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SYNTHESIS OF QUATERNARY AMMONIUM CHITOSAN IN IONIC LIQUID

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Petrochemistry and Polymer Science

Faculty of Science

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ลิควิด (SYNTHESIS OF QUATERNARY AMMONIUM CHITOSAN IN
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ในงานวิจัยนี้ 1-บิวทิล-3-เมทิลอิมิดาโซเลียมคลอไรด์ (BMIMCl) ถูกใช้เป็นตัวทำ
ละลายสำหรับการสังเคราะห์อนุพันธ์ไคโทซานที่มีประจุบวกคือ เอ็น,เอ็น,เอ็น-ไทรเมทิลแอมโม
เนียมไคโทซานคลอไรด์ (TMC), เอ็น-[(2-ไฮดรอกซิล-3-ไทรเมทิลแอมโมเนียม)โพรพิล]ไคโท
ซานคลอไรด์ (HTCC), เอ็น-บิวทิล-เอ็น-[(2-ไฮดรอกซิล-3-ไทรเมทิลแอมโมเนียม)โพรพิล]ไค
โทซานคลอไรด์ (NB-HTCC) และ เอ็น-อ็อกทิล-เอ็น-[(2-ไฮดรอกซิล-3-ไทรเมทิลแอมโมเนียม)
โพรพิล]ไคโทซานคลอไรด์ (NO-HTCC) ระดับการแทนที่ควอเทอร์ไนเซชัน (DQ) ของอนุพันธ์
ประจุบวกถูกคำนวณจากการวิเคราะห์เอ็นเอ็มอาร์ จากการทดลองพบว่าไคโทซานถูกละลายได้
อย่างสมบูรณ์ใน BMIMCl หลังจาก 16 ชั่วโมง ให้ความร้อนที่ 90°C สภาวะของปฏิกิริยาที่ดีที่สุด
ของการสังเคราะห์ TMC คือค่า DQ สูงสุด เท่ากับ 40% ซึ่งค่าที่ได้ต่ำกว่าค่า DQ ของการ
สังเคราะห์แบบวิธีเดิมที่ใช้ NMP เป็นตัวทำละลาย (75%DQ) อันเนื่องจากน้ำจากสารละลาย
โซเดียมไฮดรอกไซด์/โซเดียมไอโอไดด์ เป็นสาเหตุทำให้ไคโทซานตกตะกอนในการทำปฏิกิริยา
ทำให้หน่วงปฏิกิริยา สำหรับสาร HTCC ค่า DQ ของ HTCC, NB-HTCC และ NO-HTCC
เท่ากับ 87±4, 90±2 และ 95±1 ตามลำดับ และประเมินการสังเคราะห์ HTCC โดยใช้ BMIMCl
ที่ผ่านการทำให้บริสุทธ์อีกครั้ง ประสิทธิภาพที่ดีสุดที่แนะนำควรใช้ BMIMCl ที่ผ่านการใช้
มาแล้ว 1 ครั้งเท่านั้นของการสังเคราะห์ HTCC จากไคโทซาน และดำเนินการทดสอบฤทธิ์ยับยั้ง
แบคทีเรียสำหรับอนุพันธ์ HTCC พบว่า HTCC, NB-HTCC และ NO-HTCC มีฤทธิ์ยับยั้ง
S.aureus (แบคทีเรียแกรมบวก) สูงกว่าไคโทซานเดิม จึงระบุว่าประจุบวกบนไคโทซานสามารถ
ช่วยปรับปรุงฤทธิ์ยับยั้งแบคทีเรียของพอลิเมอร์ได้อย่างมีประสิทธิภาพ

สาขาวิชา   ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์   ลายมือชื่อนิสิต ....

ปีการศึกษา 2558   ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

5572076423 : MAJOR PETROCHEMISTRY AND POLYMER SCIENCE
KEYWORDS: IONIC LIQUID / QUATERNARY AMMONIUM CHITOSAN / DEGREE OF QUATERNIZATION

MANEERAT WANGSIRIPAISARN: SYNTHESIS OF QUATERNARY AMMONIUM CHITOSAN IN IONIC LIQUID. ADVISOR: ASST. PROF. VARAWUT TANGPASUTHADOL, Ph.D., 39 pp.

In this study, 1-butyl-3-methylimidazolium chloride (BMIMCl) was used as a solvent for synthesis of positive-charged chitosan derivatives, *N,N,N*-trimethylammonium chitosan chloride (TMC), *N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTCC), *N*-butyl-*N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (NB-HTCC) and *N*-octyl-*N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (NO-HTCC). The degree of quaternization (DQ) of the charge derivatives were calculated from NMR analysis. It was found that chitosan dissolved completely in BMIMCl after 16 h of heating at 90°C. The optimized reaction condition of TMC synthesis gave a maximum DQ of 40%, which was lower than the original method of using NMP as reaction media (75%DQ). It was possible that water from aqueous NaOH/NaI caused the chitosan polymer to precipitate in the reaction mixture, retarding the reaction. For HTCC compounds, the DQ of HTCC, NB-HTCC, and NO-HTCC were 87±4, 90±2 and 95±1 respectively. The synthesis of HTCC by using re-purified solvent (BMIMCl) was evaluated. For the most efficiency, it is recommended that BMIMCl should be re-used only once in the production of HTCC from chitosan. The antibacterial activity test for HTCC derivatives was carried out. HTCC, NB-HTCC and NO-HTCC had higher antibacterial activity against *S.aureus* (gram positive bacteria) than the original chitosan. This indicates that the positive charge on chitosan effectively improved the antibacterial activity of the polymer.

Field of Study: Petrochemistry and   Student's Signature ....

                Polymer Science   Advisor's Signature ....

Academic Year: 2015

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LIST OF ABBREVIATIONS

AMIMCl	: 1-allyl-3-methylimidazolium chloride
BMIMAc	: 1-butyl-3-methylimidazolium acetate
BMIMCl	: 1-butyl-3-methylimidazolium chloride
CFU	: Colony forming unit
COS	: Chitosan oligosaccharide
CS	: Chitosan
DD	: Degree of deacetylation
DMF	: N,N-dimethylformamide
DMSO	: Dimethyl sulfoxide
DQ	: Degree of quaternization
DS	: Degree of substitution
FT-IR	: Fourier Transform Infrared Spectroscopy
GTMAC	: Glycidyltrimethylammonium chloride
HTCC	: N-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride
ILs	: Ionic liquids
NBCS	: N-butyl chitosan
NB-HTCC	: N-butyl-N-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride

NMP   : *N*-methyl-2-pyrrolidone

NMR   : Nuclear Magnetic Resonance Spectroscopy

NOCS   : *N*-octyl chitosan

NO-HTCC   : *N*-octyl-*N*-[(2-hydroxyl-3-trimethylammonium)
                                   propyl]chitosan chloride

OD   : Optical density

rt   : Room temperature

S.aureus   : *Staphylococcus aureus*

TMC   : *N,N,N*-trimethylammonium chitosan chloride

VOCs   : Volatile organic compounds

XH   : Xylan-rich hemicellulose

CHAPTER I

INTRODUCTION

1.1 Statement of Problems

Chitosan is a natural β-(1→4) linked glucosamine biopolymer derived from chitin by deacetylation. It is a nontoxic, biodegradable and biocompatible polymer and many interesting properties which are useful in antibacterial activity. However, due to the strong intra- and inter- hydrogen bonds between hydroxyl groups and amino groups. It is insoluble in common organic solvents and neutral conditions except acidic solution (pH<6). Hence a number of publications focused on modifying chitosan structure so it could dissolve in aqueous solvents, which would expand its usage in biomedical applications. One way is to modify the neutral amino group on the chitosan chains to carry positively charged quaternary ammonium group. There have been two types positively charged chitosan reported; *N,N,N*-trimethylammonium chitosan chloride (TMC) and *N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTCC). TMC was synthesized by methylation with iodomethane (CH_3I) in NMP as solvent [1,2,3]. HTCC was synthesized by ring-opening reaction via ring opening reaction at the epoxy group of glycidyltrimethylammonium chloride (GTMAC) by the amino group of chitosan in aqueous acetic acid as solvent [4].

Ionic liquids (ILs) are molten salts with many advantage properties, such as low vapor pressure, good chemical stability, excellent dissolution power for organic and inorganic compounds, and dissolution for polysaccharide such as cellulose, starch and chitosan. The ILs can also recovered and purified and re-used again [5,6]. Recently, it has been reported that ionic liquids can successfully dissolve chitosan [7].The dissolution of chitosan in BMIMCl IL was caused by disruption of hydrogen-bonding between chitosan chains by the $BMIM^+$ and Cl^- ions (Figure 1.1). This would cause the polymer chains to move further apart and would allow easy access of chemical reagent to react with the functional groups on the chitosan.
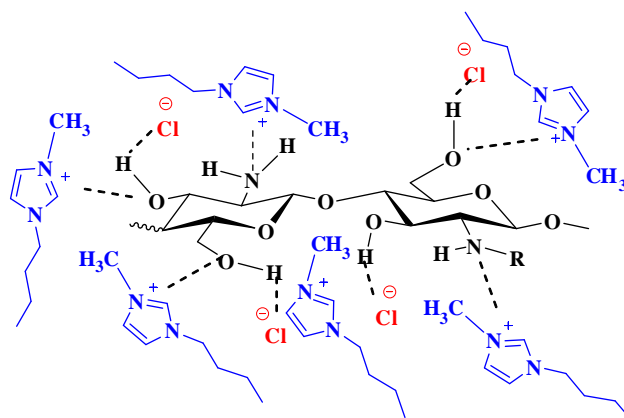
Figure 1.1 Proposed dissolution mechanism of chitosan in BMIMCl solvent

In this research, an IL, BMIMCl, was explored as an alternative solvent for synthesis of quaternary ammonium chitosan derivatives. Two derivative groups were focused; TMC and HTCC. Parameters such as dissolution temperature and time, type and amount of chemical reagents were studied. It was expected that the IL would improve the dissolution of chitosan, thus, providing easy access of reagents to react with its amino groups and leading to an increase of degree of quaternization, reduction of amount of reagent and reaction time. The antibacterial activities of the quaternary ammonium derivatives were also reported against *S.aureus* bacteria. Finally recycle of the reaction solvent, BMIMCl, was evaluated.

1.2 Objectives

1. To synthesize and study on parameter to effect on degree of quaternization of quaternary ammonium chitosan derivatives by BMIMCl as solvent

2. To test antibacterial activity between chitosan and quaternary ammonium chitosan derivatives

1.3 Scope of Investigation

1. Synthesis of *N,N,N*-trimethylammonium chitosan chloride (TMC) and synthesis of *N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTCC) and its *N*-alkylated derivatives in BMIMCl as solvent.

2. Reusability of ionic liquid.

3. Evaluation of antibacterial activity of the quaternary ammonium chitosan derivatives against *Staphylococcus aureus* (gram positive bacteria) using viable cell counts.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Ionic Liquids [8,9]

2.1.1 The Structure of Ionic Liquids

Ionic liquids (ILs) consist of positive- and negative-charged ions. The structure of ILs is similar to other salts, such as sodium chloride; however, the point difference is that ILs remains liquid at temperatures below 100°C and the melts are liquid over a wide temperature range, whereas the melting point of sodium chloride is 801°C. The most widely used ILs are based on imidazolium, pyridinium, phosphonium and ammonium cations, and tetrafluoroborate $[BF_4]^-$, hexafluorophosphate $[PF_6]^-$, methane sulfonate $[CH_3SO_3]^-$, trifluoromethane sulfonate $[CF_3SO_3]^-$, acetate, chloride anions as shown in Figure 2.1
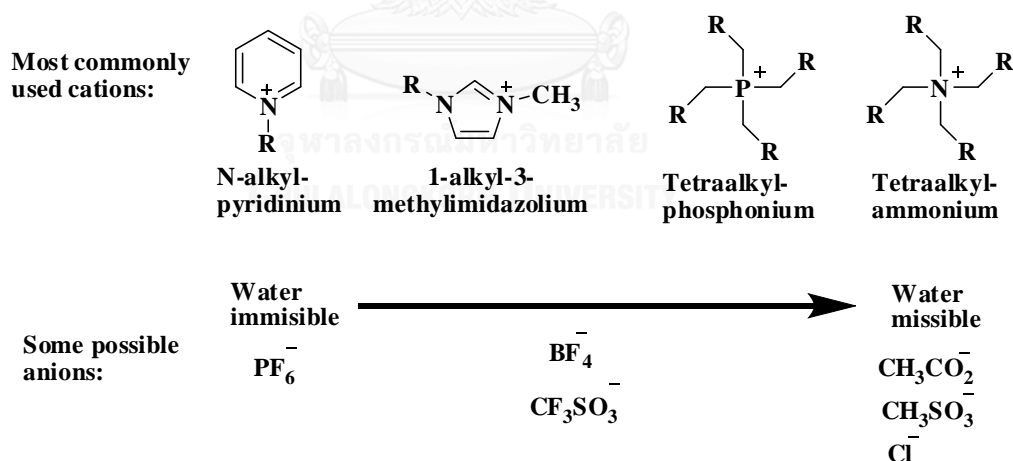
Figure 2.1 Commonly used cations and anions

2.1.2 Properties of ILs

Properties of ILs can be defined mainly by a combination of organic cation and inorganic anion. As solvents, ILs possess several advantages over conventional organic solvents, which make them environmentally compatible; ILs have the ability to soluble many different organic, inorganic and organometallic materials, high

polarity, very low vapor pressures, thermal stability, mostly are liquids from 25°C to 200°C, immiscible with low polarity organic solvents (diethyl ether, ethyl acetate, etc.) and miscible with inorganic (water, alcohols, acetone, etc.). Furthermore, the solvent properties of ILs can be tuned for a specific application by varying the anion/cation combinations.

2.1.3 ILs as Solvents

Ionic liquids are capable of the dissolution of substances such as fibers, amino acids and polysaccharides [10]. ILs are called "green" solvents because their vapor pressures are extremely low. ILs are considered "green" solvents because unlike the volatile organic compounds (VOCs) that they could replace, many of these compounds have negligible vapor pressure, not explosive and it may be feasible to recycle and repeatedly reuse them. ILs are also known as "designer solvents" since they offer the opportunity to tune their specific solvation properties for an appropriate application. Researchers can design task-specific ILs by choosing negatively charged small anions and positively charged large cations, and these specific ILs may be utilized to dissolve a certain chemicals or to extract a certain materials from a solution. By combining various kinds of cation and anion structures, it is approximated that 10^{18} ILs can be designed. The ability of ILs to dissolve solutes is defined by their ability to undergo donor acceptor interactions.

2.2 Dissolution of chitosan with ILs

2.2.1 Chitosan

Chitosan is a natural β-(1→4) linked glucosamine biopolymer derived from chitin by deacetylation. It is a nontoxic, biodegradable and biocompatible polymer and many interesting properties which are useful in antibacterial activity. However, due to the strong intra- and inter- hydrogen bonds between hydroxyl groups and amino groups (Figure 2.2). It is insoluble in common organic solvents and neutral conditions except acidic solution (pH<6). Hence there are many interest developed properties to enhance aqueous solubility and biological activity.
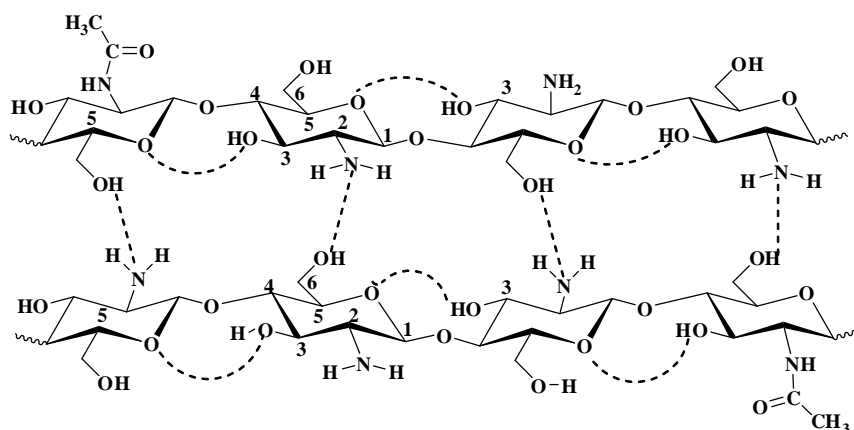
Figure 2.2 Structure of chitosan

2.2.2 Dissolution of Chitosan in ILs

In recent year, it was revealed that ionic liquids (ILs), especially those based on imidazolium cations, are capable of dissolving chitosan. Researhers are currently investigating the application of ILs as the solvent for the chemical modification of chitosan

In 2011, Hua *et al.* [7] prepared lilial (aldehyde molecule having benzene ring)-modified chitosan via Schiff base intermediates between amino group of chitosan and carbonyl group of lilial to imine form in BMIMCl as solvent. The result reported that the CS (50kDa, 95%DD) was completely dissolved in BMIMCl (1%w/w) at 90°C and DMF (16%v/v) was added for improved dissolve lilial and reduce viscosity of the reaction. The mole ratio of lilial : amino group of chitosan was 3:1 and the %DS was 43.2% of imine group.

In 2012, Peng *et al.* [11] synthesized azido-functionalized chitosan by introducing amide groups into the amine groups of chitosan and DCC as catalyst form the amide bond in AMIMCl (1-allyl-3-methylimidazolium chloride) as solvent and then DMF *(N,N*-dimethylformamide) was added to reduce the viscosity of chitosan/ionic liquid mixture. The result showed that the CS (80-100kDa, 90%DD) was completely dissolved in AMIMCl (1%w/w) at 100°C for 3 h. The %DS was 46%

In 2015, Liu *et al.* [12] synthesized linoleic acid-grafted chitosan oligosaccharide (COS) by condensation in BMIMAc (1-butyl-3-methylimidazolium acetate) as solvent. The result showed that the COS (37kDa, 95%DD) was completely dissolved in BMIMAc (8%w/w) at 100°C for 3 h. The %DS was 46%.

In 2015, Yang *et al.* [13] indicated that *N*-[(2-hydroxyl)-propyl-3-trimethyl ammonium] chitosan chloride (HTCC) was synthesized through nucleophilic substitution of glycidyltrimethylammonium chloride onto chitosan using AMIMCl as solvent. The result showed that the CS (500kDa, 91%DD) was completely dissolved in AMIMCl (2%w/w) at 80°C for 8 h. The %DS was 46%.

2.3 Other Polysaccharide in ILs

In 2012, Gericke *et al.* [14] studied the esterification of xylan-rich hemicellulose (XH) with maleic anhydride in BMIMCl using LiOH as catalyst. The result showed that the XH was completely dissolved in BMIMCl (2.5%w/w) at 90°C for 1.5 h. The proposed dissolution mechanism of XH in BMIMCl as shown in Figure 2.3
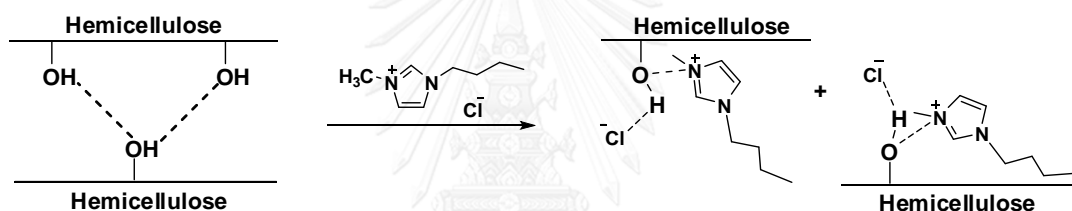
Figure 2.3 Proposed dissolution mechanism of XH in BMIMCl

In 2012, Gao *et al.* [15] reported that dissolution of corn starch and homogeneous synthesis of fatty-acid starch ester by esterification between hydroxyl group of starch and carboxyl group of fatty acid in ionic liquids, BMIMCl, EMIMAc (1-ethyl-3-methylimidazolium acetate) and the mixture of BMIMCl/DMSO as solvent. The result showed that the corn starch was completely dissolved in BMIMCl and EMIMAc at 100°C for 60 min. The degree of substitution (DS) was the highest in mixture of BMIMCl/DMSO. Because of DMSO to reduce viscosity of the reaction system providing increased fluidity of reagent in the reaction.

2.4 Reused ILs

In 2012, Gericke *et al.* [5] reported that the recycling IL of reaction of cellulose with tosyl chloride (Tos-Cl) and pyridine as base. After precipitation in ethanol and filtration of the tosyl cellulose (TOSC). Ethanol and volatile compounds were removed by evaporation under reduce pressure. The pyridinium was neutralized with solid NaHCO$_3$. Then NaCl and NaHCO$_3$ were filtrated. After that, addition

chloroform for dissolve residue pyridine were removed by reduce pressure. The obtained products containing are tosylate and IL were dissolved in water and anion exchanger (Cl⁻ loaded) was added. The tosylate ion was removed by exchange with Cl⁻ in ion exchanger. Finally, the purified IL with chloride ion was obtained.

Moreover, In 2013 Liu *et al.* [6] reported that the recycling ILs of acetylation of chitosan with acetyl chloride. After the acetylation, reaction mixture was precipitated in water and filtration of acetylated chitosan. The filtrates containing water and residue acetylated were removed by rotary evaporated under reduced pressure at 80°C and then extracted with diethyl ether for remove residue acetyl group. Because of ether has low polarity can dissolve residue acetyl group and were removed by reduce pressure at 80°C to get viscous recycle IL. Researcher indicated that the recycle IL could be reused at least for three times. The %DS show that decrease from 83 to 68%. Moreover, the color of IL becomes gradually deeper as run times going on.

2.5 Quaternary Ammonium Chitosan Derivatives

In 1998, Sieval *et al.* [1] synthesized the quaternary ammonium chitosan, *N,N,N*-trimrthyl chitosan chloride (TMC). The reaction was performed by reaction of amino group of chitosan with iodomethane (CH_3I) and sodium hydroxide controlled conditions (Figure 2.4). The reaction yield TMC with various degrees of quaternization. Furthermore, TMC can be soluble in water.
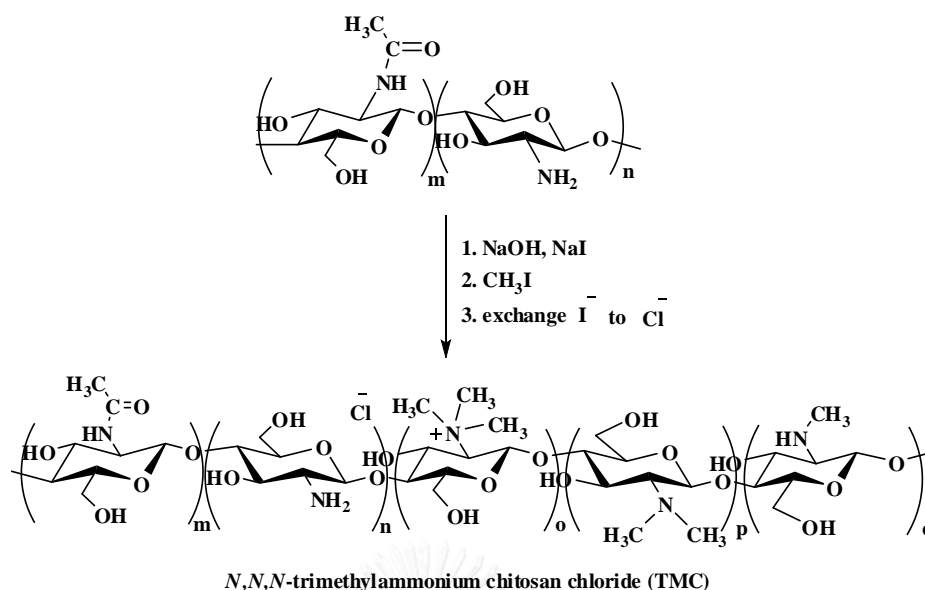
Figure 2.4 Synthesis of *N,N,N*-trimrthyl chitosan chloride (TMC)

In 2000, Seong *et al.* [4] synthesized *N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTCC) by introducing quaternary ammonium group from glycidyltrimethyl ammonium chloride (GTMAC) into the amine groups of chitosan via ring opening reaction (Figure 2.5).The complete substitution of amino group react with GTMAC was achieved when the reaction was performed at 80°C with a 4:1 mole ratio of GTMAC to amino group in the acetic acid solution
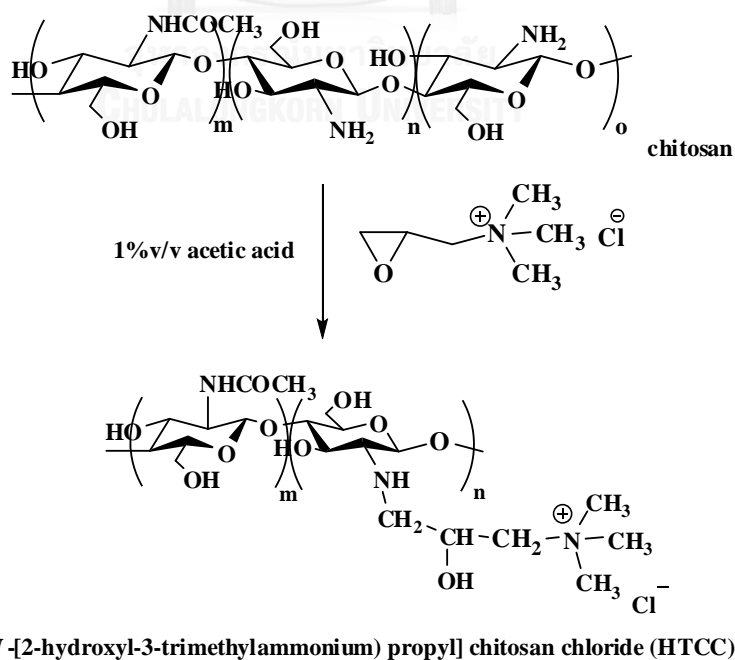
Figure 2.5 Synthesis of *N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTCC)

2.6 Quaternary Ammonium Chitosan Derivatives in Antibacterial Applications

The influence of the cationic charge and the chemical properties of the hydrophobic groups on the antimicrobial activity of chitosan derivatives has been the subject of several studies

In 2009, Sajomsang *et al.*[16] to test antibacterial activity of quaternized *N*-aryl chitosan (90%DQ) and showed very low the minimum inhibitory concentration (MIC) values, which was in the range 8-64 µg/mL for against *S.aureus* and *E.coli* bacteria. Moreover, it was found that %DS of alkyl effect to antibacterial activity should not exceed 20%DS attributed to the decreasing %DQ, which mean that less cationic charge density can be obtained on chitosan backbone. However, the mechanism of antibacterial activity of chitosan and its derivatives is proposed that the cationic charge density absorbed onto the negatively charged cell surface of bacteria leads to the cell leakage and cell membrane disruption. And the hydrophobic-hydrophobic interaction between the alkyl substituent and the hydrophobic interior of the bacteria cell wall.

In 2011, Vallapa *et al.* [17] prepared chitosan surface by introducing quaternary ammonium groups via a heterogeneous two-step process: reductive alkylation using a series of different aldehydes followed by methylation with iodomethane. The antibacterial activity of the surface-modified chitosan film against *S.aureus* and *E.coli* had higher than the chitosan film. The addition 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 negative-charged membrane of the bacteria.

In 2015, Sahariah *et al.* [18], synthesized 100%dialkylated derivatives of chitosan by varies long alkyl chains and though *N*-methylation reaction and studied relationship between length of the alkyl chains and antibacterial activity. The results were indicated that quaternary short alkyl chain derivatives showed high activity against *S. aureus*, was low MIC value (4µg/mL), whereas the longer alkyl quaternary *N*-hexyl derivatives were active against *E. coli* with MIC value of 16 µg/mL.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Chitosan with a weigh-average molecular weight (M_w) of 73kDa was purchased from Seafresh Chitosan (Lab) Co., Ltd, Thailand. The degree of deacetylation (DD) was 92% as determined by ^{1}H NMR. A cellulose dialysis tubing (Cellusep) with molecular weight cut off 3,500 g/mol was use to purify all water-soluble chitosan derivatives. 1-Methyl-3-butylimidazolium chloride, BMIMCl (Aldrich); iodomethane, CH_3I (Merck); glycidyltrimethylammonium chloride, GTMAC (Fluka); butyraldehyde (Merck); octanal (Aldrich); sodium cyanoborohydride (Aldrich); *N*-methyl-2-pyrrolidone, NMP (Merck); glacial acetic acid (Merck); deuterium oxide (D, 99.9%) (Cambridge Isotope Laboratory (CIL); trifluoroacetic acid, TFA (Fluka); d_6-dimethyl sulfoxide (D, 99.9%) (Cambridge Isotope Laboratory (CIL) was used as received. *Staphylococcus aureus* TISTR 746 was provided by Thailand Institute of Scientific and Technological Research (TISTR), Thailand. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were purchased from Difco (USA).

3.2 Characterization

3.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H-NMR and ^{1}H–^{1}H COSY spectra were recorded with either a Varian, model Mercury-400 nuclear magnetic resonance (NMR) spectrometer operating at 400 MHz and a Bruker Avance 400 operating at 300 MHz. D_2O/TFA was used to dissolve chitosan, the *N*-alkyl chitosan derivatives and the quaternary ammonium chitosan derivatives and d_6-DMSO was used to dissolve BMIMCl.

3.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

The FT-IR spectra were recorded with a Nicolet Impact 6700 FT-IR spectrometer with 64 scans at a resolution of 4 cm^{-1} in a frequency range of 400-4000 cm^{-1}.

3.3 Preparation of *N*-butyl and *N*-octyl Chitosan Derivatives (NBCS and NOCS)

Chitosan was dissolved in 1%v/v acetic acid (1%w/v). When dissolution was completed, 35 ml ethanol was added. Then the solution of aldehyde (butyraldehyde or octanal) in ethanol was added into the chitosan solution and stirred for 2 h. After 2 h, one more portions of aldehyde were added into the reaction solution and the reaction stirring for a total of 4 h. The pH of this mixture was adjusted to 5 with 1M NaOH. Then sodium cyanoborohydride (3 equivalent / -NH$_2$ group) was added and stirred for 4h. The precipitants of the *N*-alkyl chitosan derivatives were obtained from adjust the pH of the solution to 10 with 1M NaOH. These precipitant were wash with ethanol to remove unreacted aldehyde and inorganic products from the precipitant by centrifuge and wash with distillated water again. The product was characterized by NMR spectroscopy [19].

3.4 Synthesis of Quaternary Ammonium Chitosan Derivatives

3.4.1 *N,N,N*-trimethyl Chitosan Chloride (TMC)

Chitosan was completely dissolved in BMIMCl (2.5%w/v) at 90 °C and the mixture was stirred for 16 h. Then potassium carbonate (0.69 g, 9 equiv.) was added and the mixture was stirred at 55 °C for 15 min, followed by the addition of iodomethane (0.138 ml, 4 equiv.). The reaction was carried out in a closed reaction vial at 55 °C. After 2 and 4 h, two more portions of CH$_3$I (0.138 ml, 4 equiv.) were added into the reaction solution and the reaction was kept stirring for a total of 24 h. After methylation, the product was precipitated in ethanol. The solid product was dissolved in 15% (w/v) NaCl solution in order to replace the iodide counter ion with chloride ion. The suspension was dialyzed in deionized water for 2 days to remove inorganic materials and then freeze-dried overnight, giving a white and fluffy solid of trimethylated chitosan.

3.4.2 *N*-[(2-hydroxyl-3-trimethylammonium) propyl]Chitosan Chloride (HTCC)

Chitosan was completely dissolved in BMIMCl (5%w/w) at 90 °C and the mixture was stirred for 16 h. Then GTMAC (0.2528 g, 3 equiv.) was added in three portions (0.0843 g each) to the reaction mixture at 2 h intervals. After reaction for 24 h at 80 °C, the reaction solution was poured into ethanol. The isolation and purification of product was performed as described in "Isolation and purification of HTCC, NB-HTCC and NO-HTCC".

3.4.3 *N*-butyl-*N*-[(2-hydroxyl-3-trimethylammonium) propyl]Chitosan Chloride (NB-HTCC)

The *N*-butyl chitosan was completely dissolved in BMIMCl (3.75%w/w) at 90 °C and the mixture was stirred for 2 h. Then GTMAC (0.2528 g, 3 equiv.) was added in three portions (0.0843 g each) to the reaction mixture at 2 h intervals. After reaction for 24 h at 80 °C, the reaction solution was poured into ethanol. The isolation and purification of product was performed as described in "Isolation and purification of HTCC, NB-HTCC and NO-HTCC".

3.4.4 *N*-octyl -*N*-[(2-hydroxyl-3-trimethylammonium) propyl]Chitosan Chloride (NO-HTCC)

The *N*-octyl chitosan was completely dissolved in BMIMCl (3.75%w/w) at 90 °C and the mixture was stirred for 2 h. Then GTMAC (0.2528 g, 3 equiv.) was added in three portions (0.0843 g each) to the reaction mixture at 2 h intervals. After reaction for 24 h at 80 °C, the reaction solution was poured into ethanol. The isolation and purification of product was performed as described in "Isolation and purification of HTCC, NB-HTCC, and NO-HTCC".

Isolation and purification of HTCC, NB-HTCC and NO-HTCC

The precipitate was then isolated by centrifuge. The solid product was dispersed in distilled water. The final suspension was dialyzed with deionized water for two days to remove residual GTMAC and BMIMCl, followed by freeze-drying to obtain white and fluffy solid of HTCC, NB-HTCC, and NO-HTCC.

3.5 Reusability of BMIMCl

After the ring-opening reaction in BMIMCl, the positively charge chitosan derivatives were precipitated from the reaction mixture by ethanol and filtration. Then ethanol and traces of water were removed by subsequent purification process; rotary evaporation at 40°C for 1 h, vac-dry at 60°C for 4 h and vac-dry at rt for 24 h. The purified solvent was then analyzed by ^{1}H-NMR to determine the amount of leftover chemicals and it was then reused in the synthesis process.

3.6 Evaluation of Antibacterial Activity

All glassware used for the tests were sterilized in an autoclave at 121°C for 15 min prior to use. All chitosan samples were sterilized by exposing to UV radiation for 30 min prior to the tests. Mueller-Hinton broth (MHB) dissolved in deionized water was autoclaved for 15 min at 121°C. The broth was stored at 4°C before use. Mueller-Hinton agar (MHA) dissolved in deionized water was transferred into test tubes 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.

3.6.1 Preparation of Bacteria Suspension

Staphylococcus aureus TISTR 746 was used as gram positive bacteria. A loopful of bacteria were streaked on agar slant and then incubated at 37°C in an incubator for 24h. Sterile deionized water (5 mL) was added in the tube containing agar slant to obtain bacterial suspension. The optical density of the suspended bacteria in steriled deionized water was determined by UV-visible spectrophotometer (Model MV, the Bausch Lomb, USA) at a wavelength of 600 nm (OD_{600}). The value was adjusted to 0.5 by steriled deionized water.

3.6.2 Antibacterial Tests

The test was performed according to method of Wiarachai *et al*. [20]. Chitosan and quaternary ammonium chitosan derivatives were added to MHB (0.5 mg/mL) in test tubes and aseptically inoculated with 50 μL of the freshly prepared bacterial suspension in distilled water (OD_{600} = 0.5). The pH of the broth was about 7.0. The negative and positive controls were a broth solution alone and a broth solution with ampicillin (50 mg/mL), respectively. After mixing, broth cultures were incubated at

37°C in a shaking incubator, at 110 rpm for 24 h. Then, 100 µL of the bacterial solution was diluted to 10^6 times. Individual 100 µL aliquots of each diluted bacterial suspension were then spread in triplicate onto the MHA plates for total plate counting. After incubating at 37°C for 18 h, the number of colonies, and thus replication competent bacteria, were then counted as a measure of the assumed viable number of bacteria. The results, after correction for the dilution factor, are expressed as mean colony forming units per volume (CFU/mL).

CHAPTER IV

RESULTS AND DISCUSSION

This chapter was divided into four parts. The first and second part reveals the synthesis of *N,N,N*-trimethylammonium chitosan chloride (TMC) and synthesis of *N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTCC) and *N*-butyl-*N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (NB-HTCC) and *N*-octyl -*N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (NO-HTCC) in ionic liquid as solvent. The reusability of ionic liquid was reported in the third parts. The last part was explained antibacterial activity test of the quaternary ammonium chitosan derivatives having different alkyl groups.

4.1 Synthesis of *N,N,N*-trimethylammonium Chitosan Chloride (TMC)

In this work, BMIMCl was the solvent of choice to dissolve chitosan (73 kDa, 92%Degree of Deacetylation or DD) for synthesis of positive-charged chitosan derivatives. Table 4.1 exhibited the dissolution of chitosan in BMIMCl and organic solvents (5 %wt) at 90ºC. It was found that chitosan dissolved completely in BMIMCl after 16 h of heating at 90°C [7,11]. The presence of DMF or NMP did not help the dissolution. Therefore BMIMCl was used as the solvent for chitosan in the subsequent studies.

Table 4.1 Dissolution of chitosan (5 %wt) in BMIMCl and organic solvents at 90°C

solvent	Time (h)				
	6	12	16	24	48
BMIMCl	-	-	+	+	+
BMIMCl/DMF (75:25)	-	-	-	-	-
BMIMCl/NMP (75:25)	-	-	-	-	-

-, chitosan was not dissolved; +, chitosan was completely dissolved

N,N,N-trimethylammonium Chitosan Chloride (TMC) was synthesized via methylation at the amino groups of chitosan with iodomethane (CH$_3$I) in the presence of base (Figure 4.1)
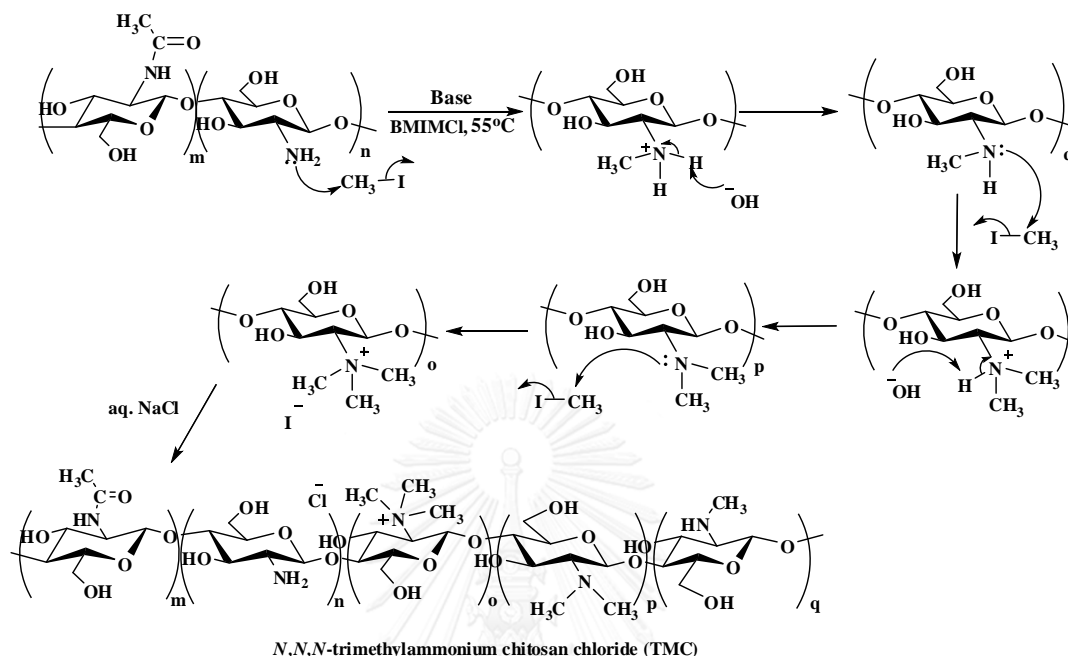
Figure 4.1 Machanism of the synthesis of *N,N,N*-trimethylammonium chitosan chloride (TMC) from chitosan by methylation with iodomethane

Figure 4.2 displays ^{1}H-NMR spectrum of TMC and chitosan. The signal of anomeric proton, H-1 and H-1', appeared at 4.9 and 5.25 ppm, respectively. The signals at 3.25-4.30 ppm were assigned to all protons in the pyranose ring of chitosan (H-2',3,3',4,4',5,5',6$_1$,6$_2$, 6$_1$',6$_2$'). The signal of H-2 appeared at 3.15 ppm. Finally the signals at 3.10, 2.85, and 2.65 ppm were assigned to *N,N,N*-trimethyl protons, *N,N*-dimethyl protons, and *N*-methyl protons on the glucosamine ring, respectively.

The structure of TMC and the individual peaks were confirmed by COSY spectrum (2-D NMR, Figure 4.3), revealed that all clearly resolved peaks of the protons in pyranose ring could be divided into two sets. The 1st set consisted of the proton signals from non-quaternized pyranose unit, labeled as H-1,2,3,4,5,6$_1$,6$_2$. The other group was the proton signals from the quaternized unit, labeled as H-1',2',3',4',5',6$_1$',6$_2$'. The degree of quaternization was calculated from the equation 4.1:

$$\%DQ \quad = \quad \left(\frac{[(\int(N^+(CH_3)_3/9)+\int(H-2))-\int(H-1)]}{\int(H-1,2',3,3',4,4',5,5',6_1,6_2,6_1',6_2'/6)\times DD}\right) \times 100 \qquad (4.1)$$

where, DQ is the degree of quaternization and was determined from the relative ratio between the integration of 9 protons from 3 methyl groups (-N(C$\underline{H}$)$_3$) and 1 proton from H-2 (δ 2.90-3.20 ppm) subtract with the integration of 1 proton from H-1 of TMC (δ 4.9 ppm) and the peak integration of 6 protons of H-1,2',3,3',4,4',5,5',6$_1$,6$_2$,6$_1$',6$_2$' of chitosan (δ 4.9 ppm and 3.25-4.30 ppm). The degree of deacetylation (DD) of chitosan determined by ^{1}H-NMR was 0.92.

For degree of dimethylation (%di-) and monomethylation (%mono-), the peak integration of protons (at 2.85 and 2.65 ppm) from the *N,N*-dimethyl protons, and *N*-methyl protons were used for calculation, respectively (equation 4.2 and 4.3).

$$\%DS_{NHCH3} \quad = \quad \left(\frac{\int(NHCH_3/3)}{\int(H-1,2',3,3',4,4',5,5',6_1,6_2,6_1',6_2'/6)\times DD}\right) \times 100 \qquad (4.2)$$

$$\%DS_{N(CH3)2} \quad = \quad \left(\frac{\int(N(CH_3)_2/6)}{\int(H-1,2',3,3',4,4',5,5',6_1,6_2,6_1',6_2'/6)\times DD}\right) \times 100 \qquad (4.3)$$

where, $\int(NHCH_3)$ is the integral of the monomethyl peak. $\int(N(CH_3)_2$ is the integral of the dimethyl peak. $\int(H-1,2',3,3',4,4',5,5',6_1,6_2,6_1',6_2'/6)$ is the integral corresponding to the H-1,2',3,3',4,4',5,5',6$_1$,6$_2$,6$_1$' and 6$_2$' protons from 3.25-4.30 ppm.
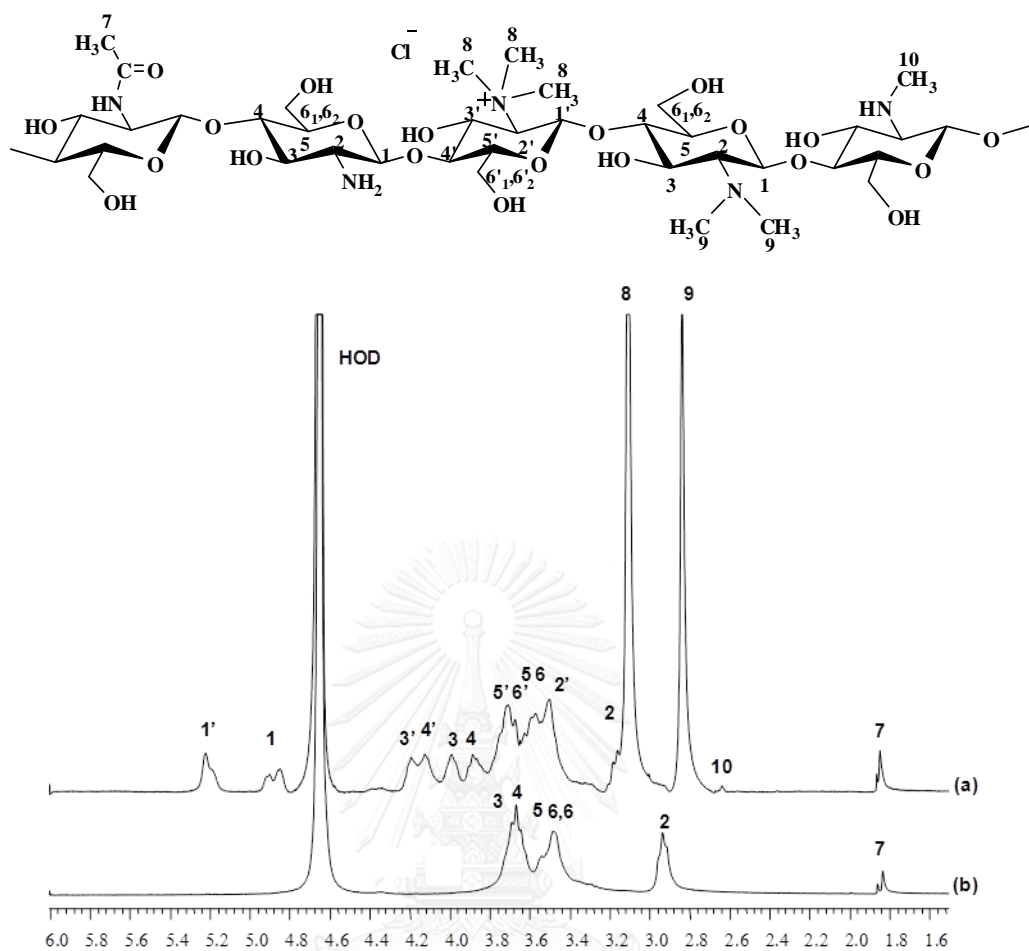
Figure 4.2 ¹H NMR spectra of (a) TMC (entry7, Table 4.2) and (b) chitosan (D₂O/CF₃COOH)
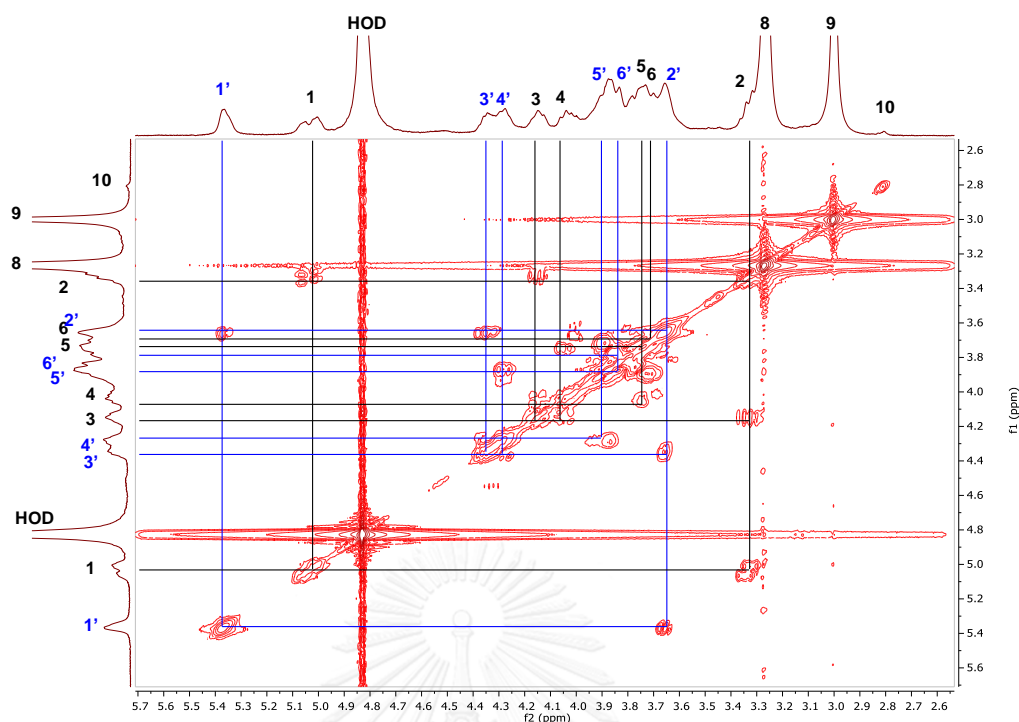
Figure 4.3 COSY spectrum of TMC (entry7, Table 4.2)

The study on the reaction condition of synthesis of TMC was carried out in different types of solvent and base as shown in Table 4.2. Comparison between the reaction carried out in NMP (entry 1) and BMIMCl (entry 2) at constant amounts of CH_3I (12 eq.) and NaOH/NaI (6/4.5eq.) revealed that, in BMIMCl, the obtained DQ was lower than that obtained from the reaction occurred in NMP. This is somewhat unexpected since chitosan was dissolved in the ionic liquid BMIMCl more than in NMP. Our trials on pyridine (entry 3) and imidazole (entry 4) as organic base in synthesis of TMC appeared to be worse due to its weak base property. This lower DQ was most likely due to high viscosity effect in the reaction mixture of BMIMCl, retarding the rate of molecular movement to achieve satisfied attacking rate of the side chain amino groups onto CH_3I. However, Gericke reported that tosylation of cellulose in mixture of ionic liquid and a co-solvent could be achieved in the presence of pyridine and imidazole as base [5]. In nucleophilic substitution of amine group by iodomethane in organic solvent, K_2CO_3 was found as a suitable base for deprotonation of amine group in acetonitrile [21]. From the result, the DQ was increased with increasing of K_2CO_3 (entry 5-8). By using 9 equiv of K_2CO_3 as base and BMIMCl as solvent, the highest DQ of 41% was obtained.

In this work it was also found that using BMIMCl as solvent for the reaction at 55°C resulted in a very viscous solution. So 1-butyl-3-methylimidazolium acetate (BMIMOAc) was also studied [12] because of its lower melting point (-20°C) than that of BMIMCl (70°C). The result, however, was not satisfied since lower substitution of ammonium group was obtained when BMIMOAc was used instead of BMIMCl (entry 9 and entry 10).

Neverthelessthe best reaction condition to synthesize TMC was obtain from BMIMCl system (40%DQ). This value was lower than that from the much reported NMP system (75%DQ). It was possible that water from NaOH/NaI solution used in the reaction caused the chitosan polymer to precipitate in the reaction mixture.

Table 4.2 Different solvents and base conditions for *N*-methylation of chitosan

Entry	Reaction condition [a]		Product		
	Solvent	Base (equiv.)	%DQ	%di-	%mono-
1	NMP	NaOH/NaI (6/4.5)	75	39	ND
2	BMIMCl	NaOH/NaI (6/4.5)	29	72	ND
3	BMIMCl	Pyridine (6)	trace	ND	ND
4	BMIMCl	Imidazole (6)	trace	ND	ND
5	BMIMCl	K_2CO_3 (2.5)	13	49	10
6	BMIMCl	K_2CO_3 (6)	38	54	1
7	BMIMCl	K_2CO_3 (9)	41	64	1
8	BMIMCl	K_2CO_3 (12)	38	40	trace
9	BMIMOAc	NaOH/NaI (9/4.5)	11	24	20
10	BMIMOAc	K_2CO_3 (9)	trace	ND	ND

[a] CH_3I 12 equivalent, reaction time 24 h and reaction temperature 55°C
[b] ND is 'not detected'.

4.2 Synthesis of *N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTCC) and its *N*-alkylated derivatives

HTCC and its two alkylated derivatives; *N*-butylated and *N*-octylated, were synthesized via ring opening reaction at the epoxy group of glycidyltrimethylammonium chloride (GTMAC) by the amino group of chitosan or *N*-alkylated chitosan in BMIMCl (Figure 4.4).
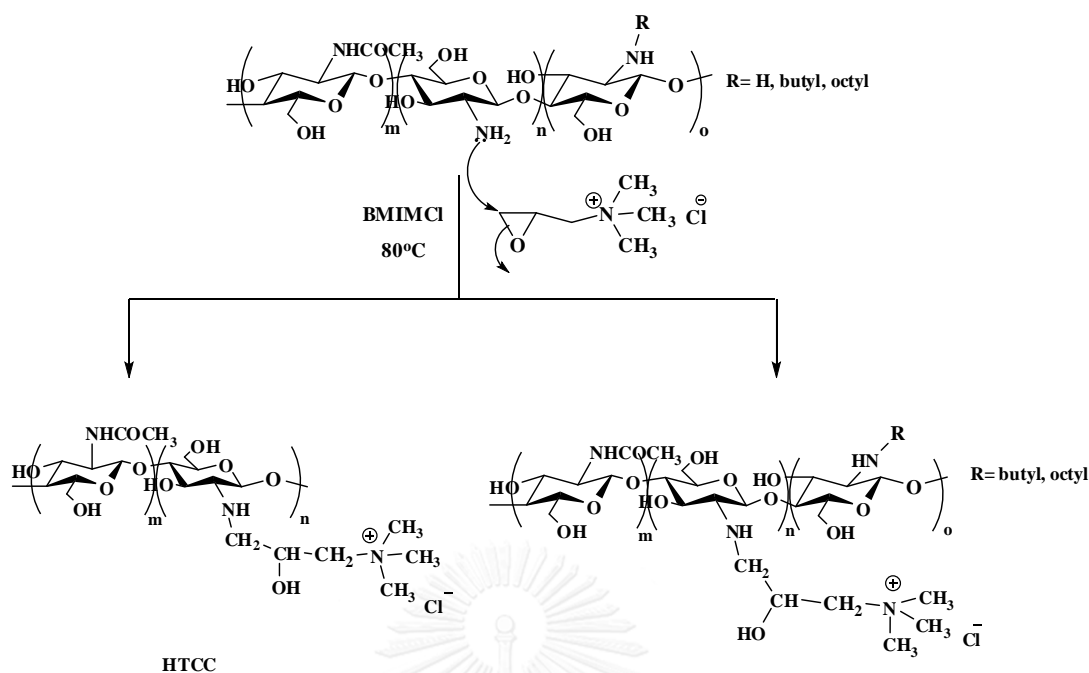
Figure 4.4 Mechanism of the *N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride (HTCC) and its *N*-alkylated derivatives

Figure 4.5 displays ¹H-NMR spectrum of HTCC, NB-HTCC and NO-HTCC as compared to chitosan. The signals of anomeric proton, H-1 appeared at 4.8 ppm and the proton signals at 3.20 ppm were assigned to H-2. The proton signals at 3.4-4.1 ppm were assigned to H-3,4,5,6,6' and the signals at 3.1 ppm were assigned to *N,N,N*-trimethyl protons of chitosan. The %DQ of methyl group on chitosan was calculated from the equation 4.4:

$$\%DQ \quad = \quad \left(\frac{\int(N^+(CH_3)_3/9)}{\int(H-1,3,4,5,6,6'/6)\times DD}\right) \times 100 \qquad (4.4)$$

where, DQ is the degree of quaternization and can be determined from the relative ratio between the integration of 9 protons from 3 methyl groups (-N(C$\underline{H}$$_3$)$_3$) and the peak integration of 6 protons of H-1,3,4,5,6,6' of chitosan (δ 3.40-4.10 ppm).

Figure 4.5 ^{1}H-NMR spectra of (a) chitosan and (b) HTCC and (c) NB-HTCC and (d) NO-HTCC

From the Table 4.3, at 2.5%wt concentration of chitosan in BMIMCl, the DQ was increased from 61 to 80% when the mole equivalent of GTMAC used in the synthesis was increased from 2 to 4. Moreover, when the concentration of chitosan was increased from 2.5 to 5, the DQ was increased from 77 to 87 (entry 4, Table 4.3). This was due to the fact that increasing the reactant concentration would increase the opportunity of amino group attacking the epoxide ring of GTMAC.

Moreover, NB-HTCC and NO-HTCC were synthesized by ring opening reaction with GTMAC. It was found that NBCS and NOCS (dissolution temperature 90°C for 2 h) was dissolved in BMIMCl faster than did chitosan (90°C for 16 h). The

small portion of butyl or octyl groups (10%substituent) possibly disrupted hydrogen-bonding between chitosan chains, allowing easy penetration of BMIMCl to break apart the polymer domain. The %DQ of NB-HTCC and NO-HTCC were 90±2 and 95±1 (entry 5 and 6), respectively when 3 equivalent of GTMAC was used in the reaction.

Table 4.3 Degree of quaternization (DQ) as determined by ^{1}H-NMR on chitosan after reacting with GTMAC in BMIMCl

Entry	Reaction condition[a]			Product		%DQ ±SD	%yield
	Initial polymer	Mole equivalent of GTMAC	Concentration of chitosan (%wt)	Integration			
				H-1,3,4,5,6,6'	-N$^+$(C$\underline{H}_3$)$_3$		
1	CS	2	2.5	106	90	61	63
2	CS	3	2.5	106	113	77	68
3	CS	4	2.5	105	116	80	68
4	CS	3	5	115	140±4	87±4	70
5	NBCS[b]	3	3.75	120	148±4	90±2	73
6	NOCS[c]	3	3.75	122	159±3	95±1	73

[a] Reaction time 24 h and reaction temperature 80°C
[b] 10%substiution of butyl group
[c] 10%substiution of octyl group

FT-IR spectroscopy was used to confirm the success of quaternization of chitosan by CH$_3$I and GTMAC. As shown in Figure 4.6, the formation of TMC, HTCC, NB-HTCC and NO-HTCC were verified by the decrement of the N-H bending peak signal at 1590 cm^{-1} of the amino group of chitosan and the appearance of the C-N stretching peak of the methyl group in quaternary ammonium groups at 1470 cm^{-1}.

Figure 4.6 FT-IR spectra of (a) chitosan, (b) TMC, (c) HTACC, (d) NB-HTACC and (e) NO-HTACC

The degree of quaternization of HTCC obtained in this work was quite closed to the product prepared in acidic aqueous solution (acetic acid) (Table 4.4). The advantage of using BMIMCl would be reusable.

Table 4.4 Synthesis of HTCC in aqueous acetic acid

	Solvent	CS	Concentration of CS (%w/v)	GTMAC (Mole equivalent)	Reaction temperature (℃)	Reaction time (h)	%DS
[4]	Acetic acid (1%v/v)	Chitosan oligosaccharide, 99%DD	5	4	80	18	102
[22]	Acetic acid (1%v/v)	100kDa, 95%DD	2	4	70	24	96

4.3 Reusability of BMIMCl

In this part, reusability of BMIMCl was evaluated. After the ring-opening reaction in BMIMCl, the positively charge chitosan derivatives were precipitated from the reaction mixture by ethanol and filtration. Then ethanol and traces of water were removed by subsequent purification process; rotary evaporation at 40°C for 1 h, vac-dry at 60°C for 4 h and vac-dry at rt for 24 h. The purified solvent was then analyzed by ^{1}H-NMR to determine the amount of leftover chemicals and it was then reused in the synthesis process. The NMR spectra of BMIMCl in every step of reuse study and GTMAC are shown in Figure 4.7.

Figure 4.7 ^{1}H-NMR spectra of (a) fresh BMIMCl, (b) first use BMIMCl, (c) second use BMIMCl, (d) third use BMIMCl and (e) GTMAC

The spectra of the post-purified reused ionic liquid (b, c, and d) displayed a few weak signals attributed to residual GTMAC and water (3.4 ppm). This suggests that GTMAC and water were not completely separated from the used BMIMCl. This is simply because both molecules having similar polarity and, especially GTMAC,

charge characteristic and the BMIMCl ionic liquid. However, the structure of BMIMCl after used in the reaction was also verified and it was found that the structure of BMIMCl remained intact since all of its proton signals (from NMR analysis) had the same pattern and integration values (Table 4.5).

Table 4.5 Integration values of (a) fresh BMIMCl, (b) first use BMIMCl, (c) second use BMIMCl, (d) third use BMIMCl and (e) GTMAC

	Integration values						
	H2	H4,5	H7	H6	H8	H9	H10
(d)	1.0	2.0	2.0	3.0	2.0	2.0	3.0
(c)	1.0	2.0	2.0	3.0	2.0	2.0	3.0
(b)	1.0	1.9	2.0	3.0	2.0	2.0	2.9
(a)	1.0	2.1	2.1	3.1	2.1	2.1	3.1

The result of chitosan-GTMAC reaction by using re-purified solvent (BMIMCl) is shown in Table 4.6. After the 1st reuse, the resulting DQ of HTCC decreased gradually from 87, 83, 72, to 57%. After two recycles, the DQ dropped about 20%. Therefore for the most efficiency, it is recommended that BMIMCl should be re-used only once in the production of HTCC from chitosan at the reaction condition.

Table 4.6 The round no. of reused BMIMCl for synthesis of HTCC by 3 equivalents of GTMAC, reaction temperature 80°C and reaction time 24h

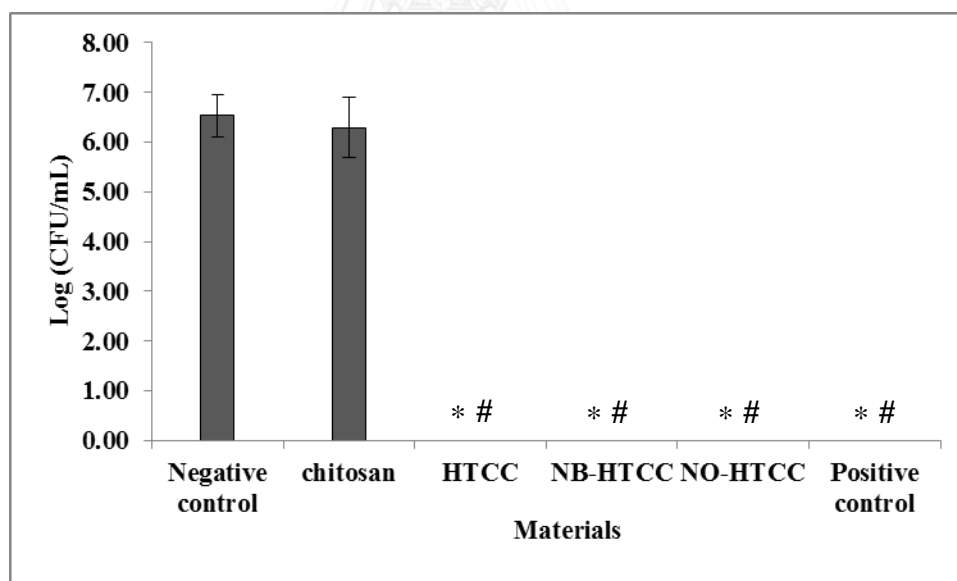| Round no.	%DQ	%yield	Color of reused BMIMCl
0	87±4	70	yellow
1	83±3	77	orange
2	72	73	orange
3	57±8	60	orange-red

4.4 Evaluation of Antibacterial Activity

The antibacterial activities of chitosan and quaternary ammonium chitosan derivatives were reported as the total number of replication competent (viable) cells as

mean colony forming units per volume (CFU/mL) against *S.aureus* bacteria at pH 7.0. The result is shown in Figure 4.8. HTCC (87%DQ), NB-HTCC (10%butylated and 90%DQ) and NO-HTCC (10%octylated and 95%DQ) (entry 4, 5 and 6, Table 4.3) had higher antibacterial activity against *S.aureus* (gram positive bacteria) than the original chitosan. This indicates that the introduction of positive charge effectively improved the antibacterial activity of chitosan. It should be noted here that the antibacterial activities of both alkylated HTCC (butyl and octyl) could not be differentiated. As reported by others, the alkylated derivatives of quaternized chitosan showed higher activity than the non-substituted one [17,20]. A modification of antibacterial test might be carried out in order to observe the effect of alkyl side group on such an activity.

Moreover the HTCC and the two alkylated derivatives could dissolve in neutral aqueous solution. This information could be used to develop antibacterial solution based on quaternary ammonium chitosan.

Figure 4.8 Viable cell counts of *S. aureus* in suspension incubated with different positive-charged chitosan (0.5 mg/mL) for 24h *$p < 0.01$ (compared with the negative control) and no viable bacteria so the count was essentially zero as shown in Table B-1 , #$p < 0.01$ (compared with the chitosan)
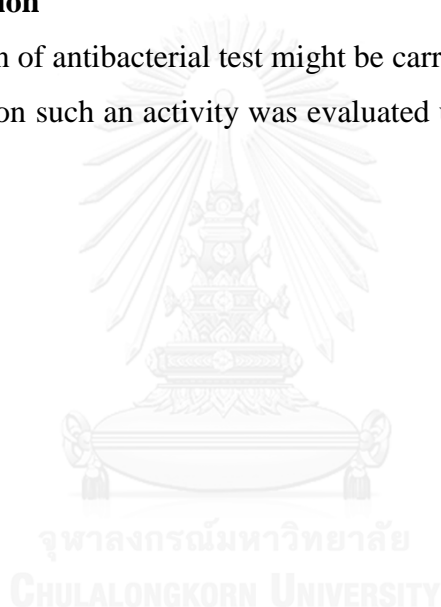
CHAPTER V

CONCLUSION AND FUTURE DIRECTION

In this work, BMIMCl was the solvent of choice to dissolve chitosan (60 kDa, 92%DD) for synthesis of positive-charged chitosan derivatives. It was found that chitosan dissolved completely in BMIMCl after 16 h of heating at 90°C. BMIMCl was used in two types of quaternization reactions of chitosan. In the 1st type, methylation by iodomethane to obtain TMC (*N,N,N*-trimethylammonium chitosan chloride), use of BMIMCl resulted in the DQ of only 40% which was lower than the value obtained from using NMP as the solvent (75%DQ) as found in this work and by others. It was possible that water from aqueous NaOH/NaI caused the chitosan polymer to precipitate in the reaction mixture. In the 2nd type, ring opening reaction by glycidyltrimethylammonium chloride to obtain HTCC (*N*-[(2-hydroxyl-3-trimethylammonium) propyl]chitosan chloride) and its *N*-alkylated derivatives (NB-HTCC and NO-HTCC) use of BMIMCl resulted in %DQ of HTCC, NB-HTCC and NO-HTCC were 87±4, 90±2 and 95±1 respectively when 3 equivalent of GTMAC was used in the reaction. The degree of quaternization of HTCC obtained in this work was quite closed to the product prepared in acidic aqueous solution (acetic acid).

Moreover, reusability of BMIMCl was evaluated. The post-purified reused ionic liquid still contained GTMAC and water as analyzed by ^{1}H-NMR. This suggests that GTMAC and water were not completely separated. This is simply because both molecules having similar polarity and, especially GTMAC, charge characteristic as the BMIMCl ionic liquid. However, the structure of BMIMCl after used in the reaction was also verified and it was found that the structure of BMIMCl remained intact since all of its proton signals (from NMR analysis). The synthesis of HTCC by using re-purified solvent (BMIMCl) was evaluated. After the 1st reuse, the resulting DQ of HTCC decreased gradually. After two recycles, the DQ dropped about 20%. Therefore for the most efficiency, it is recommended that BMIMCl should be re-used only once in the production of HTCC from chitosan.

The antibacterial activities of chitosan and quaternary ammonium chitosan derivatives were reported as the total number of replication competent (viable) cells as mean colony forming units per volume (CFU/mL). The result is suggested that HTCC (87%DQ), NB-HTCC (10%butylated and 90%DQ) and NO-HTCC (10%octylated and 95%DQ) had higher antibacterial activity against *S.aureus* (gram positive bacteria) than the original chitosan. This indicates that the introduction of positive charge effectively improved the antibacterial activity of chitosan. It should be noted here that the antibacterial activities of both alkylated HTCC (butyl and octyl) could not be differentiated.

Future direction

A modification of antibacterial test might be carried out in order to observe the effect of alkyl group on such an activity was evaluated using the minimum inhibitory concentration (MIC).

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APPENDIX

APPENDIX A

Determination of %DS of *N*-butyl Chitosan (NBCS) and *N*-octyl Chitosan (NOCS)

The degree of substitution of butyl groups on NBCS can be determined from the relative ratio between the integration of 2 protons from methylene group (-C$\underline{\text{H}}_2$-) of butyl groups (δ 1.5 ppm) and the peak integration of 1 proton of H-2 of chitosan (δ 3.0 ppm). The relative ratio from amount of butyl group on chitosan can be calculated using the follow equation:

$$\%\text{DS}_{(\text{butyl})} \quad = \quad \frac{\int(-CH_2 - of\ butyl\ group/2)}{\int(H-2\ of\ chitosan/1)} \times 100$$

The degree of substitution of octyl groups on NOCS can be determined from the relative ratio between the integration of 2 protons from methylene group (-C$\underline{\text{H}}_2$-) of octyl groups (δ 1.5 ppm) and 1 proton of H-2 of chitosan (δ 3.0 ppm) by using the follow equation:

$$\%\text{DS}_{(\text{octyl})} \quad = \quad \frac{\int(-CH_2 - of\ octyl\ group/2)}{\int(H-2\ of\ chitosan/1)} \times 100$$

The position of those signals in the NBCS and NOCS spectra is shown in Figure A-1 and %DS$_{(\text{butyl})}$ and %DS$_{(\text{octyl})}$ were determined from ^{1}H-NMR as shown in Table A-1
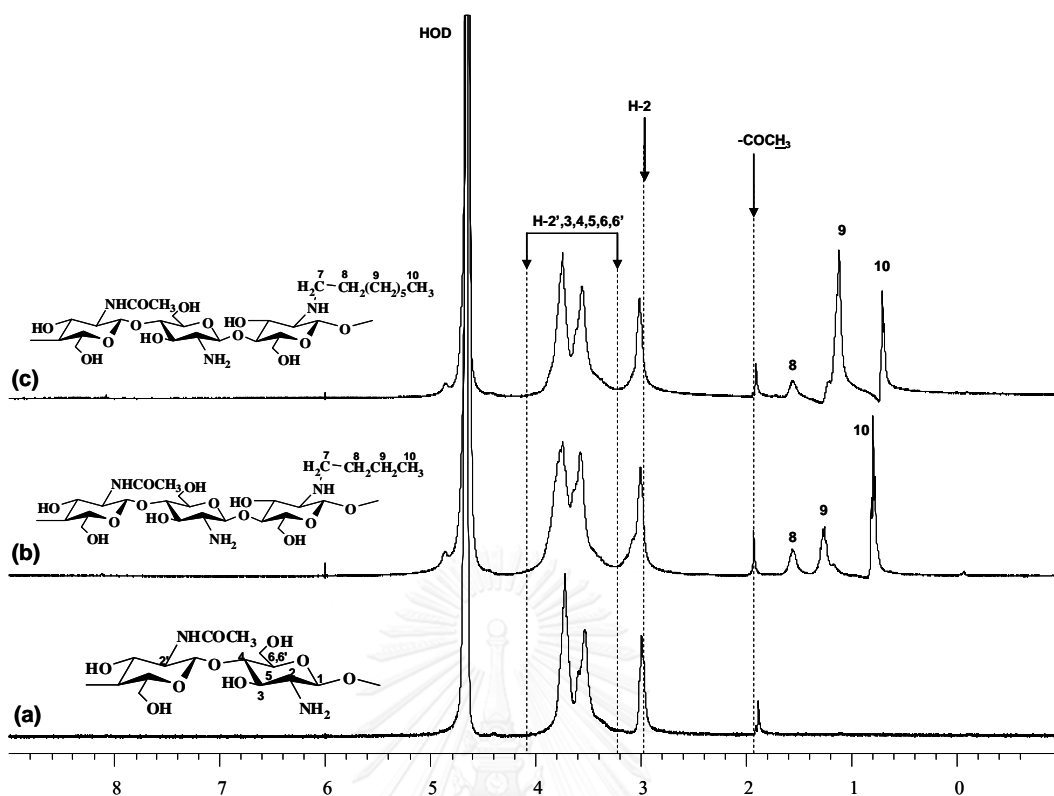
Figure A-1 ^{1}H-NMR spectra of (a) chitosan and (b) *N*-butyl chitosan and (c) *N*-octyl chitosan

Table A-1 Degree of substitution (DS) of butyl and octyl group on chitosan

substrate	Mole proportions NH$_2$:aldehyde	Integration		%DS	%yield	Physical Appearance
		H-2 of chitosan	(-CH$_2$-) @ 1.5 ppm			
NBCS	1:0.2	100	15.3	10	60	White powder
NOCS	1:0.1	100	20	10	70	White powder

APPENDIX B

Evaluation of antibacterial activity

Table B-1 Colony forming units per volume (CFU/mL) of *S.aureus* in suspension incubated with different positive-charged chitosan and chitosan for 24h.

No.	Colony forming units per volume (CFU/mL)		
	Negative Control A	Negative Control B	Negative Control C
1	65×10^5	9×10^5	65×10^5
2	93×10^5	7×10^5	51×10^5
3	48×10^5	21×10^5	52×10^5
No.	Chitosan A	Chitosan B	Chitosan C
1	4×10^5	27×10^5	61×10^5
2	7×10^5	10×10^5	141×10^5
3	5×10^5	20×10^5	65×10^5
No.	HTCC A	HTCC B	HTCC C
1	N/A	N/A	N/A
2	N/A	N/A	N/A
3	N/A	N/A	N/A
No.	NB-HTCC A	NB-HTCC B	NB-HTCC C
1	N/A	N/A	N/A
2	N/A	N/A	N/A
3	N/A	N/A	N/A
No.	NO-HTCC A	NO-HTCC B	NO-HTCC C
1	N/A	N/A	N/A
2	N/A	N/A	N/A
3	N/A	N/A	N/A
No.	Positive Control A	Positive Control B	Positive Control C
1	N/A	N/A	N/A
2	N/A	N/A	N/A
3	N/A	N/A	N/A

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

APPENDIX C

Preparation and characterization of 1-butyl-3-methylimidazolium acetate (BMIMOAc)

The preparation was performed according to a modified method of Duan *et al*. [23]. BMIMCl ethanol solution (mass ratio 100:15) was added into potassium acetate (KOAc) ethanol solution (18%w/w) until the mole ratio of BMIMCl to KOAc was 1:1.05. After being reacted for 6h at 70°C, the reaction solution was cooled down to -10°C and the precipitation of KCl were removed by filtration and the ethanol was removed by vacuum distillation. The product was dried in vacuum at 80°C for 24h before used. ^{1}H-NMR (300MHz: d_6-DMSO; δ in ppm): 0.85 (3H, t, -C$\underline{H}_3$), 1.20 (2H, m, C$\underline{H}_2$), 1.53 (3H, s, C$\underline{H}_3$CO$_2$), 1.73 (2H, m, C$\underline{H}_2$), 3.85 (3H, s, NC$\underline{H}_3$), 4.17 (2H, t, NC$\underline{H}_2$), 7.77 (1H, s, NC$\underline{H}$), 7.85 (1H, s, NC$\underline{H}$), 10.03 (1H, s, NC$\underline{H}$N)
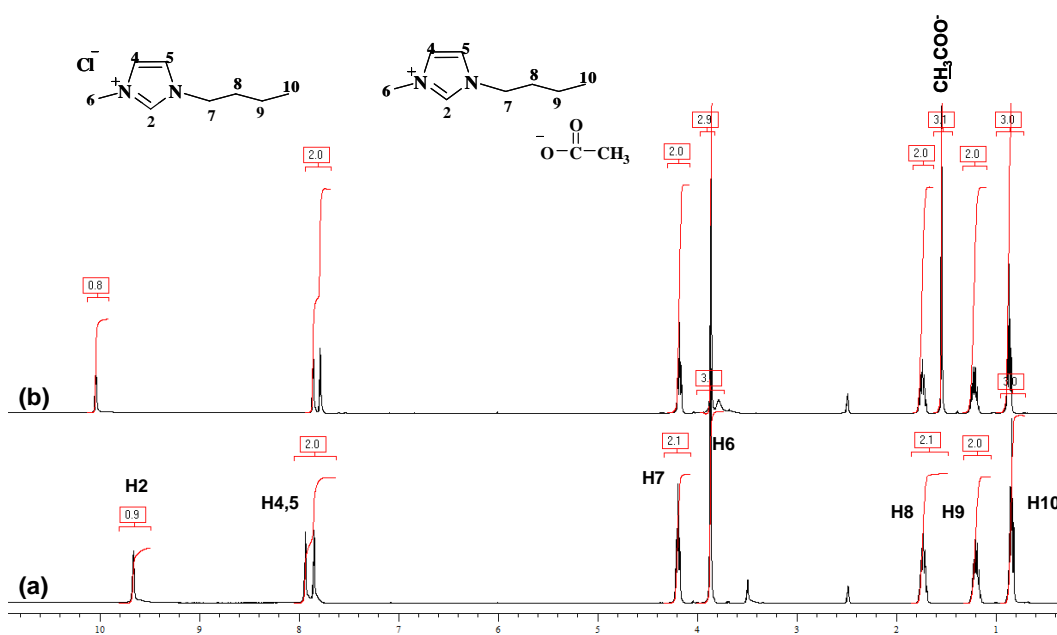

Figure C-1 Preparation of BMIMOAc

Figure C-2 ^{1}H-NMR spectrum of (a) BMIMCl and (b) BMIMOAc in d$_6$-DMSO

VITA

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