N-Alkyl, *N*-Acyl, and Triazolyl Derivatives of Chitosan: Synthesis and Antibacterial Properties

Sankar Rathinam

Thesis for the degree of Philosophiae Doctor

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N-alkýl, *N*-asýl, og triazolýlafleiður kítósans: Efnasmíð og bakteríudrepandi virkni

Sankar Rathinam

Ritgerð til doktorsgráðu

Leiðbeinandi og umsjónakennari

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Ágrip

Kítósan er líffjölliða sem virk gegn örverum og hefur jafnframt marga aðra ákjósanlega eiginleika eins og lífsamræmanleika og lífniðurbrotshæfni jafnframt því að vera óeitruð. Kítósan og afleiður þess hafa mikið verið rannsakaðar sem bakteríudrepandi efni en þrátt fyrir það er þekking á sambandi byggingar og virkni takmörkuð. Í byrjun miðaði doktorsverkefnið að því að skilgreina samband byggingar og virkni fyrir nokkar vel þekktar afleiður kítósans. Í framhaldinu voru nýjar smellefnafræðiaðferðir fyrir smíðið kítosanafleiða og konjúgata þróaðar og notaðar til að smíða nýja gerð fjölliða sem einnig voru rannsakaðar og samband byggingar og virkni ákvarðað.

Fyrsti hluti rannsóknarinnar beindist að því að skilgreina vel samband byggingar og bakteríudrepandi virkni fyrir vel þekktar og mikið notaðar kítósanafleiður og flokka þær eftir notagildi. Katjónískar (TACin, $TMC_{NH2/TM}$, $TMC_{TM/DM}$ og HTCC), anjónísk (CMC) og óhlaðnar (HPC og TGC) afleiður voru smíðaðar út frá kítósan og TBDMS-kítósan byrjunarefnum. Allar þessar afleiður voru efnagreindar með ^{1}H NMR og FT-IR til að ákvarða byggingu og með gelsúluskiljun (GPC) til að ákvarða mólþunga. Kítósanafleiðurnar voru smíðaðar með mismunandi hlutföllum af hvarfefnum þannig að myndefnin voru með setni (DS) frá 0,02 til 1,1. Virkni geng *S. aureus*, *E. coli*, og *P. aeruginosa* var ákörðuð við pH 7,2 og *5,5*. Katjónísku afleiðurnar voru virkastar gegn þessum bakteríum sérstaklega við pH 7,2. Samband mill virkni og setni (DS) var líka jákvætt fyrir TACin, $TMC_{NH2/TM}$, og $TMC_{TM/DM}$. HPC, sem var með óhlaðinn sethóp, var minna virkt og í því tilviki var neikvætt samband milli virkni og setni. CMC, sem var með anjóníska sethóp, var óvirkt gegn bakteríunum.

Núorðið er æ algengara að nota kopar hvataða azíð-alkýn-hringálagningar (CuAAC) smellefnafræði til að smíða ýmiskonar lífefnakonjúgöt, þar með talið kítósan-konjúgöt. Annar hluti doktorsverkefnisins miðaði að því að hanna og smíða nýja gerð kítósanafleiða (kítótríazólan) þar sem öllum C-2 fyrstu gráðu amínóhópunum hefur verið umbreytt í 1,2,3-tríazól hópa. Amínóhópunum í kítósani var fyrst umbreytt í azíð sem voru svo hvörfuð við endastöðu-alkýn í viðurvist Cu (II) hvata og natríum askorbats. Hlutfall umbreytingar azíðs í 1,2,3-tríazól var meira en 90%. Virkni gegn bakteríunum *S. aureus* og *E. coli* var mæld við pH 7.2. Tvö katjónísk kítótríazólan efni reyndust talsvert virk (lægsta MIC = 64 µg/mL) en anjónísk kítotríazólan efni voru ekki virk gegn bakteríum.

Þessi smellefnafræðiaðferð var líka notuð til að umbreyta fyrstu gráðu amínóhópum í hlutsetnum algengum kítósanafleiðum (TMC, TAC, HTC, HPC, and CMC) í tríazól. Virkni þessara blönduðu kítótríazólanefna gegn *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* við pH 7,2 var einnig mæld. Katjónísk kítótríazólanefnin og blönduð kítótríazólanefnin voru virk gegn bakteríunum, öll nema blönduð kítótríazólan afleidd af CMC, sem skorti virkni. CuAAC hvarfið var einnig notað til að smíða níu vatnsleysanleg kítótríazólanefni með mismunandi katjóníska eða basíska hópa. Metýlimidazól-któtrízólan afleiðan var virkust (lægsta MIC = 256 μg/mL) gegn bakteríunum.

Lokahluti rannsóknarinar hafði það markmið að nota smellefnafræðiaðferðina til að tengja örverudrepandi peptíð við kítósan. CRAMP-18 peptíð sem innhélt endastæðan alkýn hóp var smíðað. Kítósan-azíð og HPC-azíð voru smíðuð með lága setni azíðhópa, og smellefnafræðiaðferðin notuð til að tengja CRAMP-18 peptíðið við fjölliðuna. Þessi konjúgöt voru mun virkari geng gram-neikvæðum bakteríum en gegn gram-jákvæðum bakteríum.

Lykilorð:

Kítósan, trímetýlkítósan, smellefnafræði, samband byggingar og virkni, örverudrepandi virkni.

Abstract

Chitosan is a biopolymer with significant antimicrobial activity and many attractive properties such as biocompatibility, biodegradability, and non-toxicity. Chitosan and its derivatives have been widely studied as promising new antibacterial agents, but the relationship between structure and activity is still poorly understood. The Ph.D. project first sought to map the structure-antibacterial activity relationship for common and well-known chitosan derivatives. New "click chemistry" based procedures for the synthesis of derivatives and conjugates were then developed, and these novel polymeric compounds were used for further structure-activity relationship (SAR) studies.

The first part of the research focused on establishing the SAR for some of the more widely used chitosan derivatives and trying to rank them according to activity and utility. Cationic trimethylated (TACin, $TMC_{NH2/TM}$, $TMC_{TM/DM,}$ and HTCC), anionic (CMC) neutral (HPC), and TGC chitosans were synthesized using chitosan and TBDMS chitosan as precursors. All these derivatives are characterized by ^{1}H NMR, FT-IR to determine the structure, and gel permeation chromatography (GPC) to determine the molecular weight. The chitosan derivatives were synthesized with different reagent ratios to give products with degree substitution (DS) ranging from 0.02 to 1.1. Most of these derivatives displayed antimicrobial activity against *S. aureus*, *E. coli,* and *P. aeruginosa* at pH 7.2 and 5.5. Cationic derivatives were most active against these bacteria, especially at pH 7.2. The relationship with DS was also positive for TACin, $TMC_{NH2/TM}$, and $TMC_{TM/DM}$. HPC, which has a neutral substituent, was less active and had a negative relationship with DS. CMC, which has an anionic substituent, was inactive against the bacteria.

Nowadays, it is increasingly common to use copper-catalyzed azide-alkyne cycloaddition (CuAAC) "click chemistry" to prepare bioconjugates, including chitosan derivatives and conjugates. The second part of the thesis work focused on designing and synthesizing a new class of chitosan derivatives where all C-2 primary amino groups have been converted to aromatic 1,2,3-triazoles (Chitotriazolan). The chitosan amines were converted to azides and reacted with terminal alkynes in the presence of Cu (II) catalyst and sodium ascorbate. The conversion of the azide to 1,2,3-triazole was more than 90%. The antibacterial activity was evaluated against *S. aureus* and *E. coli* at pH 7.2. Two cationic

chitotriazolans exhibited (lowest MIC = 64 µg/mL) antibacterial activity, whereas the anionic chitotriazolans were inactive.

The click chemistry strategies were used to convert primary amino groups of partially substituted common chitosan derivatives (TMC, TAC, HTC, HPC, and CMC) to triazole and thus obtain mixed chitotriazolans. The antibacterial activity was evaluated against *S. aureus, E. faecalis, E. coli, and P. aeruginosa* at pH 7.2. The cationic chitotriazolans and mixed chitoriazolans were active against bacteria, except chitotriazolan derived from CMC, which lacked activity. In addition to preparing water-soluble chitosan derivatives, the CuAAC reaction was further used to synthesize nine chitotriazolans with various quaternary and basic protonable functional groups. These chitotriazolan derivatives were soluble in water. The methylimidazole-chitotriazolan derivative showed significant activity (lowest MIC = 256 µg/mL) against all bacteria and was generally the most active derivative.

The final part of the research for the thesis focused on the conjugation of antimicrobial peptides onto the chitosan backbone using the click chemistry procedure. The CRAMP-18 peptide was synthesized with a terminal alkyne group. Chitosan azide and HPC azide were prepared with a low degree of azidation, and a click reaction was performed with the modified CRAMP-18 peptide. The antimicrobial peptide chitosan conjugates were more active against gram-negative bacteria*, E. coli, and P. aeruginosa* than gram-positive bacteria.

Keywords:

Chitosan; Trimethyl chitosan; Click reaction; Structure-activity relationship; Antimicrobial activity.

Acknowledgments

First and foremost, I would like to express my heartfelt and sincere gratitude to my research supervisor Prof. Már Másson who gave me a golden opportunity to work in this exciting world of chitosan chemistry at the Faculty of Pharmaceutical Sciences, University of Iceland. His motivation, inspiration, immense knowledge, persistent guidance, and ample experience have encouraged and guided me in my academic research and daily life. It has helped me realize my dream reality. I am thankful for his patience, timely advice, and continuous support during every stage of my research work.

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I want to express my deepest thanks to my doctoral committee members Associate Prof. Berglind Eva Benediktsdóttir, Prof. Martha Ásdís Hjálmarsdóttir, Prof. Sigríður Guðrún Suman, and Associate Prof. Mikkel Boas Thygesen for their valuable guidance, insightful comments, and suggestions during my doctoral meetings. My sincere special thanks to Prof. Martha Ásdís Hjálmarsdóttir, who assisted and guided in the antimicrobial analysis.

I wish to thank our research collaborator from the University of Copenhagen sincerely. Associate Prof. Mikkel Boas Thygesen allowed me to work in his lab for a couple of months during my doctoral studies. He taught me peptide synthesis, and he provided me with constant encouragement, guidance, and support for my project. I would like to thank Kasper K. Sørensen for his support in peptide synthesis and their lab-mates during my stay in Denmark.

Special thanks to my research groupmates Vivien, Edmundo, Priyanka, Sveta, Vivek, Sigga, Romano, and Hagi friends, Maonian, Ellen, May, Sebastian, Manisha, Suppakan, Mostafa, André, Margarida, Xiaxia. We participated in

many social activities organized by the Pharmafun club in Hagi. These made the research even more enjoyable. I would like to express my gratitude to all Faculty members in Hagi Bergþóra, Berglind, Elvar, Páll, Helga, Natalia, Hákon, Lárus, Elín, Már, Sveinbjörn, Þorsteinn, and Margarét for their support and being friendly. Thanks for the technical and administrative support to Árni, Ingunna, Solla, Guðrún, Heba, and Sigrún.

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Dedicated to

My Family and My Daughter (Vizhi & Venbaa)

Contents

List of abbreviations

AMPs: Antimicrobial peptides
ATCC: American Type Culture Collection
Boc: Di-tert-butyl decarbonate
CFU: Colony-forming units
CLSI: Clinical and Laboratory Standards Institute
CMC: Carboxymethyl chitosan
CMC-CTr: CMC-Chitotriazolan
Conc. HCl: Concentrated hydro chloric acid
CRAMP: Cathelin-Related Anti-Microbial Peptide
CS: Chitosan
Cs_2CO_3: Cesium carbonate
CuAAC: Copper (I) catalyzed azide-alkyne cycloaddition reaction
$CuSO_4$ $5H_2O$: Copper (II) sulfate penta hydrate
DA: Degree of acetylation
DCM: Dichloromethane
DD: Degree of deacetylation
DIPEA: *N,N*-diisopropylethylamine
DMC: *N,N*-dimethyl chitosan
DMF: *N,N*-Dimethylformamide
DMSO: Dimethyl sulfoxide
DS: Degree of substitution
DTNB: 5,5′-dithiobis- (2-nitrobenzoic acid)
E. coli: *Escherichia coli*
EDC:1-Ethyl-3-(3-dimethylamino propyl) carbodiimide
E. faecalis: *Enterococcus faecalis*
Et_3N: Triethylamine
Fmoc: N^{α}-9-fluorenylmethoxy carbonyl
GPC: Gel permeation chromatography
GTMAC: Glycidyl trimethylammonium chloride
HBTU: N-[(1H-benzotriazol-1-yl)(dimethylamino) methylene]-N-methylmethanaminium hexafluorophosphate N-oxide
HMW: High molecular weight
HOBt: 1-Hydroxybenzotriazole
HPC: Hydroxypropyl chitosan
HPC-CTr: HPC-Chitotriazolan
HSQC: Heteronuclear single quantum coherence
HTC-CTr: HTC-Chitotriazolan
HTC: N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan
LMW: Low molecular weight
LMWC: Low molecular weight chitosan
MHB: Mueller-Hinton broth
MIC: Minimum inhibition concentration
MLC: Minimum lethal concentration
MRSA: Methicillin-resistant *Staphylococcus aureus*
MW: Molecular weight
Na_2CO_3: Sodium carbonate
Na_2SO_4: Sodium sulfate
NaCl: Sodium chloride
$NaCNBH_3$: Sodium cyanoborohydride
$NaHCO_3$: Sodium bicarbonate
NaOH: Sodium hydroxide
NMP: N-Methyl-2-pyrrolidone
N,O-CMC: N,O-Carboxymethyl chitosan

O-CMC: O-Carboxymethyl chitosan

P. aeruginosa*: Pseudomonas aeruginosa*

SAR: Structure-Activity Relationship

S. aureus*: Staphylococcus aureus*

SPPS: Solid-phase peptide synthesis

TAC-CTr: TAC-chitotriazolan

TACin: *N*-(2-(*N,N,N*-trimethyl ammoniumyl)acetyl)-chitin

TBAF: tetra-*N*-butylammonium fluoride

TBDMS: Tert-butyldimethylsilyl chloride

TBS: Tert-butyldimethylsilyl chloride

TGC: Thioglycolate chitosan

TFA: Trifluoroacetic acid

THPTA: Tris-hydroxypropyltriazolyl methylamine

TMC: Trimethyl chitosan

TMC-CTr: TMC-chitotriazolan

RT: Room temperature

List of figures

List of schemes

List of tables

List of original papers

This doctoral thesis is based on the following publications and manuscripts.

I. **Rathinam, S**., S. Ólafsdóttir, S. Jónsdóttir, M. Hjálmarsdóttir and M. Másson (2020). "Selective synthesis of N,N,N-trimethylated chitosan derivatives at different degree of substitution and investigation of structure-activity relationship for activity against P. aeruginosa and MRSA." *International Journal of Biological Macromolecules* **160**: 548-557.

II. **Rathinam, S**., S. Solodova, I. Kristjánsdóttir, M. Á. Hjálmarsdóttir and M. Másson (2020). "The antibacterial structure-activity relationship for common chitosan derivatives." *International Journal of Biological Macromolecules* **165**: 1686-1693.

III. **Rathinam, S**., M. Á. Hjálmarsdóttir, M. B. Thygesen and M. Másson (2021). "Chitotriazolan (poly(β(1-4)-2-(1H-1,2,3-triazol-1-yl)-2-deoxy-d-glucose)) derivatives: Synthesis, characterization, and evaluation of antibacterial activity." *Carbohydrate Polymers* **267**: 118162.

IV. **Rathinam, S**., M. Á. Hjálmarsdóttir, M. B. Thygesen and M. Másson (2022). "Water-Soluble Chitotriazolan's Derived from Partially Substituted Common Chitosan Derivatives: Synthesis, Characterization, and Antibacterial Activity." (Submitted manuscript).

V. **Rathinam, S**., R. Magdadaro., M. Á. Hjálmarsdóttir, and M. Másson. "Water-soluble Quaternary and Protonable Basic Chitotriazolans: Synthesis by Click Chemistry Conversion of Chitosan Azides and Investigation of Antibacterial Activity." (manuscript).

VI. **Rathinam, S**., K. K. Sørensen, M. Á. Hjálmarsdóttir, M. B. Thygesen and M. Másson. "Conjugation of CRAMP-18 Peptide to Chitosan and Hydroxypropyl Chitosan via Copper-Catalyzed Azide-Alkyne Cycloaddition and Investigation of Antibacterial Activity." (manuscript).

Declaration of contribution

I hereby declare that the thesis entitled "*N-Alkyl, N-Acyl, and Triazolyl Derivatives of Chitosan: Synthesis and Antibacterial Properties*" has now been submitted to obtain a Doctor of Philosophy degree has been based on work conducted in my Ph.D. study at the Faculty of Pharmaceutical Sciences, University of Iceland. The project's central concept and general work plan were proposed by Professor Már Másson (MM) at the Faculty of Pharmaceutical Sciences in application to the University of Iceland Research Fund and the Rannis Research Fund. I started the Ph.D. project work in June 2017. The research work was supervised and performed in collaboration with Prof. MM, Prof. Martha Ásdís Hjálmarsdóttir (MAH) Department of Biomedicine, University of Iceland, and Associate Prof. Mikkel Boas Thygesen (MBT), Department of Chemistry, University of Copenhagen Denmark. MM was the principal supervisor. I further developed the research concept and specific aims with direction from MM.

The aim of papers I & II was to synthesize common chitosan derivatives with varying degrees of substitution and investigate the structure-activity relationship. The synthesis of chitosan derivatives with different degrees of substitution and characterization by NMR and FT-IR and solubility studies were done by me. The ^{1}H NMR and 2D COSY NMR spectroscopy were done in collaboration with Dr. Sigríður Jónsdóttir Science Institute, University of Iceland. GPC analysis to determine molecular weight was performed by Dr. Svetlana Solodova. Microbiology studies to determine the MIC and MLC values were obtained by MS student Sigríður Ólafsdóttir and me under the supervision of MAH. Some preliminary studies conducted by MS student Ingibjörg Kristjánsdóttir were utilized in the research reported in paper II. I prepared the first manuscript drafts. The final version was written by me and MM and reviewed by all co-authors before submission.

The work reported in papers III & IV focused on using azide transfer reaction and copper-catalyzed azide-alkyne cycloaddition (CuAAC) to convert the primary amino group of chitosan and chitosan derivatives to 1,2,3-triazole products (Chitotriazolans). The solubility and antimicrobial activity were also investigated. The synthesis, characterization, and antibacterial activity studies were completed by me under the supervision of MM and MAH, with some additions C^{13}, COSY, and HSQC characterization performed by MBT at the University of Copenhagen,

Denmark. I first prepared the manuscript drafts. The final versions by written by me and MM and reviewed by all co-authors before submission

Paper V reports the synthesis of cationic chitotriazolan derivatives with variable structure of the cationic moiety. The synthesis was performed by me and exchange undergraduate student Romano Magdadaro from the Technological University Dublin under my supervision and also supervised by MM. I did the characterization, and the antibacterial activity study was also performed by me under the supervision of MAH. I prepared the manuscript draft, and I and MM wrote the final version.

Paper VI describes the synthesis of an antimicrobial peptide and conjugation to a chitosan backbone by CuAAC. This work was performed in collaboration with the Department of Chemistry, University of Copenhagen, Denmark. Dr. Kasper K. Sørensen synthesized the antimicrobial peptide alkyne, which was conjugated to chitosan by me, and I also performed the antimicrobial study. I prepared the manuscript draft, and I and MM wrote the final version.

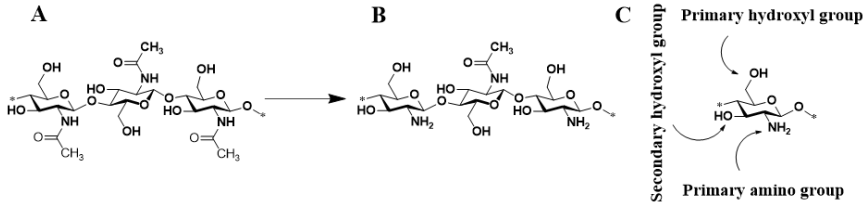
The main part of the research work was carried out at the research labs of the Faculty of Pharmaceutical Sciences, University of Iceland, in Reykjavik, Iceland. I had an opportunity to work at the lab of MBT for a couple of months during my doctoral studies at the University of Copenhagen. I got married in 2020; I wrote a manuscript and participated in the ICBMI-2020 conference during my stay in India. I have presented my research work at international conferences in Portugal, Japan, Madeira, India, and Iceland. These travels were supported by a doctoral grant from the University of Iceland Research Fund, the Icelandic Research Fund project grant (Rannis Grant No. 1709-0210), and the Scandinavia-Japan Sasakawa foundation.

Sankar Rathinam

1 Introduction

1.1 Chitin and Chitosan

Chitin is the second most abundant natural biopolymer after cellulose (Hudson and Smith 1998). Henri Braconnot first discovered chitin in 1811. It was isolated from certain types of mushrooms and first named fungine (Braconnot 1881). Chitin is a linear polymer composed of repeating units of β-1-4 linked *N*-acetyl D-glucosamine mainly found in the exoskeleton of crustaceans, insect cuticles, and arthropod shells. Chitin is also present in the cell walls of many fungi, green algae, fish scales, and yeasts. (Ravi Kumar 2000, Munro and Gow 2001, Peniche, Argüelles-Monal et al. 2008). Chitosan (CS) is a linear hydrophilic polysaccharide consisting of D-glucosamine and *N*-acetyl D-glucosamine units linked through β-(1-4) glycosidic bonds. Charles Rouget first described chitosan in 1859 (Rouget 1859) and showed that it could be derived from chitin by partial deacetylation under strong alkali. The chitin and chitosan structures are shown in the **scheme. 1** (Rinaudo 2006). The glucosamine monomers in chitosan have three nucleophilic functional groups, the C-2 primary amino group, the C-3 secondary OH group, and the C-6 primary OH group (**scheme 1. C**). Two parameters mainly define chitosan materials, the degree of deacetylation (DD) and the molecular weight (Mw). These are the most important aspects influencing physicochemical properties (Yuan, Chesnutt et al. 2011, Jiang, Fu et al. 2017). There is much interest in the biomedical application of chitosan due to its good biocompatibility, biodegradability, nontoxicity, and valuable biological properties (Kumar, Muzzarelli et al. 2004). Chitosan is much more soluble than chitin. The amino groups in chitosan can be protonated, so the polymer chain



Scheme 1. Chitin, Chitosan structure, and reactive positions.

becomes polycationic, contributing to good solubility in acidic aqueous solutions. Chitosan has a number of biological properties relevant to medical applications, such as antibacterial activity (No, Young Park et al. 2002, Rabea, Badawy et al. 2003, Dutta, Tripathi et al. 2009), antifungal activity (Roller and Covill 1999, Martínez-Camacho, Cortez-Rocha et al. 2010), anticancer activity (S. Wimardhani, F. Suniarti et al. 2014), stimulation of tight-junction opening to aid drug delivery (Ilium 1998, Agnihotri, Mallikarjuna et al. 2004), as well as stimulation of bone regeneration (Aguilar, Zein et al. 2019), and wound healing (Jayakumar, Prabaharan et al. 2011, Qu, Zhao et al. 2018). It has also been used in gene delivery(Hejazi and Amiji 2003, Mao, Sun et al. 2010), and cosmetics applications (Aranaz, Acosta et al. 2018).

Two monomer units are present in the chitin and chitosan backbone structure: *N*-acetyl-D-glucosamine and *N*-amino-D-glucosamine. The *N*-acetyl-D-glucosamine will be the main component in polymers defined as chitin. This will cause insolubility due to the strong hydrogen bonds between the acetyl groups of the same or adjacent chitin chains. On the other hand, 2-amino-D-glucosamine is more hydrophilic, especially when it becomes positively charged in an acidic solution. Chitin structure has a highly acetylated polymer and is insoluble in aqueous solvents and the most common organic solvents, limiting the application. Therefore, chitin polymer is mainly used as raw material for depolymerization or deacetylation to give chitosan. Chitosan is soluble in acidic aqueous solutions, but chemical modification can be used to improve water solubility further and enhance its biological properties. In most cases, the synthesis procedure target one or all three nucleophilic groups for functionalization.

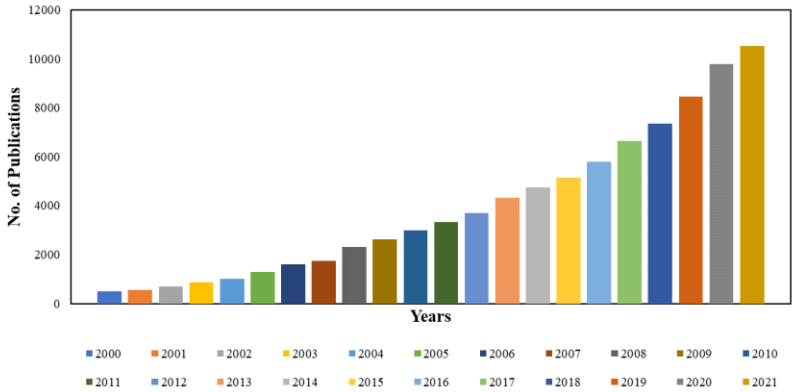


Figure 1. Chitosan publications per year from the source of the web of Science

The interest in various applications, properties, and modifications of chitosan has continually increased. **Figure 1** illustrates the number of publications listed on the Web of Science in the period from 2000 to 2021. In 2000 just 500 papers were published, whereas in 2021, more than 10,000 papers were published.

1.2 Chitosan and antimicrobial properties

Antimicrobial properties of chitosan were first reported in 1950 (Hatta, Kuwabara et al. 1950), but the interest and the number of studies have increased very significantly in the last two decades (Másson 2021). About 10-20% of all publications on chitosan focus on antimicrobial properties. Chitosan is active against various microorganisms such as bacteria, fungi, algae, and viruses (Rabea, Badawy et al. 2003, Raafat and Sahl 2009, Lopez-Moya, Suarez-Fernandez et al. 2019). According to most publications, the antibacterial and antifungal activity is triggered by the binding of the positively charged protonated amino groups in chitosan to negatively charged groups in lipopolysaccharides, membrane proteins, and phospholipids found in the cell wall or the cell membrane of the microorganism (Liu, Du et al. 2004, Chung and Chen 2008, Másson 2021), which will cause the cell membrane disintegration and damage to the bacterial cell wall. The exact mechanism is still unknown. Most studies propose that electrostatic interaction with negatively charged cell surface increases cell membrane permeability leading to cell death (Raafat, Bargen et al. 2008, Krajewska, Wydro et al. 2011, Li and Zhuang 2020). Others suggest that chitosan can penetrate the cytoplasmic membrane and bind to the DNA. The inhibition of DNA replication will then cause cell death (Xiu, Zhang et al. 2012, Yu, Zhang et al. 2018). Various possible antibacterial mechanisms have been proposed, including that chitosan acts as a chelating agent that binds to an essential metal ion to inhibit microbial growth.

Gram-positive and Gram-negative bacteria exhibit remarkable differences in their cell wall structure, in which gram-positive bacteria have thicker peptidoglycans and no outer lipid membrane. In contrast, gram-negative bacteria have a thin peptidoglycan layer and an outer lipid membrane (Beveridge 1999, Pasquina-Lemonche, Burns et al. 2020) (Beveridge 1999, Pasquina-Lemonche, Burns et al. 2020). It has been proposed that the antibacterial action of chitosan is different for Gram-positive and Gram-negative bacteria (**Fig. 2**) (Rabea, Badawy et al. 2003, Goy, Britto et al. 2009, Kong, Chen et al. 2010). However, Másson (Másson 2021) has criticized this idea as lacking experimental support.

The antimicrobial activity of chitosan is influenced by pH value, molecular weight (MW), degree of deacetylation, and temperature. Many publications have demonstrated that chitosan exhibits excellent antimicrobial activity under acidic conditions (Erdem, Kariptas et al. 2016, Varlamov and Mysyakina 2018). The pKa for the amino groups of chitosan is 6.3–6.5. Therefore, it will lack cationic charge and solubility in alkaline solutions and aqueous solutions with a pH higher than 6.5. The solubility increases with decreasing pH, as it leads to an increase in the positive charge of the primary $-NH_2$ groups of chitosan and greater antimicrobial activity (TSAI and SU 1999).

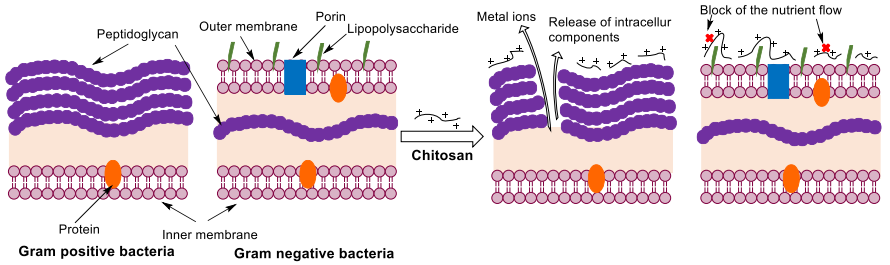


Figure 2. Proposed antibacterial action of chitosan on Gram positive bacteria and Gram negative bacteria

Chitosan properties depend on the average MW of the polymer in the material, including antibacterial properties. Several studies have focused, solely or in part, on the relationship between antibacterial activity and chitosan molecular weight. However, the conclusions from these studies have been inconsistent. Some studies have concluded that increasing the chitosan MW reduced the activity (Zheng and Zhu 2003). In contrast, other studies have found that high MW chitosan exhibits better activity than low molecular weight chitosan (No, Young Park et al. 2002). Jeon et al. reported that three fractions of chitosan oligosaccharide relative to MW (10, 5, 1 kDa) investigated the antibacterial activity. Oligosaccharide with MW is over 10000 Da required for the activity (Jeon, Park et al. 2001). Low MW chitosan 4.6 KDa and its derivatives exhibit better activity against bacteria *E. coli*, *P. aureofaciens*, yeast, and fungi (Tikhonov, Stepnova et al. 2006). Chang et al. studied six chitosan samples with MW ranging from 3.3 to 300 kDa. These were tested for activity against *S. aureus* and *E. coli*, which increased with increasing MW at low pH. Conversely, chitosan lost activity when MW >29.2 kDa at pH 7 (Chang, Lin et al. 2015). High MW chitosan (100 and 210 KDa) shows better antibacterial activity against *S. aureus* and *E. coli* than low MW chitosan (1800) (Shin, Yoo et al. 2001).

The preparation method will influence the DD of chitosan, especially the processing time and temperature used for chemical deacetylation. High DD polymers exhibit a more positive charge than low DD polymers in the same acidic medium. The chitosan with higher DD polymers has a higher positive charge will have a strong electrostatic interaction with anionic microbial cell surfaces and, which leads to stronger antibacterial activity (Jung, Youn et al. 2010, Foster, Ho et al. 2015)

The effect of temperature and pH was investigated for different molecular weight samples of chitosan. The results showed that the antibacterial activity increased when the temperature increased and the pH decreased (Chang, Lin et al. 2015). Temperature also influences the antimicrobial activity of chitosan. It has better antimicrobial activity at higher temperatures (37 °C) than at refrigeration temperatures (Erdem, Kariptas et al. 2016).

Chitosan is also active against fungi. Both chitin and chitosan can inhibit the growth of many fungi, but chitosan has a stronger fungicidal effect than chitin (Allan and Hadwiger 1979). A recent study demonstrated the antifungal efficacy of different chitosan and various concentrations against *Colletotrichum alatae* fungi (L, G et al. 2021). The low molecular weight chitosan (LMWC) has been investigated for antifungal activity against 105 clinical isolates of *Candida* spp and exhibited antifungal activity inhibiting over 89.9% of the clinical isolates examined at pH 4 (Alburquenque, Bucarey et al. 2010). Another study investigated the antibacterial and antifungal efficacy of 15 samples of chitosan that varied in molecular weight and DA in homogeneous conditions (Younes, Sellimi et al. 2014). Low MW chitosan (1000 to 10000) displayed better antifungal activity against various pathogenic yeasts and hyphae-forming fungi without hemolytic effect (Park, Kim et al. 2008).

The use of chitosan to aid drug delivery has been investigated by academics and the pharmaceutical industry (Ilium 1998). S.N.Chirkov showed that chitosan could inhibit viral infections in animal cells and investigated the effect of a physicochemical factor on antiviral activity (Chirkov 2002). Another study showed that chitosan could effectively inhibit the infection of *Autographa californica* Multicapsid Nucleopolyhedrovirus (AcMNPV), *Bombyx mori* nuclear polyhydrosis virus (BmNPV) (Ai, Wang et al. 2012). Tan et al. have reviewed chitosan and its structurally modified derivatives and found that (hydroxypropyl trimethylammonium, sulfate, carboxymethyl, bromine, peptide, phosphonium conjugates, and sialyllactose exhibit anti-viral activity against SARS-CoV-2, herpes simplex virus, influenza virus, NDV, human papillomavirus and F-MuLV (Tan, Hassandarvish et al. 2022). Chitosan has also been used in vaccines against viral

infections. One study reported the production of a vaccine candidate composed of a recombinant matrix protein-2 (sM2) loaded into poly-γ-glutamic acid (γ-PGA)-chitosan nanoparticles (PC NPs). The vaccine used cholera toxin subunit A1 (CTA1), and a fusion peptide of hemagglutinin (HA_2) as a mucosal adjuvant was found to be effective as a broadly cross-protective influenza vaccine (Chowdhury, Kim et al. 2017).

1.2.1 Antimicrobial applications

Chitosan and its derivatives have been widely used in many fields, such as plant protection, wound treatment, food preservatives, and wastewater treatment. Chitosan is also cross-linked with other materials to form hydrogel, coatings, and nanoparticles.

In agriculture, chitosan is used in many ways; one of the first applications that were considered was its use as a fungicidal antipathogen in plants (Allan and Hadwiger 1979). Chitosan is induced in the elicitation chain and could be used in plant disease control as a powerful elicitor for *Mimosa pudica* motor cells (Amborabé, Bonmort et al. 2008). The effect of chitosan on viral infection was investigated in potato plants, and it was found it could increase resistance to such infection (Chirkov, Il'ina et al. 2001). A study of strawberry plants exposed to probiotic bacteria Bph-4 and BTLK6a showed a significant increase in vegetative growth 56% and fruit yield 43 % compared to untreated control (Akter Mukta, Rahman et al. 2017).

Wound treatment is possibly the most promising medical application for chitosan. A wound dressing material was prepared using commercial polyurethane film (Tegaderm) as a backing with a composition of chitosan (83% DD) film and 2 mg of minocycline hydrochloride. The substance exhibited a stronger wound-healing effect (Aoyagi, Onishi et al. 2007). The mortality of mice with *P. aeruginosa* infected wounds was reduced from 90% to 14.3% when treated with chitosan polyphosphate that incorporated silver nanoparticles (Ong, Wu et al. 2008). Jayakumar et al. have reviewed various forms (hydrogels, fibers, membranes, scaffolds, and sponges) of chitin chitosan and its derivatives as biomaterials for wound dressings (Jayakumar, Prabaharan et al. 2011).

Chitosan can be used as a preservative, either in the form of films or coatings in food packing or preparation. A coating containing 2% chitosan and 0.2% of gallic acid incorporated coating proved to be a good preservative for fresh pork stored at 4 °C to ensure product safety and quality (Fang, Lin et al. 2018). The chitosan-sulfur nanoparticles composite film exhibited antimicrobial activity against food-borne pathogenic *E. coli* and *Listeria monocytogenes* bacteria (Shankar and Rhim 2018).

1.3 Antimicrobial chitosan derivatives

Nowadays, many researchers are interested in chitosan derivatives obtained by modifying at least one of the three reactive functional groups. The aim is to improve physicochemical or biological properties, such as antibacterial activity and suitability for biomedical applications. Chitosan is insoluble under neutral physiological conditions, limiting antibacterial applications. This issue can be addressed by introducing hydrophilic and charged moieties. The antimicrobial activity can also be improved by conjugating antimicrobial substances.

1.3.1 Quaternary chitosan derivatives

Chitosan can be modified on the *C*-2 amino group and hydroxyl groups, especially the more nucleophilic *C*-6 hydroxyl group, to introduce quaternary ammonium groups with a permanent cationic charge. The resulting chitosan quaternization improves the water solubility in a broad pH range and enhances the antimicrobial action compared to unmodified chitosan (Rúnarsson, Holappa et al. 2007, Sajomsang, Gonil et al. 2009). The *N,N,N*-trimethyl chitosan (TMC) is the most studied quaternary chitosan derivative (Muzzarelli and Tanfani 1985, Sieval, Thanou et al. 1998, Wu, Long et al. 2017), and 2-hydroxyl propyl-3-trimethyl ammonium chitosan (HTC or HTCC) is second most studied quaternary ammonium salts derived from chitosan (Seong, Whang et al. 2000). The other quaternized chitosan derivatives have also been investigated the antimicrobial activity including *N*-betaine (Holappa, Hjálmarsdóttir et al. 2006), pyridinium salts (Omidi and Kakanejadifard 2019), phosphonium salts (Wang, Xu et al. 2011), and fluorinated chitosan quaternary derivatives (Cele, Somboro et al. 2020).

1.3.1.1 N,N,N-Trimethyl chitosan (TMC) and N,N,N- trialkyl chitosan

A cationic charge on the polymer backbone can be introduced by quaternizing the 2-amino groups in chitosan. The *N,N,N*-trimethyl chitosan (TMC) is the most common derivative. TMC can be obtained by treating chitosan excess methyl iodide or dimethyl sulfate in the presence of strong alkali using *N*-methyl pyrrolidone (NMP) as a solvent shown in **Scheme 2**. (Domard, Rinaudo et al. 1986, Sieval, Thanou et al. 1998, Curti, de Britto et al. 2003, Goy, Morais et al. 2016, Wu, Long et al. 2016). This method has been further improved by using DMF/H_2O (Rúnarsson, Holappa et al. 2008) as solvent or DMSO and a mild base (Másson 2021) to obtain products with a high degree of trimethylation and no *O*-methylation. An alternative method is first to generate a Schiff's base

generated by reaction with formaldehyde, which is converted to *N,N*-dimethyl chitosan (DMC) using a suitably reducing agent (Verheul, Amidi et al. 2008). DMC is then reacted with methyl iodide to give trimethyl chitosan, shown in **Scheme 2**. The *N,N,N*-trimethylation is often accompanied by *O*-alkylation on *C*-6 and C-3 positions, especially when the reaction is carried out in NMP with a strong base (Curti, de Britto et al. 2003). This will reduce solubility and may affect biological properties (Sieval, Thanou et al. 1998, Polnok, Borchard et al. 2004). The *C*-6 position will also be blocked, preventing further chemical modification. This can be avoided by changing the reaction conditions, as previously mentioned, or by using protection groups. The *C*-3 and *C*-6 hydroxy groups have been selectively protected by reaction with *tert*-butyldimethylsilyl chloride (TBDMS) and imidazole in DMSO protection to obtain 3,6-*O*-diTBDMS chitosan (TBDMS-Chitosan) (Rúnarsson, Malainer et al. 2008). The advantage of using TBDMS as a protection group is not only that it allows selective *N*-alkylations (Benediktsdóttir, Gaware et al. 2011) and *N*-acylation's (Rúnarsson, Malainer et al. 2008, Rúnarsson, Holappa et al. 2010) but also that it changes the physicochemical characteristics of the polymer(Song, Gaware et al. 2010, Már Másson 2013) so that reactions can be carried out in moderately polar organic solvents like dichloromethane (DCM). TBDMS-chitosan has been reacted with methyl iodide using Cs_2CO_3 as a base and deprotected with *tetra*-n-butylammonium fluoride (TBAF) to obtain TMC with 100 % *N,N,N*-trimethylation and no *O*-methylation (Benediktsdóttir, Gaware et al. 2011).

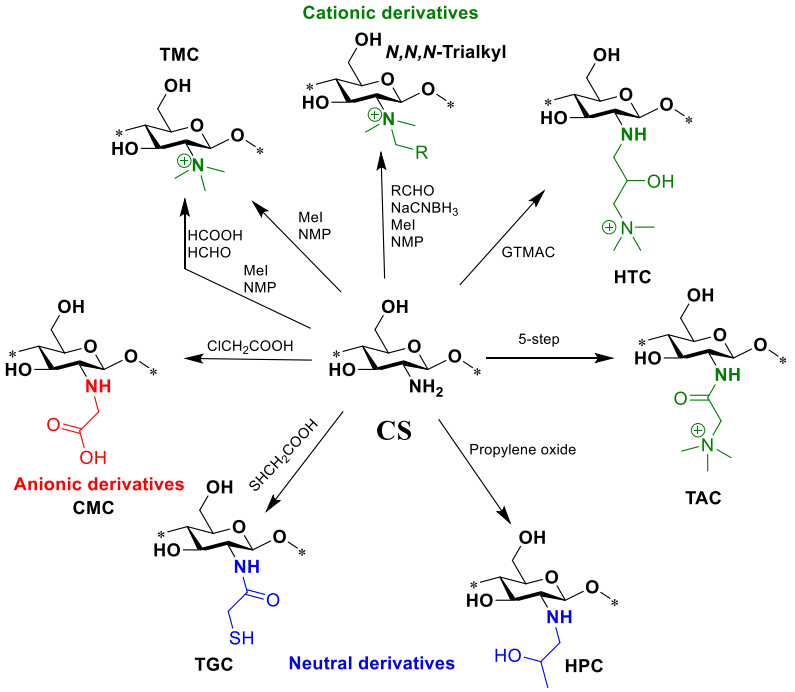
Chitosan can also be quaternized with by *N*-alkylation with more than one alkane. For example, the mono *N*-alkylation was performed by making a Schiff-base intermediate by reaction with aldehydes (**Scheme 2)** and reduction. This was followed by quaternization with methyl iodide (Kim, Choi et al. 1997). Similarly, other quaternized *N*-alkyl chitosan derivatives like *N*—*N*-propyl-*N,N*-dimethyl chitosan, and *N*-furfuryl-*N,N*-dimethyl synthesized using a similar procedure (Jia, shen et al. 2001) or starting from TBDMS-chitosan (Sahariah, Benediktssdóttir et al. 2015).

Quaternized *N,N,N*-trimethylation improves solubility in aqueous solutions relative to native chitosan and is soluble in a wide pH range (Mourya and Inamdar 2008). TMC is polycationic with a high density of positive charges on the polymer backbone, and strong electrostatic interaction with anionic cell membranes is therefore expected.

TMC biopolymers have been investigated for activity against gram-positive and gram-negative bacteria and found to be more active than chitosan against both types of bacteria (Rúnarsson, Holappa et al. 2010, Xu, Xin et al. 2010, Sahariah, Cibor et al. 2019). TMC has also been used in other biomedical applications such as enhancing drug delivery through mucosal membranes (Benediktsdóttir, Baldursson et al. 2014, Pardeshi and Belgamwar 2018), gene delivery(Kean, Roth et al. 2005), and wound treatment (Zhou, Yan et al. 2016, Abueva, Ryu et al. 2021).

1.3.1.2 2-hydroxyl propyl-3-trimethyl ammonium chitosan (HTC)

A quarternary ammonium moiety with a cationic charge can be introduced by linking it through a spacer group to the polymer backbone. The most common such is 2-hydroxyl propyl-3-trimethyl ammonium chitosan (HTC or HTCC for the



Scheme 2. Synthesis of antimicrobial common chitosan derivatives based on literatures

chloride salt), which is synthesized by reacting glycidyl trimethylammonium chloride (GTMAC) with chitosan (Seong, Whang et al. 2000, Cho, Grant et al. 2006). (**Scheme 2)**. This nucleophilic reaction with the epoxide group favors the *C*-2 amine under acidic conditions. On the other hand, the *C*-6 hydroxyl

group will be favored under basic conditions (Freitas, Moura et al. 2020). The HTCC DS was increased with increasing the reagent. GTMAC was used with various ratios, reaction times, and temperatures to obtain different degrees of substitutions (Seong, Whang et al. 2000). The *O*-HTCC compound was synthesized by reacting *N*-benzylidene chitosan with GTMAC and then removing the *N*-benzylidene substituent (protecting group) by treatment with 0.25 mol/L HCl alcohol solution (Sun and Wan 2007). A derivative similar to HTC can be synthesized by reaction with glycidyl triethylammonium chloride (GTEAC) to obtain *N*-(2-hydroxyl) propyl-3-triethyl ammonium chitosan chloride (Wan, Xu et al. 2013). Various derivatives of HTC have also been reported. One such derivative was synthesized by reductive *N*-alkylation with benzaldehyde and then by *C*-6-quaternization of the resulting *N*-benzyl chitosan with GTMAC. This derivative was investigated as an antibacterial finishing for cotton fabrics (Fu, Shen et al. 2011). HTC has been studied in several applications, including nanoparticles for controlled drug delivery (Li, Li et al. 2014), as an antimicrobial agent (Kim, Nam et al. 2003), permeation enhancer for mucosal membranes (Hecq, Siepmann et al. 2015), and for gene delivery (Xiao, Wan et al. 2012).

1.3.1.3 N-(2-(N,N,N-Trimethylammoniumyl)acetyl)-chitosan (TAC) and other quaternary N-acyl derivatives

N-(2-(*N,N,N*-trimethylammoniumyl)acetyl)-chitosan (TAC) is another relatively common quaternary ammonium derivative, although less studied than TMC and HTC derivatives. It is also known as betaine chitosan derivative or chitosan betaine because it can be considered a betaine (*N,N,N*-trimethylglycine) derivative. Holappa et al. reported the synthesis of chitosan *N*-betaine (**Scheme 2)** in five steps; First three-step reaction to obtain 6-*O*-triphenylmethyl chitosan and then *N*-acylation and deprotection. The derivative was prepared with various degrees of substitution (DS). The highest DS (0.90 was obtained in reaction with four equivalents of *N*-chlorobetainyl chloride (Holappa, Nevalainen et al. 2004). Later the antimicrobial activity was studied and found that it increased with decreasing DS (Holappa, Hjálmarsdóttir et al. 2006). Rúnarsson et al. synthesized betaine chitosan starting from TBDMS chitosan and TBDMS chitosan oligomer. The minimal inhibitory concentration (MIC) for the polymer derivative was 32-128, ≥8192, 512, and 64 µg/mL against *S. aureus, E. coli*, *E. faecalis,* and *P. aeruginosa*, respectively (Rúnarsson, Holappa et al. 2010). The oligomer derivatives were generally less active with MIC = 4096 µg/mL or more. The *N*-betaine derivative with a low degree of substitution (0.05) was effective, and the activity decreased with increasing DS (Korjamo, Holappa et al. 2008). The *N*-betaine chitosan derivative shows good antibacterial activity (Rúnarsson, Holappa

et al. 2010, Blagodatskikh, Vyshivannaya et al. 2018). TAC derivatives are also used as drug delivery (Mannila, Järvinen et al. 2009) and gene delivery (Gao, Zhang et al. 2009).

1.3.2 Water-soluble chitosan derivatives with neutral hydrophilic substituents

The water solubility of polymers can be improved by introducing neutral hydrophilic groups. One example is hydroxypropyl methylcellulose (HPMC), a water-soluble cellulose derivative. HPMC is a common excipient in drugs (Levina and Rajabi‐Siahboomi 2004) and is used as thickener food (Burdock 2007) and other consumer products (Laguna, Primo-Martín et al. 2014). Hydroxypropyl chitosan (HPC) is a well know and commercially available derivative. Glycol chitosan is another derived from chitin that is also commercially available (Saravanakumar, Min et al. 2009, Mitra, Han et al. 2014). Thioglycolate chitosan (TGC) has been studied, mainly focusing on its biological properties. The added functional groups in these derivatives can help to improve water solubility. These derivatives can be defined as "neutral" chitosan derivatives, although they contain protonatable amino groups, like chitosan, and will therefore be positively charged at low pH (Kast and Bernkop-Schnürch 2001).

1.3.2.1 Hydroxypropyl chitosan (HPC) and glycol chitosan

Hydroxypropyl groups can be introduced by alkylation of the *C*-2 amino the *C*-3, and *C*-6 hydroxyl groups in chitosan by reaction with propylene oxide under the alkaline. (**Scheme 2)** Investigation of hydroxypropyl chitosan (HPC) synthesized with DS ranging from 1.5 to 3.1 showed no antibacterial effect against *S. aureus* and *E. coli*. In contrast, antifungal activity was observed against *A. mali*, *C. diplodiella*, *F. oxysporum*, and *P. piricola* (Peng, Han et al. 2005). Xie et al. reported the synthesis of HPC derivative and the grafted with maleic acid sodium, showing good antibacterial activity against *S. aureus* and *E. coli* bacteria by the cut plug method (inhibition zone method) (Xie, Xu et al. 2002). However, most investigations involving the use of HPC have not focused on antimicrobial activity. Wan et al. prepared the hydroxyethyl chitosan and HPC with a maximum degree of substitution of 25%. The modified chitosan showed increasing ionic conductivity and did not exhibit significant changes in its tensile strength properties (Wan, Creber et al. 2004). HPC polymer has been photo-crosslinked to make wound dressing (Lu, Ling et al. 2010) and grafted with cyclodextrin copolymer for drug delivery applications (Xie, Qin et al. 2019).

Glycol chitosan is a derivative of chitosan with ethylene glycol branches linked through the *C*-6 oxygen. Iron oxide nanoparticles coated with glycol chitosan have shown good antibacterial activity against *S. aureus, E. coli,* and *S. enteritidis* (Stephen Inbaraj, Tsai et al. 2012). Glycol chitosan-based materials are used for encapsulation and drug delivery (Lin, Jia et al. 2019, Yu, Shi et al. 2020).

1.3.2.2 Thioglycolic chitosan (TGC)

The thiol functional group (-SH) is present in many natural compounds. The amino acid cysteine has a methanethiol side group that plays a significant role in its biological properties. The thiols present in our body are very reactive, so they are mostly found in the oxidized form as disulfide linkages. A thiol derivative of chitosan (**Scheme 2)**, chitosan can be obtained by reaction with thioglycolic acid in the presence of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling reagent(Geisberger, Gyenge et al. 2013). This derivative is active against the bacteria *Streptococcus sobrinus* and *Neisseria subflava* and the fungi *C. albicans*. The analysis is based on the reaction of thiolate anions. Thiolated chitosan increases water solubility and enhances antimicrobial activity (Croce, Conti et al. 2016), showing several pharmaceutical applications (Kast, Frick et al. 2003). TGC polymers with the thiol group interact with cysteine-rich glycoprotein in mucous membranes, forming a disulfide bond between the polymer and enhanced mucoadhesive property (Kast and Bernkop-Schnürch 2001).

1.3.3 Anionic chitosan derivatives

Carboxymethylcellulose is a well-known water-soluble and anionic derivative of cellulose. It is used as a pharmaceutical excipient (Li, Song et al. 2010) and in other applications (Ogushi, Sakai et al. 2007). Carboxymethyl chitosan is probably the best-known chitosan derivative commercially available from many vendors. The carboxyl groups will become anionic at pH between 4 and 5, whereas the amino groups of carboxymethyl chitosan will become cationic at low pH (below 6-7), so the net charge of this polymer will depend on pH. The abbreviation CMC is used for carboxymethyl chitosan, which can be confusing because the same abbreviation is also used for carboxymethyl cellulose. Anionic sulfonated (Lima, Pereira et al. 2013) and phosphorylated (Wang and Liu 2014) chitosan derivatives have also been reported.

1.3.3.1 Carboxymethyl chitosan (CMC)

Carboxymethyl chitosan is the most well-known chitosan derivative. It is widely used for research and various applications. It has an amphoteric character as it contains amino groups that can be protonated to become cationic and carboxylic

groups that can be deprotonated to become anionic. The net charge will depend on the degree of substitution and pH. Monochloroacetic is reacted with chitosan to obtain CMC (**Scheme 2**). The selectivity of the reaction can be controlled by adjusting the alkalinity, solvent system, and temperature to give mainly *N*-CMC, *O*-CMC, or *N,O*-CMC (Jayakumar, Prabaharan et al. 2010, Mourya, Inamdara et al. 2010, Song, Zhang et al. 2011). *N*-CMC can also be prepared by reductive alkylation (Muzzarelli, Tanfani et al. 1982, Di Colo, Zambito et al. 2004). El-Shafei found that CMC was active against *E. coli* (DSMZ 498) and *Micrococcus luteus* and could be used for multifunctional cotton finishing (El-Shafei, Fouda et al. 2008). *O*-CMC and *N,O*-CMC nanoparticles were prepared by ionic gelation with $CaCl_2$ and TPP, respectively, and studied for antibacterial activity against *S. aureus*. Nanoparticles prepared from *N,O*-CMC were more active, and activity increased with concentration. (Anitha, Divya Rani et al. 2009). Carboxymethyl chitosan (CMC) was prepared, and then the quaternized carboxymethyl chitosan (QCMC) was synthesized by using glycidyl trimethyl ammonium chloride reagent, QCMC was evaluated for antibacterial activity; the results show that it had stronger activity and it can be used as a dental pulp-cap (Sun, Du et al. 2006). CMC has been used in a broad range of biomedical and pharmaceutical applications, including its use as an anticancer agent (Jeong, Jin et al. 2010), an antioxidant (Zhao, Huang et al. 2011), for tissue engineering (Jayakumar, Rajkumar et al. 2009), wound healing (Weng, Romanov et al. 2008), drug delivery (Wang, Chen et al. 2010), and cosmetics (Jimtaisong and Saewan 2014).

1.3.4 Other chitosan derivatives

Various other chitosan derivatives have been reported and investigated for biological activity, including antibacterial activity. A reaction with cinnamaldehyde can form Schiff's base derivatives of chitosan. Similarly, sorbyl chitosan, and p-aminobenzoyl chitosan derivativescan be prepared by reacting chitosan with sorbic acid and p-aminobenzoic acid, respectively. These derivatives have been investigated for antibacterial activity against *S. aureus* and *E. coli*. They showed good activity, especially against *S. aureus* (Wang, Lian et al. 2012). (**Fig. 3B**) Various chitosan-sulfonamide derivatives have been synthesized (**Fig. 3A**) and reported to show good activity against *S. aureus, E. coli, Sarcina lutea,* and *Bacillus cereus,* as well as effect wound healing effects (Dragostin, Samal et al. 2016). To obtain *N*-heterocyclic chitosan (**Fig. 3C**), chitosan can be with furan-2-carbaldehyde, 5-methylfuran-2-carbaldehyde, 3-pyridine carboxaldehyde, benzo[*d*][1,3]dioxole-5-carbaldehyde and 4-oxo-4*H*-chromene-3-carbaldehyde by reductive amination reaction. This modification of

the chitosan structure was found to improve the biological activity against plant pathogenic fungi *F. oxysporum*, *P. debaryanum* and *P. grisea* (Badawy 2008).

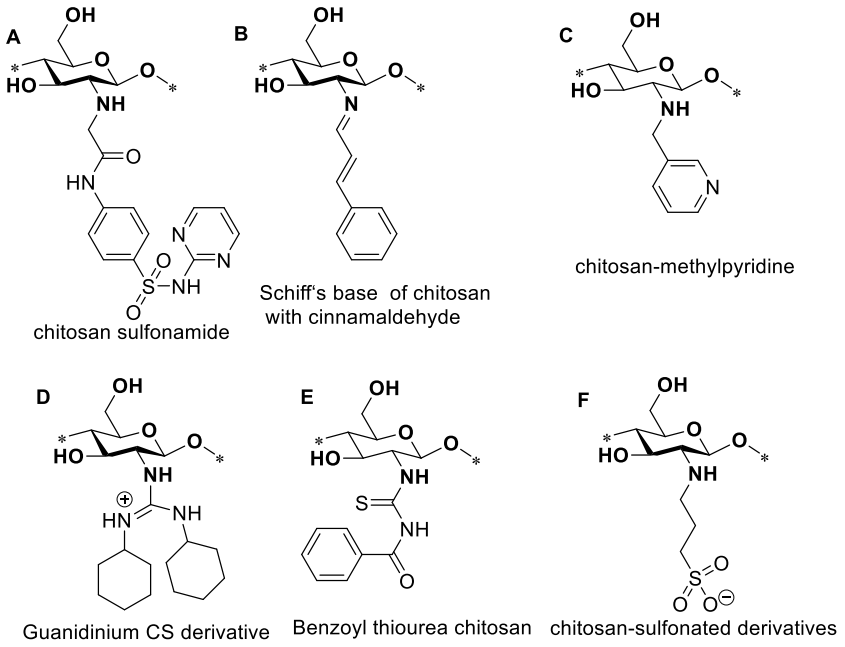


Figure 3. Chitosan with various structures

N-guanidinium chitosan derivatives were prepared and reported to show good activity against various strains of *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis,* and *C. albicans* (**Fig. 3D**) (Salama, Hasanin et al. 2020). Benzoyl, acetyl, and chloroacetyl thiourea derivatives of chitosan (**Fig. 3E**) were synthesized and tested for their antibacterial effect. These derivatives exhibited better antibacterial activity against various bacteria *E. coli*, *P. aeruginosa*, *S. aureus*, and *Sarcina sp,* and plant pathogenic fungi *Alternaria solani*, *Fusarium oxysporum f.* sp. *vasinfectum*, *Colletotrichum gloeosporioides* (*Penz.*) *Saec*, and *Phyllisticta zingiberi* than chitosan (Zhong, Xing et al. 2008). The anionic *N*-(3-sulfonic) chitosan (sulfonated chitosan) derivative was synthesized by using 1,3-propane sultone, and the sulfonated derivative was tested for antimicrobial activity against *E. coli*, *S. aureus*, *Arthrinium sacchari*, and *Botrytis cinerea* (Sun, Shi et al. 2017).

1.4 Antimicrobial chitosan conjugates

Biopolymers that are produced by linking some biologically active substance to the chitosan backbone can be defined as chitosan conjugates. These chitosan conjugates have been prepared in order to introduce new or enhanced

biological properties such as antioxidant, antibacterial, antiviral, and anti-inflammatory activity. The bioactive molecules that have been linked to chitosan are mainly polyphenols, amino acids, peptides and proteins, natural products, and small bioactive molecules (Berezin, Lomkova et al. 2012).

Polyphenols are antioxidant compounds found in many plants, fruits, vegetables, herbs, and spices. This includes flavonoids (catechin), and phenolic acids (cinnamic acid, salicylic acid, gallic acid, ferulic acid, caffeic acid, and p-coumaric acid) that have been grafted onto chitosan. The substituents have been introduced to improve the antioxidant effect and to realize novel functional properties such as radical scavenging activity. The methods used for grafting include enzyme-mediated synthesis, activated ester-mediated synthesis, and free-radical grafting (Hu and Luo 2016, Qin and Li 2020). These conjugates have been investigated as potential food packaging materials, preservatives, drug delivery, and antimicrobials (Božič, Gorgieva et al. 2012, Jiang, Lin et al. 2012, Woo and Je 2013, Lei, Wang et al. 2014). Cho et al. investigated the activity of chitosan-catechin conjugates against 20 methicillin-resistant *S. aureus* (MRSA) strains, three-gram positive bacteria, and six gram-negative bacteria. The MIC for MRSA was 64 – 128 µg/mL, 64 µg/mL for the gram-positive bacteria *B. subtilis*, *E. faecalis*, and *L. monocytogenes,* and 256 – 512 µg/mL for gram-negative bacteria. (Cho, Lee et al. 2013). Chitosan conjugated with caffeic acid, ferulic acid, or sinapic acid was investigated for activity against acne-related bacteria, such as *P. acnes*, *S. epidermidis, S. aureus*, and *P. aeruginosa*. The MIC was between 8 and 512 µg/mL. Caffeic acid conjugates were most effective against acne-related bacteria (Kim, Yu et al. 2017). Nagy et al. synthesized a series of chitosan hydroxycinnamic acid conjugates and investigated them for antibacterial activity (Nagy, Sahariah et al. 2022). In contrast to the previously mentioned publication, the conjugation of antioxidants did increase the activity against *E. coli* and *S. aureus*. Conversely, the activity decreased with increasing DS.

Antimicrobial peptides (AMPs) are oligopeptides, also called host defense peptides, consisting of between 10 to 100 amino acid units. These peptides generally have two or more positively charged groups and a substantial portion of hydrophobic residues (Karle, Gopi et al. 2003). They are found in a wide variety of bacteria, fungi, plants, invertebrates, and vertebrates (Hancock and Sahl 2006, Jenssen, Hamill et al. 2006). Most AMPs can directly interact with the microbial cell membrane. The cationic peptides are also known as innate immune modulators. AMPs are classified based on their secondary structure, which can be α-helical, β-sheet, β-hairpin, or extended. (Zasloff 2002, Bowdish, Davidson et al. 2005). Antimicrobial peptides show diversity in structure and broad

antimicrobial spectrum. Human AMPs include cathelicidin and defensins. The gene family of cathelicidin is identified from a full-length cDNA sequence of mouse marrow cells, and it's named as CRAMP, a cathelicidin-related antimicrobial peptide (Gallo, Kim et al. 1997). The CRAMP peptides consisting of amino acids segments 16 to 33 and 18 to 35, display potent antimicrobial activity without hemolytic activity and antibiofilm activity against *E. coli, P.aeruginosa* (Ha, Shin et al. 1999, De Brucker, Delattin et al. 2014).

Antimicrobial peptides have been conjugated to chitosan biopolymer and investigated antimicrobial activity. Sahariah et al. used CuAAC to conjugate anoplin to chitosan with the peptide linked through the *N* or *C*-terminal. A series of conjugates were synthesized with DS varying from 6 – 23 % and were evaluated for activity against *S. aureus*, *E. coli*, *E. faecalis*, and *P. aeruginosa*. The results showed that conjugates with linkage through the *N*-terminal were significantly more active against *E. faecalis* and *P. aeruginosa*, whereas conjugates with *C*-terminal linkage were more active against *E. coli*. An increase in the degree of substitution (DS) for the anoplin peptide significantly increased activity against *S. aureus* and reduced hemolytic activity (HC_{50}) for human erythrocytes (Sahariah, Sørensen et al. 2015). The potent antimicrobial peptide Dhvar-5 was grafted onto chitosan via copper (I) catalyzed azide-alkyne cycloaddition reaction (CuAAC) (Barbosa, Vale et al. 2017). Dhvar-5 was linked through the *N*-terminal and the *C*-terminal residues. The *C*-terminal Dhvar-5 conjugates showed more antimicrobial activity against Gram-positive bacteria *S.epidermidis*, and *S.aureus* and reduced adhesion of Gram-negative bacteria *E.coli,* and *P.aeruginosa* than the *N*-terminal Dhvar-5 conjugates. Antibiofilm properties were confirmed for chitosan-conjugate thin-films, and the Dhvar-5 conjugates showed no cytotoxic effect against HFF-1 cells (Barbosa, Costa et al. 2019). In another study, chitosan was first functionalized with *N*-succinimidyl-*S*-acetylthiopropionate and glutathione and short peptides (RWAAC-$_{NH2}$ CAAWR-$_{NH2,}$ and PWKISIHLAAC-$_{NH2}$), grafted with the formation of disulfide bond linkage. These conjugates showed antimicrobial activity against *S. aureus* and *E. coli*. The activity was enhanced relative to unmodified chitosan and peptide and showed selectivity towards *S. aureus* with low cytotoxicity (Petrin, Fadel et al. 2019).

The AMP's ε-poly-L-lysine was grafted onto chitosan polymer using copper-free thiol–ene 'click' chemistry. The peptide was conjugated with low, medium, and high molecular-weight chitosan. Conjugates with low MW chitosan (CSL-g-$EPL_{50\%}$) showed good antibacterial activity towards gram-negative *E. coli* and *P. aeruginosa*, gram-positive bacteria *E. faecalis* and *S. aureus,* and antifungal activity against *C. albicans* and *F. solani* and very low hemolytic activity (Su, Tian et al. 2017).

1.5 The structure-activity relationship for antimicrobial chitosan derivatives

Medicinal chemistry research aims to establish the relationship between molecular structure and biological activity, the so-called structure-activity relationship (SAR). This approach is mainly used for small molecules but can be applied to chitosan derivatives and conjugates. Many factors affect the SAR for chitosan derivatives. This includes the molecular weight, the structure of the substituent, the degree of acetylation (DA), and DS. The pH of the medium can also influence charge density and pH, and derivatives can have more than one type of substituent. The number of possible variations in structure and other factors influencing activity is limitless, but SAR studies can help understand the contribution and importance of all these variants.

Several studies have focused on the influence of molecular weight activity on antimicrobial activity, but conflicting results have been reported. The MW of chitosan is thought to determine to what extent it can penetrate the cell surface and cause an intracellular effect (Sudarshan, Hoover et al. 1992). Chitosan with an MW below 305 kDa has been investigated for antibacterial activity against *S. aureus* and *E. coli*. The antimicrobial activity against *S. aureus* increased with increasing MW of chitosan. In contrast, for *E. coli* bacteria, the effect was enhanced with degreasing MW of chitosan (Zheng and Zhu 2003).

Three chitosan polymers with average MW 628, 591, and 107 kDa and two chitooligosaccharides with average MW 5 and 3 kDa have investigated the relationship between molecular weight and antimicrobial activity. The activity increased when MW was less in the case of *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, and the reverse relationship in the case of the Gram-positive *S. aureus* and *S. epidermidis* bacteria (Fernandes, Tavaria et al. 2010). Similarly, Younes et al. found, based on the investigation of samples in the MW range from 42.5 to 135 kDa chitosan, that activity against gram-negative bacteria increased with decreasing MW and a reverse trend for gram-positive bacteria (Younes, Sellimi et al. 2014). Másson's group studied 49 hydrolyzed TMC and chitosan samples with molecular weight (MW) ranging from 2 to 144 kDa (Sahariah, Cibor et al. 2019). They showed that the activity against *S. aureus* increased with MW until a specific MW (CMW) was reached and that further increase did not affect the activity. The CMW was found to be 20 and 50 kDa for TMC and chitosan, respectively.

A chitosan polymer's degree of acetylation (DA) can influence the solubility in an aqueous solution and antimicrobial activity. Highly *N*-acetylated chitosan

increased is more hydrophobic and has less antimicrobial activity. Younes et al. investigated the antimicrobial activity of fifteen chitosan samples with different DA and MW. The DA ranged from 2 to 61%, the activity of chitosan with 2 ≤ DA ≤ 24 (%) showed a strong bactericidal effect, higher for Gram-negative than for Gram-positive bacteria and chitosan with DA above 41% no activity (Younes, Sellimi et al. 2014). Omura et al. have also evaluated the antimicrobial activity of chitosan with various MW and DA against Gram-positive and Gram-negative bacteria and yeasts. They found that increased DA reduced activity in all cases (Omura, Shigemoto et al. 2003). Another study investigated *N*-reacetylated oligochitosan (MW ≤ 11 kDa) with DA from 1 to 35 % for antibacterial activity against *E. coli* and *S. aureus*. The maximum activity for oligochitosan was found for DA from 16 to 28 % (Blagodatskikh, Kulikov et al. 2017).

The degree of substitution (DS) is one of the main factors to consider for the structure-activity relationship of chitosan derivatives. In general, it should be expected that the biological effect of a substituent will increase with the DS, but the possibility that there is some optimal value cannot be excluded either. In a study reported by our group, chitosan and chitooligomer were *N,N,N*-trimethylated with the degree of quaternization ranging from 0 – 70 %. The degree of *N,N*-dimethylation, *N*-monomethylation, and *O*-methylation also varied in the samples. The antibacterial activity was investigated against *S. aureus* at pH 5.5 and 7.2. The significant activity showed against *S. aureus* at pH 5.5. At pH 7.2, non-quaternized derivatives were inactive, but their highly *N*-quaternized derivatives showed MIC as low as 8 µg/mL (Rúnarsson, Holappa et al. 2007). Chitosan-arginine derivatives with DS ranging from 8.7 to 28.4 % have been studied for antibacterial activity against *S. aureus* and *E. coli*. The results showed that the higher DS of the CS-*N*-Arg samples, the better the antimicrobial ability (Xiao, Wan et al. 2011). In another study, *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan synthesized with five different DS values from 0.09 to 0.86 and evaluated against plant pathogenic bacteria *Erwinia carotovora*, *Ralstonia solanacearum*, *Rhodococcus fascians*, and *Rhizobium radiobacter*. The highest DS derivative was more active than the lowest DS derivative (Badawy and Rabea 2016). Peng et al. synthesized hydroxypropyl chitosan derivatives with DS ranging from 1.5 to 3.1. These derivatives showed no antibacterial activity against *S. aureus* and *E. coli,* but DS correlated less with enhanced antifungal activity (Peng, Han et al. 2005). A series of guanidinylated chitosan derivatives synthesized with DS ranging from 0.1 to 1.0 and chitosan *N*-acyl trimethylammonium derivatives with DS 0.15 to 1.0 were tested for antibacterial activity against *S. aureus* and *E. coli;* the activity increased with DS up to 0.55,

and then activity reached a plateau in most cases (Sahariah, Óskarsson et al. 2015).

The structure, charge, size, and physicochemical properties of the substituents added to make chitosan derivatives are very important when considering the structure-activity relationship. Rúnarsson et al. investigated the antibacterial activity of quaternary *N*-(2-(*N*,*N*,*N*-trimethylammiumyl)acetyl) chitosan, *N*,*N*-dimethyl-*N*-dodecylammoniumyl and *N*,*N*-dimethyl-*N*-butylammoniumyl derivatives of chitosan against *S. aureus, S. aureus* (MRSA) and *E. faecalis, E. coli* and *P. aeruginosa*. They have increased alkyl chain length with improved activity for the monomer, in contrast to chitosan polymer derivatives, where long alkyl chains reduced the activity (Rúnarsson, Holappa et al. 2010). *N,N*-dialkyl, and mono *N*-alkyl chitosan derivatives synthesized and then quaternized with different alkyl chain lengths and investigated antibacterial activity against Gram-positive *S. aureus* and *E. faecalis* and Gram-negative *E. coli* and *P. aeruginosa*. The short alkyl chain derivatives showed high activity against *S. aureus,* whereas more hydrophobic *N*-hexyl derivatives were most active against *E. coli* and *E. faecalis* (Sahariah, Benediktssdóttir et al. 2015). The activity of chitosan acyl thiourea derivatives against *E. coli, P. aeruginosa, S. aureus*, and *Sarcina sp* (Gram-positive cocci bacteria) was investigated. The results show that most acyl thiourea derivatives were more active than native chitosan; the MIC value against *E. coli* was 15.62 μg/mL. In addition, the antibacterial activity of these derivatives against *E. coli, P. aeruginosa* was stronger than against *S. aureus* and *Sarcina sp* (Zhong, Xing et al. 2008).

1.6 Synthesis of chitosan derivatives

The *C*-2 amino group in chitosan is more nucleophilic than the *C*-2 and *C*-6 hydroxyl groups. Modifications based on reactions with electrophiles such as alkyl halides, carboxylic acids, and epoxides often target this group (Másson 2021). This reaction will take place under basic conditions. *N,N,N*-trialkylation of chitosan to give TMC is possible by reaction with methyl iodide or another methylation reagent (**Fig. 4**). Product of this reaction will be partially substituted with *N*-methyl, *N,N,*-dimethyl, *C*-3-*O*-methylated, and *C*-6-*O*-methylated also produced (Curti, de Britto et al. 2003).

Various approaches have been used to control the specificity and regioselectivity of chitosan modification reactions to avoid side reactions and polymer chain degradation. One example is the condensation between the reaction carbonyl (aldehyde and ketone) groups and the *C*-2 amino group to form imines (Schiff bases) (Antony, Arun et al. 2019). (**Fig. 4)**. The imine (C=N) bond is easily

reduced with a suitable reducing agent, such as sodium cyanoborohydride (Verheul, Amidi et al. 2008), to get *N*-alkyl derivatives. Mono and dialkyl chitosan derivatives can be synthesized by such reductive alkylation. These products can then be converted by reaction with alkyl halides to give *N,N,N*-trialkylated quaternized derivatives (Benediktsdóttir, Gaware et al. 2011). The *C*-2 amino group of chitosan can react with carboxylic acids in the presence of 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) activator under mild conditions to give *N*-acylated products (Khan, Ullah et al. 2016). The chitosan amino group can also be reacted with acyl halides to obtain *N*-acylated chitosan derivatives (**Fig. 4)** (Rúnarsson, Holappa et al. 2010).

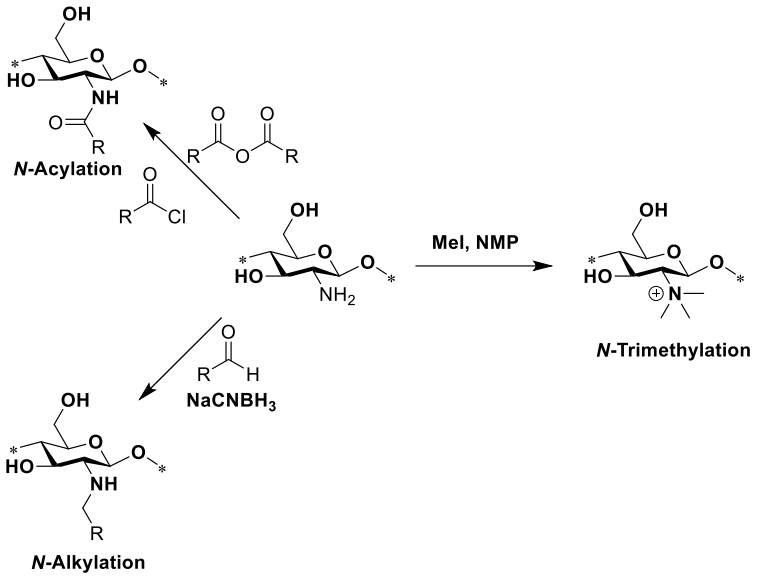


Figure 4. Synthesis of *N*-Trimethylation, Alkylation, and acylation derivatives.

1.6.1 Structural elucidation of chitosan derivatives

Many published studies reporting new chitosan derivatives only use IR spectroscopy or elemental analysis for structure elucidation, which is probably not sufficient to confirm successful synthesis. The studies often also fail to provide information about DA and MW. With such limited structure information, the value of the biological data, for example, antimicrobial activity, is questionable. Therefore, it is important to provide NMR data with a detailed interpretation to confirm the actual structure and gel permeation chromatography analysis to determine the molecular weight.

1.7 Synthesis of chitosan derivatives employing protection groups

Chemoselective (*N/O* selective) and regioselective modifications of chitosan are possible by adjusting the reaction conditions. Still, as detailed in the previous section, achieving a fully selective reaction can be challenging, especially when the DS is high. Protection groups, commonly used in the organic synthesis of small molecules, have been introduced to achieve this objective (Ágoston, Streicher et al. 2016). The advantage of using protection groups in chitosan chemistry is that it allows selective modification of the polymer and can also improve solubility in organic solvents (Kurita, Ikeda et al. 2002, Már Másson 2013). This is especially useful when basic conditions are needed as this is incompatible with the solubility of chitosan aqueous solutions. The disadvantage of this approach is that more than one deprotection step may be required to fully remove the protection groups from all monomer units in the polymer chain. This can reduce yield and molecular weight due to the degradation of the polymer chain. The most common approaches are phthaloyl protection of the amino groups, triphenylmethyl (Trityl) protection of the *C*-6 hydroxyl group, and TBDMS protection of *C*-3 and *C*-6 hydroxy groups.

1.7.1 Phthaloyl protection

The amino group of chitosan can be protected by a reaction with phthalic anhydride in *N,N*-dimethylformamide (DMF) to give phthaloyl chitosan. This conversion will also improve solubility in organic solvents (**Scheme 3**) (Nishimura, Kohgo et al. 1991, Kurita, Ikeda et al. 2002). The phthaloyl protection is beneficial to aiding regioselective modification of the *C*-6 position. (Kurita 2006). *N*-phthaloyl-chitosan has thus been used as a precursor for the regioselective introduction of ortho ester of d-mannose at the *C*-6 position (Kurita, Shimada et al. 1998). The deprotection to remove phthalate from the amino and *O*-acetyl groups is achieved by treatment with hydrazine. This method has been used to synthesize chitosan 1,2,3-triazole derivatives (Gao, Zhang et al. 2009, Ifuku, Wada et al. 2011) and *O*-alkylated chitosan derivatives (Liu, Wang et al. 2018). The selective introduction of *N,N,N*-trimethylammonium triazole moieties (**Scheme 3)** was employed successfully at the *C*-6 position through click chemistry. *N*-phthaloyl chitosan is one of the precursors to preparing selective modification of chitosan derivatives (Gao, Zhang et al. 2009). The derivative 6-amino-6-deoxychitosan, where the 6-hydroxy groups in chitosan have been substituted by amino groups, was obtained by *N*-phthalate protection, tosylation on C-6 hydroxyl group, azidation, and finally reduction of azide group

to an amine. This derivative was active against *S. aureus*, *E. coli*, *P. aeruginosa*, and *A. niger* (Yang, Cai et al. 2012).

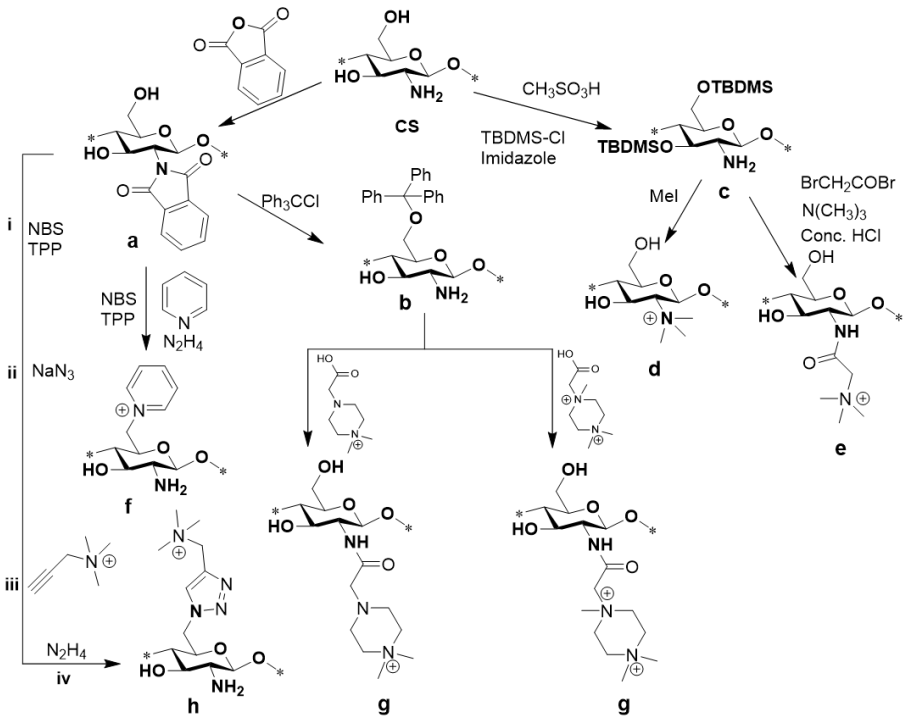
1.7.2 Trityl protection

Triphenylmethyl (trityl) is used to protect the *C*-6 hydroxyl group in carbohydrate chemistry. This group is introduced by reacting trityl chloride with phthaloyl chitosan and then deprotecting with hydrazine to obtain *C*-6 *O*-trityl chitosan. Trityl-protected chitosan has been used to synthesize *N*-acylated chitosan derivatives such as *N*-acyl, betaine derivatives (Kurita 2006), and *N*-alkylated chitosan derivatives (Rúnarsson, Holappa et al. 2007). Trityl chitosan has also been used to connect laminin pentapeptide was conjugated to chitosan. The chitosan peptide shows better inhibitory activity against experimental lung metastasis of B16BL6 melanoma cells in mice (Nishiyama, Yoshikawa et al. 2000). Prepare quaternary chitosan piperazine derivatives using the *C*-6 *O*-trityl chitosan method (**Scheme 3)** (Holappa, Nevalainen et al. 2006). The *N*-phthalyl protection and *C*-6 *O*-trityl protection strategies were used for the chemoselective conjugate of chitosan with L-leucine (Boc-L-leucine hydroxysuccinimide ester) in pyridine; the conjugates were utilized in drug delivery applications (Muhsin, George et al. 2014).

1.7.3 TBDMS protection

The synthesis of trimethylsilyl ether derivatives of starch, amylose, amylopectin, and glycogen polysaccharides using hexamethyldisilazane dissolved in formamide as the silylating reagent was first reported nearly a half-century ago (Harmon, De et al. 1973). Later, full *O*-trimethylsilylation of chitin was successfully achieved with hexamethyldisilazane and chlorotrimethylsilane in pyridine (Keisuke, Masaaki et al. 1999). Trimethylsilylated chitin exhibited higher reactivity than native chitin. It was evaluated as a reagent for reactions with triphenylmethyl chloride and acetic anhydride (Kurita, Sugita et al. 2005). The trimethylsilylated chitin was found to be readily soluble in acetone and pyridine but insoluble in other organic solvents. The trimethyl ethers are rather unstable and have limited use, but other silyl ether are commonly used as protection groups. (Már Másson 2013). To perform TBDMS protection reaction on chitosan. Rúnarsson et al. (Rúnarsson, Malainer et al. 2008) introduced the first *O*-selective protection on chitosan using *tert*-butyldimethylsilyl chloride (TBDMS-Cl) as a reagent. In the reported procedure, chitosan was converted to chitosan mesylate salt, which is soluble in DMSO solvent. The protection was carried out by chitosan salt in DMSO in the presence of TBDMS-Cl and imidazole. The TBDMS-protected chitosan is soluble in an organic solvent such as

dichloromethane and chloroform (Rúnarsson, Malainer et al. 2008). The TBDMS chitosan could be used in durable superhydrophobic films (Song, Gaware et al. 2010). and for chemoselective alkylation and acylation of the amino group. The deprotection to remove the TBDMS can be done by treatment with hydrochloric acid or *tetra*-n-butylammonium fluoride (TBAF) (quarternary ammonium salt) in methanol(Gaware, Håkerud et al. 2013). The TBDMS chitosan has been used for fully selective synthesis of *N*-alkyl, *N,N*-dialyl, *N,N,N*-trialkyl, and *N*-acyl chitosan derivatives (**Scheme 3)** with a 100% degree of substitution (DS) (Benediktsdóttir, Gaware et al. 2011, Sahariah, Benediktssdóttir et al. 2015). This method is very beneficial and has been used by Mássons research group to synthesize chitosan derivatives which have been evaluated for antibacterial activity (Sahariah, Gaware et al. 2014, Sahariah, Benediktssdóttir et al. 2015, Sahariah, Óskarsson et al. 2015) and drug delivery applications (Gaware, Håkerud et al. 2013, Benediktsdóttir, Gudjónsson et al. 2014).



Scheme 3. Synthesis of chitosan derivatives by using protection strategies.

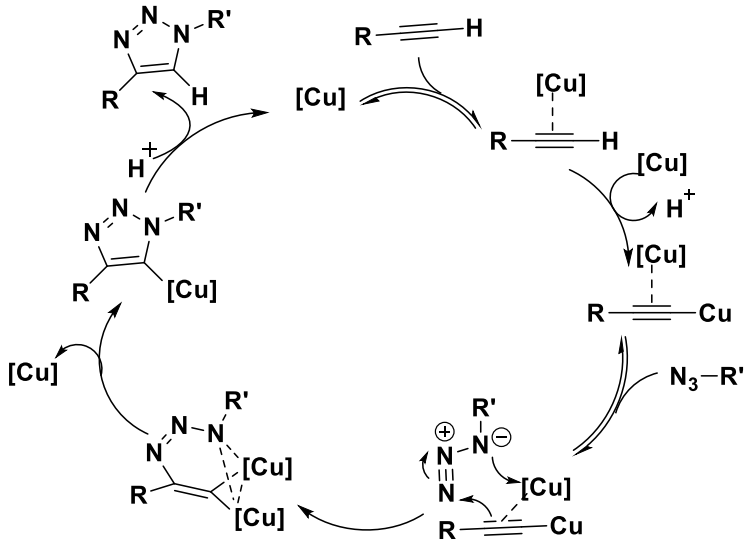
Ref **a**: (Kurita, Ikeda et al. 2002), Ref **b**: (Nishimura, Kohgo et al. 1991), Ref **c**: (Rúnarsson, Malainer et al. 2008), Ref **d**: (Benediktsdóttir, Gaware et al. 2011), Ref **e**: (Sahariah, Gaware et al. 2014), Ref **f**: (Hu, Meng et al. 2016), Ref **g**: (Holappa, Nevalainen et al. 2006), Ref **h**: (Gao, Zhang et al. 2009).

1.8 Click Chemistry

"Click Chemistry" is a term that K.B. Sharpless introduced in 2001. The term describes an organic reaction that gives a very high yield and product selectivity to form carbon-carbon or carbon-heteroatom bonds (Kolb, Finn et al. 2001).

Figure 5. Click chemistry by using CuAAC reactions

The Nobel price in chemistry in 2022 was awarded to scientists K. Barry Sharpless, Morten Meldal, and Carolyn R. Bertozzi for their development of click chemistry and biorthogonal chemistry. The click is a term used to describe a reaction where two molecules are quickly joined together to produce a single molecule, like fastening two straps together in a seat-belt buckle (Rostovtsev, Green et al. 2002, Tornøe, Christensen et al. 2002). More than 100 years ago, Posner reported that thiols could react by addition to carbon-carbon double bonds (Posner 1905). This work has been frequently cited, and the reaction is

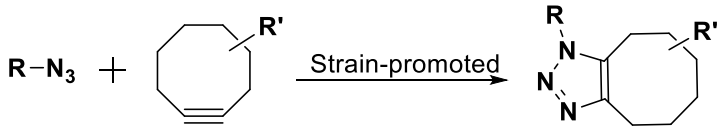


Scheme 4. Mechanism of the Copper-catalyzed azide-alkyne cycloaddition reaction

commonly used, especially in biochemistry, and nowadays, sometimes referred to as thiol-ene click chemistry (Hoyle and Bowman 2010, Lowe 2010).

One of the most widely employed click reactions is the copper (I) catalyzed azide-alkyne cycloaddition reaction (CuAAC). This reaction joins an azide and a terminal alkyne to produce 1,2,3-triazole five-membered ring derivatives (**Fig. 5**) (Tornøe, Christensen et al. 2002). The reaction gives a high conversion yield without by-products in practically any solvent, including most organic and aqueous solvents requiring only simple purification. It can also be performed at a wide range of temperatures (0-160 °C) and over a wide pH range. The reaction mechanism (**Scheme 4**) (Worrell, Malik et al. 2013) involves the formation of π-alkynyl with copper, followed by the complexation of azide by a copper π-coordinated triple bond. After cyclization, a metallacycle is formed, followed by reductive elimination to afford the relevant 1,2,3-triazole (Himo, Lovell et al. 2005, Rodionov, Fokin et al. 2005). CuAAC has impacted many research fields, such as polymer chemistry (Nielsen, Wintgens et al. 2010), biochemistry (Best 2009), medicinal chemistry (Tron, Pirali et al. 2008, Hou, Liu et al. 2012), and surface chemistry (Yaakov, Chaikin et al. 2017). Because of its advantages and increased potential applications relative to other chemical transformations, it has become popular in many fields. This reaction proceeds under various conditions and accommodates multiple functional groups to provide quantitative yield and stereospecific product.

Another reaction called strain-promoted [3 + 2] cycloaddition click reaction (Kim and Koo 2019), also known as copper-free click reaction (**Scheme 5**), is a bioorthogonal reaction to overcome the cytotoxicity of the CuAAC reaction due to Cu(I) (Agard, Baskin et al. 2006). In the copper-free reaction, cyclooctenes are activated by ring strain and electron-withdrawing groups. The strain-

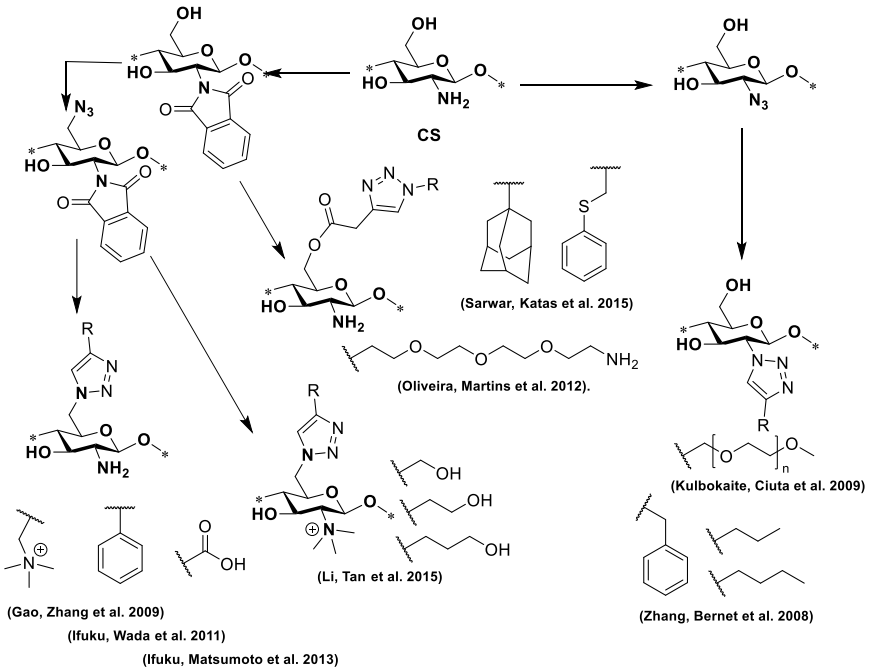


Scheme 5. Strain-promoted azide-alkyne cycloaddition reaction

promoted click reaction does not require toxic reagents and has therefore been used in the metabolic incorporation of an azide and alkyne probe into a biomolecule (Agnew, Buck et al. 2008).

1.9 CuAAC Click chemistry for the synthesis of chitosan derivatives and conjugates

The CuAAC has been used to synthesize chitosan derivatives containing 1,2,3-triazole groups. Small molecules, antimicrobial peptides, and other biological



Scheme 6. Synthesis of chitosan derivatives by using CuAAC reaction

molecules have been linked to chitosan using the click chemistry procedure. The triazole moiety has been introduced to the *C*-6 position or *C*-2 amino group by various approaches. Cationic chitosan derivative with 6-*N*,*N*,*N*-trimethyltriazole groups was synthesized via click chemistry performed on *N*-phthaloyl-protected chitosan. The 6-*N*,*N*,*N*-trimethyltriazole-chitosan (**Scheme 6**) could bind strongly to DNA and increase cellular uptake of DNA to transfect HEK 293 cells and MDA-MB-468 cells (Gao, Zhang et al. 2009).

The synthesis of chitosan-1,2,3-triazole derivatives has also been performed by CuAAC reaction. First, the *N*-phthaloyl chitosan and the *C*-6 hydroxyl group were converted to azidation through bromination. Then the azide was successfully converted to chitosan-1,2,3-triazole with ethynyl compounds having hydroxymethyl and phenyl groups (**Scheme 6**) (Ifuku, Wada et al. 2011). The same research group has published the synthesis of other chitosan derivatives via click reaction (Ifuku, Matsumoto et al. 2013).

Sarwar et al. reported that chitosan triazolyl derivatives on *C*-6 hydroxyl position via CuAAC, five different chitosan derivatives were synthesized, and the chitosan triazolyl derivatives and their nanoparticles showed enhanced antibacterial and antifungal activities (Sarwar, Katas et al. 2015). The chitin *C*-6 hydroxyl group was successfully converted to azide by nucleophilic substitution, and then the β-cyclodextrin was conjugated via click reaction (chitin-6-cyclodextrin).

Deacetylation was then carried out to obtain chitosan-6-cyclodextrin (Chen, Ye et al. 2015). The chitosan *C*-6 hydroxyl group was converted to azide by bromination, and then click reaction, a chitosan-1,2,3-triazolyl ring containing aliphatic alcohol (**Scheme 6**) with various chain lengths was used as a functional group to enhance the antifungal activity. These derivatives show antifungal activity increased with increasing hydrophobic chain lengths (Li, Tan et al. 2015). The chemical modification of the trimethyl chitosan-1,2,3-triazole group exhibited increased radical scavenging activity than unmodified chitosan (Li, Sun et al. 2018). The preparation of chitosan *O*-prop-2-ynyl carbamate was achieved by *N*-phthaloyl protection; the PEG-azide (**Scheme 6**) was conjugated to the alkynylated polymer through copper-catalyzed Huisgen 1,3-dipolar cycloaddition (Oliveira, Martins et al. 2012).

Zhang et al. investigated the conversion of the amino group in chitosan to azide, prepared chitosan azide via *C*-6 *O*-trityl protection, and then Click reaction by using various alkynes to obtain chitosan-1,2,3-triazole derivatives, the derivatives were insoluble, so the structure was confirmed by solid-state NMR (Zhang, Bernet et al. 2008). The chitosan azide was prepared from four different methods by using azide epichlorohydrin, sodium azide with sodium nitrite, trifluoromethane sulfonyl azide, and imidazole-1-sulfonyl azide hydrochloride. The degree of azidation was calculated by FTIR, and the range was 28 – 65 %; the *N*-azidated chitosan conversion above 60 % was insoluble in aqueous and organic solvents but dissolved in 5% LiCl solution in *N*-methyl-2-pyrrolidone (NMP). The click reaction was carried out by chitosan azide and methoxy poly(ethylene glycol) alkyne (**Scheme 6**) (Kulbokaite, Ciuta et al. 2009). The synthesis of *N*-azidated chitosan in the *C*-2 position and then conversion of triazole derivatives obtained by using Cu (I) catalyzed cycloaddition reaction of azide with various alkynes (aromatic and aliphatic chain lengths) (Zhang, Bernet et al. 2008).

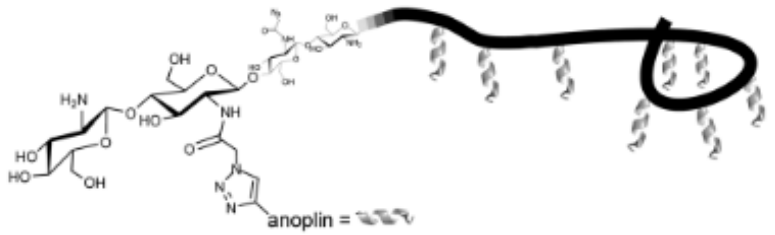


Figure 6. Chitosan antimicrobial peptide conjugation through CuAAC reaction (Sahariah et al. 2015).

There were some reports for the chitosan peptide derivatives, the conjugation of antimicrobial peptide (**Fig. 6**) to chitosan backbone was successfully performed by click reaction as mentioned in section 1.4 (Sahariah, Sørensen et al. 2015, Barbosa, Vale et al. 2017, Barbosa, Costa et al. 2019).

2 Aims

The project aimed to develop antimicrobial conjugates of the biocompatible biopolymer chitosan derived from chitin. This investigation of chitosan conjugates was expanded in the project to include conjugates of short peptides and other cationic compounds. The synthesis was to be based on TBDMS protection to allow chemoselective modification and precise control of the degree of substitution and direct modification of chitosan amino-functional moieties. These materials were to be tested for activity against clinically essential strains of Gram-positive and Gram-negative bacteria. A detailed investigation of the structure-activity relationship was also to be carried out in this study.

The project utilized a newly developed "click chemistry" procedure for efficient one-pot synthesis of chitosan conjugates with different functional groups linked through aromatic 1,2,3-triazole moiety in the C-2 amino group position on the biopolymer. The conjugates were synthesized in different functional moieties such as cationic, anionic, and neutral side chains, contributing to improved aqueous solubility and enhanced antimicrobial activity. The click chemistry strategies were used to conjugate the antimicrobial peptide (CRAMP-18) on the chitosan biopolymer, and these derivatives were evaluated for activity against Gram-positive bacteria and Gram-negative bacteria.

The six studies listed below were undertaken to successfully achieve these aims of the study.

I. The first study is the synthesis of cationic chitosan derivatives like TMC, TACin, and TMC/DMC by utilizing TBDMS and Boc protection strategies at different degrees of substitution and investigating the antibacterial activity to determine the structure-activity relationship.

II. The synthesis of common chitosan derivatives with cationic, anionic, and neutral moieties. Then investigation of the structure-antibacterial activity relationship for common chitosan derivatives.

III. Optimization and synthesis of chitosan-1,2,3-triazole (chitotriazolan) derivatives via click reaction using starting TBDMS chitosan and native-chitosan and investigation of antibacterial activity.

IV. Further extension of III that involved the synthesis of partially substituted chitotriazolan starting from common chitosan derivatives. Then the remaining *C*-2 amino group will be converted to the 1,2,3-triazole group on chitosan structure and evaluate antibacterial activity.

V. The fifth study was the synthesis of a series of quaternary and basic water-soluble chitosan-1,2,3-triazole derivatives to investigate the structure-activity relationship and antimicrobial activity.

VI. The final study was the conjugation of antimicrobial peptides (CRAMP-18) on chitosan via click reaction strategies and investigation of antimicrobial activity.

3 Experimental section

3.1 Materials

Chitosan (S160302-1-2-3-4, Degree of acetylation 17% and Mw 108 kDa) and (TM3623) was provided by Primex ehf Siglufjördur, Iceland. Reagent grade methanesulfonic acid, acetic acid, tert-butyldimethylsilyl chloride (TBDMS-Cl), imidazole, bromoacetyl bromide, acetyl chloride, trimethylamine solution, hydrochloric acid, di-tert-butyl dicarbonate, N-Methyl-2-pyrrolidone (NMP), cesium carbonate (Cs_2CO_3), sodium chloride (NaCl), iodomethane, formic acid, formaldehyde solution, sodium hydroxide (NaOH), glycidyltrimethylammonium chloride (GTMAC), propylene oxide, thioglycolic acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC), chloroacetic acid, sodium azide, sulfuryl chloride, copper (II) sulfate pentahydrate ($CuSO_4$ $5H_2O$, purity ≥98.0%), sodium ascorbate, propargyl bromide, *N*-methylpropargylamine, *N,N*-dimethylpropargylamine, 3-butynoic acid, 3-methyl-1-pentyn-3-ol, 2-methyl-3-butyn-2-ol, 3-butyn-2-ol, sodium sulfite, *N,O*-bis(trimethylsilyl)acetamide, tris(trimethylsilyl) phosphite, 4-bromo-1-butyne, triethylamine, triethanolamine, diethanolamine, piperazine, *N*-methylpiperazine, 1,4-dimethylpiperazine, pyridine, 1-methylimidazole, potassium carbonate, sodium sulphate, trifluoracetic acid, 4- pentynoic acid, N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) amino acids, *N,N*-dimethylformamide (DMF), *N*-methyl-pyrrolidone (NMP), *N*-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate N-oxide (HBTU), 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), piperidine and *N,N*-diisopropylethylamine (DIPEA), tris-hydroxypropyltriazolylmethylamine (THPTA) were purchased from Sigma Aldrich (Germany). All solvents, including dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), isopropyl alcohol, acetone, methanol, ethanol, and acetonitrile were also obtained from Sigma Aldrich. De-ionized water produced from tap water using a Milli-Q™ filtration system. Dialysis membranes (RC, Spectra/Por, MW cutoff 3500 Da 45 mm) were purchased from Spectrum® Laboratories Inc. (Rancho Dominguez, USA) and Pur-A-Lyser Mega 3500 dialysis kit.

3.2 Methods and Characterization

3.2.1 NMR Spectroscopy

The chitosan derivatives were characterized by ^{1}H NMR and 2D NMR techniques, COSY and HSQC. ^{1}H and most COSY NMR spectra were recorded on a Bruker Avance 400 spectrophotometer operating at 400 MHz. The ^{13}C NMR and HSQC spectra were recorded on a Bruker 500 MHz spectrometer equipped with a cryoprobe at the University of Copenhagen, Denmark. NMR samples were prepared in $CDCl_3$, D_2O, or D_2O/DCl in concentrations of 8-15 mg/mL. The samples were measured at 298 K. The *N*-acetyl peak at 2.08 ppm was used as an internal reference in all proton NMR spectra of chitosan derivatives and conjugates.

3.2.2 DS Calculation

The degree of substitution (DS) and acetylation (DA) for all chitosan derivatives was calculated based on the integral values of relevant peaks in the ^{1}H NMR spectra. The following equations were used to calculate the DS and DA for TACin polymers.

$A_1 = \int(H_2\text{-}H_6)/6$

$DS = \int TM/(A_1 \times 9)$

$DA = \int Ac/(A_1 \times 3)$

Where ∫H2 – H6 is the integral of hydrogen at *C*-2 to *C*-6 (observed as a multiplet at 3.54 to 3.83 ppm) in the glucosamine and acetylglucosamine units; ∫TM is integral of the 9 protons in the *N,N,N*-trimethyl group (singlet at 3.33 ppm) and ∫Ac is the integral of the 3 protons in the *N*-Acetyl group (singlet at 2.08 ppm).

The following equations were used to determine the DS and DA for $TMC_{NH2/TM}$ polymer and the relative number (D_{NH2}) of unmodified glucosamine monomer units with primary amino groups.

$A_2 = \int TM_2/9 + \int H_{2\ Pri\ NH2} + \int Ac/3$

$DS = \int TM_2/(A_2 \times 9)$

$D_{NH2} = \int H_{2\ Pri\ NH2}/(A_2)$

$DA = \int Ac/(A_2 \times 3)$

Where $\int TM_2$ is the integral of *N,N,N*-trimethyl group (singlet at 3.35 ppm) and $\int H_{2\ pri\ NH2}$ is the integral of the *C*-2 hydrogen peak (observed at 2.9 ppm) on monomers with primary amino groups.

The following equations evaluate the DS, DDM and DA for $TMC_{DM/TM}$ polymer.

$A_3 = \int TM_2/9 + \int DM/6 + \int Ac/3$

$DS = \int TM_2/(A_3 \times 9)$

$DDM = \int TM_2/(A_3 \times 6)$

$DA = \int Ac/(A_3 \times 3)$

$\int DM$ is the integral of *N,N*-dimethyl group (observed as a broad singlet at 2.6-2.8 ppm) DDM is the degree of dimethylation.

The following equations are used for evaluating the DS and DA for HTC polymer.

$A_1 = (\int H_3\text{–}H_6^1 - \int TM_3/9 - \int Ac/3)/5$

$DS = \int TM_3/(A_1 \times 9)$

$DA = \int Ac/(A_1 \times 3)$

Where $\int H_3\text{–}H_6^1$ is the integral of hydrogen at *C*-3 to *C*-6 (observed as a multiplet at 3.67 to 3.86 ppm) in the glucosamine unit as well as the C**H**(OH) proton from the HTC side chain and *C*-2 hydrogen for *N*-acetylated units; TM_3 is the integral of *N,N,N*-trimethyl group of glycidyl trimethylammonium 9 protons (singlet at 3.25 ppm).

The following equations are used for evaluating the DS and DA for HPC polymer.

$A_2 = (\int H_3\text{–}H_6^2 - \int M_{HP}/3 - \int Ac/3)/5$

$DS = \int M_{HP}/(A_2 \times 3)$

$DA = \int Ac/(A_2 \times 3)$

Where $\int H_3\text{–}H_6^2$ is the integral of hydrogen at *C*-3 to *C*-6 (observed as a multiplet at 3.69 to 4.05 ppm) in the glucosamine unit as well as the C**H**(OH) proton from the HPC side chain and *C*-2 hydrogen for N-acetylated units; M_{HP} is the integral of hydroxypropyl methyl proton (singlet at 1.19 ppm).

The following equations evaluate the DS and DA for TGC polymer.

$A_3 = (\int H_3 – H_6^3 – \int Ac/3)/5$

$DS = \int Ac_{TG}/(A_3 \times 2)$

$DA = \int Ac/(A_3 \times 3)$

Ac_{TG} is the integral of thioglycolic acyl protons (singlet peak at 2.1 ppm). The DS for the CMC polymer was calculated according to the equation previously reported in the literature (M El-Nesr, Raafat et al. 2014).

3.2.3 IR Spectroscopy

The infrared (IR) spectra of the CS and chitosan derivatives were measured by Thermo Scientific™ Nicolet™ iZ10 FT-IR spectrometer with provided "Omnic" software in the 500-4000 cm^{-1} wavelength regions at room temperature. The set number of scans was 64, and the resolution was 4.0 cm^{-1}. A few milligrams of the sample material were used for each IR spectra, and all compounds were measured against a blank background.

3.2.4 Gel permeation chromatography

Average Molecular weight (Mw) determination was carried out using gel permeation chromatography (GPC). GPC measurements were done using the Polymer Standards Service (PSS) (GmbH, Mainz, Germany) Dionex Ultimate 3000 HPLC system (Thermo Scientific-Dionex Softron GmbH, Germering, Germany), Dionex Ultimate 3000 HPLC pump, and Dionex Ultimate 3000 autosampler (Thermo Scientific-Dionex Softron GmbH, Germering, Germany), Shodex RI-101 refractive index detector (Shodex/Showa Denko Europe GmbH, Munich, Germany), PSS's ETA-2010 viscometer and MALLS detector (PPC SLD 7100). WINGPC Unity 7.4 software (PSS GmbH, Mainz, Germany) was used for data collection and processing. A series of three columns [PSS Novema 10 µ guard (50 × 8 mm), PSS Novema 10 µ 30 Å (150 × 8 mm) and PSS Novema 10 µ 1000 Å (300 × 8 mm)] (PSS GmbH, Mainz, Germany) were used in the HPLC system. Ready Cal-Kit Pullulan standards with M_p (180-708000 Da) from PSS (GmbH, Mainz, Germany) were used for calibration. The eluent used was 0.1 M NaCl/0.1% TFA solution. Each sample was dissolved in the same eluent as mentioned above at a concentration of 1 mg/mL at 25 °C using a flow rate of 1 mL/min. Each sample had an injection volume of 100 µL, and the time between injections was 30 min.

3.2.5 Solubility analysis

The polymer solubility was measured in water at room temperature. The CS and

their derivatives were weighed, and milli-Q water pipetted to give a 2.5% concentration (12.5 mg/0.5 mL). The sample solutions were stirred at room temperature for 1 h to 24 h to dissolve the solid material. Samples that, by visual inspection, were not fully dissolved (became a gel or were partially soluble) were tested again at lower concentrations (1.25, 0.625, and 0.416%). Materials were recorded as soluble when the sample was visually observed as a clear solution with no undissolved material detected. The chitotriazolan derivatives were tested in distilled water and samples measured at 8 mg/mL, and soluble material was noted by visualization.

3.2.6 Antibacterial assay

Minimal inhibition concentration (MIC) was measured according to the Clinical and Laboratory Standards Institute (CLSI) standard (CLSI 2009). The antibacterial activity was tested against different bacterial species, Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300) the American Type Culture Collection (ATCC) *Staphylococcus aureus* (*S. aureus)*, (ATCC 29213), *Enterococcus faecalis* (*E. faecalis), (*ATCC *29212),* and Gram-negative bacteria *Escherichia coli* (*E. coli)*, (ATCC 25922), *and Pseudomonas aeruginosa (P. aeruginosa), (*ATCC *27853)*. Prior to MIC testing, the bacterial strains were cultured on blood agar at 37 °C for 12-18 h. The bacterial colonies were suspended in saline water, adjusted to 0.5 McFarland, and further diluted in Mueller-Hinton broth (MHB) to reach a final concentration of 5×10^5 colony-forming units (CFU) /mL in the test wells. The MHB media was used for MIC measurement at pH 7.2. Gentamicin is a well-known antibiotic used as a performance control, broth is a sterility control, and broth with the bacterial solution is a growth control. The stock solution of compounds was prepared in sterile water at an 8192 µg/mL concentration. Serial dilution was performed, and 50 µL of the sample was added to 50 µL of MHB using microtiter 96-well plates, including the controls. When the dilutions were done, 50 µL of bacterial 5×10^5 (CFU)/mL suspension was added to each well. The microtiter plates were incubated at 37 °C for 20 to 24 h. The MIC values were determined as the lowest concentrations of the antibacterial agent to completely inhibit the visible growth of microorganisms in the microtiter 96-well plate, as observed by the naked eye. For minimum lethal concentration (MLC) measurement, 10 µL × 2 of each dilution that showed no visible growth was plated on an agar plate and incubated at 35 °C for 20 – 24 h. MLC was defined as the lowest concentration that achieved a 99.9% decrease in viable cells.

3.3 Synthesis

3.3.1 Di-3,6-OTBDMS chitosan (TBDMS chitosan)

Chitosan (5.0 g, 29.58 mmol) was dissolved in 0 °C methanesulfonic acid (48 mL, 739.5 mmol) and 50 mL of water. The mixture was stirred for 30 min in an ice bath until a clear solution was obtained. Then an excess of ethanol (100 ml) was added, and the gel-like precipitated was filtered, washed with acetone, and dried under suction for over one h. The crude material was dissolved in water, re-precipitated with acetone, and washed with acetone (3 × 100 mL). The material was dried under a vacuum for six h at 40 °C to obtain chitosan mesylate salt as a white powder (**A1**). ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.83 (CH_3SO_3), 3.20 (H2), 3.75-3.94 (H3, H4, H5, H6), 4.88 (H1).

Chitosan mesylate salt (**A1**) (2.5 g, 8.04 mmol) was dissolved in dry DMSO (30 mL) at room temperature until a clear solution was obtained. In another round bottom flask, TBDMS-Cl (6.05 g, 40.20 mmol) and imidazole (9.30 g, 136.7 mmol) were dissolved in DMSO (30 mL). This solution was added to the chitosan mesylate solution and stirred vigorously. After completing the addition of the reagent, the reaction mixture turned into solid gel material and was stirred at room temperature for 16 h. The crude material was filtered through sintered funnel washed with water (excess) and acetonitrile (3 × 100 mL). The material was dried for one h in the suction and then dried under vacuum for six h at 40 °C to obtain white powder TBDMS – Chitosan (**A2**). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.11-0.24 (Si [$(CH_3)_2$]), 0.94-1.00 ([$(CH_3)_3$]) 2.08 (*N*-$COCH_3$), 2.82 (H2), 3.41-3.95 (H3, H4, H5, H6), 4.39 (H1).

3.3.2 TACin synthesis via acetylation (1a – 1f)

TBDMS-Chitosan (**A2**) (0.50 g, 1.25 mmol) was dissolved in dichloromethane (15 mL), and the solution cooled to –-20 °C. Triethylamine (0.87 mL, 6.3 mmol) bromoacetyl bromide (0.011 mL, 0.125 mmol for **1a**, 0.027 mL, 0.314 mmol for **1b**, 0.093 mL, 1.06 mmol for **1c**, 0.131 mL, 1.51 mmol for **1d**, 0.508 mL, 2.51 mmol for **1e**, 0.87 mL, 10.07 mmol for **1f**) was added. After 5 mins, acetyl chloride (0.18 mL, 2.5 mmol) was added at -20 °C under the N_2 atmosphere. The reaction mixture was stirred at -20 °C for one h and then concentrated under reduced pressure on a rotary evaporator. The crude product was stirred with acetonitrile, filtered on a sintered filter funnel, and washed with acetonitrile. The solid material was dried under suction. The dry material was dissolved in CH_2Cl_2 and extracted with water and brine solution. The organic layer was dried over sodium sulfate (Na_2SO_4) and concentrated under reduced pressure to afford yellow solid

intermediate **A3** ($BrCH_2COTBDMS$-chitosan).

Trimethylammonium solution (31-35 % in ethanol, 5 mL) was added, at room temperature, to a solution of intermediate **A3** (0.30 g) in dichloromethane (15 mL). The reaction mixture was stirred at room temperature for 24 h. After that, the reaction mixture was concentrated under reduced pressure to obtain crude intermediate **A4** ($(CH_3)_3NCH_2CO$-TBDMS-chitosan$^+$ Br^-). The crude intermediate **A4** was used for the next step without purification and analysis.

Conc. HCl (2 mL) was added to a solution of intermediate **A4** (0.40 g) in methanol (10 mL). The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was diluted with deionized water (10 mL) and ion-exchanged by adding (10% w/v) sodium chloride (NaCl) (15 mL). The resulting mixture was stirred at 25 °C for one h, followed by dialysis in cellulose membrane tubing with a molecular weight cut-off at 3.5 kDa. The solution was dialyzed against (5% w/v) NaCl solution for one day and then against deionized water for two days (the media was changed twice per day) and freeze-dried to afford the product. Yield: 90 mg for **1a**, 100 mg for **1b**, 215 mg for **1c**, 275 mg for **1d**, 275 mg for **1e**, 280 mg for **1f** ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 3.35 [$(CH_3)_3$], 3.54-3.88 (H2, H3, H4, H5, H6), 4.17 (*N*-CH_2-), 4.66 (H1).

3.3.3 Quaternized *N,N,N*-trimethyl chitosan and *N,N*-dimethyl chitosan

3.3.3.1 $TMC_{NH2/TM}$ (2a-2f)

TBDMS-Chitosan (**A2**) (0.60 g, 1.51 mmol) was dissolved dichloromethane (20 mL). Triethylamine (0.42 mL, 3.0 mmol) was added at 0 °C and di-*tert*-butyl dicarbonate (0.347 mL, 1.5 mmol for **2a**, 0.243 mL, 1.05 mmol for **2b**, 0.144 mL, 0.623 mmol for **2c**, 0.104 mL, 0.453 mmol for **2d**, 0.034 mL, 0.15 mmol for **2e**, 0 mL for **2f**) was added at 0 °C under the N_2 atmosphere. The reaction mixture was stirred at room temperature for 16 h. The crude mixture was concentrated under reduced pressure on a rotary evaporator and dried under a high vacuum to afford the solid intermediate **A5**.

Intermediate **A5** (0.43 g) was dissolved in NMP (10 mL), and cesium carbonate (1.33 g, 4.0 mmol) was added. The reaction mixture was stirred for three h, followed by the addition of iodomethane (0.31 mL, 5.1 mmol). This solution was stirred at 50 °C. After 24 and 48 h, additional iodomethane (0.31 mL) was added to the reaction, and the stirring continued at 50 °C for 48 h. The solution

was dialyzed against deionized water for four days and freeze-dried to obtain the intermediate **A6**.

Conc. HCl (2 mL) was added to a solution of intermediate **A6** (0.300-0.400 g) in methanol (10 mL) at room temperature. The reaction mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with deionized water (10 mL) and ion-exchanged by adding (10% w/v) NaCl (15 mL). The resulting mixture was stirred at 25 °C for one h, followed by dialysis in cellulose membrane tubing with a molecular weight cut-off at 3.5 kDa. The solution was dialyzed against (5% w/v) NaCl solution for one day and then against deionized water for two days (the media was changed twice per day) and freeze-dried to afford the $TMC_{NH2/TM}$. Yield: 215 mg for **2a**, 235 mg for **2b**, 160 mg for **2c**, 215 mg for **2d**, 240 mg for **2e**, 200 mg for **2f**, (^{1}H NMR (400 MHz, D_2O)): δ 2.08 (*N*-$COCH_3$), 2.85 (H2), 3.35 [$(CH_3)_3$], 3.69-3.95 (H3, H4, H5, H6), 5.46 (H1, H1′).

3.3.3.2 $TMC_{DM/TM}$ – Chitosan (3a-3e)

Chitosan (2.0 g, 11.83 mmol) was dissolved in formic acid (7 mL). Then 35% formaldehyde (10 mL) and 40 mL of deionized water were added. The reaction mixture was stirred at 70 °C with reflux for five days. Then, the slightly yellow viscous solution was formed, and it was concentrated by rotary evaporation, and then the pH was increased to 12 by adding 1 M sodium hydroxide (NaOH) solution to the crude solution causing gel formation. The gel was washed with deionized water and filtered to remove impurities. Then the material was dissolved in deionized water at a pH adjusted to 4 (1 M HCl solution), followed by dialysis in cellulose membrane tubing with a molecular cut-off of 3.5 kDa. The dialysis media was deionized water for three days (the media was changing twice per day). Finally, the product was freeze-dried to afford *N,N*-dimethyl chitosan (DMC). ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.99 [*N*$(CH_3)_2$], 3.26 (H2), 3.57-4.19 (H3, H4, H5, H6), 5.05 (H1).

DMC (200 mg) was dissolved in 40 ml of deionized water, and the pH was adjusted to 11 with NaOH causing gel formation. The gel was washed with water and finally three times with acetone. Next, DMC was suspended in NMP (50 mL) and iodomethane (0.1 mL for **3a**, 0.5 mL for **3b**, 1.0 mL for **3c**, 1.5 mL for **3d**, 2.0 mL for **3e**) was added. The dispersion was stirred at 40 °C for 30 h. Then ethanol and diethyl ether (50:50) (150 mL) was added. The TMC was precipitated out and washed with diethyl ether, and dried completely. To perform ion exchange, TMC was dissolved in 100 mL of an aqueous 10% NaCl solution and stirred overnight. Finally, the TMC was dialyzed against deionized

water for three days by changing the buffer twice per day and then freeze-dried to afford the product. Yield: 165 mg for **3a**, 170 mg for **3b**, 210 mg for **3c**, 215 mg for **3d**, 130 mg for **3e**, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.89 [*N*$(CH_3)_2$], 2.99 (H2), 3.36 [*N*$(CH_3)_3$], 3.74-4.47 (H3, H4, H5, H6), 5.4 (H1).

3.3.4 HTC (4a – 4f)

Chitosan (1.0 g, 5.91 mmol) was dissolved in deionized water (40 mL) then glycidyltrimethylammonium chloride (0.8 mL, 5.9 mmol for **4a**, 1.58 mL, 11.83 mmol for **4b**, 2.38 mL, 17.74 mmol for **4c**, 3.17 mL, 23.66 mmol for **4d**, 3.9 mL, 29.58 mmol for **4e**, 4.76 mL, 35.49 mmol for **4f**) was added under the nitrogen atmosphere and the reaction mixture stirred at 50 °C for 24 h. After this, the reaction mixture was poured into cold acetone to form a white precipitate. The crude precipitate was filtered through the sintered funnel and washed with methanol to remove excess GTMAC and allow the material to dry under suction for one h, further drying it in a vacuum oven at 40 °C for six h. The HTC was dissolved in water and hydrochloric acid, and the solution was dialyzed against deionized water for three days by changing the water twice per day and then freeze-dried to obtain a white solid. Yield: 1.08 g for **4a**, 1.16 g for **4b**, 1.34 g for **64c**, 1.33 g for **4d**, 1.46 g for **4e**, 1.24 g for **4f**, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.58–2.96 (H2, *N*-CH_2), 3.25 [*N*$(CH_3)_3$], 3.44–3.79 (H3, H4, H5, H6), 4.34 (CH_2), 4.57 (H1).

3.3.5 Hydroxypropyl chitosan (5a – 5f)

Chitosan (0.5 g) was alkalized by adding 5 mL of 30% NaOH aqueous solution and stirred at room temperature for two h. Then the reaction mixture was kept at -18 °C for 24 h. After that, the mixture was thawed, isopropyl alcohol 15 mL was added and stirred at room temperature for 1 h, then propylene oxide (0.5 mL for **5a**, 1.0 mL for **5b**, 3.0 mL for **5c**, 6.0 mL for **5d**, 10.0 ml for **5e**, 15.0 mL for **5f**) was added with stirring over a time. The suspension was further stirred at 45 °C for 16 h. The resulting product was neutralized by the addition of hydrochloric acid and dialyzed using a cellulose tube against deionized water for four days, then freeze-dried to afford colorless fluffy HPC. Yield: 545 mg for **5a**, 525 mg for **5b**, 540 mg for **5c**, 580 mg for **5d**, 670 mg for **5e**, 690 mg for **5f**, ^{1}H NMR (400 MHz, D_2O): δ 1.14–1.27 (HP-CH_3), 2.08 (*N*-$COCH_3$), 3.20–4.14 (H2, H3, H4, H5), 4.91 (H1).

3.3.6 Thiolated chitosan (6a – 6b)

Chitosan (1.0 g, 5.92 mmol for **6a**, 0.5 g, 2.95 mmol for **6b**) was dissolved in

dilute HCl (100 mL, 0.05 M) to obtain 1% solution of chitosan hydrochloride. 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (1.10 g, 7.10 mmol) was added to the under-stirring solution, and subsequently, thioglycolic acid (0.41 mL, 5.91 mmol) was added at room temperature. The pH was adjusted to 5 by the addition of NaOH (0.5 M), and the reaction mixture was stirred at room temperature for five h, followed by dialysis in cellulose membrane tubing with a molecular cutoff of 3.5 kDa (4 days). The dialysis media were applied as follows: Day 1, HCl (5 mmol); days 2 and 3, HCl (5 mmol) containing 1% NaCl; day 4, HCl (1 mmol). The medium was exchanged twice per day. Afterward, the product was lyophilized and stored at 4 °C in the dark. Yield: 1.15 g for **6a,** 0.77 g for **6b**, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.10 (TG-CH_2) 3.20 (H2), 3.56–3.93 (H3, H4, H5, H6), 4.90 (H1).

3.3.7 Carboxymethylated chitosan (7a – 7e)

To a solution of chloroacetic acid (2.80 g, 29.58 mmol) in deionized water (30 mL), the resulting mixture was adjusted to pH 7.0 with NaOH (1 M) solution. Then chitosan (1.0 g, 5.92 mmol) was added to the solution. The reaction mixture was heated to 90 °C with vigorous stirring and maintained for four h. During the reaction, the pH was controlled at pH 7.0 by adding a 20% aqueous sodium carbonate (Na_2CO_3) solution every 30 min. Then the solution was filtered through the sintered funnel, washed with ethanol (3 × 50 mL), and allowed to dry under suction for one h. The material was further dry in a vacuum oven at 40 °C for eight hours to afford the product. The material was dialyzed against water for four days and lyophilized to get the pure material ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 3.20 (H2), 3.33 (*N*-CH_2), 3.74–4.02 (H3, H4, H5, H6), 4.63 (H1).

Chitosan (1.0 g, 6.08 mmol) was alkalized by 50% NaOH solution (18 mL) and stirred at room temperature for 24 h after that filtered. The alkalized chitosan was taken in the flask, and in another flask monochloroacetic acid (2.5 g, 26.5 mmol) was dissolved in isopropanol (13.0 mL). This solution was added to the alkalized chitosan under the nitrogen atmosphere over some time. The reaction mixture was stirred at room temperature for 1 to 24 h. The pH of the solution was adjusted to 7 using a 2.5 M HCl solution. The material was dialyzed against water for four days and lyophilized to get the pure material (1 h for **7b**, 4 h for **7c**, 8 h for **7d**, 24 h for **7e**). ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 3.21 (H2), 3.42 (*N*-CH**2**), 3.78–3.94 (H3, H4, H5, H6), 4.27–4.36 (O-CH_2), 4.91 (H1).

3.3.8 Preparation of imidazole sulfonyl azide hydrochloride salt (A7)

The imidazole sulfonyl azide hydrochloride salt (**A7**) was prepared following a previously published procedure (Goddard-Borger and Stick 2007). Warning: Imidazole sulfonyl azide hydrochloride salt is explosive. Handle with care.

Synthesis of *N*-propargyl *N,N,N*-trimethylammonium bromide salt (A8): The title compound was synthesized according to a previously published procedure (Nguyen, Fournier et al. 1999).

Synthesis of sodium salt of prop-2-yne-1-sulfonate (A9): The molecule was synthesized according to a reported procedure (Ouadahi, Allard et al. 2012).

Synthesis of but-3-yn-1-yl phosphonate (A10): This compound was synthesized by the following procedure reported in a previous publication (Wanat, Walczak et al. 2015).

3.3.9 TBDMS-Chitosan azide (A11)

TBDMS-chitosan (500 mg, 1.26 mmol) was dissolved in 15 mL of DCM and 15 mL of MeOH. After that, imidazole sulfonyl azide hydrochloride (**A7**) (0.395 g, 1.89 mmol) and triethylamine (Et_3N) (0.26 mL, 1.89 mmol) were added to the solution. A solution of $CuSO_4$ $5H_2O$ (31 mg, 0.125 mmol dissolved in 1 mL water) was added to the reaction mixture. The color of the reaction mixture changed to a blue tinge, and the product started to precipitate. The reaction was further stirred at room temperature for 60 h under an N_2 atmosphere. The material was concentrated under reduced pressure. A precipitate was filtered, washed with ethanol, and dried for more than one hour by suction. The resulting material had a light bluish color, and the product formation was confirmed by IR spectroscopy.

3.3.10 TBDMS- Chitosan 4-(*N,N*-dimethylaminomethyl) triazole (A12)

TBDMS-Chitosan azide (**A11**) (700 mg, (1.75 mmol) was stirred into DMF (20 mL). Then $CuSO_4$ $5H_2O$ (56 mg, 0.23 mmol in 2.5 mL water) and sodium ascorbate (174 mg, 0.87 mmol in 2.5 mL water) were added to the reaction mixture, followed by *N,N*-dimethylamino-1-propyne (0.94 mL, 8.76 mmol) under nitrogen atmosphere. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for three days and freeze-dried. Full conversion of starting material to the product was confirmed by the absence of the azide peak in the FT-IR.

3.3.11 4-(*N,N*-dimethylaminomethyl) chitotrizazolan (8)

TBDMS-Chitosan 4-(*N,N*-dimethylaminomethyl) triazole (**A12**) (600 mg) was dissolved in methanol (30 mL) and Conc. HCl (5 mL was diluted with 10 mL of methanol) was added slowly. The reaction mixture was then stirred at room temperature for 24 h. After that, the reaction mixture was dialyzed against water for three days (first day 5% NaCl, next two days water) and then freeze-dried. Yield: 325 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.81 (H6'), 2.95 [*N*-$(CH_3)_2$], 3.14 (H6), 3.52 (H5), 3.77 (H4), 3.94 (H3) 4.40 (H2), 4.56 (triazole CH_2), 5.17 (H1), 8.46 (triazole CH).

3.3.12 TBDMS-Chitosan 4-(*N,N,N*-trimethylamoniummethyl) triazole (A13)

TBDMS-Chitosan azide (**A11**) (350 mg, 0.87 mmol) was stirred in DMF (10 mL). Then $CuSO_4$ $5H_2O$ (28 mg, 0.11 mmol in 2.5 mL water) and sodium ascorbate (86 mg, 0.44 mmol in 2.5 mL water) were added to the reaction mixture, followed by *N*-propargyl-*N,N,N*-trimethylammonium bromide (**A8**) (429 mg, 4.38 mmol) under nitrogen atmosphere. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for three days and freeze-dried. Full conversion of starting material to the product was confirmed by the absence of the azide peak in the FT-IR.

3.3.13 4-(*N,N,N*-trimethylamoniummethyl) chitotriazolan (9)

TBDMS-Chitosan 4-(*N,N,N*-trimethylamoniummethyl) triazole (**A13**) (250 mg) was stirred in methanol and Conc. (25 mL, and Conc. HCl (4 mL was diluted with 5 mL of methanol) was added slowly. The reaction mixture was stirred at room temperature for 24 h. After that, the reaction mixture was dialyzed against water for three days (first day 5% NaCl, next two days water) and then freeze-dried. Yield: 185 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.90 (H6'), 3.20 [H6, *N*-$(CH_3)_3$], 3.52 (H5), 3.77 (H4), 4.44 (H3), 4.58 (H2), 4.76 (triazole CH_2), 5.18 (H1), 8.58 (triazole CH).

3.3.14 Chitosan azide (A14)

Chitosan (500 mg, 2.958 mmol) was dissolved in 40 mL of 0.1 M HCl solution, then $NaHCO_3$ (0.248 g, 1.0 equiv) was added to the solution, and the mixture was stirred vigorously for 30 mins. After that, imidazole sulfonyl azide hydrochloride (**A7**) (0.93 g, 4.437 mmol) and sodium bicarbonate ($NaHCO_3$) (2.48 g 10.0 equiv) were added slowly in small portions. Then a solution of copper (II) sulfate pentahydrate ($CuSO_4$ $5H_2O$) (95 mg, 0.384 mmol) in 1 mL of

water and 10 mL of methanol solution was added to the reaction mixture. The reaction mixture was turned to bluish color and was stirred at room temperature for 24 h. Finally, the material was precipitated out from the solution using acetone. The precipitate was filtered and washed with water five times and acetone. The product was dried, and the presence of the azide group was confirmed by IR spectroscopy.

3.3.15 Chitotriazolan (10 – 18)

Chitosan azide (**A14**) (1 equiv) was dissolved in DMSO (15 mL) at 50 °C. Then $CuSO_4$ $5H_2O$ (0.13 equiv in 2.5 mL water) and sodium ascorbate (0.5 equiv. in 2.5 mL water) were added to the reaction mixture, followed by the addition of alkynes (5.0 equiv) under nitrogen atmosphere. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for three days (first day 5% NaCl, following two days water) and freeze-dried. The products were confirmed by FT-IR to show that the azide peak (at 2109 cm^{-1}) had completely disappeared and by ^1H NMR when solutions in D_2O could be prepared.

3.3.16 Synthesis of derivative (10)

Chitosan azide (**A14**) (200 mg, 1.07 mmol) and *N*-propargyl-*N,N,N*-trimethylammonium bromide (**A8**) (523 mg, 5.34 mmol), derivative **10** was obtained. Yield: 270 mg, ^1H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.90 (H6′), 3.20 [H6, *N*$(CH_3)_3$], 3.52 (H5), 3.78 (H4), 4.44 (H3), 4.58 (H2), 4.77 (triazole CH_2 was merging with D_2O peak), 5.18 (H1) 8.59 (triazole CH).

Derivative (11): Chitosan azide (**A14**) (200 mg, 1.0686 mmol) and sodium salt of prop-2-yne-1-sulfonate (**A9**) (759 mg, 5.343 mmol), derivative **11** was obtained Yield: 230 mg**.** ^1H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.77 (H6′), 3.10 (H6), – 3.51 (H5), 3.75 (H4), 4.40 – 4.54 (H2, H3, triazole CH_2), 5.10 (H1) 8.27 - 8.44 (broad triazole CH).

Derivative (12): Chitosan azide (**A14**) (200 mg, 1.0686 mmol) and sodium prop-2-yn-1-ylphosphonate (636 mg, 5.343 mmol), derivative **12** was obtained. Yield: 340 mg**.** The NMR was not obtained because of partial solubility.

Derivative (13): Chitosan azide (**A14**) (200 mg, 1.0686 mmol) and sodium but-3-yn-1-ylphosphonate (1.25 g, 5.343 mmol), derivative **13** was obtained. Yield: 336 mg**.** The NMR was not obtained because of partial solubility.

Derivative (14): Chitosan azide (**A14**) (200 mg, 1.0686 mmol) and *N*-methylpropargylamine (0.45 mL, 5.343 mmol), derivative **14** was obtained. Yield: 190 mg**.** The structure was confirmed by IR spectroscopy, but NMR was not obtained because of poor solubility.

Derivative (15): Chitosan azide (**A14**) (200 mg, 1.0686 mmol) and 3-butynoic acid (449 mg, 5.343 mmol), derivative **15** was obtained. Yield: 220 mg**.** The structure was confirmed by IR spectroscopy, but NMR was not obtained because of poor solubility.

Derivative (16): Chitosan azide (**A14**) (200 mg, 1.0686 mmol) and 3-methyl-1-pentyn-3-ol (0.6 mL, 5.343 mmol), derivative **16** was obtained. Yield: 220 mg**.** The structure was confirmed by IR spectroscopy, but NMR was not obtained because of poor solubility.

Derivative (17): Chitosan azide (**A14**) (200 mg, 1.0686 mmol) and 2-methyl-3-butyn-2-ol (0.52 mL, 5.343 mmol), derivative **17** was obtained. Yield: 190 mg. The structure was confirmed by IR spectroscopy, but NMR was not obtained because of poor solubility.

Derivative (18): Chitosan azide (**A14**) (200 mg, 1.0686 mmol) and 3-butyn-2-ol (0.42 mL, 5.343 mmol), derivative **18** was obtained. Yield: 195 mg**.** The structure was confirmed by IR spectroscopy, but NMR was not obtained because of poor solubility.

3.3.17 Azides of common chitosan derivatives

The synthetic procedure was followed by our recently reported procedure (**A14**). The azide group was confirmed by IR spectroscopy at 2109 cm^{-1}. The common chitosan azide yield: 95 mg for trimethyl chitosan azide (**A15**), 125 mg for TAC azide (**A16**), 140 mg for HTC azide (**A17**), 280 mg for HPC azide (**A18**), and 190 mg for CMC azide (**A19**).

3.3.18 Mixed chitotriazolan (19 – 23)

The azides of common chitosan derivatives (**A15**, **A16**, **A17**, **A18**, and **A19**) were dissolved in DMSO at 50 °C. Then $CuSO_4$ $5H_2O$ (in 2.5 mL water), sodium ascorbate (in 2.5 mL water), and *N*-propargyl-*N,N,N*-trimethylammonium bromide (520 mg) (**A8**) were added to the reaction mixture under nitrogen atmosphere. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for four days (Ion exchanged by 5

% NaCl) and freeze-dried. The product was confirmed by FT-IR, the complete disappearance of the azide peak at 2109 cm^{-1}.

TMC-4-(*N,N,N*-trimethylamoniummethyl) chitotriazolan (**19**): Yield: 27 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.92 (H6′$_{(tr)}$), 3.21 (*N*-$(CH_3)_{3(tr)}$], 3.32 [*N*-$(CH_3)_{3(gl)}$], 3.57 – 4.01 (H2$_{(gl)}$, H5-H6$_{(gl)}$)H4-H6$_{(tr)}$), 4.22 (H3), 4.47 (H2), 4.75 (triazole CH_2 merges with the HDO peak), 5.41 (H1$_{(tr)}$, H1$_{(gl)}$), 8.60 (triazole $CH_{(tr)}$). The (gl) subscript identifies peaks assigned to the alkylated glucosamine monomer, and (tr) identifies peaks assigned to the triazolyl-2-deoxyglucose monomers.

TAC-4-(*N,N,N*-trimethylamoniummethyl) chitotriazolan (**20**): Yield: 100 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.91 (H6′$_{(tr)}$), 3.20 [*N*-$(CH_3)_{3(tr)}$], 3.35 [*N*-$(CH_3)_{3(ga)}$], 3.56 – 4.07 (H2-H6$_{(ga)}$, H4-H6$_{(tr)}$), 4.20 (TAC-$CH_{2(ga)}$), 4.65 ($CH_{2(tr)}$), 5.20 (H1$_{(tr)}$), 8.61 (triazole $CH_{(tr)}$). The (ga) subscript identifies peaks assigned to the acylated glucosamine monomer.

HTC-4-(*N,N,N*-trimethylamoniummethyl) chitotriazolan (**21**): Yield: 170 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.57 – 2.90 (H6′$_{(tr)}$, *N*-$CH_{2(gh)}$), 3.19 [*N*-$(CH_3)_{3(tr)}$], 3.25 [*N*-$(CH_3)_{3(gh)}$], 3.44 – 3.96 (H2-H6$_{(gh)}$, H4-H6$_{(tr)}$), 4.34 (–$CH_{2(gh)}$), 4.59 (triazole $CH_{2(tr)}$), 5.19 (H1$_{(tr)}$), 8.59 (triazole $CH_{(tr)}$). The (gh) subscript identifies peaks assigned to the HTC glucosamine monomer.

HPC-4-(*N,N,N*-trimethylamoniummethyl) chitotriazolan (**22**): Yield: 220 mg,^{1}H NMR (400 MHz, D_2O): δ 1.10 – 1.19 (HPC-$CH_{3(gh)}$), 2.08 (*N*-$COCH_3$), 2.79 – 2.93 (H6′, H2$_{(gh)}$), 3.20 [*N*-$(CH_3)_{3(tr)}$], 3.52 – 3.97 (H3-H6$_{(gh)}$), H4-H6$_{(tr)}$), 4.41 – 4.57 (triazole CH_2, H3$_{(tr)}$), 5.18 (H1$_{(tr)}$), 8.59 (triazole $CH_{(tr)}$). The (gh) subscript identifies peaks assigned to the HPC glucosamine monomer.

CMC-4-(*N,N,N*-trimethylamoniummethyl) chitotriazolan (**23**): Yield: 215 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.84 – 4.01 [triazole *N*-$(CH_3)_{3(tr)}$, H2-H6$_{(gc)}$, H4-H6$_{(tr)}$], 4.36 – 4.58 (H3$_{(tr)}$, triazole $CH_{2(tr)}$ was merging with HD_2O peak), 5.18 (H1$_{(tr)}$), 8.58 (triazole $CH_{(tr)}$). The (gc) subscript identifies peaks assigned to the CMC glucosamine monomer.

Chitosan azide (**A20**)**:** The synthesis of chitosan azide (**A20**) was followed by the synthesis of derivative **A14**. The azide group was confirmed by IR spectroscopy at 2109 cm^{-1}.

3.3.19 Synthesis of different alkynes (A8, A21 – A28)

N,N,N-trimethylprop-2-yn-1-aminium (**A8**), *N,N,N*-tris(2-hydroxyethyl)prop-2-yn-1-aminium (**A22**), 1,4-dimethyl-1-(prop-2-yn-1-yl)piperazin-1-ium (**A23**), and 2,2′-

(prop-2-yn-1-ylazanediyl)bis(ethan-1-ol) (**A28**) were synthesized by according to derivative **A8**. Other alkynes were synthesized by following reported procedure *N,N,N*-triethylprop-2-yn-1-aminium (**A21**) and 1-methyl-3-(prop-2-yn-1-yl)-1H-imidazol-3-ium (**A25**) (Gaitor, Paul et al. 2017), 1-(prop-2-yn-1-yl)pyridin-1-ium (**A24**) (Kanitskaya, Elokhina et al. 2002), 1-methyl-4-(prop-2-yn-1-yl)piperazine (**A26**), 1-(prop-2-yn-1-yl)piperazine (**A27**) (Karoli, Mamidyala et al. 2012).

3.3.20 Chitotriazolan derivatives (24 - 32)

The synthesis of triazole conversion from chitosan azide was followed by our previous **3.3.15** section. Briefly, the chitosan azide (**A20**) (200 mg, 1.07 mmol) was dissolved in DMSO at 50 °C. Then $CuSO_4$ $5H_2O$ (34 mg in 2.5 mL water), sodium ascorbate (106 mg in 2.5 mL water), and different alkynes 523 mg of **A8** for **24**, 749 mg of **A21** for **25**, 1.005 g of **A22** for **26**, 818 mg of **A23** for **27**, 631 mg of **A24** for **28**, 646 mg of **A25** for **29**, 738 mg of **A26** for **30**, 497 mg of **A27** for **31**, 764 mg of **A28** for **32** were added to the reaction mixture under nitrogen atmosphere. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for four days (Ion exchanged by 5 % NaCl) and freeze-dried. The product was confirmed by FT-IR, the complete disappearance of the azide peak at 2109 cm^{-1}

Chitotriazolan derivative (**24**): Yield: 250 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.90 (H6′), 3.20 [H6, *N*$(CH_3)_3$], 3.52 (H5), 3.78 (H4), 4.44 (H3), 4.58 (H2), 4.77 (triazole CH_2 was merging with D_2O peak), 5.18 (H1) 8.59 (triazole CH).

Chitotriazolan derivative (**25**): Yield: 320 mg, ^{1}H NMR (400 MHz, D_2O): δ 1.45 [*N*(Ethyl-$CH_3)_3$], 2.08 (*N*-$COCH_3$), 2.89 (H6′), 3.11 (H6), 3.33 [*N*$(CH_2)_3$], 3.49 (H5), 3.77 (H4), 4.42 (H3), 4.52 (H2), 4.66 (triazole CH_2 was merging with D_2O peak), 5.17 (H1) 8.57 (triazole CH).

Chitotriazolan derivative (**26**): Yield: 310 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.88 (H6′), 3.12 (H6), 3.51 (H5), 3.68 [(H4), [(*N*$(CH_2$-$CH_2OH)_3$] 4.21 [(*N*$(CH_2$-$CH_2OH)_3$] 4.41 (H3), 4.56 (H2), 5.03 (triazole CH_2), 5.14 (H1) 8.56 (triazole CH).

Chitotriazolan derivative (**27**): Yield: 315 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.44 (*N*-CH_3) 2.90 – 3.04 [(H6′), (H6), Pip-3,5-CH_2] 3.16 quaternary-*N*(CH_3), 3.54 – 3.60 [(H5), quaternary-Pip-2,6-CH_2] 3.78 (H4), 4.42 (H3), 4.57 (H2), 4.88 (triazole CH_2 was merging with D_2O peak), 5.16 (H1) 8.59 (triazole CH).

Chitotriazolan derivative (**28**): Yield: 305 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.59 (H6′), 3.88 (H6), 3.38 (H5), 3.69 (H4), 4.38 (H3), 4.51 (H2), 5.02 [(H1) was merging with D_2O peak)], 6.06 (triazole CH_2), 8.15 [pyridine (C3-H & C5-H)], 8.49 (triazole CH), 8.63 [pyridine (C4-H)], 9.05 [pyridine (C2-H & C6-H)].

Chitotriazolan derivative (**29**): Yield: 275 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.67 (H6′), 2.94 (H6), 3.44 (H5), 3.71 (H4), 3.90 (imidazole *N*-CH_3), 4.42 – 4.56 [(H3), (H2)], 5.10 (H1), 5.64 (triazole CH_2), 7.49 – 7.58 [imidazole (C1-H, C4-H, C5-H)], 8.41 (triazole CH).

Chitotriazolan derivative (**30**): Yield: 260 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.79 – 3.12 [(H6′), (H6), *N*(CH_3)], 3.34 – 3.94 [(Pip-2,3,5,6-CH_2) (H5), (H4)], 4.39 (H3), 4.56 (H2), 4.75 (triazole CH_2), 5.11 (H1) 8.51 (triazole CH).

Chitotriazolan derivative (**31**): Yield: 150 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.71 – 3.28 [(H6′), (H6), (Pip-2,3,5,6-CH_2)], 3.47 – 3.93 [(H5), (H4), (triazole CH_2)], 4.33 – 4.58 [(H3), (H2)], 5.12 (H1), 8.59 (triazole CH).

Chitotriazolan derivative (**32**): Yield: 215 mg. Due to the solubility issue could not be confirmed by proton NMR, so it was confirmed by IR spectroscopy.

3.3.21 Synthesis of Antimicrobial peptide (pentynoyl-CRAMP-18) (33)

The antimicrobial peptide was constructed by solid-phase peptide synthesis (SPPS) on a Biotage Alstra+ synthesizer by using the standard method Fmoc-protocol using Tendagel S Rink Amide resin with 0.23 mmol/g loading capacity. The resin (0.2 mmol) was placed in a 20 mL syringe rector with a Teflon filter. Swelling and washing the resin with DMF and the Fmoc group's deprotection was carried out at room temperature by treating with 20 % solution piperidine in DMF for 3 mins and followed by 20 % solution piperidine in DMF for 5 mins. The resin was washed with NMP (3 x 5 mL), DCM (3 x 5mL), and NMP (5 x 5 mL) and then dried. The peptide couplings were performed by treating with N^{α} -9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids (4 equiv), HBTU (3.8 equiv), HOBt (4 equiv), and DIPEA (7.8 equiv) were dissolved in NMP then reacted with resin in the shaker at room temperature for 45 mins. After that, the resin was washed with NMP (3 x 5 mL), DCM (3 x 5 mL), and NMP (5 x 5 mL) then dried. The deprotection and coupling were repeated till the final amino acids. The final coupling was peptide modification with pentynoic acid. Then finally, the resin cleavage and side-chain protecting group removal were performed simultaneously by treating with TFA: H_2O (95:5) at room temperature

for 2 hrs. The TFA filtrate was precipitated using diethyl ether, then centrifugated, and collected the peptide. The peptide was purified by a Biotage Selekt flash purification system and a linear gradient flow of CH_3CN-H_2O (0.1% formic acid). (Pentynoyl-KLKKIGQKIKNFFQKLVP) ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.21 – 7.30 (m, 6H of phenyl), 7.15 (d, *J* = 7.7 Hz, 2H of phenyl), 7.06 (d, *J* = 7.2 Hz, 2H of phenyl), 4.55 (t, *J* = 6.9 Hz, 2H), 4.41 – 4.48 (m, 2H), 4.38 (d, *J* = 8.0 Hz, 1H), 4.31 – 4.09 (m, 13H), 4.06 (d, *J* = 8.2 Hz, 1H), 3.94 – 3.73 (m, 4H), 3.66 – 3.58 (m, 3H), 3.04 – 2.99 (m, 1H), 2.91 – 2.82 (m, 15H), 2.65 – 2.55 (m, 2H), 2.44 (s, 2H), 2.32 (s, 1H alkyne CH), 2.28 – 2.17 (m, 5H), 2.08 – 1.80 (m, 10H), 1.77 – 1.05 (m, 50H methylene protons of Lys, Leu, Ile, Pro), 0.90 – 0.75 (m, 30H Methyl protons of Leu, Ile, Val).

Chitosan azide (A29): The synthesis of chitosan azide **(A29)** with a low degree of azidation was prepared by following section 3.3.14. The product was obtained, Yield: 190 mg, and the sharp azide peak was confirmed by IR spectroscopy at 2113 cm^{-1} wavenumbers.

3.3.22 Synthesis of chitosan – CRAMP-18 peptide conjugation via CuAAC reaction (34)

Chitosan azide **(A29)** (10 mg, 0.0534 mmol) was dissolved in DMSO (2 mL) at 50 °C in the sealed tube. Then $CuSO_4$ $5H_2O$ (0.13 equiv. in 0.5 mL water) and sodium ascorbate (0.5 equiv. in 0.5 mL water) were added, and then pentynoyl-CRAMP-18 peptide (119 mg, 0.0534 mmol) was added to the reaction mixture followed by THPTA ligand (23 mg, 0.0534 mmol) was added. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for four days (first day 5% NaCl, following three days water) and freeze-dried. Yield: 30 mg. ^{1}H NMR (400 MHz, D_2O): δ 7.92 (triazole CH), 7.32 – 7.03 (3H peptide phenyl protons), 4.63 – 4.04 (H2, H3, Triazole CH_2, and peptide protons 6H), 3.92 – 3.32 (H4, H5, H6, and peptide protons 7H), 3.05 – 2.83 (H6', and peptide protons 5H), 2.69 – 2.55 (peptide proton 2H), 2.31 – 2.17 (peptide proton 2H), 2.09 – 1.10 (CS-(*N*-$COCH_3$), and peptide protons 17H), 0.94 – 0.73 (peptide proton 7H).

Hydroxypropyl chitosan azide (HPC-N_3) (A30): The synthesis of HP-chitosan azide **(A30)** procedure was followed by section 3.3.14. The product was obtained Yield: 90 mg; the sharp azide peak was confirmed by IR spectroscopy at 2113 cm^{-1} wavenumbers.

3.3.23 Synthesis of HPC-CRAMP peptide conjugation via CuAAC reaction (35)

HPC azide **(A30)** (5 mg, 0.0267 mmol) was dissolved in DMSO (2 mL) at 50 °C in the sealed tube. Then $CuSO_4$ $5H_2O$ (0.13 equiv. in 0.5 mL water) and sodium ascorbate (0.5 equiv. in 0.5 mL water) were added, and then pentynoyl-CRAMP-18 peptide (59 mg, 0.0267 mmol) was added to the reaction mixture, followed by THPTA ligand (12 mg, 0.0267 mmol) was added. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for four days (first day 5% NaCl, following three days water) and freeze-dried. Yield 17 mg. ^{1}H NMR (400 MHz, D_2O): δ 7.94 (triazole CH), 7.32 – 7.03 (1.4H peptide phenyl protons), 4.62 – 4.01 (H2, H3, Triazole CH_2, and peptide protons 4H), 3.96 – 3.22 (H4, H5, H6, and peptide protons 7H), 3.07 – 2.81 (H6′, and peptide protons 3.3H), 2.69 – 2.50 (peptide proton 2H), 2.34 – 2.17 (peptide proton 1H), 2.20 – 1.10 (CS-(*N*-$COCH_3$), and peptide protons 9.2H), 1.18 – 0.98 (HP-CS proton 2.5H), 0.95 – 0.70 (peptide proton 5H).

Chitotriazolan (36): The synthesis of triazole conversion procedure was followed by section 3.3.15. Briefly, Chitosan azide (**A29**) (50 mg, 0.267) was dissolved in DMSO (5 mL) at 50 °C. Then $CuSO_4$ $5H_2O$ (0.13 equiv. in 1 mL water), sodium ascorbate (0.5 equiv. in 1 mL water) and *N*-propargyl-*N,N,N*-trimethylammonium bromide (**A4**) (1.5 equiv.) were added. The reaction mixture was stirred at 50 °C for 48 h. FT-IR spectra confirmed the disappearance of azide peak (at 2113 cm^{-1}). Yield: 45 mg. ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.87 (H6′), 3.20 [*N*$(CH_3)_3$], 3.48 – 4.01 (H4, H5, H6), 4.47 (H3), 4.60 (H2), 4.76 (triazole CH_2 was merging with D_2O peak), 5.19 (H1) 8.59 (triazole CH).

Synthesis of HP-Chitotriazolan (37): Briefly, HPC azide (**A30**) (50 mg, 0.267) was dissolved in DMSO (5 mL) at 50 °C. Then $CuSO_4$ $5H_2O$ (8 mg, 0.13 equiv. in 1 mL water), sodium ascorbate (26 mg, 0.5 equiv. in 1 mL water) and *N*-propargyl-*N,N,N*-trimethylammonium bromide (**A4**) (5 equiv.) were added. The reaction mixture was stirred at 50 °C for 48 h. FT-IR spectra confirmed the disappearance of azide peak (at 2113 cm^{-1}) Yield 37 mg. ^{1}H NMR (400 MHz, D_2O): δ 1.12 – 1.19 (HPC-$CH_{3(gh)}$), 2.08 (*N*-$COCH_3$), 2.60 – 2.90 (H6′, $H2_{(gh)}$), 3.21 [*N*$(CH_3)_{3(tr)}$], 3.56 – 3.92 (H3-$H6_{(gh)}$), H4-$H6_{(tr)}$), 4.43 – 4.77 (triazole CH_2, $H3_{(tr)}$), 5.22 ($H1_{(tr)}$), 8.58 (triazole $CH_{(tr)}$). The (gh) subscript identifies peaks assigned to the HPC glucosamine monomer.

Synthesis of hydroxypropyl chitosan (HPC) (38): The synthetic procedure was followed by section 3.3.5. Yield: 580 mg. ^{1}H NMR (400 MHz, D_2O): δ 1.16 – 1.21 (HP-CH_3), 2.08 (*N*-$COCH_3$), 2.82 – 4.00 (H2, H3, H4, H5, H6), 4.75 (H1 merging with D_2O peak).

4 Results and Discussion

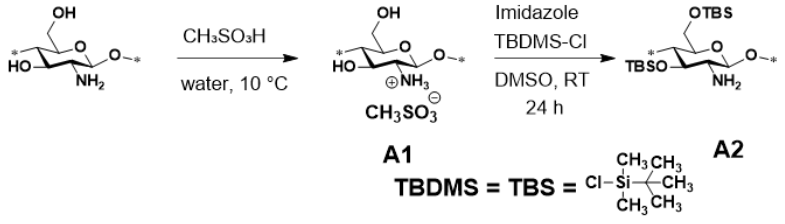
In this chapter, all the results are discussed to give an appropriate compiled summary of the project. These results and discussions are divided into two categories. The first part includes synthesis and characterization (NMR, IR, and molecular weight determination), and the second part mainly focuses on antimicrobial activity.

4.1 Chitosan modification on amino functional group

The Ph.D. study was mainly focused on the chemical modification of chitosan biopolymer, specifically at the *C*-2 position of the amino group, to obtain the chitosan derivatives. The chitosan derivatives were designed to be soluble in an aqueous solution and improved antimicrobial activity relative to native chitosan. Two strategies were used for synthesizing chitosan derivatives, (i) modification of the amino group using TBDMS chitosan as a precursor and (ii) direct modification of the chitosan amino group without using protection groups. Functional groups were introduced using different ratios of reagent and substrate to obtain derivatives with the degree of substitution varying from 0.02 to 1.1. The molecular weights and antimicrobial properties were assessed, as discussed in the later section.

4.1.1 Cationic quaternized chitosan derivatives (Paper I)

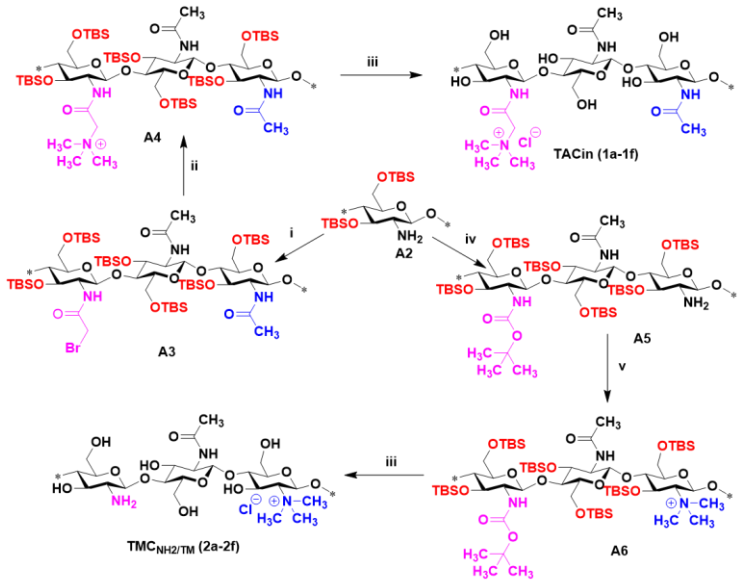
Chitosan was converted to TBDMS-chitosan (**Scheme 7**), which is soluble in dichloromethane and other organic solvents. This was done in two steps. First, chitosan was converted to chitosan-mesylate salt and formulated into a fine



Scheme 7. Synthetic route for the preparation of chitosan mesylate (**A1**) OTBS chitosan (**A2**)

powder that dissolved without difficulty in water and DMSO. In the second step, C-3 and C-6 hydroxyl groups were protected by reaction with TBDMS-imidazole at room temperature.

TBDMS chitosan was then used to synthesize quaternary chitin and chitosan derivatives with degrees of substitution (DS) from 0.1 to 1.0 (**Scheme 8**).



Scheme 8. Synthetic route for the preparation of TACin (**1a-1f**), and TMC$_{NH2/TM}$ (**2a-2f**)

Synthesis of *N*-(2-(*N,N,N*-trimethyl ammoniumyl)acetyl)-chitin (TACin): The procedure was based on a previously reported method for the synthesis of N-(2-(*N,N,N*-trimethyl ammoniumyl)acetyl)-chitosan (TAC) (Sahariah, Gaware et al. 2014) OTBS-chitosan was reacted with bromoacetyl bromide followed by a reaction with trimethylamine. Then all protonable groups were removed by fully acetylating the remaining primary amino groups before deprotection with Conc. hydrochloric acid (HCl) in methanol (**Scheme 8**). The TACin structure was confirmed by proton NMR, and the degree of substitution (DS) was calculated to be from 0.07 to 0.88 (**Table 1)**. The *N,N,N*-trimethyl, and *N*-acetyl peaks in the NMR spectra were observed at 3.3 ppm and 2.08 ppm, respectively (**Fig. 7**). The analysis confirmed that the trimethyl DS was consistent with the bromoacetyl bromide/acetyl chloride ratio and a high degree of *N*-acylation. However, after deprotection, some residual peaks of TBDMS groups were

retained in the structure. The calculated substitution for this group ranged from 0.005 to 0.08 (**Table 1**). Previous work in our research group has shown (Gaware, Håkerud et al. 2013, Gaware, Håkerud et al. 2017) that it may be necessary to repeat the deprotection step to fully remove TBDMS. However, this was not done as it could have caused further degradation of the polymer chain, reducing the molecular weight and yield.

Synthesis of TMC derivatives ($TMC_{NH2/TM}$): The trimethylated chitosan derivatives with 100% DS without *O*-methylation have previously been synthesized using the TBDMS protection strategy (Benediktsdóttir, Gaware et al. 2011). Here, the aim was to synthesize derivatives with partial quaternization and the remaining amino groups unmodified. Therefore, the TBDMS-chitosan was reacted with Boc anhydride in order to protect part of the amino groups. This was followed by a methylation step and a final step deprotection step, whereby the *N*-Boc and TBDMS groups were removed using Conc. hydrochloric acid (HCl) in methanol (**Scheme 8)**.

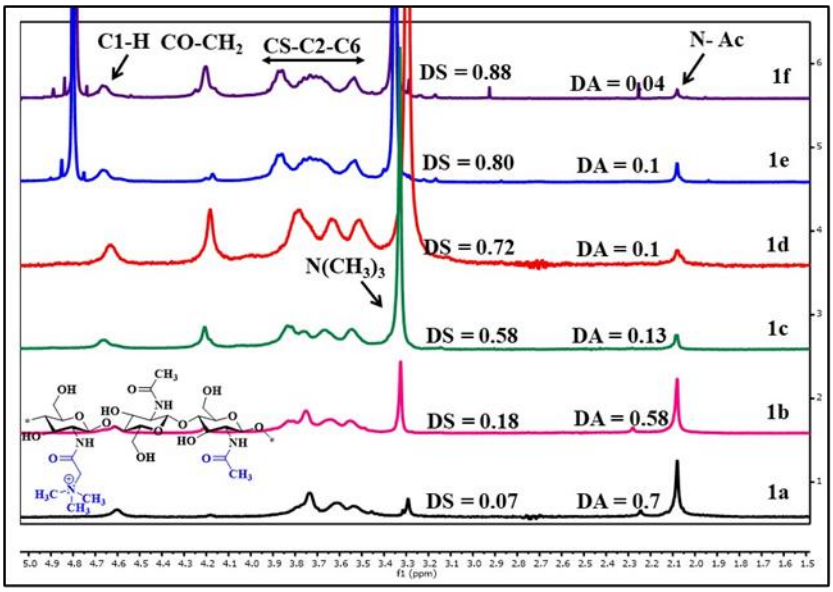


Figure 7. ^{1}H NMR spectra of TACin all compounds (**1a-1f**)

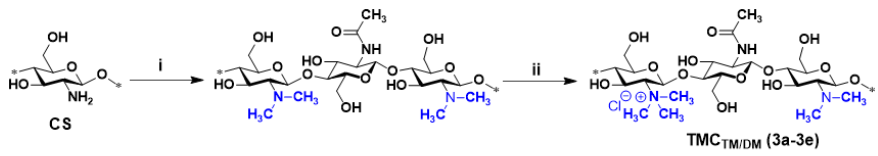
Table 1. Physicochemical properties of chitosan and chitosan derivatives

Compd	DS	DA	D_{NH2} or DDM	Molecular Weight (Da)	Polydispersity Index (D)	TBDMS Residue *
Chitosan	-	0.17	-	1.1×10^5	2.0	-
1a	0.07	0.7	-	1.5×10^4	1.2	0.04
1b	0.18	0.58	-	7.7×10^3	3.8	0.01
1c	0.58	0.13	-	6.4×10^4	2.0	0.01
1d	0.72	0.1	-	7.9×10^4	4.4	0.01
1e	0.8	0.1	-	4.6×10^4	2.8	0.008
1f	0.88	0.04	-	4.7×10^4	2.7	0.005
			D_{NH2}			
2a	0.06	0.1	0.83	2.4×10^4	2.0	0.008
2b	0.25	0.09	0.65	1.0×10^4	1.9	0.018
2c	0.26	0.1	0.63	1.2×10^4	2.8	0.017
2d	0.41	0.09	0.48	1.4×10^4	1.8	0.009
2e	0.53	0.09	0.37	1.2×10^4	1.6	0.012
2f	0.89	0.1	0	3.1×10^4	1.4	0.08
			DDM			
3a	0.18	0.2	0.6	1.7×10^5	2.1	0
3b	0.32	0.19	0.48	1.1×10^5	1.9	0
3c	0.44	0.21	0.33	1.3×10^5	1.9	0
3d	0.34	0.2	0.45	1.0×10^5	1.9	0
3e	0.28	0.19	0.52	8.6×10^4	1.9	0

This allowed us to report, for the first time, partially quaternized TMC that was free of *N,N*-di and *N*-mono methylated primary amino groups. The trimethyl DS ranged from 0.06 up to 0.89 (**Table 1).** The partial quaternization was confirmed by the presence of both the trimethyl peak at 3.3 ppm and the *C*2-H peak at 2.9 pp specifying the presence of a primary amino group (**Fig. 8A**). In contrast, the *C*2-H proton peak is absent in the fully *N,N,N*-trimethylated derivative **2f** (**Fig. 8C**). There was no indication of *N,N*-di, *N*-mono or *O*-methylation peaks in the NMR spectra. The TBDMS group was not completely

removed by a single deprotection step, as in the case of TACin. The Mw of the starting material was 108 kDa, whereas it was 10-31 kDa for $TMC_{NH2/TM}$ after deprotection. (**Table 1**).

$TMC_{DM/TM}$ was synthesized by a previously reported procedure (Xu, Xin et al. 2010). Chitosan was reacted with formaldehyde in formic acid to produce dimethyl chitosan. The obtained dimethyl chitosan was then reacted with different equivalent ratios of iodomethane in NMP to produce $TMC_{DM/TM}$ derivatives. Some of the amino groups in these derivatives were *N,N,N*-trimethylated, and the remainder was *N,N*-dimethylated (**Scheme 9**). The dimethyl peak was observed at 2.80 ppm and the quaternized trimethyl peak at 3.35 ppm in the proton NMR spectra of $TMC_{DM/TM.}$ The highest DS for trimethylation in the obtained products was only 0.44 only, whereas the lowest DS for trimethylation was 0.18. The DS did not correlate well with the quantity of iodomethane reagent used for each reaction (**Fig. 8B** and **8D**). This showed that the literature procedure for $TMC_{DM/TM}$ did not offer much control of the DS, whereas it was relatively easy to control the DS when protection groups were used in the synthesis of $TMC_{DM/TM}$ and TACin. In contrast, the direct modification did not cause much cleavage of the glycosidic bond, and the MW did not change significantly.



Reagemts and conditions: (i) formic acid, formaldehyde solution (35 %), deionized water 70 °C; (ii) NaOH (1M), Iodomethane, NMP 40 °C.

Scheme 9. Synthetic route for the preparation of $TMC_{DM/TM}$ (**3a-3e**)

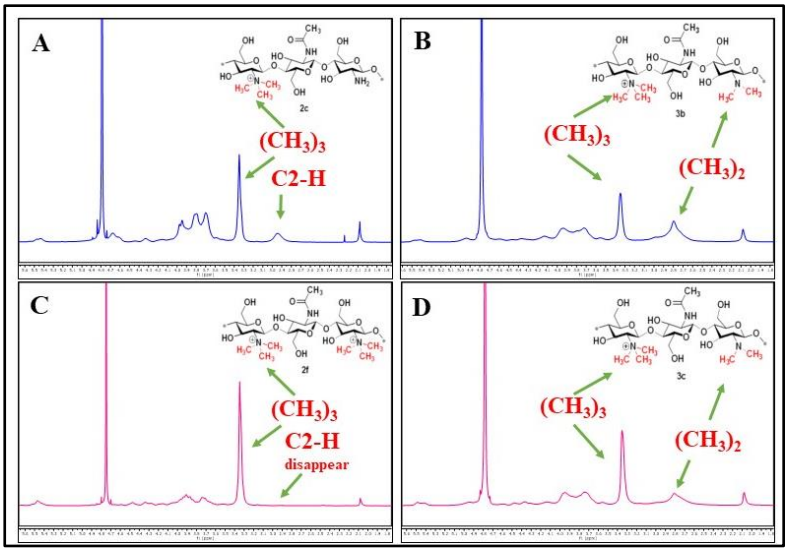
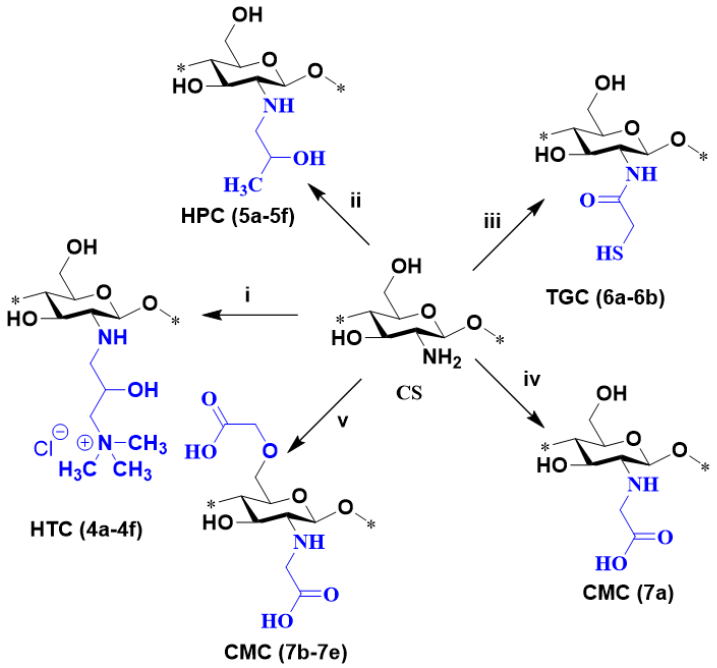


Figure 8. ^{1}H NMR of $TMC_{NH2/TM}$ compound **2c** (A) $TMC_{DM/TM}$ compound **3b** (B) $TMC_{NH2/TM}$ compound **2f** (C) and $TMC_{DM/TM}$ compound **3c** (D)

4.1.2 Common chitosan derivatives with a diverse degree of substitution (Paper II)

The HTC, HPC, TGC, and CMC derivatives were synthesized using known procedures (Lim and Hudson 2004, Peng, Han et al. 2005, Mourya and Inamdar 2008, Geisberger, Gyenge et al. 2013) as shown in **scheme 10.** The HTC derivative was confirmed by ^{1}H NMR spectroscopy. A prominent trimethyl ammonium peak was observed at 3.25 ppm (**Fig. 9)**. The DS of the derivatives increased with an increasing ratio of GTMAC reagent relative to the chitosan substrate to give derivatives with DS ranging from 0.16 to 1.07. The HPC derivative DS increased with the ratio of the propylene oxide reagent to give compounds with DS from 0.08 to 1.05. The HPC polymer molecular weight increased with increasing DS of the polymer. The thiolated chitosan derivatives were synthesized using EDC as the coupling reagent. A new peak was observed in the proton NMR spectra at 2.1 ppm. This peak corresponds to the TGC-CH_2 proton (Han, Wei et al. 2012), but the DS for **8a** and **8b** was only 0.02 and 0.04, respectively.



Reagents and conditions : (i) Glycidyltrimethylammonium chloride, deionised water at 60 °C for 18 h; (ii) propylene oxide, isopropyl alcohol, NaOH (30%); (iii) HCl solution (0.5 M), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.2 eq), thioglycolic acid and NaOH (0.5 M) at RT for 5 h. (iv) Chloroacetic acid, deionised water, NaOH (1.0 M) and Na_2CO_3 (20%) solution at 50 °C for 4 h; (v) Chloroacetic acid, NaOH (50%), isopropanol at RT for 1 to 24 h.

Scheme 10. Synthesis of HTC (**4a-4f**), HPC (**5a-5f**) TGC (**6a-6b**) and CMC (**7a-7e)**

The CMC derivatives were confirmed by proton NMR, and DS was calculated according to an equation used by Sun et al. (Sun, Du et al. 2006). The DS for *N/O*-CMC derivatives (0.47 to 1.06) increased with increasing reaction time, and *N*-CMC DS was 0.31. The MW of the CMC derivative's molecular weight is generally correlated with DS. The calculated DS, DA, and molecular weight for all common derivatives are shown in **Table 2**.

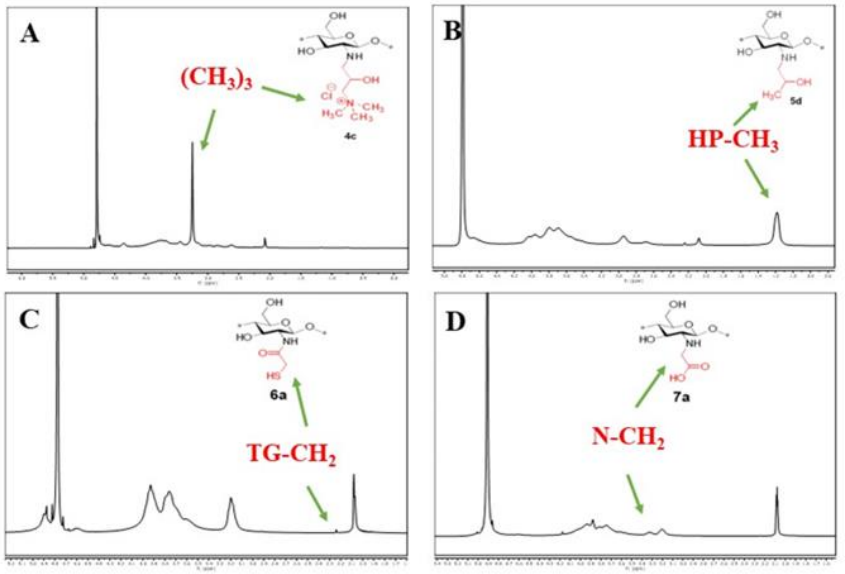


Figure 9. ^{1}H-NMR of HTC compound **4c** (A), HPC compound **5d** (B), TGC compound **6a** (C), CMC compound **7a** (D).

Solubility Analysis

The solubility of one high DS derivative and one low DS derivative for each structure was investigated by visual inspection. Low DS TACin was slightly soluble, whereas high DS TACin was fully soluble in water. The $TMC_{NH2/TM}$ derivatives were highly soluble, whereas $TMC_{TM/DM}$ was only slightly soluble. The HTC and HPC derivatives had good solubility in water, whereas CMC had low solubility (**Table 3**).

Table 2. Identification and properties (DS, DA, and Mw) of the HTC, HPC, TGC, and CMC samples

Compd	DS	DA	Molecular Weight (Da)	Polydispersity Index (D)
4a	0.16	0.16	2.5×10^5	2.0
4b	0.37	0.15	2.5×10^5	2.1
4c	0.55	0.16	2.6×10^5	2.1
4d	0.83	0.18	2.9×10^5	2.1
4e	1.07	0.21	3.3×10^5	2.2
4f	1.1	0.17	2.7×10^5	2.0
5a	0.08	0.09	1.2×10^5	1.8
5b	0.17	0.07	1.2×10^5	1.9
5c	0.36	0.08	1.4×10^5	1.9
5d	0.61	0.06	1.6×10^5	2.0
5e	0.84	0.07	1.7×10^5	1.8
5f	1.05	0.07	2.0×10^5	1.5
6a	0.04	0.18	3.3×10^4	6.4
6b	0.02	0.17	1.9×10^5	1.8
7a	0.31	0.24	2.4×10^5	2.4
7b	0.47	0.11	3.3×10^5	2.9
7c	0.7	0.13	3.3×10^5	3.8
7d	1.06	0.16	5.7×10^5	3.3
7e	0.84	0.14	2.6×10^5	3.5

Table 3. Solubility test of chitosan and synthesized chitosan derivatives in water

Compounds	Concentration (2.5 %)	
	1 h	24 h
CS	-	-
TACin 1a	+	+
TACin 1f	++	
$TMC_{NH2/TM}$ 2a	++	
$TMC_{NH2/TM}$ 2f	++	
$TMC_{TM/DM}$ 3a	-	-
$TMC_{TM/DM}$ 3c	+	+
HTC 4a	+	+
HTC 4f	++	
HPC 5a	++	
HPC 5f	++	
TGC 6a	++	
CMC 7a	+	+
CMC 7d	+	+

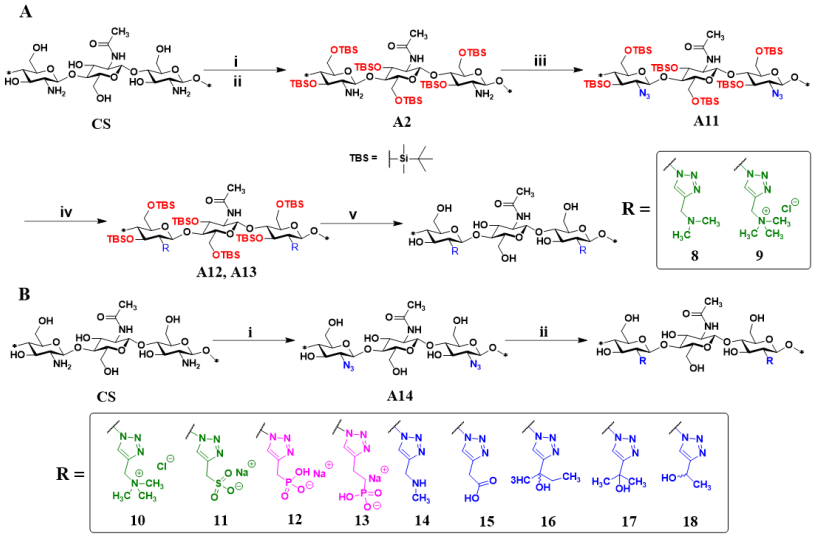
(++) Soluble (clear solution), (+) Slightly soluble (gel or partially soluble),

(-) Not soluble.

4.1.3 Chitotriazolan derivatives (Paper III)

The main aim of this work was to fully convert the primary amino groups of chitosan to azide and then use the CuAAC reaction to convert them to water-soluble 1,2,3-triazoles. Kulbokaite coworkers have reported that chitosan azides are insoluble in aqueous solutions and organic solvents (Kulbokaite, Ciuta et al. 2009), limiting the conversion of the amino groups (Zhang, Bernet et al. 2008). In order to address this issue, the synthesis was started from TBDMS chitosan. The protected TBDMS chitosan is soluble in organic solvents such as chloroform and dichloromethane (Rúnarsson, Malainer et al. 2008) (Sahariah, Másson et al. 2018). and it was expected that TBDMS chitosan azides would be soluble like other protected derivatives **Scheme 11A.** The conversion to TBDMS-protected chitosan azide was confirmed by IR spectroscopy (**Fig. 10).** Still, to our surprise,

it turned out that the TBDMS chitosan azide had low solubility in organic solvents and thus could not be fully characterized by NMR. It was attempted to dissolve TBDMS chitosan azide in aqueous and organic solvents including water, 0.1 M HCl, 0.1 M NaOH, MeOH, acetonitrile, chloroform, dichloromethane, and NMP also further tested in mixed solvents like 1:1 ratio of MeOH:0.1 M HCl, acetonitrile:0.1 M HCl but the solid did not dissolve in any these solvents. However, the solid material was partially soluble in DMF and DMSO (this required the sample to be stirred for 1–2 h at room temperature or 50 °C), thus the subsequent "click reaction" was carried out in DMF to obtain 4-(*N,N*-dimethylaminomethyl) **8** and 4-(*N,N,N*-trimethylaminiummethyl) **9** citotriazolan followed by deprotection to remove TBDMS.



Routes and conditions: **11A** (i) methane sulfonic acid, deionized water, 10 °C; (ii) imidazole, TBDMS-Cl, DMSO, RT; (iii) imidazole sulfonyl azide HCl salt, triethylamine, $CuSO_4$ $5H_2O$, DCM, methanol, RT; (iv) $CuSO_4$ $5H_2O$, sodium ascorbate, terminal alkyne, DMF 50 °C; (v) Conc. HCl, methanol RT. Synthetic routes and conditions: **11B** (i) 0.1 M HCl solution, sodium bicarbonate, imidazole sulfonyl azide HCl salt, $CuSO_4$ $5H_2O$, water, methanol, RT; (ii) $CuSO_4$ $5H_2O$, sodium ascorbate, terminal alkynes, DMSO, 50 °C

Scheme 11. Synthesis of chitotriazolan via TBDMS (TBS) protection (**A**). Synthesis of chitotriazolan via without TBDMS protection (**B**)

Direct conversion of chitosan to azide without the use of protection groups was also tested. The conversation to azide could be confirmed with FT-IR, and the aromatic triazole conversion. As previously reported (Kulbokaite, Ciuta et al. 2009), the material was insoluble in an aqueous solution and organic solvents. Zhang et al. reported that similar chitosan-1,2,3-triazole materials where the click reaction was done in DMSO. The products were insoluble in NMR solvents, but full conversion could be confirmed by solid-state NMR (Zhang, Bernet et al.

2008). The CuAAC reaction was therefore done with this material and *N,N,N*-trimethylprop-2-yn-1-ammonium bromide in DMSO. The complete conversion of azide to chitotriazolan was confirmed by the disappearance of the azide peak in the IR spectra and the appearance of a triazole peak at 8.5 ppm in ^{1}H NMR, corresponding to a 90 % degree of substitution for the triazole group.

This direct synthesis procedure was also used to synthesize other chitotriazolan derivatives with *N*-methylaminomethyl, carboxymethyl, 2-hydroxybut-2-yl, 2-hydroxyprop-2-yl, and 1-hydroxyethyl side groups. This procedure was also used to introduce anionic sulfonate and phosphonate groups. The propargyl sulfonate and propargyl phosphonates were synthesized according to reported procedures (Ouadahi, Allard et al. 2012, Wanat, Walczak et al. 2015) and used to synthesize 4-substituted sulfomethyl, phosphomethyl, and phosphoethyl chitotriazolan derivatives (**Scheme 11B).** Chitotriazolan derivatives **14-18** were not soluble in any of the solvents tested, and the conversion could only be confirmed by IR spectroscopy.

4.1.3.1 Characterization of chitotriazolan by FT-IR and NMR spectroscopy

The FT-IR spectra of chitosan, TBDMS chitosan azide (**A11**), chitosan azide (**A14**), and chitotriazolans **10**, **12**, and **14-17** are shown in **Fig. 10**. The characteristic C=O stretching vibration band at 1652 cm^{-1} for the N-acetyl group was observed in all spectra. Following the conversion to azide, a new band characteristic for the azide (N_3) group appeared at 2109 cm^{-1} (**Fig. 10 B** and **C**). The azide band disappeared after the CuAAC reaction confirming the formation of 1,2,3-triazole on the chitosan backbone at the C-2 position. In **Fig. 10C,** strong bands at 775 cm^{-1} and 831 cm^{-1} correspond to Si-C stretching vibrations. The chemical modification of a new band occurs at 1475 cm^{-1} (**Fig. 10**) assigned to the weak peak of *N*-CH_3 absorbance, and a new band appeared at 795 cm^{-1}, confirming the P-O bond for the phosphonate group (**Fig. 10. E**). The insoluble chitotriazolan derivatives were confirmed by the disappearance of the sharp azide peaks (**Fig. 10. F, G, H, I)**.

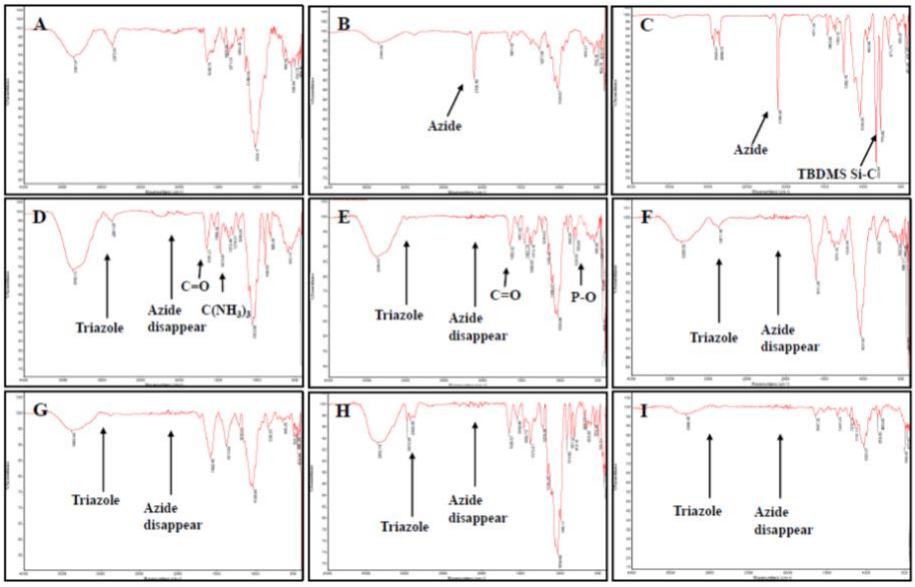


Figure 10. FT-IR spectra for chitosan and chitotriazolan derivatives: **CS** (A), derivative **A14** (B), derivative **A11** (C), derivative **10** (D), derivative **12** (E). FT-IR spectra for insoluble chitotriazolan derivative **14** (F), derivative **15** (G), derivative **16** (H), derivative **17** (I).

The ^{1}H NMR, ^{13}C NMR, COSY, and heteronuclear single quantum coherence (HSQC) spectra of soluble chitotriazolan derivatives are shown in **Fig. 11**. and **Fig. 12.** The 1,2,3-triazole proton is observed at 8.59 ppm it corresponds to the 1,2,3-triazole proton it was further confirmed the CuAAC reaction on the chitosan backbone structure **10** and **11** in **Fig. 11.** The new peak for the quarternary trimethylammonium group for derivative **10** appeared at 3.2 ppm, and the methylene (CH_2) group at 4.8 ppm merged with the HDO peak; however, it was clearly visible in the HSQC spectrum. The HSQC spectra for derivatives **10** and **11** could be used to confirm the assignment of the proton peaks (**Fig. 12C** and **Fig. 12D).** The conversion of azide to triazole leads to a dramatic shift in the *C*-2 proton peak from around 2.8 ppm to 4.58 ppm. At the same time, peaks are also shifted significantly from shift values for unmodified chitosan. The *C*-6 protons were observed at 2.90 ppm and 3.2 ppm (merged with the $N(CH_3)_3$ peak), and the *C*-5, *C*-4, and *C*-3 protons at 3.52, 3.78, and 4.44 ppm, respectively. The anionic derivative **11** triazole peak was broadened and appeared in a slightly up-field position (8.13 – 8.43 ppm) relative to **10.** The CH_2 signal adjacent to the sulfonate groups was observed at 4.27 – 4.42 ppm.

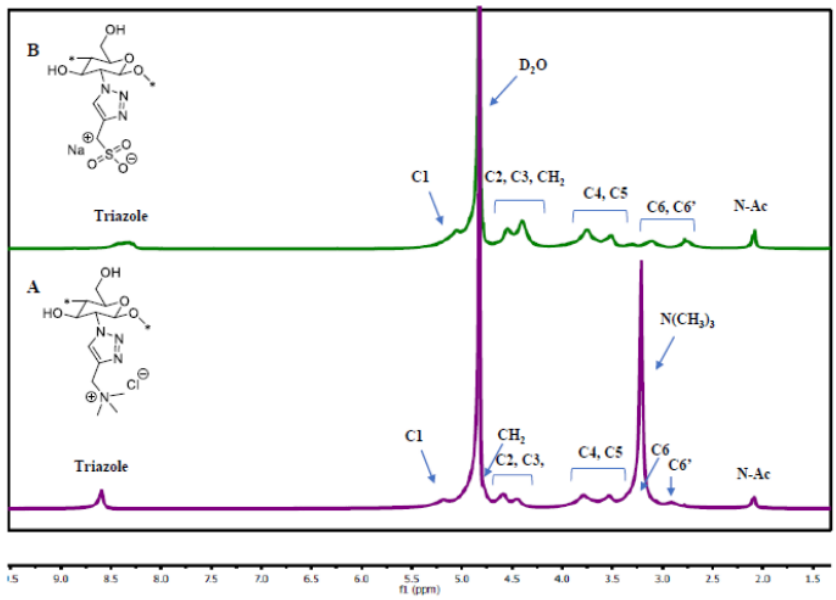


Figure 11 ^{1}H NMR spectra for derivative **10** (A) and derivative **11** (B).

The ^{13}C APT NMR showed the protonated 1,2,3-triazole carbon at 137 ppm (**Fig. 12A**). The confirmed the aromatic triazole ring presented in the chitosan *C*-2 position. The chitosan carbon signals for *C*-2 to *C*-6 appeared between 60 – 80 ppm and *C*-1 at 100 ppm. The COSY spectra further confirmed the assignment of the 1,2,3-triazole peak at 8.59 ppm and the *N*-acetyl peak at 2.08 ppm (**Fig. 12B)**. The complete interpretation of all peaks also confirmed that the azide had been fully converted to the chitotriazolan structure**.** The HSQC spectrum clearly shows the trimethylammonium protons at 3.2 ppm for cationic 4-(*N,N,N*-trimethylammonium methyl) chitotriazolan, whereas this peak was not present in the anionic 4-sulfomethyl chitotriazolan spectrum.

Table 4 reports the degree of substitution (DS), degree of acetylation (DA), and molecular weight (MW) for all water-soluble derivatives. The calculation based on integrals of the proton peaks indicated more than 90 % conversion from the free amino group in chitosan to 1,2,3 triazole in the cationic chitotriazolan **10**. Only one peak could be observed for each monomer proton of the chitotriazolan backbone, and this was consistent with 100% conversion. The molecular weights were reduced for derivatives **8** and **9** to more than four times less than the Mw of the chitosan starting material.

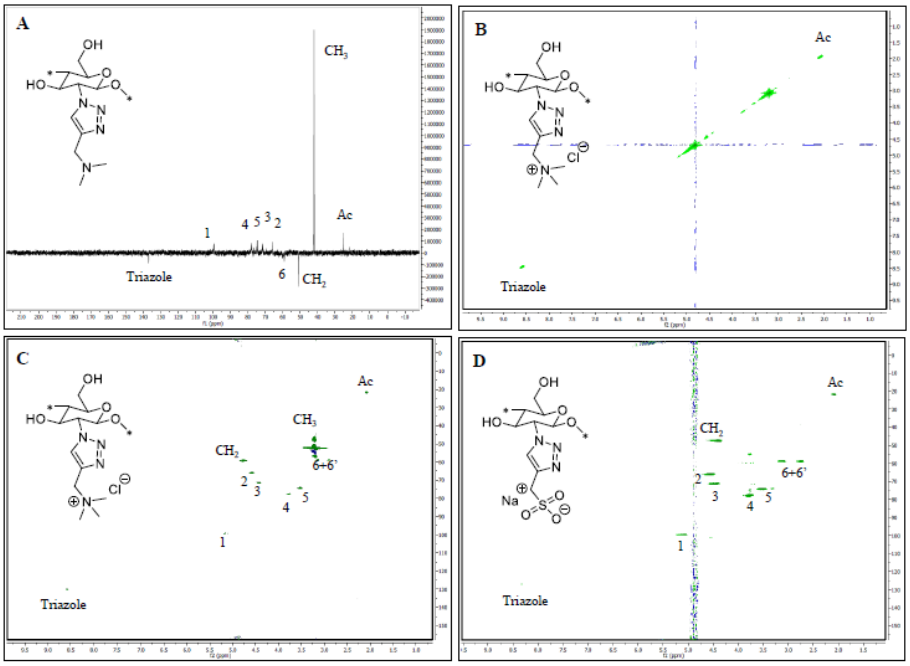


Figure 12. ^{13}C APT NMR for derivative **8** (A), COSY NMR for derivative **10** (B), HSQC NMR for derivative **10** (C), and derivative **11** (D).

The dramatic change in MW was caused by the polymer chain's protection and deprotection, which occurs when the chitosan mesylate salt is prepared and in the deprotection reaction to remove TBDMS (Sahariah, Gaware et al. 2014). The average MW was determined for derivatives **10** and **12**, increased about twice the unmodified chitosan, and synthesized without using of protecting groups, which was consistent with the increase in the MW of the monomer units when chitosan was converted to chitotriazolan derivatives. The MW of 4-sulfomethyl chitotriazolan **11** and 4-phosphoethyl chitotriazolan **13** were found to be around 6 KD which was much less than expected. This was probably due to low solubility in the mobile phase, and the higher MW material was removed in the filtration of the samples.

Table 4 The degree of substitution (DS), degree of acetylation (DA), and molecular weight analysis for chitotriazolan derivatives.

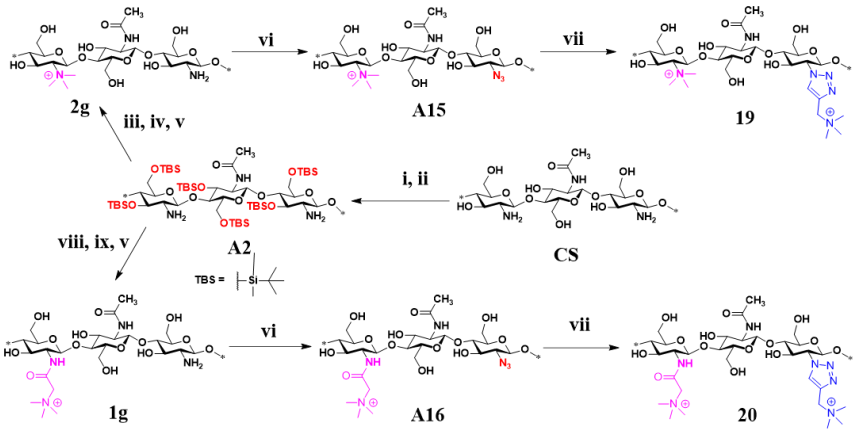
Derivatives	DS-TM [a b]	DA [a]	DS-Triazole [a]	MW (kDa)	Polydispersity Index (D)
8	0.98	0.08	0.86	28.94	1.76
9	0.73	0.09	0.68	17.05	1.72
10	0.98	0.18	0.9	214.59	1.97
11	NA [c]	0.17	0.8	(6.26) [d]	(1.69) [d]
12	NA	ND [e]	ND	220.02	2.69
13	NA	ND	ND	(5.58) [d]	(1.04) [d]

[a] degree of substitution (DS), degree of acetylation (DA), and DS-triazole were calculated based on ^{1}H NMR spectroscopy. [b] TM-Trimethylation, [c] Not available, [d] These samples were poorly soluble in the mobile phase but could be analyzed after the insoluble material (about 80%) had been removed by filtration. [e] Not determined.

4.1.4 Mixed chitotriazolan derivatives (Paper IV)

In this work, the synthesis of mixed chitotriazolan was started from *N,N,N*-trimethyl chitosan (TMC), *N*-(2-*N,N,N*-trimethyl ammoniumyl)-acetyl chitosan (TAC), 2-hydroxypropyl trimethylammonium chitosan (HTC), 2-hydroxypropyl chitosan (HPC), and carboxymethyl chitosan (CMC). The study was done to investigate if the click chemistry procedure could be applied to common chitosan derivatives.

Common chitosan derivatives TMC, TAC, HTC, HPC, and CMC with a partial degree of substitution were synthesized so that they also contained some primary groups that could be converted to triazole. The synthesis of these common derivatives was described in previous sections 4.1.1 and 4.1.2. The degree of substitution and degree of acetylation for all common chitosan derivatives are shown in **table 5**.



Routes and conditions: (i) methane sulfonic acid, deionized water, 10 °C; (ii) imidazole, TBDMS-Cl, DMSO, RT; (iii) Boc anhydride, Et_3N, DCM, 0 °C; (iv) iodomethane, Cs_2CO_3, NMP 40 °C; (v) Conc. HCl, methanol, RT; (vi) 0.1 M HCl solution, sodium bicarbonate, imidazole sulfonyl azide HCl salt, $CuSO_4$ $5H_2O$, water, methanol, RT; (vii) $CuSO_4$ $5H_2O$, sodium ascorbate, N-propargyl-*N,N,N*-trimethylammonium bromide, DMSO, 50 °C; (viii) Bromoacetyl bromide, Et_3N, DCM, -10 °C; (ix) Trimethylammonium solution, DCM, RT.

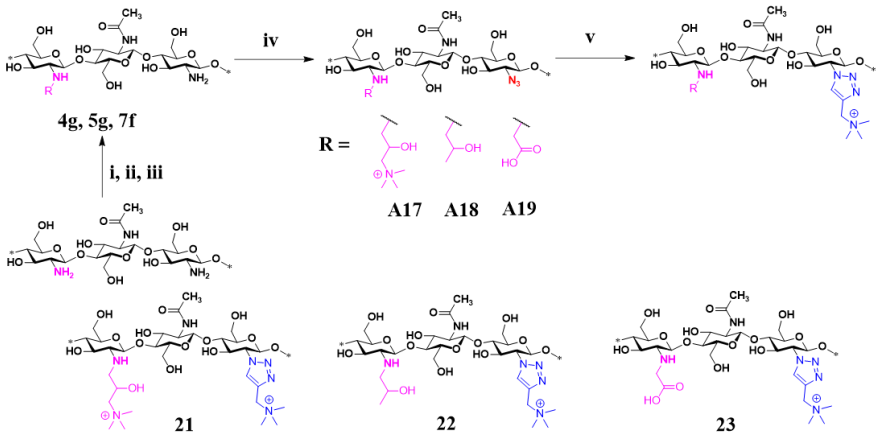
Scheme 12. Synthesis of mixed TMC and TAC chitotriazolan via TBDMS (TBS) protection

Table 5. Degree of substitution for common chitosan derivatives.

Common CS derivatives	DS	DA
TMC (**2g**)	0.30	0.08
TAC (**1g**)	0.46	0.06
HTC (**4g**)	0.37	0.15
HPC (**5g**)	0.36	0.08
CMC (**7f**)	0.31	0.15

In chitosan chemistry, the synthesis of multiple substituents on chitosan backbone and its structure-activity relationship, antibacterial activity studies are rare. Our goal was to synthesize common chitosan derivatives with 30 to 50 % DS. The remaining amino group was to be converted to 1,2,3-triazole moieties in chitosan, leading to 100 % total DS for the chitosan derivatives. The reaction scheme for the synthesis of the common chitosan derivatives (**2g**, **1g**, **4g**, **5g**, and **7f**), conversion to common chitosan azide intermediates (**A15**, **A16**, **A17**, **A18**, and **A19**) by reaction with imidazole sulfonyl azide HCl salt is shown in **Scheme 13**.

These common chitosan azides intermediates were more soluble than simple chitosan azide (**A14**) in DMSO at room temperature and 50 °C. The solubility might be the partial substitution of common chitosan derivatives or a low degree of azidation, but it was still not possible to characterize chitosan derivatives by solution-based proton NMR. The azide-alkyne reaction subsequently showed that only the unsubstituted primary amino groups were converted to azide. The "mixed" TMC-chitotriazolan **(TMC-CTr)** (**19**), TAC-chitotriazolan **(TAC-CTr) (20**), HTC-chitotriazolan **(HTC-CTr)** (**21**), HPC-chitotriazolan **(HPC-CTr)** (**22**), and CMC-chitotriazolan **(CMC-CTr)** (**23**) derivatives were obtained by CuAAC reaction with *N,N,N*-trimethylprop-2-yn-1-aminium (**Scheme 12** and **13**).



Routes and conditions: (i) Glycidyltrimethylammonium chloride, water, 50 °C; (ii) propylene oxide, isopropyl alcohol, NaOH (30 %); (iii) chloroacetic acid, water, NaOH (1 M), Na_2CO_3 (20 %) 50 °C; (iv) 0.1 M HCl solution, sodium bicarbonate, imidazole sulfonyl azide HCl salt, $CuSO_4$ $5H_2O$, water, methanol, RT; (v) $CuSO_4$ $5H_2O$, sodium ascorbate, N-propargyl-*N,N,N*-trimethylammonium bromide, DMSO, 50 °C.

Scheme 13. Synthesis of mixed chitotriazolan derivatives

The partially substituted derivatives have some unsubstituted amino group at *C*-2 position which was converted to azide. The yield for **A15** was 95 mg or slightly below 50%, and 125 mg for **A16** or just above 50%. The yield for other azides **A17-19** was higher or between 55 - 95 %.

The azide was soluble, as mentioned above, and was carried forward to CuAAC reaction in DMSO at 50 °C. The yield for the TMC-chitotriazolan was less than 30%, and only 27 mg was obtained. This reaction was repeated, and the yield was similarly low. The other click product of mixed chitotriazolans **20**-**23** yield was considerably better or between 56-90 %. The overall yield in the two steps was high for the final three (**21**-**23**) products, and the weight of the final **HTC-CTr**, **HPC-CTr,** and **CMC-CTr** products was similar to the weight of the HTC,

HPC, and CMC starting materials. These mixed chitotriazolan polymers were more water soluble than common chitosan derivatives and unmodified chitosan.

4.1.4.1 *Characterization of mixed chitotriazolans by IR and NMR spectroscopy*

The FT-IR spectra confirmed the prominent azide peak vibrational stretching frequency at 2114 cm^{-1}. It was the strong absorbance. The azide peak disappeared after the click reaction, and an additional band at the 1474 cm-1 peak was assigned *N*-CH_3 bending vibration (Zhang, Tan et al. 2021). All the mixed chitotriazolan derivatives clearly showed vibrational frequency at 1475 $cm^{-1,}$ corresponding to triazole trimethyl *N*-CH_3, which was not present in the starting materials.

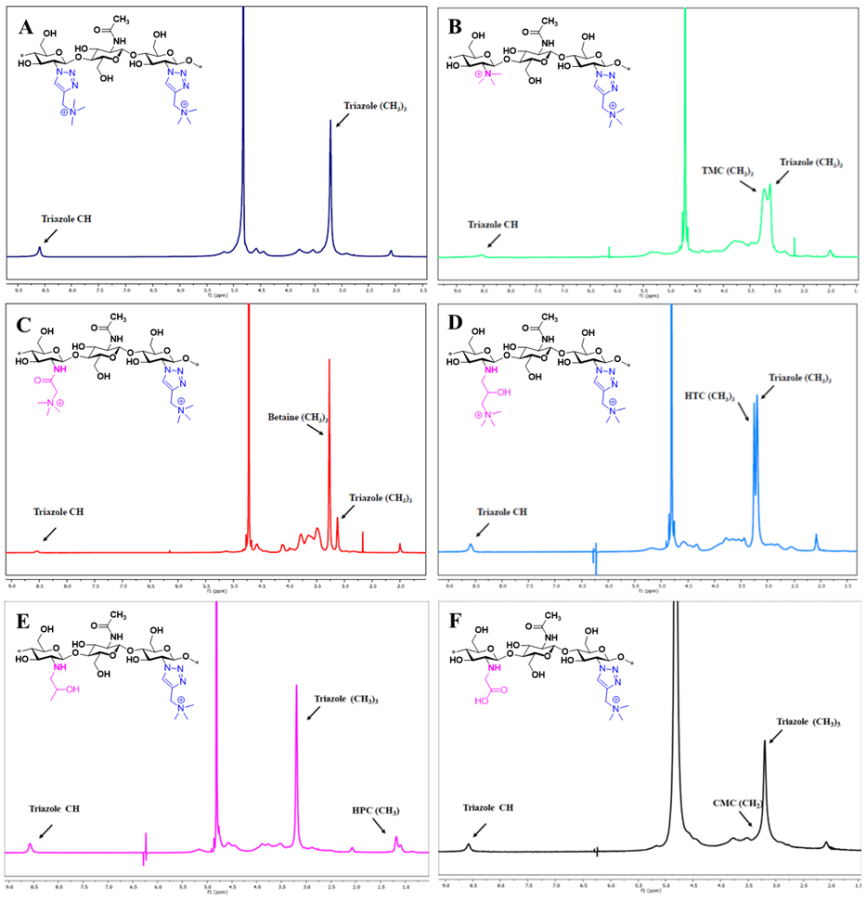


Figure 13. Proton NMR of Chitotriazolan derivatives: Simple chitotriazolan **10** (A), derivative **19** (B), derivative **20** (C), derivative **21** (D), derivative **22** (E), derivative **23** (F).

The mixed chitotriazolan derivatives were characterized by NMR spectroscopy shown in **Fig. 13**. The simple chitotriazolan **10** proton NMR has been used to interpret other spectra by superimposing it on the ^{1}H NMR spectra. The triazole trimethylammonium prominent peak was assigned at 3.20 ppm for all derivatives. In contrast, the peak set to the trimethylammonium part of the TMC, TAC (betaine), and HTC structure can be observed at 3.31, 3.35, and 3.25 ppm, respectively (**Fig. 13-B, C, D).** The HPC structure CH_3 peak could be observed at 1.19 ppm (**Fig. 13E**). For the anionic derivative of carboxymethylated chitotriazolan derivative CMC, the characteristic CH_2 peak is merged with triazole $(CH_3)_3$ peaks at 3.20 ppm **Fig. 13F**.

The H-2, H-3, H-4, H-5, H-6, and H-6´ protons of the chitotriazolan structure will be observed at 4.58, 4.44, 3.78, 3.52, 3.20, and 2.90 ppm, respectively. The H-2 to H-6 protons in the TMC structure will be observed between 3.75 and 4.47 ppm (Benediktsdóttir, Gaware et al. 2011) and at 3.5-3.9 the TAC structure, where the *N,N,N*-trimethyl acyl CH_2 protons are also observed at 4.17 ppm (Sahariah, Gaware et al. 2014, Sahariah, Óskarsson et al. 2015). The H-3 to H-5 protons of the alkylated derivatives HTC, HPC, and CMC will be in the range of 3.20 to 4.14 ppm, and the H-2 proton peaks should be observed at 2.80 to 2.90 ppm.

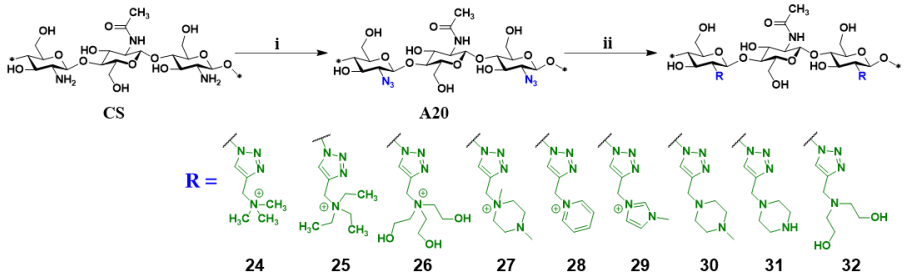
Generally, when there are multiple substituents in a polymer chain quite challenging to interpret the position of signals and intensities of different peaks in the proton spectra. The spectra signals are consistent with the expected structure of the mixed chitotriazolans. However, many peaks are merged or flattened due to significant peak broadening in the polymer spectra. The peaks are assigned and integrated based on the integral of the most prominent peaks, such as the aromatic triazole proton at 8.6 ppm, trimethylammonium proton at (3.2 and 3.25-3.35 ppm), the *N*-acetyl peak at 2.1 ppm, and the hydroxypropyl CH_3 protons in HPC-CTr **22** at 1.2 ppm. Based on these integral values, the degree of substitution (DS) and degree of acetylation (DA) were calculated by using proton NMR (**Table 6**). The DS for the triazole trimethyl group for polymers **19** and **20** obtained 0.4 and 0.15, respectively. The other derivative **21**, **22**, and **23** DS was more than 0.58 to 0.78. The molecular weight and polydispersity index were determined using size exclusion chromatography (T**able 6**). There are some degradations in the polymer chain, so the molecular weight was reduced as comparing native chitosan.

Table 6. DS, DA, and Molecular weight for chitotriazolan derivatives

Chitotriazolan derivatives	DS for triazole TM	DS for triazole CH	Common CS deri. DS	DA	M.Wt (kDa)	Polydispersity index
10	0.98	0.9	-	0.18	214.59	1.97
19	0.4	0.24	0.25	0.08	13.24	1.12
20	0.15	0.12	0.35	0.06	15.99	1.20
21	0.58	0.50	0.30	0.12	94.59	1.74
22	0.78	0.67	0.34	0.06	76.37	1.89
23	0.69	0.48	0.31	0.12	87.22	1.82

4.1.5 Chitotriazolans with various alkynes (Paper V)

The development of the synthesis procedure chitosan-1,2,3-triazole (chitotriazolan) derivatives was described in the previous section, 4.1.3. This work was followed further study where the procedure was to synthesize water-soluble quaternary and basic protonable chitotriazolan derivatives with diverse structures. The click modifications utilized to obtain these derivatives by CuAAC are illustrated in **Scheme 14**. The conversion of chitosan azide was confirmed by FT-IR spectroscopy, and the azide band shows the vibrational frequency at 2109 cm^{-1}.



Routes and conditions; (i) 0.1 M HCl solution, sodium bicarbonate, imidazole sulfonyl azide HCl salt, $CuSO_4$ $5H_2O$, water, methanol, RT; (ii) $CuSO_4$ $5H_2O$, sodium ascorbate, various alkynes, DMSO, 50 °C.

Scheme 14. Synthesis of chitotriazolan derivatives with various structures

Various alkynes were prepared using propargyl bromide reacted with trimethylamine solution, triethylamine, triethanolamine, *N,N*-dimethylpiperazine, *N*-methyl imidazole, pyridine, *N*-methylpiperazine, piperazine, and *N,N*-diethanolamine (see in **Fig. 14)**. These alkynes were conjugated to chitosan backbone structure via CuAAC reaction to give chitotriazolan derivatives. The conversion of triazole and quaternized functional groups was confirmed by proton NMR and by the disappearance of azide peak by FT-IR spectroscopy. The triethylamine and *N,N,N*-triethanolamine alkyne derivatives were selected based on previously synthesized *N,N,N*-trimethylamine alkyne derivatives. The alkynes with piperazine, methylimidazole, and pyridine were also selected based on similarity with the quaternized moiety in some of the chitosan derivatives previously our research group and Finish collaborators (Holappa, Nevalainen et al. 2006, Holappa, Nevalainen et al. 2006).

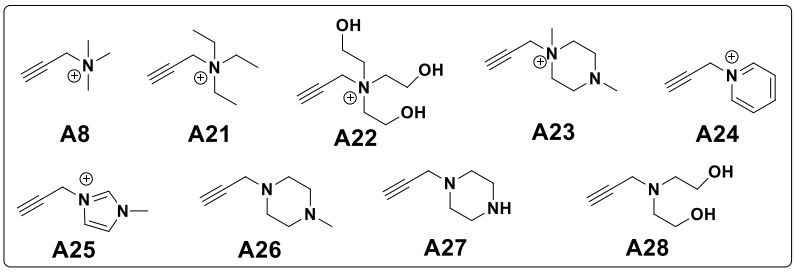


Figure 14. Structure of different alkynes

4.1.5.1 Characterization of cationic chitotriazolan by IR and NMR Spectroscopy

The chitotriazolan derivatives were characterized by IR and NMR spectroscopy (see **Fig. 15**). In the FT-IR spectrum of chitosan azide intermediate, the azide moiety is evident from the vibrational frequency at 2109 cm^{-1}. The peak at 2109 cm^{-1}in the spectrum of chitosan azide disappeared when the *C*-2 azido was transformed to 1,2,3-triazoles. A new peak appearance for C-H alkene proton 1,2,3-triazole derivatives between 8.19 to 8.59 ppm further proved the successful click reaction in the proton NMR. The chitosan *N*-acetyl peak at 2.08 ppm for the reference protons for all derivatives. The *N*-$(CH_3)_3$ proton peak was observed at 3.21 ppm for derivative **24** (**Fig. 15A**), and the peak was consistent with our previous report. The *N*-$(CH_2CH_3)_3$ proton peaks were observed at 1.45 ppm and 3.33 ppm for ethyl CH_3 and ethyl CH_2 protons, respectively (**Fig. 15B**). *N,N,N*-triethanolammonium *N*-$(CH_2CH_2OH)_3$ protons peaks were observed at 3.68 ppm and 4.21 ppm (**Fig. 15C**).

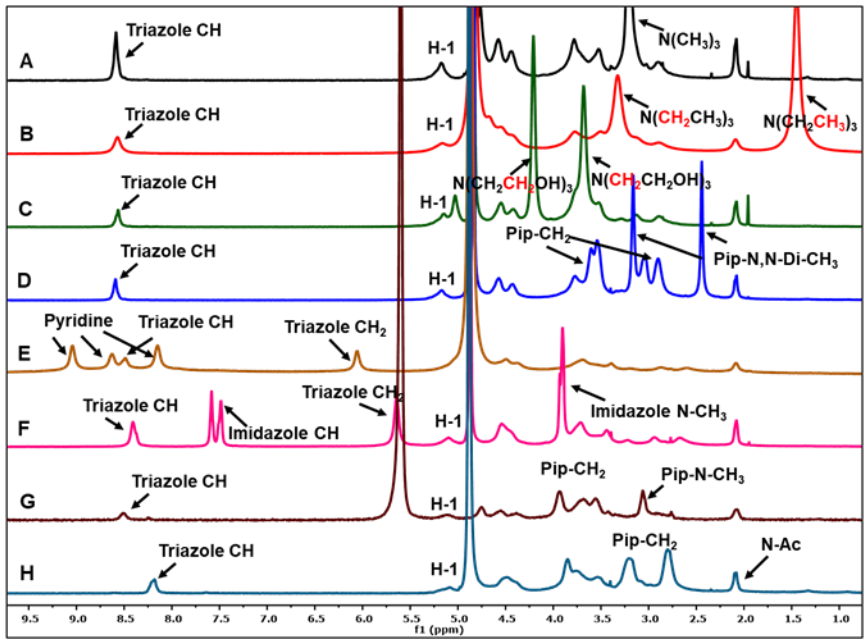


Figure 15. Proton NMR spectra of all chitotriazolan derivatives: Derivative **24** (A), Derivative **25** (B), Derivative **26** (C), Derivative **27** (D), Derivative **28** (E), Derivative **29** (F), Derivative **30** (G), Derivative **31** (H).

The characteristic signals of the piperazine *N,N*-dimethyl derivative **27,** for the Pip-*N*-CH_3, Pip-3,5-CH_2, quaternary-*N*(CH_3), and quaternary-Pip-2,6-CH_2 were

observed at 2.44 ppm, 2.90 – 3.04 ppm, 3.16 ppm, and 3.54 – 3.60 ppm, respectively. The proton spectra **fig. 15E** represents pyridine derivative **28**, a new signal attributed to the aromatic region for pyridine five protons, and the triazole -CH_2 protons shifted slightly upfield at 6.06 ppm. The imidazole click derivative **29** shows the signal for the imidazole (C1-H, C4-H, C5-H) alkene three protons at 7.49 – 7.58 ppm (**Fig. 15F**). These spectra further confirmed that the signal of *N*-methyl peak appeared at 3.90 ppm and triazole CH_2 protons shifted to upfield at 5.64 ppm. The derivative *N*-methyl piperazine **30** peaks were obtained at 3.34 – 3.94 ppm for (Pip-2,3,5,6-CH_2) and 3.06 for *N*-methyl protons, and the derivative **31** showed a signal for the piperazine in the range between 2.71 – 3.28 (Pip-2,3,5,6-CH_2) merging with chitosan H-6 and H-6′ protons.

Water solubility

Solutions prepared from the chitotriazolan derivatives in distilled water at 25 °C are shown in **Fig. 16.** Chitosan was soluble in acidic solutions at room temperature, and chitosan azide was soluble only in DMSO/DMF at 50 °C. All the chitotriazolan derivatives were somewhat soluble in water at room temperature. The solubilities of all derivatives were measured at 8 mg/mL, and the clearest visible soluble derivatives are quaternary or cationic chitosan triazole derivatives. Other derivatives that do not contain quaternary groups showed somewhat reduced solubility. The derivatives **24**−**29** were soluble in water, **30** and **31** were slightly soluble in water, and in derivative **32,** some undissolved particles were present in the solution.

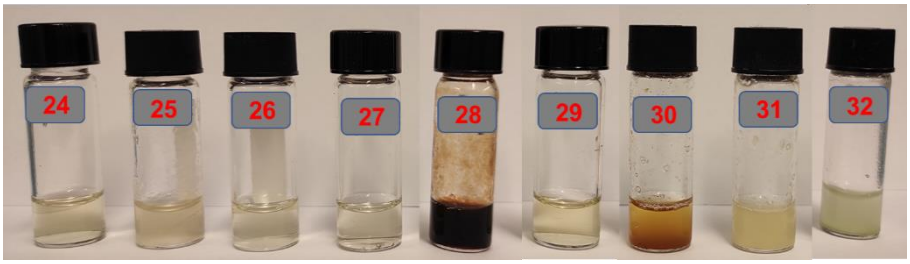


Figure 16. Solubility test in water for all chitotriazolan derivatives (**24 – 32**)

The determination of the degree of substitution (DS) and the degree of acetylation (DA) for all derivatives was done by proton NMR (see in **table 7)**. The DS, when the was calculated based on the triazole -CH proton signal, has a value between 0.5 to 0.68 was obtained. The DS was calculated based on the signal for conjugated functional groups such as trimethyl, triethyl, triethanol, dimethyl piperazine, pyridine, methylimidazole, methylpiperazine, and

piperazine and ranged between 0.75 to 0.83. Based on the DS value and degree of acetylation, the conversion of azide to triazole was more than 90%. The isolated yield was calculated based on one monomer unit, and most of the derivatives obtained a good yield of 80 – 94 %, except derivatives 31 and 32 were the yield was 60 % and 61 %, respectively.

Table 7. The DS and DA for all chitotriazolan derivatives

Chitotriazolan derivatives	Yield (%)	DS for triazole CH	DS for other functional groups	DA
24	82	0.63	0.82	0.17
25	91	0.68	0.79	0.19
26	80	0.4	0.76	0.17
27	88	0.5	0.80	0.18
28	94	0.67	0.80	0.10
29	84	0.65	0.83	0.17
30	75	0.5	0.75	0.20
31	60	0.55	0.77	0.20
32	61	NA	NA	NA

NA – Not available

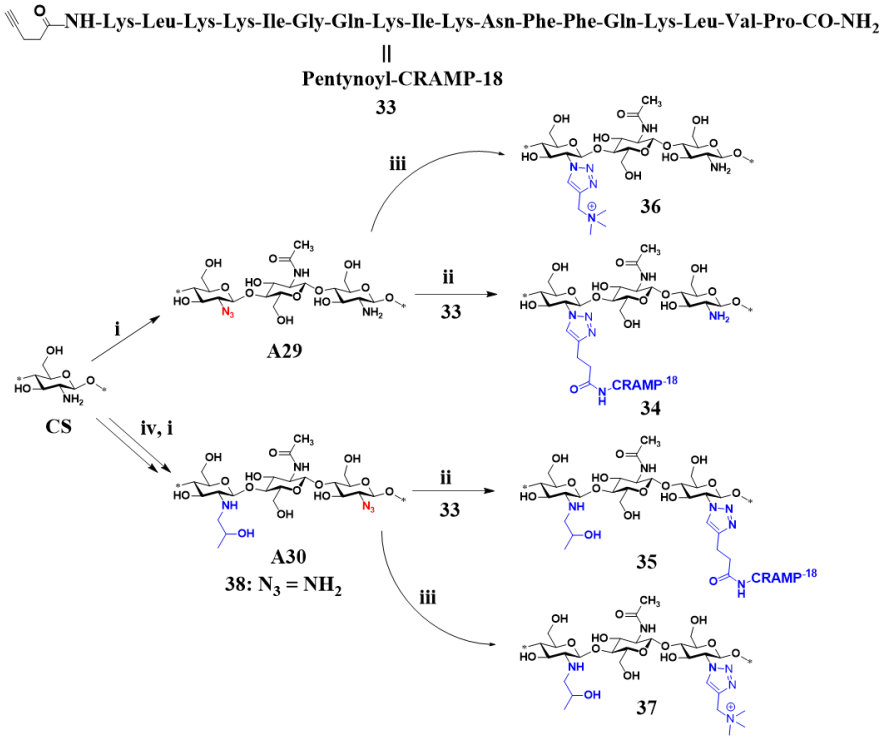
4.1.6 Chitotriazolan CRAMP-18 peptide conjugates (Paper VI)

Antimicrobial peptides (AMPs), also known as host defense peptides, are diverse in structure and exhibit a broad spectrum of antimicrobial properties against a wide range of bacteria, fungi, and viruses. One class of AMPs in humans is defensins and cathelicidin. The Cathelin-related antimicrobial peptide (CRAMP) is the mouse version of the human LL-37 peptide.

The work reported in this section aimed to conjugate the antimicrobial peptide (AMP) onto the chitosan backbone structure via a copper-catalyzed azide-alkyne cycloaddition reaction.

In order to synthesize biopolymer **34**, the chitosan *C*-2 amino group was converted to azide with a low degree of azidation. For this purpose, only 0.4 equivalent of imidazole sulfonyl azide HCl salt was used for the synthesis. The N-terminus Cathelin-Related Anti-Microbial Peptide (CRAMP-18) peptide sequence

is KLKKIGQKIKNFFQKLVP and shows good antimicrobial activity against *P. aeruginosa* (De Brucker, Delattin et al. 2014). And we have synthesized CRAMP-18 peptide using the Biotage® Initiator+ Alstra™, a fully automated microwave peptide synthesizer. The N-terminus of CRAMP-18 was coupled with 4-pentynoic acid to obtain the alkyne-CRAMP-18 (**33**) (**scheme 15**).



Reagent and conditions: (i) 0.1 M HCl solution, sodium bicarbonate, imidazole sulfonyl azide HCl salt, $CuSO_4$ $5H_2O$, water, methanol, RT; (ii) $CuSO_4$ $5H_2O$, sodium ascorbate, pentynoyl-CRAMP-18, DMSO, THPTA, 50 °C; (iii) $CuSO_4$ $5H_2O$, sodium ascorbate, N-propargyl-*N,N,N*-trimethylammonium bromide, DMSO, 50 °C; (iv) propylene oxide, isopropyl alcohol, NaOH (30 %).

Scheme 15. Conjugation of CRAMP-18 peptide on chitosan through click reaction

Similar to our previous results, the chitosan azide was not soluble in aqueous and organic solvents. Still, due to the low degree of azidation was soluble in DMSO at 50 °C. The chitosan azide reacted with **33** using CuAAC reaction in the presence of THPTA. In the reaction, one equivalent of pentynoyl-CRAMP-18 was used to obtain the CRAMP-18 chitotriazolan (**34**) (**Scheme 15**); however, some azide residue was retained in the product, as shown by IR spectroscopy. A similar procedure was followed for the conjugation of CRAMP-18-hydroxypropyl chitotriazolan (**35**) (**scheme 15**). The peptide chitotriazolan **34** and **35** contains an unreacted azide in a low percentage, and it was confirmed by IR

spectroscopy. The simple structure of chitotriazolan **36** and HP-chitotriazolan **37** derivatives were synthesized (**Scheme 15**) to evaluate the structure-activity relationship.

Table 8. The DS, DA, and molecular weight for CRAMP-18-chitotriazolan derivatives

Chitotriazolan derivatives	DS for triazole CH	DS for pep-Phenyl	DS for pep-Me	DS for HP-Me	DS for triazole TM	DA
34	0.2	0.23	0.22	NA	NA	ND
35	0.13	0.12	0.12	0.65	NA	ND
36	0.26	NA	NA	NA	0.34	0.11
37	0.22	NA	NA	0.61	0.25	0.05
38	NA	NA	NA	0.80	NA	0.06

4.1.6.1 Characterization of IR and NMR spectroscopy

The CRAMP-18 grafted chitosan derivatives were characterized by IR spectroscopy. The strong stretching vibrational frequency by the diazo transfer reaction of azide peaks for the chitosan azide **A29** and HPC azide **A30** were confirmed at 2113 cm^{-1}. The following CuAAC reaction gave the CRAMP-18 grafted chitosan derivatives an enhanced peak of carbonyl stretching frequency at 1652 cm^{-1}; it was a merging of chitosan (C=O) carbonyl peak and peptide carbonyl (C=O) amide peaks. The peak at 1537 cm^{-1} corresponds to aromatic phenyl (C=C) bending frequency, and the azide peak was retained at 2113 cm^{-1} low percentage after the click reaction.

An analysis of CRAMP-18 grafted chitosan **34** and **35** derivatives by NMR spectroscopy is shown in **Fig. 17**. The triazole peak of CRAMP-18 conjugated chitosan backbone via CuAAC reaction was observed at 7.94 ppm. The peptide aromatic peak of phenylalanine was observed at 7.08 – 7.30 ppm **Fig. 17A**. Further, the CRAMP-18 peptide peaks ranged from 0.79 to 4.48 ppm for all aliphatic side chains. The CRAMP-18-HP-chitotriazolan derivative **35** confirmed the structure at 1.09 to 1.13 ppm for hydroxypropyl methyl group merging along with peptide peaks **Fig. 17B**.

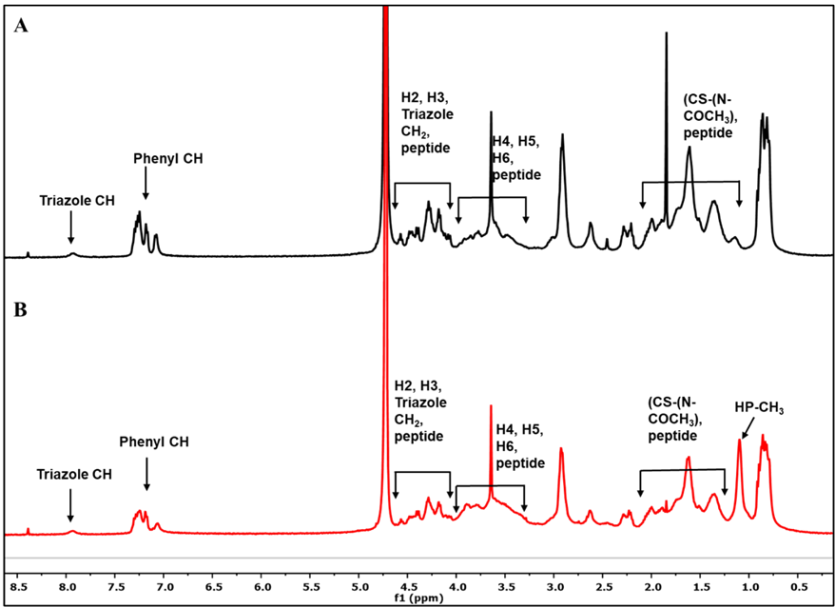


Figure 17. Proton NMR of CRAMP-18 conjugated on CS **34** (A) and CRAMP-18 conjugated on HPC **35** (B)

4.2 Investigation of Structure-Activity relationship for Chitosan derivatives

4.2.1 *N,N,N*-Trimethylated Chitosan with different Degree of substitution (Paper I)

The antimicrobial activity of cationic trimethylated chitosan derivatives was evaluated against *Pseudomonas aeruginosa* and MRSA at physiological pH (7.4) according to the CLSI procedure for broth microdilution assay in (**Table 9).** There was a noticeable correlation between the DS and antibacterial activity for the TACin derivatives **Fig. 18.** The activity increased with increased DS and was more active against MRSA. The higher DS had better 256 and 64 µg/mL activity against *P. aeruginosa* and MRSA, respectively. The highest DS TACin derivatives were more than 32 times more active than the lowest (0.17) DS derivatives against *P. aeruginosa,* and 64 times more active against MRSA.

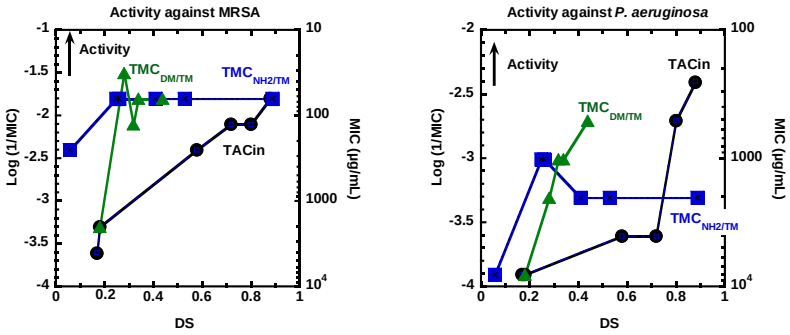


Figure 18. The relationship between antibacterial activity and Degree of Substitution for TACin (●), $TMC_{NH2/TM}$ (■) and $TMC_{DM/TM}$ (▲)

The activity for $TMC_{NH2/TM}$ increased up to DS 0.2 with no further increase in the antibacterial activity when DS further increased. The $TMC_{NH2/TM}$ derivatives were more active against *P. aeruginosa* than TACin derivatives with comparable DS but significantly active when the DS was high. The $TMC_{NH2/TM}$ derivatives were more active than TACin against MRSA except at the highest DS (0.9), where the two derivatives had equal activity. The $TMC_{DM/TM}$ derivative was less active than $TMC_{NH2/TM}$ at low DS, and the activity against MRSA was equal to $TMC_{NH2/TM}$ at higher DS.

The $TMC_{NH2/TM}$ derivatives **2b** and **2c** with DS equal to 0.25 and 0.26 were more active against *P. aeruginosa* than the higher DS derivatives but differed only by one dilution (1024 µg/mL vs. 2048 µg/mL), which is not considered significant. The MIC for 0.44 DS $TMC_{DM/TM}$ **3c** was 512 µg/mL, a two-dilution difference that may be considered significant.

Chito-oligomer derivatives have less antibacterial activity than chitosan polymer derivatives with identical structure modification (Rúnarsson, Holappa et al. 2007, Rúnarsson, Holappa et al. 2010, Sahariah, Cibor et al. 2019). However, differences in molecular weight polymer derivatives do not cause significant differences in activity (Sahariah, Gaware et al. 2014, Sahariah, Cibor et al. 2019). These previous findings seem to be supported by the current result, especially for MRSA, where the higher molecular weight $TMC_{DM/TM}$ has an equal activity to the lower molecular $TMC_{NH2/TM}$ at high DS.

Table 9. MIC analysis for modified chitosan derivatives against *P. aeruginosa* and MRSA bacteria.

Compounds	Degree of Substitution (DS)	*P. aeruginosa* (ATCC 27853) (μg/mL)	MRSA (ATCC 43300) (μg/mL)
1a	0.17	≥8192	4096
1b	0.18	≥8192	2048
1c	0.58	4096	256
1d	0.72	4096	128
1e	0.8	512	128
1f	0.88	256	64
2a	0.06	≥8192	256
2b	0.25	1024	64
2c	0.26	1024	64
2d	0.41	2048	64
2e	0.53	2048	64
2f	0.89	2048	64
3a	0.18	≥8192	2048
3b	0.32	1024	128
3c	0.44	512	64
3d	0.34	1024	64
3e	0.28	2048	32

4.2.2 Antibacterial activity for common chitosan derivatives (Paper II)

The cationic, neutral, and anionic chitosan derivatives **1a** to **7e** were investigated for antibacterial activity against Gram-positive *S. aureus* and Gram-negative *E. coli* at pH 7.2 and 5.5 (**Table 10**). The antimicrobial effect has been attributed to the electrostatic interaction between positively charged chitosan polymer chains and negatively charged microbial cell membranes (Helander, Nurmiaho-Lassila et al. 2001, Kong, Chen et al. 2010, Dash, Chiellini et al. 2011). Chitosan, HPC, and TGC have protonated amines, with pKa around 6, and these

groups are expected to be mostly protonated cationic form at pH 5.5, whereas the positive charge is much diminished at pH 7.2. The quaternary derivatives $TMC_{NH2/TM}$, and $TMC_{TM/DM}$ should have a cationic charge largely independent of DS at pH 5.5, whereas the DS should determine the cationic charge density at pH 7.2. The DS should only determine the cationic charge of TACin as the structure does not contain any protonable groups. The CMC derivative should be zwitterionic with cationic charges dominating at pH 5.5, whereas the charge should be mostly anionic at pH 7.2.

The antibacterial activity of the TACin shows good activity against *S. aureus* and *E. coli* at pH 7.2 than unmodified chitosan at pH 7.2. However, at a lower pH of 5.5, these derivatives were less active than chitosan. The general trend for $TMC_{NH2/TM}$ and $TMC_{TM/DM}$ was an increase in the activity with DS, which is more marked at pH 7.2 and especially for *S. aureus*. The most active structures were significantly more active than chitosan, and TMC tended to be more active than TACin.

The lowest DS structure of the HTC derivative (DS 0.16, **4a**) was the most active. The activity decreased with an increase in the DS except for *S. aureus* at pH 7.2, where the DS did not influence the MIC values. These results indicated that the HTC side chain does not contribute to increased antibacterial activity. This was surprising as HTC is often reported as an antibacterial chitosan derivative (Cheah, Show et al. 2019). The HTC side chain will, however, contribute to improved solubility. This can influence the measured antimicrobial effect under some testing conditions.

Table 10. MIC analysis for chitosan and synthesized chitosan derivatives against *S. aureus* and *E. coli* bacteria.

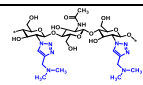
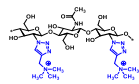
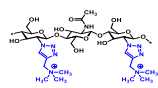
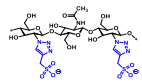
Compounds	DS	*S. aureus* (μg/mL)		*E. coli* (μg/mL)	
		pH: 7.2	pH: 5.5	pH: 7.2	pH: 5.5
Chitosan		256	256	2048	512
TACin 1a	0.07	2048	4096	≥8192	≥8192
1b	0.18	1024	2048	4096	4096
1c	0.58	128	4096	512	4096
1d	0.8	64	≥8192	128	1024
1e	0.88	64	≥8192	64	2048
$TMC_{NH2/TM}$ 2a	0.06	128	2048	128	1024
2b	0.25	128	512	2048	512
2c	0.26	64	128	512	256
2d	0.41	32	64	256	256
2e	0.53	32	512	512	512
2f	0.89	32	2048	128	512
$TMC_{TM/DM}$ 3a	0.18	≥8192	256	≥8192	128
3b	0.32	256	256	512	128
3c	0.44	64	256	64	128
3d	0.34	32	128	256	64
3e	0.28	8	32	256	128
HTC 4a	0.16	8	32	64	256
4b	0.37	64	256	2048	2048
4c	0.55	64	256	1024	2048
4d	0.83	64	1024	4096	4096
4e	1.07	64	4096	64	≥8192
4f	1.1	64	1024	4096	4096
HPC 5a	0.08	256	512	256	512
5b	0.17	256	512	512	256
5c	0.36	1024	512	2048	256
5d	0.61	4096	2048	4096	1024
5e	0.84	2048	512	1024	1024
5f	1.05	4096	4096	≥8192	≥8192
TGC 6a	0.02	256	128	512	256
6b	0.04	256	512	512	512
CMC 7a	0.31	≥8192	≥8192	≥8192	≥8192
7b	0.47	≥8192	≥8192	≥8192	≥8192
7c	0.7	≥8192	≥8192	≥8192	≥8192
7d	0.84	≥8192	≥8192	≥8192	≥8192
7e	1.06	≥8192	≥8192	≥8192	≥8192

The low DS (0.08) HPC derivative exhibited activity that was similar to unmodified chitosan. However, when DS increased, the activity decreased. The DS for the two TGC derivatives was negligible, and the activity was generally very similar to chitosan, as expected. Carboxymethylated chitosan is the most studied chitosan derivative. Some studies have reported antimicrobial activity (Gupta and Haile 2007, Upadhyaya, Singh et al. 2013, Zhou, Shi et al. 2015). However, derivatives **7a-e** showed no effect in the broth microdilution assay (MIC ≥ 8192 µg/mL). These compounds are zwitterionic and soluble in 0.01 M HCl solution. It is also possible that the ionic interaction between polymer chains also inhibits these structures' antimicrobial action. Our findings are supported and the finding that CMC is inactive against *Candiat* species (Seyfarth, Schliemann et al. 2008).

4.2.3 Effect of chitotriazolan derivatives on antibacterial activity (Paper III)

The antibacterial activity of water-soluble chitotriazolan derivatives was evaluated against *S. aureus* and *E. coli* bacteria at pH 7.2 (**Table 11**). The cationic chitotriazolan derivative **10** was the most active, with a MIC value equal to 64 ug/mL, whereas the anionic derivative **11** was inactive. The structure of derivative **9** was identical to the structure of derivative **10.** Still, it was more than 30 times less active against the strain. TBDMS chitosan was the starting material for the synthesis of **9,** and this derivative had some residual TBDMS groups (DS TBDMS ~ 0.004) on the polymer backbone. This was also the case for compound **8** (DS TBDMS < 0.03)**,** which was not quaternized with one less *N*-methyl group and inactive against the bacteria. The TBDMS groups are highly lipophilic and may negatively impact the activity. The protection and deprotection steps reduce the Mw, which may also affect the activity. The MW of **10** was 214.59 kDa**,** whereas the Mw of **8** and **9** were 28.94 kDa and 17.05 kDa, respectively.

Table 11. Antibacterial activity of water-soluble chitotriazolan derivatives

Derivatives	Structure	MIC (μg/mL)	
		S. aureus	*E. coli*
8		≥8192	≥8192
9		2048	2048
10		64	64
11		≥8192	≥8192
Gentamicin	-	0.25	1

4.2.4 Antibacterial properties for mixed chitotriazolan derivatives (Paper IV)

Investigation of the antimicrobial properties and mode of action of chitosan has been a long journey of scientific exploration that began decades ago; there is still much to be learned. There have been some reports on the antimicrobial properties of chitosan containing 1,2,3-triazole moieties obtained by CuAAC, including Chitosan *N*-propyl and hydroxypropyl triazolyl betaine ester (Kritchenkov, Egorov et al. 2019) chitin 6-*O*-hydroxypropyl triazolyl betaine ester (Kritchenkov, Egorov et al. 2021) chitosan *N*-alkyl triazolyl ethyltrimethylammoniumn (Tan, Zhang et al. 2018), chitosan *N*-alkyl triazolinum pyridinium (Tan, Li et al. 2018), chitosan *N*-alkyl triazolyl triphenylphosphonium (Tan, Zhang et al. 2020) TMC – *C*-6-halogenotriazole (Li, Tan et al. 2016) and chitosan *N*-, 6-*O* triazolyl methyl nicotinate (Qin, Liu et al. 2013).

Table 12. Antibacterial activity for mixed chitotriazolan derivatives

Derivatives	MIC (μg/mL)			
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*
10	64	1024	128	128
19	64	2048	512	4096
20	128	2048	256	1024
21	128	2048	128	512
22	1024	4096	512	1024
23	≥4096	≥4096	≥4096	≥4096

The mixed chitotriazolans were investigated for activity against Gram-positive *S. aureus (29213) and E. faecalis (29212),* Gram-negative *E. coli (25922), and P. aeruginosa (27853)* (**Table 12)**. The cationic chitotriazolan derivative **10** was equally or more active than the mixed chitotriazolans **19-23** against all the strains. The cationic mixed chitotriazolans **19-21** were equally or slightly less active (1-2 dilutions) than **10** against *S. aureus*, *E. faecalis*, and *E. Coli*, but **19** and **20** were considerably less active (5 and 3 dilutions difference) against *P. aeruginosa*. In contrast, **21** was less active (2 dilutions) against *P. aeruginosa* than **10**. This is somewhat consistent with previous work, where we have also observed that the structure-activity relationship for quaternary *N*-alkyl chitosan against *P. aeruginosa* was different from the relationship for *S. aureus* and *E. coli* but similar to the relationship for *E. faecalis* (Sahariah, Benediktssdóttir et al. 2015).

The CMC-chitotriazolan **23** lacked activity against all tested microorganisms. The anionic derivative from the previous section **11** was inactive against bacteria, and CMC derivatives **7a-7e** with DS ranging from 0.3 to 1.1 lacked activity against *S. aureus* and *E. coli*. These results finding that the anionic chitosan derivative. The DS for triazole *N,N,N*-trimethyl group was 68 %, so a net positive charge for 23 is expected, contributing to excellent solubility. Other CMC derivatives with quaternary ammonium substituents have been reported and investigated for antimicrobial activity. Sun et al (Sun, Du et al. 2006) said that hydroxypropyl trimethylammonium (HT) CMCs were more active against *S. aureus* and *E. coli*

than CMC and HTC. In contrast, Xu et al. have reported that 6-*O* carboxymethyl TMC was less active against the same bacteria than the parent TMC polymer (Xu, Xin et al. 2010), which is more consistent with the results for mixed chitotriazolan **23**.

4.2.5 Effect of different chitotriazolan derivatives on antibacterial activity (Paper V)

This study evaluated quaternized and basic chitotriazolan derivatives against Gram-positive and Gram-negative microorganisms (*S. aureus (29213)*, *E. faecalis (29212)*, *E. coli (25922)*, and *P. aeruginosa (27853*). The MIC and MLC (µg/mL) values were determined and are shown in **Table 13**. The cationic quaternary chitotriazolan derivatives worked effectively against all bacterial strains; the values range from 256 to 8192 µg/mL. The trimethylammonium chitotriazolan derivatives exhibit better activity against all tested bacteria. Still, surprisingly, in this study, derivative **24** had 512 µg/mL against *S. aureus,* which was three times less activity than **10**. The *N,N,N*-triethylammonium derivative **25** was similar activity except for *S.aureus*. The piperazine derivatives **27**, **30**, and **31** were tested against all bacteria. The activity shows **27,** and others were not active against all tested bacteria. The derivatives **26**, **28**, and **29** had better activity against all bacteria; the activity value ranged between 256 to 8192 µg/mL, and derivative **29** was the most active in this series of studies. It had 256 µg/mL except for *E. faecalis* bacteria. The diethanolammonium derivative **32** was not active at all against bacteria. MLC value for all derivatives had similar activity except **24**, **25**, and **31** against *P. aeruginosa*.

Table 13. Antibacterial activity of chitotriazolan derivatives for MIC and MLC values

Derivatives	Structure	MIC (µg/mL)			
		S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*
24		512	8192	512	1024 *MLC 2048
25		8192	8192	512	1024 *MLC 2048
26		256	8192	4096	512
27		4096	8192	1024	4096
28		512	2048	512	1024
29		256	4096	256	256
30		8192	8192	8192	≥8192
31		≥8192	≥8192	≥8192	8192 *MLC ≥8192
32		≥8192	≥8192	≥8192	≥8192

4.2.6 Antibacterial analysis of antimicrobial peptide chitosan conjugates (Paper VI)

The antibacterial activity of CRAMP-18 grafted chitotriazolan derivatives was measured by serial dilution method at physiological pH 7.2 against Gram-positive *S. aureus (29213), E. faecalis (29212),* and Gram-negative bacteria *E. coli (25922), and P. aeruginosa (27853)* and the resulting MIC values are presented in **Table 14.** The CRAMP-18 grafted chitotriazolan **34** and **35** derivatives were active against *E. coli* bacteria at 256 µg/mL and active against *P. aeruginosa* 1024 and 512 µg/mL, respectively, which shows that CRAMP-18

grafted chitotriazolan derivatives active against Gram-negative bacteria whereas not active or equal to 4096 µg/mL against both Gram-positive bacteria.

Table 14. Antibacterial activity for CRAMP-18 conjugated chitosan biopolymers

Derivatives	MIC (µg/mL)			
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*
34	≥4096	≥4096	256	1024
35	≥4096	≥4096	256	512
36	1024	4096	4096	4096
37	4096	4096	2048	4096
38	4096	4096	4096	4096
33*	128	128	8	64

***Pentynoyl-CRAMP-18 peptide

The azide might lack the activity for both **34** and **35** biopolymers because a low percentage of azide residue was retained in the antimicrobial peptide chitosan polymers. The parent structure **36** reduced the activity relative to chitotriazolan **10** because **36** was not fully substituted. The derivatives **37** and **38** activity were consistent with the mixed HPC-chitotriazolan **22** and HPC derivatives against Gram-positive and Gram-negative bacteria. The structures of antimicrobial chitosan conjugates are shown in **Fig. 19**.

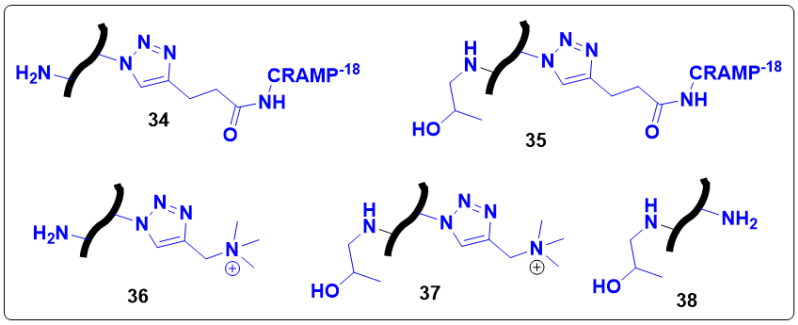


Figure 19. Structures of antimicrobial chitosan conjugates

5 Summary and Conclusions

Chitosan is a linear renewable biopolymer that has a unique polycationic chemical structure with a high charge density, reactive hydroxyl, and amino groups. The low solubility of chitosan in neutral and alkaline solutions limits its application. Nevertheless, the chemical modification of chitosan can be used to make derivatives with new functional properties for a wide range of applications.

Synthetic modification of chitosan

The new $TMC_{NH2/TM}$ derivatives were synthesized through TBDMS and Boc protection strategies $TMC_{NH2/TM,}$ where the C-2 amino groups were partially in the primary state and partially *N*,*N*,*N*-trimethylated. The first synthesis of cationic chitin (TACin) derivatives synthesized by TBDMS protection and re-acetylation was reported in this work. TBDMS protection strategy allowed better control of the DS than the conventional reductive alkylation and methylation strategy for the synthesis of $TMC_{DM/TM}$.

The common chitosan derivatives such as $TMC_{NH2/TM}$, TACin, $TMC_{DM/TM}$, HTC, HPC, TGC, and CMC were synthesized (**Fig. 20**) in each structure with various ranges (0.02 – 1.1) of the degree of substitution by different ratios of the reagent.

The chitosan *C*-2 amino group was converted to azide and then modified to 1,2,3-triazole (**Fig. 20**), and we successfully obtained the complete conversion til we gave the first water-soluble chitotriazolan derivatives. The chitotriazolan derivatives could be synthesized without using TBDMS protection strategies.

We aimed to synthesize mixed chitotriazolan structures with common chitosan derivatives (TMC, TAC, HTC, HPC, and CMC) **Fig. 20**. The partial degree of substitution for all common chitosan derivatives, such as TMC, TAC, HTC, HPC, and CMC was synthesized. Then, the remaining primary amino group in these derivatives was converted to 1,2,3-triazole moieties via click chemistry.

The click chemistry strategy was further explored by synthesizing a series of new quaternized and basic protonable chitotriazolan derivatives (**Fig. 20**). The CuAAC reaction was carried out with various alkynes; in every case, a quantitative conversion of the chitosan azide was achieved. Quaternized derivatives were highly water soluble.

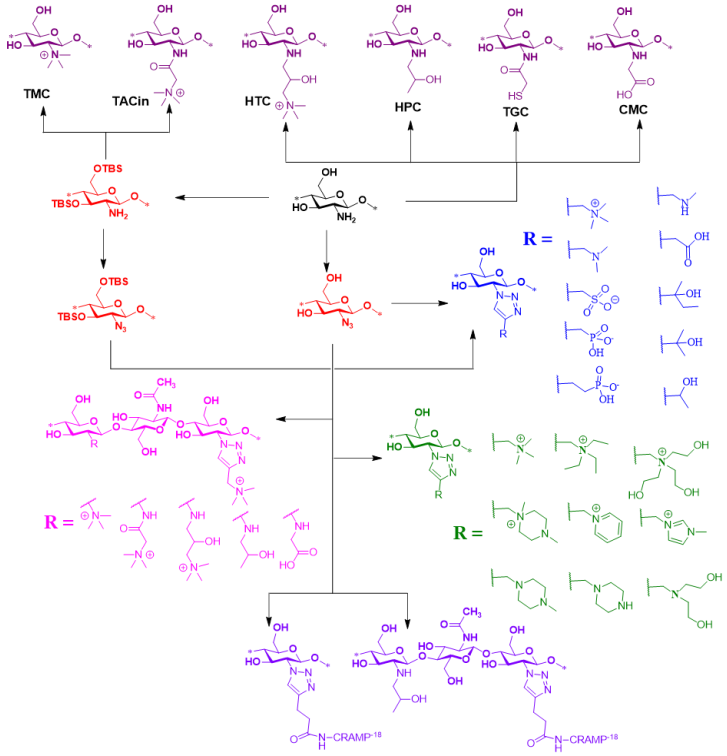


Figure 20. The summary of modified chitosan derivatives with different structures

The chitosan azide and HPC azide with a low degree of azidation were synthesized to conjugate an antimicrobial peptide. The antimicrobial CRAMP-18 peptide was synthesized via the solid-phase peptide synthesis method. The pentynoyl-CRAMP-18 peptide was conjugated to chitosan azide and HPC azide via CuAAC reaction (**Fig. 20**).

The structure-activity relationship for chitosan derivatives

The structure activity-relationship for the $TMC_{NH2/TM}$ and TACin derivatives was remarkably different. The $TMC_{NH2/TM}$ antibacterial activity was increased at low DS after 0.2–0.3 DS maintained the activity, and there was no change in activity up to DS 0.89. In contrast, the activity of TACin increased continuously with DS from 0.07 to 0.88. The TACin derivative had more activity with MIC equal to 256 µg/mL at the highest DS than $TMC_{NH2/TM}$ derivative against *P. aeruginosa*. The highest DS $TMC_{NH2/TM}$ and TACin derivatives were equal in activity against MRSA, with MIC equal to 64 µg/mL.

The most active compounds were $TMC_{NH2/TM}$ and $TMC_{TM/DM}$, and there was a positive correlation between DS and activity, especially at pH 7.2. TACin was less active, especially at low DS, but there was a correlation between DS for the quarternized 2-(*N,N,N*-trimethylammoniumyl)acetyl) group and activity. These results are consistent with our previous investigations. The activity of HTC derivatives was considerably mixed as the structure with the lowest DS had more activity than unmodified chitosan, but an increase in DS generally led to a decrease in the activity. The neutral HPC derivatives had a clear inverse relationship between DS and activity. The TGC structure had very low DS, and the activity was not different from chitosan. All CMC derivatives were inactive against the bacteria; thus, no correlation with DS could be observed (**Fig. 21**).

The antibacterial activity was evaluated against *S. aureus* and *E. coli* at pH 7.2. The cationic chitotriazolan derivatives with *N,N,N*-trimethylammoniummethyl had significant antibacterial activity, whereas the anionic chitotriazolans were inactive.

Mixed chitotriazolan derivatives were partially substituted with TMC, TAC, HTC, and HPC, showing enhanced antibacterial activity against *S. aureus* and *E. coli* relative to *E. faecalis* and *P. aeruginosa*. However, the chitotriazolan derived from anionic CMC was not active against any of the strains.

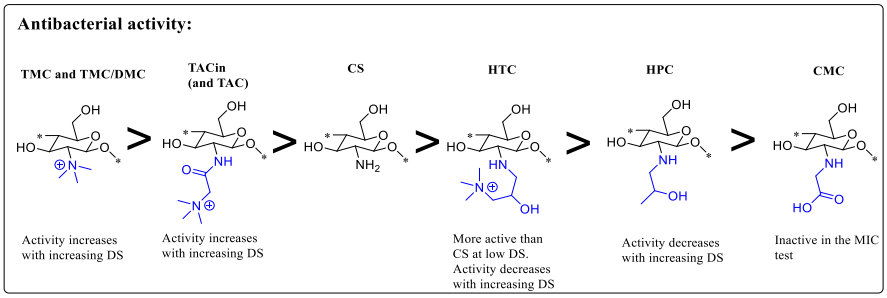


Figure 21. Summary of SAR for chitosan derivatives against *S. aureus* and *E. coli* at pH 7.2.

There is a clear structure-antibacterial activity relationship for chitotriazolan derivatives. Chitotriazolans structure with quaternary ammonium groups displayed more activity than chitotriazolans with basic protonable amino groups. The chitotriazolan with methylimidazole was most active (256 µg/mL) against *S. aureus, E. coli,* and *P. aeruginosa* for *E. faecalis* 4096 µg/mL.

The chitosan-CRAMP-18 conjugated biopolymer showed significant activity against *E. coli* (256 µg/mL) than unmodified chitosan. The HP-chitotriazolan-CRAMP-18 peptide showed similar activity.

6 Future perspectives

The study focused on synthesizing and structural modifying a new class of chitosan biopolymer derivatives using TBDMS-chitosan and unmodified chitosan. The chitosan derivatives were investigated for antibacterial activity against Gram-positive and Gram-negative bacteria. The structure-activity relationship has been studied for the chitosan derivatives to understand the influence of side chain structure with different degrees of substitution and antibacterial activity. These results found that the structure with the quaternary ammonium group displayed water solubility and the most active derivatives against all bacteria. The synthesis and structural modification of this new class of biopolymers should stimulate further research into the biological properties and utility of diverse applications. Further work could focus on developing chitosan derivatives or conjugates to eradicate and prevent bacterial biofilm formation. These could, for example, be used for coating medical implants. In the investigation of the antimicrobial mode of action, the chitosan derivatives could be synthesized with fluorescent-molecular labels. Thus, it would be possible to study the localization in the cell membrane and other parts of the microorganism to elucidate the mechanism of action. Another possible future work would be to develop antimicrobial nanoparticles based on chitotriazolan conjugates with antimicrobial substances other than AMP, including known drugs. In the antimicrobial mode of action, future work should clarify the molecular details of the underlying mechanisms and their relevance to the antimicrobial activity of chitosan derivatives.

References

Abueva, C., H. S. Ryu, J. W. Min, P. S. Chung, H. S. You, M. S. Yang and S. H. Woo (2021). "Quaternary ammonium N,N,N-trimethyl chitosan derivative and povidone-iodine complex as a potent antiseptic with enhanced wound healing property." International Journal of Biological Macromolecules **182**: 1713-1723.

Agard, N. J., J. M. Baskin, J. A. Prescher, A. Lo and C. R. Bertozzi (2006). "A Comparative Study of Bioorthogonal Reactions with Azides." ACS Chemical Biology **1**(10): 644-648.

Agnew, B., S. Buck, T. Nyberg, J. Bradford, S. Clarke and K. Gee (2008). Click chemistry for labeling and detection of biomolecules, SPIE.

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

Ágoston, K., H. Streicher and P. Fügedi (2016). "Orthogonal protecting group strategies in carbohydrate chemistry." Tetrahedron: Asymmetry **27**(16): 707-728.

Aguilar, A., N. Zein, E. Harmouch, B. Hafdi, F. Bornert, D. Offner, F. Clauss, F. Fioretti, O. Huck, N. Benkirane-Jessel and G. Hua (2019). "Application of Chitosan in Bone and Dental Engineering." Molecules (Basel, Switzerland) **24**(16): 3009.

Ai, H., F. Wang, Y. Xia, X. Chen and C. Lei (2012). "Antioxidant, antifungal and antiviral activities of chitosan from the larvae of housefly, Musca domestica L." Food Chemistry **132**(1): 493-498.

Akter Mukta, J., M. Rahman, A. As Sabir, D. R. Gupta, M. Z. Surovy, M. Rahman and M. T. Islam (2017). "Chitosan and plant probiotics application enhance growth and yield of strawberry." Biocatalysis and Agricultural Biotechnology **11**: 9-18.

Alburquenque, C., S. A. Bucarey, A. Neira-Carrillo, B. Urzúa, G. Hermosilla and C. V. Tapia (2010). "Antifungal activity of low molecular weight chitosan against clinical isolates of Candida spp." Medical Mycology **48**(8): 1018-1023.

Allan, C. R. and L. A. Hadwiger (1979). "The fungicidal effect of chitosan on fungi of varying cell wall composition." Experimental Mycology **3**(3): 285-287.

Amborabé, B.-E., J. Bonmort, P. Fleurat-Lessard and G. Roblin (2008). "Early events induced by chitosan on plant cells." Journal of Experimental Botany ***59***(9): 2317-2324.

Anitha, A., V. V. Divya Rani, R. Krishna, V. Sreeja, N. Selvamurugan, S. V. Nair, H. Tamura and R. Jayakumar (2009). "Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, O-carboxymethyl and N,O-carboxymethyl chitosan nanoparticles." Carbohydrate Polymers **78**(4): 672-677.

Antony, R., T. Arun and S. T. D. Manickam (2019). "A review on applications of chitosan-based Schiff bases." International Journal of Biological Macromolecules **129**: 615-633.

Aoyagi, S., H. Onishi and Y. Machida (2007). "Novel chitosan wound dressing loaded with minocycline for the treatment of severe burn wounds." International Journal of Pharmaceutics **330**(1): 138-145.

Aranaz, I., N. Acosta, C. Civera, B. Elorza, J. Mingo, C. Castro, M. D. L. L. Gandía and A. Heras Caballero (2018). "Cosmetics and Cosmeceutical Applications of Chitin, Chitosan and Their Derivatives." Polymers **10**(2): 213.

Badawy, M. E. (2008). "Chemical modification of chitosan: synthesis and biological activity of new heterocyclic chitosan derivatives." Polymer International ***57***(2): 254-261.

Badawy, M. E. I. and E. I. Rabea (2016). "Synthesis and Antimicrobial Activity of N-(6-Carboxyl Cyclohex-3-ene Carbonyl) Chitosan with Different Degrees of Substitution." International Journal of Carbohydrate Chemistry **2016**: 6046232.

Barbosa, M., F. Costa, C. Monteiro, F. Duarte, M. C. L. Martins and P. Gomes (2019). "Antimicrobial coatings prepared from Dhvar-5-click-grafted chitosan powders." Acta Biomaterialia **84**: 242-256.

Barbosa, M., N. Vale, F. M. T. A. Costa, M. C. L. Martins and P. Gomes (2017). "Tethering antimicrobial peptides onto chitosan: Optimization of azide-alkyne "click" reaction conditions." Carbohydrate Polymers **165**: 384-393.

Benediktsdóttir, B. E., Ó. Baldursson and M. Másson (2014). "Challenges in evaluation of chitosan and trimethylated chitosan (TMC) as mucosal permeation enhancers: From synthesis to in vitro application." Journal of Controlled Release **173**: 18-31.

Benediktsdóttir, B. E., V. S. Gaware, Ö. V. Rúnarsson, S. Jónsdóttir, K. J. Jensen and M. Másson (2011). "Synthesis of N,N,N-trimethyl chitosan homopolymer and highly substituted N-alkyl-N,N-dimethyl chitosan derivatives with the aid of di-tert-butyldimethylsilyl chitosan." Carbohydrate Polymers **86**(4): 1451-1460.

Benediktsdóttir, B. E., T. Gudjónsson, Ó. Baldursson and M. Másson (2014). "N-alkylation of highly quaternized chitosan derivatives affects the paracellular permeation enhancement in bronchial epithelia in vitro." European Journal of Pharmaceutics and Biopharmaceutics **86**(1): 55-63.

Berezin, A. S., E. A. Lomkova and Y. A. Skorik (2012). "Chitosan conjugates with biologically active compounds: design strategies, properties, and targeted drug delivery." Russian Chemical Bulletin **61**(4): 781-795.

Best, M. D. (2009). "Click Chemistry and Bioorthogonal Reactions: Unprecedented Selectivity in the Labeling of Biological Molecules." Biochemistry **48**(28): 6571-6584.

Beveridge, T. J. (1999). "Structures of gram-negative cell walls and their derived membrane vesicles." J Bacteriol **181**(16): 4725-4733.

Blagodatskikh, I. V., S. N. Kulikov, O. V. Vyshivannaya, E. A. Bezrodnykh and V. E. Tikhonov (2017). "N-Reacetylated Oligochitosan: pH Dependence of Self-Assembly Properties and Antibacterial Activity." Biomacromolecules **18**(5): 1491-1498.

Blagodatskikh, I. V., O. V. Vyshivannaya, A. V. Alexandrova, E. A. Bezrodnykh, P. V. Zelenikhin, S. N. Kulikov and V. E. Tikhonov (2018). "Antibacterial Activity and Cytotoxicity of Betainated Oligochitosane Derivatives." Microbiology **87**(5): 725-731.

Bowdish, D. M., D. J. Davidson and R. E. Hancock (2005). "A re-evaluation of the role of host defence peptides in mammalian immunity." Curr Protein Pept Sci **6**(1): 35-51.

Božič, M., S. Gorgieva and V. Kokol (2012). "Laccase-mediated functionalization of chitosan by caffeic and gallic acids for modulating antioxidant and antimicrobial properties." Carbohydrate Polymers **87**(4): 2388-2398.

Braconnot, H. J. A. C. (1881). "Sur la nature des champibnons." **79**: 265-304.

Burdock, G. A. (2007). "Safety assessment of hydroxypropyl methylcellulose as a food ingredient." Food and Chemical Toxicology **45**(12): 2341-2351.

Cele, Z. E. D., A. M. Somboro, D. G. Amoako, L. F. Ndlandla and M. O. Balogun (2020). "Fluorinated Quaternary Chitosan Derivatives: Synthesis, Characterization, Antibacterial Activity, and Killing Kinetics." ACS Omega **5**(46): 29657-29666.

Chang, S.-H., H.-T. V. Lin, G.-J. Wu and G. J. Tsai (2015). "pH Effects on solubility, zeta potential, and correlation between antibacterial activity and molecular weight of chitosan." Carbohydrate Polymers **134**: 74-81.

Cheah, W. Y., P.-L. Show, I. S. Ng, G.-Y. Lin, C.-Y. Chiu and Y.-K. Chang (2019). "Antibacterial activity of quaternized chitosan modified nanofiber membrane." International Journal of Biological Macromolecules **126**: 569-577.

Chen, Y., Y. Ye, Y. Jing, Y. Gao, Y. Guo and H. Tan (2015). "The Synthesis of the Locating Substitution Derivatives of Chitosan by Click Reaction at the 6-Position of Chitin." International Journal of Polymer Science **2015**: 419506.

Chirkov, S. N. (2002). "The Antiviral Activity of Chitosan (Review)." Applied Biochemistry and Microbiology **38**(1): 1-8.

Chirkov, S. N., A. V. Il'ina, N. A. Surgucheva, E. V. Letunova, Y. A. Varitsev, N. Y. Tatarinova and V. P. Varlamov (2001). "Effect of Chitosan on Systemic Viral Infection and Some Defense Responses in Potato Plants." Russian Journal of Plant Physiology **48**(6): 774-779.

Cho, J., J. Grant, M. Piquette-Miller and C. Allen (2006). "Synthesis and Physicochemical and Dynamic Mechanical Properties of a Water-Soluble Chitosan Derivative as a Biomaterial." Biomacromolecules **7**(10): 2845-2855.

Cho, Y.-S., D.-S. Lee, Y.-M. Kim, C.-B. Ahn, D.-H. Kim, W.-K. Jung and J.-Y. Je (2013). "Protection of hepatic cell damage and antimicrobial evaluation of chitosan-catechin conjugate." Journal of the Korean Society for Applied Biological Chemistry **56**(6): 701-707.

Chowdhury, M. Y. E., T.-H. Kim, M. B. Uddin, J.-H. Kim, C. Y. Hewawaduge, Z. Ferdowshi, M.-H. Sung, C.-J. Kim and J.-S. Lee (2017). "Mucosal vaccination of conserved sM2, HA2 and cholera toxin subunit A1 (CTA1) fusion protein with poly gamma-glutamate/chitosan nanoparticles (PC NPs) induces protection against divergent influenza subtypes." Veterinary Microbiology **201**: 240-251.

Chung, Y.-C. and C.-Y. Chen (2008). "Antibacterial characteristics and activity of acid-soluble chitosan." Bioresource Technology **99**(8): 2806-2814.

CLSI, I. (2009). "CLSI document M07-A8." Clinical Laboratory Standards Institute, Wayne, Pennsylvania, USA **29**.

Croce, M., S. Conti, C. Maake and G. R. Patzke (2016). "Synthesis and screening of N-acyl thiolated chitosans for antibacterial applications." Carbohydrate polymers **151**: 1184-1192.

Curti, E., D. de Britto and S. P. Campana-Filho (2003). "Methylation of Chitosan with Iodomethane: Effect of Reaction Conditions on Chemoselectivity and Degree of Substitution." **3**(10): 571-576.

Dash, M., F. Chiellini, R. M. Ottenbrite and E. Chiellini (2011). "Chitosan—A versatile semi-synthetic polymer in biomedical applications." Progress in Polymer Science **36**(8): 981-1014.

De Brucker, K., N. Delattin, S. Robijns, H. Steenackers, N. Verstraeten, B. Landuyt, W. Luyten, L. Schoofs, B. Dovgan, M. Fröhlich, J. Michiels, J. Vanderleyden, B. P. Cammue and K. Thevissen (2014). "Derivatives of the mouse cathelicidin-related antimicrobial peptide (CRAMP) inhibit fungal and bacterial biofilm formation." Antimicrob Agents Chemother **58**(9): 5395-5404.

De Brucker, K., N. Delattin, S. Robijns, H. Steenackers, N. Verstraeten, B. Landuyt, W. Luyten, L. Schoofs, B. Dovgan, M. Fröhlich, J. Michiels, J. Vanderleyden, B. P. A. Cammue and K. Thevissen (2014). "Derivatives of the mouse cathelicidin-related antimicrobial peptide (CRAMP) inhibit fungal and bacterial biofilm formation." Antimicrobial agents and chemotherapy **58**(9): 5395-5404.

Di Colo, G., Y. Zambito, S. Burgalassi, I. Nardini and M. F. Saettone (2004). "Effect of chitosan and of N-carboxymethylchitosan on intraocular penetration of topically applied ofloxacin." International Journal of Pharmaceutics **273**(1): 37-44.

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

Dragostin, O. M., S. K. Samal, M. Dash, F. Lupascu, A. Pânzariu, C. Tuchilus, N. Ghetu, M. Danciu, P. Dubruel, D. Pieptu, C. Vasile, R. Tatia and L. Profire (2016). "New antimicrobial chitosan derivatives for wound dressing applications." Carbohydrate Polymers **141**: 28-40.

Dutta, P. K., S. Tripathi, G. K. Mehrotra and J. Dutta (2009). "Perspectives for chitosan based antimicrobial films in food applications." Food Chemistry **114**(4): 1173-1182.

El-Shafei, A. M., M. M. G. Fouda, D. Knittel and E. Schollmeyer (2008). "Antibacterial activity of cationically modified cotton fabric with carboxymethyl chitosan." **110**(3): 1289-1296.

Erdem, B., E. Kariptas, T. Kaya, S. Tulumoglu and Ö. Görgülü (2016). "Factors influencing antibacterial activity of chitosan against Aeromonas hydrophila and Staphylococcus aureus." International Current Pharmaceutical Journal **5**(5): 45-48.

Fang, Z., D. Lin, R. D. Warner and M. Ha (2018). "Effect of gallic acid/chitosan coating on fresh pork quality in modified atmosphere packaging." Food Chem **260**: 90-96.

Fernandes, J. C., F. K. Tavaria, S. C. Fonseca, O. S. Ramos, M. E. Pintado and F. X. Malcata (2010). "In vitro screening for anti-microbial activity of chitosans and chitooligosaccharides, aiming at potential uses in functional textiles." J Microbiol Biotechnol **20**(2): 311-318.

Foster, L. J. R., S. Ho, J. Hook, M. Basuki and H. Marçal (2015). "Chitosan as a Biomaterial: Influence of Degree of Deacetylation on Its Physiochemical, Material and Biological Properties." PLOS ONE **10**(8): e0135153.

Freitas, E. D., C. F. Moura, Jr., J. Kerwald and M. M. Beppu (2020). "An Overview of Current Knowledge on the Properties, Synthesis and Applications of Quaternary Chitosan Derivatives." Polymers (Basel) **12**(12).

Fu, X., Y. Shen, X. Jiang, D. Huang and Y. Yan (2011). "Chitosan derivatives with dual-antibacterial functional groups for antimicrobial finishing of cotton fabrics." Carbohydrate Polymers **85**(1): 221-227.

Gaitor, J. C., L. M. Paul, M. M. Reardon, T. Hmissa, S. Minkowicz, M. Regner, Y. Sheng, S. F. Michael, S. Isern and A. Mirjafari (2017). "Ionic liquids with thioether motifs as synthetic cationic lipids for gene delivery." Chemical Communications **53**(59): 8328-8331.

Gallo, R. L., K. J. Kim, M. Bernfield, C. A. Kozak, M. Zanetti, L. Merluzzi and R. Gennaro (1997). "Identification of CRAMP, a Cathelin-related Antimicrobial Peptide Expressed in the Embryonic and Adult Mouse*." Journal of Biological Chemistry **272**(20): 13088-13093.

Gao, Y., Z. Zhang, L. Chen, W. Gu and Y. Li (2009). "Chitosan N-betainates/DNA self-assembly nanoparticles for gene delivery: In vitro uptake and transfection efficiency." International Journal of Pharmaceutics **371**(1): 156-162.

Gao, Y., Z. Zhang, L. Chen, W. Gu and Y. Li (2009). "Synthesis of 6-N,N,N-Trimethyltriazole Chitosan via "Click Chemistry" and Evaluation for Gene Delivery." Biomacromolecules **10**(8): 2175-2182.

Gaware, V. S., M. Håkerud, A. Juzeniene, A. Høgset, K. Berg and M. Másson (2017). "Endosome Targeting meso-Tetraphenylchlorin−Chitosan Nanoconjugates for Photochemical Internalization." Biomacromolecules **18**(4): 1108-1126.

Gaware, V. S., M. Håkerud, K. Leósson, S. Jónsdóttir, A. Høgset, K. Berg and M. Másson (2013). "Tetraphenylporphyrin Tethered Chitosan Based Carriers for Photochemical Transfection." Journal of Medicinal Chemistry **56**(3): 807-819.

Geisberger, G., E. B. Gyenge, D. Hinger, A. Käch, C. Maake and G. R. Patzke (2013). "Chitosan-Thioglycolic Acid as a Versatile Antimicrobial Agent." Biomacromolecules **14**(4): 1010-1017.

Goddard-Borger, E. D. and R. V. Stick (2007). "An Efficient, Inexpensive, and Shelf-Stable Diazotransfer Reagent: Imidazole-1-sulfonyl Azide Hydrochloride." Organic Letters **9**(19): 3797-3800.

Goy, R. C., D. D. Britto and O. B. G. Assis (2009). "A review of the antimicrobial activity of chitosan." Polímeros **19**(3): 241-247.

Goy, R. C., S. T. B. Morais and O. B. G. Assis (2016). "Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on E. coli and S. aureus growth." Revista Brasileira de Farmacognosia **26**: 122-127.

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

Ha, J. M., S. Y. Shin and S. W. Kang (1999). "Synthesis and antibiotic activities of CRAMP, a cathelin-related antimicrobial peptide and its fragments." Bulletin of the Korean Chemical Society **20**(9): 1073-1077.

Han, B., Y. Wei, X. Jia, J. Xu and G. Li (2012). "Correlation of the structure, properties, and antimicrobial activity of a soluble thiolated chitosan derivative." Journal of Applied Polymer Science **125**(S2): E143-E148.

Hancock, R. E. W. and H.-G. Sahl (2006). "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies." Nature Biotechnology **24**(12): 1551-1557.

Harmon, R. E., K. K. De and S. K. Gupta (1973). "Preparation of Trimethylsilyl Derivatives of Polysaccharides." **25**(12): 429-431.

Hatta, S., S. Kuwabara, H. Miyamoto, K. Aoyama, N. Utsunomiya and S. Tanji (1950). "Studies on macramin, a new high-molecular antibacterial substance derived from chitin." Jpn Med J (Natl Inst Health Jpn) **3**(2): 119-123.

Hecq, J., F. Siepmann, J. Siepmann, K. Amighi and J. Goole (2015). "Development and evaluation of chitosan and chitosan derivative nanoparticles containing insulin for oral administration." Drug Development and Industrial Pharmacy **41**(12): 2037-2044.

Hejazi, R. and M. Amiji (2003). "Chitosan-based gastrointestinal delivery systems." Journal of Controlled Release **89**(2): 151-165.

Helander, I. M., E. L. Nurmiaho-Lassila, R. Ahvenainen, J. Rhoades and S. Roller (2001). "Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria." International Journal of Food Microbiology **71**(2): 235-244.

Himo, F., T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless and V. V. Fokin (2005). "Copper(I)-catalyzed synthesis of azoles. DFT study predicts unprecedented reactivity and intermediates." J Am Chem Soc **127**(1): 210-216.

Holappa, J., M. Hjálmarsdóttir, M. Másson, Ö. Rúnarsson, T. Asplund, P. Soininen, T. Nevalainen and T. Järvinen (2006). "Antimicrobial activity of chitosan N-betainates." Carbohydrate Polymers **65**(1): 114-118.

Holappa, J., T. Nevalainen, R. Safin, P. Soininen, T. Asplund, T. Luttikhedde, M. Másson and T. Järvinen (2006). "Novel Water-Soluble Quaternary Piperazine Derivatives of Chitosan: Synthesis and Characterization." **6**(2): 139-144.

Holappa, J., T. Nevalainen, J. Savolainen, P. Soininen, M. Elomaa, R. Safin, S. Suvanto, T. Pakkanen, M. Másson, T. Loftsson and T. Järvinen (2004). "Synthesis and Characterization of Chitosan N-Betainates Having Various Degrees of Substitution." Macromolecules **37**(8): 2784-2789.

Holappa, J., T. Nevalainen, P. Soininen, M. Másson and T. Järvinen (2006). "Synthesis of Novel Quaternary Chitosan Derivatives via N-Chloroacyl-6-O-triphenylmethylchitosans." Biomacromolecules **7**(2): 407-410.

Hou, J., X. Liu, J. Shen, G. Zhao and P. G. Wang (2012). "The impact of click chemistry in medicinal chemistry." Expert Opinion on Drug Discovery **7**(6): 489-501.

Hoyle, C. E. and C. N. Bowman (2010). "Thiol–Ene Click Chemistry." **49**(9): 1540-1573.

Hu, L., X. Meng, R. Xing, S. Liu, X. Chen, Y. Qin, H. Yu and P. Li (2016). "Design, synthesis and antimicrobial activity of 6-N-substituted chitosan derivatives." Bioorganic & Medicinal Chemistry Letters **26**(18): 4548-4551.

Hu, Q. and Y. Luo (2016). "Polyphenol-chitosan conjugates: Synthesis, characterization, and applications." Carbohydrate Polymers **151**: 624-639.

Hudson, S. M. and C. Smith (1998). Polysaccharides: Chitin and Chitosan: Chemistry and Technology of Their Use As Structural Materials. Biopolymers from Renewable Resources. D. L. Kaplan. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 96-118.

Ifuku, S., C. Matsumoto, M. Wada, M. Morimoto and H. Saimoto (2013). "Preparation of highly regioselective amphiprotic chitosan derivative via "click chemistry"." International Journal of Biological Macromolecules **52**: 72-76.

Ifuku, S., M. Wada, M. Morimoto and H. Saimoto (2011). "Preparation of highly regioselective chitosan derivatives via "click chemistry"." Carbohydrate Polymers **85**(3): 653-657.

Ilium, L. (1998). "Chitosan and Its Use as a Pharmaceutical Excipient." Pharmaceutical Research **15**(9): 1326-1331.

Jayakumar, R., M. Prabaharan, S. V. Nair, S. Tokura, H. Tamura and N. Selvamurugan (2010). "Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications." Progress in Materials Science **55**(7): 675-709.

Jayakumar, R., M. Prabaharan, P. T. Sudheesh Kumar, S. V. Nair and H. Tamura (2011). "Biomaterials based on chitin and chitosan in wound dressing applications." Biotechnology Advances **29**(3): 322-337.

Jayakumar, R., M. Rajkumar, H. Freitas, P. T. Sudheesh Kumar, S. V. Nair, T. Furuike and H. Tamura (2009). "Bioactive and metal uptake studies of carboxymethyl chitosan-graft-d-glucuronic acid membranes for tissue engineering and environmental applications." International Journal of Biological Macromolecules **45**(2): 135-139.

Jenssen, H., P. Hamill and R. E. W. Hancock (2006). "Peptide Antimicrobial Agents." **19**(3): 491-511.

Jeon, Y.-J., P.-J. Park and S.-K. Kim (2001). "Antimicrobial effect of chitooligosaccharides produced by bioreactor." Carbohydrate Polymers **44**(1): 71-76.

Jeong, Y.-I., S.-G. Jin, I.-Y. Kim, J. Pei, M. Wen, T.-Y. Jung, K.-S. Moon and S. Jung (2010). "Doxorubicin-incorporated nanoparticles composed of poly(ethylene glycol)-grafted carboxymethyl chitosan and antitumor activity against glioma cells in vitro." Colloids and Surfaces B: Biointerfaces **79**(1): 149-155.

Jia, Z., D. shen and W. Xu (2001). "Synthesis and antibacterial activities of quaternary ammonium salt of chitosan." Carbohydrate Research **333**(1): 1-6.

Jiang, G. B., Z. T. Lin, X. J. Xu, Z. Hai and K. Song (2012). "Stable nanomicelles based on chitosan derivative: In vitro antiplatelet aggregation and adhesion properties." Carbohydrate Polymers **88**(1): 232-238.

Jiang, Y., C. Fu, S. Wu, G. Liu, J. Guo and Z. Su (2017). "Determination of the Deacetylation Degree of Chitooligosaccharides." Marine drugs **15**(11): 332.

Jimtaisong, A. and N. Saewan (2014). "Utilization of carboxymethyl chitosan in cosmetics." **36**(1): 12-21.

Jung, E. J., D. K. Youn, S. H. Lee, H. K. No, J. G. Ha and W. Prinyawiwatkul (2010). "Antibacterial activity of chitosans with different degrees of deacetylation and viscosities." **45**(4): 676-682.

Kanitskaya, L. V., V. N. Elokhina, S. V. Fedorov, A. M. Shulunova, A. S. Nakhmanovich, V. K. Turchaninov and V. A. Lopyrev (2002). "Spectroscopic Study of Reaction of Propargyl Bromide with Pyridine." Russian Journal of General Chemistry **72**(5): 778-784.

Karle, I. L., H. N. Gopi and P. Balaram (2003). "Crystal structure of a hydrophobic 19-residue peptide helix containing three centrally located D amino acids." Proc Natl Acad Sci U S A **100**(24): 13946-13951.

Karoli, T., S. K. Mamidyala, J. Zuegg, S. R. Fry, E. H. L. Tee, T. A. Bradford, P. K. Madala, J. X. Huang, S. Ramu, M. S. Butler and M. A. Cooper (2012). "Structure aided design of chimeric antibiotics." Bioorganic & Medicinal Chemistry Letters **22**(7): 2428-2433.

Kast, C. E. and A. Bernkop-Schnürch (2001). "Thiolated polymers — thiomers: development and in vitro evaluation of chitosan–thioglycolic acid conjugates." Biomaterials **22**(17): 2345-2352.

Kast, C. E., W. Frick, U. Losert and A. Bernkop-Schnürch (2003). "Chitosan-thioglycolic acid conjugate: a new scaffold material for tissue engineering?" International Journal of Pharmaceutics **256**(1): 183-189.

Kean, T., S. Roth and M. Thanou (2005). "Trimethylated chitosans as non-viral gene delivery vectors: Cytotoxicity and transfection efficiency." Journal of Controlled Release **103**(3): 643-653.

Keisuke, K., H. Masaaki and N. Yasuhiro (1999). "Silylated Chitin: A New Organosoluble Precursor for Facile Modifications and Film Casting." **28**(8): 771-772.

Khan, I., S. Ullah and D.-H. Oh (2016). "Chitosan grafted monomethyl fumaric acid as a potential food preservative." Carbohydrate Polymers **152**: 87-96.

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

Kim, E. and H. Koo (2019). "Biomedical applications of copper-free click chemistry: in vitro, in vivo, and ex vivo." Chem Sci **10**(34): 7835-7851.

Kim, J.-H., D. Yu, S.-H. Eom, S.-H. Kim, J. Oh, W. K. Jung and Y.-M. Kim (2017). "Synergistic Antibacterial Effects of Chitosan-Caffeic Acid Conjugate against Antibiotic-Resistant Acne-Related Bacteria." Marine Drugs **15**(6): 167.

Kim, Y. H., C. W. Nam, J. W. Choi and J. Jang (2003). "Durable antimicrobial treatment of cotton fabrics using N-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride and polycarboxylic acids." **88**(6): 1567-1572.

Kolb, H. C., M. G. Finn and K. B. Sharpless (2001). "Click Chemistry: Diverse Chemical Function from a Few Good Reactions." Angewandte Chemie International Edition **40**(11): 2004-2021.

Kong, M., X. G. Chen, K. Xing and H. J. Park (2010). "Antimicrobial properties of chitosan and mode of action: A state of the art review." International Journal of Food Microbiology **144**(1): 51-63.

Korjamo, T., J. Holappa, S. Taimisto, J. Savolainen, T. Järvinen and J. Mönkkönen (2008). "Effect of N-betainate and N-piperazine derivatives of chitosan on the paracellular transport of mannitol in Caco-2 cells." European Journal of Pharmaceutical Sciences **35**(3): 226-234.

Krajewska, B., P. Wydro and A. Jańczyk (2011). "Probing the modes of antibacterial activity of chitosan. Effects of pH and molecular weight on chitosan interactions with membrane lipids in Langmuir films." Biomacromolecules **12**(11): 4144-4152.

Kritchenkov, A. S., A. R. Egorov, R. A. Abramovich, A. V. Kurliuk, T. V. Shakola, E. K. Kultyshkina, M. J. Ballesteros Meza, A. V. Pavlova, E. P. Suchkova, G. Le Nhat Thuy, N. Van Tuyen and V. N. Khrustalev (2021). "Water-soluble triazole chitin derivative and its based nanoparticles: Synthesis, characterization, catalytic and antibacterial properties." Carbohydrate Polymers **257**: 117593.

Kritchenkov, A. S., A. R. Egorov, A. P. Dysin, O. V. Volkova, L. A. Zabodalova, E. P. Suchkova, A. V. Kurliuk and T. V. Shakola (2019). "Ultrasound-assisted Cu(I)-catalyzed azide-alkyne click cycloaddition as polymer-analogous transformation in chitosan chemistry. High antibacterial and transfection activity of novel triazol betaine chitosan derivatives and their nanoparticles." International Journal of Biological Macromolecules **137**: 592-603.

Kulbokaite, R., G. Ciuta, M. Netopilik and R. Makuska (2009). "N-PEG'ylation of chitosan via "click chemistry" reactions." Reactive and Functional Polymers **69**(10): 771-778.

Kumar, M. N. V. R., R. A. A. Muzzarelli, C. Muzzarelli, H. Sashiwa and A. J. Domb (2004). "Chitosan Chemistry and Pharmaceutical Perspectives." Chemical Reviews **104**(12): 6017-6084.

Kurita, K. (2006). "Chitin and Chitosan: Functional Biopolymers from Marine Crustaceans." Marine Biotechnology **8**(3): 203.

Kurita, K., H. Ikeda, Y. Yoshida, M. Shimojoh and M. Harata (2002). "Chemoselective Protection of the Amino Groups of Chitosan by Controlled Phthaloylation: Facile Preparation of a Precursor Useful for Chemical Modifications." Biomacromolecules **3**(1): 1-4.

Kurita, K., K. Shimada, Y. Nishiyama, M. Shimojoh and S.-I. Nishimura (1998). "Nonnatural Branched Polysaccharides: Synthesis and Properties of Chitin and Chitosan Having α-Mannoside Branches." Macromolecules **31**(15): 4764-4769.

L, V., R. G, M. Y, S. C, C. P, R. I, C. R and C. D (2021). "Antifungal effect of chitosan of different molecular weight against Colletotrichum alatae under in vitro conditions." Journal of Microbiology & Experimentation **9**(1): 9-13.

Laguna, L., C. Primo-Martín, P. Varela, A. Salvador and T. Sanz (2014). "HPMC and inulin as fat replacers in biscuits: Sensory and instrumental evaluation." LWT - Food Science and Technology **56**(2): 494-501.

Lei, F., X. Wang, C. Liang, F. Yuan and Y. Gao (2014). J. Appl. Polym. Sci.: 131.

Levina, M. and A. R. Rajabi-Siahboomi (2004). "The Influence of Excipients on Drug Release from Hydroxypropyl Methylcellulose Matrices." Journal of Pharmaceutical Sciences **93**(11): 2746-2754.

Li, H.-Y., X. Song and P. C. Seville (2010). "The use of sodium carboxymethylcellulose in the preparation of spray-dried proteins for pulmonary drug delivery." European Journal of Pharmaceutical Sciences **40**(1): 56-61.

Li, J. and S. Zhuang (2020). "Antibacterial activity of chitosan and its derivatives and their interaction mechanism with bacteria: Current state and perspectives." European Polymer Journal **138**: 109984.

Li, Q., X. Sun, G. Gu and Z. Guo (2018). "Novel Water Soluble Chitosan Derivatives with 1,2,3-Triazolium and Their Free Radical-Scavenging Activity." Mar Drugs **16**(4).

Li, Q., W. Tan, C. Zhang, G. Gu and Z. Guo (2015). "Novel triazolyl-functionalized chitosan derivatives with different chain lengths of aliphatic alcohol substituent: Design, synthesis, and antifungal activity." Carbohydrate Research **418**: 44-49.

Li, Q., W. Tan, C. Zhang, G. Gu and Z. Guo (2016). "Synthesis of water soluble chitosan derivatives with halogeno-1,2,3-triazole and their antifungal activity." International Journal of Biological Macromolecules **91**: 623-629.

Li, S.-D., P.-W. Li, Z.-M. Yang, Z. Peng, W.-Y. Quan, X.-H. Yang, L. Yang and J.-J. Dong (2014). "Synthesis and characterization of chitosan quaternary ammonium salt and its application as drug carrier for ribavirin." Drug Delivery **21**(7): 548-552.

Lim, S.-H. and S. M. Hudson (2004). "Synthesis and antimicrobial activity of a water-soluble chitosan derivative with a fiber-reactive group." Carbohydrate Research **339**(2): 313-319.

Lima, P. H. L., S. V. A. Pereira, R. B. Rabello, E. Rodriguez-Castellón, M. M. Beppu, P. Chevallier, D. Mantovani and R. S. Vieira (2013). "Blood protein adsorption on sulfonated chitosan and κ-carrageenan films." Colloids and Surfaces B: Biointerfaces **111**: 719-725.

Lin, F., H.-R. Jia and F.-G. Wu (2019). "Glycol Chitosan: A Water-Soluble Polymer for Cell Imaging and Drug Delivery." **24**(23): 4371.

Liu, C., J. Wang, S. Huang, L. Yu, Y. Wang, H. Chen and D. Wang (2018). "Self-assembled nanoparticles for cellular delivery of peptide nucleic acid using amphiphilic N,N,N-trimethyl-O-alkyl chitosan derivatives." Journal of Materials Science: Materials in Medicine **29**(8): 114.

Liu, H., Y. Du, X. Wang and L. Sun (2004). "Chitosan kills bacteria through cell membrane damage." Int J Food Microbiol **95**(2): 147-155.

Lopez-Moya, F., M. Suarez-Fernandez and L. V. Lopez-Llorca (2019). "Molecular Mechanisms of Chitosan Interactions with Fungi and Plants." Int J Mol Sci **20**(2).

Lowe, A. B. (2010). "Thiol-ene "click" reactions and recent applications in polymer and materials synthesis." Polymer Chemistry **1**(1): 17-36.

Lu, G., K. Ling, P. Zhao, Z. Xu, C. Deng, H. Zheng, J. Huang and J. Chen (2010). "A novel in situ-formed hydrogel wound dressing by the photocross-linking of a chitosan derivative." **18**(1): 70-79.

M El-Nesr, E., A. Raafat, S. Nasef, E. A Soliman and E.-S. Hegazy (2014). "Radiation Synthesis and Characterization of N,O-Carboxymethyl Chitosan/poly(vinylpyrrolidone) Copolymer Hydrogel." **47**: 14-27.

Mannila, J., K. Järvinen, J. Holappa, L. Matilainen, S. Auriola and P. Jarho (2009). "Cyclodextrins and chitosan derivatives in sublingual delivery of low solubility peptides: A study using cyclosporin A, alpha-cyclodextrin and quaternary chitosan N-betainate." International journal of pharmaceutics **381**(1): 19-24.

Mao, S., W. Sun and T. Kissel (2010). "Chitosan-based formulations for delivery of DNA and siRNA." Advanced Drug Delivery Reviews **62**(1): 12-27.

Már Másson, V. S. G., Berglind Eva Benediktsdóttir (2013). Utilization of Silyl Ethers and Other Protection Groups in the Synthesis of Chitosan Derivatives. Chitin and Chitosan Derivatives: Advances in Drug Discovery and Developments. S.-K. Kim, CRC Press**:** 69-91.

Martínez-Camacho, A. P., M. O. Cortez-Rocha, J. M. Ezquerra-Brauer, A. Z. Graciano-Verdugo, F. Rodriguez-Félix, M. M. Castillo-Ortega, M. S. Yépiz-Gómez and M. Plascencia-Jatomea (2010). "Chitosan composite films: Thermal, structural, mechanical and antifungal properties." Carbohydrate Polymers **82**(2): 305-315.

Másson, M. (2021). Antimicrobial Properties of Chitosan and Its Derivatives. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 1-38.

Másson, M. (2021). Chapter 33 - Chitin and chitosan. Handbook of Hydrocolloids (Third Edition). G. O. Phillips and P. A. Williams, Woodhead Publishing**:** 1039-1072.

Másson, M. (2021). Chitosan derivatives and methods for preparing the same. US20210253745A1. Iceland, HASKOLI ISLANDS (UNIVERSITY OF ICELAND) Primex ehf.

Mitra, R. N., Z. Han, M. Merwin, M. Al Taai, S. M. Conley and M. I. Naash (2014). "Synthesis and characterization of glycol chitosan DNA nanoparticles for retinal gene delivery." ChemMedChem **9**(1): 189-196.

Mourya, V. K. and N. N. Inamdar (2008). "Chitosan-modifications and applications: Opportunities galore." Reactive and Functional Polymers **68**(6): 1013-1051.

Mourya, V. K. and N. N. Inamdar (2008). "Trimethyl chitosan and its applications in drug delivery." Journal of Materials Science: Materials in Medicine **20**(5): 1057.

Mourya, V. K., N. Inamdara and N. Ashutosh Tiwari (2010). "Carboxymethyl Chitosan And Its Applications %J Advanced Materials Letters." **1**(1): 11-33.

Muhsin, M. D. A., G. George, K. Beagley, V. Ferro, C. Armitage and N. Islam (2014). "Synthesis and Toxicological Evaluation of a Chitosan-l-Leucine Conjugate for Pulmonary Drug Delivery Applications." Biomacromolecules **15**(10): 3596-3607.

Munro, C. A. and N. A. Gow (2001). "Chitin synthesis in human pathogenic fungi." Med Mycol **39 Suppl 1**: 41-53.

Muzzarelli, R. A. A. and F. Tanfani (1985). "The N-permethylation of chitosan and the preparation of N-trimethyl chitosan iodide." Carbohydrate Polymers **5**(4): 297-307.

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

Nagy, V., P. Sahariah, M. Á. Hjálmarsdóttir and M. Másson (2022). "Chitosan-hydroxycinnamic acid conjugates: Optimization of the synthesis and investigation of the structure activity relationship." Carbohydrate Polymers **277**: 118896.

Nguyen, H.-K., O. Fournier, U. Asseline, N. T. Thuong and D. Dupret (1999). "Smoothing of the thermal stability of DNA duplexes by using modified nucleosides and chaotropic agents." Nucleic Acids Research **27**(6): 1492-1498.

Nielsen, T. T., V. Wintgens, C. Amiel, R. Wimmer and K. L. Larsen (2010). "Facile Synthesis of β-Cyclodextrin-Dextran Polymers by "Click" Chemistry." Biomacromolecules **11**(7): 1710-1715.

Nishimura, S., O. Kohgo, K. Kurita and H. Kuzuhara (1991). "Chemospecific manipulations of a rigid polysaccharide: syntheses of novel chitosan derivatives with excellent solubility in common organic solvents by regioselective chemical modifications." Macromolecules **24**(17): 4745-4748.

Nishiyama, Y., T. Yoshikawa, N. Ohara, K. Kurita, K. Hojo, H. Kamada, Y. Tsutsumi, T. Mayumi and K. Kawasaki (2000). "A conjugate from a laminin-related peptide, Tyr-Ile-Gly-Ser-Arg, and chitosan: efficient and regioselective conjugation and significant inhibitory activity against experimental cancer metastasis,1." Journal of the Chemical Society, Perkin Transactions 1(7): 1161-1165.

No, H. K., N. Young Park, S. Ho Lee and S. P. Meyers (2002). "Antibacterial activity of chitosans and chitosan oligomers with different molecular weights." International Journal of Food Microbiology **74**(1): 65-72.

Ogushi, Y., S. Sakai and K. Kawakami (2007). "Synthesis of enzymatically-gellable carboxymethylcellulose for biomedical applications." Journal of Bioscience and Bioengineering **104**(1): 30-33.

Oliveira, J. R., M. C. L. Martins, L. Mafra and P. Gomes (2012). "Synthesis of an O-alkynyl-chitosan and its chemoselective conjugation with a PEG-like amino-azide through click chemistry." Carbohydrate Polymers **87**(1): 240-249.

Omidi, S. and A. Kakanejadifard (2019). "Modification of chitosan and chitosan nanoparticle by long chain pyridinium compounds: Synthesis, characterization, antibacterial, and antioxidant activities." Carbohydrate Polymers **208**: 477-485.

Omura, Y., M. Shigemoto, A. Takahiro, H. Saimoto, Y. Shigemasa, I. Nakamura and T. Tsuchido (2003). "Antimicrobial Activity of Chitosan with Different Degrees of Acetylation and Molecular Weights." Biocontrol Science **8**: 25-30.

Ong, S.-Y., J. Wu, S. M. Moochhala, M.-H. Tan and J. Lu (2008). "Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties." Biomaterials **29**(32): 4323-4332.

Ouadahi, K., E. Allard, B. Oberleitner and C. Larpent (2012). "Synthesis of azide-functionalized nanoparticles by microemulsion polymerization and surface modification by click chemistry in aqueous medium." Journal of Polymer Science Part A: Polymer Chemistry **50**(2): 314-328.

Pardeshi, C. V. and V. S. Belgamwar (2018). "N,N,N-trimethyl chitosan modified flaxseed oil based mucoadhesive neuronanoemulsions for direct nose to brain drug delivery." International Journal of Biological Macromolecules **120**: 2560-2571.

Park, Y., M. H. Kim, S. C. Park, H. Cheong, M. K. Jang, J. W. Nah and K. S. Hahm (2008). "Investigation of the antifungal activity and mechanism of action of LMWS-chitosan." J Microbiol Biotechnol **18**(10): 1729-1734.

Pasquina-Lemonche, L., J. Burns, R. D. Turner, S. Kumar, R. Tank, N. Mullin, J. S. Wilson, B. Chakrabarti, P. A. Bullough, S. J. Foster and J. K. Hobbs (2020). "The architecture of the Gram-positive bacterial cell wall." Nature **582**(7811): 294-297.

Peng, Y., B. Han, W. Liu and X. Xu (2005). "Preparation and antimicrobial activity of hydroxypropyl chitosan." Carbohydrate Research **340**(11): 1846-1851.

Peniche, C., W. Argüelles-Monal and F. M. Goycoolea (2008). Chapter 25 - Chitin and Chitosan: Major Sources, Properties and Applications. Monomers, Polymers and Composites from Renewable Resources. M. N. Belgacem and A. Gandini. Amsterdam, Elsevier**:** 517-542.

Petrin, T. H. C., V. Fadel, D. B. Martins, S. A. Dias, A. Cruz, L. M. Sergio, M. Arcisio-Miranda, M. A. R. B. Castanho and M. P. dos Santos Cabrera (2019). "Synthesis and Characterization of Peptide−Chitosan Conjugates (PepChis) with Lipid Bilayer Affinity and Antibacterial Activity." Biomacromolecules **20**(7): 2743-2753.

Polnok, A., G. Borchard, J. C. Verhoef, N. Sarisuta and H. E. Junginger (2004). "Influence of methylation process on the degree of quaternization of N-trimethyl chitosan chloride." European Journal of Pharmaceutics and Biopharmaceutics **57**(1): 77-83.

Posner, T. (1905). "Beiträge zur Kenntniss der ungesättigten Verbindungen. II. Ueber die Addition von Mercaptanen an ungesättigte Kohlenwasserstoffe." **38**(1): 646-657.

Qin, Y. and P. Li (2020). "Antimicrobial Chitosan Conjugates: Current Synthetic Strategies and Potential Applications." International journal of molecular sciences **21**(2): 499.

Qin, Y., S. Liu, R. Xing, K. Li, H. Yu and P. Li (2013). "Synthesis and antifungal evaluation of (1,2,3-triazol-4-yl)methyl nicotinate chitosan." International Journal of Biological Macromolecules **61**: 58-62.

Qu, J., X. Zhao, Y. Liang, T. Zhang, P. X. Ma and B. Guo (2018). "Antibacterial adhesive injectable hydrogels with rapid self-healing, extensibility and compressibility as wound dressing for joints skin wound healing." Biomaterials **183**: 185-199.

Raafat, D., K. v. Bargen, A. Haas and H.-G. Sahl (2008). "Insights into the Mode of Action of Chitosan as an Antibacterial Compound." **74**(12): 3764-3773.

Raafat, D. and H. G. Sahl (2009). "Chitosan and its antimicrobial potential–a critical literature survey." Microb Biotechnol **2**(2): 186-201.

Rabea, E. I., M. E. T. Badawy, C. V. Stevens, G. Smagghe and W. Steurbaut (2003). "Chitosan as Antimicrobial Agent: Applications and Mode of Action." Biomacromolecules **4**(6): 1457-1465.

Ravi Kumar, M. N. V. (2000). "A review of chitin and chitosan applications." Reactive and Functional Polymers **46**(1): 1-27.

Rinaudo, M. (2006). "Chitin and chitosan: Properties and applications." Progress in Polymer Science **31**(7): 603-632.

Rodionov, V. O., V. V. Fokin and M. G. Finn (2005). "Mechanism of the ligand-free CuI-catalyzed azide-alkyne cycloaddition reaction." Angew Chem Int Ed Engl **44**(15): 2210-2215.

Roller, S. and N. Covill (1999). "The antifungal properties of chitosan in laboratory media and apple juice." International Journal of Food Microbiology **47**(1): 67-77.

Rostovtsev, V. V., L. G. Green, V. V. Fokin and K. B. Sharpless (2002). "A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes." Angew Chem Int Ed Engl **41**(14): 2596-2599.

Rouget, C. J. C. R. (1859). "Des substances amylacées dans les tissus des animaux, spécialement des Articulés (chitine)." **48**: 792-795.

Rúnarsson, Ö. V., J. Holappa, S. Jónsdóttir, H. Steinsson and M. Másson (2008). "N-selective 'one pot' synthesis of highly N-substituted trimethyl chitosan (TMC)." Carbohydrate Polymers **74**(3): 740-744.

Rúnarsson, Ö. V., J. Holappa, C. Malainer, H. Steinsson, M. Hjálmarsdóttir, T. Nevalainen and M. Másson (2010). "Antibacterial activity of N-quaternary chitosan derivatives: Synthesis, characterization and structure activity relationship (SAR) investigations." European Polymer Journal **46**(6): 1251-1267.

Rúnarsson, Ö. V., J. Holappa, T. Nevalainen, M. Hjálmarsdóttir, T. Järvinen, T. Loftsson, J. M. Einarsson, S. Jónsdóttir, M. Valdimarsdóttir and M. Másson (2007). "Antibacterial activity of methylated chitosan and chitooligomer derivatives: Synthesis and structure activity relationships." European Polymer Journal **43**(6): 2660-2671.

Rúnarsson, Ö. V., C. Malainer, J. Holappa, S. T. Sigurdsson and M. Másson (2008). "tert-Butyldimethylsilyl O-protected chitosan and chitooligosaccharides: useful precursors for N-modifications in common organic solvents." Carbohydrate Research **343**(15): 2576-2582.

S. Wimardhani, Y., D. F. Suniarti, H. J. Freisleben, S. I. Wanandi, N. C. Siregar and M.-A. Ikeda (2014). "Chitosan exerts anticancer activity through induction of apoptosis and cell cycle arrest in oral cancer cells." Journal of Oral Science **56**(2): 119-126.

Sahariah, P., B. E. Benediktssdóttir, M. Á. Hjálmarsdóttir, O. E. Sigurjonsson, K. K. Sørensen, M. B. Thygesen, K. J. Jensen and M. Másson (2015). "Impact of Chain Length on Antibacterial Activity and Hemocompatibility of Quaternary N-Alkyl and N,N-Dialkyl Chitosan Derivatives." Biomacromolecules **16**(5): 1449-1460.

Sahariah, P., D. Cibor, D. Zielińska, M. Á. Hjálmarsdóttir, D. Stawski and M. Másson (2019). "The Effect of Molecular Weight on the Antibacterial Activity of N,N,N-Trimethyl Chitosan (TMC)." International Journal of Molecular Sciences **20**(7): 1743.

Sahariah, P., V. S. Gaware, R. Lieder, S. Jónsdóttir, M. Á. Hjálmarsdóttir, O. E. Sigurjonsson and M. Másson (2014). "The Effect of Substituent, Degree of Acetylation and Positioning of the Cationic Charge on the Antibacterial Activity of Quaternary Chitosan Derivatives." Marine Drugs **12**(8): 4635-4658.

Sahariah, P., M. Másson and R. L. Meyer (2018). "Quaternary Ammoniumyl Chitosan Derivatives for Eradication of Staphylococcus aureus Biofilms." Biomacromolecules **19**(9): 3649-3658.

Sahariah, P., B. M. Óskarsson, M. Hjálmarsdóttir and M. Másson (2015). "Synthesis of guanidinylated chitosan with the aid of multiple protecting groups and investigation of antibacterial activity." Carbohydr Polym **127**: 407-417.

Sahariah, P., K. K. Sørensen, M. Á. Hjálmarsdóttir, Ó. E. Sigurjónsson, K. J. Jensen, M. Másson and M. B. Thygesen (2015). "Antimicrobial peptide shows enhanced activity and reduced toxicity upon grafting to chitosan polymers." Chemical Communications **51**(58): 11611-11614.

Sajomsang, W., P. Gonil and S. Tantayanon (2009). "Antibacterial activity of quaternary ammonium chitosan containing mono or disaccharide moieties: Preparation and characterization." International Journal of Biological Macromolecules **44**(5): 419-427.

Salama, A., M. Hasanin and P. Hesemann (2020). "Synthesis and antimicrobial properties of new chitosan derivatives containing guanidinium groups." Carbohydrate Polymers **241**: 116363.

Saravanakumar, G., K. H. Min, D. S. Min, A. Y. Kim, C.-M. Lee, Y. W. Cho, S. C. Lee, K. Kim, S. Y. Jeong, K. Park, J. H. Park and I. C. Kwon (2009). "Hydrotropic oligomer-conjugated glycol chitosan as a carrier of paclitaxel: Synthesis, characterization, and in vivo biodistribution." Journal of Controlled Release **140**(3): 210-217.

Sarwar, A., H. Katas, S. N. Samsudin and N. M. Zin (2015). "Regioselective Sequential Modification of Chitosan via Azide-Alkyne Click Reaction: Synthesis, Characterization, and Antimicrobial Activity of Chitosan Derivatives and Nanoparticles." PloS one **10**(4): e0123084-e0123084.

Seong, H.-S., H. S. Whang and S.-W. Ko (2000). "Synthesis of a quaternary ammonium derivative of chito-oligosaccharide as antimicrobial agent for cellulosic fibers." **76**(14): 2009-2015.

Seyfarth, F., S. Schliemann, P. Elsner and U. C. Hipler (2008). "Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and N-acetyl-D-glucosamine against Candida albicans, Candida krusei and Candida glabrata." Int J Pharm **353**(1-2): 139-148.

Shankar, S. and J.-W. Rhim (2018). "Preparation of sulfur nanoparticle-incorporated antimicrobial chitosan films." Food Hydrocolloids **82**: 116-123.

Shin, Y., D. I. Yoo and J. Jang (2001). "Molecular weight effect on antimicrobial activity of chitosan treated cotton fabrics." **80**(13): 2495-2501.

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

Song, Q., Z. Zhang, J. Gao and C. Ding (2011). "Synthesis and property studies of N-carboxymethyl chitosan." **119**(6): 3282-3285.

Song, W., V. S. Gaware, Ö. V. Rúnarsson, M. Másson and J. F. Mano (2010). "Functionalized superhydrophobic biomimetic chitosan-based films." Carbohydrate Polymers **81**(1): 140-144.

Stephen Inbaraj, B., T.-Y. Tsai and B.-H. Chen (2012). "Synthesis, characterization and antibacterial activity of superparamagnetic nanoparticles modified with glycol chitosan." Science and Technology of Advanced Materials **13**(1): 015002.

Su, Y., L. Tian, M. Yu, Q. Gao, D. Wang, Y. Xi, P. Yang, B. Lei, P. X. Ma and P. Li (2017). "Cationic peptidopolysaccharides synthesized by 'click' chemistry with enhanced broad-spectrum antimicrobial activities." Polymer Chemistry **8**(25): 3788-3800.

Sudarshan, N. R., D. G. Hoover and D. Knorr (1992). "Antibacterial action of chitosan." Food Biotechnology **6**(3): 257-272.

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

Sun, Y. and A. Wan (2007). "Preparation of nanoparticles composed of chitosan and its derivatives as delivery systems for macromolecules." **105**(2): 552-561.

Sun, Z., C. Shi, X. Wang, Q. Fang and J. Huang (2017). "Synthesis, characterization, and antimicrobial activities of sulfonated chitosan." Carbohydrate Polymers **155**: 321-328.

Tan, R. S. L., P. Hassandarvish, C. F. Chee, L. W. Chan and T. W. Wong (2022). "Chitosan and its derivatives as polymeric anti-viral therapeutics and potential anti-SARS-CoV-2 nanomedicine." Carbohydrate Polymers **290**: 119500.

Tan, W., Q. Li, F. Dong, J. Zhang, F. Luan, L. Wei, Y. Chen and Z. Guo (2018). "Novel cationic chitosan derivative bearing 1,2,3-triazolium and pyridinium: Synthesis, characterization, and antifungal property." Carbohydr Polym **182**: 180-187.

Tan, W., J. Zhang, Y. Mi, F. Dong, Q. Li and Z. Guo (2018). "Synthesis, characterization, and evaluation of antifungal and antioxidant properties of cationic chitosan derivative via azide-alkyne click reaction." Int J Biol Macromol **120**(Pt A): 318-324.

Tan, W., J. Zhang, Y. Mi, F. Dong, Q. Li and Z. Guo (2020). "Enhanced antifungal activity of novel cationic chitosan derivative bearing triphenylphosphonium salt via azide-alkyne click reaction." International Journal of Biological Macromolecules **165**: 1765-1772.

Tikhonov, V. E., E. A. Stepnova, V. G. Babak, I. A. Yamskov, J. Palma-Guerrero, H.-B. Jansson, L. V. Lopez-Llorca, J. Salinas, D. V. Gerasimenko, I. D. Avdienko and V. P. Varlamov (2006). "Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl)succinoyl/-derivatives." Carbohydrate Polymers **64**(1): 66-72.

Tornøe, C. W., C. Christensen and M. Meldal (2002). "Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides." The Journal of Organic Chemistry **67**(9): 3057-3064.

Tron, G. C., T. Pirali, R. A. Billington, P. L. Canonico, G. Sorba and A. A. Genazzani (2008). "Click chemistry reactions in medicinal chemistry: applications of the 1,3-dipolar cycloaddition between azides and alkynes." Med Res Rev **28**(2): 278-308.

TSAI, G.-J. and W.-H. SU (1999). "Antibacterial Activity of Shrimp Chitosan against Escherichia coli." Journal of Food Protection **62**(3): 239-243.

Upadhyaya, L., J. Singh, V. Agarwal and R. P. Tewari (2013). "Biomedical applications of carboxymethyl chitosans." Carbohydrate Polymers **91**(1): 452-466.

Varlamov, V. P. and I. S. Mysyakina (2018). "Chitosan in Biology, Microbiology, Medicine, and Agriculture." Microbiology **87**(5): 712-715.

Verheul, R. J., M. Amidi, S. van der Wal, E. van Riet, W. Jiskoot and W. E. Hennink (2008). "Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan." Biomaterials **29**(27): 3642-3649.

Wan, A., Q. Xu, Y. Sun and H. Li (2013). "Antioxidant Activity of High Molecular Weight Chitosan and N,O-Quaternized Chitosans." Journal of Agricultural and Food Chemistry **61**(28): 6921-6928.

Wan, Y., K. A. M. Creber, B. Peppley and V. Tam Bui (2004). "Ionic conductivity and tensile properties of hydroxyethyl and hydroxypropyl chitosan membranes." **42**(8): 1379-1397.

Wanat, P., S. Walczak, B. A. Wojtczak, M. Nowakowska, J. Jemielity and J. Kowalska (2015). "Ethynyl, 2-Propynyl, and 3-Butynyl C-Phosphonate Analogues of Nucleoside Di- and Triphosphates: Synthesis and Reactivity in CuAAC." Organic Letters **17**(12): 3062-3065.

Wang, J., J.-S. Chen, J.-Y. Zong, D. Zhao, F. Li, R.-X. Zhuo and S.-X. Cheng (2010). "Calcium Carbonate/Carboxymethyl Chitosan Hybrid Microspheres and Nanospheres for Drug Delivery." The Journal of Physical Chemistry C **114**(44): 18940-18945.

Wang, J., Z. Lian, H. Wang, X. Jin and Y. Liu (2012). "Synthesis and antimicrobial activity of Schiff base of chitosan and acylated chitosan." Journal of Applied Polymer Science **123**(6): 3242-3247.

Wang, K. and Q. Liu (2014). "Chemical structure analyses of phosphorylated chitosan." Carbohydrate Research **386**: 48-56.

Wang, L., X. Xu, S. Guo, Z. Peng and T. Tang (2011). "Novel water soluble phosphonium chitosan derivatives: Synthesis, characterization and cytotoxicity studies." International Journal of Biological Macromolecules **48**(2): 375-380.

Weng, L., A. Romanov, J. Rooney and W. Chen (2008). "Non-cytotoxic, in situ gelable hydrogels composed of N-carboxyethyl chitosan and oxidized dextran." Biomaterials **29**(29): 3905-3913.

Woo, J. Y. and J. Y. Je (2013). "Antioxidant and tyrosinase inhibitory activities of a novel chitosan-phloroglucinol conjugate." International Journal of Food Science and Technology **48**(6): 1172-1178.

Worrell, B. T., J. A. Malik and V. V. Fokin (2013). "Direct Evidence of a Dinuclear Copper Intermediate in Cu(I)-Catalyzed Azide-Alkyne Cycloadditions." **340**(6131): 457-460.

Wu, M., Z. Long, H. Xiao and C. Dong (2016). "Recent research progress on preparation and application of N, N, N-trimethyl chitosan." Carbohydrate Research **434**: 27-32.

Wu, M., Z. Long, H. Xiao and C. Dong (2017). "Preparation of N, N, N-trimethyl chitosan via a novel approach using dimethyl carbonate." Carbohydrate Polymers **169**: 83-91.

Xiao, B., Y. Wan, X. Wang, Q. Zha, H. Liu, Z. Qiu and S. Zhang (2012). "Synthesis and characterization of N-(2-hydroxy)propyl-3-trimethyl ammonium chitosan chloride for potential application in gene delivery." Colloids and Surfaces B: Biointerfaces **91**: 168-174.

Xiao, B., Y. Wan, M. Zhao, Y. Liu and S. Zhang (2011). "Preparation and characterization of antimicrobial chitosan-N-arginine with different degrees of substitution." Carbohydrate Polymers **83**(1): 144-150.

Xie, J., D. Qin, Y. Han and L. Wang (2019). "Synthesis and characterization of a novel hydroxypropyl chitosan-graft-β-Cyclodextrin copolymer as potential drug carrier." Journal of Carbohydrate Chemistry **38**(5): 383-397.

Xie, W., P. Xu, W. Wang and Q. Liu (2002). "Preparation and antibacterial activity of a water-soluble chitosan derivative." Carbohydrate Polymers **50**(1): 35-40.

Xiu, Z.-m., Q.-b. Zhang, H. L. Puppala, V. L. Colvin and P. J. J. Alvarez (2012). "Negligible Particle-Specific Antibacterial Activity of Silver Nanoparticles." Nano Letters **12**(8): 4271-4275.

Xu, T., M. Xin, M. Li, H. Huang and S. Zhou (2010). "Synthesis, characteristic and antibacterial activity of N,N,N-trimethyl chitosan and its carboxymethyl derivatives." Carbohydrate Polymers **81**(4): 931-936.

Yaakov, N., Y. Chaikin, E. Wexselblatt, Y. Tor, A. Vaskevich and I. Rubinstein (2017). "Application of Surface Click Reactions to Localized Surface Plasmon Resonance (LSPR) Biosensing." **23**(42): 10148-10155.

Yang, J., J. Cai, Y. Hu, D. Li and Y. Du (2012). "Preparation, characterization and antimicrobial activity of 6-amino-6-deoxychitosan." Carbohydrate Polymers **87**(1): 202-209.

Younes, I., S. Sellimi, M. Rinaudo, K. Jellouli and M. Nasri (2014). "Influence of acetylation degree and molecular weight of homogeneous chitosans on antibacterial and antifungal activities." International Journal of Food Microbiology **185**: 57-63.

Yu, A., H. Shi, H. Liu, Z. Bao, M. Dai, D. Lin, D. Lin, X. Xu, X. Li and Y. Wang (2020). "Mucoadhesive dexamethasone-glycol chitosan nanoparticles for ophthalmic drug delivery." International Journal of Pharmaceutics ***575***: 118943.

Yu, W.-Z., Y. Zhang, X. Liu, Y. Xiang, Z. Li and S. Wu (2018). "Synergistic antibacterial activity of multi components in lysozyme/chitosan/silver/hydroxyapatite hybrid coating." Materials & Design **139**: 351-362.

Yuan, Y., B. M. Chesnutt, W. O. Haggard and J. D. Bumgardner (2011). "Deacetylation of Chitosan: Material Characterization and in vitro Evaluation via Albumin Adsorption and Pre-Osteoblastic Cell Cultures." Materials (Basel, Switzerland) **4**(8): 1399-1416.

Zasloff, M. (2002). "Antimicrobial peptides of multicellular organisms." Nature **415**(6870): 389-395.

Zhang, F., B. Bernet, V. Bonnet, O. Dangles, F. Sarabia and A. Vasella (2008). "2-Azido-2-deoxycellulose: Synthesis and 1,3-Dipolar Cycloaddition." Helvetica Chimica Acta **91**(4): 608-617.

Zhang, J., W. Tan, Q. Li, X. Liu and Z. Guo (2021). "Preparation of Cross-linked Chitosan Quaternary Ammonium Salt Hydrogel Films Loading Drug of Gentamicin Sulfate for Antibacterial Wound Dressing." **19**(9): 479.

Zhao, D., J. Huang, S. Hu, J. Mao and L. Mei (2011). "Biochemical activities of N,O-carboxymethyl chitosan from squid cartilage." Carbohydrate Polymers **85**(4): 832-837.

Zheng, L.-Y. and J.-F. Zhu (2003). "Study on antimicrobial activity of chitosan with different molecular weights." Carbohydrate Polymers **54**(4): 527-530.

Zhong, Z., R. Xing, S. Liu, L. Wang, S. Cai and P. Li (2008). "Synthesis of acyl thiourea derivatives of chitosan and their antimicrobial activities in vitro." Carbohydrate Research **343**(3): 566-570.

Zhou, Y., L. Shi, F. Li, H. Yang, X. Liu, J. Mao and W. Xu (2015). "Preparation and Characterization of Carboxymethyl-Functionalized Chitosan Fiber." Journal of Natural Fibers **12**(3): 211-221.

Zhou, Z., D. Yan, X. Cheng, M. Kong, Y. Liu, C. Feng and X. Chen (2016). "Biomaterials based on N,N,N-trimethyl chitosan fibers in wound dressing applications." Int J Biol Macromol **89**: 471-476.

Original publications

Paper I

Paper II

Paper III

Paper IV

Paper V

Paper VI