9] Sharma, V. K.; Yngard, R. A.; Lin, Y., Silver nanoparticles: Green synthesis and their antimicrobial activities. *Advances in Colloid and Interface Science* 145 (1-2) (2009): 83-96.

[10] Hachem, R. Y.; Wright, K. C.; Zermeno, A.; Bodey, G. P.; Raad, I. I., Evaluation of the silver iontophoretic catheter in an animal model. *Biomaterials* 24 (20) (2003): 3619-3622.

[11] Furno, F.; Morley, K. S.; Wong, B.; Sharp, B. L.; Arnold, P. L.; Howdle, S. M.; Bayston, R.; Brown, P. D.; Winship, P. D.; Reid, H. J., Silver nanoparticles and polymeric medical devices: A new approach to prevention of infection *Journal of Antimicrobial Chemotherapy 54* (6) (2004): 1019-1024.

[12] Kassaee, M. Z.; Akhavan, A.; Sheikh, N.; Sodagar, A., Antibacterial effects of a new dental acrylic resin containing silver nanoparticles. *Journal of Applied Polymer Science* 110 (3) (2008): 1699-1703.

[13] Kumar, A.; Vemula, P. K.; Ajayan, P. M.; John, G., Silver-nanoparticle-embedded antimicrobial paints based on vegetable oil. *Nature Materials* 7 (3) (2008): 236-241.

[14] Wagener, M., Antimicrobial coatings. *Polymers Paint Colour Journal* 196 (4503) (2006): 34-37.

[15] Leaper, D. J., Silver dressings: Their role in wound management. *International Wound Journal* 3 (4) (2006): 282-294.

[16] Duran, N.; Marcato, P. D.; De Souza, G. I. H.; Alves, O. L.; Esposito, E., Antibacterial effect of silver nanoparticles produced by fungal process on textile fabrics and their effluent treatment. *Journal of Biomedical Nanotechnology* 3 (2) (2007): 203-208.

[17] Lee, H. J.; Jeong, S. H., Bacteriostasis and skin innoxiousness of nanosize silver colloids on textile fabrics. *Textile Research Journal* 75 (7) (2005): 551-556.

[18] Chen, X.; Schluesener, H. J., Nanosilver: A nanoproduct in medical application. *Toxicology Letters* 176 (1) (2008): 1-12.

[19] Benn, T. M.; Westerhoff, P., Nanoparticle silver released into water from commercially available sock fabrics. *Environmental Science and Technology* 42 (18) (2008): 7025-7026.

[20] Kumar, M. N. V. R.; Muzzarelli, R. A. A.; Muzzarelli, C.; Sashiwa, H.; Domb, A. J., Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews* 104 (12) (2004): 6017-6084.

[21] Jia, Z. S.; Shen, D. F.; Xu, W. L., Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydrate Research* 333 (1) (2001): 1-6.

[22] Runarsson, O. V.; Holappa, J.; Nevalainen, T.; Hjalmarsdottir, M.; Jrvinen, T.; Loftsson, T.; Einarsson, J. M.; Jonsdottir, S.; Valdimarsdottir, M.; Masson, M., Antibacterial activity of methylated chitosan and chitooligomer derivatives: Synthesis and structure activity relationships. *European Polymer Journal* 43 (6) (2007): 2660-2671.

[23] Avadi, M. R.; Sadeghi, A. M. M.; Tahzibi, A.; Bayati, K.; Pouladzadeh, M.; Zohuriaan-Mehr, M. J.; Rafiee-Tehrani, M., Diethylmethyl chitosan as an antimicrobial agent: Synthesis, characterization and antibacterial effects. *European Polymer Journal* 40 (7) (2004): 1355-1361.

[24] Sun, L.; Du, Y.; Fan, L.; Chen, X.; Yang, J., Preparation, characterization and antimicrobial activity of quaternized carboxymethyl chitosan and application as pulp-cap. *Polymer* 47 (6) (2006): 1796-1804.

[25] Qin, C.; Xiao, Q.; Li, H.; Fang, M.; Liu, Y.; Chen, X.; Li, Q., Calorimetric studies of the action of chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride on the growth of microorganisms. *International Journal of Biological Macromolecules* 34 (1-2) (2004): 121-126.

[26] Chi, W.; Qin, C.; Zeng, L.; Li, W.; Wang, W., Microbiocidal activity of chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride. *Journal of Applied Polymer Science* 103 (6) (2007): 3851-3856.

[27] Sajomsang, W.; Tantayanon, S.; Tangpasuthadol, V.; Daly, W. H., Synthesis of methylated chitosan containing aromatic moieties: Chemoselectivity and effect on molecular weight. *Carbohydrate Polymers* 72 (4) (2008): 740-750.

[28] Qi, L. F.; Xu, Z. R.; Jiang, X.; Hu, C. H.; Zou, X. F., Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydrate Research* 339 (16) (2004): 2693-2700.

[29] Du, W. L.; Xu, Y. L.; Xu, Z. R.; Fan, C. L., Preparation, characterization and antibacterial properties against *E. coli K88* of chitosan nanoparticle loaded copper ions. *Nanotechnology* 19 (8) (2008): 5.

[30] Du, W. L.; Niu, S. S.; Xu, Y. L.; Xu, Z. R.; Fan, C. L., Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. *Carbohydrate Polymers* 75 (3) (2009): 385-389.

[31] Shi, Z. L.; Neoh, K. G.; Kang, E. T.; Wang, W., Antibacterial and mechanical properties of bone cement impregnated with chitosan nanoparticles. *Biomaterials* 27 (11) (2006): 2440-2449.

[32] Talaro, K. P., *Foundations in microbiology*. 5th ed. New York: McGraw-Hill, 2005.

[33] Bacterial growth [online]. 2003. http://en.wikipedia.org/wiki/Bacterial_ growth [2009]

[34] Kumar, M., A review of chitin and chitosan applications. *Reactive & Functional Polymers* 46 (1) (2000): 1-27.

[35] Prashanth, K. V. H.; Tharanathan, R. N., Chitin/chitosan: modifications and their unlimited application potential - an overview. *Trends in Food Science & Technology* 18 (3) (2007): 117-131.

[36] Domard, A.; Rinaudo, M.; Terrassin, C., New method for the quaternization of chitosan. *International Journal of Biological Macromolecules* 8 (2) (1986): 105-107.

[37] Jia, Z.; Shen, D.; Xu, W., Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydrate Research* 333 (1) (2001): 1-6.

[38] Seong, H. S.; Whang, H. S.; Ko, S. W., Synthesis of a quaternary ammonium derivative of chito-oligosaccharide as antimicrobial agent for cellulosic fibers. *Journal of Applied Polymer Science* 76 (14) (2000): 2009-2015.

[39] Muzzarelli, R. A. A.; Tanfani, F.; Emanuelli, M.; Mariotti, S., *N*-(carboxymethylidene)chitosans and *N*-(carboxymethyl)chitosans: Novel chelating polyampholytes obtained from chitosan glyoxylate. *Carbohydrate Research* 107 (2) (1982): 199-214.

[40] Muzzarelli, R. A. A., Modified chitosans carrying sulfonic acid groups. *Carbohydrate Polymers* 19 (1992): 231-236.

[41] Chen, C. S. S., J.C.; Tsai,G.J., Antimicrobial effect and physical properties of sulfonated chitosan. *In Advances in Chitin Science* 3 (1998): 273–277.

[42] Chen, C. S. S., J.C.; Tsai,G.J. , Antimicrobial effect and physical properties of sulfonbenzoyl chitosan. *In Advances in Chitin Science* 3 (1998): 278–282.

[43] Cuero, R. G.; Osuji, G.; Washington, A., *N*-Carboxymethylchitosan inhibition of aflatoxin production: role of zinc. *Biotechnology Letters* 13 (6) (1991): 441-444.

[44] Tsai, G. J.; Su, W. H., Antibacterial activity of shrimp chitosan against *Escherichia coli*. *Journal of Food Protection* 62 (3) (1999): 239-243.

[45] Shahidi, F.; Arachchi, J. K. V.; Jeon, Y. J., Food applications of chitin and chitosans. *Trends in Food Science & Technology* 10 (2) (1999): 37-51.

[46] Lim, S. H.; Hudson, S. M., Review of chitosan and its derivatives as antimicrobial agents and their uses as textile chemicals. *Journal of Macromolecular Science - Polymer Reviews* 43 (2) (2003): 223-269.

[47] Kurita, K.; Kojima, T.; Nishiyama, Y.; Shimojoh, M., Synthesis and some properties of nonnatural amino polysaccharides: branched chitin and chitosan. *Macromolecules* 33 (13) (2000): 4711-4716.

[48] Hwang, J. K. K., H. J.; Yoon, S. J.; Pyun, Y. R. , *Advances in Chitin Science* Taiwan: Chen, R.H.; Chen, H.C., Eds.; Rita Advertising Co. Ltd, 1998.

[49] Liu, N.; Chen, X. G.; Park, H. J.; Liu, C. G.; Liu, C. S.; Meng, X. H.; Yu, L. J., Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydrate Polymers* 64 (1) (2006): 60-65.

[50] Rabea, E. I.; Badawy, M. E.-T.; Stevens, C. V.; Smagghe, G.; Steurbaut, W., Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules* 4 (6) (2003): 1457-1465.

[51] Ye, W. J.; Leung, M. F.; Xin, J.; Kwong, T. L.; Lee, D. K. L.; Li, P., Novel core-shell particles with poly(*n*-butyl acrylate) cores and chitosan shells as an antibacterial coating for textiles. *Polymer* 46 (23) (2005): 10538-10543.

[52] Ye, W. J.; Xin, J. H.; Li, P.; Lee, K. L. D.; Kwong, T. L., Durable antibacterial finish on cotton fabric by using chitosan-based polymeric core-shell particles. *Journal of Applied Polymer Science* 102 (2) (2006): 1787-1793.

[53] Xie, Y.; Liu, X.; Chen, Q., Synthesis and characterization of water-soluble chitosan derivate and its antibacterial activity. *Carbohydrate Polymers* 69 (1) (2007): 142-147.

[54] Li, Z.; Zhuang, X. P.; Liu, X. F.; Guan, Y. L.; Yao, K. D., Study on antibacterial *O*-carboxymethylated chitosan/cellulose blend film from LiCl/N, *N*-dimethylacetamide solution. *Polymer* 43 (4) (2002): 1541-1547.

[55] Gupta, D.; Haile, A., Multifunctional properties of cotton fabric treated with chitosan and carboxymethyl chitosan. *Carbohydrate Polymers* 69 (1) (2007): 164-171.

[56] Jung, B. O.; Kim, C. H.; Choi, K. S.; Lee, Y. M.; Kim, J. J., Preparation of amphiphilic chitosan and their antimicrobial activities. *Journal of Applied Polymer Science* 72 (13) (1999): 1713-1719.

[57] Hu, Y.; Du, Y.; Yang, J.; Kennedy, J. F.; Wang, X.; Wang, L., Synthesis, characterization and antibacterial activity of guanidinylated chitosan. *Carbohydrate Polymers* 67 (1) (2007): 66-72.

[58] Xing, K.; Chen, X. G.; Li, Y. Y.; Liu, C. S.; Liu, C. G.; Cha, D. S.; Park, H. J., Antibacterial activity of oleoyl-chitosan nanoparticles: A novel antibacterial dispersion system. *Carbohydrate Polymers* 74 (1) (2008): 114-120.

[59] Kim, C. H.; Choi, J. W.; Chun, H. J.; Choi, K. S., Synthesis of chitosan derivatives with quaternary ammonium salt and their antibacterial activity. *Polymer Bulletin* 38 (4) (1997): 387-393.

[60] Ignatova, M.; Starbova, K.; Markova, N.; Manolova, N.; Rashkov, I., Electrospun nano-fibre mats with antibacterial properties from quaternised chitosan and poly(vinyl alcohol). *Carbohydrate Research 341* (12) (2006): 2098-2107.

[61] Vallapa, N. Preparation and antibacterial activity of quaternary ammonium-containing chitosan surface. Master's thesis, Petrochemistry and Polymer Science, Chulalongkorn University, Bangkok, Thailand, 2006.

[62] Belalia, R.; Grelier, S.; Benaissa, M.; Coma, V., New bioactive biomaterials based on quaternized chitosan. *Journal of Agricultural and Food Chemistry* 56 (5) (2008): 1582-1588.

[63] Sadeghi, A. M. M.; Dorkoosh, F. A.; Avadi, M. R.; Saadat, P.; Rafiee-Tehrani, M.; Junginger, H. E., Preparation, characterization and antibacterial activities of chitosan, *N*-trimethyl chitosan (TMC) and *N*-diethylmethyl chitosan (DEMC) nanoparticles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods. *International Journal of Pharmaceutics* 355 (1-2) (2008): 299-306.

[64] Sieval, A. B.; Thanou, M.; Kotze, A. F.; Verhoef, J. C.; Brussee, J.; Junginger, H. E., Preparation and NMR characterization of highly substituted *N*-trimethyl chitosan chloride. *Carbohydrate Polymers* 36 (2-3) (1998): 157-165.

[65] Agnihotri, S. A.; Mallikarjuna, N. N.; Aminabhavi, T. M., Recent advances on chitosan-based micro- and nanoparticles in drug delivery. *Journal of Controlled Release* 100 (1) (2004): 5-28.

[66] Amidi, M.; Romeijn, S. G.; Borchard, G.; Junginger, H. E.; Hennink, W. E.; Jiskoot, W., Preparation and characterization of protein-loaded *N*-trimethyl chitosan nanoparticles as nasal delivery system. *Journal of Controlled Release* 111 (1-2) (2006): 107-116.

[67] Janes, K. A.; Calvo, P.; Alonso, M. J., Polysaccharide colloidal particles as delivery systems for macromolecules. *Advanced Drug Delivery Reviews* 47 (1) (2001): 83-97.

[68] Tang, Z. X.; Qian, J. Q.; Shi, L. E., Preparation of chitosan nanoparticles as carrier for immobilized enzyme. *Applied Biochemistry and Biotechnology* 136 (1) (2007), 77-96.

[69] Gan, Q.; Wang, T.; Cochrane, C.; McCarron, P., Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery. *Colloids and Surfaces B-Biointerfaces* 44 (2-3) (2005): 65-73.

[70] Zhao, J. Y.; Wu, J. M., Preparation and characterization of the fluorescent chitosan nanoparticle probe. *Chinese Journal of Analytical Chemistry* 34 (11) (2006): 1555-1559.

APPENDICES

APPENDIX A

Table A-1 Optical density (OD_{600}) of *S. aureus* and *E.coli* in suspension incubated with different quaternary ammonium-containing chitosan particles (0.5 mg/mL) for 24 h.

Sample	Optical density (OD_{600})	
	S.aureus	*E.coli*
Negative control	0.33 ± 0.00	0.68 ± 0.04
Chitosan particles	0.35 ± 0.01	0.61 ± 0.05
TMC particles	0.04 ± 0.01	0.69 ± 0.04
QAC particles	-0.02 ± 0.02	0.59 ± 0.01
MEC particles	0.02 ± 0.01	0.73 ± 0.03
MPC particles	0.01 ± 0.01	0.03 ± 0.02
MBzC particles	-0.01 ± 0.00	-0.01 ± 0.00
EMC particles	-0.00 ± 0.02	0.93 ± 0.04
Positive control	0.01 ± 0.00	0.00

Table A-2 Viable cell counts of *S.aureus* and *E. coli* in suspension incubated with different quaternary ammonium-containing chitosan particles (0.5 mg/mL) for 24 h.

Sample	Viable cell counts (Log (CFU/mL)	
	S.aureus	*E.coli*
Negative control	8.38 ± 0.03	8.97 ± 0.01
Chitosan particles	8.45 ± 0.09	9.09 ± 0.13
TMC particles	7.05 ± 0.02	9.04 ± 0.08
QAC particles	N/A	8.18 ± 0.18
MEC particles	5.88 ± 0.23	9.16 ± 0.07
MPC particles	2.83 ± 0.46	7.22 ± 0.07
MBzC particles	N/A	3.73 ± 0.05
EMC particles	N/A	7.75 ± 0.05
Positive control	N/A	N/A

N/A = There were no viable bacteria so the count was essentially zero.

Table A-3 Viable cell counts of *S. aureus* in suspension incubated with chitosan and QAC particles (0.5 mg/mL) for 24 h.

Time (h)	Viable cell counts (Log(CFU/mL)			
	Negative control	Chitosan particles	QAC particles	Positive control
0	4.71 ± 0.02	1.20 ±0.17	N/A	N/A
6	7.27 ± 0.02	3.19 ± 0.08	N/A	N/A
12	8.72 ± 0.03	5.16 ± 0.06	N/A	N/A
18	8.56 ± 0.02	6.79 ± 0.16	N/A	N/A
24	7.38 ± 0.03	7.20 ± 0.08	N/A	N/A

N/A = There were no viable bacteria so the count was essentially zero.

Table A-4 Optical density (OD_{600}) and viable cell counts of *S. aureus* and *E.coli* in suspension incubated with different concentration of chitosan particles (mg/mL) for 24 h.

Concentration of particles (mg/mL)	Optical density (OD_{600})		Viable cell counts Log(CFU/mL)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
Negative control	0.58 ± 0.01	0.61 ± 0.03	8.38 ± 0.03	9.34 ± 0.03
8	-[a]	-0.05 ± 0.10	-[a]	8.90 ± 0.10
4	0.01 ± 0.03	-0.01 ± 0.02	3.96 ± 0.01	8.94 ± 0.02
2	0.03 ± 0.04	0.29 ± 0.01	6.54 ± 0.01	9.16 ± 0.01
1	0.39 ± 0.05	0.35 ± 0.04	8.09 ± 0.04	9.25 ± 0.04
0.5	0.52 ± 0.02	0.41 ± 0.02	8.60 ± 0.05	9.22 ± 0.02
0.25	0.63 ± 0.04	0.45 ± 0.01	8.69 ± 0.05	9.24 ± 0.01
0.125	0.62 ± 0.02	0.49 ±0.02	-[a]	9.36 ± 0.02
0.0625	0.62 ± 0.03	0.54 ± 0.04	-[a]	9.35 ± 0.04
Positive control	0.01 ± 0.00	0.01± 0.00	N/A	N/A

[a] The experiment using this particular concentration was not performed.

N/A = There were no viable bacteria so the count was essentially zero.

Table A-5 Optical density (OD_{600}) and viable cell counts of *S.aureus* and *E. coli* in suspension incubated with different concentration of TMC particles (mg/mL) for 24 h**.**

Concentration of particles (mg/mL)	Optical density (OD_{600})		Viable cell counts Log(CFU/mL)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
Negative control	0.58 ± 0.01	0.61 ± 0.03	9.26 ± 0.03	9.34 ± 0.04
2	0.03 ± 0.04	0.02 ± 0.03	4.03 ± 0.32	6.53 ± 0.03
1	0.02 ± 0.01	0.56 ± 0.05	5.34 ± 0.02	8.90 ± 0.04
0.5	0.07 ± 0.00	0.69 ± 0.05	7.90 ± 0.23	8.98 ± 0.10
0.25	0.54 ± 0.04	0.65 ± 0.01	8.73 ± 0.08	9.20 ± 0.05
0.125	0.66 ± 0.01	0.62 ± 0.02	9.12 ± 0.10	9.18 ± 0.04
0.0625	0.67 ± 0.01	0.65 ± 0.01	9.11 ± 0.03	9.26 ± 0.06
Positive control	0.01 ± 0.00	0.01 ± 0.00	N/A	N/A

N/A = There were no viable bacteria so the count was essentially zero.

Table A-6 Optical Density (OD_{600}) and viable cell counts of *S. aureus* and *E.coli* in suspension incubated with different concentration of QAC particles (mg/mL) for 24 h.

Concentration of particles (mg/mL)	Optical density (OD_{600})		Viable cell counts Log(CFU/mL)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
Negative control	0.58 ± 0.00	0.77 ± 0.03	9.28 ± 0.05	9.34 ± 0.03
2	-[a]	0.77 ± 0.03	0.00	8.88 ± 0.02
1	-[a]	0.82 ± 0.00	0.00	8.70 ± 0.12
0.5	0.01 ± 0.01	0.80 ± 0.01	5.28 ± 0.11	8.74 ± 0.12
0.25	0.04 ± 0.03	0.75 ± 0.04	6.97 ± 0.03	8.81 ± 0.14
0.125	0.16 ± 0.02	0.74 ± 0.01	7.71 ± 0.37	9.13 ± 0.11
0.0625	0.70 ± 0.06	0.71 ± 0.01	8.83 ± 0.03	9.21 ± 0.03
0.03125	0.80 ± 0.02	0.71 ± 0.02	9.39 ± 0.00	9.31 ± 0.05
0.015625	0.82 ± 0.04	0.67 ± 0.02	-[a]	9.35 ± 0.06
Positive control	0.01 ± 0.00	0.01 ± 0.00	N/A	N/A

[a] The experiment using this particular concentration was not performed.

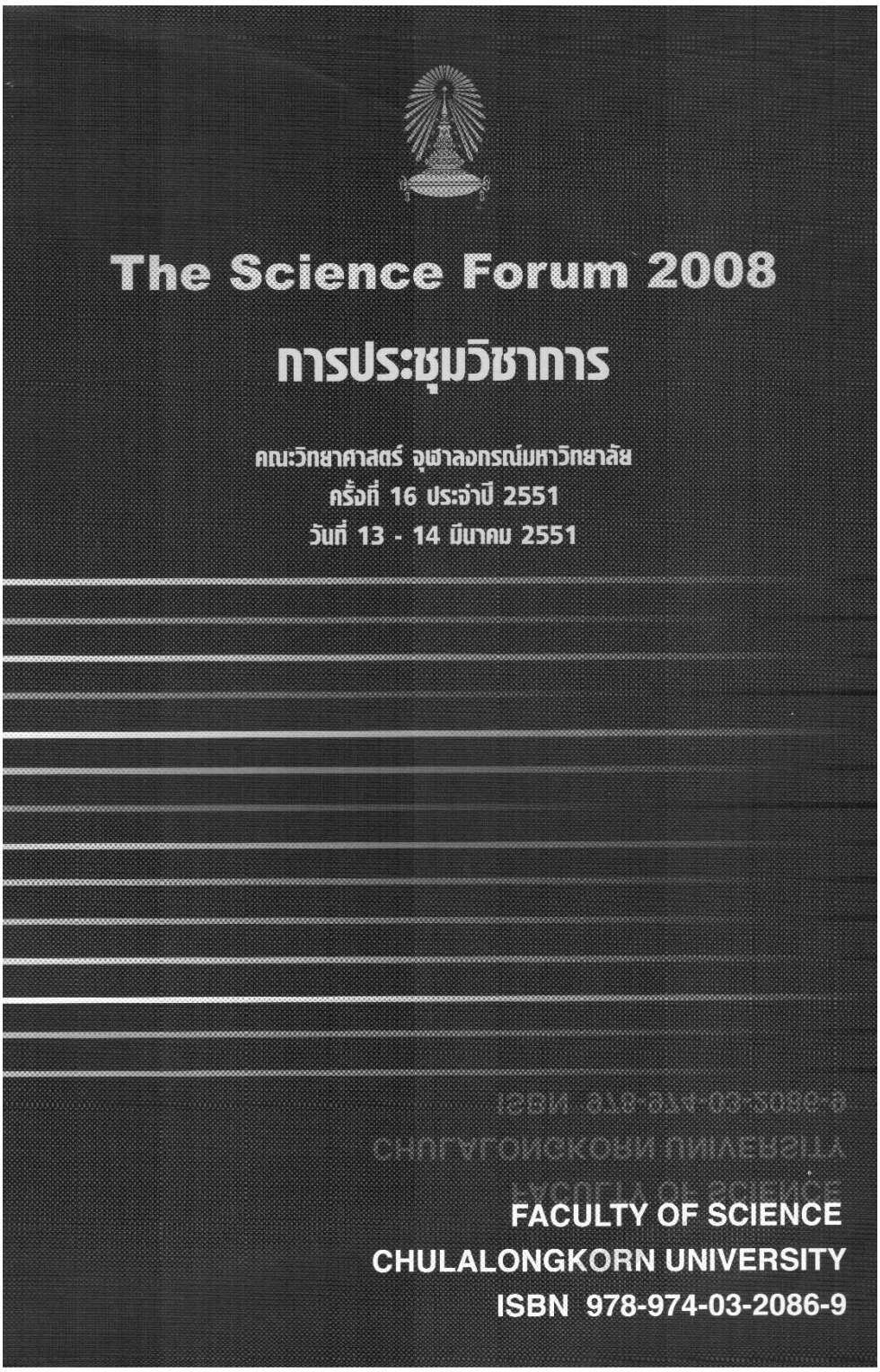
N/A = There were no viable bacteria so the count was essentially zero.

APPENDIX B

B-1 The 16th Science Forum, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Best Poster Award in the Symposium: Nanomaterials

B-2 The 5th National Chitin-Chitosan Conference, Center for Chitin-Chitosan Biomaterials (CCB), Metallurgy and Materials Science Research Institute (MMRI), Chulalongkorn University, Bangkok, Thailand.

B-3 Pure and Applied Chemistry International Conference 2009, Faculty of Science, Naresuan University, Phitsanulok, Thailand.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POSTERS

Development of antibacterial fillers from quaternized chitosan particles

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This research aims to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles that may be applicable for biomedical devices, health, food, textile, and personal hygiene industries. Two routes have been proposed to introduce quaternary ammonium groups in order to enhance the antibacterial activity of chitosan particles. Route 1 is to prepare chitosan particles by ionic gelation and modify the particle surface by heterogeneous quaternization using methyl iodide (MeI). Route 2 involves the synthesis of *N,N,N*-trimethyl chitosan by homogeneous quaternization using MeI followed by particle formation using ionic gelation. Results from FT-IR and ^{1}H-NMR analyses confirmed the success of the quaternary ammonium-containing particle formation. The antibacterial activity, tested against *Staphylococcus aureus* (a gram positive bacteria) by viable cell counting, indicated that the additional positive charges introduced to the chitosan particles provide the quaternary ammonium-containing chitosan particles with a superior antibacterial activity compared to the native chitosan particles in a neutral pH range.

Keywords: chitosan, antibacterial activity, quaternary ammonium group

Gold nanoparticles stabilized by chitosan and its derivatives

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A new series of polysaccharide-stabilized gold nanoparticles (AuNPs) were synthesized by two methods. The first method employs chitosan as both a reducing and a stabilizing agent. The second method uses sodium borohydride as a reducing agent and chitosan derivatives; *N,N,N*-trimethyl chitosan (TMC), *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride (HTACC), or *N*-succinyl chitosan (SCC), as a stabilizing agent. The morphology, size and stability of AuNPs were evaluated by UV-vis spectroscopy, photon correlation spectroscopy (PCS), transmission electron microscopy (TEM), and Fourier transform infrared spectroscopy (FTIR). The AuNPs have a spherical morphology and a size range of 5-14 nm. The size, size distribution and stability of the particles varied with the molar ratio of polymer to $HAuCl_4$. It is anticipated that the chitosan and its derivatives surrounding the AuNPs will allow shell functionalization, biomolecule conjugation and the formation of AuNPs-labeled biomolecules that can be applied for biosensing applications.

Keywords: gold nanoparticles, chitosan, chitosan derivatives

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A_08

การพัฒนาสารตัวเติมที่มีฤทธิ์ในการยับยั้งแบคทีเรียจากอนุภาคควอเทอไนซ์ไคโทซาน

Development of antibacterial fillers from quaternized chitosan particles

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บทคัดย่อ: งานวิจัยนี้สนใจที่จะพัฒนาสารตัวเติมอินทรีย์ที่มีฤทธิ์ในการยับยั้งแบคทีเรีย เป็นมิตรกับสิ่งแวดล้อม จากอนุภาคควอเทอไนซ์ไคโทซาน ซึ่งน่าจะสามารถประยุกต์ในอุตสาหกรรมที่เกี่ยวข้องกับวัสดุทางชีวการแพทย์ สุขภาพ และสิ่งทอ เตรียมอนุภาคควอเทอไนซ์ไคโทซานโดยปฏิกิริยาเมทิเลชันด้วยเมทิลไอโอไดด์ผ่านปฏิกิริยาแบบเอกพันธ์และวิวิธพันธ์ ผลการทดลองที่วิเคราะห์ด้วย เอฟที-ไออาร์ และโปรตอนเอ็นเอ็มอาร์ยืนยันความสำเร็จในการเตรียมอนุภาคควอเทอร์ไนซ์ไคโทซาน ทดสอบฤทธิ์ในการยับยั้งแบคทีเรียกับ *Staphylococcus aureus* และ *Escherichia coli* โดยพบว่าการเพิ่มประจุบวกทำให้อนุภาคควอเทอไนซ์ไคโทซานมีฤทธิ์ในการยับยั้งแบคทีเรียที่เหนือกว่าอนุภาคไคโทซานในสภาวะที่มีพีเอชเป็นกลาง

Abstract: This research aims to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles that may be applicable for biomedical device, health, and textile industries. Quaternized chitosan particles were prepared by methylation using methyl iodide (MeI) via homogeneous and heterogeneous reactions. Results from FT-IR and ^{1}H-NMR analyses confirmed the success of the quaternized chitosan particles formation. Antibacterial activity tests were performed against *Staphylococcus aureus* (*S.aureus*) and *Escherichia coli* (*E.coli*). The results indicated that the additional positive charges introduced to the chitosan particles rendered the quaternized particles superior antibacterial activity to the native chitosan particles at neutral pH.

Introduction

Incorporation of antibacterial fillers is an efficient approach to introduce antibacterial activity to materials. It is highly desirable if these fillers are nontoxic, affordable, and environmentally friendly. Chitosan is a natural nontoxic biopolymer derived by deacetylation of chitin, an abundantly available biopolymer found in exoskeletons of insects, shell of crustaceans, and fungal cell walls.[1] Chitosan in its soluble form (in acidic media) exhibits antibacterial activity due to the fact that its amino group ($-NH_2$) is converted to an ammonium ion

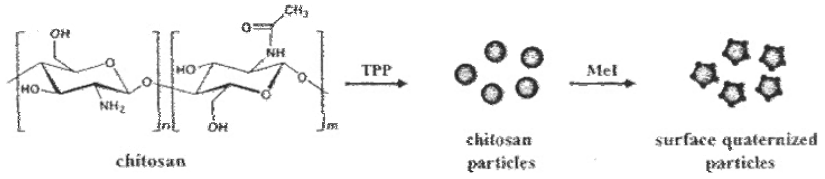
groups on chitosan has been recognized as a potential way to enhance the antibacterial activity of chitosan in a broader pH range.[3] This research aims to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles by introducing quaternary ammonium groups via heterogeneous and homogeneous quaternization with methyl iodide (MeI). It is anticipated that these particles should exhibit reasonably good antibacterial activity and can be used as an alternative to silver nanoparticles, conventional inorganic antibacterial fillers.

Materials and Methods:

Materials: Chitosan flakes (DAC 85%, and molecular weight = 45,000 Da) was purchased from Seafresh Chitosan (Lab) Co., Ltd (Thailand). Methyl iodide (MeI), sodium hydroxide (NaOH), sodium iodide (NaI) and tripolyphosphate (TPP) were purchased from Fluka (Switzerland). Acetic acid, ethanol (EtOH), and methanol (MeOH) were purchased from Merck (Germany). All reagents and materials are analytical grade and used without further purification.

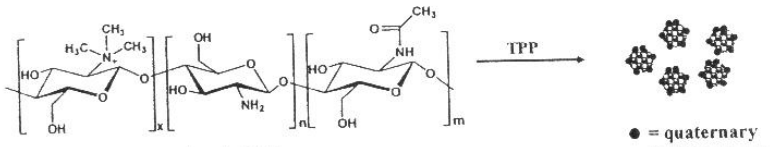
Methods: Two routes have been employed for the preparation of quaternized chitosan particles.

***Heterogeneous Route*:** Chitosan particles were first prepared by ionic gelation. Chitosan was dissolved at selected concentration with 1% (v/v) acetic acid. pH of the solution was raised to 4.6-4.8 with 10 N NaOH. Chitosan particles formed spontaneously upon an addition of 3 mL of an aqueous TPP solution to 3 mL of chitosan solution under magnetic stirring. The particles were isolated by centrifugation. Supernatants were discarded and the particles were extensively rinsed with distilled water and then freeze-dried before further use or analysis. Later, surface-quaternized chitosan particles (QAC) were obtained by heterogeneous quaternization. An anhydrous methanol solution of NaI (0.2 M) was added into a flask containing chitosan particles (0.03 g) and NaOH (0.13 g, 0.30 mol). MeI was added via syringe every 4 h. The total volume of the reaction mixture was 10 mL. The reaction mixture was stirred at 40 °C for 8 h. The particles were isolated by centrifugation. Supernatants were discarded and the particles were extensively rinsed with methanol and dried under vacuum.



***Homogeneous Route*:** *N,N,N*-trimethyl chitosan was synthesized according to a modified method of Sieval et al.[4] Chitosan (2 g) was dispersed in 80 mL of *N*-methyl-2-pyrrolidone followed by the addition of NaOH (0.81 g, 0.02 mol) and NaI (1.51 g, 0.01 mol). The reaction mixture was stirred at 40 °C under magnetic stirring. MeI (4.32 g, 0.03 mol) was added every 4 h. The mixture was stirred at 40 °C for 8 h. The product was precipitated using acetone. The precipitate was dissolved in 15% (w/v) NaCl solution in order to replace the iodide counterion with a chloride counterion. The suspension was then transferred to the dialysis membrane

where it was dialyzed against distilled water. The final product was obtained after freeze-dried. Quaternized chitosan particles (TMC) were prepared by ionic gelation method with TPP under magnetic stirring using the same method described above. The particles were isolated by centrifugation. Supernatants were discarded and the particles were extensively rinsed with distilled water and then freeze-dried before further use or analysis.



N, N, N - trimethylchitosan

Evaluation of Antibacterial Activity

Mueller-Hinton broth was used as a growth medium for the antibacterial assays, *Staphylococcus aureus (S. aureus)* and *Escherichia coli* (*E. coli*) were used as gram positive and gram negative bacteria, respectively. All glassware used for the tests was sterilized in an autoclave at 121°C for 15 min prior to use. The quaternized chitosan particles were sterilized by exposing to UV radiation for 60 min prior to the tests.

***Turbidimetric method*:** Quaternized chitosan particles were accurately quantfied and added to the test tubes each containing 5 mL of Muller–Hinton broth. The tubes were inoculated under aseptic conditions with 50 µL of the freshly prepared bacteria suspension. The positive and negative controls were with ampicillin and blank, respectively. After mixing, the tubes were incubated at 37°C, 110 rpm for 24 h. The optical density of the solution was measured at 600 nm (OD_{600}) by UV-Vis spectroscopy to determine a density of bacteria in the broth.

***Viable cell count*:** Quaternized chitosan particles were accurately quantfied and added to the test tubes each containing 5 mL of Muller–Hinton broth. The tubes were inoculated under aseptic conditions with 50 µL of the freshly prepared bacteria suspension. After mixing, the tubes were incubated at 37°C, 110 rpm for 24 h. Then, the mixture (100 µL) of the bacterial solution was diluted to 10^6 times. A 100 µL of the diluted bacterial solution was then spread onto the Mueller-Hinton agar. After incubating at 37°C for 18 h, a number of viable bacteria were then counted. The results after multiplication with the dilution factor were expressed as mean colony forming units per volume (CFU/mL).

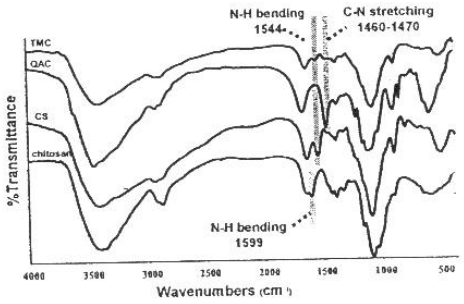


Fig.1: FT-IR spectra of chitosan, CS, QAC and TMC particles

Results and Discussion: FT-IR spectroscopy was used to confirm the success of quaternization of chitosan particles by MeI both homogeneously and heterogeneously. As shown in Fig. 1, the intensity of the characteristic N-H bending peak of chitosan (CS) particles at 1544 cm^{-1} correspondingly decreased after the methylation (See spectra of QAC and TMC particles). It should be noted that the N-H bending signal of chitosan

was shifted from 1599 cm^{-1} of virgin chitosan to 1544 cm^{-1} of chitosan particles. This is a consequence of the ammonium groups being ionically crosslinked with TPP molecules. The peaks in a range of 1470-1460 cm^{-1} appearing on the spectra of QAC and TMC particles are the characteristic peaks of C-N stretching, indicating that the bonding between methyl groups and amino groups of chitosan were formed.

^{1}H NMR spectra of quaternized chitosan particles are illustrated along with that of the CS particle in Fig.2. The presence of signal at 2.9-3.1 ppm, assigned to the methyl protons of the quaternary ammonium group and the signal at 2.8 ppm, assigned to the methyl protons of the disubstituted amino groups also verified the existence of quaternary ammonium groups both in QAC and TMC particles.

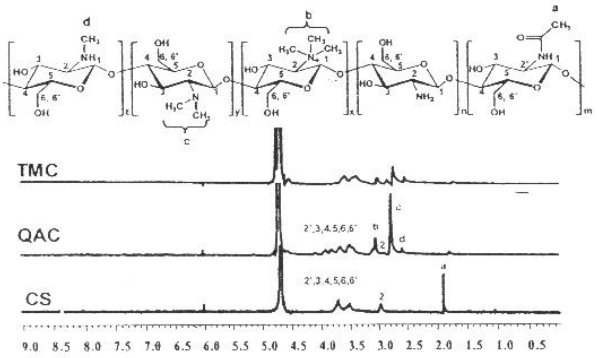
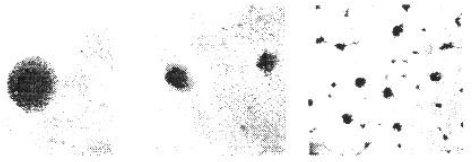


Fig.2: ^{1}H-NMR spectra of CS, QAC, and TMC particles

TEM images shown in Fig.3 reveal that CS, QAC and TMC particles are quite spherical and have a size in a range of 100 nm to 1 μm.



CS particles **QAC particles** **TMC particles**

Fig.3: TEM micrographs of CS, QAC, and TMC particles

To test the antibacterial activity of all chitosan particles, ampicillin and blank were used as a positive and negative control, respectively. Data from turbidimetric and viable cell count shown in Fig. 4-5 indicate that QAC and TMC particles exhibited significantly higher antibacterial activity against *S. aureus* (gram positive bacteria) than the CS particles at pH 7. Despite the death of *E. coli* (gram negative bacteria), all media appeared opaque. This is the reason why the ODs of all the solutions were similar. In the case of *E. coli*, the results from viable cell counting were thus more reliable than those obtained from the turbidimetric method. Apparently, the QAC particles were more active in prohibiting the growth of *S. aureus* and *E. coli* than the TMC particles.

This may be explained by the higher surface charge density of QAC particles, which were prepared by the heterogeneous methylation, than the TMC particles of which the methylation was performed prior to the particle formation. It is assumed that the absence of outer membrane and the presence of negatively charged teichoic acid on the surface of *S. aureus* made them more vulnerable against the quaternized chitosan particles than *E. coli*.[4] Their smaller dimension may partly account for more intimate contact with the quaternized particles, making the antibacterial activity more effective (Fig 6).

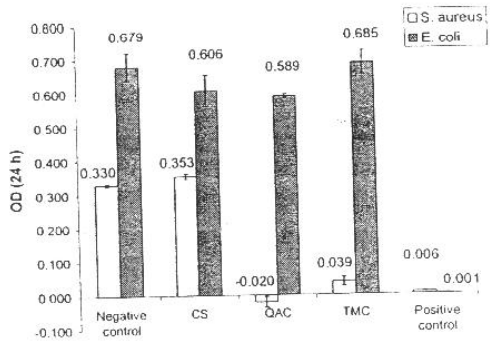


Fig.4: Optical density of *S. aureus* and *E. coli* in the presence of CS, QAC and TMC particles.

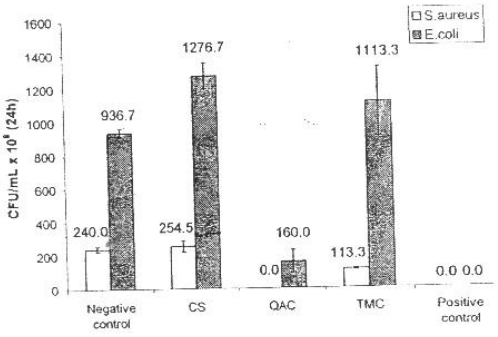
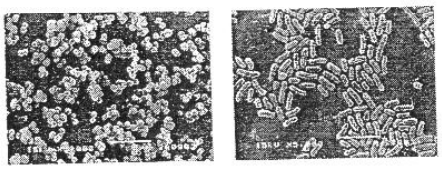


Fig.5: Viable cell counts of *S. aureus* and *E. coli* in the presence o TMC particles.



S. aureus ***E. coli***

Fig. 6: Morphologies of *S. aureus* and *E. coli*.

Conclusion

It has been demonstrated from ^{1}H-NMR and FT-IR analyses that the quaternized chitosan particles, QAC and TMC particles can be successfully prepared. The sub-micron quaternized particles were spherical in shape. Data from antibacterial activity tests showed that the QAC and TMC particles have higher antibacterial activity against both *S. aureus* and *E. coli* than the CS particles at neutral pH. The additional positive charge introduced to the chitosan particles by methylation apparently make the particles more favorable substrates for interacting with the negatively charged membrane of the bacteria. The superior antibacterial activity renders the quaternized chitosan particles potential antibacterial fillers for many applications. It turns out that the surface quaternization is the more effective route to enhance antibacterial activity of the chitosan particles than the homogeneous route.

Acknowledgement

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References:

1. Kumar, MNVR, Muzzarelli, RAA, Muzzarelli, C, Sashiwa, H, Domb, AJ. (2004) Chitosan chemistry and pharmaceutical perspectives. *Chemical Review,* 104, 6017-6084.
2. Tsai, GJ, Su, WH. (1999) Antibacterial activity of shrimp chitosan against *Escherichia coli*. *Journal of Food Protection*, 62, 239-243.
3. Jia, Z, Shen, D, Xu, W. (2001) Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydrate Research,* 333, 1-6.
4. Sieval, AB, Thanou, M, Kotze, AF, Verhoef, JC, Brussee, J, Junginger, HE. (1998) Preparation and NMR characterization of highly substituted *N*-trimethyl chitosan chloride. *Carbohydrate Polymers*, 36, 157-165.
5. Talaro, KP. (2005) Foundations in microbiology. 5 th ed. Boston: McGraw-Hill.

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ABSTRACTS

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S7-PO-27

Synthesis and Characterization of Biofunctionalized Gold Nanoparticles Stabilized by Chemically Modified Chitosan

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Gold nanoparticles (AuNPs) were first synthesized by employing chitosan as both reducing and stabilizing agent. Gold salts can be reduced to zerovalent gold nanoparticles by amino groups in the main chains of chitosan. The morphology, size and stability of AuNPs were evaluated by UV-vis spectroscopy, dynamic light scattering, transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FT-IR). The results showed that the AuNPs have spherical morphology and a size range of 11.5±3.5 nm. They are stable in aqueous solution due to the repulsion between the charged chitosan which acts as shell coating. It was also found that the size, size distribution and stability of the AuNPs varied with volume ratio of chitosan to $HAuCl_4$, concentration of surfactant, and molecular weight of chitosan. As analyzed by UV-vis spectroscopy, bovine serum albumin (BSA), a model of antigen, can successfully be attached to the amino groups of chitosan. Moreover, a one-step synthesis of AuNPs can be achieved using BSA-modified chitosan as both reducing and stabilizing agent. These results strongly indicate the possibility of using the synthesized AuNPs for chromogenic biosensor based on the agglomeration of AuNPs.

REFERENCES

1. Huang, H.; Yang, X. *Carbohydr. Res.* **2004**, 339, 2627-2631.
2. Ho, K. C.; Tsai, P. J.; Lin, Y. S.; Chen, Y. C. *Anal. Chem.* **2004**, 76, 7162-7168.

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Quaternary Ammonium-Containing Chitosan Particles: Preparation and Antibacterial Activity

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This research aims to develop environmentally friendly, organic antibacterial fillers from quaternized chitosan particles that may be applicable for biomedical device, health, food, textile, and personal hygiene industries. Two routes have been proposed to introduce quaternary ammonium groups in order to enhance the antibacterial activity of chitosan particles. Route 1 is to firstly prepare chitosan particles by ionic gelation and then modify the surface of the particles by heterogeneous quaternization using selected aldehydes and alkyl iodides. Route 2 involves the synthesis of *N,N,N*-trimethyl chitosan by homogeneous quaternization using MeI. The particles were then formed by ionic gelation. Results from FT-IR and ^{1}H-NMR analyses confirmed the success of the quaternary ammonium-containing particle formation. The antibacterial activity tested against *S.aureus* (gram positive bacteria) and *E.coli* (gram negative bacteria) by optical density (OD_{600}) and viable cell counting methods indicated that the additional positive charges introduced to the chitosan particles rendered the quaternary ammonium-containing chitosan particles superior antibacterial activity to the native chitosan particles in a neutral pH range. Furthermore, the particles prepared by heterogeneous route exhibited higher antibacterial activity than those prepared by homogeneous route.

REFERENCES

1. Kumar, M. N. V. R.; Muzzarelli, R. A. A.; Muzzarelli, C.; Sashiwa, H.; Domb, A. J. *Chem Rev.* **2004**, 104, 6017-6084.
2. Tsai, G. J.; Su, W. H. *J. Food. Protect.* **1999**, 62, 239-243.
3. Jia, Z.; Shen, D.; Xu, W. *Carbohydr. Res.* **2001**, 333, 1-6.

VITAE

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July 2008	The 5th National Chitin-Chitosan Conference, Center for Chitin-Chitosan Biomaterials (CCB), Metallurgy and Materials Science Research Institute (MMRI), Chulalongkorn University, Bangkok, Thailand.
January 2009	Pure and Applied Chemistry International Conference 2009, Faculty of Science, Naresuan University, Phitsanulok, Thailand.