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The synthesis of water soluble N-acyl chitosan derivatives for characterization as antibacterial agents

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THE SYNTHESIS OF WATER SOLUBLE *N*-ACYL CHITOSAN DERIVATIVES FOR CHARACTERIZATION AS ANTIBACTERIAL AGENTS

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ABSTRACT

Currently, natural polysaccharides are being utilized increasingly in the markets because they exhibit biodegradability, biocompatibility, versatility, and are found abundant in nature. The diversity of natural polysaccharides provides the chemist with a broad spectrum of raw materials that can be used in many biological applications. Chitosan is a natural polysaccharide that possesses excellent biological properties. It has been recognized for its antibacterial activity, in that it is destructive towards the bacterium *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*).

The antibacterial property of chitosan can be enhanced by changing the hydrophobic/hydrophilic nature of the polysaccharide backbone. This research entails the chemical modification of chitosan with distinct linear aliphatic, cyclic, and novel hydrophilic anhydrides, followed by quaternization using 3-chloro-2-hydroxyl propyl trimethylammonium chloride (Quat-188) to enhance its antibacterial properties. It is believed that these types of hydrophobic/hydrophilic derivatives inhibit the growth of bacteria by initial electrostatic interactions with negatively charged groups of the bacterial cell surface.

The antibacterial activities of the *N*-acyl chitosan Quat-188 derivatives were investigated using the minimum inhibitory concentration (MIC) method. In most cases, it was found that an increase in the percent extent of *N*-acyl substitution (% ES) led to a decrease in the percent extent of quaternization (% EQ), which resulted in an increase in the MIC of the derivative. For example, 2-glycerol glutamide chitosan Quat-188 with 5% ES and 61% EQ exhibited a MIC of 64 μg/mL against *E. coli* and 32 μg/mL against *S. aureus*. At 25% ES and 31% EQ, the derivative exhibited a MIC of 128 μg/mL against both *E. coli* and *S. aureus*. These results suggest that an increase in the extent of

N-acylation results in a decrease in the amount of cationic charge that can placed on the polysaccharide backbone, which leads to a decline in the antibacterial activity.

CHAPTER 1. INTRODUCTION

1.1 Utility of Natural Polysaccharides

Billions of dollars are spent annually to ensure the success and longevity of biotechnical products. For many years, the vast majority of these products include those made from petroleum synthetic polymers. However, the potential market for products made from natural polysaccharides (polymers) is growing rapidly. Carbohydrate based polymers provide the chemist with a broad spectrum of raw materials that exhibit biodegradability, biocompatibility, and versatility. The properties of natural polysaccharides can be exploited in the derivation of many bio-synthetic polymer conjugates, that will biodegrade and pose no threat to the environment after their use.

One very interesting inherent property of natural polysaccharides is their biological functionality. These materials have been utilized in medical therapy, which includes cardiology, dentistry, hematology[1], and they have important roles as vaccines.[2] Polysaccharides used in these biomedical applications are largely found in sea animals, plants, and bacterial sources,[3] as shown in Table 1.1.

A very important area where natural polysaccharides have been receiving attention is their uses as biocides. These polysaccharides are classified as biocidal polymers. Some characteristics for an ideal biocidal polymer include: it should be easily and inexpensively synthesized; it should be stable in long-term usage and storage at the temperature of its intended application; it should not be soluble in water for water-disinfection applications; it should not decompose to and emit toxic products; it should not be toxic or irritating to those handling it; it should be regenerable upon loss of activity; and it should be biocidal to a broad spectrum of pathogenic microorganisms in brief times of contact.[4] Biocidal polymers are incorporated into textile fibers, paints,

waxes, oils, disinfectants, and antimicrobial products,[4] and they also have great utility in the areas of healthcare, hygiene, and biomedicine.

Table 1.1 Main sources of polysaccharides for biomedical applications

Sources of polysaccharides	Polysaccharides produced
Plants: Algae Tubers Seeds Primary cell walls	 Alginates, carrageenans, agarose Konjac gum Galactomannans (tara, carob, locust bean, guar) Pectins
Animal: Crustaceous Umbilical rod, vitreous humor and roster combs Bacteria	 Chitin and derivative chitosan Hyaluronan Xanthan, hyaluronan, gellan, succinoglycan

1.2. The Scope of Polysaccharide Chemistry

Polysaccharides are poly-acetals formed by condensation reactions of sugar molecules. Polysaccharides are also known as glycans, because they consist of monosaccharides joined together by glycosidic linkages. Polysaccharides that comprise one monosaccharide are termed homopolysaccharides (homopolymers). Cellulose, a naturally occurring polysaccharides, is a classic example of a homopolymer. It is found in plants such as microfibrils (2-20 nm diameter and 100 - 40 000 nm long).[5] These form the structurally strong framework in the cell walls. Cellulose consists of monomeric glucose units, linked by β-glycosidic linkages (Figure 1.1). The linear arrangement of the β-linked glucose units in the polymer is a result of the configuration of the anomeric carbon atoms.

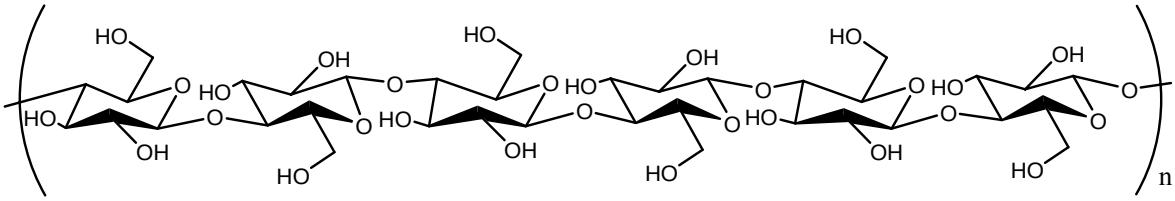


Figure 1.1 The structure of cellulose

Polysaccharides comprising two distinct monosaccharides that are of the same sugar (e.g. a hexose sugar) are termed copolymers. A very well known naturally occurring copolymer composed of monosaccharides that have amino groups in the nonanomeric position is chitosan. Chitosan is a widely investigated copolymer whose monomeric units are made of 2-amino-2-deoxy-D-glucopyranose and residual 2-acetamido-2-deoxy-D-glucopyranose units. The structure of chitosan is shown in Figure 1.2. Like cellulose, the monomeric units are linked by β-glycosidic linkages, and the configuration of the anomeric carbon atoms in chitosan makes the polymer chains essentially linear. Unlike cellulose, chitosan is not common in nature. Some reports of its existence has been associated with the cell walls of fungi[6] but it is primarily a principle derivative of chitin. Therefore, prior to discussing the science that will elucidate the importance of chitosan, it is essential to explore its derivation. This begins with a comprehensive study of chitin.

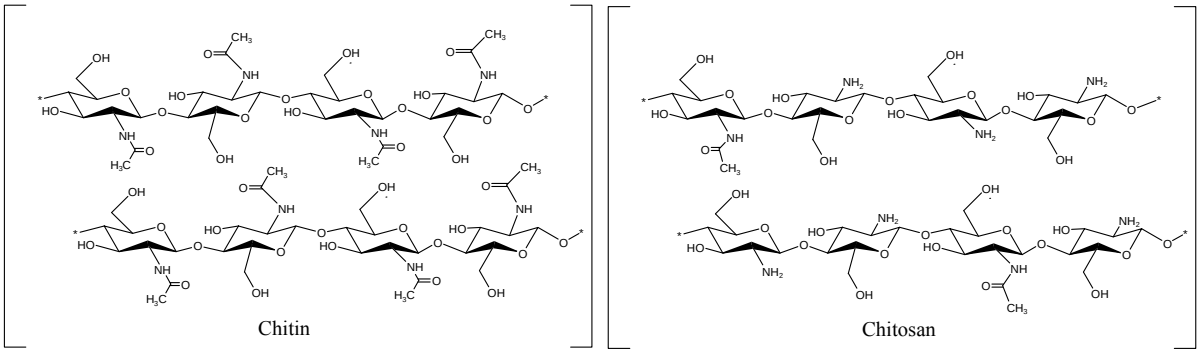


Figure 1.2 The polymeric structures of chitin and chitosan

1.3 Introduction of Chitin

Chitin, a naturally abundant homopolymer, consists of β-(1→4) linked 2-acetamido-2-deoxy-D-glucopyranose units (Figure 1.2). Chitin has been found in a wide range of natural sources such as squid, fungi, insects, and some algae.[7,8] A large quantity of chitin is manufactured from the exoskeleton of crustacean sources (shrimp, crab, lobster, crayfish), and from the shells of mollusks.[9] In the shells of mollusks, chitin is closely associated with proteins, where it provides adhesion between fiber beds of stacked laminas. Chitin exists as part of a chitinoproteic complex in the shells of mollusks, where the shell matrix is composed of 2 structural units[9] (Figure 1.3). The first unit is called the mineralization matrix (MM), which consists of an acidic polypeptide fraction with a strong affinity for Ca^{2+} ions. The arrangement of this fraction is speculated as a spiraled peptide chain.[10] The second unit contains a high molecular weight chitinoproteic complex that has no affinity for Ca.[2+] This unit is called the carrier protein (CP), and it is arranged in the form of sheets and layers. The attachment of the mineralization matrix to the carrier protein complex will activate the mineralizing substrate, leading to epitaxial $CaCO_3$ deposition.

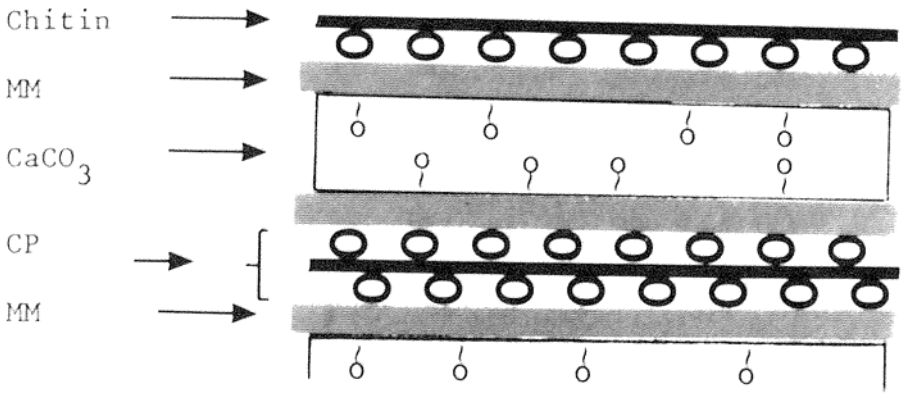
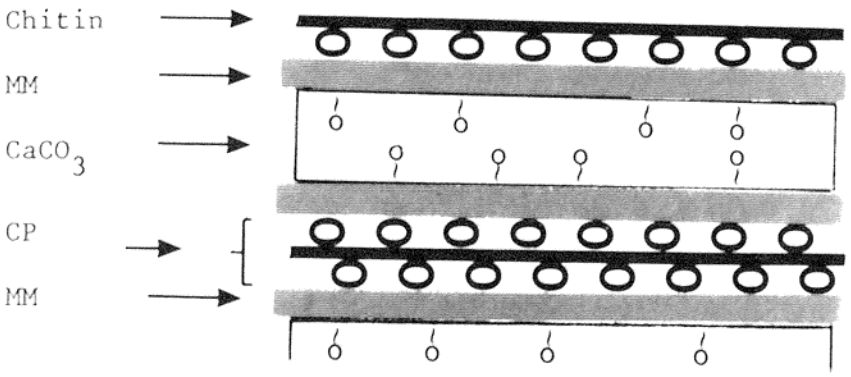


Figure 1.3[9] Schematic of the structure of the organic matrix in mollusk shells

1.4 Isolation of Chitin

Chitin can be isolated from the shells of mollusks via chemical procedures. The shells are initially treated with 5% NaOH, which denatures proteins, resulting in chitin + $CaCO_3$ + lipids. Treatment with 30% HCl hydrolyzes the lipids, dissolves calcium salts (demineralization), and other minor inorganic constituents. This process isolates chitin (Scheme 1.1).

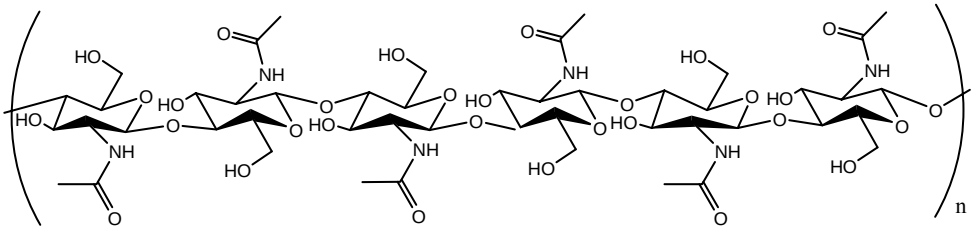
Scheme 1.1 Isolation of chitin from mollusk shells



5% NaOH
(removes proteins)

Chitin + $CaCO_3$ + lipids

30% HCl
(removes $CaCO_3$ + lipids)



(isolated chitin)

1.5 Economical Aspects of Chitin

The production of chitin is currently based on crustacean shells discarded by the canning industries in Oregon, Washington, and Virginia[7] and the availability of crustaceans shells from fisheries and tinned food industries. In India, the Central Institute of Fisheries Technology, Kelara conducted research on chitin. From their findings, they discovered that dry pawn waste contained 23% chitin and dry squid contained 15% chitin.[11] Chitin is now produced commercially in India, Japan, Poland, Norway, and Australia. Some factors that control the cost of the production of chitin include availability of seafood wastes, chemical costs, nitrogen gas, transportation, energy costs, and labor.

1.6 Properties and Characterization

Chitin is regarded as a suitable functional material because it exhibits excellent properties such as biocompatibility, biodegradability, non-toxicity, and adsorption properties.[12,13] However, this bio-functional polymer exhibits a limitation in processibility due to problems related to its solubility. The highly ordered crystalline structure of chitin (Figure 1.4) originates from extensive hydrogen bonding that occurs between the hydroxyl groups and the *N*-acetamido groups in the repeating units. Intramolecular C3-OH hydroxyl to C5-O ring oxygen hydrogen bonds across each $\beta(1\rightarrow4)$-glycosidic linkage restrict chitin units to the low-energy chair conformation, resulting in a rigid and linear polymer backbone.[14] This rigidity prevents the polymer's complete dissolution in common organic solvents (DMSO, DMF, DCM, NMP) and aqueous solvents.

Chitin has been reported to show solubility in concentrated acidic solvents such as H_3PO_4, HCl, and H_2SO_4, and amide/LiCl systems[15] (e.g. *N,N*-dimethylacetamide/LiCl

and *N*-methyl-2-pyrrolidone/LiCl). However, these solvents can lead to de-polymerization of the polymer chain[16] and difficulty in removing residual solvent molecules. Because of the problems related to its solubility in aqueous and organic solvents, chemical modification of chitin to generate new bio-functional materials is of primary interest, where such modification would not change the fundamental skeleton of the polymer. A very elaborate procedure includes alkaline *N*-deacetylation of the *N*-acetamido functional groups of chitin (Scheme 1.2). Modification of the *N*-acetamido groups by *N*-deacetylation results in functional amines that can undergo nucleophilic substitution reactions.

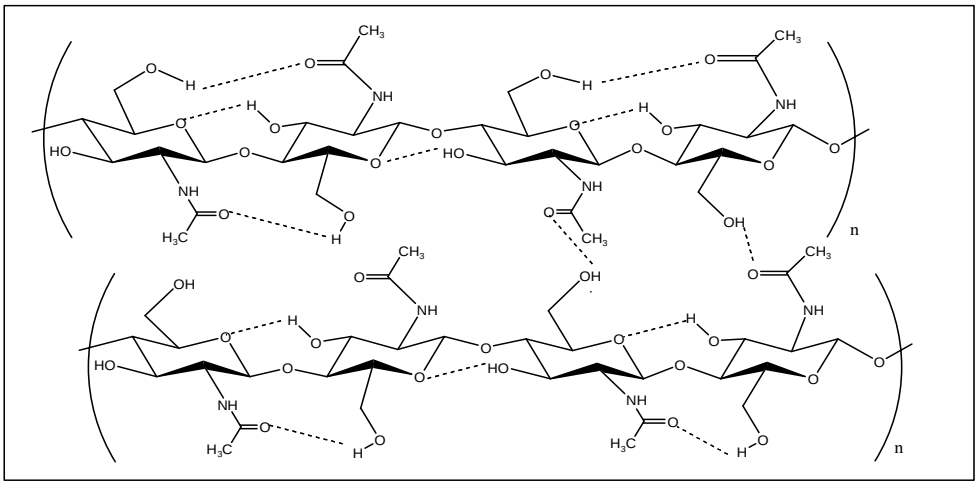


Figure 1.4 Crystalline structure of chitin

1.7 *N*-deacetylation of Chitin

Chitosan, the principle derivative of chitin, exists naturally in the cell walls of fungi, but its occurrences is much less widespread in biomass than that of chitin.[6, 17] Due to chitin's abundance in nature, chitosan is usually derived from this source, mainly through alkaline *N*-deacetylation of the *N*-acetamido functional groups. An alternative procedure to modify the *N*-acetamido groups is enzymatic *N*-deacetylation of chitin, using the enzyme chitin deacetylase.[18] This enzyme is produced primarily by fungi such

as *Absidia coerulea, Colletotrichum lindemuthianu,* and *Mucor rouxiss*.[19-22] However, it was postulated that the enzyme cannot penetrate into the interior of chitin's crystalline structure, and only the peripheral *N*-acetamido groups are amenable for enzymatic *N*-deacetylation.[23] Hence, chemical modification is commonly employed for the preparation of chitosan. Chemical modification gives the scientist a handle on governing the 3 factors that influence the *N*-deacetylation process; they are NaOH concentration, reaction temperature, and reaction time.[8, 14, 24] Methacanon et al. examined the *N*-deacetylation process to determine the optimal conditions for the conversion of the *N*-acetamido groups into *N*-amino groups.[8] The data obtained from this investigation implied that at lower NaOH concentrations, there was no significant change in the degree of deacetylation (DDA), despite elevated temperature and time of the reaction. On the other hand, DDA rapidly increased and leveled off, as a function of increased alkaline concentration, temperature and time (Table 1.2).

Scheme 1.2 *N*-deacetylation of chitin

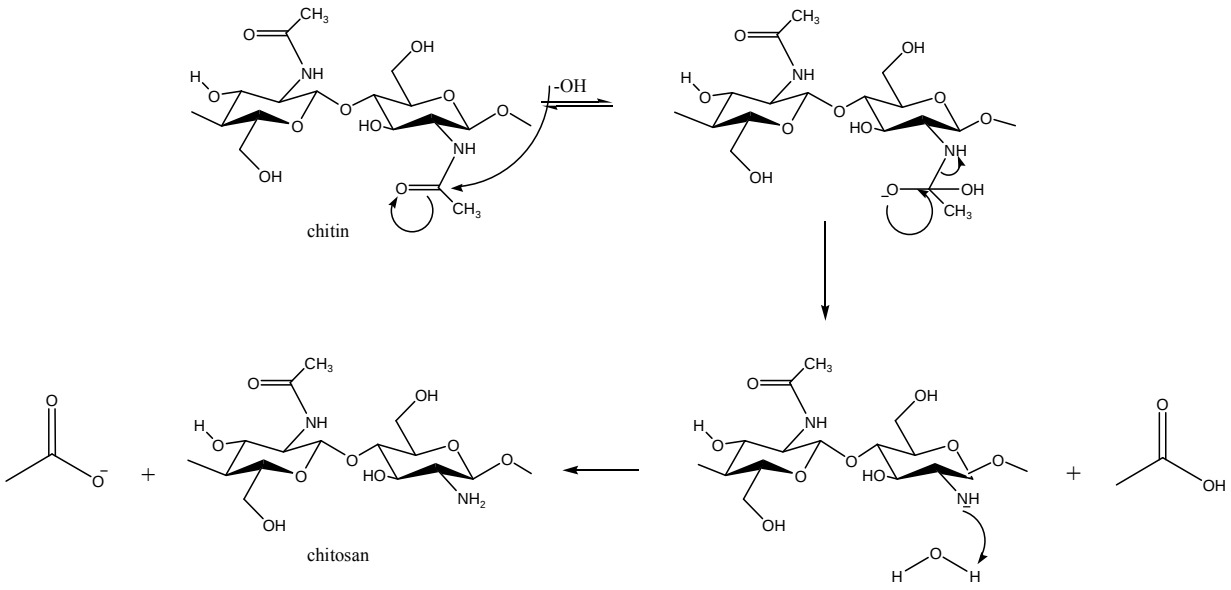


Table 1.2 Degree of *N*-deacetylation of chitin obtained from various conditions

Chitin/solution ratio	NaOH concentration (%)	Temperature (^{0}C)	Time (min)	%DDA (DDA x 100)
1:20	20	100	15	21.6
			120	22.8
1:10	40	40	15	21.6
			120	43.3
		60	15	34.8
			60	52.4
			120	68.0
		80	15	56.7
			60	70.9
			120	84.0
		100	60	73.6
			120	88.7
	60	40	120	27.5
		60	120	70.7
		80	45	84.2
			60	90.2
			120	94.7
		100	30	94.0
			60	97.3

1.7.1 Effects of *N*-deacetylation Experimental Conditions

In addition to investigating the influence of alkaline concentration, temperature, and time on the % DDA, Methacanon et al. have shown that these factors can affect the molecular weight of resulting chitosan. It is well known that the experimental conditions for *N*-deacetylation can lead to degradation of the polymer main chain.[16] The results shown in Table 1.3 reveal the inverse relationship between NaOH concentration, temperature, time, and molecular weight; that is, as the basicity, temperature, and time of the reaction was increased, the molecular weight of the polymer decreased. In attempts to address the problem involving the degradation of chitin during the *N*-deacetylation process, it has been reported that the addition of thiophenol reduces chain scission by trapping molecular oxygen and exerting a catalytic effect.[25] Bough et al. minimized chain degradation during *N*-deacetylation by purging the reaction with N_2 to flush out

air.[26] Also, treating chitin with concentrated NaOH in the presence of $NaBH_4$ can minimize degradation of the polymer.[27]

Table 1.3 Molecular weight of chitosan obtained from various *N*-deacetylation conditions

Condition	MW x 10^5
40% NaOH, 80 ^{0}C, 120 min	8.74
40% NaOH, 100 ^{0}C, 60 min	4.58
60% NaOH, 80 ^{0}C, 60 min	10.9
60% NaOH, 80 ^{0}C, 120 min	8.87
60% NaOH, 100 ^{0}C, 60 min	4.53
60% NaOH, 100 ^{0}C, 120 min	3.22

1.7.1.1 Homogenous and Heterogeneous *N*-deacetylation

N-deacetylated chitosan can be prepared under homogenous or heterogeneous experimental conditions, thus affecting the sequencing arrangements of the 2-acetamido-2-deoxy-D-glucopyranose (*N*-acetamido) units and 2-amino-2-deoxy-D-glucopyranose (*N*-amino) units, and the solubility of the polymer. Two types of sequencing arrangements of the monosaccharide units of chitosan include blocks (termed block copolymers) and/or random (termed random copolymers). In theory, block copolymers contain a block of one monomeric unit connected to a block of another monomeric unit.[28] The sequence is illustrated below:

-A-A-A-A-A-A-A-A-A-B-B-B-B-B-B-B-B-

Random copolymers have no specific sequence of the monomers.[28] As the name implies, the monomers appear in a random fashion:

- A-B-A-A-B-A-B-A-B-B-A-B-A-B-A-B-A-

The parameters that influence homogenous experimental conditions include alkaline media, low temperature, and prolonged reaction time.[14, 29, 30] Under these conditions, there is a random scission of the *N*-acetyl groups along the polymer chain,

which results in a random sequencing of the 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. In investigating the experimental conditions that affect the solubility of *N*-deacetylated chitosan, Sannan et al. prepared chitosans under homogenous conditions, and reported that the materials were water soluble.[16] Kurita also prepared water soluble chitosans under homogenous conditions, where he reported that the water solubility is attributed to the random deacetylation along the chains of chitin.[31]

The parameters that influence heterogeneous experimental conditions include an alkaline solvent system, elevated temperatures, and short reaction times,[8, 24, 31] (when compared to the reaction times for homogenous experimental conditions). Under these conditions, there is an ordered scission of the *N*-acetamido groups. This results in blocks of water soluble fractions (*N*-amino units) connected to water insoluble fractions (*N*-acetamido units). In their study of the chemical composition and sequencing of the *N*-acetamido and *N*-amino units of chitosan, Vaarum et al. prepared materials under heterogeneous conditions, and obtained acid-soluble and acid insoluble fractions.[32] It has been reported that chitosans prepared under heterogeneous conditions during deacetylation have a block-wise distribution of acetamido units,[30] resulting in aqueous soluble and insoluble fractions.

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CHAPTER 2. CHITOSAN CHEMISTRY

2.1 Solubility of Chitosan

Chitosan, a biomaterial obtained via alkaline *N*-deacetylation of chitin, has recently attracted much attention from scientists across the globe. It is a copolymer that is primarily composed of $\beta(1\rightarrow4)$ linked 2-amino-2-deoxy-D-glucopyranose units, and residual 2-acetamido-2-deoxy-D-glucopyranose units. Although the polymer backbone consists of hydrophilic functional groups and is hydrophobic in nature, chitosan is normally insoluble in water and most common organic solvents (e.g. DMSO, DMF, NMP, organic alcohols, pyridine). The insolubility of chitosan in aqueous and organic solvents is a result of its crystalline structure, which is attributed to extensive intramolecular and intermolecular hydrogen bonding between the chains and sheets, respectively[1] (Figure 2.1).

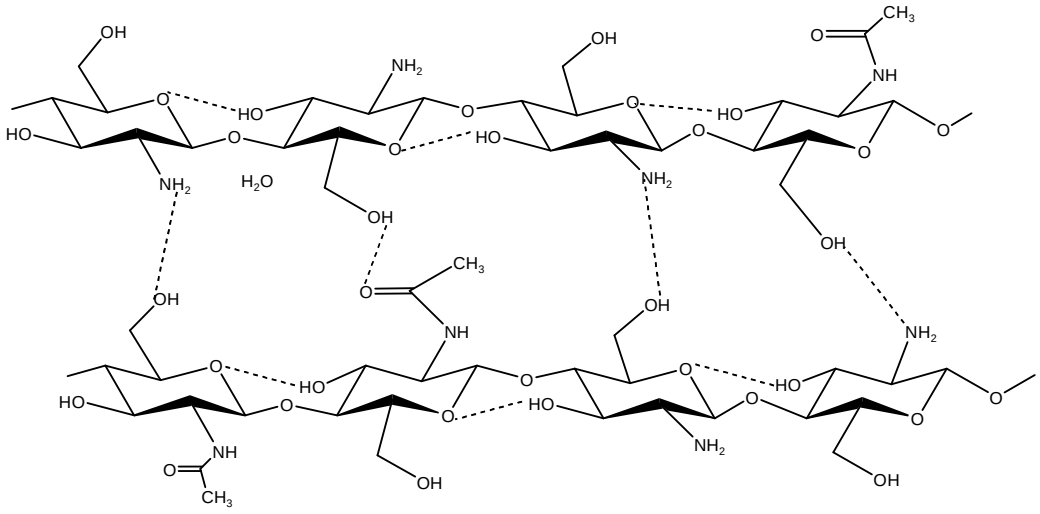
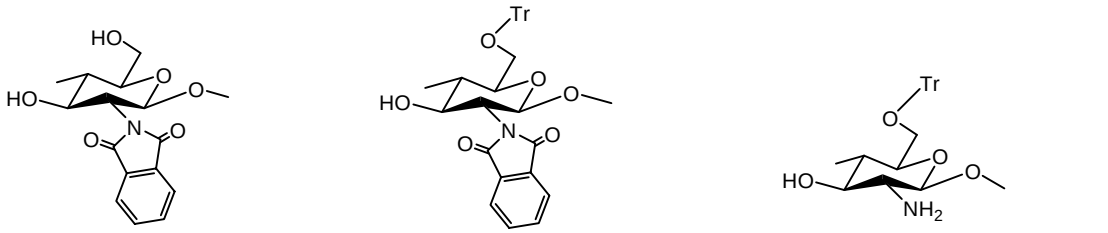


Figure 2.1 Crystalline structure of chitosan

A few attempts have been made to enhance chitosan's solubility in organic solvents.[2-4] Organic soluble chitosan derivatives previously synthesized mainly served as precursors or standard intermediates for the preparation of finely designed chitosan

biomaterials.[3, 5-7] Some representative precursors that are amenable to further chemical modification are shown in Figure 2.1. Their syntheses are given in Schemes 2.9



N-phthaloyl chitosan *N*-phthaloyl-3-hydroxy-6-*O*-trityl chitosan 2-amino-3-hydroxy-6-*O*-trityl chitosan

Figure 2.2 Standard chitosan precursors for further modification

Many attempts have been made to enhance chitosan's solubility in water however. One major reason is because most biological applications for chemical substances require the material to be processible and functional at neutral pH. Thus, obtaining a water soluble derivative of chitosan is an important step towards the further application of the polymer as a biofunctional material.

2.2 Enhancing the Solubility Property

The pKa of the *N*-amino groups of chitosan is reported as 6.5.[8] Therefore, chitosan is soluble in dilute organic acidic solutions where the pH is < 6.5 (e.g. formic, acetic, pyruvic, 10% citric, and lactic acid).[9, 10] Although chitosan dissolves in aqueous medium at pHs less than or equal to 6.5, acidic solutions may not be desirable in many of chitosan's applications (e.g. cosmetics, food, and biomedicines). Hence, the goal is to enhance chitosan's solubility at neutral pH. The procedures to enhance the polymer's solubility include chemical modification of the *N*-amino functional groups, resulting in *N*-substituted derivatives with improved solubilities in aqueous medium. Some *N*-substituted reactions include *N*-alkylation, *N*-acylation, and *N*-hydroxyacylation.

2.3 Effect of *N*-substitution

Prior to discussing the details of *N*-alkylation, *N*-acylation, and *N*-hydroxyacylation, it is necessary to mention that the type of organic compound used in each of these *N*-substitution reactions is chosen to ultimately enhance the bioactivity of chitosan (as will be discussed in chapters 4 and 5). Then, it is important to understand how the organic substituent remarkably changes the solubility of chitosan in water. As mentioned, excessive intramolecular and intermolecular hydrogen bonding between the chains and sheets of chitosan plays a major role for the polymer's insolubility in water. Through substitution of the *N*-amino groups, the normal regularity of intermolecular hydrogen bonding is reduced, which creates space for water molecules to fill in and solvate the hydrophilic groups of the polymer backbone (and the substituent if it comprises hydrophilic components). Substitution with bulky substituents further enhances chitosan's solubility in water.[11, 12] This is because the large size of the substituent creates more space between the polymer's sheets, thus weakening intermolecular hydrogen bonding to a greater extent. This allows more water molecules to fill in these spaces, leading to an increase in the polymer's solubility in the medium.

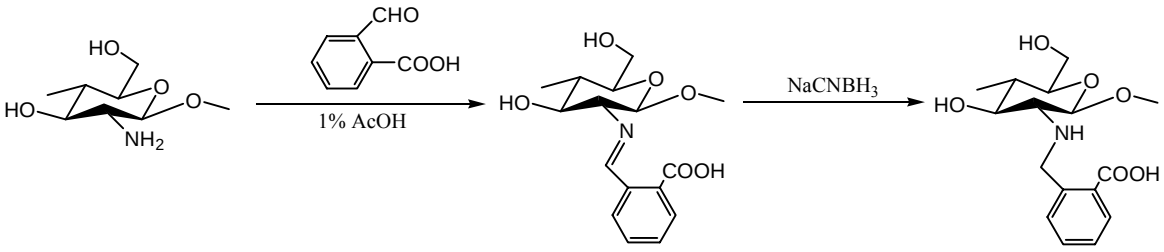
2.4 *N*-substitution via *N*-alkylation

The conversion of chitosan to a variety of *N*-alkylated derivatives can be effected by treatment of the polymer with aldehydes or ketones. Condensation of chitosan with these functional groups affords the Schiff base intermediates aldimines (from reactions with aldehydes) or ketimines (from reactions with ketones). Most of these types of reactions proceed smoothly in a binary solvent mixture of aqueous acetic acid and methanol. Initially the reaction is carried out under homogenous experimental conditions, but over time, gelation occurs due to the Schiff bases' poor solubility in the

medium.[13] The Schiff base formation is followed by reduction of the imine linkage with sodium cyanoborohydride or sodium borohydride, resulting in *N*-alkylated chitosan.

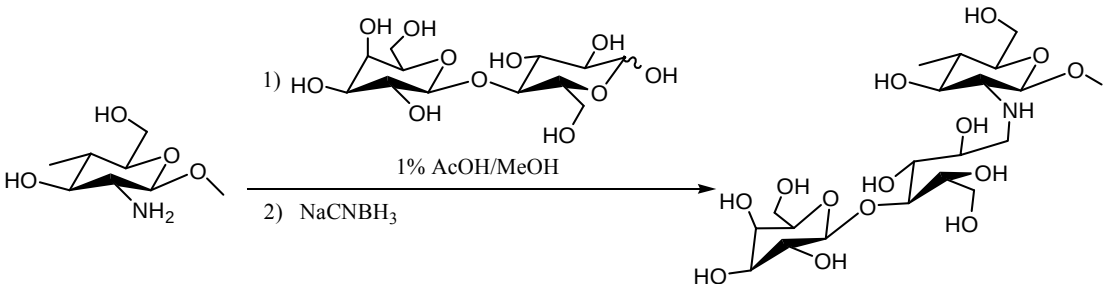
In a chemical modification reaction of chitosan, Kurita synthesized an *N*-alkylated derivative from the addition of phthalaldehydic acid to an aqueous suspension of chitosan, followed by reduction of the Schiff base to afford *N*-*o*-carboxybenzyl chitosan[14] (Scheme 2.1). This derivative displayed solubility in neutral media.

Scheme 2.1 Reductive alkylation of chitosan with phthalaldehydic acid



N-alkylated derivatives can also be prepared by the introduction of sugar branches at the *N*-amino groups. These reactions involve reductive alkylation of chitosan using sodium cyanoborohydride and diverse reducing sugars such as D-galactose, D-glucose, cellobiose, and lactose.[15, 16] Yalpani et al. prepared chitosan derivatives by reductive alkylation with lactose[17] (Scheme 2.2). This hydrophilic derivative was reported to be soluble in water.

Scheme 2.2 Reductive alkylation of chitosan with lactose



2.5 *N*-substitution via *N*-acylation

N-acylation of chitosan is the most typical and extensively studied modification reaction. The process involves a reaction between chitosan and an acid anhydride or acyl halide. The reaction proceeds through an addition/elimination type mechanism, where amide functionality of the *N*-amino groups is restored. These reactions are driven toward amide formation because amides are more stable molecules (compared to acyl carbonyls) as explicable in terms of resonance localization of the lone pair electrons on nitrogen into the carbonyl pi system (Figure 2.3).

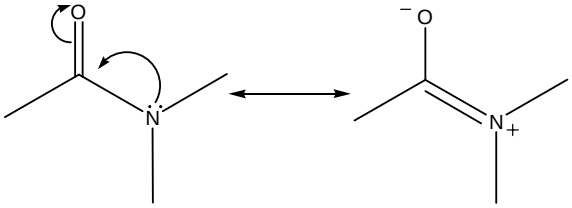


Figure 2.3 Resonance stabilization of amides

N-acylation of chitosan has been achieved with various kinds of acid anhydrides. Because most acid anhydrides exhibit very little solubility in aqueous media, the reactions between chitosan and acid anhydrides are conducted under heterogeneous experimental conditions. These conditions could lead to fractions of water soluble and water insoluble products, and/or regioselectivity issues such as *O*-acylation. For this reason, methanol is usually added to help solvate the anhydride in the medium[12, 14, 18] and the reaction is carried out under homogenous experimental conditions. The *N*-acylation of chitosan with anhydrides in a mixture of aqueous acetic acid and methanol at room temperature proceeds selectively at the *N*-amino functional groups.[11,12]

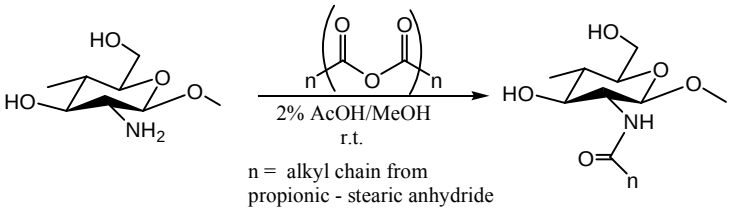
Several *N*-acyl derivatives comprising aliphatic side chains have been prepared.[2, 19, 20] For example, Hirano et al. synthesized novel water soluble *N*-saturated fatty acyl derivatives of chitosan through reactions with propionic, butyric, pentanoic, hexanoic,

and octanoic anhydride, and the longer chain acid anhydrides decanoic, lauric, myristic, palmitic, and stearic anhydride[21] (Scheme 2.3). The authors reported that the chain length of the substituent and the degree of substitution (DS) have a considerable influence on the solubility of chitosan. The shorter chain derivatives (*N*-acetyl to *N*-octanoyl chitosan) with low to moderate DSs all exhibited solubility in water. However, at higher DSs, the derivatives displayed very little to no solubility in water. The longer fatty acyl derivatives (*N*-decanoyl to *N*-stearoyl) were all insoluble in water regardless of the DS, due to an overwhelming increase in hydrophobicity.

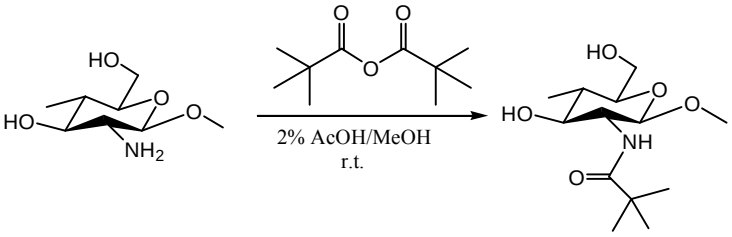
N-acylation with bulky and cyclic anhydrides exploit the ease in which the substituents greatly reduce the normal regularity of intermolecular hydrogen bonding of chitosan, resulting in derivatives with very good solubility in water. Zhang and Hirano prepared *N*-trimethylacetyl chitosan derivatives from a homogenous reaction between chitosan and trimethylacetic anhydride[11] (Scheme 2.4). The derivatives (DS = 0.30-0.54) were reported as water soluble materials.

Hirano and Moriyasu studied ring opening reactions of chitosan with various cyclic anhydrides.[22] The structure of the attachment is shown in Scheme 2.5. The authors reported that all of the derivatives (DS = 0.45-0.80) displayed solubility in water at various pHs. All of the products exhibited solubility in the pH region below 4.0 and above pH 7.0. The solubility in the acidic region would be caused by the protonation of the *N*-amino groups ($-NH_2$ to $-NH_3^+$), and the solubility in the basic region would be caused by the change of the carboxy groups to carboxylate ions (–COOH to $-COO^-$). The derivatives did not exhibit complete solubility in the pH range of 4.0 – 7.0, which corresponded to the isoelectric point of the products. In this pH range, an equimolar of $-NH_3^+$ and $-COO^-$ groups existed in the macromolecule.

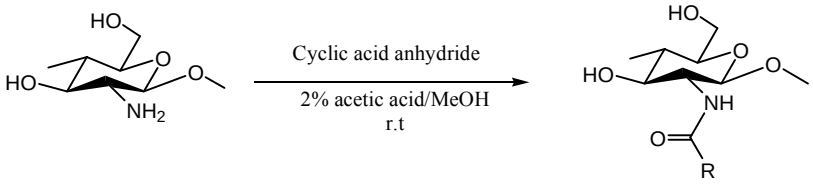
Scheme 2.3 The synthesis of *N*-saturated fatty-acyl chitosan derivatives



Scheme 2.4 The synthesis of *N*-trimethylacetyl chitosan



Scheme 2.5 *N*-acylation of chitosan with cyclic acid anhydrides



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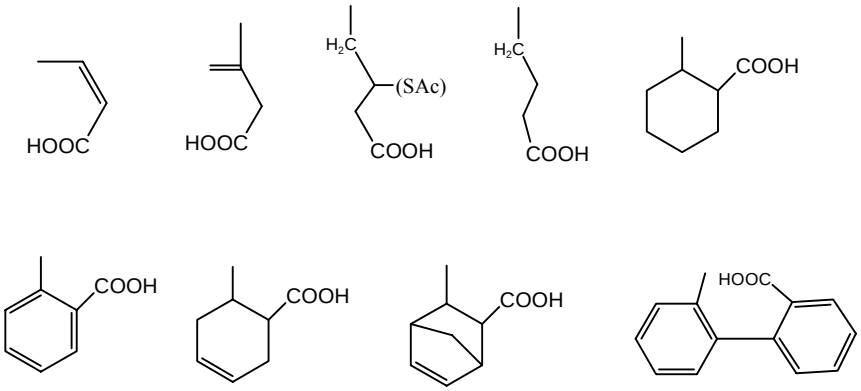
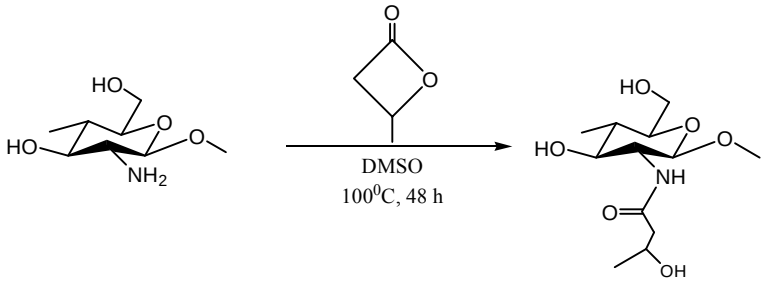


2.6 *N*-substitution via *N*-hydroxyacylation

N-hydroxyacylation introduces hydroxyacyl substituents to the backbone of chitosan, which can effectively increase the hydrophilicity owing to the hydrophilic hydroxyacyl group. However, only a few reports on the *N*-hydroxyacylation of chitosan

with lactones have been documented due to the low reactivity and stability of most lactone compounds. Kurita et al. attempted the preparation of *N*-hydroxyacylated derivatives from reactions of chitosan with β-butyrolactone and γ-butyrolactone[23] (Scheme 2.6), but reported that only β-butyrolactone was reactive towards nucleophilic attack, on the account of the high reactivity of β-lactones.

Scheme 2.6 *N*-hydroxyacylation of chitosan with β-butyrolactone

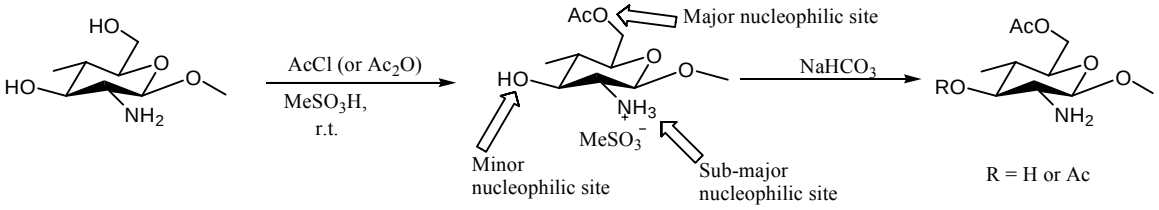


2.7 *O* verses *N* Substitution

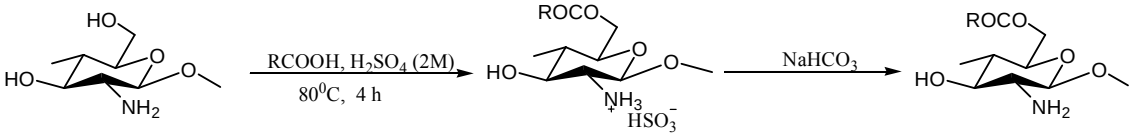
Chitosan is a multi-nucleophilic polymer due to the presence of the *N*-amino and hydroxyl functional groups. The initial sites where substitution occurs are the more nucleophilic *N*-amino groups. However, the experimental conditions and protection of the *N*-amino groups can influence *O*-substitution. Sashiwa et al. have prepared *O*-acetylated chitosan from a reaction of acetyl chloride in methanesulfonic acid at low temperature[24] (Scheme 2.7). A series of *O*-acyl derivatives were synthesized from reactions of alkanoic acid compounds with chitosan in the presence of H_2SO_4 as a catalyst,[25] without an additional protection step of the NH_2 groups (Scheme 2.8).

Protection of the *N*-amino groups via complete *N*-phthaloylation of chitosan permits rapid *O*-substitution of the C6 hydroxyl groups. Selective tritylation (triphenylmethylation) at the C-6 position followed by removal of the phthalamide group by hydrazinolysis affords 2-amino-3-hydroxy 6-*O*-trityl chitosan, which serves as an intermediate for further preparation of chitosan derivatives[3, 7] (Scheme 2.9).

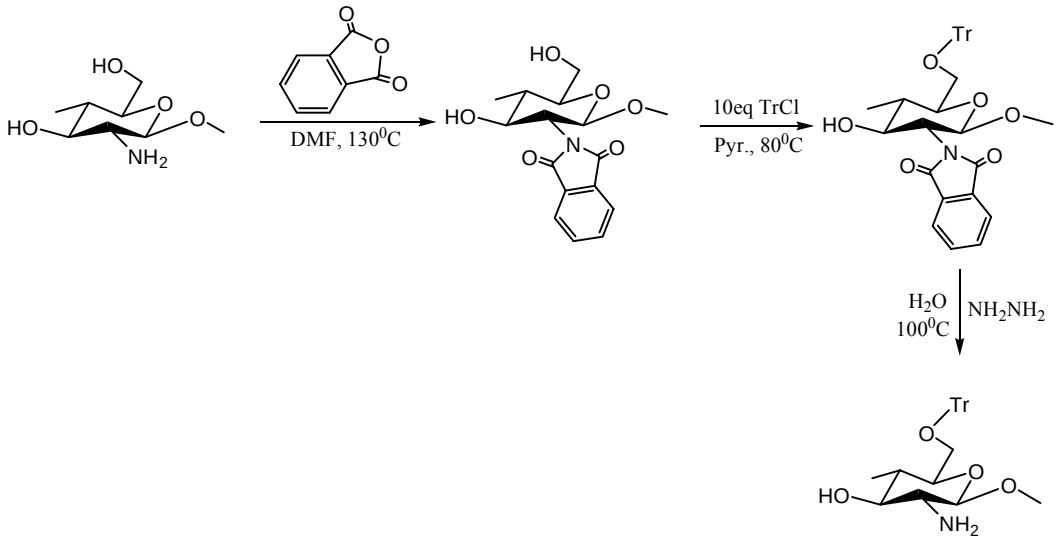
Scheme 2.7 The synthesis of water-soluble chitosan derivatives by *O*-acetylation



Scheme 2.8 Synthetic route to *O*-acyl chitosan



Scheme 2.9 The synthesis of 2-amino-3-hydroxy 6-*O*-trityl chitosan



2.8 The Preparation of Water Soluble Ionic Chitosan Derivatives

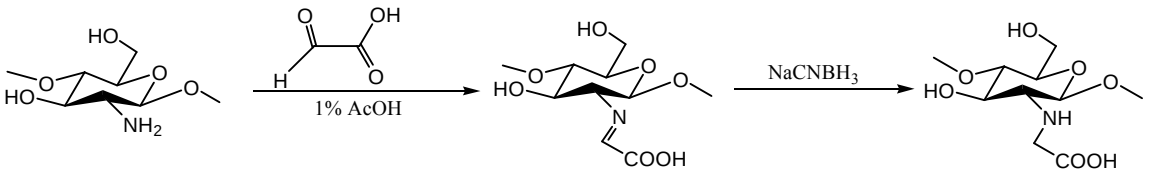
Chitosan has many biological applications, but the polymer's uses are underutilized because of the problems associated with its solubility. The goal in the

modification of chitosan is to enhance its solubility over an entire range of pHs. A common approach in achieving this is to introduce an ionic moiety onto the polymer backbone. Previously synthesized chitosan derivatives with improved solubilities include those that contain carboxyalkyl and sulfate anionic substituents, and quaternary ammonium substituents.

2.8.1 *N*-carboxyalkylation

N-carboxyalkylation of chitosan involves the introduction of acidic (anionic) groups onto the polymer backbone. Kurita prepared *N*-carboxymethyl chitosan from the addition of glyoxylic acid to an aqueous suspension of chitosan, followed by reduction of the Schiff base intermediate with sodium cyanoborohydride[14] (Scheme 2.10). This method, based on reductive alkylation, results in regioselective *N*-carboxymethylation of the *N*-amino groups.

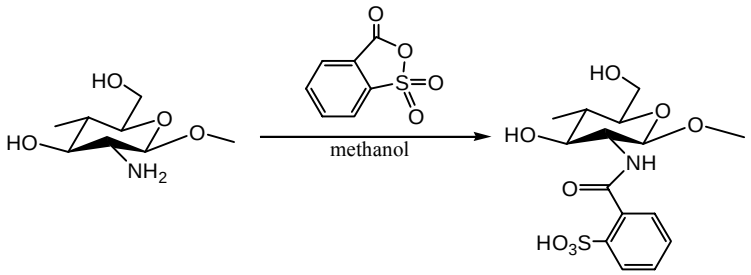
Scheme 2.10 The synthesis of *N*-carboxymethyl chitosan



2.8.2 *N*-sulfation

N-sulfation of chitosan is another example of introducing anionic charge to the polymer backbone. *N*-sulfonation of chitosan can be achieved via a reaction of chitosan with 2-sulfobenzoic acid anhydride[26] (Scheme 2.11). Sulfated chitosans owe to the possibility of preparing polymers that are analogues to the natural blood anticoagulant herapin.[27] Heparin is a highly sulfated polysaccharide used medicinally as an anticoagulant in the treatment of various cardiovascular diseases.

Scheme 2.11 *N*-sulfonation of chitosan with 2-sulfobenzoic acid anhydride



2.8.3 Quaternization

Quaternary ammonium derivatives of chitosan are interesting in view of their industrial and pharmaceutical applications.[28-30] These types of derivatives have major advantages over the parent chitosan in that they have a permanent positive charge on their polymer backbone. The simplest synthetic approach to give chitosan quaternary ammonium functionality is via conversion of the *N*-amino groups into *N*-trimethylammonium halide salts. *N*-trimethylammonium groups are the products of nucleophilic substitution reactions from alkyl halides with tertiary amines. The general structure for an *N*-alkylammonium functional group is shown in Figure 2.4. The counter-ion attached to the nitrogen by an electrovalent bond could be any anion, but is usually chloride or bromide to form the salt.

Quaternary ammonium functionality could be introduced to chitosan either by direct alkylation of the *N*-amino functional groups, or via covalent attachment of quaternary ammonium substituents to the *N*-amino groups. These processes are referred to as quaternization. With excess methyl iodide and sodium hydroxide, the *N*-amino groups of chitosan can be directly trimethylated (Scheme 2.12). Using this approach, Domard et al. reported that the cationic derivative was soluble in water over a wide pH range.[31] Quaternary ammonium groups can also be introduced to the backbone as side chains. For example, Lang et al. prepared quaternary ammonium derivatives from

reactions of chitosan with glycidyl trimethylammonium chloride.[28] In an alternative approach, Daly and Guerrini have prepared cationic chitosan derivatives using a solution of N-3-chloro-2-hydroxyl propyl trimethylammonium chloride salt (Quat-188)[32] (Scheme 2.13). These type of derivatives have very important roles in biological systems due to their aliphatic hydroxy side chains and their cationic moieties.

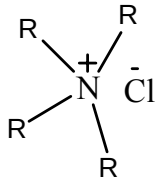
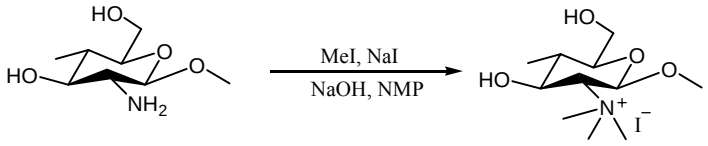
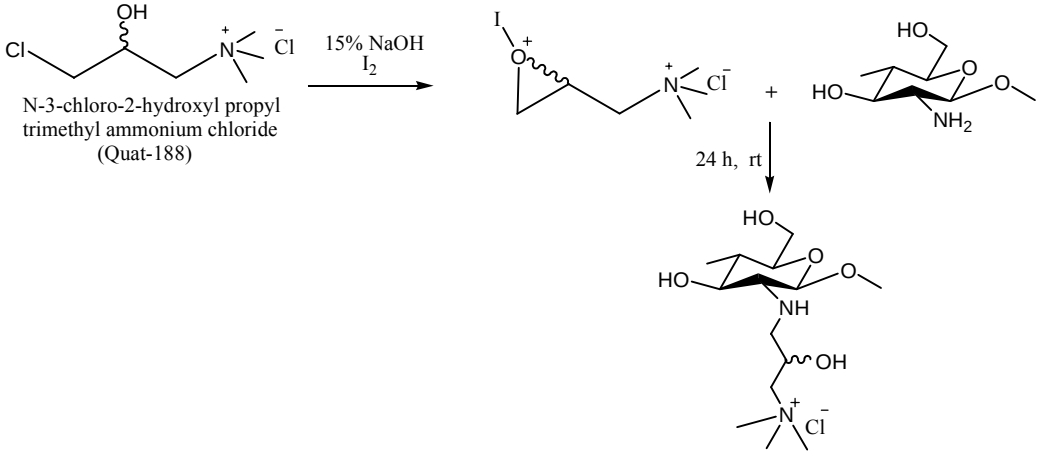


Figure 2.4 Quaternary ammonium functional group.

Scheme 2.12 Quaternization of chitosan with methyl iodide



Scheme 2.13 Quaternization of chitosan with Quat-188



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CHAPTER 3. BIOLOGICAL APPLICATIONS OF CHITOSAN

3.1 Introduction

The major driving force in the development of new applications for chitosan lies in the fact that the polymer is biodegradable, biocompatible, and possesses selective adsorption properties. Its biological nature has spurred an interest in various scientific fields, some of which include agriculture, food-preservation, and bio-medicine. Its adsorption ability has been examined for applications in water treatment, cosmetics, and biotechnical areas. The investigation of the biological properties of chitosan has led to the creation of finely designed bio-polymeric materials that have contributed towards improving the quality of life. This chapter seeks to explore the aforementioned applications of chitosan, with the intent of understanding its role in biological systems.

3.2. Biodegradable and Biocompatible Properties

3.2.1 Agriculture

Chitosan has many potential applications in agriculture because the polymer is essentially biodegradable. One application that has been widely employed is plant protection. Hadwiger and Beckman have applied chitosan to plants infected with the fungus *Fusarium solani*, and discovered that chitosan was effective in inhibiting fungal growth.[1] The results of chitosan's antifungal activity against *Fusarium solani sp. pisi* (a fungal pathogen of peas) and *Fusarium solani f. sp. phaseoli* (a fungal pathogen of beans) are shown in Table 3.1. The concentrations shown in Table 3.1 are the minimal concentrations which gave complete inhibition. The samples were prepared by dissolving shrimp chitosan in 1% acetic acid, followed by slow dissolution of 1% sodium nitrite with chitosan, and neutralizing the solution with 1N NaOH. The nitrous acid treatment depolymerizes polymers containing hexosamines with a primary amino group

and discriminates between chitin and chitosan because the acid only affects deamination and depolymerization if the polymer carries free amino groups.

Other effects of chitosan treatments on plants have been observed. For example, Pospieszny and Atabekov sprayed bean leaves infected with afalfa mosaic virus (AIMV) with 0.1% chitosan solutions.[2] This led to a significant reduction of lesions produced by AIMV. The data in Table 3.2 show that chitosan is an effective inhibitor of AIMV infections when applied days before and days after AIMV inoculation. The chitosan solutions used for the investigation were prepared by dissolving chitosan in 0.05% acetic acid and adjusting the pH to 6 with 1N NaOH.

Table 3.1 Chitosan as an inhibitor of *F. solani*

Additive to medium	Minimum Growth-inhibiting Concentration			
	F. solani f. sp. *phaseoli*		*F. solani* f. sp. *pisi*	
	Within 24 h (μg/mL)	Within 66 h (μg/mL)	Within 24 h (μg/mL)	Within 66 h (μg/mL)
Chitosan	31	62	62	125

Table 3.2 Effects of pre- and post-treatment of *Phaseolus vulgaris* with 0.1 chitosan on the local lesions induced by AIMV; the control is 0.05% acetic acid adjusted to pH 6.

Interval between treatment and inoculation	No. of local lesions per half-leaf		% Decrease in the no. of lesions
	Chitosan	Control	
Pretreatment			
5 days	0.2	24.5	99.2
4 days	0.3	34.7	99.2
3 days	0.1	51.0	99.8

(Table 3.2 continued)			
2 days	0.2	58.6	99.6
1 day	0.2	40.3	99.5
3 h	0.4	19.8	98.0
Post-treatment			
1 h	3.0	36.0	91.7
2 h	18.5	62.9	70.6
3 h	30.6	78.2	60.9
4 h	40.2	93.2	56.9

3.2.2 Food Science

Chitosan and chitosan glutamate are worthy of further study as natural preservatives for foods prone to fungal spoilage. Roller and Covill investigated the antimicrobial properties of chitosan glutamate in laboratory media and apple juice against yeasts and molds associated with food spoilage.[3] In this study, chitosan glutamate was tested at pH 3.4 against yeast and pHs 5.2 and 4.5 against mold. The growth rate of *Mucor racemosus*, a filamentous fungi, was reduced by nearly 75% in the presence of chitosan glutamate at concentrations of 1 and 2 g/L. The presence of chitosan in apple juice (pH 3.4) at levels ranging from 0.1 to 5 g/L inhibited the growth of spoilage yeasts (e.g. *Zygosaccharomyces bailii* and *Saccharomycodes ludwigii*).

The effect of chitosan in meat preservation has been examined. Darmadji and Izumimoto have studied the inhibitory effect of different concentrations of chitosan on the growth of some meat spoilage bacteria and some meat starter cultures.[4] It was reported that 0.01% chitosan at pH 6.8 inhibited the growth of *Pseudomonas fragi, Bacillus subtilis, Escherichia coli,* and *Staphylococcus aureus.* The growth of the meat starter cultures *Lactobacillus plantarium, Pediococcus pentosaceus,* and *Micrococcus varians* were inhibited by 0.1% and 1.0% chitosan. Also, the addition of chitosan to meat had a good effect on the development of the red color.

3.2.3 Biomedicine

Assorted biomedical applications have been reported for chitosan and chitosan derivatives. There are extensive data in the literature related to biomedical applications in mammalian cells. For example, chitosan derivatives can be used for tissue engineering such as skin or hard tissue replacement. Muzzarelli reported on the preparation of *N*-carboxybutyl chitosan[5] and Biagini et al. discovered that this derivative was capable of reconstructing dermal tissue architecture.[6] The derivative also organized repair tissue with ordered architecture and deprived the tissue of scar features. Muzzarelli applied *N*-carboxybutyl chitosan to the skin of humans and animals to order tissue reconstruction associated with surgical wounds, ulcers, burns, and infections,[7] and to assist the spontaneous tissue repair of the meniscus.[8]

Chitosan is a hemostatic agent (stops bleeding). The hemostatic property of chitosan has rendered its usefulness as dressings (e.g. sponges and bandages) for the treatment of wounds from infections. Motoski et al. have prepared chitosan sponges by dispersing chitosan in aqueous acetic acid and treating the solution with a solution of dodecyl sulfate and methanol/NaOH.[9] The sponge was applied to wounds on the backs of rabbits and covered; the sponge kept porosity after 7 days of its application. HemCon bandage is a compressed chitosan acetate dressing that was developed as a hemostatic agent.[10] Burkatovsky et al. tested its ability to kill bacteria in the infected wounds of mice[11] and discovered that chitosan acetate rapidly killed the bacteria in the wounds before systematic invasion took place.

Due to the high *N*-amino content, chitosan acts as a powerful natural magnetic attraction for lipids, fats, and bile in the digestive tract, and actually binds with them to prevent their absorption into the bloodstream.[12] The attracting ability of chitosan can

possibly reduce cholesterol and triglycerides blood plasma levels, which contribute to obesity and cardiovascular disease. Various hypolipemic formulations including particles, powders, solutions, and injections containing chitosan, were prepared for oral administration.[13] Suzuki et al. reported that oral administration of chitosan to mice effectively decreased blood cholesterol levels by 66.2%.[13] The lipid lowering ability of chitosan and its protective effect against cholesterol gallstone formation was investigated by Trautwein et al.[14] In this study, male Golden Syrian hamsters were fed gallstone-inducing diets containing cholesterol, where after administration of 79% deacetylated chitosan, cholesterol accumulated in the liver was reduced by 35-38%.

3.3 Adsorption Properties and Application

The adsorption property of chitosan is one of its most attractive functions. It is a natural poly-ligand rich in *N*-amino content and is used in affinity interactions because of its specificity. The concept of using affinity interactions is very attractive because the use of these techniques, for example, can be applied for isolation and purification applications.

3.3.1 Chelation

One of the earliest applications of chitosan was for chelating harmful metal ions such as copper, lead, mercury, and uranium from waste water. Studies on the chelation property has been documented by many scientists.[15, 16] Muzzarelli investigated the chelation ability of alginic acid, chitin, and chitosan, and reported that chitosan exhibited the best chelation of transition metal ions.[17, 18] This is explicable in terms of the polymer's high *N*-amino group content, which acts as electron rich donors. Muzzarelli et al. assessed the chelating ability of chitosan-glucan, which is derived via alkaline treatment of waste mycelia of *Aspergillus niger*.[16] Chitosan-glucan exhibited selective

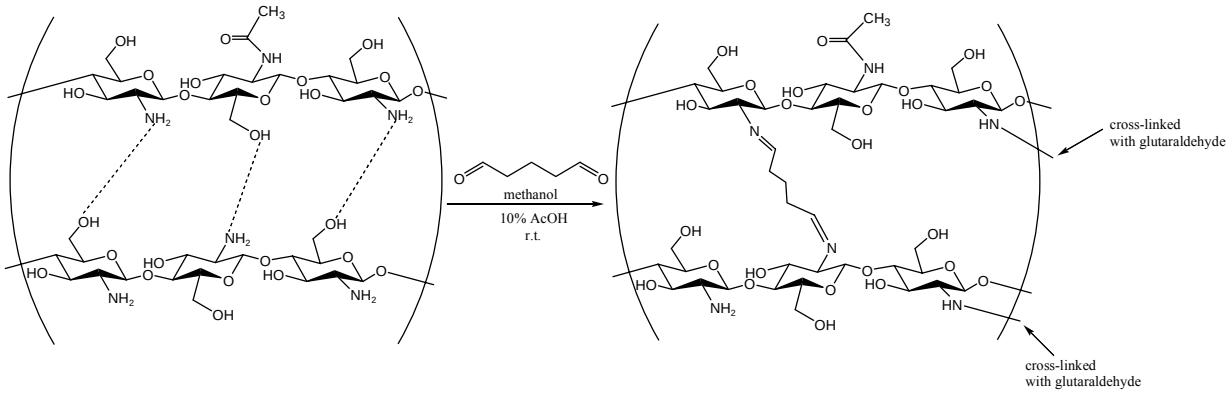
collection of Cr^{3+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} from solution. Nair and Madhavan utilized chitosan for the removal of Hg^{2+} from solution[19] and the adsorption kinetics of mercuric ions by chitosan were reported by Peniche-Covas et al.[20]

Kurita et al. have studied chitosan's adsorption of Cu^{2+} and $Hg,^{2+}$ and reported that the chitosan's adsorption capacity depends on its crystallinity, degree of deacetylation (DDA), and affinity for water.[15] In this study, chitosan materials were derived under homogenous experimental conditions, where some of the materials contained 50% *N*-amino content. These samples displayed the highest adsorption ability for the ions when compared to other chitosan derivatives that contained higher DDAs. The samples also displayed the highest solubilities in water. The authors indicated that chitosan's affinity for water is closely associated with its adsorption ability; that is, it is probable that the polymer's increase in the affinity for water resulted in a larger surface area for chelation, which resulted in an increased uptake of the metal ions.

3.3.1.1 Adsorption Enhancement: Cross-linking

The work of Kurita et al. suggested that chitosan's high crystallinity adversely effects its adsorption ability.[15] Problems associated with its crystallinity could be addressed by cross-linking the polymer under homogenous experimental conditions. Performance of the reactions under homogenous conditions would enable a random substitution of the *N*-amino group functional groups along the polymer backbone, resulting in effective destruction of the tight arrangement of the rigid polymer molecules, leading to increased hydrophilicity and easy accessibility of the chelating *N*-amino functional groups.[15] Koyama and Taniguchi improved chitosan's adsorption capacity by cross-linking with glutaraldehyde under homogenous conditions[21](Scheme 3.1). Here, the cross-linked chitosan collected 96% Cu^{2+} ions, where chitosan collected 74% Cu^{2+}.

Scheme 3.1 Cross-linking chitosan with glutaraldehyde



3.3.1.2 Adsorption Enhancement: *N*-acylation

Kurita et al. have shown that selective introduction of nonanoyl groups at the *N*-amino groups of chitosan via *N*-acylation, enhances the adsorption capacity.[22] *N*-acylation was achieved by reacting chitosan with nonanoyl chloride under homogenous conditions (Scheme 3.2). From this reaction, the adsorption capacity of chitosan (with a DDA of 88%) was improved from 75% to 98% with samples that comprised 5 to 29% *N*-nonanoyl substitution. It was noted that the maximum adsorption capacity was reached at low degrees of *N*-acyl substitution because higher substitution led to an increase in the hydrophobicity (lower water solubility), leading to lower adsorption. This indicates that a balance between the destruction of chitosan's high crystallinity and the hydrophobicity brought about by chemical modification is important in adjusting the adsorption capacity.

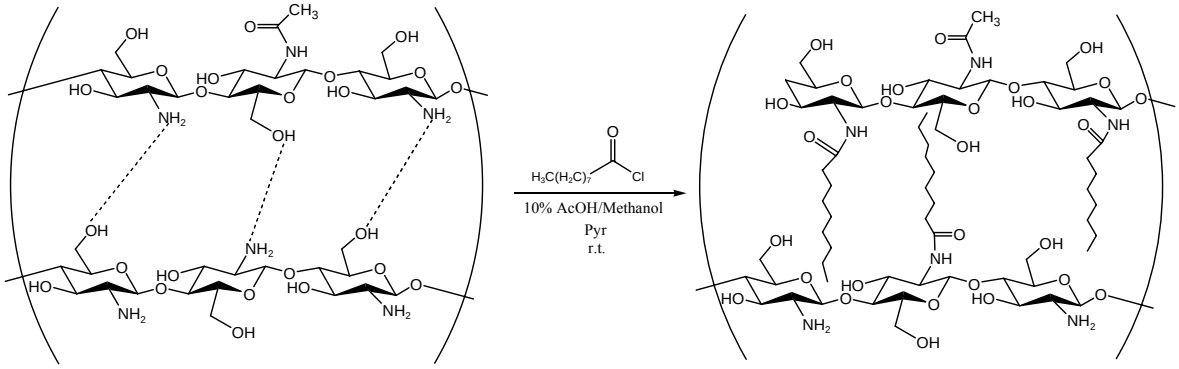
3.3.2 Coagulation

Chitosan can effectively function as a polycationic coagulant in waste-water treatment.[23] Several reports have demonstrated the effectiveness of chitosan for the coagulation of anionic substances (e.g. proteins, solids, and dyes),[24] and/or organic compounds in food-processing wastes.[24-27] It has been reported that coagulated

byproducts from food processing wastes generally contain significant amounts of protein (30-75%) that can have potential applications in animal feeds.[28]

No and Meyers utilized chitosan for the coagulation of amino acids (arginine, alanine, glutamic acid, serine, and glycine) from seafood-processing waste-water.[29] Concentrations of suspended solids and turbidity in crawfish waste-water were reduced to 97% and 83%, respectively, by treatment with 150 mg/L chitosan at pH 6. Bough and Landes investigated the coagulating ability of chitosan and reported that the polymer effectively removed suspended solids in cheese whey, a non-living processing waste from cheese making operations.[30] Concentrations of suspended solids were reduced >90% at pH 6. The approximate composition of the solids was 73% protein, 6% lactose, 10% ash, and 7% moisture. In a similar investigation, Wu et al. measured chitosan's effectiveness in coagulating proteins and removing turbidity from cheese whey.[24] The optimal concentrations for the chitosans tested ranged from 7 to 150 mg/mL with a resultant reduction of more than 90% in turbidity.

Scheme 3.2 *N*-acylation of chitosan with nonanoyl chloride



3.3.3 Cosmetics

A cosmetic is defined as any substance to be placed in contact with various surface parts of the human body (e.g. epidermis, hair systems, nails, lips, and external

genital organs), or with teeth and the mucous membranes of the oral cavity with a view exclusively or principally to perfume them, protect them, and keep them in good condition, to change their appearance, or to correct body odors.[31] Chitosan is a natural cationic gum that has been used for various cosmetic applications, particularly for hair and skin treatment[32, 33] and many other personal care cosmetic applications. Muzzarelli reviewed chitosan's applications in cosmetics, and indicated that its uses in shampoos can help remove left-over starch.[12] A line of products including three types of shampoos containing 0.5-6.0% chitosan salt, have the effect of conferring shine and strength to hair due to the ionic interactions between chitosan and hair proteins.[34] When applied to the surface of the skin, chitosan forms a protective and moisturizing elastic film. This makes chitosan useful in the formulation of moisturizing agents (e.g. lotions, sunscreens).[35] Bandai et al. prepared a bath lotion containing chitosan lactate, chitosan succinate, and chitosan alkyl phosphate (Figure 3.1, I–III respectively).[36] This cosmetic increased skin softness.

3.4 Antibacterial Properties

One of the most unique biological properties of chitosan is its antibacterial activity. Chitosan inhibits the growth of a wide variety of bacteria,[37] as shown in Table 3.3. However, chitosan exhibits its antibacterial effect at pHs < 6.5 (acidic range) due to its poor solubility above pH 6.5.

To analyze chitosan's antibacterial activity at pHs above 6.5, chitosan derivatives are usually prepared. There are a plethora of literature and books that explore the antibacterial property of several chitosan derivatives. The most popular derivatives include those that comprise acidic (anionic) or quaternary ammonium (cationic) moieties on the polymer backbone.

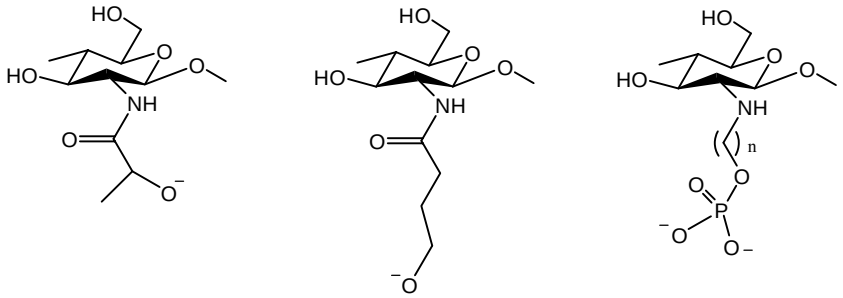
The preparation of water-soluble *N*-carboxybutyl chitosan has been reported by Muzzarelli et al.[5](Scheme 3.3), and its antibacterial activity was investigated in view of its uses in wound management.[38] The antibacterial activity against a variety of gram (+) and gram (-) bacteria was assessed by a quantitative assay based on conventional agar dilution tests, as described in detail in the literature.[38] The results of the antibacterial activity are shown in Table 3.4 and Table 3.5, where the concentration of *N*-carboxybutyl chitosan ranged from 9 to 2 mg/mL and 9 to 4 mg/mL for gram (+) and gram (-) bacteria, respectively. The majority of the gram (+) strains tested were inhibited at a concentration of 8 mg/mL. One-half to three-quarters of the gram (-) strains were inhibited by 6 mg/mL and 90 - 100% were inhibited by 9 mg/mL.

N,N,N-trimethyl chitosan was prepared from a reaction of chitosan with excess methyl iodide and sodium hydroxide under controlled conditions[39] (Scheme 3.4). The antibacterial activity of this derivative was tested against *E. coli* and compared to the antibacterial activity of *N*-propy*l*-*N,N*-dimethyl chitosan (Scheme 3.5) to investigate the effect of the alkyl chain length of the substituent.[40] Table 3.6 shows the antibacterial activity of the derivatives against *E. coli* in water.

The results in Table 3.6 show that the quaternized derivatives at the highest molecular weight displayed the highest antibacterial activity, meaning these derivatives gave the lowest minimum inhibitory concentrations overall. When comparing the antibacterial activities of both derivatives at high molecular weight, *N*-propyl-*N,N*-dimethyl chitosan was more effective, demonstrating that the length of the alkyl chain strongly affected the antibacterial property.[41] This is probably explicable in terms of increased hydrophobic-hydrophobic interactions between the alkyl chain and the hydrophobic interior of the bacterial cell wall.

3.4.1 Mode of Action of Antibacterial Activity

The mode in which the chitosan derivatives that bear ionic moieties inhibit the growth of bacterial cells has been proposed, but not fully understood. It is believed that charged groups in the bacterial cell wall interact electrostatically with, for example, the positively charged quaternary ammonium nitrogens of chitosan. The lipophilic chain or the hydrophobic substituent diffuses through the bacterial cell wall causing disruption of the cytoplasmic membrane, and eventually, cell death.[42]



I II III

Figure 3.1 Structures of chitosan lactate (I), chitosan succinate (II), and chitosan alkyl phosphate (III)

Table 3.3 Antibacterial activity of chitosan

Bacteria	MIC (μg/mL)
Agrobacterium tumefaciens	100
Bacillus cereus	1000
Corinebacterium michiganence	10
Erwinia sp.	500
Erwinia carotovora subsp.	200
Escherichia coli	20
Klebsiella pneumoniae	700
Micrococcus luteus	20
Pseudomonas fluorescens	500
Staphylococcus aureus	20
Xanthomonas campestris	500

Scheme 3.3 Preparation of *N*-carboxybutyl chitosan

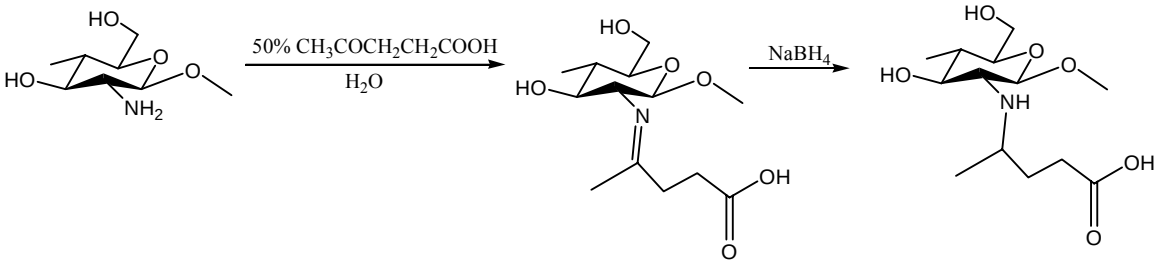


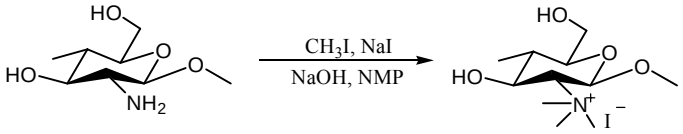
Table 3.4 Activity of *N*-carboxybutyl chitosan against gram (+) bacteria as determined by the agar dilution technique

	% Inhibition at the following polymer concentration (mg/mL)			
Concentrations → **2**	**4**	**6**	**8**	**9**
Staphylococcus aureus	11	87	92	94
Coagulase-negative Staphylococci	71	92	100	
Streptococcus spp.		20	100	
Enterococcus faecalis		9	100	

Table 3.5 Activity of *N*-carboxybutyl chitosan against gram (-) bacteria as determined by the agar dilution technique

	% Inhibition at the following polymer concentration (mg/mL)			
Concentrations →	**4**	**6**	**8**	**9**
Escherichia coli	19	52	81	90
Klebsiella spp.	39		82	96
Enterobacter spp.	40	60	80	100
Serratia spp.	15	31		53
Citrobacter freundii		50		100
Proteus spp.	25	46	96	100
Morganella morganii		33	66	
Salmonella spp.				
Pseudomonas aeruginosa	40	76	88	96
Acinetobacter anitratus			40	60
Haemophilus influenzae	6	47	72	75

Scheme 3.4 Preparation of *N,N,N*-trimethyl chitosan



Scheme 3.5 Preparation of *N*-propyl-*N,N*-dimethyl chitosan

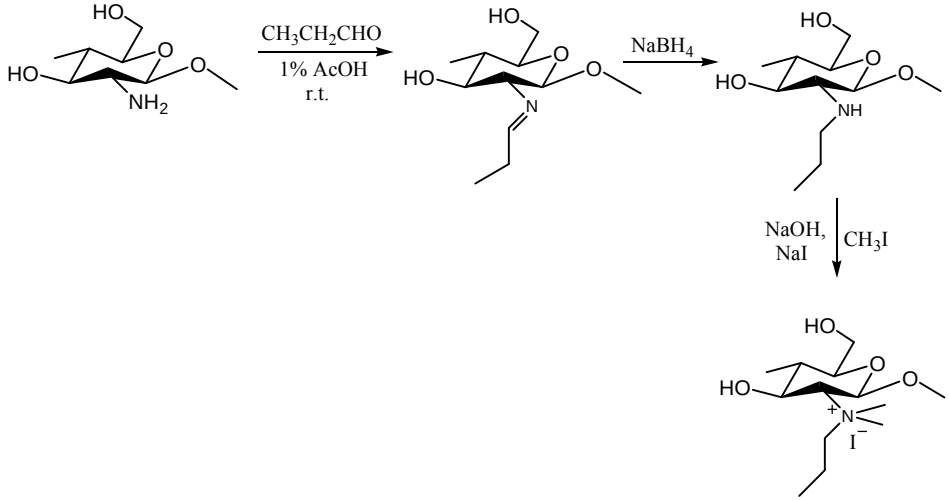


Table 3.6 MIC of quaternized chitosan against *E. coli* in water medium

Quaternized chitosan	MIC (μg/mL)		
	I	**II**	**III**
N,N,N-Trimethyl chitosan	1	1	2
N-Propyl-*N,N*-dimethyl chitosan	0.5	1	1

Quaternized chitosan prepared by chitosan with MW 2.13 x 10^5 (**I**)
Quaternized chitosan prepared by chitosan with MW 1.90 x 10^4 (**II**)
Quaternized chitosan prepared by chitosan with MW 7.80 x 10^3 (**III**)

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CHAPTER 4. SYNTHESIS OF *N*-ACYL QUAT-188 CHITOSAN DERIVATIVES

4.1. A Review

To the synthetic polymer chemist, the most important aspect of their work is in the organic synthesis of materials. The chemist manipulates the molecular structure of the polymer to develop functional characteristics in the end product by chemical modification or through other modification conditions. The resulting polymeric material can be used to make ingredients for products with unique physical, chemical, and/or biological properties.

Polymeric materials are created for specific applications and the synthetic route leading to their creation is chosen based on the desirable properties to be imparted. In the preparation of chitosan derivatives, the synthetic route involves the chemical modification of chitosan with organic compounds. The reactions leading to the end products are relatively simple, exploiting the nucleophilicity of the electron rich *N*-amino functional groups. The reactions can occur under homogenous or heterogeneous conditions, which thus affect the distribution of substitution and ultimately the solubility of the polymer. To obtain water soluble chitosan over a wide range of pHs, the process involves the introduction of polar ionic groups onto the polymer backbone.

4.2 Objective of Study

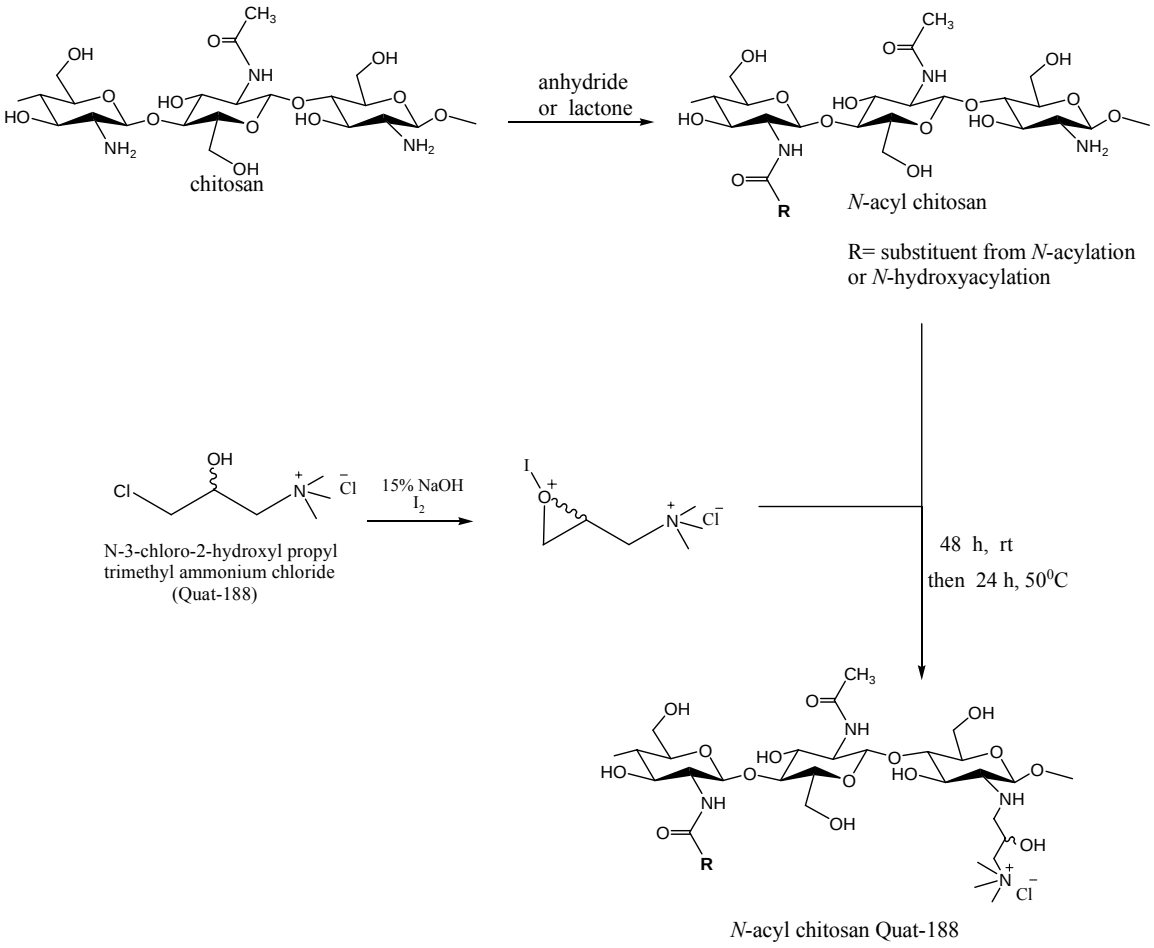
This research entails the synthesis and characterization of hydrophobic water soluble *N*-acyl chitosan derivates and the investigation of their antibacterial activity. From a chemical point of view, the main objective in the modification of chitosan with organic compounds is to increase the polymer's hydrophobic character, while enhancing its solubility in water. Aliphatic-hydroxy quaternary ammonium substituents are covalently attached to the *N*-acyl derivatives to render the hydrophobic derivatives

completely soluble at physiological pH. The biological significance behind increasing the hydrophobic character of the polymer is to enhance its antibacterial properties; that is through hydrophobic-hydrophobic interactions believed to occur between the hydrophobic substituent and the hydrophobic interior of the bacterial cell wall.[1] The quaternary ammonium substituent exhibits an antibacterial effect through electrostatic interaction with the negatively charged groups of the bacterial cell surface.[2] The biological mode of antibacterial activity is described more in detail in Chapter 5.

4.3 Overall Syntheses Performed in the Present Project

The *N*-acyl derivatives are prepared from reactions of chitosan with aliphatic and cyclic anhydrides, the novel anhydrides 2-(*cis*-1,3-O-benzylidene glycerol) succinic and glutaric acid mono ester anhydride, and lactones. The reactions of chitosan with the lactones were performed at room temperature and under mildly acidic conditions (1% AcOH), where the lactones exhibited solubility in the aqueous medium. The reactions of chitosan with the commercial and novel anhydrides were carried out at room temperature in a 1:1 v/v solvent mixture of 1% AcOH and DMF or DMSO. The addition of DMF or DMSO was to completely dissolve the anhydride in the medium. An equal amount of DMF or DMSO was added to keep chitosan in solution. The homogenous experimental conditions used for chitosan's reaction with the anhydrides and lactones were to produce a statistically controlled random distribution of the substituents along the polymer chain. Subsequent to their derivation, the *N*-acyl derivatives were subjected to quaternization using 3-chloro-2-hydroxyl propyl trimethylammonium chloride (Quat-188) (Scheme 4.1). The quaternization reactions were performed at pH 8 for 48 h and initially at room temperature. After a 48 h stir in the basic medium, the temperature of the reactions was then increased to 50 ^{0}C and stirred for an additional 24 h.

Scheme 4.1 Preparation of *N*-acyl chitosan Quat-188



4.4 Isolation and Purification of Products

The *N*-acyl products formed as precipitates upon neutralization of the reactions with 15 % NaOH. The precipitates were filtered, washed with water and carried forward for quaternization, or they were washed with acetone and dried overnight under a stream of nitrogen gas.

Quaternization of the *N*-acyl derivatives resulted in products that exhibited solubility in the aqueous environment used for their syntheses. These solutions were purified by dialysis. The process of dialysis involves the separation of low molecular weight inorganic and un-reacted organic materials by diffusion through a semi-permeable

membrane, while retarding the diffusion of the *N*-acyl derivative (due to its larger molecular weight).

4.5 Instrumental Analysis and Characterization

4.5.1 FT-IR Spectroscopy

FT-IR spectroscopy provides information through band properties, frequencies and intensities, and can therefore be used to identify species and predict chemical processes. For this research, formation of the *N*-acyl bond was determined by FT-IR spectroscopy. The N-H absorption of the *N*-acetamido (*N*-amide) group of chitosan corresponds to a frequency of 1586 cm^{-1}, as shown in Figure 4.7. Upon the modification of chitosan with one of the organic compounds, the N-H absorption of the new *N*-acyl bond overlaps the N-H absorption of the *N*-acetamido group, and the absorptions shift to a lower frequency.

4.5.2 ^{1}H NMR Spectroscopy

^{1}H-NMR spectroscopy has been recognized as the method of choice for the quantitative determination of the degree of deacetylation (DDA) and extent of *N*-substitution (ES) of the chitosan derivatives. This method is also particularity useful for studying the macromolecular structure of chitosan and its derivatives and for quick identification of impurities sometimes present in commercial chitosan and chitosan derivatives. For this research, ^{1}H NMR was used to determine the % ES of the *N*-acyl substituent and to confirm the macromolecular structures of the derivatives.

4.5.3 Gel Permeation Chromatography/Light Scattering

Gel Permeation Chromatography (GPC) is essentially a process for separating macromolecules according to their size. Its general applications to synthetic polymer chemistry have revolutionized the procedures for polymer molecular weight

determination. Quaternization of the *N*-acyl derivatives was performed under alkaline conditions and the reactions were carried out for several days at different temperatures. Sannan et al. have reported that chitosan reactions performed under alkaline conditions for long reaction times can possibly degrade the polymer main chain and result in a reduction in the molecular weight.[3] Therefore, GPC data were obtained for some of the quaternized *N*-acyl derivatives to determine their extents of molecular weight reduction.

4.6 Experimental

4.6.1 Materials

Chitosan was purchased from the Sigma Aldrich Chemical Company, with a molecular weight of ~130,800 g/mole. The solvents were of HPLC grade and used without further purification. The lactones, acid anhydrides, *cis*-1,3-*O*-benzylidene glycerol, and dicyclohexylcarbodiimide (DCC) were purchased from Sigma Aldrich. A 65% solution of 3-chloro-2-hydroxyl propyl trimethylammonium chloride (Quat-188) was obtained from the Dow Chemical Company. Semi-permeable membranes used for dialysis of the chitosan derivatives were Spectra Por, manufactured by Spectrum Laboratories In., with a molecular weight cut-off (MWCO) of 6,000-8,000.

4.6.2 Instrumentation

The NMR spectra were recorded with a Bruker AC300 in D_2O/d_4-CD_3COOD or D_2O, depending on the solubility of the product. FT-IR spectra were recorded using a Bruker Tensor 27 instrument with an Attenuate Total Reflectance cell. The molecular weights of the quaternized *N*-acyl derivatives were determined by a gel permeation chromatography/light scattering (GPC/MALS) system consisting of a Agilent 1100 Series generic pump and injector, three Viscotek Columns (Viscogel) at 20 ^{0}C, a Wyatt Optilab rEX refractive index detector, and a Wyatt Dawn Heleos light scattering detector.

The mobile phase used was 5% acetic acid (pH 4) at a flow rate of 1 mL/min. The chromatograms were collected by Astra V software, and analyzed with the Astra 5.3.1.5 program. The differential refractive indices (d*n*/d*c*) of commercial chitosan were estimated to be 0.180 assuming complete recovery of the sample injected.

4.6.3 Syntheses

Preparation of the *N*-acyl chitosan derivatives was attempted by either *N*-acylation or *N*-hydroxyacylation. In most cases, chitosan was reacted with a anhydride or lactone 3 times in 3 separate trials, to obtain 3 products per *N*-acyl derivative. All 3 products comprised a different extent of hydrophobicity.

4.6.3.1 Representative Procedure for *N*-acylation of Chitosan with Acyclic Anhydrides

Chitosan (0.500 g, 3.106 mmol) was dissolved in 50 mL 1% AcOH. To this was added 50 mL of DMF and then the acyclic anhydride (e.g. butyric anhydride 0.221 g, 1.397 mmol). The solution was stirred 24 h before the pH was increased from 4.5 to 9 using 15% NaOH and the product precipitated. The product was washed with water, until the pH of the filtrate was adjusted to 7. The product was later carried forward while still moist for quaternization. The macromolecular structures and % ESs of the *N*-aliphatic and benzoyl acyl derivatives are shown in Figure 4.4 and Table 4.1, respectively.

4.6.3.2 Representative Procedure for *N*-acylation of Chitosan with Cyclic Anhydrides

Chitosan (0.500 g, 3.106 mmol) was dissolved in 50 mL 1% AcOH. To this was added 50 mL of DMF and then the cyclic anhydride (e.g. cis-1,2,3,6-tetrahydrophthalic anhydride 0.142 g, 0.933 mmol). The solution was stirred 24 h before increasing the pH from 4.5 to 7 with 15% NaOH and the product precipitated. The product was washed with copious amounts of acetone, filtered, and dried overnight under a steady stream of

N_2. The structures and % ESs of the *N*-ionic acyl derivatives are shown in Figure 4.2 and Table 4.2, respectively.

4.6.3.3 The Synthesis of 2-(*cis*-1,3-O-benzylidene glycerol) Succinic (or Glutaric) Acid Mono Ester Anhydride

The 2-(*cis*-1,3-O-benzylidene glycerol) acid mono ester anhydrides were prepared using the cyclic anhydrides succinic[4,5] and glutaric anhydride. Scheme 4.4 shows the synthetic route towards the preparation of 2-(*cis*-1,3-O-benzylidene glycerol) succinic acid mono ester anhydride. *Cis*-1,3-*O*-benzylidene glycerol (3.000 g, 16.648 mmol) and succinic anhydride (2.658 g, 26.372 mmol) were dissolved in 15 mL of anhydrous pyridine and the reaction was heated to 50 ^{0}C for 16 h under a stream of N_2. After a 16 h stir, the pH of the solution was adjusted to 4 using a 1N HCl solution and extracted three times with DCM. The combined organic extracts were dried over Na_2SO_4, gravity filtered and evaporated. The white solid was stirred in 108 mL of ethyl ether for 1 h and cooled to -52 ^{0}C for 3 h before collecting 2.932 g of the monoacid succinate (white powder, 63% yield). ^{1}H NMR data ($CDCl_3$) (Figure 4.26): δ (ppm) 2.75 (s, 4, -C$\underline{H}_2$-C$\underline{H}_2$), 4.16 (m, 4, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 4.29 (m, 4, -C$\underline{H}_2$-CH- C$\underline{H}_2$), 4.73 (m, 2, -CH_2- C$\underline{H}$-CH_2-), 5.53 (s, 2, C$\underline{H}$), 7.34 (m, 6, arom. C$\underline{H}$), 7.47 (m, 4, arom. C$\underline{H}$). ^{13}C NMR ($CDCl_3$) (Figure 4.22): δ 178.39 (C$\underline{O}$OH), 172.52 (-C$\underline{O}$OR-), 139.20 (C$\underline{H}$), 129.52 (C$\underline{H}$), 128.72 (C$\underline{H}$), 126.44 (C$\underline{H}$), 101.65 (C$\underline{H}$), 69.38 (C$\underline{H}$), 66.76 (C$\underline{H}_2$), 29.43 (C$\underline{H}_2$), 29.25 (C$\underline{H}_2$).

The succinate monoacid (2.000 g, 7.136 mmol) and DCC (0.884 g, 4.280 mmol) were dissolved in 13 mL of DCM and stirred for 18 h at room temperature (Scheme 4.4). The DCU precipitate was collected by gravity filtration and washed with 25 mL DCM. The organic phase was added to 95 mL of hexane for precipitation of the product. The precipitate and hexane were cooled to -50 ^{0}C, stirred for 6 h, and filtered. The product

was dried under a stream of N_2 and 1.604 g of the anhydride was isolated. The product appeared as a white powder. The % yield was computed as 83%.

4.6.3.4 Representative Procedure for *N*-acylation of Chitosan with 2-(*cis*-1,3-O-benzylidene glycerol) Succinic (or Glutaric) Acid Mono Ester Anhydride

Chitosan (0.500 g, 3.106 mmol) was dissolved in 50 mL 1% AcOH. To this was added 50 mL of DMSO and then a solution of 2-(*cis*-1,3-O-benzylidene glycerol) succinic (or glutaric) acid mono ester anhydride in DMSO (0.206 g, 0.380 mmol in 10 mL DMSO) (Scheme 4.5). The solution was stirred 24 h before the reaction was neutralized with 15% NaOH (pH 7) and the product precipitated. The product was washed with excess acetone, filtered, and dried overnight under a steady stream of N_2. The % ESs and structures are shown in Table 4.3 and Figure 4.3, respectively.

4.6.3.5 Representative Procedure for *N*-hydroxyacylation of Chitosan with Lactones

Chitosan (0.500 g, 3.106 mmol) was dissolved in 50 mL 1% AcOH. To this was added 1.5 mL of glacial acetic acid and then the lactone (e.g. ε-caprolactone, 0.142 g, 1.242 mmol). The solution was stirred 24 h before the reaction was neutralized with 15% NaOH (pH 7) and the material precipitated. The material was washed several times with copious amounts of acetone, filtered, and dried overnight under a steady stream of N_2.

4.6.3.6 Representative Procedure for Quaternization of the *N*-acyl Chitosan Derivatives

A 10 mL (11.600 g, 61.669 mmol) solution of 65% Quat-188 was added to a beaker and the pH of the solution was adjusted to 8 using 15% NaOH. A catalytic amount of I_2 (0.250 grams, 0.985 mmol) was added followed by the addition of the *N*-acyl chitosan derivative (Scheme 4.1). After a 48 h stir, 50 mL of deionized water was added to the solution, and the reaction was heated at 50 ^{0}C and stirred for 24 h. The reaction was then cooled to room temperature and the clear light tan brown solution was

dialyzed in 4 liters of deionized water for 4 days. After dialysis, the solution was added dropwise to acetone and the product precipitated. The product was dried under N_2. The appearance of the product was either a white flaky solid or a tan powder. The structures of the *N*-acyl Quat-188 products are shown in Figures 4.4 through 4.6. The % ES and grams of the products recovered are summarized in Tables 4.4 through 4.6.

Table 4.1 % ES of *N*-aliphatic and benzoyl acyl chitosan

Name of Derivative	grams of anhydride used	% ES targeted	% ES obtained
N-propionoyl chitosan	0.121 0.202 0.323	30 50 80	21 47 45
N-butyroyl chitosan	0.098 0.221 0.418	20 45 85	34 41 30
N-valeroyl chitosan	0.127 0.266 0.521	22 46 90	8 0 11
N-hexanoyl chitosan	0.093 0.153 0.299	14 23 45	13 18 21
N-heptanoyl chitosan	0.113 0.226 0.339	15 30 45	23 31 50
N-benzoyl chitosan	0.316 0.527 0.703	45 75 100	14 30 50

Table 4.2 % ES and recovery of the *N*-ionic acyl chitosan derivatives

Name of derivative (entry)	grams of anhydride Used	% ES targeted	% ES obtained	crude *N*-acyl product recovered (g)	FW of MR (g/mol)	crude % recovery (wt% (mol/mol))
N-succinoyl chitosan (1)	0.093	30	14	0.510	261.0	94
N-maleoyl chitosan (2)	0.198	65	2	0.580	259.0	115
N-glutaroyl chitosan (3)	0.089 0.213 0.361	25 60 102	5 10 14	0.598 0.541 1.064	275.1	116 95 194
N-3,3-dimethyl glutaroyl chitosan (4)	0.231 0.250 0.441	52 57 100	10 3 6	0.512 0.540 0.522	303.2	94 105 99
N-cis-1,2 cyclohexanedicarboxoyl chitosan (5)	0.144 0.287	30 60	6 0	0.450 0.575	315.1	85 115
N-cis 1,2,3,6-tetrahydrophthaloyl chitosan (6)	0.095 0.142 0.354	20 30 75	7 11 20	0.542 0.525 0.719	313.1	95 102 106
N-3-NO_2 phthaloyl chitosan (7)	0.120 0.390 0.540	20 65 90	5 9 n/a	0.519 0.526 0.526	354.1	98 95 n/a
N-trimellitoyl chitosan (8)	0.179	30	19	0.523	353.1	86

FW of MR = formula weight of the monosaccharide residue

Yield (%) = {recovered grams of product/ [(FW of monosaccharide residue of chitin x ES of acetylation) + (FW of monosaccharide residue of chitosan x extent of *N*-glucosamine remaining after *N*-acylation) + FW of monosaccharide residue of product x ES of *N*-acylation)]}／ moles of sample

moles of sample = 0.5 g of commercial chitosan / (FW of monosaccharide residue of chitin x ES of acetylation) + (FW of monosaccharide residue of chitosan x extent of *N*-glucosamine remaining after *N*-acylation)

Table 4.3 % ES and recovery of 2-glycerol succinamide chitosan and 2-glycerol glutamide chitosan

Name of derivative	grams of anhydride used	% ES targeted	% ES obtained	crude *N*-acyl product recovered (g)	FW of MR (g/mol)	crude % recovery (wt% (mol/mol))
2-glycerol succinamide chitosan (1)	0.206 0.350 0.500	12 21 30	11 20 28	0.614 0.636 0.703	335.2	110 105 109
2-glycerol glutamide chitosan (2)	0.177 0.248 0.461	10 14 26	5 5 25	0.711 0.800 0.719	345.2	134 151 112

Table 4.4 % ES and recovery of *N*-aliphatic and benzoyl acyl chitosan Quat-188

Name of *N*-acyl Quat derivative	% ES of *N*-acyl substituent	grams of quaternized *N*-acyl derivative recovered
N-propionoyl chitosan Quat-188	21 47 45	0.179 0.310 0.256
N-butyroyl chitosan Quat-188	34 41 30	0.449 0.314 0.374
N-valeroyl chitosan Quat-188	8 0 11	0.760 0.666 0.578
N-hexanoyl Chitosan Quat-188	13 18 21	0.439 0.427 0.307
N-heptanoyl chitosan Quat-188	23 31 50	0.350 0.203 0.198
N-benzoyl chitosan Quat-188	14 30 50	0.198 0.184 0.371

Table 4.5 % ES and recovery of *N*-ionic acyl chitosan Quat-188

Name of *N*-acyl Quat derivative	% ES of *N*-acyl substituent	grams of quaternized *N*-acyl derivative recovered
N-succinoyl chitosan Quat-188 (1)	14	0.244
N-maleoyl chitosan Quat-188 (2)	2	0.120
N-glutaroyl chitosan Quat-188 (3)	5 10 14	0.105 0.163 0.137
N-3,3-dimethyl glutaroyl chitosan Quat-188 (4)	10 3 6	0.250 0.462 0.617
N-cis-1,2 cyclohexanedicar-boxoyl chitosan Quat-188 (5)	6 0	0.270 0.257
N-cis1,2,3,6-tetrahydrophthaloyl chitosan Quat-188 (6)	7 11 20	0.406 0.340 0.099
N-3-NO_2 phthaloyl chitosan Quat-188 (7)	5 9 n/a	0.534 0.251 0.599
N-trimellitoyl chitosan Quat-188 (8)	19	0.203

Table 4.6 % ES and recovery of 2-glycerol succinamide and glutamide chitosan Quat-188

Name of *N*-acyl Quat derivative	% ES of *N*-acyl substituent	grams of quaternized *N*-acyl derivative recovered
2-glycerol succinamide chitosan Quat-188 (1)	11 20 28	0.304 0.188 0.031
2-glycerol glutamide chitosan Quat-188 (2)	5 6 25	0.093 n/a 0.035

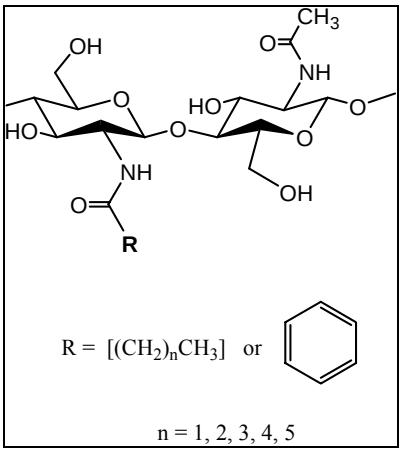


Figure 4.1 Macromolecular structures of the *N*-aliphatic and benzoyl acyl chitosan derivatives

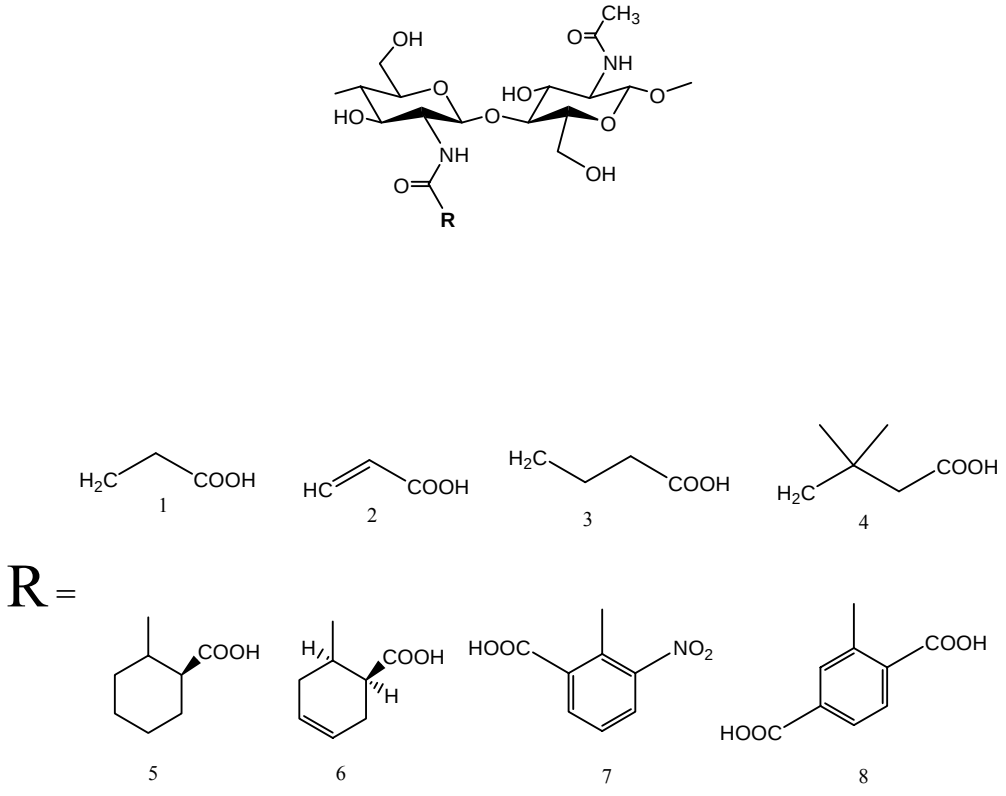


Figure 4.2 Macromolecular structures of the *N*-ionic acyl chitosan derivatives

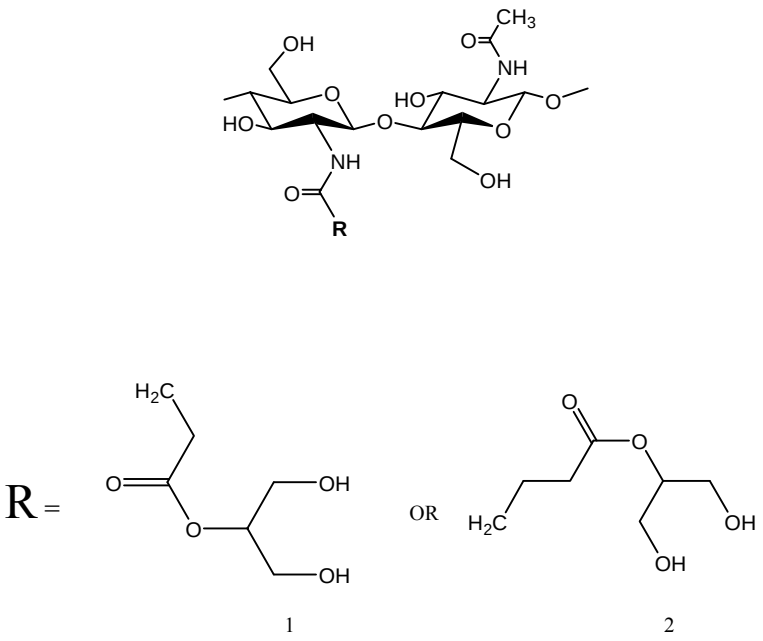


Figure 4.3 Macromolecular structure of 2-glycerol succinamide chitosan (1) and 2-glycerol glutamide chitosan (2)

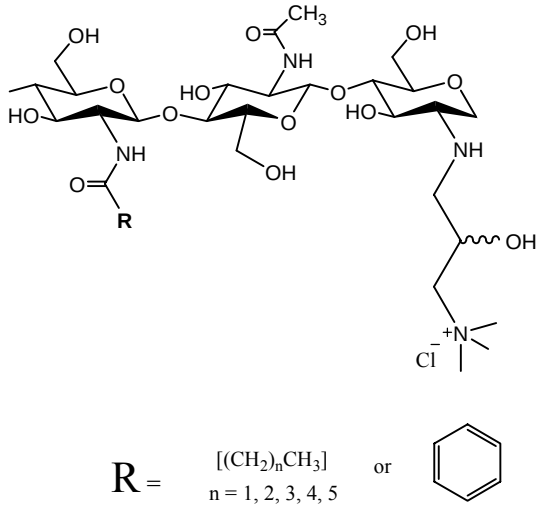


Figure 4.4 Macromolecular structures of the *N*-aliphatic and benzoyl acyl chitosan Quat-188 derivatives

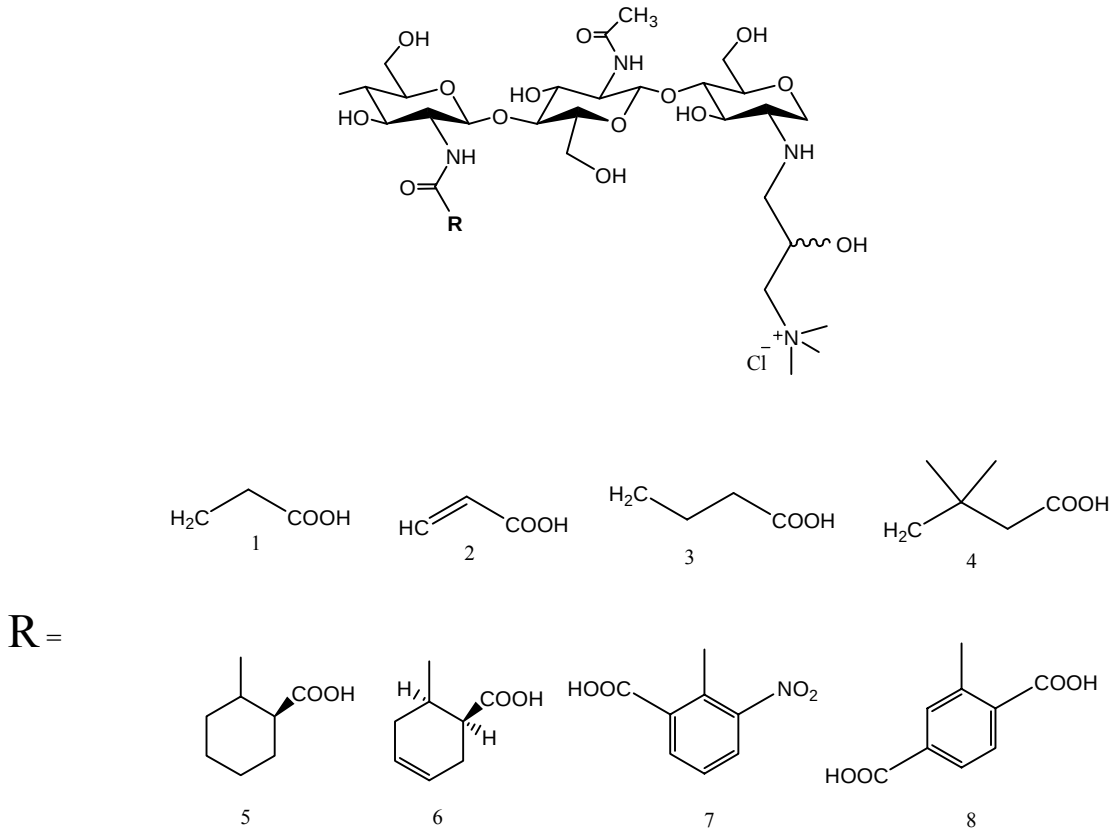


Figure 4.5 Macromolecular structures of the *N*-ionic acyl chitosan Quat-188 derivatives

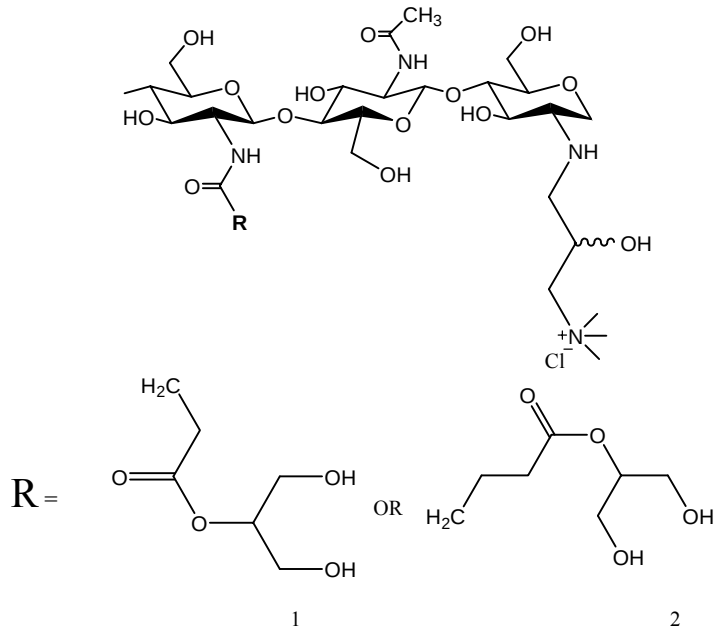


Figure 4.6 Macromolecular structure of 2-glycerol succinamide (1) and glutamide chitosan (2) Quat-188

4.7 Results/Discussion

4.7.1 FT-IR Analysis of Chitosan

Figure 4.7 shows the FT-IR of commercial chitosan. The double peak of frequencies 1647.14-1586.81 cm^{-1} corresponds to the *N*-acetamido (*N*-amide) absorption, where the C=O absorption occurs at 1647.14 cm^{-1}, and the N-H bend of the amide occurs at 1586.81 cm^{-1}. The N-H stretch of the amide occurs to the left of 3100 cm^{-1}, overlapping the amino N-H absorption (or the $–NH_2$ functional groups) at 3291.76 cm^{-1}. Normally, carbonyl absorptions occur at higher frequencies, roughly greater than or equal to 1700 cm^{-1}. However, the frequency of the C=O absorption is explicable in terms of the "back donating" effect of the unpaired electrons on the nitrogen, which conjugates with the carbonyl group, resulting in increased single bond character between the carbon and the oxygen and a lowering of the C=O absorption frequency. The aliphatic C-H stretch occurs at 2867.37 cm^{-1}. The absorption appearing at the frequency of 1024.71 cm^{-1} corresponds to the C-O group.

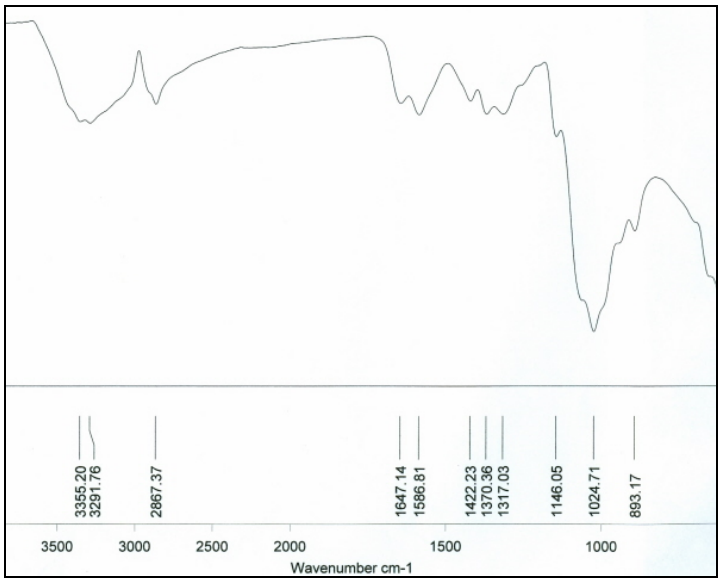


Figure 4.7 FT-IR of commercial chitosan

4.7.2 ^{1}H NMR Characterization of Chitosan

The ^{1}H NMR spectrum of commercial chitosan is shown in Figure 4.8. The internal standard used for assigning the chemical shifts of the protons was D_2O/d_4-CD_3COOD. The chemical shift of the internal standard appears at 4.67 ppm. The chemical shift of the acetal proton (-C<u>H</u>) of the glucosamine overlaps the chemical shift of the internal standard and appears at 4.58 ppm. The -C<u>H</u>-NH_2 proton appears at 3.01 ppm. The -C<u>H</u>-OH, HOHC-C<u>H</u>-$CHCH_2$-, and -C<u>H_2</u>-OH protons overlap and are assigned to the chemical shift at 3.75 ppm. The chemical shifts of -C<u>H</u>-CH_2 and -C<u>H_2</u>*OH appear at 3.59 ppm. The acetamido protons (-NHCO-C<u>H_3</u>) appear furthest upfield at 1.94 ppm. The % DDA of commercial chitosan was computed as 88% by applying the following Equation 4.1:

$$\left[1-\left(\frac{\text{(1/\# of acetyl protons) x integral of acetyl protons}}{\text{(1/\# of glucosamine protons) x integral of glucosamine protons}}\right)\right] \text{X } 100$$

$$= \% \text{ DDA}$$

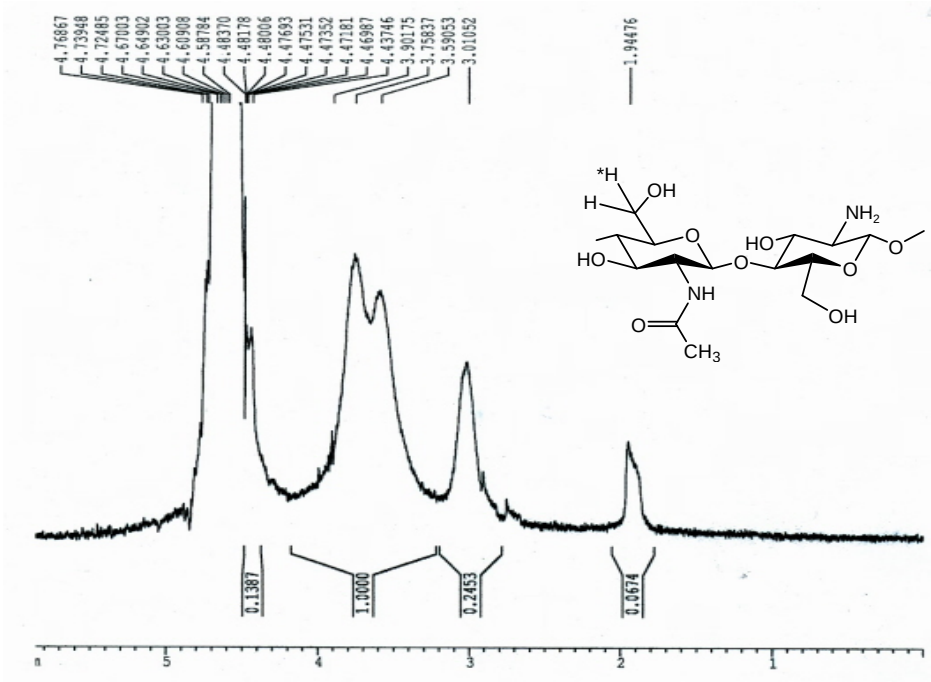


Figure 4.8 ^{1}H NMR of commercial chitosan (88% DDA) in D_2O/d_4-CD_3COOD.

4.7.3 Formation of *N*-aliphatic and Benzoyl Acyl Chitosan

Acyclic acid anhydrides are reactive acylating reagents because of a combination of the polar effect of the oxygen substituent which enhances reactivity of the carbonyl group. The nucleophilic *N*-amino groups of chitosan undergo addition at the carbonyl groups of the anhydrides, followed by elimination of the carboxylate (-O-COR) leaving group. The mechanistic route towards formation of the end product via this reaction is summarized in Scheme 4.2. The identity of the rate-determining step (slow step) appears to be the expulsion of the carboxylate group from the tetrahedral intermediate (B).[6-8] The expulsion step leading to *N*-acyl formation is the driving force of the reaction.

4.7.3.1 Experimental Preparation of the *N*-aliphatic and Benzoyl Acyl Chitosan Derivatives

The *N*-acylation of chitosan with the acyclic anhydrides was carried out in a binary solvent mixture of 1% AcOH/DMF (1:1 v/v). The addition of DMF helped

dissolve the anhydride in the aqueous medium. An equal amount of the co-solvent (to that of AcOH) was added to maintain chitosan's solubility in the medium, thus influencing homogenous experimental conditions. To minimize the possibility of depolymerization and the occurrence of side reactions, the reactions were carried out at room temperature. The experiments were conducted for 24 h.

Prior to precipitation, the *N*-acyl products appeared as white or tan viscous solutions. Using 15 % NaOH for precipitation, the pH of the solutions were increased to pH 9. To remove excess base, the precipitates formed were first filtered and then washed with water until the pH of the filtrate was adjusted to 7. They were later carried over while still moist for quaternization.

4.7.3.2 FT-IR Analysis of *N*-aliphatic and Benzoyl Acyl Chitosan

FT-IR data was obtained for the *N*-aliphatic and benzoyl acyl derivatives for qualitative analysis of their macromolecular structures. Upon modification of chitosan with one of the acyclic anhydrides, the band assigned to the NH bend shifts to a lower frequency. For example, the FT-IR of *N*-heptanoyl chitosan is shown in Figure 4.9. When compared to commercial chitosan, the NH bend shifts from 1586 cm^{-1} (chitosan) to 1542 cm^{-1} (*N*-heptanoyl chitosan). This is probably attributed to intermolecular hydrogen bonding between the carbonyl of the *N*-acyl functional group and the hydrophilic groups of the polymer backbone. The C=O absorption of *N*-heptanoyl is more defined and overlaps less with the NH bend, when compared to the commercial chitosan. The appearance of the peak at 2923 cm^{-1} corresponds to the aliphatic C-H stretch of the methyl group ($-CH_3$ functional group) of the substituent. The methylene absorption of the substituent ($-CH_2$ functional group) overlaps with the methylene absorption of chitosan at 2863 $cm.^{-1}$

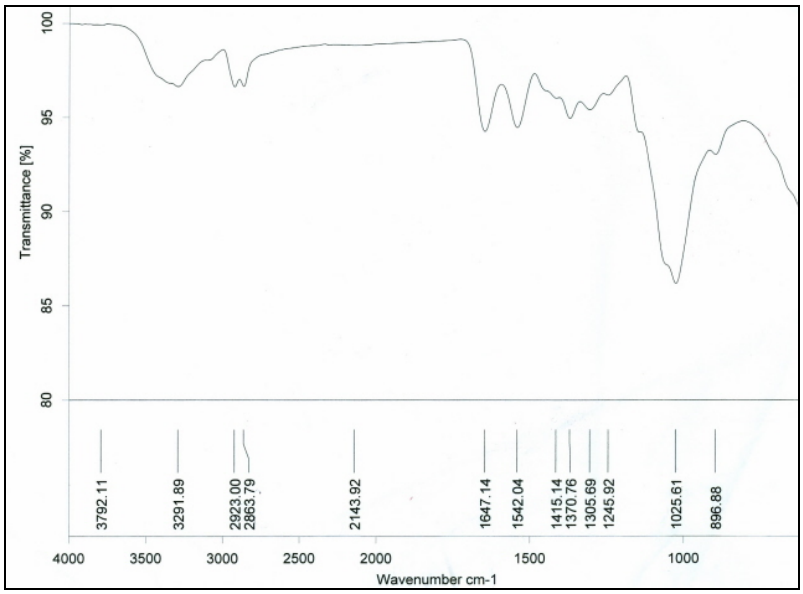


Figure 4.9 FT-IR of *N*-heptanoyl chitosan

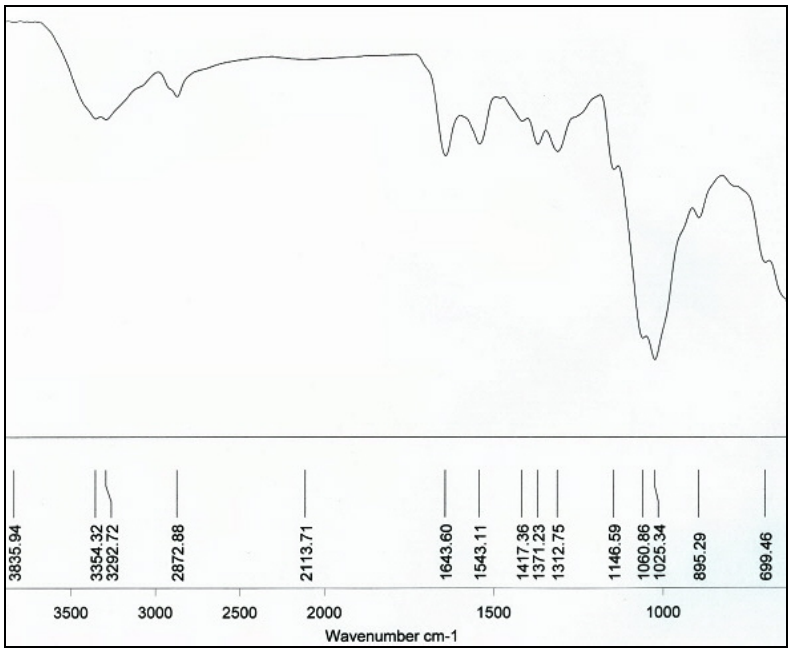
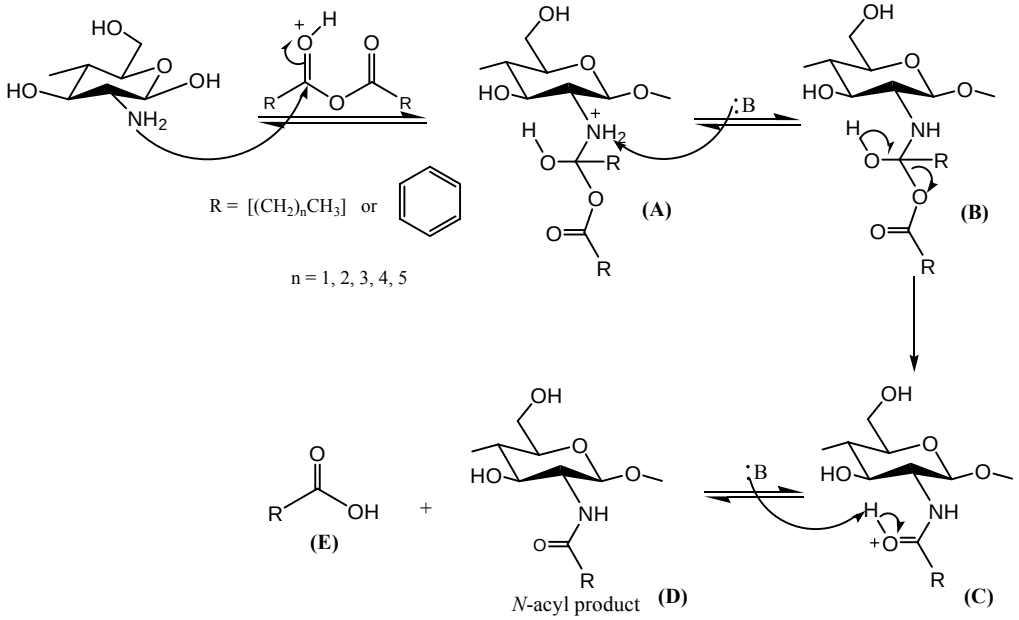


Figure 4.10 FT-IR of *N*-benzoyl chitosan

The FT-IR for benzoyl chitosan is shown in Figure 4.10. The NH bend has a frequency of 1543 cm^{-1}. The C=C ring stretch absorption occurs at 1417 cm^{-1}. The =C-H stretch appears to the left of 3000 cm^{-1}, and the =C-H out-of-plane (oop) bending has a frequency range of 900-690 cm^{-1}.

Scheme 4.2 Mechanistic route towards the synthesis of *N*-aliphatic and benzoyl acyl chitosan



4.7.3.3 ^{1}H NMR Characterization of the *N*-aliphatic and Benzoyl Acyl Chitosan Derivatives

^{1}H NMR data were obtained for qualitative analysis of the macromolecular structure and for quantitative determination of the % ESs of the derivatives. A representative proton NMR spectrum of one of the *N*-aliphatic acyl derivatives (*N*-propionoyl chitosan) in D_2O/d_4-CD_3COOD is shown in Figure 4.11. Before assigning the chemical shifts of the substituent protons, it is first important to note the change in the appearance of the glucosamine backbone protons of *N*-propionoyl chitosan when compared to chitosan. This observable difference may be due to intermolecular hydrogen bonding between the carbonyl groups of the *N*-acyl substituents and the backbone hydroxyl groups of chitosan. The chemical shift at 2.21 ppm corresponds to -CO-C$\underline{H}_2$-CH_3. Slightly upfield is the chemical shift assigned to the methyl protons (-CO-CH_2-CH_3), at 0.99 ppm. The % ES was found to be 47%, by applying Equation 4.2.

Equation 4.2:

$$\left[\left(\frac{\text{integral of substituent protons}}{(1/(\#\text{ of glucosamine protons}))\quad x\quad \#\text{ of protons of substituent}}\right)\right] X\ 100$$

$$= \left[\left(\frac{0.2821}{(1/5)\quad x\ \ 3\ (-CH_3)}\right)\right] X\ 100$$

$$= 47\ \%\ ES$$

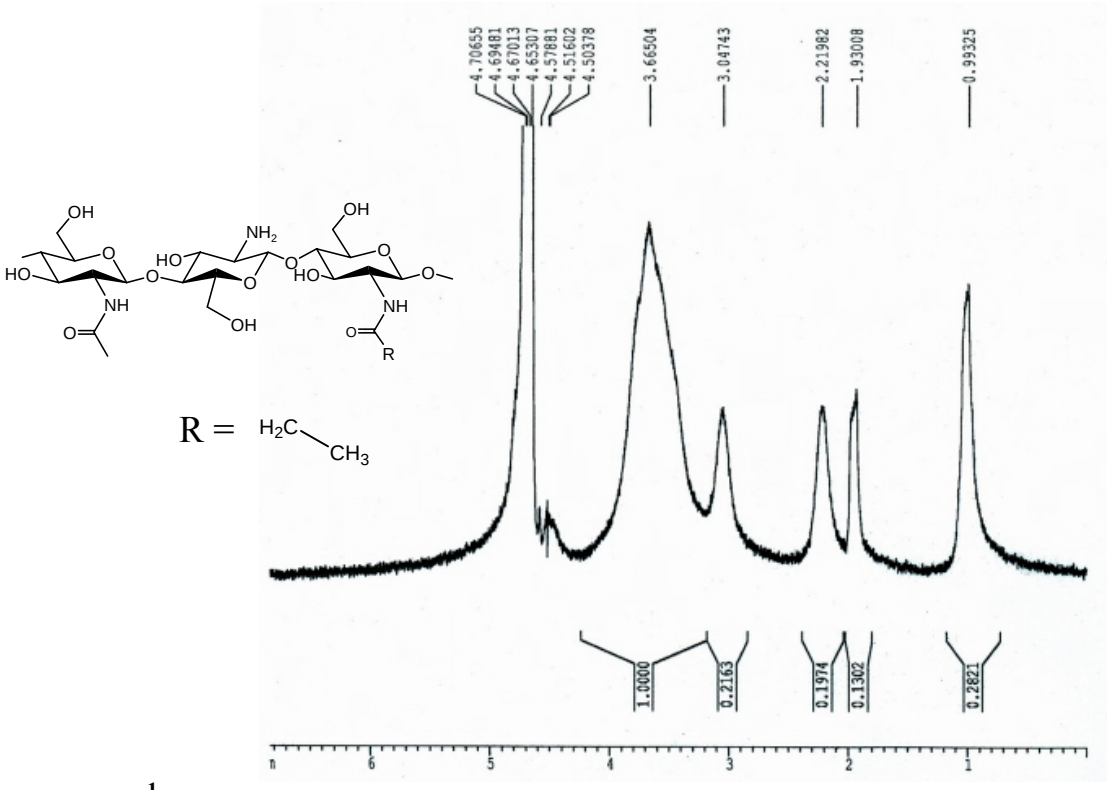


Figure 4.11 ^{1}H NMR of *N*-propionoyl (47% ES) chitosan in D_2O/d_4-CD_3COOD.

The proton NMR spectrum of benzoyl chitosan in D_2O/d_4-CD_3COOD is shown in Figure 4.12. The aromatic substituents were advantageous in determining the ES because their proton resonances appeared downfield (with respect to the backbone protons), which allowed better integration of the peaks with minimum interference. The chemical shifts at 7.42, 7.52, and 7.66 are assigned to the aromatic protons of the substituent. By applying Equation 4.2, the % ES was found to be 14%.

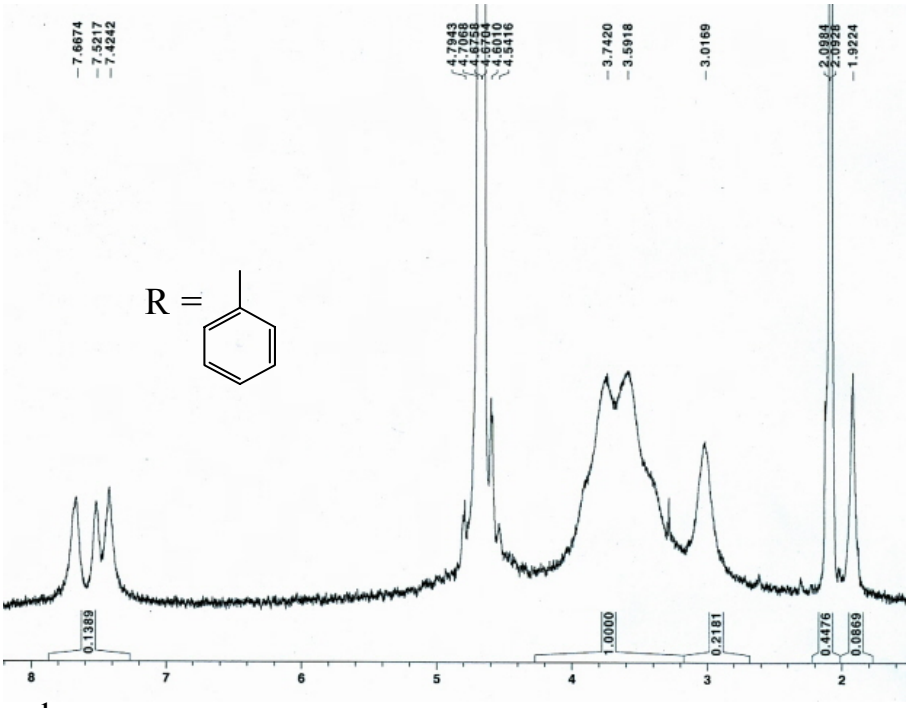


Figure 4.12 ^{1}H NMR of *N*-benzoyl (14% ES) chitosan in D_2O/d_4-CD_3COOD

4.7.3.4 % ES of the *N*-aliphatic and Benzoyl Acyl Chitosan Derivatives

The % ES targeted and the % ES obtained for the *N*-aliphatic and benzoyl acyl derivatives are shown in Table 4.1. Most of the reactions between chitosan and the aliphatic anhydrides resulted in derivatives with % ES values nearly equal to or greater than half their targeted % ESs. The % ES values obtained exploit the high reactivity of the acyclic anhydrides towards nucleophilic attack by the *N*-amino functional groups of chitosan.[9] In some cases, the % ES computed using the ^{1}H NMR data was higher than the targeted % ES. This may be due to an integration of the protons of starting material (un-reacted anhydride) or the aliphatic acid protons (Scheme 4.2, (E)) along with the protons of the *N*-acyl substituent.

The % ES values obtained for *N*-valeroyl chitosan were all much lower than the targeted % ESs. These results suggest that valeric anhydride is not very reactive towards chitosan, despite the anhydride's structural similarity to the other linear aliphatic

anhydrides. The reaction of chitosan with benzoic anhydride resulted in derivatives with % ES values close to their targeted % ESs. Despite the large size of benzoic anhydride (compared to the linear aliphatic anhydrides), the compound did not present sterics to the approaching *N*-amino groups of chitosan because like the linear aliphatic anhydrides, it can rotate itself about its carbon-oxygen single bond in a way to accommodate the nucleophile (Figure 4.13).

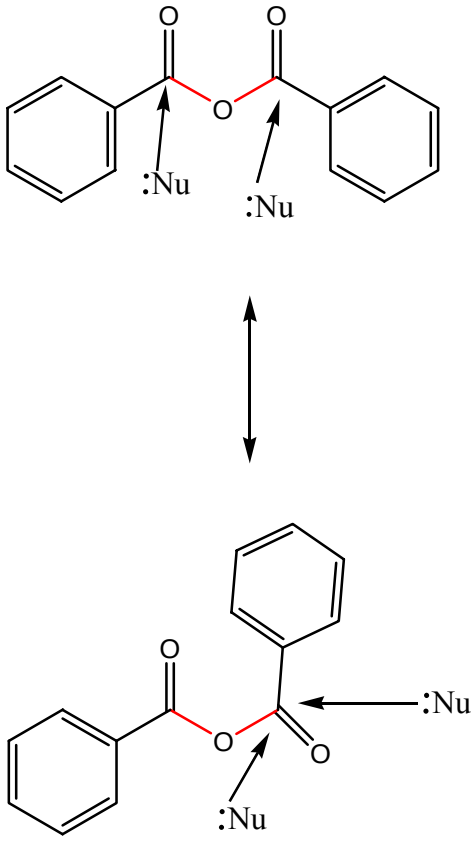


Figure 4.13 Free rotation of benzoic anhydride

4.7.3.5 Effects of ES and Structure on the Solubility of the *N*-aliphatic and Benzoyl Acyl Chitosan Derivatives

Homogenous experimental conditions were employed in the reactions to promote a random distribution of the substituents along the chains of chitosan, which is known to increase the solubility of the polymer in water.[10,11] The solubility of the *N*-aliphatic and

benzoyl acyl derivatives in water is shown in Table 4.7. For qualitative analysis of their solubilization in water and 1 % acetic acid, 10 mg of each derivative was dissolved in 1 mL of water or 1 mL of 1% acetic acid. The results in Table 4.7 show that the derivatives displayed different solubilities in the aqueous mediums. The solubility was designated +++ for the derivatives that exhibited complete aqueous solubility, ++ for derivatives that were nearly completely aqueous soluble, + for derivatives that showed partial solubility and insolubility in the aqueous medium, - for derivatives that exhibited very little to almost no solubility in the medium, and -- for derivatives that were completely aqueous insoluble.

The results in Table 4.7 imply that the solubility is affected by the % ES and/or structure (chain-length) of the aliphatic substituent.[9] For example, with respect to the % ES on the solubility, *N*-propionoyl chitosan substituted at 21% exhibited swelling in water (enhanced solubility), but was completely insoluble when substituted at 47% ES. With respect to the structure on the solubility, *N*-propionoyl chitosan at 21% ES swells in water, whereas *N*-hexanoyl chitosan substituted at 21% ES exhibits low swelling in water.

The solubility of *N*-benzoyl chitosan in water was mainly affected by the % ES. *N*-benzoyl chitosan substituted at 14% ES exhibited swelling in water. A 16% increase in the ES (from 14-30% ES) resulted in a product with low water solubility. Beyond this % ES, benzoyl chitosan was insoluble in water.

The solubility of the *N*-aliphatic and benzoyl acyl derivatives in 1% acetic acid is shown in Table 4.7. Most of the derivatives were completely soluble in the acidic medium. The derivatives that were insoluble in water (due to their high extent of hydrophobicity) showed partial solubility in acetic acid. The solubility in the acidic

environment would be caused by ionization of the $-NH_2$ groups to $-NH_3^+$ ions. The partial solubility of the derivatives with high ESs was attributed to a decrease in the availability of the *N*-amino groups that can be converted into $-NH_3^+$ groups and also due to a high increase in the hydrophobic character of the polymer.

Table 4.7 Solubility of *N*-aliphatic and benzoyl acyl chitosan in water and 1% acetic acid (AcOH)

Name of Derivative	%ES	solubility in water before Quaternization (10 mg/mL)	solubility in 1% AcOH before Quaternization (10 mg/mL)
N-propionoyl chitosan	21 47 45	++ + -	+++ +++ +++
N-butyroyl chitosan	34 41 30	+ - -	+++ +++ +++
N-valeroyl chitosan	8 0 11	+ - -	+++ +++ +++
N-hexanoyl chitosan	13 18 21	+ + -	+++ +++ +++
N-heptanoyl chitosan	23 31 50	- - --	+++ +++ ++
N-benzoyl chitosan	14 30 50	++ - -	+++ ++ ++

+++ = completely soluble
++ = high aqueous enhanced, but not completely soluble
\+ = moderate aqueous enhanced
\- = low aqueous enhanced
-- = insoluble

4.7.4 Mechanistic Route Towards the Synthesis of the *N*-ionic Acyl Chitosan Derivatives

The mechanism of the reaction between chitosan and the cyclic anhydrides is shown in Scheme 4.3. For the 5-member ring cyclic anhydrides, the rate-determining step for the forward reaction may involve the breakdown of the tetrahedral intermediate (B) formed by the intramolecular attack of the hydroxyl oxygen on the anhydride.[6-8] This ring-opening step relieves the anhydride of ring strain. In the case with 3,3-dimethyl glutaric anhydride, nucleophilic attack of the anhydride by chitosan may have been hindered due to the arrangement of the axial geminal substituent (Figure 4.24).

4.7.4.1 Experimental Preparation of the *N*-ionic Acyl Derivatives

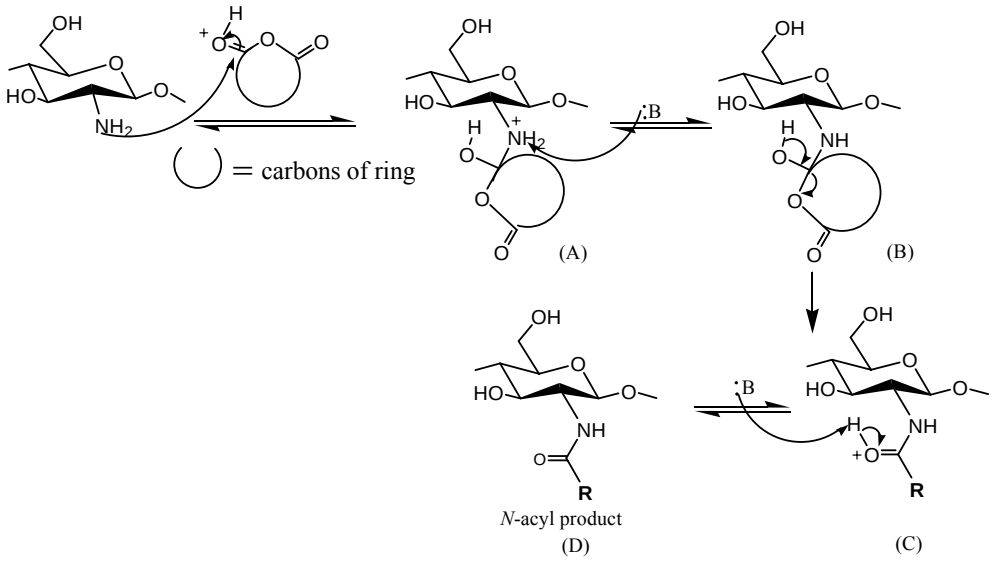
Prior to precipitation, the products from the reactions of chitosan with the cyclic anhydrides appeared as white viscous solutions. Following precipitation, the products appeared as white gels. The products were filtered and washed with copious amounts of acetone to remove organic reagents and by-products. In addition to removing undesirable material, the use of excess acetone changed the texture and appearance of the product, when compared to using smaller amounts of acetone. When less acetone was used, the products appeared as hard, rough intractable solids that were difficult to grind into fine powders. When more acetone was used, the products appeared as fibrous solids that were easy to grind into powders. It is believed that the excess acetone possibly removed water molecules trapped between the chains of the derivatives, thus affecting inter-chain aggregation.

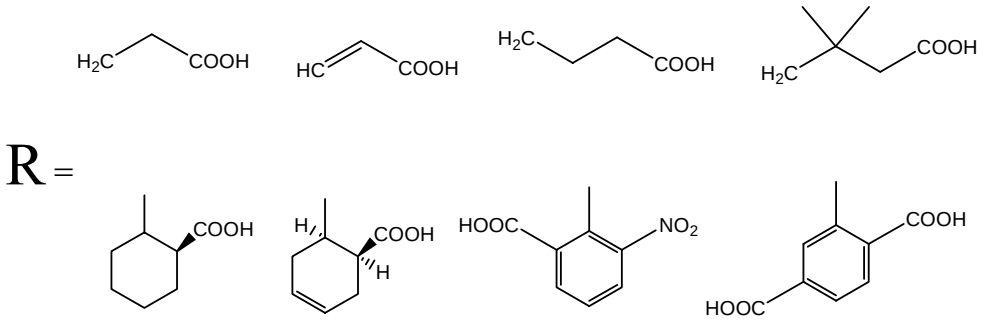
4.7.4.2 FT-IR Spectroscopy of the *N*-ionic Acyl Derivatives

FT-IR data were obtained for the *N*-ionic acyl derivatives to rule out the possibility of imide formation and thus confirm the arrangement of the substituent. Imides give absorption bands in the range of 1775 and 1720 cm^{-1}, due to the

asymmetrical and symmetrical stretch of the C=O functional group, respectively. The absence of the imide absorption in each FT-IR spectrum confirmed *N*-acyl bond formation (-HN-CO-R), versus imide bond formation (O=C-NR-C=O). As observed in the FT-IR spectra of the *N*-aliphatic and benzoyl acyl derivatives, the NH bend of the *N*-ionic derivatives shifted to a lower frequency, indicating *N*-acyl bond or product formation. Two representative FT-IR spectra are shown in Figures 4.14 and 4.15. The absorptions corresponding to the functional groups of each derivative are summarized immediately following the FT-IR spectrum of the appropriate derivative.

Scheme 4.3 Mechanistic route towards the synthesis of *N*-ionic acyl chitosan





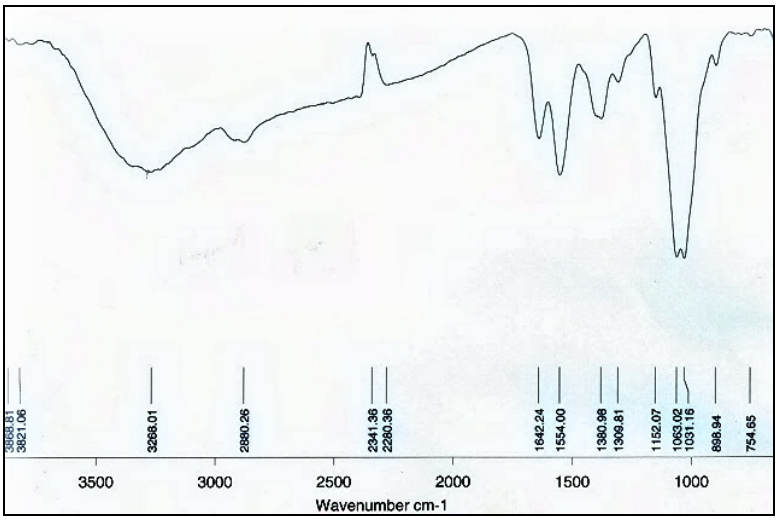


Figure 4.14 FT-IR of *N*-succinoyl chitosan

FT-IR data: NH bend 1554 cm^{-1}; C=O absorption of *N*-acyl 1642 cm^{-1} (overlaps with C=O of acid); aliphatic methylene CH stretch 2880 cm^{-1}.

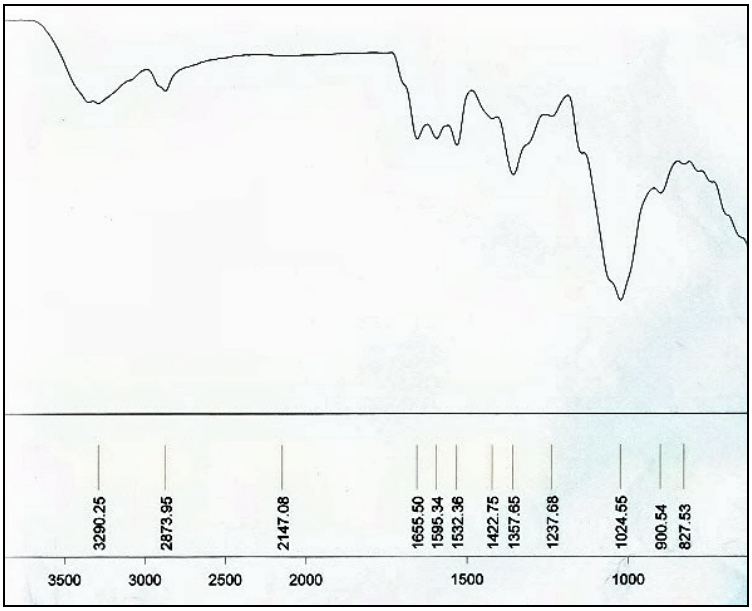


Figure 4.15 FT-IR of *N*-3NO_2-phthaloyl chitosan

FT-IR data: NH bend 1532 cm^{-1}; C=O absorption of *N*-acyl 1655 cm^{-1} (overlaps with C=O of acid); N=O stretch (of nitro group) 1595 cm^{-1}; C=C ring stretch 1422 cm^{-1}; =CH stretch to the left of 3000 cm^{-1}; =CH out-of-plane (oop) bending 900-690

4.7.4.3 ^{1}H NMR Characterization of the *N*-ionic Acyl Chitosan Derivatives

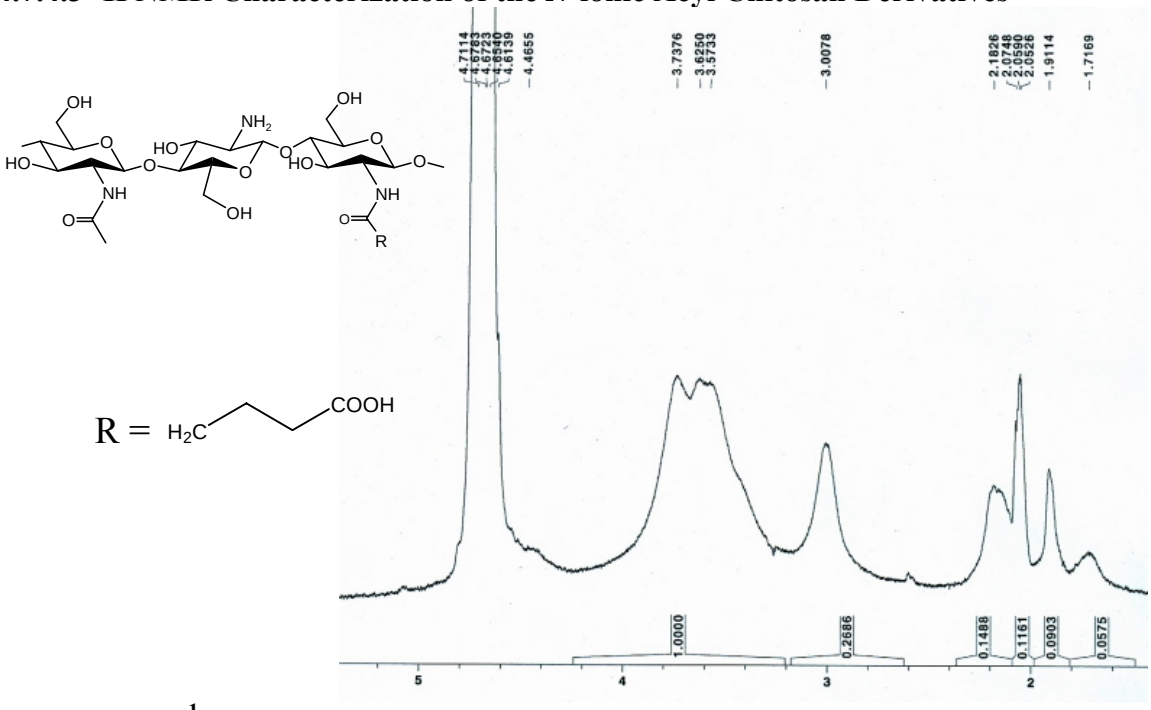


Figure 4.16 ^{1}H NMR of *N*-glutaroyl (14% ES) chitosan in D_2O/d_4-CD_3COOD

^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 1.71 (2H, -CH_2-C<u>H</u>$_2$-CH_2), 2.07 (2H, -CO-C<u>H</u>$_2$-CH_2-) 2.18 (2H, -C<u>H</u>$_2$-COOH), % ES = 14%.

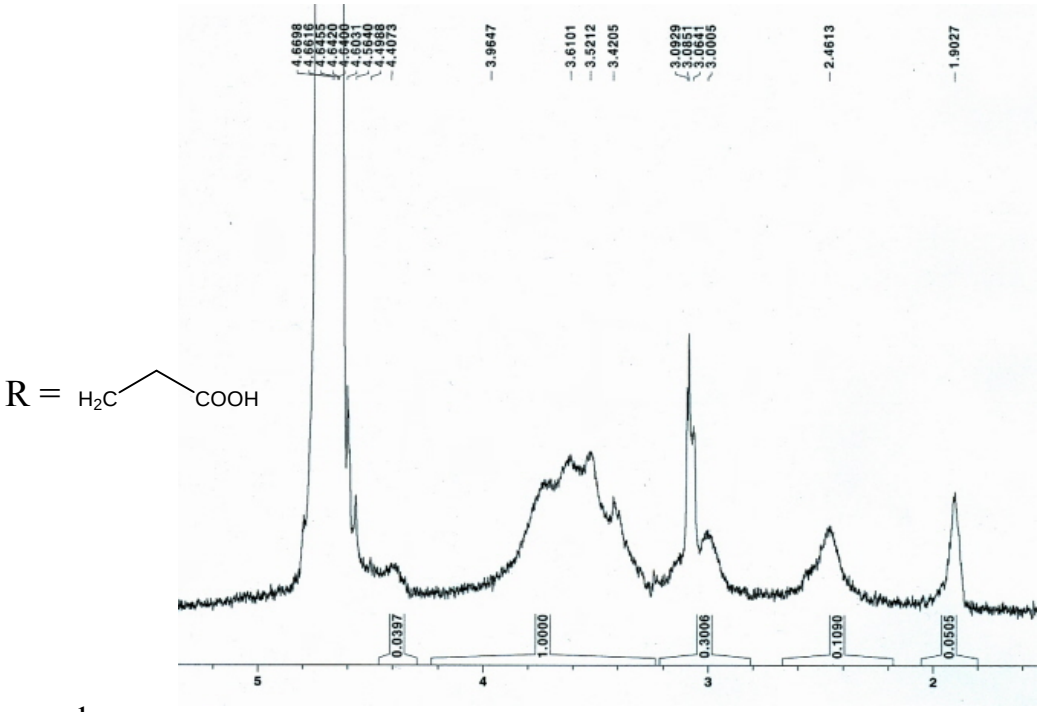


Figure 4.17 ^{1}H NMR of *N*-succinoyl (14% ES) chitosan in D_2O/d_4-CD_3COOD

^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 2.46 (4H, -C<u>H</u>$_2$-C<u>H</u>$_2$-), % ES = 14%.

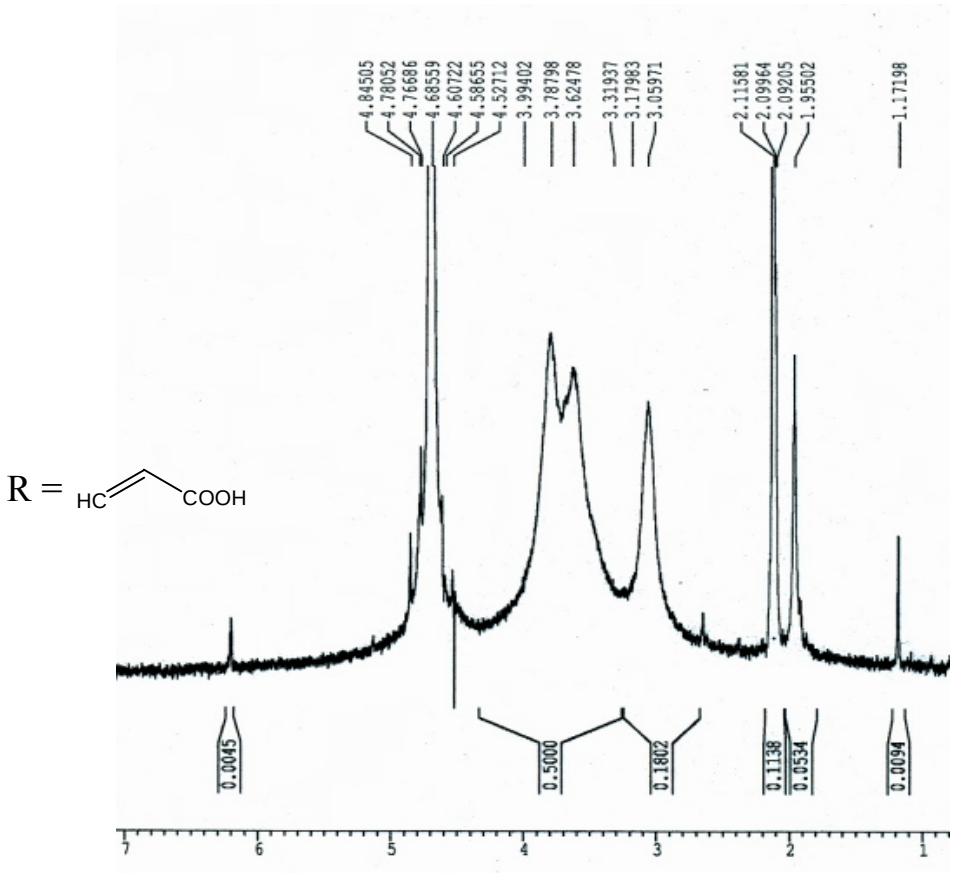


Figure 4.18 ^{1}H NMR of *N*-maleoyl (2% ES) chitosan in D_2O/d_4-CD_3COOD.

^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 6.14 (2H, -CH=CH-), % ES = 2%.

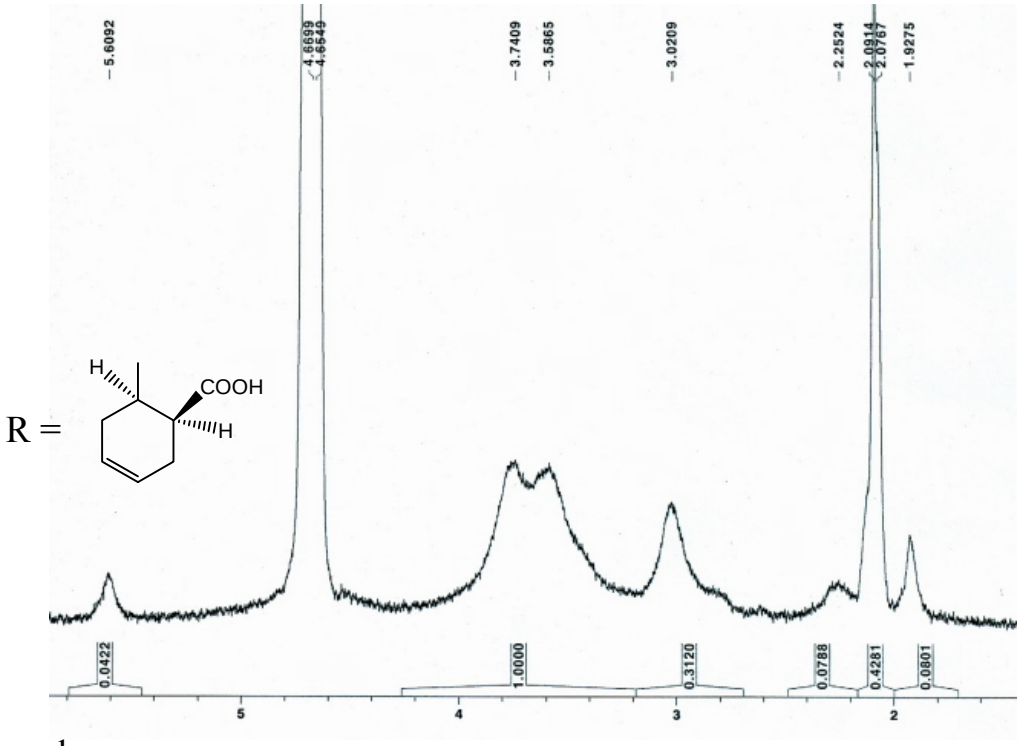


Figure 4.19 ^{1}H NMR of *N*-cis-1,2,3,6 tetra hydrophthaloyl (11% ES) chitosan in D_2O/d_4-CD_3COOD

^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 2.25 (2H, -C<u>H</u>-CH_2-), 2.25(4H, C<u>H</u>$_2$-CH=), 5.60 (2H, vinyl -C<u>H</u>=C<u>H</u>), % ES = 11%.

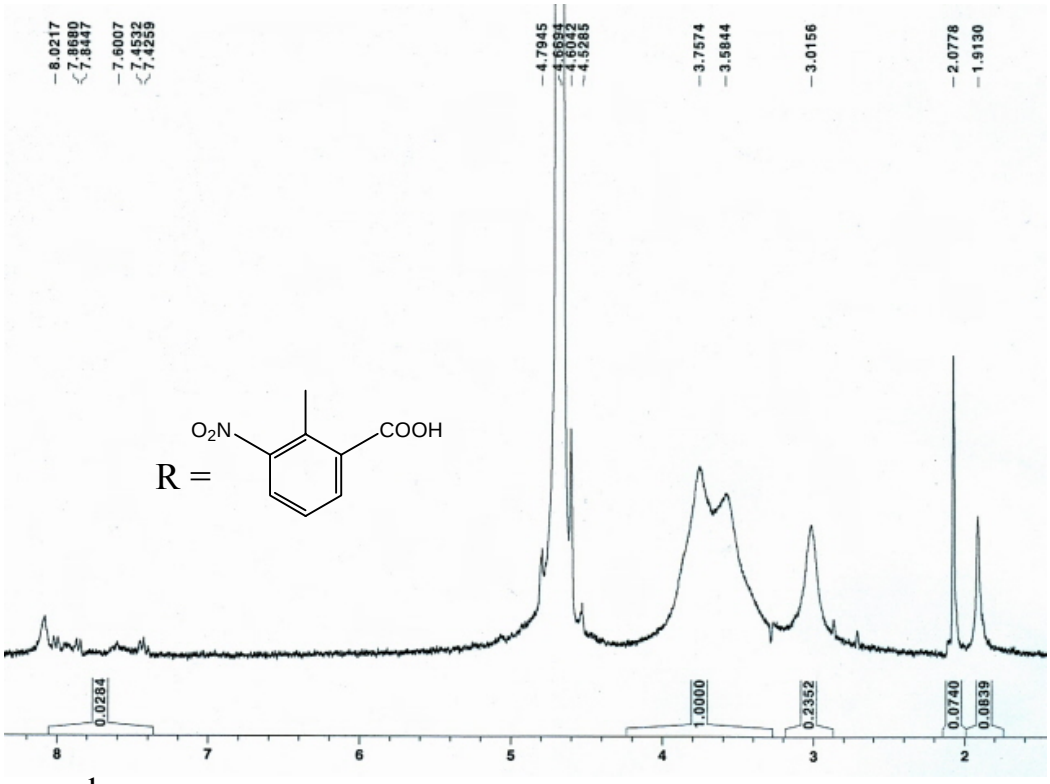


Figure 4.20 ^{1}H NMR of *N*-3NO_2 phthaloyl (5% ES) chitosan in D_2O/d_4-CD_3COOD

^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 7.45 (1H, arom -C<u>H</u>), 7.84 (1H, arom -C<u>H</u>), 7.86 (1H, arom -C<u>H</u>), % ES = 5%.

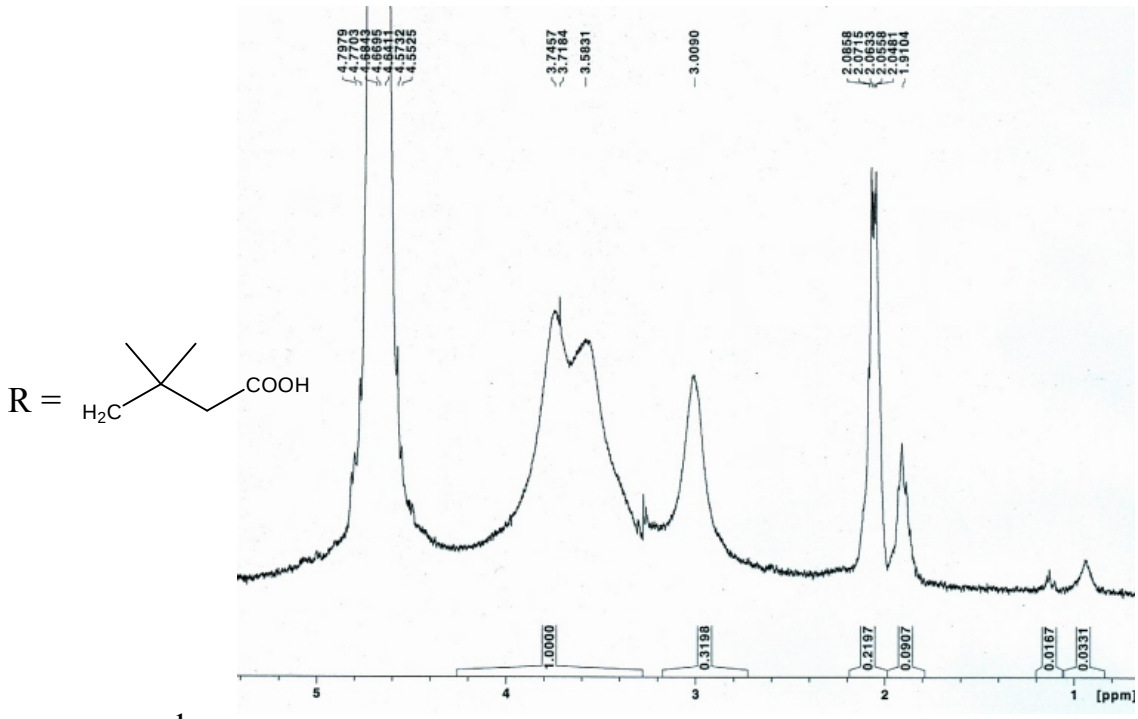


Figure 4.21 ^{1}H NMR of *N*-3,3 dimethyl glutaroyl (3% ES) chitosan in D_2O/d_4-CD_3COOD

^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 1.73 (6H, geminal -C<u>H</u>$_3$), 2.07 (2H, –C<u>H</u>$_2$-COOH) 2.08 (2H, –C<u>H</u>$_2$-CONH-), % ES = 3%.

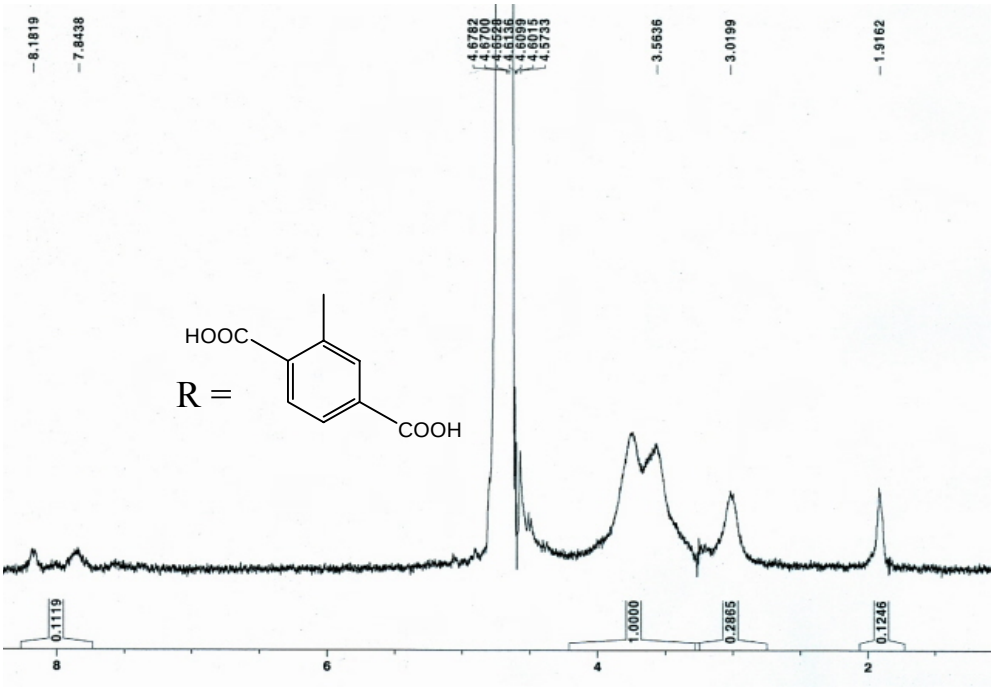


Figure 4.22 ^{1}H NMR of *N*-trimellitoyl (19% ES) chitosan in D_2O/d_4-CD_3COOD

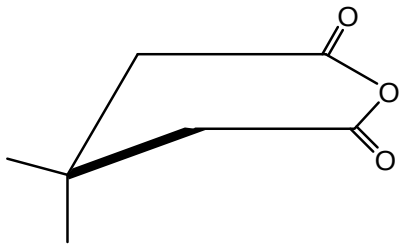
^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 7.84 (2H, arom -C<u>H</u>), 8.18 (1H, arom -C<u>H</u>), % ES = 19%.

A representative ^{1}H NMR spectrum of each type of *N*-ionic acyl derivative in D_2O/d_4-CD_3COOD is shown in Figures 4.16 through 4.22. Their % ES values were obtained by applying Equation 4.2. The chemical shifts corresponding to the substituent protons are summarized immediately following the ^{1}H NMR spectrum of the appropriate *N*-acyl derivative.

4.7.4.4 % ES of the *N*-ionic Acyl Chitosan Derivatives

The % ESs obtained from the reactions of chitosan with the cyclic anhydrides were much lower than their targeted % ESs, as shown in Table 4.2. This can be attributed to the molecular structure and/or size of the anhydrides, which reflected their reactivity towards nucleophilic attack by chitosan. In interpreting the % ES obtained for *N*-3,3-dimethyl glutaroyl chitosan, the low % ES can be explained in terms of the conformation of the anhydride, which closely resembles the chair conformation of

cyclohexane (Figure 4.23). In this conformation, the arrangement of the axial geminal substituent presents some sterics to the approaching *N*-amino groups of chitosan (Figure 4.24).



3,3-dimethyl glutaric anhydride

Figure 4.23 Conformation of 3,3-dimethyl glutaric anhydride

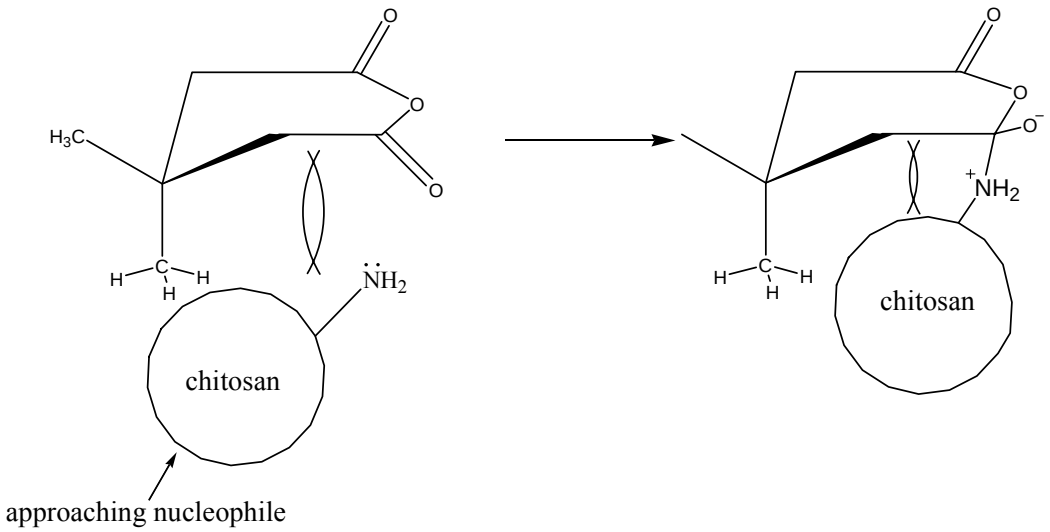
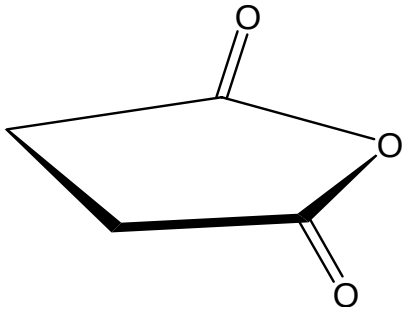


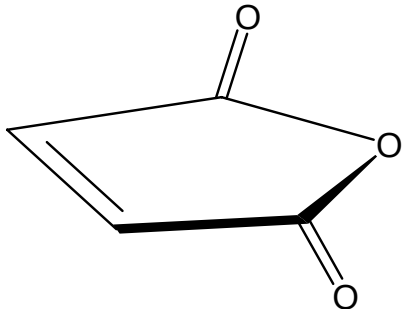
Figure 4.24 Nucleophilic attack of 3,3-dimethyl glutaric anhydride by the *N*-amino groups of chitosan

Chitosan was then allowed to react with glutaric anhydride to determine if absence of the geminal substituents would lead to derivatives with higher extents of glutaroyl substitution. The resulting glutaroyl chitosan derivatives however, had % ESs that were less than half their targeted % ESs as shown in Table 4.2 (although higher than the % ES values for 3,3-dimethyl glutaroyl chitosan). These results imply that the low % ES obtained is mainly attributed to the low reactivity of the anhydride.

Chitosan's reaction with succinic anhydride (Figure 4.25) resulted in a derivative with half its targeted % ES. The % ES obtained is a reflection of the high reactivity of succinic anhydride. When compared to the reactivity of 6-member ring anhydrides, the higher reactivity of 5-member ring anhydrides is due to the degree of ring strain in the molecule.



succinic anhydride



maleic anhydrie

Figure 4.25 Conformations of succinic and maleic anhydride

Chitosan's reactivity with maleic anhydride (Figure 4.25) resulted in derivative with 2% ES out of 65% targeted ES. Although the anhydride has some degree of ring strain, the vinyl functional group decreases electrophilicity at the carbonyl centers, thus decreasing its reactivity. Also, nucleophilic attack of maleic anhydride would disrupt the delocalization of the π-system of the molecule, which is disfavorable.

The % ESs obtained from the reactions of chitosan with *cis*-1,2 cyclohexanedicarboxylic, *cis*-1,2,3,6-tetrahydrophthalic, 3-nitro phthalic, and 1,2,4-benzenetricarboxylic (trimellitic) anhydride are shown in Table 4.2. The reactions yielded products with % ESs much lower than their targeted % ESs. The bulky sizes of the anhydrides were believed to have affected the % ESs obtained. Although the anhydrides comprise 5-member rings which make them reactive, their large sizes presented steric hindrance to the approaching *N*-amino groups of chitosan.[12]

4.7.4.5 Effects of the ES and Structure on the Solubility of the *N*-ionic Acyl Derivatives

While *N*-succinoyl chitosan exhibited swelling in water, *N*-maleoyl chitosan and the *N*-glutaroyl chitosan derivatives exhibited very little swelling. The % ESs obtained with *N*-maleoyl and *N*-glutaroyl chitosan may have led to insufficient disruption or minimal reduction in the normal regularity of intermolecular H-bonding between the polymer chains,[13] resulting in products with low water solubility. Despite the low % ESs obtained for *N*-3,3-dimethyl glutaroyl, *N*-cis-1,2 cyclohexanedicarboxoyl, *N*-cis 1,2,3,6-tetrahydrophthaloyl, *N*-3-NO_2 phthaloyl and *N*-trimellitoyl chitosan, the derivatives showed swelling in water. A possible explanation for the enhanced solubility of these derivatives could be attributed to the size and/or bulkiness of the *N*-acyl substituent. As mentioned in chapter 2, the modification of chitosan with bulky groups enhances the polymer's solubility in water due to the substituents' ability to create a large distance between the polymer chains, thus allowing water molecules to fill in the spaces created. This causes the polymer to exhibit greater enhancement in water. All of the *N*-ionic acyl derivatives displayed good solubility in 1% acetic acid (Table 4.8). The solubility in the acidic environment would be caused by ionization of the $-NH_2$ groups to $-NH_3^+$.

Table 4.8 Solubility of *N*-ionic acyl chitosan in water and 1% AcOH

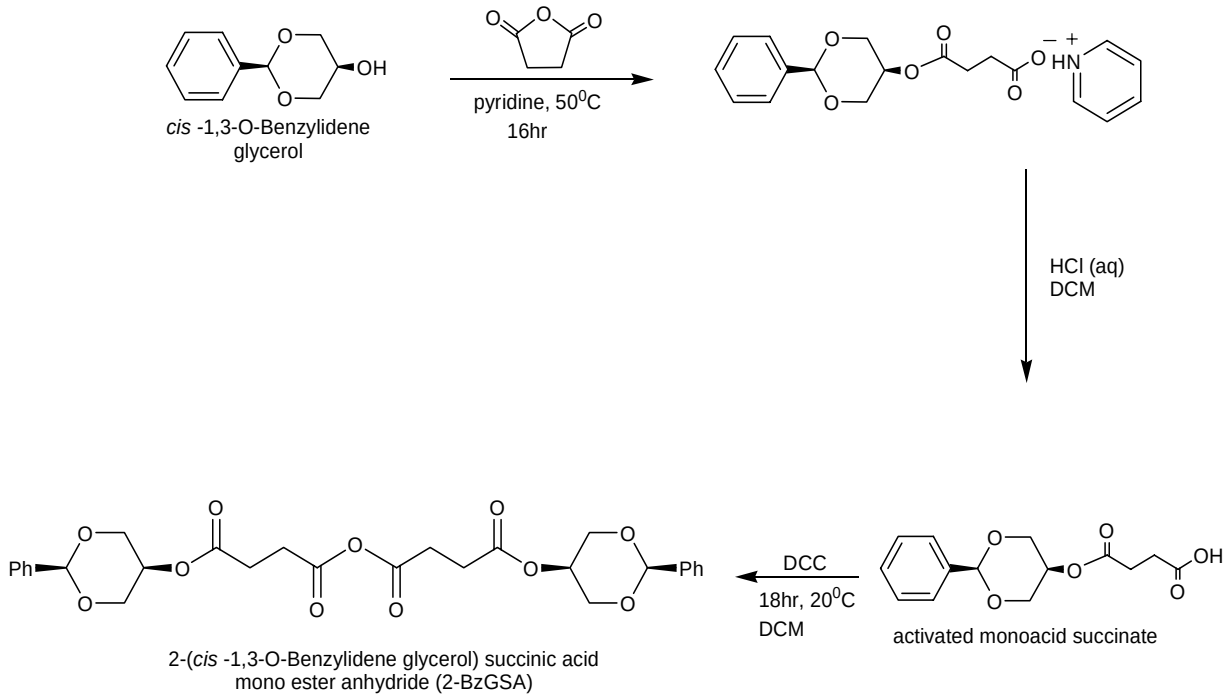
Name of derivative	% ES	solubility in water before Quaternization (10 mg/mL)	solubility in 1% AcOH before Quaternization (10 mg/mL)
N-succinoyl chitosan (1)	14	+	+++
N-maleoyl chitosan (2)	2	-	+++
N-glutaroyl chitosan (3)	5 10 14	- - -	+++ +++ +++
N-3,3-dimethyl glutaroyl chitosan (4)	10 3 6	+ + +	+++ +++ +++
N-cis-1,2 cyclohexanedicarboxoyl chitosan (5)	6 0	+ --	+++ +++
N-cis 1,2,3,6-tetrahydrophthaloyl chitosan (6)	7 11 20	++ ++ -	+++ +++ +++
N-3-NO_2 phthaloyl chitosan (7)	5 9 n/a	++ ++ ++	+++ +++ +++
N-trimellitoyl chitosan (8)	19	+	+++

+++ = completely soluble
++ = high aqueous enhanced, but not completely soluble
+ = moderate aqueous enhanced
- = low aqueous enhanced
-- = insoluble

4.7.5 The Preparation of 2-Glycerol Succinamide and Glutamide Chitosan

The reaction between *cis*-1,3-*O*-benzylidene glycerol and succinic or glutaric anhydride yielded the novel anhydrides 2-(*cis* -1,3-O-benzylidene glycerol) succinic acid mono ester anhydride (2-BzGSA) or 2-(*cis* -1,3-O-benzylidene glycerol) glutaric acid mono ester anhydride (2-BzGGA), respectively. A representative reaction is shown in Scheme 4.4. The reaction of *cis*-1,3-*O*-benzylidene glycerol with succinic anhydride yielded the pyridinium salt intermediate. Following the acidic workup, the activated monoacid succinate was isolated. This product was fully characterized by ^{1}H and ^{13}C NMR as shown in Figures 4.26 and 4.27, respectively, using $CDCl_3$ as the internal standard. The activated monoacid succinate was then converted to the anhydride using the procedure reported by Carnahan et al.[5] Spectral data for the anhydride were consistent with those reported.

Scheme 4.4 Synthesis of 2-(*cis*-1,3-O-benzylidene glycerol) succinic acid mono ester anhydride (2-BzGSA).



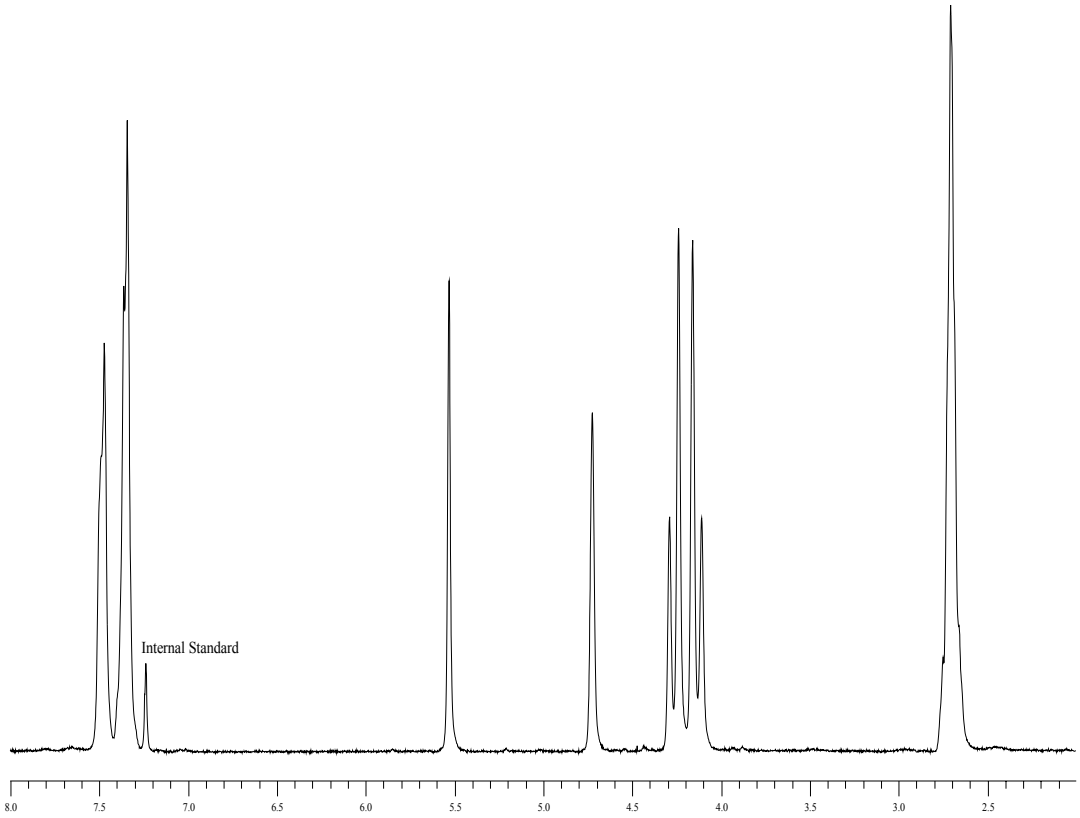


Figure 4.26 ^{1}H NMR of the monoacid succinate in $CDCl_3$

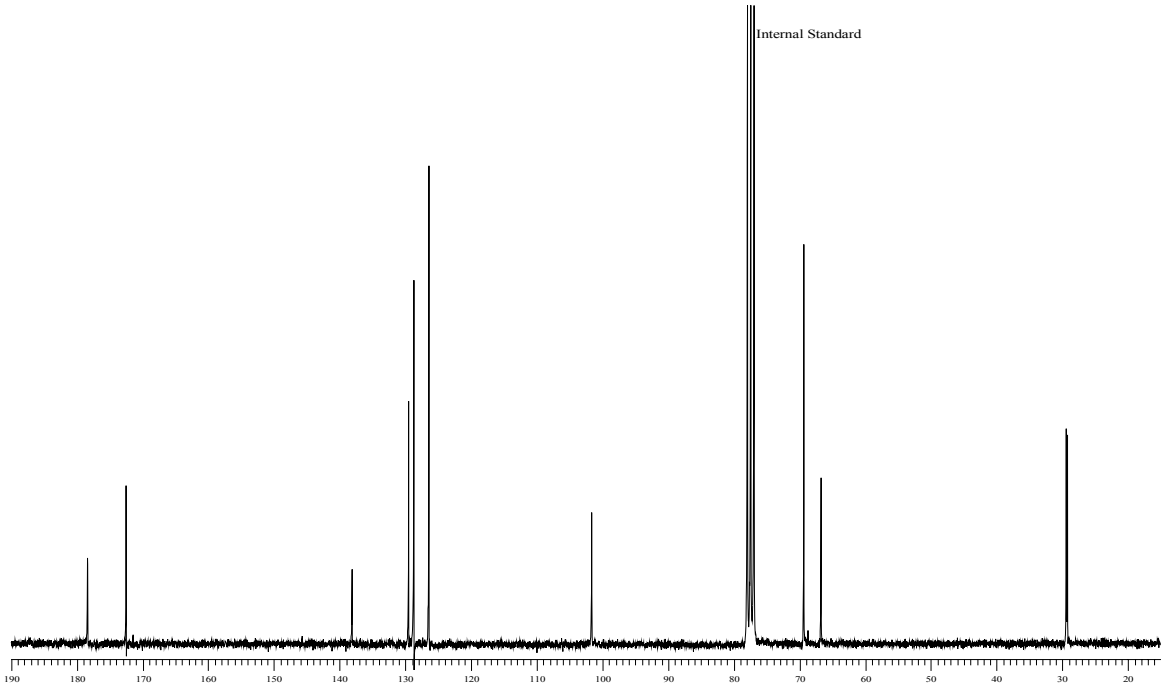


Figure 4.27 ^{13}C NMR of the monoacid succinate in $CDCl_3$

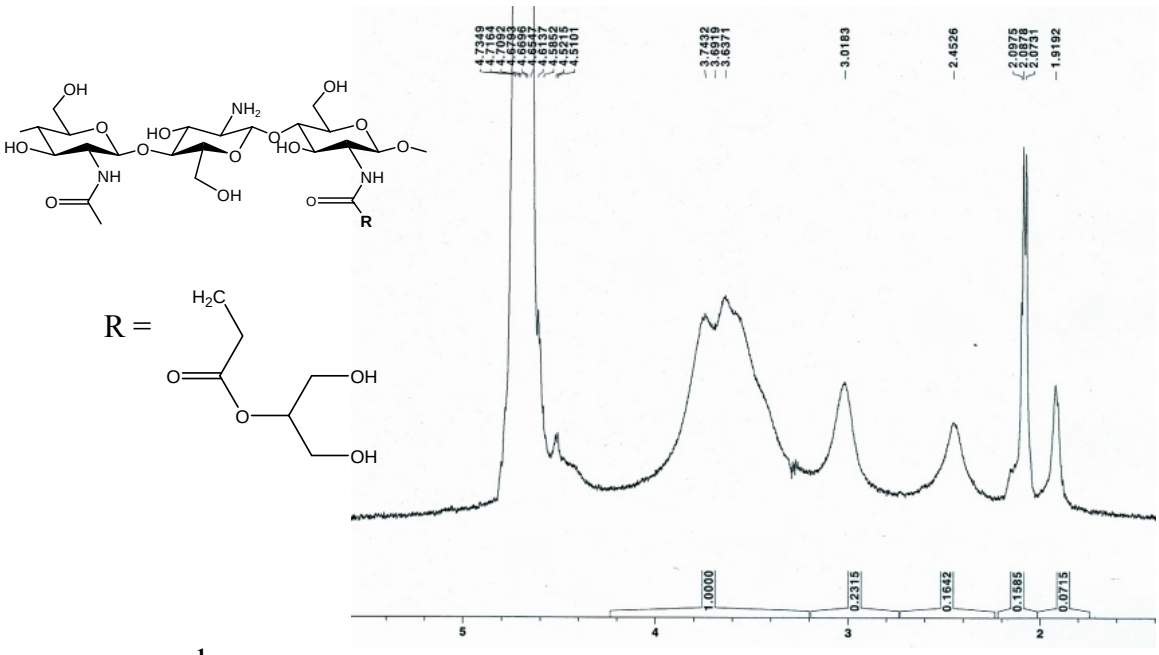


Figure 4.28 ^{1}H NMR of 2-glycerol succinamide (20% ES) chitosan in D_2O/d_4-CD_3COOD

^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 2.45 (2H, -HNCO-C<u>H</u>$_2$-CH_2-), 2.45 (2H, -CH_2-C<u>H</u>$_2$-COO-), 3.63 (4H,-C<u>H</u>$_2$-CH-C<u>H</u>$_2$-), 4.52 (1H, -CH_2-C<u>H</u>-CH_2-), % ES = 25%.

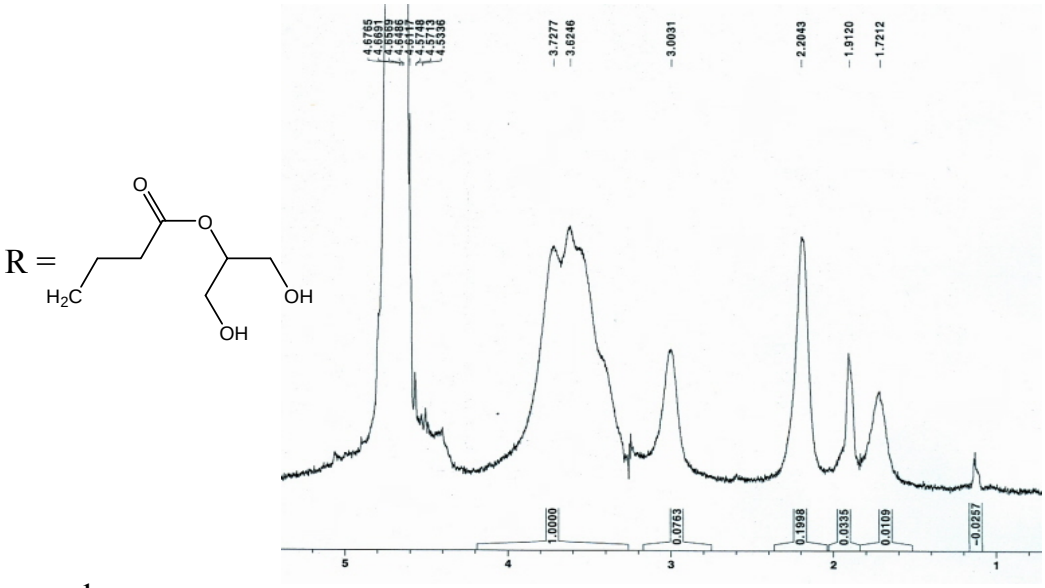


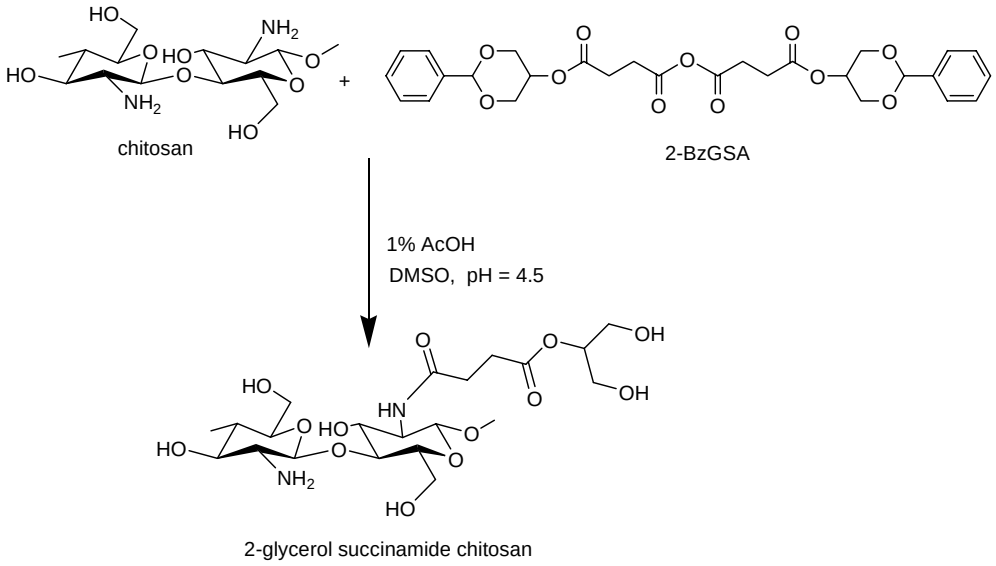
Figure 4.29 ^{1}H NMR of 2-glycerol glutamide (25% ES) chitosan in D_2O/d_4-CD_3COOD

^{1}H NMR data (D_2O/d_4-CD_3COOD): δ (ppm) 1.72 (2H, -CH_2-C<u>H</u>$_2$-CH_2), 2.20 (2H, -HNCO-C<u>H</u>$_2$-CH_2-), 2.20 (2H, -CH_2-C<u>H</u>$_2$-COO-), 3.61 (4H,-C<u>H</u>$_2$-CH-C<u>H</u>$_2$-), 4.53 (1H, -CH_2-C<u>H</u>-CH_2-), % ES = 25%.

4.7.5.1 Mechanistic Route Towards the Synthesis of 2-Glycerol Succinamide and Glutamide Chitosan

Scheme 4.5 shows the synthetic route towards the *N*-acylation of chitosan with 2-BzGSA. The reaction was conducted in a solvent mixture of acetic acid and DMSO due to the limited solubility of 2-BzGSA in the medium. Reaction with the anhydride is accompanied by removal of the benzylidene blocking group, which ultimately places aliphatic hydroxy substituents pendant to the polymer backbone, i.e., 2-glycerol succinamide chitosan.

Scheme 4.5 Synthesis of 2-glycerol succinamide chitosan



4.7.5.2 ^{1}H NMR Analysis of 2-Glycerol Succinamide and Glutamide Chitosan

A representative proton NMR of 2-glycerol succinamide chitosan in D_2O/d_4-CD_3COOD is shown in Figure 4.28. The appearance of the peak at 2.45 ppm corresponds to the two glyceryl α-protons of the substituent. The chemical shift of the α-hydroxy protons (3.63 ppm, $C\underline{H}_2$-CH-$C\underline{H}_2$) overlaps the backbone protons of chitosan. The CH_2-$C\underline{H}$-CH_2 proton appears slightly downfield at 4.52 ppm, overlapping the acetal backbone protons and the chemical shift of the internal standard. The absence of the

aromatic protons of the anhydride confirms concomitant hydrolysis of the benzyl acetal under the acidic experimental conditions used for the *N*-acylation. The % ES was found to be 20%, by applying Equation 4.2.

The % ESs of 2-glycerol succinamide and glutamide chitosan are summarized in Table 4.3. The reaction between chitosan and the novel anhydrides resulted in derivatives with nearly half or close to their exact targeted % ESs.

4.7.5.3 Effects of the ES and Structure on the Solubility of 2-Glycerol Succinamide and Glutamide Chitosan

Table 4.9 Solubility of 2-glycerol succinamide and glutamide chitosan in water and 1% AcOH

Name of derivative	% ES	solubility in water before Quaternization (10 mg/mL)	solubility in 1% AcOH before Quaternization (10 mg/mL)
2-glycerol succinamide chitosan	11 20 28	++ + +	+++ +++ +++
2-glycerol *glutamide* chitosan	5 5 25	+ + +	+++ +++ +++

+++ = completely soluble
++ = high aqueous enhanced, but not completely soluble
+ = moderate aqueous enhanced

The solubility of chitosan can be enhanced by incorporating OH-groups pendant to the polymer backbone.[14] The reaction of chitosan with 2-(*cis* -1,3-O-benzylidene glycerol) succinic acid mono ester anhydride and 2-(*cis* -1,3-O-benzylidene glycerol) glutaric acid mono ester anhydride resulted in chitosan derivatives with enhanced solubility at neutral pH (Table 4.9). Upon increasing the % ES, the solubility of 2-glycerol succinamide and glutamide chitosan was decreased when tested in water, despite the increase in the number of hydrophilic groups on the polymer backbone. The decrease

in the solubility was probably a result of an increase in the hydrophobicity of polymer, which dominated the hydroxyl groups (of the substituent) affinity for water.

4.7.6 Quaternization of the *N*-aliphatic, -Benzoyl, -Ionic, 2-Glycerol Succinamide, and Glutamide Acyl Chitosan Derivatives

Quaternization of the *N*-acyl derivatives was conducted under heterogeneous conditions using a commercially available solution of 65% (w/w) Quat-188 (Scheme 4.1). The chlorohydrin was probably converted to the corresponding epoxide *in situ* under the alkaline conditions employed. A catalytic amount of I_2 was added to enhance the electrophilicity of the epoxide[15] towards nucleophilic attack by the amines of chitosan, which allows a greater extent of cationic substitution to occur.

A representative ^{1}H NMR spectrum of *N*-acyl quaternized chitosan is shown in Figure 4.30 (*N*-propionoyl chitosan Quat-188). The appearance of the peak at 4.16 ppm corresponds to the CH_2-C<u>H</u>-CH_2 proton of the 3-(N,N,N-trimethylammonium chloride)-2-hydroxyl substituent. The methylene protons (C<u>H</u>$_2$) of this substituent expected at 3.51 ppm and 2.80 ppm overlap the backbone protons of chitosan. The strong intense chemical shift at 3.09 ppm corresponds to the methyl protons (N^+-C<u>H</u>$_3$) adjacent to the quaternary nitrogen.

Quaternization of the *N*-acyl derivatives resulted in derivatives with partial or complete solubility in water (Tables 4.10 through 4.12). The *N*-aliphatic and benzoyl derivatives that displayed low swelling and insolubility in water before quaternization were partially soluble in the medium after they were quaternized. The *N*-aliphatic and benzoyl acyl derivatives that exhibited high and moderate swelling in water prior to quaternization exhibited complete solubility in water following quaternization. The *N*-ionic acyl derivatives that were water insoluble prior to quaternization displayed partial

solubility in water following quaternization. The *N*-ionic acyl derivatives that displayed high, moderate, and low swelling in water prior to quaternization were rendered completely water soluble after the Quat reaction, with the exception of *N*-succinoyl (14% ES) and *N*-cis 1,2,3,6-tetrahydrophthaloyl (20% ES) chitosan Quat-188. The 2-glycerol succinamide and glutamide chitosan derivatives that showed high and moderate swelling in water prior to quaternization were rendered completely water soluble after their quaternization.

N-succinoyl (14% ES) chitosan Quat-188, *N*-cis 1,2,3,6-tetrahydrophthaloyl (20% ES) chitosan Quat-188, and the partial soluble *N*-aliphatic, benzoyl and *N*-ionic quaternized derivatives exhibited partial solubility in water after quaternization. This was probably attributed to an excessive increase in the hydrophobicity of the polymers via quaternization. Although quaternization of the *N*-acyl derivatives was to increase the hydrophilic nature of the polymers, in these cases, the aliphatic portion of the Quat substituents may have additionally increased the hydrophobic character of the polymer and dominated its potential hydrophilicity.

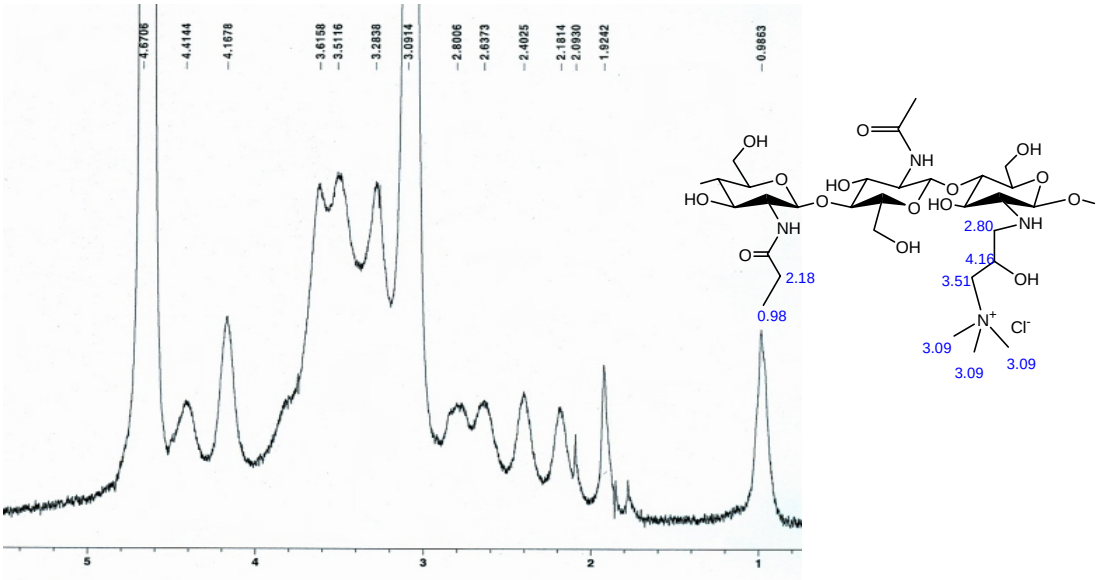


Figure 4.30 ^{1}H NMR of *N*-propionoyl chitosan Quat-188 in D_2O

4.8 Molecular Weight Determination

To determine if the molecular weight of the *N*-acyl Quat derivatives changed as a result of the quaternization step, GPC/MALS data was obtained. The results are summarized in Table 4.13. When compared to chitosan, the weight-average molecular weights (M_w) of the derivatives were much lower. The significant decrease in molecular weight is mainly attributed to the basic environment used to prepare the *N*-acyl chitosan Quat-188 derivatives (Scheme 4.1). Sannan et al. have reported that treating chitosan under alkali conditions could result in a decrease in the molecular weight.[3,10] In this case, the protocol required the reactants to be stirred for a total of 72 h, where after 48 h, the reaction was heated to 50 ^{0}C for an additional 24 h. The basic media, slightly elevated temperature, and the prolonged reaction time influenced degradation of the polymer main chain. Kurita has reported that chitosan reactions performed under basic conditions can result in reduction in molecular weight, which increases with increasing reaction time.[16]

4.9 Attempted *N*-hydroxyacylation of Chitosan

The reactions between chitosan and ε-caprolactone, δ-gluconolactone, γ-octanoyl lactone, and α,α-diphenyl γ-lactone were performed at room temperature under acidic homogenous conditions (pH 2). Following the reactions, the materials obtained were analyzed by ^{1}H NMR. Absence of the substituent protons in each of the NMR spectra indicated that chitosan was not *N*-hydroxyacylated with the lactones. The reactions were then performed at pH 6 to ensure that the *N*-amino groups were mostly in their deprotonated form. Analysis of the resulting materials showed that the modification of chitosan with the lactones was still not achieved. The reactions were attempted at pH 7 and at 70 ^{0}C, but there was no product formation. This was probably due to the high stability or low reactivity of the lactones[17] towards nucleophilic attack by chitosan.

Table 4.10 The solubility of the *N*-aliphatic and benzoyl acyl derivatives in water before and after quaternization

Name of derivative	% ES	solubility in water before quaternization	solubility in water after quaternization
N-propionoyl chitosan Quat-188	21 47 45	++ + -	+++ +++ *
N-butyroyl chitosan Quat-188	34 41 30	+ - -	+++ * *
N-valeroyl chitosan Quat-188	8 0 11	+ - -	+++ * *
N-hexoyl chitosan Quat-188	13 18 21	+ + -	+++ +++ *
N-heptanoyl chitosan Quat-188	23 31 50	- - --	* * *
N-benzoyl chitosan Quat-188	14 30 50	++ - -	+++ * *

+++ = completely soluble
++ = high aqueous enhanced, but not completely soluble
+ = moderate aqueous enhanced
- = low aqueous enhanced
-- = insoluble
* = partial solubility

Table 4.11 The solubility of the *N*-ionic acyl derivatives in water before and after quaternization

Name of derivative	% ES	solubility in water before quaternization	solubility in water after quaternization
N-succinoyl chitosan Quat-188	14	+	*
N-maleoyl chitosan Quat-188	2	-	+++
N-glutaroyl chitosan Quat-188	5 10 14	- - -	+++ +++ +++
3,3-dimethyl *N*-glutaroyl chitosan Quat-188	10 4 6	+ + +	+++ +++ +++
N-cis 1,2,3,6-tetrahydrophthaloyl chitosan Quat-188	7 11 20	++ ++ -	+++ +++ *
N-3-NO_2 phthaloyl chitosan Quat-188	5 9 n/a	++ ++ ++	+++ +++ +++
N-cis-1,2 cyclohexanedicarboxoyl chitosan Quat-188	6 0	+ --	+++ +++
N-trimellitoyl chitosan Quat-188	19	+	+++

+++ = completely soluble
++ = high aqueous enhanced, but not completely soluble
+ = moderate aqueous enhanced
- = low aqueous enhanced
-- = insoluble
* = partial solubility

Table 4.12 The solubility of the 2-glycerol succinamide and glutamide chitosan in water before and after quaternization

Name of derivative	% ES	solubility in water after quaternization	solubility in water after quaternization
2-glycerol succinamide chitosan Quat-188	11 20 28	++ + +	+++ +++ +++
2-glycerol *glutamide* chitosan Quat-188	5 5 25	+ + +	+++ +++ +++

+++ = completely soluble
++ = high aqueous enhanced, but not completely soluble
\+ = moderate aqueous enhanced

Table 4.13 Molecular Weight of chitosan and the chitosan Quat-188 derivatives via GPC/MALS

Name of Compound	M_n	M_w	M_w/M_n	d*n*/d*c*
chitosan (commercial)	106,100	130,800	1.233	0.180
chitosan Quat-188	20,090	26,703	1.329	0.147
N-3NO_2 phthaloyl chitosan Quat-188 (9% ES)	37,883	77,010	2.033	0.142
2-glycerol succinamide chitosan Quat-188 (20% ES)	27,270	36,420	1.336	0.144

4.10 References

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CHAPTER 5. THE ANTIBACTERIAL ACTIVITY OF *N*-ACYL CHITOSAN QUAT-188

5.1 Introduction

Studies on the antibacterial activity of chitosan derivatives have revealed that the polymer is effective in inhibiting the growth of bacterial cells.[1-3] The antibacterial activities have been described mostly with chitosan derivatives that contain quaternary ammonium functional groups. Chapter 5 investigates the antibacterial activity of the *N*-acyl chitosan Quat-188 derivatives against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) and describes the possible mode of action in which the derivatives inhibit the growth of these bacterial cells. Prior to discussing the antibacterial data obtained for the derivatives, it is first important to understand how the bacteria's framework influences the antibacterial activity. This begins by contrasting gram-negative bacterial cells with gram-positive bacterial cells, which differ primarily in their bacterial surfaces.

5.1.1 The Cell Wall of Gram-Negative and Gram-Positive Bacteria

Many gram-negative bacterial cells have complex cell walls. Wide-ranging studies on the structure of gram-negative cells have been concentrated on *E. coli* in particular. The structure of a typical gram-negative cell is shown in Figure 5.1.[4] The outermost regions of the cell wall consist of various components that form a structure called the outer-membrane. This membrane is a lipid bilayer which contains phospholipids (phosphatidylethanolamine and phosphatidyl-glycerol) on its inner surface and lipopolysaccharide (3-deoxy-D-*manno*-octulosonic acid, hexoses, heptoses, ethanolamine, and phosphoric acid) exclusively on its outer surface. The core lipopolysaccharide is linked to an antigenic side chain. The side chain comprises sugar units, which form the outmost layer of the cell and is the main source of its antigenic

characteristics. The arrangement of the lipoprotein (made of amino and fatty acids) anchors the outer-membrane to the peptidoglycan layer. Peptidoglycan is the component of the cell which contributes to wall strength and constitutes about 10% of the wall mass of gram-negative cells. Parallel to the peptidoglycan is the cytoplasmic membrane, which like the outer-membrane, is a lipid bilayer. It consists of a double layer of phospholipids and has proteins embedded in it. The membrane surrounds the cytoplasm of the cell, separating it from the environment.

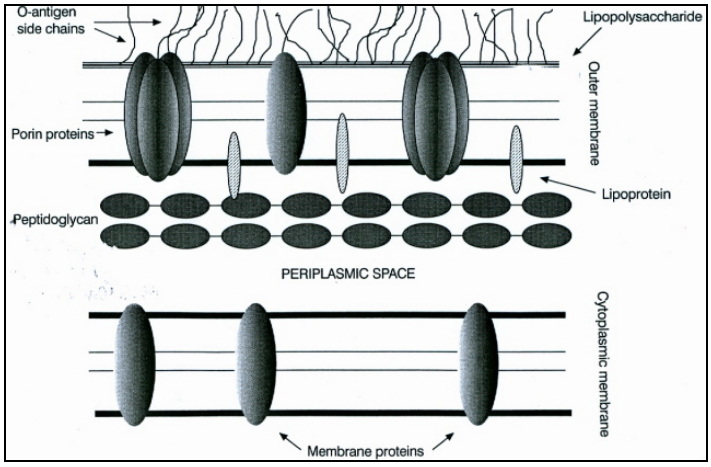

Figure 5.1[4] The arrangement of a gram-negative bacterial cell

Many gram-positive bacteria have relatively simple cell walls (Figure 5.2).[4] These cells have much thicker peptidoglycan in their cell walls and no outer-membrane external to this structure. The peptidoglycan provides a physical barrier for protection from the environment and gives strength and shape to the cell. Attached to the peptidoglycan are acidic polymers, which account for 30-40 % of the wall mass.[5] These polymers are teichoic acid (a substituted poly-D-ribitol 5-phosphate) and lipoteichoic acid (a substituted glycerol 3-phosphate). The acidic character of the polymers is to ensure that the cell surface is strongly polar and carriers a negative charge. The polarizability can influence the entry of ionized molecules into the cell. Proteins

covalently linked to the peptidoglycan provide the main source of the antigenic properties of the bacteria. Gram-positive bacteria also have proteins that expand the cytoplasmic membrane.

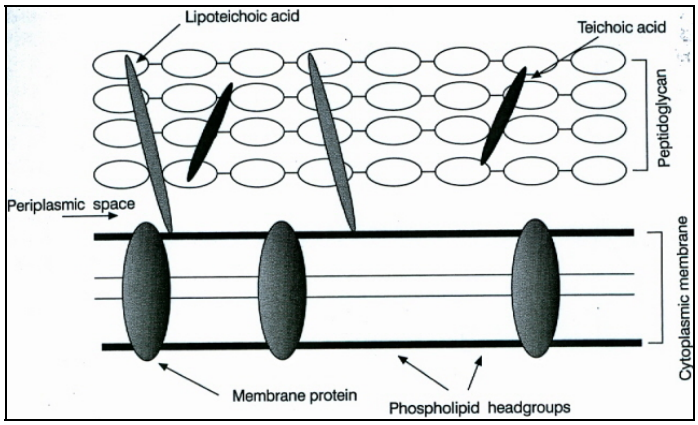

Figure 5.2[4] The arrangement of a gram-positive bacterial cell

5.2 Antibacterial Activity: Mode of Action

Nurdin et al. have proposed a possible mechanism in how polymeric quaternary ammonium materials exhibit an antibacterial effect against bacteria.[6] It is believed that the negatively charged cell surface interacts electrostatically with the positively charged quaternary nitrogen on the polymer. The lipophilic or hydrophobic chain then diffuses through the bacterial cell wall causing disruption of the cytoplasmic membrane, loss of cytoplasmic constituents, and eventually, cell death. Nakae and Nikaido have reported however, that as a polymeric macromolecule, chitosan is unable to pass the outer-membrane of gram-negative bacteria because the membrane functions as an outer permeability barrier against macromolecules.[7] Therefore, the possibility of chitosan's direct access to the intracellular parts of the cell (e.g. cytoplasmic membrane) is unlikely. Helander et al. believe that the mode of antibacterial activity against gram-negative bacteria involves the binding of cationic chitosan to the anionic cell surface. This

disrupts the integrity of the outer-membrane which functionally weakens it and sensitizes the bacteria to other inhibitory substances (e.g. detergent-induced lysis, hydrophobic antibiotics, or probes).[5]

5.3 Minimum Inhibitory Concentration Tests

The antibacterial activity of the *N*-acyl chitosan Quat-188 derivatives was studied by employing the Minimum Inhibitory Concentration (MIC) method. A MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.[8] The test organisms used in the assessments were *E.coli* (ATCC 25922) and *S. aureus* (ATCC 29213). The inhibitory effect of the derivatives against the bacterium was determined on nutrient broth containing a specific concentration of the agent (e.g. 256, 128, 64, 32, 16, 8, 4, and 2 μg/mL).

5.4 Targeted MICs

The MICs of chitosan Quat-188 against *E. coli* and *S. aureus* were used as standard MICs to judge the quality of the MIC data obtained for the *N*-acyl chitosan Quat-188 derivatives. The MIC of chitosan Quat-188 was 64 μg/mL against *E. coli* and 32 μg/mL against *S. aureus*. Therefore, the *N*-acyl Quat derivatives were to target MIC values lower than 64 μg/mL and 32 μg/mL against *E. coli* and *S. aureus*, respectively.

5.5 Experimental

5.5.1 Materials

The test organisms *E. coli* and *S. aureus* were obtained from Louisiana State University's Protein Facility lab. Potassium monobasic phosphate (KH_2PO_4, ACS crystal) and potassium dibasic phosphate (K_2HPO_4, ACS powder) were purchased from Fluka in the highest level of purity. Sterile translucent Costar® 96-well cell culture cluster plates were manufactured by Coring Inc. Polystyrene sterile Falcon tubes and

disposable polystyrene cuvettes were manufactured by Becton Dickinson and Company. Difco nutrient broth (NB) was provided by Dickinson and Company. Ethanol was purchased from Sigma Aldrich. Silver nitrate ($AgNO_3$) and potassium chromate (K_2CrO_4) were purchased from Lancaster.

5.5.2 Instrumentation

Ultrospec 4050, manufactured by LKB Biochrom, was the spectrometer used for the antibacterial assessments.

5.5.3 Preparation for Performing the MIC Tests

The test organisms *E. coli* and *S. aureus* were stored in a freezer at $^{-}80\ ^{0}C$. A 50 mM phosphate buffer solution was used to maintain the pH of the solutions. The buffer solution was made by combining 78 mL of 0.1M potassium monobasic phosphate (KH_2PO_4,) with 122 mL of 0.1M potassium dibasic phosphate (K_2HPO_4), and 400 mL of water. The bacterial testing was performed in sterile translucent Costar® 96-well cell culture cluster plates. Polystyrene sterile Falcon tubes were used to prepare the bacterial solutions and also a series of different concentrations of the antibacterial agent. Difco nutrient broth was used for the bacterial inoculations. The optical density (OD) measurements were performed in disposable polystyrene cuvettes. Prior experiments conducted by Dr. Martha Juban (of Louisiana State University's Protein Facility) determined the level of OD required for the bacterial solutions in order to attain specific cell density and the time it took to attain mid-log phase growth for the specific organisms used in this study. All of the solutions prepared (except those containing microorganisms and the antibacterial agent), equipment, and glassware used were autoclaved at 121 ^{0}C for 20 min prior to their use. In order to help maintain the sterility during the assessments, a clean hood was sprayed with sufficient ethanol to cover the surface and flame-dried.

Additionally, the assessments were carried out in the proximity of heat convection generated with the help of a Bunsen burner.

5.5.4 Antibacterial Assessments

The *N*-acyl chitosan Quat-188 derivative (2.1 mg) was dissolved in 4 mL of sterile water to make a stock solution of the concentration 512 μg/mL. This solution was diluted with water by a factor of 2 (256 μg/mL). The most concentrated solution (256 μg/mL) was diluted in the multiples of 2 until the least concentrated solution was 2 μg/mL. 8% (w/v) nutrient broth was made using phosphate buffer as the solvent. The bacteria of choice was thawed from -80 ^{0}C to room temperature. The bacteria was then inoculated into 5 mL of nutrient broth and this solution was incubated at 37 ^{0}C for 12 h. After incubation, 1 mL of this solution was transferred into a culture flask containing 25 mL sterile nutrient broth and the flask was incubated at 37 ^{0}C on a shaker for 3.5 h. Approximately 2 mL of this solution was diluted with sterile nutrient broth, such that the optical density of the diluted solution was 0.200 for *E. coli* and 0.400 for *S. aureus*. Once the desirable optical density was obtained, it was further diluted with four times its volume with sterile nutrient broth. This resulting solution appeared clear and was immediately stored at 0^{0}C prior to use. The bacterial cell density in the clear solution was determined to be 4 x 10^7 cells/mL. This solution was further diluted to 2 x 10^6 cells once placed in the wells used for the bacterial assessment (as determined by the work of Dr. Martha Juban). The cell control well contained 50 μL phosphate buffer + 50 μL cell solution (containing 2x 10^6 cells) + 100 μL of deionized water. The agent control well contained 50 μL phosphate buffer + 50 μL nutrient broth + 100 μL of the *N*-acyl chitosan Quat-188 derivative. The test well contained 50 μL phosphate buffer + 50 μL cell solution + 100 μL of the *N*-acyl chitosan Quat-188 derivative. The cell and agent

controls were run simultaneously with the test wells to ensure proper growth of the bacteria within the diluted bacterial culture in the absence of the chitosan derivative and to be sure that there was no bacterial growth in the solutions of the derivative in the absence of bacterium, respectively. Following preparation of the 96-well plate by the procedure above, it was incubated at 37 ^{0}C for 14 h. A schematic of a 96-well plate used in the antibacterial assessment plate is shown in Figure 5.3.[9]

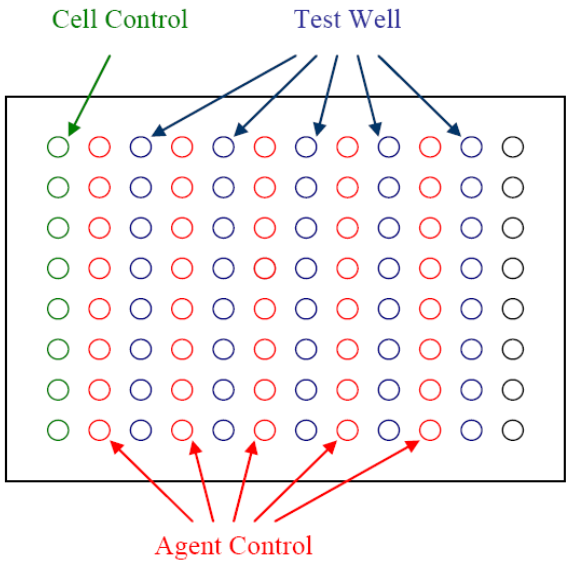

Figure 5.3[9] Schematics of a 96-well plate used in the antibacterial assessment

5.5.5 Mohr's Method: Determination of Chloride Ion Concentration

The extent of cationic substitution of the *N*-acyl chitosan Quat-188 derivatives was determined by the Mohr Method Titration, using $AgNO_3$ as the titrant. Only those derivatives that gave MICs less than or equal to the MICs of chitosan Quat-188 (64 µg/mL against *E. coli* and 32 µg/mL against *S. aureus*) were studied. The *N*-acyl derivative (0.035 g) was dissolved in 25 mL of deionized H_2O and then 0.5 mL of 0.064 mM K_2CrO_4 was added as an indicator. After stirring for 5 min, the solution was titrated with 0.017M $AgNO_3$ until the endpoint of the reaction was reached, as evidenced by the formation of the red-brown precipitate silver chromate.

Table 5.1 Minimum Inhibitory Concentration of *N*-aliphatic acyl and benzoyl chitosan Quat-188

Name of chitosan agent	*N*-acyl % ES	MIC against *E. coli* (μg/mL)	MIC against *S. aureus* (μg/mL)
N-propionoyl chitosan Quat-188	21 47 45	128 128 256	128 256 > 256
N-butyroyl chitosan Quat-188	34 41 30	128 256 64	32 > 256 128
N-valeroyl chitosan Quat-188	8 0 11	64 n/a 256	64 n/a > 256
N-hexanoyl chitosan Quat-188	13 18 21	64 64 128	64 128 > 256
N-heptanoyl chitosan Quat-188	23 31 50	64 64 > 256	256 64 > 256
N-benzoyl chitosan Quat-188	14 30 50	64 256 > 256	64 > 256 > 256

Table 5.2 Minimum Inhibitory Concentration of *N*-ionic acyl chitosan Quat-188

Name of chitosan agent	*N*-acyl % ES	MIC Against *E. coli* (μg/mL)	MIC Against *S. aureus* (μg/mL)
N-succinoyl chitosan Quat-188	14	n/a	n/a
N-maleoyl chitosan Quat-188	2	64	16
N-glutaroyl chitosan Quat-188	5 10 14	32 64 64	32 32 32
N-3,3-dimethyl glutaroyl chitosan Quat-188	10 3 6	64 64 > 256	32 64 > 256
N-cis-1,2 cyclohexanedicar-boxoyl chitosan Quat-188	6 0	128 32	32 16
N-cis1,2,3,6- tetrahydrophthaloyl chitosan Quat-188	7 11 20	64 64 n/a	32 32 n/a
N-3-NO_2 phthaloyl chitosan Quat-188	5 9 n/a	32 128 64	32 128 64
N-trimellitoyl chitosan Quat-188	19	128	32

Table 5.3 Minimum Inhibitory Concentration of 2-glycerol succinamide and glutamide chitosan Quat-188

Name of chitosan agent	*N*-acyl % ES	MIC Against *E. coli* (μg/mL)	MIC Against *S. aureus* (μg/mL)
2-glycerol succinamide chitosan Quat-188	11 20 28	64 64 256	32 64 256
2-glycerol glutamide chitosan Quat-188	5 5 25	64 128 128	32 32 128

Table 5.4 Antibacterial activity of the *N*-acyl chitosan Quat-188 derivatives with targeted MICs

Name of chitosan agent	% ES *N-acyl*	% EQ	MIC against *E. coli* (μg/mL)	contributing Quat concentration *E. coli* (μg/mL)	MIC against *S. aureus* (μg/mL)	contributing Quat concentration *S. aureus* (μg/mL)
Chitosan Quat-188	n/a	97	64	62	32	31
N-butyroyl chitosan Quat-188	34 30	35 34	128 64	45 22	32 128	11 82
N-valeroyl chitosan Quat-188	8	83	64	53	64	53
N-hexoyl chitosan Quat-188	13 18	46 33	64 64	29 21	64 128	29 42
N-heptanoyl chitosan Quat-188	23 31	35 48	64 64	22 31	256 64	90 31

(Table 5.4 continued)						
N-benzoyl chitosan Quat-188	14	33	64	21	64	21
N-maleoyl chitosan Quat-188	2	57	64	36	16	10
N-glutaroyl chitosan Quat-188	5 10 14	70 49 47	32 64 64	22 31 30	32 32 32	22 16 15
N-3,3-dimethyl glutaroyl chitosan Quat-188	10 3	74 70	64 64	47 45	32 64	24 45
N-cis-1,2 cyclohexanedicar-boxoyl chitosan Quat-188	6 0	74 72	128 32	95 23	32 16	24 12
N-cis1,2,3,6-tetrahydrophthaloyl chitosan Quat-188	7 11	59 58	64 64	38 37	32 32	19 19
N-3-NO_2 phthaloyl chitosan Quat-188	5 n/a	78 51	32 64	25 33	32 64	25 33
N-trimellitoyl chitosan Quat-188	19	34	128	44	32	11
2-glycerol succinamide chitosan Quat-188	11 20	55 51	64 64	35 33	32 64	18 33
2-glycerol *glutamide* chitosan Quat-188	5 5	61 49	64 128	39 63	32 32	20 16

5.5.6 Analysis of the 96-Well Plate

Following the incubation of the 96-well plate for 14 h, individual results were examined visually for qualitative analysis of the antibacterial activity of the agents. Clear and translucent wells indicated antibacterial activity and the lowest concentration of the agent was recorded as the MIC. Cloudy wells indicated continual bacterial growth in the presence of the agent, indicating that the agent at that particular concentration was not effective in exhibiting antibacterial activity. A representative developed 96-well plate used for the antibacterial assessment is shown in Figure 5.4.[9]

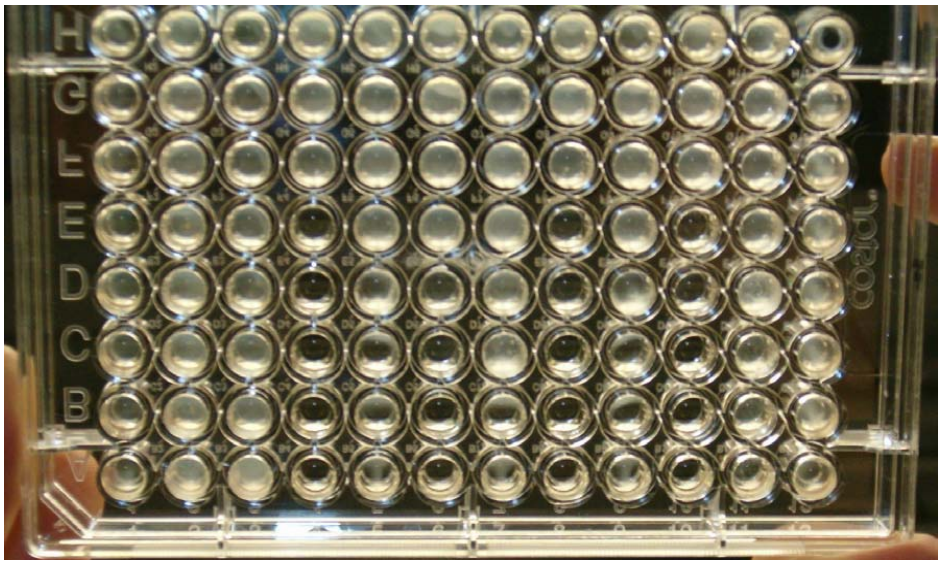

Figure 5.4[9] Developed 96-well plate for the antibacterial assessment

5.5.7 Titrations

The Mohr titration method involves the titration of the chloride counter ion of the Quat substituent. The percent extent of quaternization (% EQ) was calculated by applying Equation 5, proposed by Wu et al.[10]

Equation 5:

$$\%\ EQ = \frac{(Vc/1000)}{(Vc/1000) + (W_1(1- DD))/M_1 + (W_1DD- W_2)/M_2}$$

V and *c* are the volume and concentration of the titrant used in the Mohr titration, respectively. DD is the degree of deacetylation of chitosan. M_1 and M_2 are the molecular weights of one *N*-acetylglucosamine and one *N*-deacetylglucosamine unit, respectively. W_1 is the weight of the test sample. $W_2 = (Vc M_3)/1000$, where M_3 is the molecular weight of one monomeric unit of 3-(N,N,N-trimethylammonium chloride)-2-hydroxyl chitosan (312.18 g/mol).

Table 5.4 gives the % EQ of the *N*-acyl Quat-188 derivatives and their corresponding contributing Quat MICs. These MICs represent the antibacterial activity that the Quat substituents contributed to the total MIC of the sample.

5.6 Results and Discussion

5.6.1 Antibacterial Activity

The MICs of the *N*-acyl chitosan Quat-188 derivatives are shown in Tables 5.1 through 5.3. The MIC data shows that the % ES of the *N*-acyl attachment has a considerable influence on the antibacterial activity against *E. coli* and *S. aureus*. In most cases, the antibacterial activity of the derivative declined with greater levels of *N*-acyl substitution. For example, *N*-propionoyl chitosan Quat-188 substituted at 21% ES inhibited the growth of *E. coli* at 128 μg/mL. At 47% ES, the MIC of *N*-propionoyl chitosan Quat-188 increased to 256 μg/mL. 2-glycerol glutamide chitosan Quat-188 substituted at 5% ES inhibited the growth of *S. aureus* at a concentration of 32 μg/mL and the MIC was increased to 128 μg/mL when substituted at 25% ES. It was found that the % EQ corresponding to the derivative with 5% ES, was 61% EQ (shown in Table 5.4) and the derivative with 25% ES had a % EQ of 31% (not shown in Table 5.4). The data may be explicable in terms of an 'antagonist' effect of *N*-acyl substitution to that of cationic substitution. When the levels of *N*-acyl substitution were increased, the number

of available amines for quaternization was decreased, which meant that less cationic charge density could be placed on the polymer's backbone. This phenomenon suggests that the major determining factor for the variation in the antibacterial activity is the change in the hydrophobic-hydrophilic balance of the derivative. A permeability barrier anticipated for activity against *E. coli* was not apparent; the derivatives exhibited activity comparable to that observed with *S. aureus*.

5.7 References

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CHAPTER 6. CONCLUSION

Novel water soluble *N*-acyl chitosan Quat-188 materials were prepared from reactions of chitosan with linear aliphatic, acyclic, cyclic, and hydrophilic anhydrides, followed by quaternization with a solution of 3-chloro-2-hydroxyl propyl trimethylammonium chloride (Quat-188). Following their derivation, the antibacterial activities were investigated. Using the MIC method, the highest antibacterial activity was found for derivatives with low extents of *N*-acyl substitution (e.g. 5 – 10% ES), while derivatives with higher % ESs exhibited a decline in the activity. For example, the MICs of 2-glycerol succinamide chitosan Quat-188 at 11% ES was 64 μg/mL and 32 μg/mL against *E. coli* and *S. aureus*, respectively; at 28 % ES, the derivative gave MICs of 256 μg/mL against both bacteria. The results have shown that increases in the hydrophobic character of the polymer led to a decrease in the antibacterial activity, which was indicated as an increased MIC.

The parent compound chitosan Quat-188 exhibited MICs of 64 μg/mL and 32 μg/mL against *E. coli* and *S. aureus*, respectively. When the antibacterial activity of the *N*-acyl chitosan Quat-188 derivatives was corrected with the contributing Quat concentrations, derivatives exhibited MICs as low as 25 μg/mL against *E. coli* (i.e. *N*-benzoyl (14% ES) chitosan Quat-188) and 16 μg/mL against *S. aureus* (i.e. *N*-trimellitoyl (19% ES) chitosan Quat-188). The contributing Quat MICs of 2-glycerol succinamide and glutamide chitosan Quat-188 MICs were comparable to the contributing Quat MICs of the *N*-aliphatic, benzoyl, and ionic acyl chitosan Quat-188 derivatives.

VITA

Lakia M. Champagne was born in New Orleans, Louisiana, to the proud parents of Mr. and Mrs. Tony O. Champagne Sr. From August 1993 to May 1997, Lakia attended Warren Easton Fundamental Senior High School where she participated in various extracurricular activities. She was a member of the chemistry, biology and Beta club, and a captain on the Easton Eagles flag team. Following her senior year, she graduated with honors. Following graduation, she enrolled at Xavier University of Louisiana, where she obtained the Bachelor of Science degree in biochemistry with a minor in biology. It was at Xavier University where she developed a passion for chemistry, eminently for organic chemistry.

In August 2002, Lakia was accepted into Louisiana State University's graduate program in the Department of Chemistry. Under the guidance of Dr. William H. Daly, her research focuses on the synthesis of hydrophobic water soluble polymeric materials that exhibit antibacterial activity against human pathogenic bacteria. During her graduate training, she became a member of the American Red Cross Society Club (LSU), American Chemical Society (ACS), and the National Organization for the Professional Advancement of Black Chemists and Chemical Engineers (NOBCChE), where she served as Vice-President for the 2005-2006 academic year. Lakia has had the opportunity to present her research at several national conferences, including ACS and NOBCChE. She has received honorable awards including the 2006 Charles E. Coates Memorial Fund Travel Award, the 2006 NOBCChE National Conference Award, the 2006 President's Hurricane Relief Fund Award (LSU), and the 2004 Colgate-Palmolive Teaching Scholar Award in Organic Chemistry 2364. At the May 2008 commencement, Lakia M. Champagne will receive the degree of Doctor of Philosophy in chemistry.