อนุภาคนาโนของควอเทอนารีแอมโมเนียมไคตินสำหรับยานาโนต้านแบคทีเรีย

นายณัฐกันตภ์ คุ้มพะเนียด

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Quaternary ammonium chitin nanoparticles for antibacterial nanomedicine

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	Quaternary ammonium chitin nanoparticles for antibacterial nanomedicine
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ณัฐกันตภ์ คุ้มพะเนียด : อนุภาคนาโนของควอเทอนารีแอมโมเนียมไคตินสำหรับยานาโนต้านแบคทีเรีย (Quaternary ammonium chitin nanoparticles for antibacterial nanomedicine) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. วันเพ็ญ เตชะบุญเกียรติ, 65 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาวิธีการเตรียมอนุพันธ์ของไคตินเป็นควอเทอนารีแอมโมเนียมไคตินสำหรับต้านแบคทีเรียสำหรับประยุกต์ด้านชีวการแพทย์ เช่น วัสดุปิดแผล ยาอนุภาคระดับนาโน หรือตัวนำส่งยา เป็นต้น โดย (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมคลอไรด์ ได้ถูกเลือกเพื่อนำมาปรับปรุงสมบัติของไคตินโดยปฏิกิริยาเอซิเลชัน ได้เป็น (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคตินทำให้เกิดประจุบวกขึ้นบนพื้นผิวของไคติน ซึ่งสามารถทำปฏิกิริยากับประจุลบบนเยื่อหุ้มเซลล์ของแบคทีเรียทำให้แบคทีเรียตายลง เพื่อเพิ่มประสิทธิภาพความสามารถในการต้านแบคทีเรียจึงได้ศึกษาอิทธิพลของขนาดและรูปร่างของวัสดุ โดยตั้งสมมุติฐานว่าการเพิ่มพื้นที่ผิวของวัสดุสามารถขยายพื้นที่การเผยประจุบวกให้แสดงบนพื้นผิวได้มากขึ้น และสามารถเพิ่มความสามารถในการต้านแบคทีเรียได้มากขึ้น ดังนั้นจึงเตรียม (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคตินในรูปของอนุภาคระดับนาโนและไฮโดรเจล โดยวิธีการสั่นด้วยคลื่นความถี่สูงและการขึ้นรูปด้วยการแลกเปลี่ยนตัวทำละลาย จากสเปกทรัมฟูเรียร์ทรานฟอร์มอินฟราเรดสเปกโทรสโกปีของควอเทอนารีแอมโมเนียมไคติน พบพีคพันธะเอสเทอร์ที่ 1735 เลื่อนจากคาร์บอกซีลิกเอสเทอร์ของ (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมคลอไรด์ที่ 1726 และจากสเปกทรัมคาร์บอน-นิวเคลียร์แมกเนติกเรโซแนนซ์ (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคติน พบพีคของ CH_2- ที่ 66.85 และพีคของ $+NCH_3$ ที่ 63.85 ชี้ให้เห็นถึงความสำเร็จของปฏิกิริยาเอซิเลชัน ระดับปฏิกิริยาเอซิเลชัน หรือ ระดับการแทนที่ถูกวิเคราะห์ได้ 1.67 จากเทคนิคการกระเจิงแสง ได้รายงานผลขนาดอนุภาคของ (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคตินที่ถูกเตรียมด้วยเทคนิคคลื่นเสียงความถี่สูงที่ประมาณ 284.8±65.42 นาโนเมตร และมีปริมาณประจุบนพื้นผิวอนุภาค 0.61±0.06 (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคตินถูกขึ้นรูปด้วยเทคนิคคลื่นเสียงความถี่สูงเป็นสารละลายอนุภาคนาโนและอนุภาคนาโนบนกระจกกลม และถูกขึ้นรูปด้วยเทคนิคการแลกเปลี่ยนตัวทำละลายเป็นไฮโดรเจล ถูกทดสอบสมบัติต้านแบคทีเรียต่อ *Escherichia Coli* และ *Staphylococcus aureus* วิเคราะห์จาก %Reduction และ antibacterial activity ไม่พบความสามารถในการต้านแบคทีเรียของสารละลายอนุภาคนาโนและไฮโดรเจล แต่อนุภาคนาโนบนกระจกกลมสามารถต้านแบคทีเรียได้ประมาณ 14.62% ในขณะที่ (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคตินในรูปแบบดั้งเดิมก่อนผ่านขั้นตอนการเพิ่มประสิทธิภาพยังคงความสามารถในการต้านแบคทีเรีย ซึ่งอาจเกิดจากการย่อยสลายพันธะเอสเทอร์ด้วยน้ำระหว่างกระบวนการขึ้นรูป และจากการทดสอบความสามารถในการสลายตัวของ (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคตินและไคตินต่อสารละลายเอนไซม์ไลโซไซม์ (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคตินสามารถสลายตัวได้เร็วกว่าไคตินที่ไม่ผ่านการดัดแปร อธิบายจากการทดสอบความเป็นผลึกด้วยเทคนิคเอกซ์เรย์ดิฟแฟรกชัน พบว่าความเป็นผลึกของ (คาร์บอกซีเมทิล) ไตรเมทิลแอมโมเนียมไคตินลดลง จากปริมาณผลึกของไคตินที่ประมาณ 82.94% เหลือเพียง 68.79% ทำให้สลายตัวได้เร็วกว่าไคตินที่ไม่ผ่านการดัดแปร ดังนั้นอนุภาคนาโนของควอเทอนารีแอมโมเนียมไคตินนี้น่าจะสามารถประยุกต์เป็นสารต้านแบคทีเรียทางชีวการแพทย์ได้แต่จำเป็นต้องทำการแปรรูปให้มีขนาดอนุภาคพร้อมใช้งานหลังจากสังเคราะห์ทันที เพื่อรักษาความสามารถในการต้านแบคทีเรียเอาไว้

ภาควิชา	วัสดุศาสตร์	ลายมือชื่อนิสิต	.....
สาขาวิชา	วิทยาศาสตร์พอลิเมอร์ประยุกต์และเทคโนโลยีสิ่งทอ	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2559		

5771971023 : MAJOR APPLIED POLYMER SCIENCE AND TEXTILE TECHNOLOGY
KEYWORDS: CHITIN / QUATERNARY AMMONIUM / ANTIBACTERIAL / CTCMA

NATTHAKUN KUMPANEAD: Quaternary ammonium chitin nanoparticles for antibacterial nanomedicine. ADVISOR: ASST. PROF. WANPEN TACHABOONYAKIAT, Ph.D., 65 pp.

This research was purposed to synthesize quaternary ammonium chitin as an antibacterial agent for biomedical applications such as wound dressing, nanomedicine or drug carrier. (Carboxymethyl)trimethyl ammonium chloride (CMA) was selected to modified onto chitin (CT) via acylation resulted in carboxymethyl trimethyl ammonium chitin (CTCMA). CTCMA possessed positive charges to interact with negatively charged bacterial cell membrane, leading to cell death. In order to study the effect of material size and shape to antibacterial activity with an expectation that an increasing in surface area should enlarge positively charged exposure in a consequence of enhancing antibacterial activity, thus, CTCMA nanoparticles and hydrogels were fabricated in this research via ultra-sonication technique and self-assembly. From FTIR spectra, CTCMA exhibited the characteristic peak of ester linkage at 1735 cm^{-1} shifted from carboxylic ester of CMA at 1726 cm^{-1} and from ^{13}C-NMR spectra, CTCMA exhibited the chemical shift of CH_2- at 66.85 ppm and +NCH_3 at 63.85 ppm, indicating the successful acylation. The degree of acylation or degree of substitution was determined as 1.67. Dynamic light scattering technique showed that particle size and surface charged of CTCMA were about 284.8±65.42 nm and 0.61±0.06, respectively. CTCMA was prepared into colloidal nanoparticles dispersed in distilled water and nanoparticles dried on glass slide. Besides, hydrogel was prepared via solvent exchange method. Antibacterial activity of samples was tested against both *Escherichia coli* (*E. coli*) and *Staphylococcus aureus (S. aureus)* by %reduction under ASTM E2149-10. It was found that CTCMA after fabrication into nanoparticles and hydrogels did not exhibited antibacterial activities. This might due to the hydrolysis of ester linkage during fabrication. Besides, the antibacterial activity of CTCMA nanoparticles dried on glass slide was also evaluated under AATCC100. It was found that CTCMA showed antibacterial activity against *S. aureus* around 14.62%. Degradation of CTCMA and CT hydrogels were investigated against lysozyme. CTCMA showed faster degradability than unmodified chitin. Explained from X-ray diffractogram of CTCMA, it represented crystallinity index about 68.79% which less than CT that represented about 82.94%. Thus, quaternary ammonium chitin could be applied for antibacterial biomedical applications. But, it has to be fabricated into ready to use applications instantly after synthesis for preserving its antibacterial activity.

Department:	Materials Science	Student's Signature	.....
Field of Study:	Applied Polymer Science and Textile Technology	Advisor's Signature	.....
Academic Year:	2016		

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I would like to deepest appreciation to my committee; thanks to Assistant Professor Dr. Sireerat Charuchinda, Associate Professor Onusa Saravari, Associate Professor Dr. Onruthai Pinyakong and Dr. Rawiwan Maniratanachote. And I gratefully thank to members in the Department of Materials Science especially my friends for their cheerfulness, assistance, promoting and suggestion since starting point until the last.

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It would not be possible to be successful without love, sustainable being educated and promotion from my family all over my study.

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Chapter 1
Introduction

1.1 Introduction

Bacteria are normally found living around in the environment. Some of them develop and establish ability to produce toxic through many directions. Thus, they can cause symptoms by invading into body through skin or soft tissue leading to infection [1-4]. On these days, pathogenic bacteria (e.g. *Staphylococcus aureus (S. aureus)*, *Streptococcus pneumoniae (S. pneumoniae)* and *Escherichia coli (E. coli))* have evolved continuously due to various factors such as adaption for living against global warming effect, resistant to antibiotics, etc. Thus, they can be more effective in causing diseases, developed themselves against antibiotics treatment. Antibiotics are always applied within many biomedical applications to eliminate pathogens when body get infection or prevent bacterial infection. However, it does not only damage body's cells, but it also causes pathogens development and resistant to antibiotics treatment[5-9].

Some inventions are developed to overcome those issues, one of our interesting is to use polymer as an active substance to reduce side effects of small molecular antibiotics. Much attention had been paid to chitin (CT), natural polysaccharide extracted from shrimp or carb shell, due to its advantages such as easy process, biocompatibility, non-toxicity and biodegradability[10]. Chitin can be fabricated in many forms for varieties of biomedical applications such as scaffolds for tissue engineering[11], nano-scale drug carriers[12], thermoresponsive hydrogels[13], sponge for wound dressings[14, 15], and so on. From our previous research, chitin was successfully established antibacterial property by adding quaternary ammonium salt through acylation. Carboxymethyl trimethyl ammonium chitin (CTCMA), one kind of quaternary ammonium chitin, was obtained and applied as a polymeric biodegradable antibacterial substance in form of powder[16]. CTCMA showed antibacterial property against pathogens, since positively electrical charges of quaternary ammonium can cause electrostatic interaction with negatively charged bacterial cell membranes which

damaged the cell membranes. Some evidences explicate the antibacterial mechanisms of chitosan that it can damage pathogen's cell wall by electrostatic interaction between positively electrical charges of CTCMA surface and negatively charged cell membranes[17]. Another is binding bacterial cells and stacking them together causing cell's inactivity[18]. Moreover, it can penetrate through pathogen's cell wall and disrupt RNA reproduction in case of very small particles size[19].

Preparation of CTCMA in purpose for biomedical applications was considered. Therefore, CTCMA should be fabricated in various forms such as nanoparticles or hydrogels. However, the processability would influence to exposure surface areas of the positively electrical charges of CTCMA to bacterial cell membranes. In order to evaluate the efficiency of antibacterial property of CTCMA in various forms, the antibacterial activities after fabrication of CTCMA nanoparticles and hydrogels were considered in this research.

With an approach for biomedical applications such as nanomedicine, tissue engineering and wound dressing, the antibacterial polymeric substances were fabricated as nanoparticles and hydrogels. In order to fabricate nanoparticles, nanotechnology would be applied via several methods, solvents exchanging and self-assembly were applied for preparation into various shape of products, several kinds of acids were used to hydrolyze polymer into nanoparticles[20] . Molecular weight and size of polymer were reduced through enzymatic degradation[21] or using mechanical force to disperse polymer in suspension by ultra-sonication[22, 23]. Among various methods, solvent exchanging and ultra-sonication were used to enhance the nanotechnological performance with no damage appeared to the modified functions of the polymers. Also, several methods were used to fabricate hydrogels such as chemical crosslink[24], self-assembly[16], and so on. Among various methods, self-assembly was considered to be a mild condition without damage the modified functional groups.

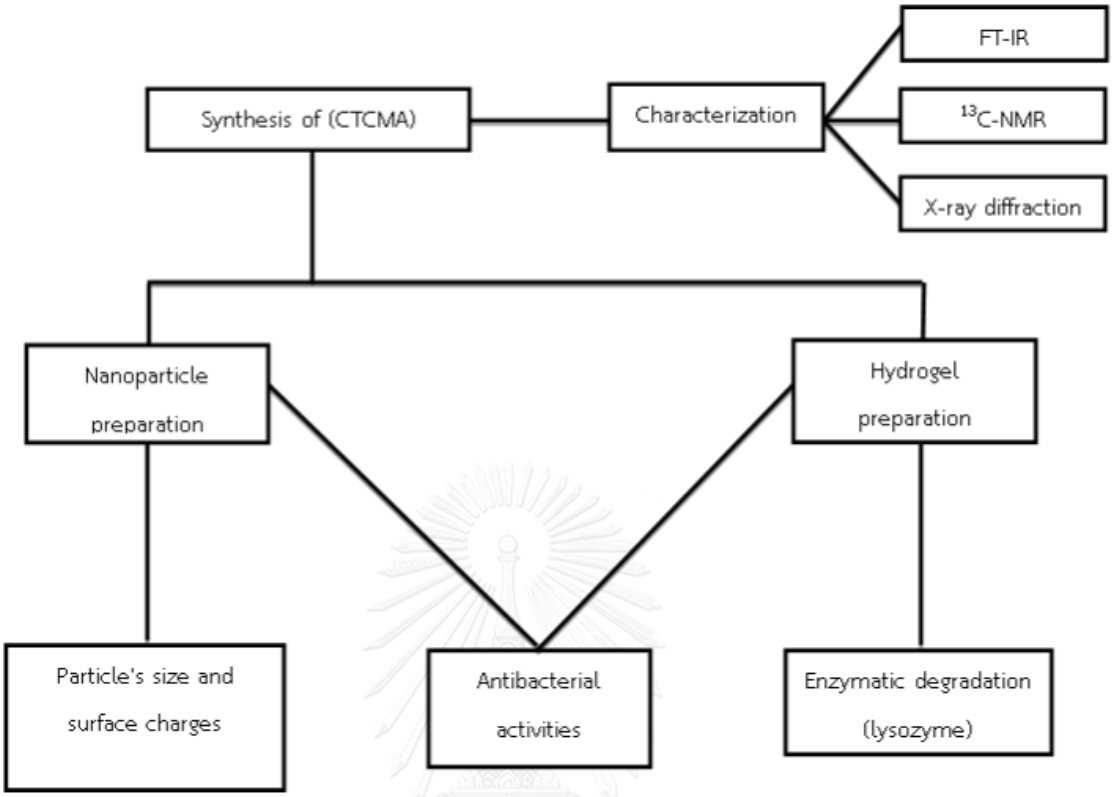
In this research, CTCMA was synthesized through acylation and structural characterized by Fourier Transform Infrared (FT-IR) spectroscopy and carbon nuclear magnetic resonance (^{13}C-NMR) spectroscopy. Crystallinity of CTCMA and CT was measured by X-ray diffraction technique (XRD). CTCMA was redissovled in

dimethylacetamide (DMAC)/lithium chloride (LiCl) to prepare CTCMA solution. For nanoparticle preparation, the solution was diluted to the suitable concentrations, then drop-wised into distilled water to obtain nanoparticle agglomeration. The agglomeration was distributed into individual nanoparticles dispersed in distilled water under probe sonication. For preparation of hydrogels, the CTCMA solution was then casted into various sizes hydrogel by self-assembly. The particle sizes and charges of CTCMA nanoparticles were determined through dynamic light scattering (DLS) technique. Antibacterial properties of CTCMA hydrogel, CTCMA nanoparticles were evaluated comparing to unprocessed powder form against *S. aureus* and *E. coli*. under ASTM E2149-10. Also, the nanoparticles were redispersed in ethanol and imbedded on glass cover slip (15 mm in diameter) and dried under vacuum. The dried nanoparticles on glass cover slip were also tested for antibacterial activity by direct contact test against *S. aureus* and *E. coli*. under AATCC100. In order to approach for biomedical applications, the biodegradability must be concerned. The enzymatic degradation CT and CTCMA hydrogels was performed with different disc sizes in the representative of different forms under physiological condition, since the biodegradation of nanoparticles was not possible to be measured by weight reduction.

1.2 Objectives

- Synthesis of carboxymethyl trimethyl ammonium chitin (CTCMA)
- Evaluation of antibacterial activities of CTCMA nanoparticles and hydrogels
- Enzymatic degradation of CTCMA hydrogels under physiological condition.

1.3 Scope of the works



Chapter 2
Literature reviews

2.1 Bacteria

Bacteria are groups of prokaryotic component single cellular microorganism spreading and can be found in all parts of the world, surface of land, very deep beneath the ground, even in ice of arctic, heating hot springs, or the deepest of the ocean. They were classified in the domain of archaebacteria and eubacteria. The first life on earth over 4 billion years ago seems to be a unicellular organism that lacks a membrane-bound nucleus. The genetic substances (DNA) of prokaryotic creatures preserve in shape of plasmid in cytoplasm emerge with other organelles inside of their cell membrane[25]. This plasmid gives advantages to bacteria such as containing antibacterial resistant genes.

Various shapes of bacteria are acquired from rigidity of their cell wall. Most of them have invariable shape, but some of them are polymorphism. They vary in three major shapes from spherical (Coccus), rods (Bacillus) and helically (Spirillum). Cell wall of bacteria is rigid structure that provides the strength of bacteria cell. Cell wall stabilizes bacterial shape, protects them from outside harm and prevents cell expansion and shrinkage from uptake water due to osmotic pressure from environment.

Bacteria are divided into Gram-negative and Gram-positive bacteria based on their different cell wall compositions. The cell wall of bacteria was developed to protect the cell from internal turgor pressure and consists of insoluble porous, rigid matter such as peptidoglycan and cross–linked polymer which provides voluminous strength including *N*-acetylglucosamine, *N*-acetylmuramic acid, L-alanine, D-alanine, D-glutamatic and a diamino acid (LL or meso diaminopimelic acid, L-lysine, L-orthinine or L-diaminobutryic acid).

For Gram-positive bacteria, their cell walls can be stained with crystal violet dye due to thick layer of peptidoglycan. The dry weight of cell wall constitutes of peptidoglycan up to 50%. Whilst, Gram-negative bacteria consist only 10% of

peptidoglycan. Additionally, there are other substances such as peptidoglycan like polysaccharides in *Streptococcus pyogenes (S. pyogenes),* teichoic acids in *Staphylococcus aureus (S. aureus),* lipids as mycolic acids in *Corynebacterium* and *Mycobacterium.* They are toxic and play a role of causing diseases.

Gram-negative bacteria consist of a thin peptidoglycan layer surrounded by an outer membrane which rich in lipids that make them does not be stained the violet, but stained red or pink colored. The dry weight of cell wall constitutes of lipid of around 11-12%. The outer membrane is an impermeable barrier preventing the escape of important enzymes from the periplasmic space between the cytoplasmic membrane and outer membrane. The outer membrane also prevents external chemicals and enzymes that can damage cells. Lysozyme, which dissolves selectively the peptidoglycan can damage Gram-positive bacteria.

A bilayer outer menbrane consisting many of phospholipids, proteins, and polysaccharides is anchored to the peptidoglycan layer by means of Braun' s lipoprotein. The outer membrane of lipopolysaccharide (LPS) layer has toxic substances and known as endotoxins which are composed of lipid A, core polysaccharide and O antigen. The outer membrane does not allow large molecules such as protein to permeate into cells, but allow small molecules such as monosaccharides peptides and amino acids permeate through channels called porins. Porins span the membrane and are specific for different kinds of small molecules[26].

Bacteria are generally one of main decomposers found in the environment. They assimilate organic matters into soil nutrients and always synergize with other microbial such as woods and carrions[27]. On the other hand, bacteria are used for benefits for many centuries such as food fermentation, enzymes extraction or hormones production. By the way they can be harmful to living creatures as well.

2.1.1 Pathogenic bacteria

Some of bacteria have developed their DNA to establish ability to survive against various inhabit conditions such as terrible environment in hot spring or highly

pressure of the ocean floor, especially the immune system of other creatures which they dwelling called “host”[28].

There are several instrumentalities that were selected by bacteria to use against host’s immune system. Producing toxins from proteinaceous or non-proteinaceous matters that allow the bacteria to evade through immune system, kill host’s cells and cause the diseases. Non- proteinaceous toxins are produced from both of Gram-negative and Gram-positive bacteria, such as lipopolysaccharide (endotoxin) and teichoic acid. Actually, proteinaceous toxins (exotoxins) are the enzymes synthesized by bacteria. Proteinaceous toxins react with specific proteins of host, thus, causing in different symptoms such as botulinum, toxin produced by *Clostridium botulinum* and tetanus from *Clostridium tetani*[29]. The bacteria which cell wall itself contain various toxins which can cause in abnormal operation in circulation system are so-called endotoxin. Unlike endotoxin bacteria, exotoxin bacteria will secret toxin or release toxin during lysis of the cell. In this case, using antibiotics may collapse the cell wall and release more amount of toxins[30, 31]. Form this phenomenon, bacteria were derived into two kinds (Gram-positive and Gram-negative) from components on surface of their cell wall. Capsulation is one of some bacteria retaliation by producing high molecular weight polysaccharide and defecating out to coat itself. This ability protects and disguises bacteria from host’s immune system (for example: neutrophils and macrophages) as well as antibiotics. Capsulation is the one of major virulence determinants since it leads to over phagocytosis while invading of bacteria, which resulted in inflammation. Adhesins is the term of adherence that bacteria use to bind itself to host’s cell before beginning its operations (invasion into host’s cell, toxin secretion or stimulate host’s cell to send signal). Adhesins made of polypeptides or polysaccharides in the shape of hair-like or helical cylinder shape like *Pseudomonas aeruginosa*[32]. After adherence, some pathogens begin to invade host’s cell. Invasion can be derived into two types. One is extracellular invasion. These pathogens stay alive at the surface of host’s tissues. They secrete enzymes into host’s cell to decompose components and absorb for feeding. Other is intracellular invasion, these pathogens penetrate into host’s cell and stay alive inside. They also secrete enzymes

like extracellular invasion and spread to other cells. *S. aureus* is one of species that do the extracellular invasion[33].

2.1.2 Infectious diseases

Bacteria which develop themselves and establish pathogenicity can cause various kinds of infectious diseases. Infection is the establishment of the organisms in the tissues resulting in injury or harmful effect to the host. Infectious diseases are caused by toxins and exudates from bacteria. Some of them can cause one or more diseases and can be spread straight from animals to animals, animals to humans or humans to humans. There are various methods of spreading diseases such as bites form animals or insects, contamination in food and water, transmitting by contacting patients, even exposing to bacteria dwelling in the environment. They also normally found living harmless on skin, digestive system, respiratory system, etc. But, they can cause diseases in some conditions. They start from penetrate through surface of tissue and colonize[1-4].

Infectious diseases caused by bacteria were reported in many researches. *Escherichia coli (E. coli)* is indicated to be the major cause of food poisoning and diarrhea. Moreover, other species including *Cronobacter spp., Salmonella, Shigella*, and so on. are also foodborne pathogens[34]. Surprisingly, there is a first report that *Proteus mirabilis* which normally cause kidney stones can also cause diarrhea[35]. *Helicobacter pylori* (*H. Pylori)* is proved existence in children with gastritis symptom[36]. In asymptomatic gonorrhea men patients, *Neisseria gonorrhoeae* (*N. Gonorrhoeae)* is detected and specified as original cause[37]. Meningitis is one of high risk diseases for new born because of not fully development in immune system. Cerebrospinal fluid was collected from patients and assayed by polymerase chain reaction technique. *Haemophilus influenzae (H. influenzae type b), Streptococcus agalactiae (S. Agalactiae)* and *Neisseria meningitides (N. Meningitides)* are reported as associate in causing meningitis[38]. *S. aureus* is primary cause to various diseases. For examples: toxic shock syndrome, pneumonia, food poisoning and especially wound infection.

Secondary infection which occurs after wound treatment is indicated to be effect by *S. aureus*[5].

To treat and relieve infectious diseases, variety medicines were developed and tested in clinique. From several researches and experiments, various antibiotics were invented and generally applied to eliminate various types of microorganisms.

2.2 Antibiotics

Before antibiotics were found, the first antimicrobial agent "Prontosil" was synthesized by German chemist, Gerhard Domagk. It had activity against Gram-positive bacteria. The active functional group was sulphanilamide. Its derivatives were also synthesized onward. Even though sulphamilamide and its derivatives have plenty of potential against bacteria, their side effects are also great as well. The major breakthrough in the treatment of infectious diseases was development of benzylpenicillin, the first penicillin. It was discovered by Sir Alexander Fleming in 1928[39].

Once antibiotics were established, they should have several required properties to be effective against microorganisms.

- Antibiotics should be soluble in body fluids for sake of diffuse into body's tissues and must not be digestible before exerting its activity.
- In case of orally applied, they must not be inactivated under acid condition of digestive system and still can be absorbed in intestine.
- They should not harm the resident microflora.
- They should not be resisted by target pathogen's development so easily.
- Their side effects should be minimal to patients.
- Long shelf life is preferred for antibiotics.

Antibiotics have the property to interfere target pathogens in some ways with normal or critical functions. Exertions of antibiotics were described in four groups following.

Group one: Inhibit bacterial cell wall

Antibiotics in this group contain functionality to cause growth inhibition or weaken bacterial cell wall. Such as β-lactam which has ability to form covalent bond with active site of bacteria's transpeptidase enzyme. Bacterial cell wall is continued to be produced but in the way much weaker. Due to hypotonic habitats, bacteria were up taken with water through osmotic pressure, swelling and lysis or explode.

According to this group, penicillin, the first β-lactam antibiotic. It has effectively potential against Gram-positive bacteria. But, it is not effective to Gram-negative bacteria because it is unable to penetrate through Gram-negative's bacterial cell wall and it is not stable in acid condition. Afterward, its derivative was developed. For example: ampicillin, this derivative has more advantages, wider specificity, more effective to Gram-negative and be stable under acid condition.

Cephalosporins also have β-lactam like penicillin. But, it has wider specificity and more resistant to β-lactamases. It is used to treat infectious diseases that developed resistant to penicillin such as gonorrhea.

Even though penicillin was developed for decades, it is still being used and developed. Recently, penicillin is used synergism with baicalein against *S. aureus* which produce enzyme penicillinase. It seems to be very effective because baicalein can protects penicillin from penicillinase and can inhibits activity of penicillinase[40].

Group two: Antibiotics that disrupt cell membranes

Antibiotics in this group establish the ability to disrupt phospholipids of bacterial cell membrane in a consequence of leaking of its cytoplasm and death. Polypeptides, polymixins which is produced by species of *Bacillus*, are belonged to this group. It always used with other antibiotics for external apply due to its toxicity. This is tracing of exudate that *E. coli* release when they were treated with polymixin together with gramicidin. Owing to treatment, the more exudate is released, the more strain of bacteria is detected[41].

Group three: Inhibitors of protein synthesis

Antibiotics in this group exert in binding active site of bacterial ribosome preventing from proteins synthesis. Streptomycin, the one belongs to this group, can

bind 30S subunit of bacterial ribosome thus 50S cannot attach to initiate protein complex.

Due to streptomycin unwelcome side effects, antibiotics with similar function were developed instead. For example: Gentamicin was applied and modified to improve its antibacterial activity. Montmorillonite was used as drug carrier and test *in vitro* against *E. coli*. From the research reports, Montmorillonite-gentamicin complex had highest potential in releasing drug at temperature 50° C[42].

Group four: inhibition of nucleic acid synthesis

The characteristic of this group is to inhibit activity of enzyme RNA polymerase, thus mRNA reproducing is inhibited. Model of this group is Rifampin which used to treat mycobacteria that cause tuberculosis because of its ability to penetrate through cell wall. But it has undesirable ability to react with other antibiotics, thereby it decreases or negates effect of other drugs.

Another model of this group is quinolone. Quinolone was modified to increase antibacterial activity by synthesize with metronidazole as a hybrid. Metronidazole-quinolone hybrids seem to have more effectiveness compare to reference drug due to more functional groups to bind active sites of bacteria[43]

. 2.2.1 Undesirable effects of antibiotics

Even though antibiotics have colossal benefaction to all those patients who get infected by bacteria. Not only they terminate bacteria, but also cause side effects to patients. There are several reports in being primary cause of abnormal status in patients.

Various researches were key reported that penicillin can cause penicillin allergy and 5.6% of patients have this symptom. The first-time patients who take penicillin, the immune system considered it as a harmful substance and created antibody against antibiotics. Penicillin allergy can result in hives, rash and itching, thus penicillin can’t be used for treatment to whom shown allergic symptom. In contrast of clinical treatment, penicillin is the most common, low price and useful against various diseases, therefore, penicillin is always selected to use with patients first. On the other

hand, other antibiotics in the same groups of penicillin may also activate this symptom. Thereby patients must be treated with other antibiotics which have higher price[44-46].

Quinolones is the group of antibiotics including Ofloxacin, Ciprofloxacin, Levofloxacin, and so on. Antibiotics of this group are mostly not caused seriously symptoms. For example: nausea, vomiting, insomnia, abdominal pain and headache[47-49].

Moreover, some of antibiotics can harm to host's cell as well as harm to bacteria. A human mammary epithelial cell line (MCF10A) was served as *in vitro* model to investigate the influence of antibiotics (ciprofloxacin, ampicillin, and kanamycin) to mammalian cells. The results shown that antibiotics showed an effect to mammalian cells by inducing oxidative stress when antibiotic was applied for a period of time, furthermore mitochondria also was induced to be dysfunction[50].

2.3 Developing of bacteria against immune system and treatment

After invasion, bacteria itself can develop to survive and duplicate inside the host's cells. Even though, there are several methods that immune system works against bacteria such as phagolysosomal vacuole that eliminate bacterial cells. Bacteria are still continuing to evolve to avoid being terminated by immune mechanism. *Coxiella burnetti* evolves to gain ability to dwell within acidic environment incurred by phagolysosomal vacuole and initiates its duplication when pH gets low. Some of them develop to live inside and combine with non-lysosomal vacuoles such as *Mycobacterium* spp and *Salmonella* spp. Moreover, in case of *Shigella flexneri*, *L monocytogenes* and *Rickettsia rickettsia*, they live in host's cell cytosol, consume surrounding matters and use host's cell cytoskeleton to spread intercellular invasion[51].

Bacteria can develop themselves against antibiotics. Mechanisms to resist antibiotics were classified into three types. The amount of antibiotics required for killing bacteria was fluctuated depending on different types of bacteria. Targeted sites of bacteria bound by antibiotics were changed and developed. And antibiotics were

modified or degraded by enzymes produced by bacteria and then they were inactivated[39]. These overall phenomena were caused by variant genetics. Bacteria can receive antibiotics resistance genes from various opportunities that can be occurred spontaneously. Some of them receive from other organisms, no matter donators are interspecies or intraspecies, from transposons or suspended components, from up taking DNA, form transformation (example: pathogenicity island that contain antibiotics resistance genes) and from conjugation (sexual transfer). For example, *M. tuberculosis* has developed the binding sites of itself to hinder the sensitivity of antibiotics that mean resistance was established[6]. *S. aureus* has developed itself against various kinds of antibiotics especially penicillin, methicillin, oxacillin, azithromycin, ciprofloxacin, tetracyclin and newly found to resist vancomycin[7-9]. Besides, antibiotic resistant ability can be shared between microbes in form of biofilms or microbial communities. From the investigation in healthcare environment, antibiotics resistant genes can be transmitted to other species[5]. Thus, when one specie of microbe develops antibiotics resistance, it can spread this ability overflowing to other species.

This is the major problems for infectious diseases which cannot be effectively treated and world-wide spread. Even there are enormous showing up of alternatives every day, but they are still mutating day by day.

2.4 Alternative antibacterial substances in various biomedical applications

Recently, alternative ways to solve the problems of undesirable effects of antibiotics had been paid much attention. One of them is to use polymers that have antibacterial activities. Polymers with antibacterial activities were used in various applications. For example:

Antibacterial surface coating was developed pervasively and applied to various applications such as medical devices coating[52]. Derivatives of polynorbornene were synthesized and used as an antibacterial surface coating, using positive charges on their structure to cause damages on bacterial' cell membrane[53]. Copolymer coating of 4-vinyl-n-hexylpyridinium bromide and dimethyl(2-methacryloyloxyethyl) phosphonate

can inhibit various of pathogenic bacterial and has minimal effect on soft human tissues[54].

Non-quaternary ammonium also interested to use in biomedical applications, thiazole was modified onto methacrylate monomer, then polymerized to produce antibacterial dental resin to extend lifetime of product which could be degraded by bacteria[55].

Wound dressing also is one of the major biomedical applications of antibacterial substances. Due to its external usage, it has to deal with various and pathogens from environment and has to frequently be changed. Not only it has to be effective to pathogen, it also has to be decomposed and environmental friendly material. Natural polymer and natural compounds always be applied to this application these day. Chitosan, the natural polymer with antibacterial property, it was modified to amplify its antibacterial property. Poly(aminoethyl) was grafted onto hydroxyl groups of chitin and eliminated acetyl afterward. Then, it was casted as hydrogel by changed its solvent into pour solvent. It possessed excellent properties both of antibacterial activity (against *E. coli*, *S. aureus* and *S. epidermidis*) and cytocompatibility[56]. Natural compounds also were applied for wound dressing matrix as bioactive substance against pathogens. Polyvinyl alcohol which embedded with soursop leaves extract was processed into nanofiber via electrospinning technique and used as wound dressing. It showed the inhibition against *S. aureus*[57]. Moreover, high water absorbability polymer, sponge-like materials was applied as wound dressing as well. Chitosan-cyclodextrin sponge like wound dressing was prepared by gelation with physical cross-linked in acid. It showed antibacterial activity against *S. aureus*[14]. In order to enhance their antibacterial activity, antibacterial substance was encapsulated in polymer matrix. Silver sulfadiazine was loaded in chitosan solution and solidified by changing solvent. This sponge like wound dressing could inhibited pathogen by releasing active substance confirmed by appearance of clear zone[15].

It is enormous utilities in field of nanomedicine. Nanomedicine is the nanoscale bioactive material carrier which has more efficiency and accuracy than normal medicine[58]. One example of using polymer in nanomedicine was ofloxacin modified with poly(lactic-co-glycolic acid) and methoxy poly(ethylene glycol)-b-poly(lactic-co-

glycolic acid) using as an antibacterial nanoparticles against various kinds of human pathogens and found in more efficiency compare to ofloxacin free drug[59]. Moreover, 5-FU loaded carboxymethyl chitin nanoparticles were prepared through solution-emulsion mixing using calcium chloride and Ferric chloride as crosslinking agent for usage in treating cancer[60].

However, there is no report about using polymer itself as active antibacterial nanomedicine that can exert without releasing any more active substances as well as antibacterial hydrogel. Through chemical modification, polymer establishes antibacterial property on its structure and can be used as antibacterial agent.

2.5 Chitin-chitosan

Chitin-chitosan copolymer is the second abundant natural polymer extracted from shell of crustacean, thus it is waste from sea food industry. Chitin-chitosan copolymer has similar structure as cellulose with β-(1-4) glycosidic linkage but different only the functional groups at carbon in position two. Chitin, *N*-acetyl-D-glucosamine, has acetamido group as a functional group, whilst chitosan, D-glucosamine, has amine group as a functional group. The chemical structure of chitin-chitosan copolymer was shown in Figure 2.1

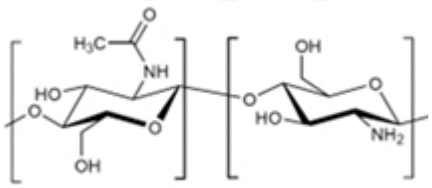


Figure 2. 1 Chitin-chitosan copolymer

Chitin- chitosan copolymer is selected to use in biomedical applications because of its advantages: non-toxicity, biodegradability, biocompatibility to tissues and easy to process into various forms[10] such as tissue engineering scaffold[11] , drug carrier[12], thermoresponsive hydrogel[13] and so on.

From our previous research, chitin- chitosan copolymer was successfully modified to establish antibacterial activity onto its structure. Quaternary ammonium chitin provided antibacterial property because of the positively charges on its structure. According to W. Tachaboonyakiat[16], Carboxymethyltrimethylammonium chloride

(CMA), a member of quaternary ammonium substances, was selected to modified with chitin-chitosan copolymer via acylation using dicyclohexylcarbodiimide (DCC) as a coupling agent. Thus, carboxymethyltrimethyl-ammonium chitin (chitin betainate or CTCMA) was obtained as a product and dicyclohexylurea as a byproduct. Synthesis pathway of CTCMA was shown in Figure 2.2 Antibacterial activity of CTCMA was investigated against *E. coli* and *S. aureus* and found that at minimum concentration of CTCMA powder in bacterial broth (10 mg/mL) can completely inhibited *E. coli* at 10 minutes. For *S. aureus,* inhibition of CTCMA is about 80% after test for 24 hrs.

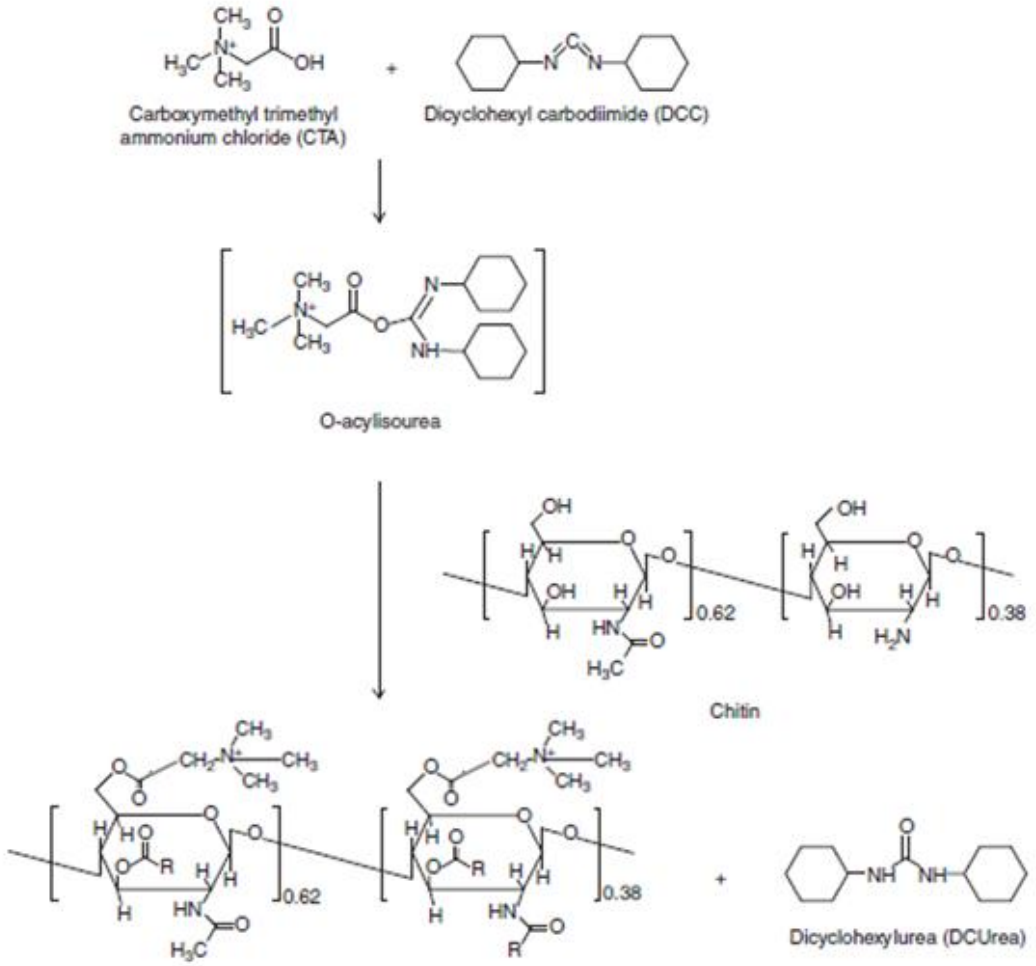


Figure 2. 2 Acylation reaction of chitin betainate or CTCMA

The antibacterial mechanism of chitin/chitosan copolymer was reported as its cationic property could stack with negatively charged cell membrane by electrostatic interaction[17], leading to flocculation of bacterial cells as well as undernourishment causing death rather than directly killing bacteria. Moreover, chitin/chitosan main chain

bind many bacterial cells together that cause bacteria instantly inactivation. Besides, the lower M_w of chitin/ chitosan have more flexibility to bridge bacterial cells together[18]. Furthermore, the reproduction of RNA was blocked when chitin/chitosan penetrated through bacterial cell wall. In this case chitin/chitosan M_w must lower than critical value (~5000 Da), so called chitooligosaccharide[19]. According to the study of Liu *et al.*, the mechanism of antibacterial action was investigated by confocal laser scanning microscopy and found the existing of chitooligosaccharide inside bacterial cell and revealed that chitooligosaccharide passed through the bacterial cell wall[61]

2.6 Optimization of CTCMA antibacterial property

Even though CTCMA has its own antibacterial activity, but it is necessary to be used in high dose usage in powder form. Therefore, the enhancement of antibacterial activities of CTCMA was possible to be developed by change its form or increasing surface area by reducing its particle size or it molecular weight. Thereby improvement of the polymeric antibacterial agents is still needed to reach much effective antibacterial activities.

The processability methods for preparation of chitin for using in biomedical applications were described in two main methods. One is using nanotechnology to produce chitin nanoparticles[60] and another is forming chitin as sponge like matrix and use as wound dressing[14].

In order to optimize antibacterial property of chitin- chitosan copolymer, reducing particle size of chitin- chitosan was considered. Several methods were suggested. Chemical hydrolysis via acid hydrolysis, this method can produce very low M_w of polymer until 1-15 degree of polymerization[20]. Chitin was treated with enzyme chitinase to obtain chitoligosaccharide as the result[21]. Physical method by using mechanical force to disperse polymer into nanoparticles in suspension[22, 23].

Sponge like materials are the one that suitable for biomedical applications providing the increment of surface contact area., good adsorption for absorbing exudate, keeping moisture and covering the wound area protecting the infection [15, 56].

It was our interest to prepare chitin derivative nanoparticles by self-assembly under ultrasonication and sponge-like hydrogel by self-assembly owing to less chemicals, less damage, less toxic and controllable process. Then, self-assembly of CTCMA nanoparticles in aqueous suspension will be prepared under sonication. And CTCMA sponge-like hydrogels will be performed by self-assembly via solvent exchanging with humidity in air.

Chapter 3

Experiments

3.1 Materials

Chitin [degree of acetylation (DA) of 71 which determined by FTIR] was purchased from A.N. Lab (Samutsakorn, Thailand).

Carboxyl methyl trimethyl ammonium chloride (CMA; betaine hydrochloride) was purchased from Fluka (Munich, Germany).

Dicyclohexyl carbodiimide (DCC) was purchased from Sigma- aldrich (Buch,Switzerland).

Dimethylacetamide (DMAc) (AR) was purchased from RCI Lab scan (Samut Sakhon, Thailand)

Lithium chloride (LiCl) was purchased from Ajax (Victoria, Australia)

Potassium chloride was purchased from Ajax (Victoria, Australia)

Potassium di- hydrogen phosphate was purchased from RCI Labscan (Samut Sakhon, Thailand)

Sodium chloride was purchased from RCI Labscan (Samut Sakhon, Thailand)

Disodium phosphate was purchased from RCI Labscan (Samut Sakhon, Thailand)

Lysozyme enzyme from chicken egg white was purchased from Fluka (Steinheim, Germany)

3.2 Methods

3.2.1 Preparation of purified CT

Firstly, 2 g of chitin powder was dissolved in 500 mL DMAc with 5% w/v of LiCl in beaker (1 L) and stirred for 24 hours until completely dissolved. After that, the solution was precipitated in large amount of acetone, washed several times with acetone. The purified chitin was obtained after vacuum drying.

3.2.2 Modification of CTCMA

CTCMA was synthesized according to the method of Tachaboonyakiat *et al.* Carboxymethyl trimethylammonium chloride (CMA) was modified onto CT's chain via acylation. First, 2 g (0.01 equivalent mole to pyranose) of purified CT was dissolved in 150 mL DMAc with 5% w/v of LiCl in beaker (500 mL) and stirred until completely dissolved. CMA (3.07 g, 0.02 mole) was then added into CT solution and further stir until completely dissolved. Then, DCC (8.24 g, 0.04 mole) was dissolved in 120 mL DMAc with 5% w/v of LiCl in beaker (200 mL). Then, DCC solution was poured into the mixture of CT and CMA mixture which was cooled in an ice bath for 30 minutes since the reaction is exothermic. After 30 minutes, the reaction was performed at room temperature for 24 hours. The mixture was centrifuged to separate *N,N*-dicyclohexyl urea (DCUrea). The solution was then kept in refrigerator to increase the sedimentation of DCUrea. The supernatant was precipitated in large amount of acetone, rinsed with distilled water, washed with acetone several times. The product was obtained by filtering and drying under vacuum.

3.2.3 Structural Characterization

Fourier Transform Infrared Spectroscopy (FTIR): The characteristic functional groups of CTCMA was characterized by FTIR. The IR spectra were recorded in attenuated total reflectance mode (ATR) on a Nicolet 6700 Fourier Transform Infrared Spectrometer (Thermo Scientific, Wisconsin, USA. Degree of substitution was calculated from the degree of acylation which was calculated from the integral ratios of FTIR

spectra at wave number 1735 cm^{-1} (ester linkage) and 1650 cm^{-1} (amide linkage) over 1072 cm^{-1} (C-O bond of pyranose ring) as defined in Eq. (1).

$$\%DS(acylation) = \left[\frac{A_{1735}-A_{1650}}{A_{1072}}\right] \quad (1)$$

where A_{1735} is the area under ester peak

A_{1650} is the area under amide peak

A_{1072} is the area under C-O bond of pyranose ring peak

Carbon 13 nuclear magnetic resonance spectroscopy (^{13}C-NMR): The structure of the CT and CTCMA were also characterized by solid state cross-polarization/magic angle spinning (CP/MAS)^{13}C-NMR (400 MHz) using an ASCEND 400WB Bruker spectrometer (Bremen, Germany).^{13}C-NMR spectrometer resonate at frequency of 100 MHz, 3600scans. The contact and delay times were set at 2 ms and 3 s, respectively.

3.2.4 Crystallization characterization and degree of crystallinity

X-ray diffraction technique (XRD): Crystallinity of CT and CTCMA was verified through XRD (Bruker AXS Model D8 with radiation Cu-K_α (λ = 1.54060 Å) at 298 K. The relative intensity was registered in a dispersion range (2θ) of 5-55°.

The crystallinity index (CI) was calculated according to Eq. (2)

$$\%CI = \left[\frac{I_{110}-I_{am}}{I_{110}}\right] \times 100 \quad (2)$$

where; I_{110} is the maximum intensity (arbitrary units) of diffraction (110) at (2θ) = 19°

I_{am} is the intensity of the amorphous diffraction unit at (2θ) = 12.6°

3.2.5 Fabrication of CT and CTCMA into nanoparticles and hydrogels for biomedical applications

1) Colloidal nanoparticles dispersed in distilled water: CTCMA solution with a concentration of 10 mg/mL was prepared by dissolving 0.1 g of CTCMA in 10 mL DMAc with 5% w/v of LiCl in beaker (50 mL). Then, a 1 mL of 10 mg/mL of CTCMA solution was dropped into 9 mL distilled water under sonication by Bandelin SONOREX Digital 10P (Sigma-Aldich, Darmstadt, Germany) and stirred to obtain CTCMA nanoparticle agglomeration (1 mg/mL). The CTCMA nanoparticle agglomeration was dialyzed against distilled water for 3 days, in order to remove residue solvent from precipitants. Distilled water was changed every 3 hours. Finally, CTCMA nanoparticle agglomeration was dispersed into individual nanoparticles by probe sonication (Ultrasonicator probe type (Probe sonicator) [NC/VCX 130, 500, 750 High Intensity Ultrasonic Processor, BECTHAI Bangkok Equipment & Chemical.Co.Ltd] for 15 minutes (30 seconds on and 60 seconds off in each cycle).

CT nanoparticles were prepared as same procedure as CTCMA nanoparticles. However, CT solution was more viscous than CTCMA solution, thus, the concentration of colloidal CT nanoparticles was limited at 0.1 mg/mL by precipitating a 1 mL of 1 mg/mL CT solution (DMAc/LiCl) into 9 mL distilled water under sonication, and dialyzed against distilled water, then probe sonicated to obtain individual colloidal nanoparticles in distilled water.

Note: The nanoparticles can be prepared only at the specific diluted concentration of CT or CTCMA solution (DMAc/LiCl) that dropped into distilled water for solvent exchange to form particle by self-assembly. CT or CTCMA solution (DMAc/LiCl) with a concentration of 1 mg/mL or 10 mg/ mL was the highest concentration used to prepare 0.1 mg/mL or 1 mg/mL CT or CTCMA nanoparticles dispersed in distilled water, respectively. At higher concentration of CT or CTCMA solution (DMAc/LiCl), beads would be formed after dropped in distilled water instead of nanoparticle agglomeration.

On the antibacterial test (ASTM E2149-10), both colloidal CT and CTCMA nanoparticles were centrifuged to remove distilled water and change with the bacterial suspension.

On the antibacterial test (AATCC100), a 200 μL of 1 mg/mL CTCMA colloidal nanoparticles dispersed in distilled water was dropped onto 150 mm in diameter glass cover slip and dried under vacuum to obtain 0.2 mg of CTCMA nanoparticles on each glass slide. Similarly, a 200 μL of 0.1 mg/mL CT colloidal nanoparticles dispersed in distilled water was dropped onto 15 mm in diameter glass cover slip and dried under vacuum, repeated for 10 cycles to obtain total 0.2 mg of CT nanoparticles on each glass slide.

2) Hydrogel: CT and CTCMA sponge-like dried gel was prepared by self-assembly due to solvent exchange with humidity in air. A 1% (w/v) of CT or CTCMA solution was prepared by dissolving 250 mg of CT or CTCMA in 25 mL of DMAc with 5% w/v of LiCl in beaker (50 mL). Then, 1% (w/v) CT or CTCMA solution was casted in petri dish diameter 13 cm and exposed to moisture until hydrogels was formed. The hydrogels were dialyzed in distilled water for 24 hrs, then cut into disk for two diameters (0.4 and 0.8 cm) and freeze-dried to obtain sponge-like dried gel. The diameters were varied in order to study the effect of surface area on antibacterial activity.

3.2.6 Particle size and surface charges

The particle size of CTCMA nanoparticles and its surface charges were determined at room temperature by DLS (NanoZS, Malven Instrument Ltd. , Worcestershire, UK). Fresh prepared suspension of CTCMA nanoparticles (within 24 hrs. after preparation) was diluted with distilled water to the final concentration of 0.01 mg/ml was shaken by vortex before measurement to prevent particle aggregation. The droplet size was reported as the surface mean diameter (d_{32}), as defined in Eq. (3)

$$d_{32} = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \qquad (3)$$

where n_i is the number of droplet with diameter d_i.

3.2.7 Antibacterial activities of CTCMA nanoparticles and sponge-like dried gels

1) Antibacterial activity (ASTM E2149-10) was evaluated against *Escherichia coli* (Gram-negative, ATCC 25922) and *Staphylococcus aureus* (Gram-positive, ATCC 6538). Bacterial were incubated at 30°C for 24 hrs. in 5 mL nutrient broth. Then, bacterial suspension was diluted to concentration of 1x10^6 CFU/mL with phosphate buffer. (Phosphate buffer was prepared by dissolving 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium phosphate and 0.24 potassium dihydrogen phosphate in 800 mL of distilled water at room temperature, adjusted pH to 7.4 with hydrochloric acid and adjusted volume to 1000 mL with additional distilled water) The dispersion of CT or CTCMA nanoparticles in distilled water with concentrations of 0.1 mg/mL and 1 mg/mL, respectively was centrifuged to remove distilled water and replaced with bacterial broth. CT, CTCMA powder and CT, CTCMA sponge-like dried gels (diameter 0.4 and 0.8 mm) were also tested with concentration of 10 mg/mL (sample weight in bacterial suspension). The concentration of 10 mg/mL of sample weight in bacterial suspension was referred from our previous studies [16]. The bacterial suspension containing samples were shaken at 30 °C for 60 min. The remaining bacterial suspensions were spread on trypticase soy agar (TSA) plate and were incubated at 30°C for 24 hrs. The survived bacterial colonies were count and calculated the percentage of reduction as Eq. (4)

$$\%Reduction\ (\mathrm{CFU/mL}) = \left[\frac{\log(number\ of\ bacteria(CFU))_{before} - \log(number\ of\ bacteria(CFU))_{after}}{\log(number\ of\ bacteria(CFU))_{before}}\right] \times 100 \qquad (4)$$

2) Antibacterial activity (AATCC100): Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) were incubated in nutrient broth at 37°C for 18 hrs. Then, bacterial suspension was diluted to concentration of 1-5x10^5 CFU/mL with phosphate buffer. Bacteria were cultured on samples by dropped 10 μL of bacterial suspension on samples (CT and CTCMA dried nanoparticles) that contained in flask and cultured for 60 min at 37°C. After that, neutralizing solution was added into flask and shook for 1

min. *Serial dilutions were made and 100 µL aliquots were plated on TSA plates, and incubated at 37 ℃ for 24 hrs.*

Antibacterial activity was estimated from Eq. (4) as mentioned before.

3.2.8 Evaluation of biodegradability

In order to evaluate the biodegradability of CT and CTCMA, it was prepared as hydrogel disks with various diameters in a representative of various sizes for easily weight measurement. First, a 1%(w/v) of CT or CTCMA solution in DMAc/LiCl was prepared. Briefly, 0.5 g of CT or CTCMA, was dissolved in 50 mL DMAc with 5% w/v of LiCl in beaker (100 mL). Then CT or CTCMA solution was casted in 13 cm in diameter petri dish and formed into hydrogel by self- assembly due to solvent exchange (humidity in air). After hydrogels were formed, placed them in beaker (1 L) contained 1 L of distilled water to remove residue solvent. Distilled water was changed daily for 3 days. CT and CTCMA hydrogels were prepared into various sizes disk shape (0.2, 0.4, 0.6, 0.8 and 1.0 cm) by mold cutting.

Lysozyme solution with a concentration of 1.60 mg/mL with was the lysozyme concentration in tear[62, 63], was prepared freshly every day. Briefly, a 64 mg of lysozyme was dissolved in 40 mL potassium phosphate buffer (pH 6.0) in beaker (100 mL).

Potassium phosphate buffer was prepared as a stock solution by mixing 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium phosphate and 0.24 g of potassium dihydrogen phosphate in volumetric flask contained 800 mL of distill water and stirred to completely dissolved. Then, the volume was adjusted to reach 1 L.

Each of CT and CTCMA hydrogels (wet weight) with different diameters was weighted before experiment. They were immersed in 1 mL lysozyme solution in 37°C chamber for 24 hours. Hydrogels were collected and removed the bound enzyme solution softly with filter paper. Their weights were measured every day while placed hydrogels back into chamber with freshly lysozyme solution at given time and further measuring for 5 days. The degradation rate was measured by calculation of weight loss

of each disk's size against time and plotted to estimate degradation time as shown in Eq. (5).

$$\%Weight\ loss\ = \left[\frac{(Weight\ (before) - Weight\ (after))}{Weight\ (before)}\right] \times 100 \quad (5)$$

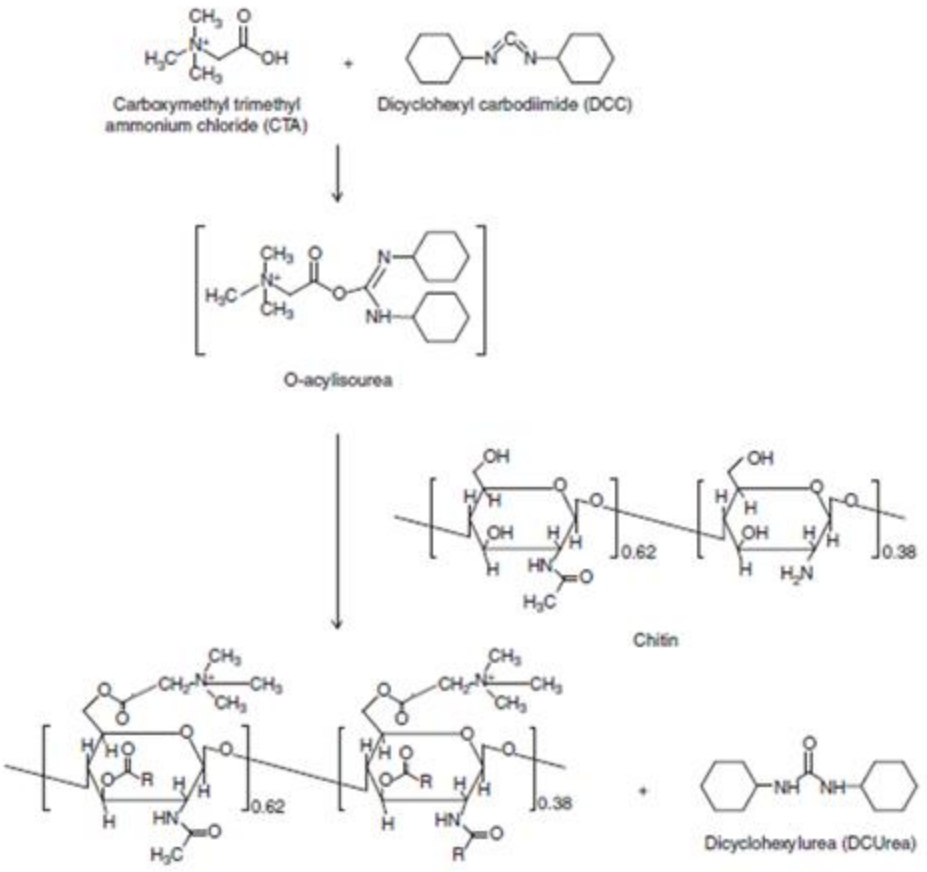
where; $Weight_{(before)}$ = wet weight of hydrogel before immerse in lysozyme solution

$Weight_{(after)}$ = wet weight of hydrogel after immerse in lysozyme solution

Chapter 4
Results and Discussion

4.1 Structural characterization of CTCMA

The synthesis pathway of CTCMA was described in our previous research[16]. Briefly, CMA has carboxylic groups which could be reacted with carbodiimide groups of *N,N'*-dicyclohexylcarbodiimide to form *O*-acylisourea (intermediate) and then react with hydroxyl group and/or amino group of partly deacetylated CT to obtain CTCMA as shown in Scheme4.1.



Scheme 4.1 Synthesis pathway of CTCMA.

FTIR was preliminary investigated for newly formed functional groups. Figure 4.1 shows FTIR spectra of CTCMA comparing with CMA and CT. CT and CTCMA exhibit

characteristic peaks of O-H and N-H stretching at 3500 cm^{-1}, C-H stretching of methyl at 2900 cm^{-1}, C=O stretching, amide I at 1650 cm^{-1}, N-H bending at 1560 cm^{-1} and C-O stretching of pyranose ring at 1072 cm^{-1}. CMA exhibit characteristic peaks of carboxylic ester at 1726 cm^{-1} and C-N stretching at 1196 cm^{-1}. CTCMA exhibits the same characteristic peak of CMA but there is a shifting of carboxylic ester of CMA at 1726 cm^{-1} to ester linkage at 1735 cm^{-1}. This can be inferred that CMA was reacted with CT via acylation. Then, CTCMA was determined for degree of acylation from Eq. (1) with degree of substitution of 1.67.

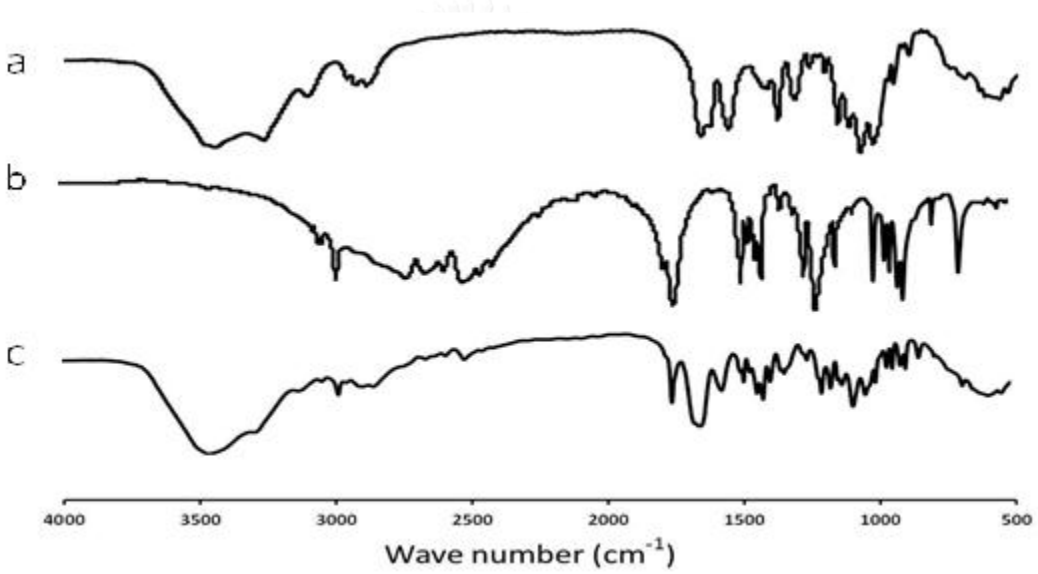


Figure 4. 1 FTIR spectra of (a) CT (b) CMA and (c) CTCMA

$$DS(acylation) = (A_{1735} + A_{1650})/ A_{1072} \quad (1)$$

$$= (4.07 + 5.68)/ 5.84$$

$$= 1.67$$

where A_{1735} is the area under ester peak

A_{1650} is the area under amide peak

A_{1072} is the area under C-O bond of pyranose ring peak

In order to confirm the structure of the synthesized CTCMA, CTCMA was also investigated by ^{13}C-NMR comparing with CT as shown in Figure 4.2. ^{13}C NMR of CT (δ:ppm) shown the characteristic peaks as follows: δ 174.39(HNC=O), 103.61(C-1 pyranose), 82.98(C-4 pyranose), 75.46(C-5 pyranose), 73.18(C-3 pyranose), 60.98(C-6 pyranose), 54.94(C-2 pyranose), 22.60(O=C**C**H$_3$). ^{13}C-NMR of CTCMA (δ:ppm) shown its characteristic following: δ 174.35(HNC=O and –OC=O),103.64(C-1 pyranose), 83.07(C-4 pyranose), 75.54(C-5 pyranose),73.29(C-3 pyranose), 66.85(CH_2, a), 63.94(+NCH_3, b), 61.02(C-6 pyranose), 54.98(C-2 pyranose), 22.53(O=C**C**H$_3$). From the evidence, CTCMA shown the same characteristic peaks as same as CT but also shown additional characteristic peaks of CH_2 (peak a) and +NCH_3 (peak b) that appeared from acylation reaction of CMA and CT. It can be confirmed the successful chemical modification.

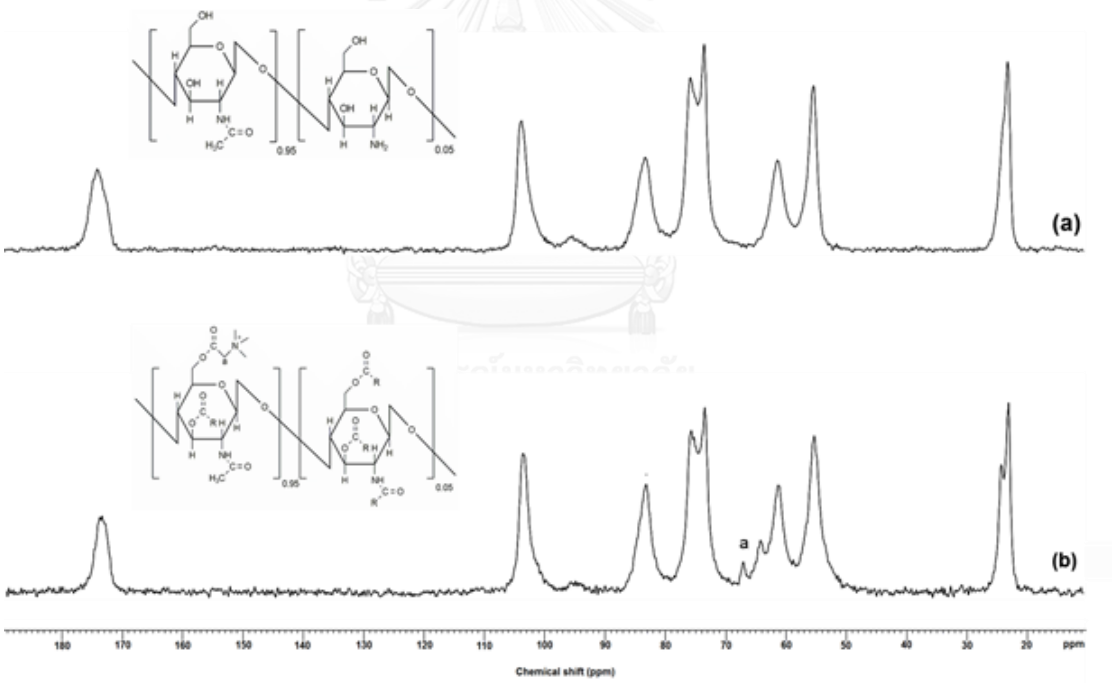


Figure 4. 2 ^{13}C-NMR spectra of (a) CT and (b) CTCMA

4.2 Crystallization characterization and degree of crystallinity

In order to fully understand the results related to crystalline structure, an X-ray technique was experimentally carried out. The X-ray diffractogram of CT (a) and CTCMA (b) is illustrated in Figure 4.3. CT and CTCMA exhibit the intensity of the amorphous diffraction unit at $2\theta = 12.6°$ and the maximum intensity (arbitrary units) of the diffraction at $2\theta = 19°$.

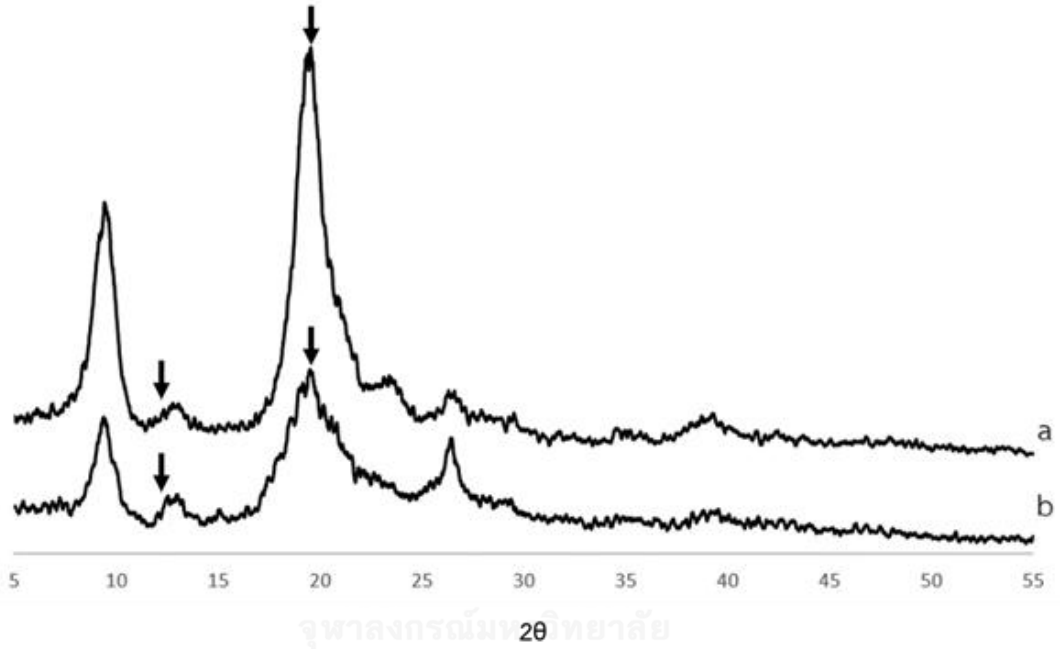


Figure 4. 3 X-ray Diffractograms of CT (a) and CTCMA (b)

Theoretically, the crystallinity index of CT and CTCMA were calculated using Eq. (2) and found to be about 82.94% and 68.79%, respectively. This indicated that CTCMA slightly loss its crystallinity after chemical modification due to steric hindrance of modified functional group (quaternary ammonium group) in a consequence of easily to be dissolved in DMAc/5% (w/v) LiCl and has lower solution viscosity than CT solution.

4.3 Fabrication of CT and CTCMA into nanoparticles and hydrogels for biomedical applications

4.3.1 Colloidal nanoparticles dispersed in distilled water

Colloidal nanoparticles in distilled water were prepared via dispersion by ultrasonication of nanoparticle agglomeration (0.1 mg/mL for CT and 1 mg/mL for CTCMA) in distilled water. Nanoparticle agglomerations were obtained from coagulation of CT and CTCMA solution in distilled water under sonication and stirring. They are white colloidal suspension containing nanoscale particles dispersed in distilled water. They were approached for antibacterial nanomedicine applications.

4.3.2 Hydrogel

Hydrogels were prepared by self-assembly of CT and CTCMA by solvent exchanging with humidity at concentration 1% w/v of CT and CTCMA solutions. After dialysis of hydrogels, they were cut into various sizes for many approached applications such as antibacterial wound dressing, antibacterial scaffold and so on.

4.4 Particle size and surface charges

The particle size and surface charges of CT and CTCMA nanoparticles were determined by DLS (NanoZS, Malven Instrument Ltd. , Worcestershire, UK) and summarized in Table 4.1. The colloidal CT or CTCMA nanoparticle was diluted to the final colloidal concentration of 0.01 mg/mL. The CTCMA nanoparticle' s size was around 284. 8±65. 42 nm much smaller than size of CT nanoparticle (463. 57±20.76). This might because of its morphology. As mentioned in Section 4.2, crystallinity of CTCMA decreased due to steric effect of modified functional groups (quaternary ammonium groups), thus CTCMA main chains were easier to rearrange its molecule by ultrasonic in a consequence of smaller particle size. The surface charge of CTCMA nanoparticles was about 0.61±0. 06 mV, increasing form CT (0.17±0. 10) due to its positively functional groups of modified quaternary ammonium groups ($+NCH_3$).

Table 4. 1 Particles size and surface charge of CT and CTCMA nanoparticles

Samples	Particles size (nm)	Surface Charge (mV)
CT (0.01 mg/mL)	463.57±20.76	0.17±0.10
CTCMA (0.01 mg/mL)	284.8±65.42	0.61±0.06

4.5 Antibacterial activities of CTCMA nanoparticles and sponge-like dried gels

Antibacterial activities were evaluated from two methods according to ASTM E2149-10 and AATCC100. In order to evaluate the effect of shape and size from different fabrication methods to antibacterial activity, the 10 mg/mL of samples was controlled factor. Unfortunately, it was not possible to prepare nanoparticles dispersed in distilled water through self-assembly. Beads were formed instead at higher concentrations than their limit concentrations. Therefore, the CT and CTCMA nanoparticles dropped onto glass slide and additional performed antibacterial test via direct AATCC100.

4.5.1 Evaluation of antibacterial activity (ASTM E2149-10)

The antibacterial activity of CT, CTCMA powder, CT, CTCMA nanoparticle suspension and CT, CTCMA sponge-like dried gel against *E. coli* and *S. aureus* measured from residue colonies of bacterial was shown in Table 4.2 CTCMA powder completely inhibited both of *E. coli* and *S. aureus*. But, after fabrication into various shapes such as nanoparticles and sponge-like dried gel, no antibacterial activities were found against both *E. coli* and *S. aureus*.

Table 4. 2 Antibacterial activity of CT, CTCMA powder, CT, CTCMA nanoparticle suspension and CT, CTCMA sponge-like dried gel against pathogenic *E. coli* and *S. aureus* (ASTM E2149-10) at incubation time of 60 min.

		Microorganisms			
		E. coli		*S. aureus*	
Samples	**Sample forms**	**CFU/mL**	**%Reduction**	**CFU/mL**	**%Reduction**
CT	Inoculum	$1\times10^6\pm0.08$	-	$2.44\times10^6\pm0.08$	-
	powder (10mg/mL)	$2.88\times10^6\pm0.07$	0.00	$1.96\times10^6\pm0.21$	0.00
	sponge-like dried gel 0.8 (10 mg/mL)	$2.18\times10^6\pm0.03$	0.00	$3.18\times10^6\pm0.07$	0.00
	sponge-like dried gel 0.4 (10 mg/mL)	$1.97\times10^6\pm0.21$	0.00	$3.05\times10^6\pm0.04$	0.00
	nanoparticle* (0.1mg/mL)	$2.82\times10^6\pm0.01$	0.00	$2.34\times10^6\pm0.43$	0.00
CTCMA	Inoculum	$1\times10^6\pm0.08$	-	$2.44\times10^6\pm0.08$	-
	powder (10mg/mL)	0.00	100	0.00	100
	sponge-like dried gel 0.8 (10 mg/mL)	$2.24\times10^6\pm0.08$	0.00	$3.19\times10^6\pm0.03$	0.00
	sponge-like dried gel 0.4 (10 mg/mL)	$1.98\times10^6\pm0.22$	0.00	$3.07\times10^6\pm0.02$	0.00
	nanoparticle* (1mg/mL)	$2.75\times10^6\pm0.08$	0.00	$2.28\times10^6\pm0.10$	0.00

*CT or CTCMA colloidal nanoparticles dispersed in distilled water can be prepared with the maximum particle concentrations at 0.1 mg/mL and 1 mg/mL, respectively, due to its high viscosity making it into beads like particles and cannot be processed into nanoparticles via ultrasonication.

Since CMA was modified onto CT through acylation (amide and ester linkages). CTCMA obviously exhibited ester linkage which was very sensitive to hydrolysis. In

order to prepare nanoparticles and sponge-like dried gels, there are several steps of dialysis or washing with distilled water that might hydrolyzed the active modified functional groups of quaternary ammonium groups. Therefore, the ester linkages of dried nanoparticle agglomeration and sponge-like dried gels were reobserved by FTIR as shown in Figure 4.4.

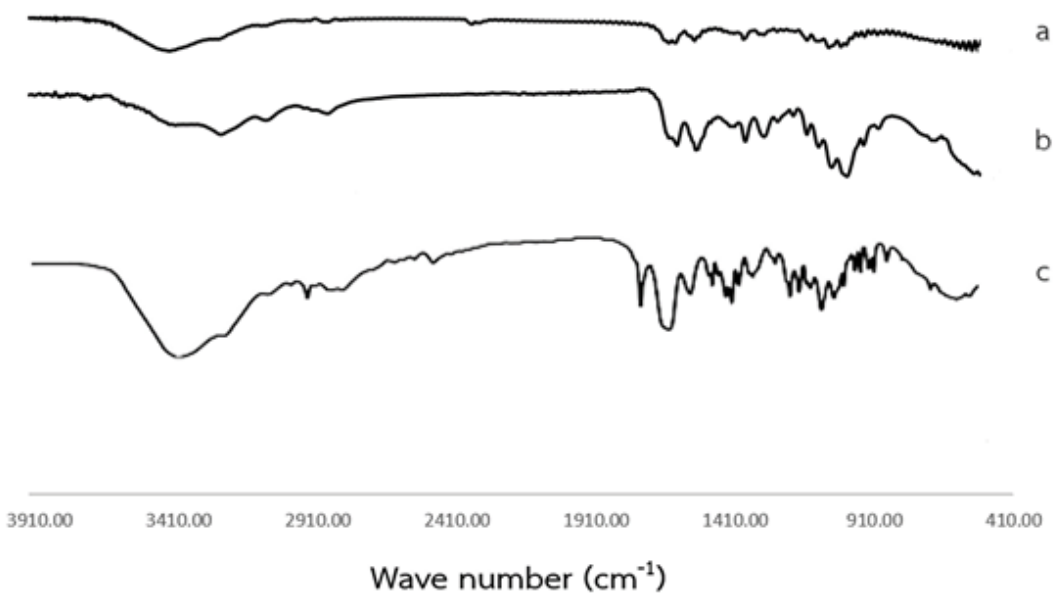


Figure 4. 4 FT-IR of CTCMA nanoparticles (a), CTCMA sponge-like dried gel (b) and CTCMA (c)

With the assumption that CTCMA after fabrication to nanoparticles and sponge-like dried gels might be hydrolyzed leading to exhibit no antibacterial property. Structures of CTCMA after fabrication into nanoparticles (a) and sponge (b) were characterized by FT-IR spectroscopy comparing to the spectrum of CTCMA powder before fabrication. From spectra, they indicated that peak of ester linkage (1735 cm^{-1}) was disappeared from both of CTCMA nanoparticles and sponge-like dried gels. Thus, processing of CTCMA might be one factor that caused ester linkage hydrolysis and influenced to no antibacterial activities.

However, with the assumption that the smaller size of the samples into nanoparticles should increase the surface exposure area to bacterial cell membranes

that should show antibacterial activity as if in low concentration samples. With the limitation of nanoparticle preparation that can be prepared only in diluted condition. Thus, the concentrations of CT (0.1 mg/mL) and CTCMA (1 mg/mL) nanoparticles obtained in this research work might be too low to inhibit bacterial growth by this standard method. Therefore, the CT and CTCMA nanoparticles were coated on 15 mm in diameter glass cover slip with the total weight of nanoparticles on glass slide of 0.2 mg, then tested antibacterial activity by AATCC100.

4.5.2 Evaluation of antibacterial activity (AATCC100)

The antibacterial activity of CT, CTCMA dried nanoparticles against *E. coli* and *S. aureus* was measured from residue colonies of bacteria as summarized in Table 4.3 Both CT and CTCMA dried nanoparticles could not exhibited antibacterial activity against *E. coli*, but exhibited antibacterial against *S. aureus* with % reduction around 15.23 and 14.62, respectively. However, the % reduction of CTCMA nanoparticles was very closed to CT which might because of some active functional groups degraded during fabrication as mentioned before.

In cases of CTCMA nanoparticle suspension and CTCMA dried nanoparticles, CTCMA was processed in water that might hydrolyze the ester linkages between CT and CMA. Furthermore, CTCMA nanoparticle suspension was carried out via ultrasonication that had high intention energy and produced heat and may accelerated hydrolysis of ester linkages.

Another case, CTCMA hydrogels were casted in open air that directly contacted to humidity. Moreover, CTCMA hydrogels were dialyzed in water for 24 hrs. that might cause the hydrolysis of ester linkages of CTCMA.

These fabrication problems have to be solved in our future aspects.

In our previous research, CTCMA showed more sensitive to inhibit *E. coli* than *S. aureus* at the concentration of samples at 10 mg/mL. But CTCMA did not exhibited antibacterial activity against *E. coli* when reduced the concentration below 10 mg/mL. (10 min), but still exhibited antibacterial activity against *S. aureus* (24 hrs.). The result showed in Table 4.3 agreed with the previous results that the concentration of

nanoparticles onto glass slide might be lower than the limit to exhibit antibacterial activity against *E. coli.* Therefore, the increment of nanoparticle weights on glass cover slip should be concerned and the effect of nanoparticle weights and incubation time to antibacterial activity should be investigated in our future aspects.

Table 4. 3 Antibacterial activity of CT, CTCMA dried nanoparticles against *E. coli* and *S. aureus* (AATCC100) at incubation time of 60 min.

	Microorganisms			
	E. coli		***S. aureus***	
Sample forms	**CFU/mL**	**%Reduction**	**CFU/mL**	**%Reduction**
Inoculum	$5.75\times10^5\pm0.02$	-	$2.29\times10^5\pm0.00$	-
CT nanoparticles (0.2 mg)	$4.70\times10^5\pm0.60$	0.00	$2.50\times10^5\pm0.98$	15.23
CTCMA nanoparticles (0.2 mg)	$5.27\times10^5\pm0.30$	0.00	$2.60\times10^5\pm0.01$	14.62

4.6 Evaluation of biodegradability

From observation of biodegradation of CT and CTCMA hydrogel against lysozyme solution, there was significantly reduction of hydrogel's weight and seem to be gradually degraded as increasing time, especially for CTCMA hydrogel. Degradation results of CT were shown in Figure 4.5, CT disk with 0.2 cm in diameter (CT0.2) has highest degradation rate and degraded fastest by time, degradation rate of others decreased while increasing size of hydrogels. CT0.2 has highest % weight loss at 50% after 5 days. Besides, degradation rate of CTCMA was shown in Figure 4.6. CTCMA0.2 also has highest reduction rate and was driven fastest by time. The results showed completely degradation of CTCMA0.2 after 5 days. From comparison with CT hydrogels, CTCMA has degradation rate much higher than CT due to modification of CT via acylation. The additional groups (carboxymethyl trimethyl ammonium) might increase

the gap between crystalline layers of polymer chains because of the steric hindrance group) causing in less crystallinity of CTCMA. Thus, CTCMA can be swelled and degraded by lysozyme enzyme more than CT.

As mentioned in XRD results that CTCMA slightly loss its crystallinity, thus, it was degraded by lysozyme solution faster than CT because enzyme could diffuse through the loosely domains of CTCMA. Then, these parts may be destroyed, exhibiting lower molecular weight.

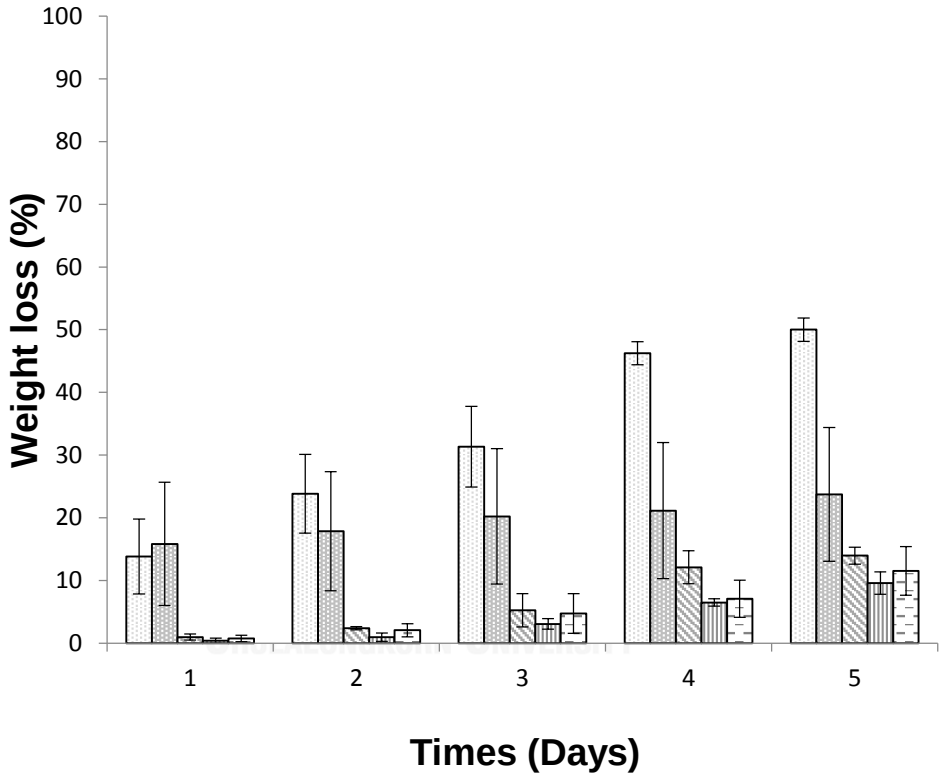


Figure 4. 5 Degradation profile of CT hydrogel by 1.6 mg/ml lysozyme. (CT diameter 0.2 cm, CT diameter 0.4 cm, CT diameter 0.6 cm, CT diameter 0.8 and CT diameter 1.0 cm)

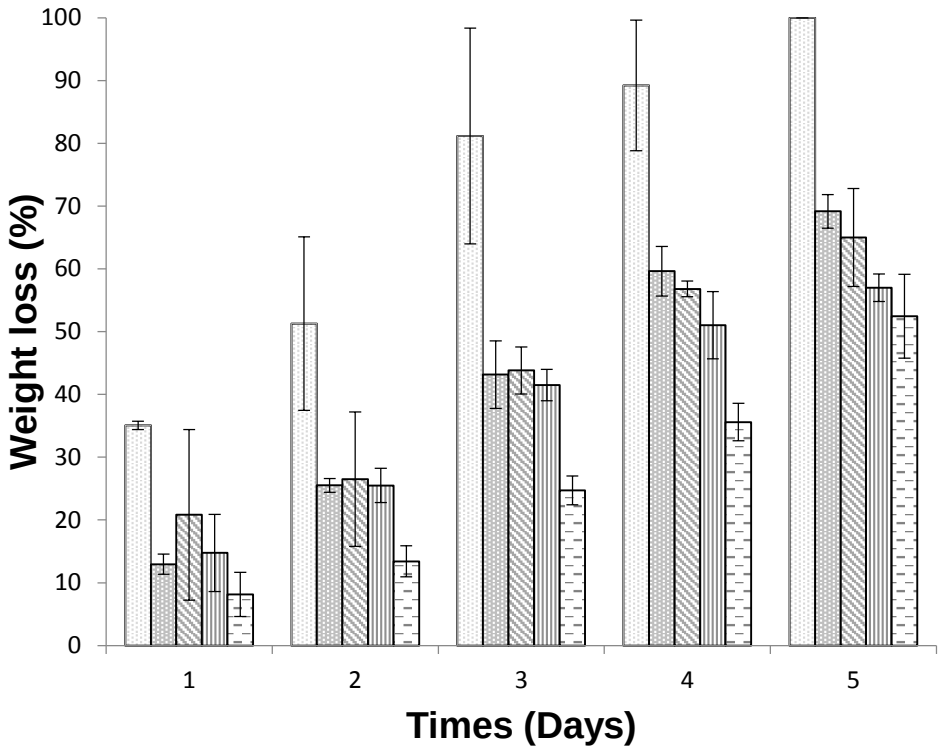


Figure 4. 6 Degradation profile of CTCMA hydrogel by 1.6 mg/ml lysozyme. (CTCMA diameter 0.2 cm, CTCMA diameter 0.4 cm, CTCMA diameter 0.6 cm, CTCMA diameter 0.8 and CTCMA diameter 1.0 cm)

Chapter 5
Conclusions and future aspects

5.1 Conclusions

5.1.1 Caboxymethyl trimethyl ammonium chitin (CTCMA) was successfully developed via acylation using dicyclohexyl carbodiimide (DCC) as coupling agent and it was defined by establishing of characteristic IR spectrum of caboxymethyl trimethyl ammonium group (CMA) in modified chitin (CT) and signal of characteristic ^{13}C-NMR of CMA also founded. Degree of substitution of CTCMA was calculated about 1.67

5.1.2 CTCMA crystallinity was decreased compared to reactant (CT) due to steric hindrance of modified functionality (CMA). The crystallinity index of CT and CTCMA were calculated from X-ray diffractogram and reported about 82.94% and 68.79%, respectively. Thus, CTCMA can be processed into nanoparticle more easily than CT

5.1.3 CTCMA was fabricated into nanoparticle in distilled water approaching for nanomedicine application and hydrogel which approached for sponge-like wound dressing.

5.1.4 Particles size of CTCMA nanoparticles was defined as 248.8±65.42 μm. Thus, surface of CTCMA reveal positive charges about 0.61±0.06. In contrast of degree of substitution, surface charge energy of CTCMA is low because surface charge energy of chitin which is the main structure is closed to 0 mV. So, the modification could increase surface charges to be positive.

5.1.5 In antibacterial activity reports, fabricated CTCMA into nanoparticles and sponge-like dried gel could not exhibit antibacterial activity compared to CTCMA powder. From FT-IR spectra of processed CTCMA. It lost its antibacterial property due to hydrolyzation of ester linkage bond between main chain and quaternary ammonium group during process. Moreover, by the reason of limitation of preparing CTCMA

nanoparticle, CTCMA nanoparticle suspension might lack of efficiency against bacteria due to its low concentration.

5.1.6 CTCMA can be degraded more rapid than chitin against lysozyme solution about 2 times and can be completely degraded within 5 days in the case of smallest size of CTCMA (diameter 0.2 cm). As the X-ray diffraction report, the crystallinity of CTCMA was decreased in a consequence of increase in spacing between main chain structure due to steric hindrance of CMA from modification. CTCMA can be dissolved in DMAc/LiCl faster and its viscosity is lower than CT, leading to easier for enzyme to attach the binding sites.

5.2 Future aspects

5.2.1 Fabrication problems

In this research, there is problem occurred during fabrication. CTCMA was prepared several steps that contacted to water or humidity occurred in hydrolysis of ester linkage and lost its antibacterial property. Firstly, CTCMA was precipitated and washed several times after synthesized before collected in the form of powder. After that, CTCMA powder was redissolved and then fabricated in water several times and contacted to humidity in air both of nanoparticle and hydrogels. Therefore, decreasing the steps that contact to water or accelerate hydrolyzation is selected.

CTCMA has to be performed into nanoparticle suspension or hydrogel suddenly after synthesis and should not contact much to water or humidity.

CTCMA nanoparticles have to be performed instantly after synthesis and eliminate by product. CTCMA solution will be diluted into suitable concentration by DMAc with 5% LiCl. Then, diluted CTCMA solution will be precipitated in water, stir and wash with acetone rapidly to obtain fine particle instead of using probe sonication.

Besides, CTCMA hydrogel preparation, CTCMA solution will be adjusted till concentration 1% (w/v) after synthesis and eliminate by product. Then, it will be casted in petri dish 13 cm., expose to moisture to form gel, immerse in distill water, acetone and dried.

5.2.2 Nanoparticle concentration problems

CTCMA dried nanoparticle has to be prepared more concentration in order to exhibit more efficiency antibacterial property. Fine particle of CTCMA will be dropped on glass slide more cycle to increase amount of CTCMA dried nanoparticle.

5.2.3 Nanoparticle size

CT or CTCMA nanoparticle size will be confirmed by SEM and TEM

5.2.4 Antibacterial Activity

Various forms of CT and CTCMA samples will be determined for antibacterial activity against *E. coli* and *S. aureus* through properly methods. (AATCC100 for sponge-like dried gel, ISO 22196:2011 or JIS Z 2801 for dried nanoparticle on glass slip, ASTM E2149-13a for both sponge-like dried gel and nanoparticle suspension)

5.2.5 Cytotoxicity test

In order to use CTCMA nanoparticle and sponge-like dried gels as antibacterial substances without damaging the soft tissue, the prepared nanoparticle and sponge-like dried gel should be evaluated for their cytotoxicity.

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APPENDIX

Appendix A

FTIR spectra

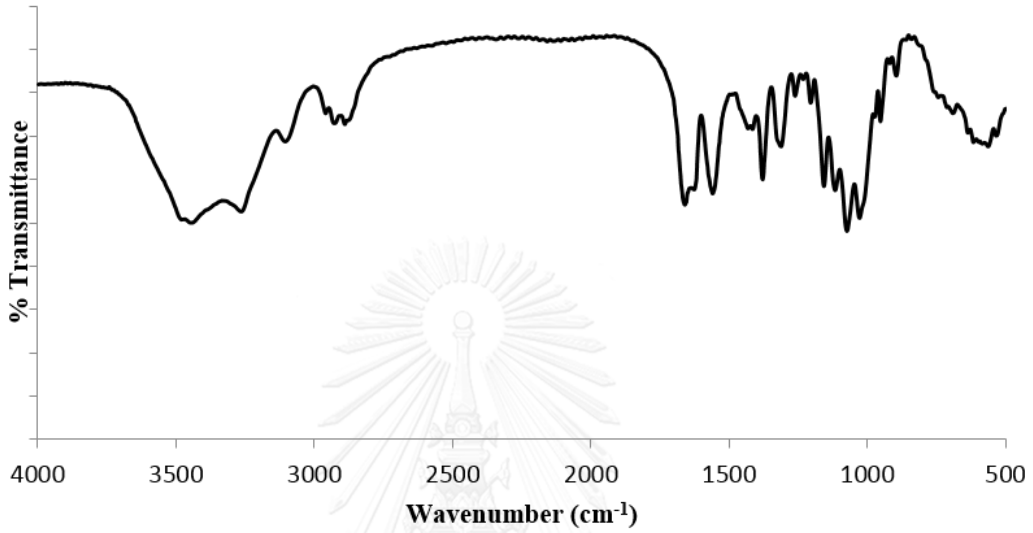


Figure A1 FTIR spectrum of CT

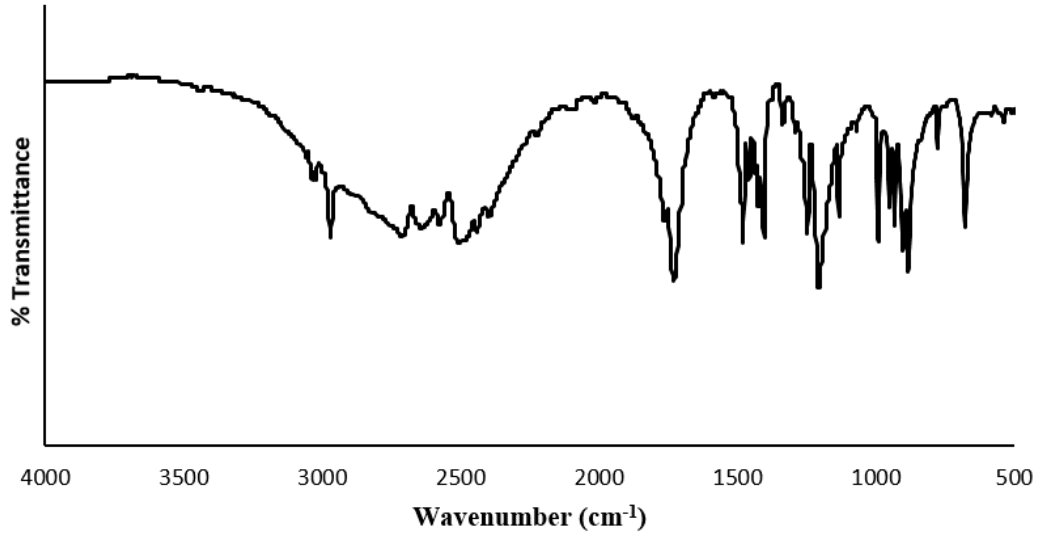


Figure A2 FTIR spectrum of CMA

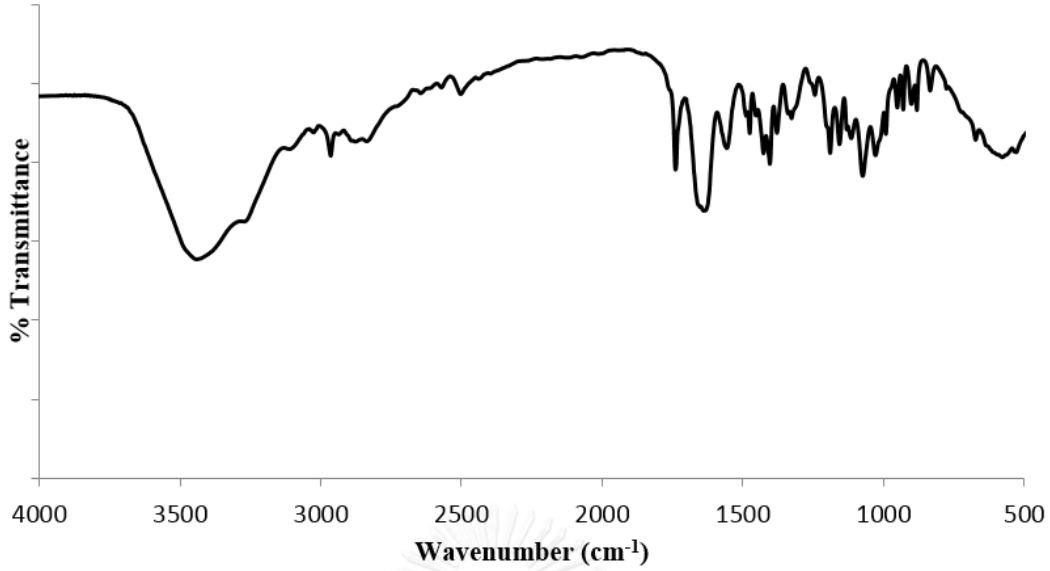


Figure A3 FTIR spectrum of CTCMA

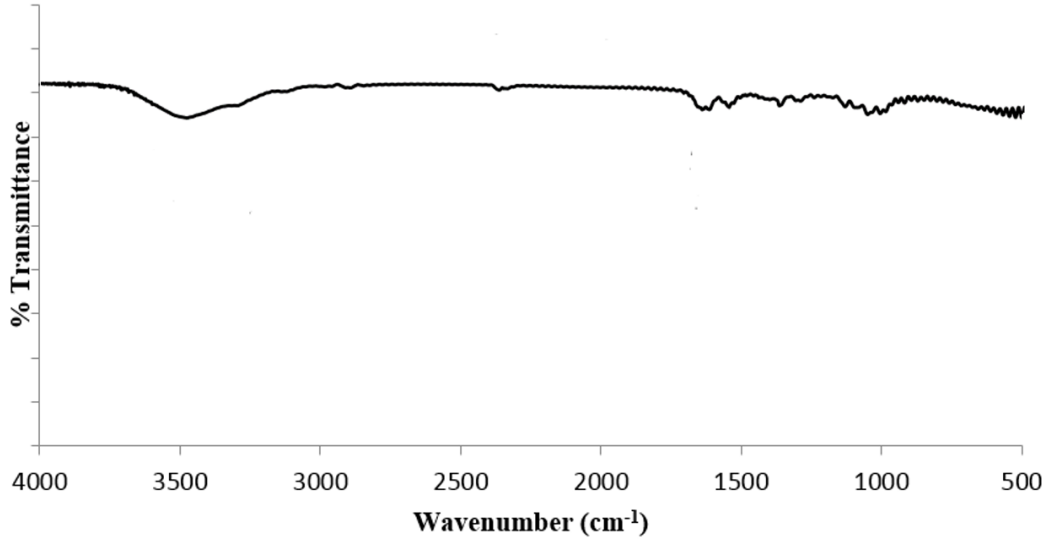


Figure A4 FTIR spectrum of CTCMA nanoparticles

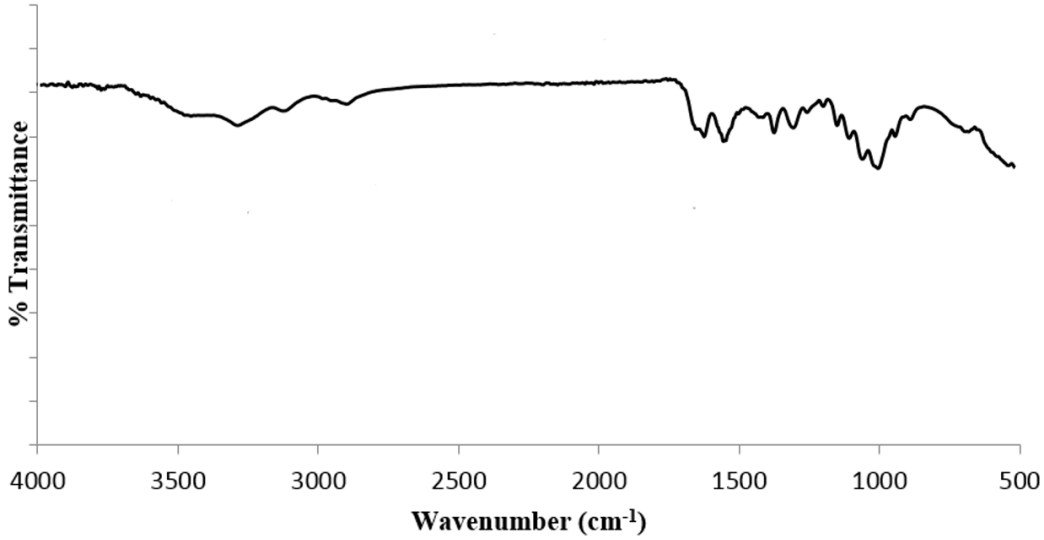


Figure A5 FTIR spectrum of CTCMA dried sponge-like hydrogel

Degree of substitution (DS) was calculated from the degree of acylation which was calculated from the integral ratios of FTIR spectra at wave number 1735 cm^{-1} (ester linkage) and 1650 cm^{-1} (amide linkage) over 1072 cm^{-1} (C-O bond of pyranose ring) as defined in Equation below

$$DS(acylation) = (A_{1735} + A_{1650})/ A_{1072}$$

where A_{1735} is the area under ester peak

A_{1650} is the area under amide peak

A_{1072} is the area under C-O bond of pyranose ring peak

Appendix B

^{13}C-NMR spectra

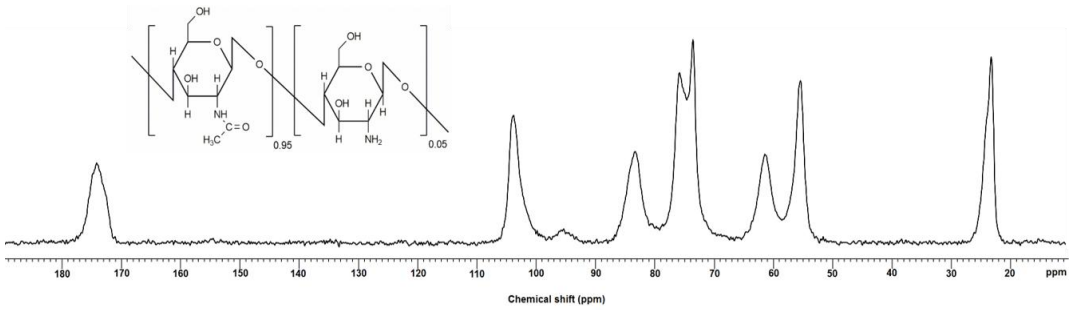


Figure B1 ^{13}C-NMR spectrum of CT

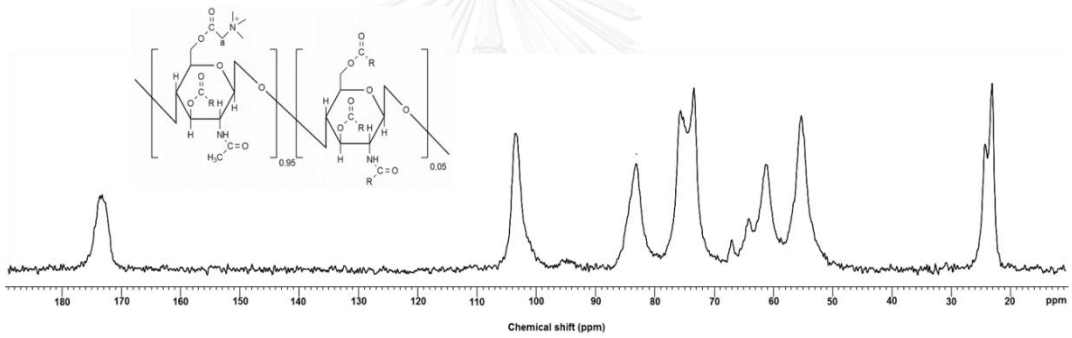


Figure B2 ^{13}C-NMR spectrum of CTCMA

Appendix C

X-ray diffractogram

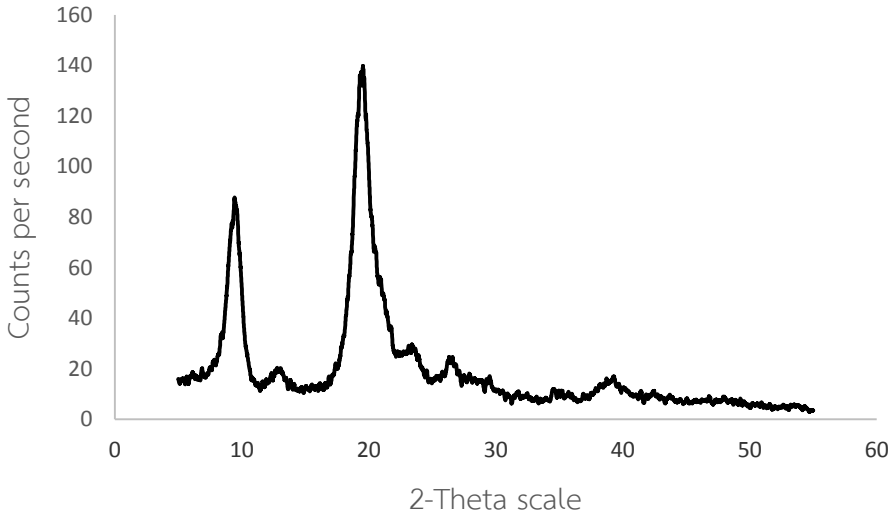


Figure C1 X-ray diffractogram of CT

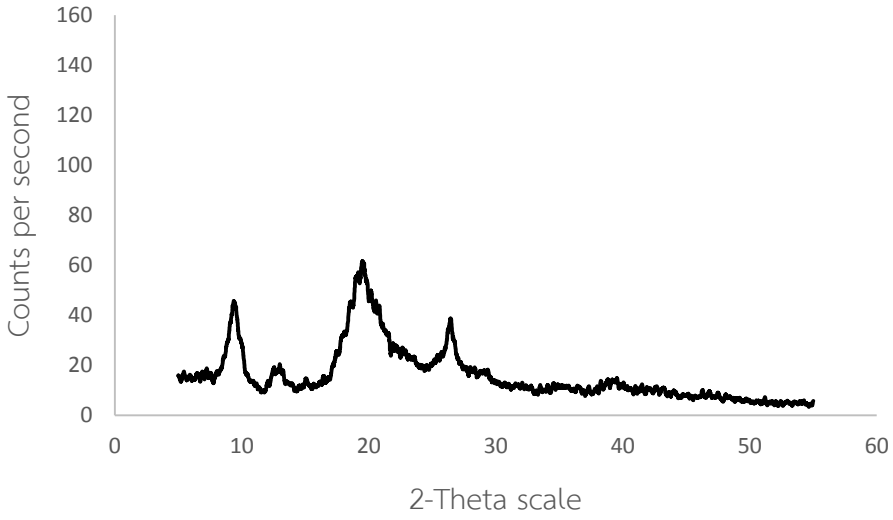


Figure C2 X-ray diffractogram of CTCMA

Appendix D

Particles size of CT and CTCMA

Table D1 Particles size of CT investigated via DLS technique

Samples	Size (nm)
CT_10^{-4}_1	440.4
CT_10^{-4}_2	469.8
CT_10^{-4}_3	480.5
Average ± Standard deviation	463.57 ± 20.76

Table D2 Particles size of CTCMA investigated via DLS technique

Samples	Size (nm)
CTCMA_10^{-4}_1	301.0
CTCMA_10^{-4}_2	340.6
CTCMA_10^{-4}_3	212.8
Average ± Standard deviation	284.8 ± 65.42

Appendix E

Surface charge of CT and CTCMA

Table E1 Surface charge of CT

Samples	Surface charge (mV)
CT_10^{-4}_1	0.09
CT_10^{-4}_2	0.13
CT_10^{-4}_3	0.28
Average ± Standard deviation	0.17±0.10

Table E2 Surface charge of CTCMA

Samples	Surface charge (mV)
CTCMA_10^{-4}_1	0.66
CTCMA_10^{-4}_2	0.55
CTCMA_10^{-4}_3	0.63
Average ± Standard deviation	0.61±0.06

Appendix F

Antibacterial activity of CT or CTCMA

Table F1 Antibacterial activity of CT, CTCMA powder, CT, CTCMA nanoparticle suspension and CT, CTCMA sponge-like dried gel against pathogenic *E. coli* and *S. aureus* (ASTM E2149-10) at incubation time of 60 min.

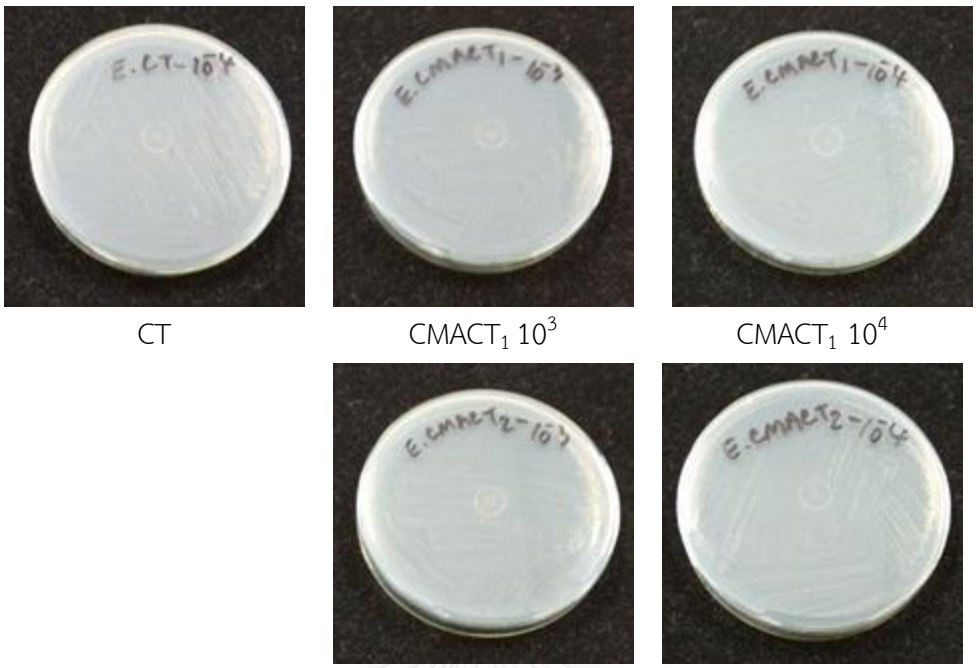
		Microorganisms					
		E. coli			*S. aureus*		
Samples	**Sample forms**		**CFU/mL**	**%Reduction**		**CFU/mL**	**%Reduction**
CT	Inoculum		1.07×10^6 1.00×10^6 1.04×10^6	-		2.48×10^6 2.37×10^6 2.44×10^6	-
		Average	1.04×10^6		Average	2.44×10^6	
		S.D.	0.08		S.D.	0.08	
	powder (10mg/mL)		2.88×10^6 2.88×10^6 2.88×10^6	0.00		1.96×10^6 1.96×10^6 1.96×10^6	0.00
		Average	2.88×10^6		Average	1.96×10^6	
		S.D.	0.07		S.D.	0.21	
	sponge-like dried gel 0.8 (10 mg/mL)		2.15×10^6 2.18×10^6 2.22×10^6	0.00		3.12×10^6 3.17×10^6 3.26×10^6	0.00
		Average	2.18×10^6		Average	3.18×10^6	
		S.D.	0.03		S.D.	0.07	
	sponge-like dried gel 0.4 (10 mg/mL)		1.80×10^6 1.92×10^6 2.20×10^6	0.00		3.01×10^6 3.05×10^6 3.09×10^6	0.00
		Average	1.97×10^6		Average	3.05×10^6	
		S.D.	0.21		S.D.	0.04	
	nanoparticle* (0.1mg/mL)		2.80×10^6 2.82×10^6 2.83×10^6	0.00		1.85×10^6 2.59×10^6 2.59×10^6	0.00
		Average	2.82×10^6		Average	2.34×10^6	
		S.D.	0.01		S.D.	0.43	

CTCMA	Inoculum		1.07×10^6 1.00×10^6 1.04×10^6	-		2.48×10^6 2.37×10^6 2.44×10^6	-
		Average	1.04×10^6		Average	2.44×10^6	
		S.D.	0.08		S.D.	0.08	
	powder (10mg/mL)		0.00 0.00 0.00	100		0.00 0.00 0.00	100
		Average	0.00		Average	0.00	
		S.D.	0		S.D.	0	
	sponge-like dried gel 0.8 (10 mg/mL)		2.14×10^6 2.28×10^6 2.29×10^6	0.00		3.15×10^6 3.20×10^6 3.22×10^6	0.00
		Average	2.24×10^6		Average	3.19×10^6	
		S.D.	0.08		S.D.	0.03	
	sponge-like dried gel 0.4 (10 mg/mL)		1.83×10^6 1.89×10^6 2.23×10^6	0.00		3.06×10^6 3.06×10^6 3.09×10^6	0.00
		Average	1.98×10^6		Average	3.07×10^6	
		S.D.	0.22		S.D.	0.02	
	nanoparticle* (1mg/mL)		2.70×10^6 2.70×10^6 2.85×10^6	0.00		2.22×10^6 2.22×10^6 2.40×10^6	0.00
		Average	2.75×10^6		Average	2.28×10^6	
		S.D.	0.08		S.D.	0.10	

*CT or CTCMA colloidal nanoparticles dispersed in distilled water can be prepared with the maximum particle concentrations at 0.1 mg/mL and 1 mg/mL, respectively, due to its high viscosity making it into beads like particles and cannot be processed into nanoparticles via ultrasonication.

Table F2 Antibacterial activity of CT, CTCMA dried nanoparticles against *E. coli* and *S. aureus* (AATCC100) at incubation time of 60 min.

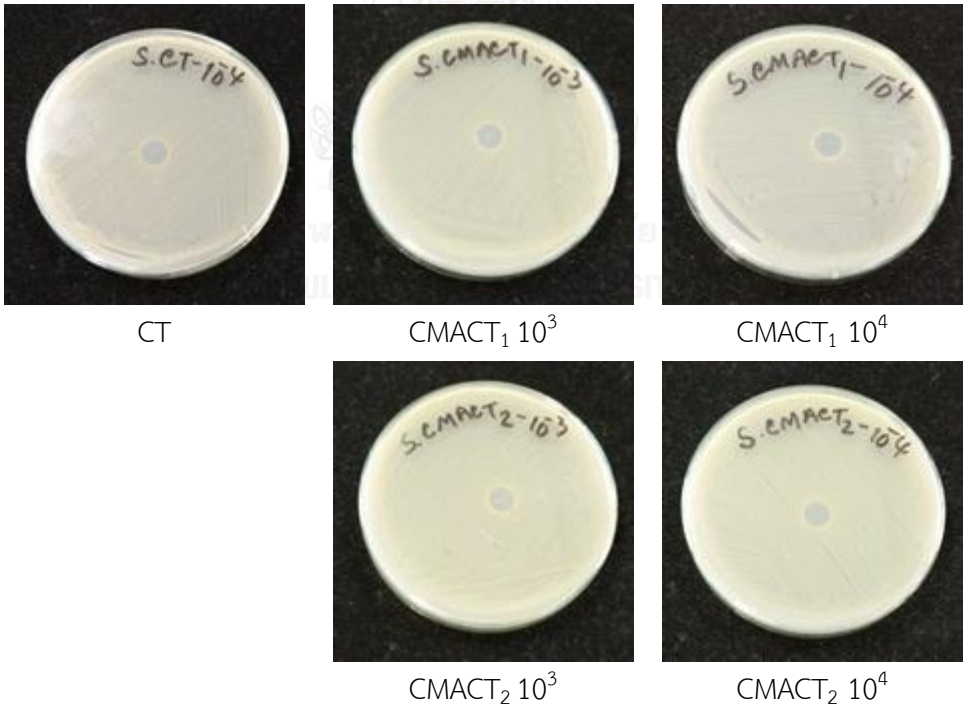
	Microorganisms					
	E. coli			*S. aureus*		
Sample forms		**CFU/mL**	**%Reduction**		**CFU/mL**	**%Reduction**
Inoculum		5.78×10^5 5.72×10^5 5.75×10^5	-		2.29×10^5 2.29×10^5 2.29×10^5	-
	Average	5.75×10^5		Average	2.29×10^5	
	S.D.	0.02		S.D.	0	
CT nanoparticles (0.2 mg)		4.00×10^5 5.00×10^5 5.10×10^5	1.54		1.70×10^5 2.20×10^5 3.60×10^5	15.23
	Average	4.70×10^5		Average	2.50×10^5	
	S.D.	0.6		S.D.	0.98	
CTCMA nanoparticles (0.2 mg)		5.00×10^5 5.20×10^5 5.60×10^5	0.73		2.60×10^5 2.60×10^5 2.60×10^5	14.62
	Average	5.27×10^5		Average	2.60×10^5	
	S.D.	0.3		S.D.	0	



CT $CMACT_1\ 10^3$ $CMACT_1\ 10^4$

$CMACT_2\ 10^3$ $CMACT_2\ 10^4$

Figure F1 Inhibition zone of nanoparticle on filter paper against *Escherichia coli* for 24 h., incubated at 37°C



CT $CMACT_1\ 10^3$ $CMACT_1\ 10^4$

$CMACT_2\ 10^3$ $CMACT_2\ 10^4$

Figure F2 Inhibition zone of nanoparticle on filter paper against on *Staphylococcus aureus* for 24 h., incubated at 37°C

Appendix G

Calculating of weight loss (%) of CT and CTCMA

Weight loss (%) of CT and CTCMA was calculated as follow;

$$\% \text{ Weight loss} = (\text{Weight}_{(before)} - \text{Weight}_{(after)}) / \text{Weight}_{(before)} \times 100$$

Where;

$\text{Weight}_{(before)}$ = weight of hydrogel before immerse in lysozyme solution

$\text{Weight}_{(after)}$ = weight of hydrogel after immerse in lysozyme solution

Table G1 Weight of CT hydrogel diameter 0.2 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CT0.2_1	0.0027	0.0025	0.0022	0.0020	0.0015	0.0014
CT0.2_2	0.0027	0.0023	0.0021	0.0019	0.0014	0.0013
CT0.2_3	0.0026	0.0021	0.0018	0.0016	0.0014	0.0013
Average	0.0027	0.0023	0.0020	0.0018	0.0014	0.0013
SD	0.0001	0.0002	0.0002	0.0002	0.0001	0.0001

Table G2 Weight of CT hydrogel diameter 0.4 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CT0.4_1	0.0186	0.0150	0.0146	0.0146	0.0143	0.0139
CT0.4_2	0.0188	0.0144	0.0141	0.0131	0.0130	0.0125
CT0.4_3	0.0170	0.0162	0.0158	0.0155	0.0154	0.0149
Average	0.0181	0.0152	0.0148	0.0144	0.0142	0.0138
SD	0.0010	0.0009	0.0009	0.0012	0.0012	0.0012

Table G3 Weight of CT hydrogel diameter 0.6 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CT0.6_1	0.0533	0.0526	0.0519	0.0489	0.0458	0.0455
CT0.6_2	0.0517	0.0511	0.0505	0.0496	0.0470	0.0453
CT0.6_3	0.0472	0.0470	0.0462	0.0456	0.0410	0.0402
Average	0.0507	0.0502	0.0495	0.0480	0.0446	0.0437
SD	0.0032	0.0029	0.0030	0.0021	0.0032	0.0030

Table G4 Weight of CT hydrogel diameter 0.8 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CT0.8_1	0.1033	0.1032	0.1028	0.1008	0.0972	0.0925
CT0.8_2	0.1051	0.1049	0.1044	0.1009	0.0983	0.0972
CT0.8_3	0.1048	0.1039	0.1030	0.1019	0.0974	0.0935
Average	0.1044	0.1040	0.1034	0.1012	0.0976	0.0944
SD	0.0010	0.0009	0.0009	0.0006	0.0006	0.0025

Table G5 Weight of CT hydrogel diameter 1.0 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CT1.0_1	0.2080	0.2055	0.2039	0.2030	0.1975	0.1854
CT1.0_2	0.2180	0.2176	0.2157	0.1998	0.1951	0.1838
CT1.0_3	0.2008	0.1991	0.1945	0.1938	0.1894	0.1847
Average	0.2089	0.2074	0.2047	0.1989	0.1940	0.1846
SD	0.0086	0.0094	0.0106	0.0047	0.0042	0.0008

Table G6 Weight of CTCMA hydrogel diameter 0.2 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CTCMA0.2_1	0.0069	0.0045	0.0034	0.0001	0.0000	0.0000
CTCMA0.2_2	0.0053	0.0034	0.0033	0.0019	0.0011	0.0000
CTCMA0.2_3	0.0026	0.0017	0.0009	0.0005	0.0003	0.0000
Average	0.0049	0.0032	0.0025	0.0008	0.0005	0.0000
SD	0.0022	0.0014	0.0014	0.0009	0.0006	0

Table G7 Weight of CTCMA hydrogel diameter 0.4 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CTCMA0.4_1	0.0227	0.0195	0.0171	0.0143	0.0098	0.0065
CTCMA0.4_2	0.0183	0.0158	0.0137	0.0097	0.0077	0.0055
CTCMA0.4_3	0.0198	0.0176	0.0145	0.0108	0.0071	0.0067
Average	0.0203	0.0176	0.0151	0.0116	0.0082	0.0062
SD	0.0022	0.0019	0.0018	0.0024	0.0014	0.0006

Table G8 Weight of CTCMA hydrogel diameter 0.6 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CTCMA0.6_1	0.0743	0.0547	0.0527	0.0436	0.0327	0.0312
CTCMA0.6_2	0.0673	0.0466	0.0433	0.0349	0.0295	0.0245
CTCMA0.6_3	0.0733	0.0694	0.0625	0.0425	0.0306	0.0195
Average	0.0716	0.0569	0.0528	0.0403	0.0309	0.0251
SD	0.0038	0.0116	0.0096	0.0047	0.0016	0.0059

Table G9 Weight of CT hydrogel diameter 0.8 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CTCMA0.8_1	0.1530	0.1376	0.1167	0.0853	0.0662	0.0625
CTCMA0.8_2	0.1460	0.1278	0.1108	0.0865	0.0787	0.0660
CTCMA0.8_3	0.1455	0.1139	0.1038	0.0881	0.0724	0.0625
Average	0.1482	0.1264	0.1104	0.0866	0.0724	0.0637
SD	0.0042	0.0119	0.0065	0.0014	0.0063	0.0020

Table G10 Weight of CTCMA hydrogel diameter 1.0 cm. measure for 5 days

Weight (g)						
Sample/days	0	1	2	3	4	5
CTCMA1.0_1	0.3218	0.2885	0.2694	0.2380	0.2127	0.1285
CTCMA1.0_2	0.2922	0.2802	0.2570	0.2278	0.1932	0.1472
CTCMA1.0_3	0.3047	0.2741	0.2684	0.2254	0.1858	0.1595
Average	0.3062	0.2809	0.2649	0.2304	0.1972	0.1451
SD	0.0149	0.0072	0.0069	0.0067	0.0139	0.0156

For example;

$$\% \text{ Weight loss}_{(\text{day1 CTCMA0.2\_1})} = (\text{Weight}_{(\text{before})} - \text{Weight}_{(\text{after})}) / \text{Weight}_{(\text{before})} \times 100$$

$$= (0.0069 - 0.0045) / 0.0069 \times 100$$

$$= 34.78\ \%$$

VITA

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Publication:

International Proceedings

N. Kumpanead and W. Tachaboonyakiat “Quaternary ammonium chitin nanoparticle approaching for effective antibacterial property” International Polymer Conference of Thailand (PCT-6), Pathumwan Princess Hotel, Bangkok, Thailand, June 30- July 1, 2016. pp. 100-103 (2016).

International Conferences

1 N. Kumpanead, W. Tachaboonyakiat. “Quaternary ammonium chitin nanoparticle approaching for an effective antibacterial activity” The 10th International Symposium in Science and Technology 2015 (ISST 2015), Chaloem Rajakumari 60 Building, Chulalongkorn University, Bangkok, Thialand, Aug 31- Sep 2, 2015.

2. N. Kumpanead and W. Tachaboonyakiat “Quaternary ammonium chitin nanoparticle approaching for effective antibacterial property” International Polymer Conference of Thailand (PCT-6), Pathumwan Princess Hotel, Bangkok, Thailand, June 30- July 1, 2016.

3. N. Kumpanead, W. Tachaboonyakiat “(Carboxymethyl) trimethylammonium chtitin nanoparticle introducing for an effective antibacterial property” 252nd American Chemical Society National Meeting and Exposition, Pennsylvania Convention Center, Philladelphia, Pennsylvania, USA, August 21-25,