REMEDIATION OF WATER USING MODIFIED CHITOSAN

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Remediation of Water using Modified Chitosan

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Declaration

I declare that **"Remediation of Water using Modified Chitosan"** is my own work and to the best of my knowledge has never been reported or submitted for any degree or examination in any university. All sources of information used are cited, acknowledged and completely referenced at the end of each chapter.

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Shakeela Sayed

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Dedication

To my parents without your support I would be nowhere. Daddy this is especially for you I wish you could have been here for this.

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Conference Contributions

October 2012 – Poster Presentation:

The Antimicrobial activities of Chitosan and derivatives, presented at **H3 – D (Holistic Drug Discovery & Development Centre) Symposium 2012** - "New Paradigms in Drug Discovery: Challenges and Opportunities in Africa", 15-18 October 2012, Cape Town, South Africa.

November 2012 – Poster Presentation:

The Antimicrobial activities of Chitosan and derivatives, presented at the **1st Pan-African Summer School in Nanomedicine** 4-10 November 2012, Pretoria, South Africa.

Abstract

Water treatment has been an area of increasing concern over the last decade. This interest is due to exponential increase in demand of already limited water sources. Therefore the treatment of wastewater for re-use is a topic of great interest. The treatment applied depends on the source and quality of the water. Common water treatment options include filtration, flocculation, coagulation, lime softening, reverse osmosis and clarification to name a few. In addition, water should also be treated for the presence of harmful micro-organisms which is normally done using chlorine-based disinfection. Water purification filters which purify water by removal of impurities and micro-organisms are in great demand. Therefore the aim of this study was to develop ion exchange polymers and antimicrobial filters using 'green' materials.

Chitosan, a linear semi-crystalline polysaccharide, is a polymer which has recently been receiving significant scientific interest. This is as a result of its unique properties including its biocompatibility, chemical versatility, biodegradability and low toxicity. Chitosan may be obtained from the partial deacetylation of chitin and is the second most abundant biopolymer in nature, after cellulose. Therefore due to these favourable properties and natural abundance of this polymer, new applications are of interest. A series of chitosan and 6-deoxy-6-amino chitosan derivatives were synthesized using reported methods. These reactions yielded derivatives containing thiol groups and quaternary ammonium functional groups. The incorporation of these functional groups onto the polymer backbone has advantages over native chitosan such as an increase in solubility, gelling properties, reversion of the net charge from polycationic to polyanionic, amphiphilic character and improved biocompatibility. Chitosan and the quaternary derivatives 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (**5A**) and trimethylchitosan chloride (**6A**) were tested as ion exchange resins for the removal of the harmful contaminant perchlorate (ClO_4^-). In addition these polymers were loaded with Fe and evaluated for ClO_4^- removal. This study found that these polymers and their Fe loaded counterparts are capable of binding ClO_4^-.

In a separate study, all chitosan and 6-deoxy-6-amino chitosan derivatives were loaded with silver (Ag) metal. Certain soluble derivatives and their silver-loaded counterparts were tested for their antimicrobial activity against *E.coli* (chitosan & Ag-loaded derivatives) and *S. aureus* (chitosan derivatives). The polymers showed some inhibitory activity against *E.coli* and *S. aureus* and the Ag loaded compounds displayed moderate to no activity. This study was extended to the testing of the polymers as anti-mycobacterial agents. It was found that thiolated derivatives displayed moderate activity while the other derivatives tested did not significantly inhibit bacterial growth.

Abbreviations

BG	brilliant green
calc.	calculated
CFU	colony-forming units
^{13}C-NMR	carbon nuclear magnetic resonance
°	degrees
°C	degrees Celsius
DDA	degree of deacetylation
DQ	degree of Quaternization
DNA	deoxyribonucleic acid
DCl	deuterated hydrochloric acid
D_2O	deuterated water
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EPA	Environmental Protection Agency
EtOH	ethanol
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
EtOAc	ethyl acetate
e.g.	example
FDA	Food & Drug Administration
FT-IR	fourier transform infrared spectroscopy
g	gram(s)
hr(s)	hour(s)
ICP-MS	inductively coupled plasma mass spectrometry
IR	infrared
INH	isoniazid
LMW	low molecular weight
LB	luria broth
w/v	mass concentration
MeOH	methanol
Me	methyl
NMP	*N*-Methyl-2-pyrrolidone

MeI	methyl iodide
µg	micrograms
µg/mL	micrograms per millilitre
*µ*L	microliters
µM	micromolar
mg	milligram(s)
mL	millilitre(s)
mM	millimolar
mmol	millimole(s)
mmol/g	millimoles per gram
MIC	minimum inhibitory concentration
min	minute(s)
M	molar
mol	mole(s)
MW	molecular weight
$mol.dm^{-3}$	moles per decimetre
M. tb	mycobacterium tuberculosis
NAc	*N*-acetyl
nm	nanometer
(-)	negative
NMR	nuclear magnetic resonance
NB	nutrient broth
p	para
ppm	parts per million
%	percent
PBS	phosphate buffered saline
pDNA	plasmid DNA
(+)	positive
^{1}H-NMR	proton nuclear magnetic resonance
cm^{-1}	reciprocal centimetres
RT	room temperature
s	singlet (NMR)
siRNA	small interfering ribonucleic acid
SANS	South African National Standard

THF	tetrahydrofuran
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl
TMS	tetramethylsilane
TSH	thyroid stimulating hormone
TNTC	too numerous to count
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
TPP	triphenylphosphine
TB	tuberculosis
UV-Vis	ultraviolet-visible spectroscopy
v/v	volume concentration
WHO	World Health Organization
ZOI	zone of inhibition

CHAPTER ONE

BIOPOLYMERS AND WATER PURIFICATION

1.1. Water

Without water there would be no life. Recent statistics has shown that more than 1 billion people globally, lack access to clean water. It is also predicted that in the next two decades the average water supply will decrease by a third thus condemning millions of people to an unfortunate and avoidable death. Due to these statistics, water treatment has been a subject of paramount importance for many years.[1]

Water is recycled in the atmosphere and follows the pathway known as the hydrological cycle. Water evaporates from the earth's surface and condenses in the atmosphere where precipitation occurs returning the water to the earth's surface.[2] This cycle gives rise to several different types of water which includes surface-, ground-, grey- and wastewater.[3] The remediation of these water sources, in particular wastewater, are of great interest. Wastewater (sewage) is defined as water that has been used. It stems from a large range of sources including, toilets, drains, rainwater, run-off, agricultural and industrial sources. Wastewater must be treated, so that pollution of clean water is prevented. The treatment applied is dependent on the water source and the contaminants present. On average, wastewater is comprised of 99.9 % water and 0.1 % dissolved or suspended matter (e.g. nutrients: phosphorus and nitrogen, fats, oils, grease: cooking oils, medicines, pesticides, personal care products and pathogens (disease-causing bacteria and viruses)). Wastewater may be recycled in a number of ways with the use of filters and/or chemical treatments.[4,5]

Drinking water, is a precious commodity which needs to be rationed in order to help meet the demand. Despite the fact that the earth is covered by 71 % water, less than 1 % of fresh water is available for human consumption.[2,6] Safe drinking water is defined as water which may be consumed by humans and used for domestic purposes such as bathing and food preparation. In addition, this water may not contain chemicals or micro-organisms at a harmful level and should ideally be appealing in appearance, taste and odour. Drinking water is sourced from surface water (rainfall and runoff into rivers or dams) and groundwater.[6,7] National drinking water standards are enforced to ensure that the drinking water supplied to the public is of a high quality. The South African National Standard (SANS) 241 Drinking Water Specification is the conclusive reference on satisfactory limits for drinking water quality in South Africa. SANS 241 limits are comparable to those set by the World Health Organisation (WHO). Water treatment is crucial in the prevention of the spread of water

borne diseases such as diarrhoea, botulism and dysentery. The greatest threat in access to clean, safe drinking water is on the African continent where 90 % of the global problem exists (Figure 1.1).[8]

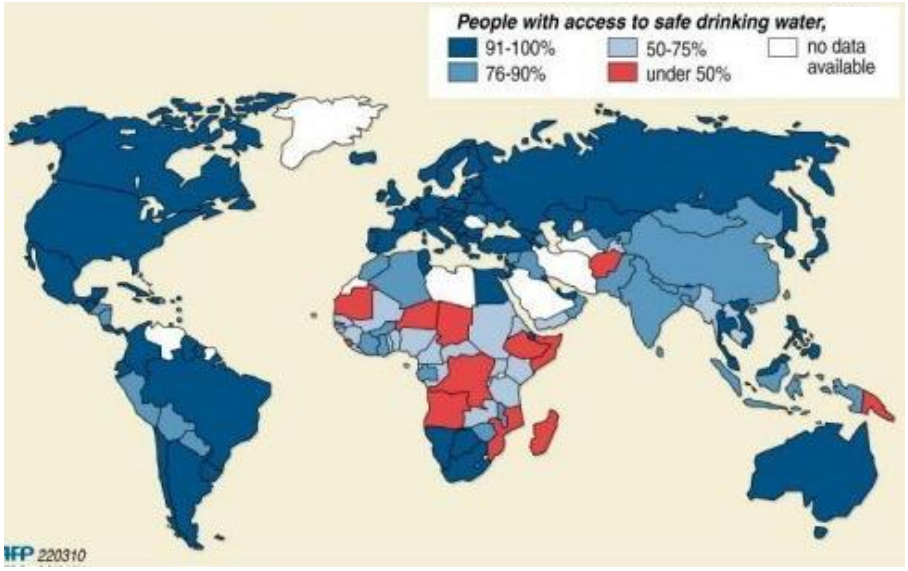


Figure 1.1: The world population with access to clean drinking water.[8]

1.1.1. Water contaminants

Water is contaminated either at the source or at the point of use. Point of source contamination implies that the water source in use is contaminated while point of use contamination occurs once the water has been transferred from the source to a storage vessel. The major contaminants present in drinking water include organic and inorganic waste as well as microbes. Organic contaminants present in drinking water include atrazine, a pesticide which is a known carcinogen, *cis*-1,2-dichloroethylene which affects the function of the liver and nervous system and dibromochloropropane which causes cancer and sterility. Common inorganic contaminants include arsenic which is toxic and causes cancer and chromium which causes skin irritation, and damage to the kidney, liver and nerve tissues. Other inorganic contaminants such as lead and cyanide are well known poisons.[9]

Microbes are ever-present in terrestrial and aquatic environments. These microorganisms are the basis of food webs and biogeochemical cycles. Most microbes are harmless except, microbes in water associated with human and animal excreta which are potentially harmful. Communicable diseases caused by pathogenic bacteria, viruses and parasites (e.g. protozoa and helminths) are a well-known health risks associated with drinking-water.[10] The severity of the infection or the occurrence of an infection in an individual who has been exposed to a waterborne pathogen is influenced by factors such as age, sex, state of health and living conditions.[10]

Indicators of bacterial contamination include total coliforms which are gram-(-ve) bacteria, faecal thermo-tolerant coliforms which are a subset of total coliform bacteria and *Escherichia coli (E. coli)* which are exclusively faecal in origin. Guidelines published by the WHO state that none of these bacteria should be detectable in a 100-mL water sample. Typically, *E. coli* is the most reliable indicator of faecal contamination and total coliforms the least reliable indicator. Therefore in most water treatment studies, *E. coli* is used as the model bacteria when testing for microbes.[11]

1.2. Water purification processes and commercial purification products

All water is not treated in the same manner, treatment depends on the source and quality of the water. Surface water in general requires more treatment steps due to greater exposure to pollutants. Water taken from a river or dam typically contains suspended material and contaminants. Coagulants or flocculants (e.g. the biopolymer chitosan, clay, aluminium sulfate, etc.) are added to the water to allow the debris to form clumps and settle. The water is then passed through a filter and subsequently disinfected, usually with chlorine which is a strong oxidant. High pollutant content requires additional treatment which leads to higher costs.[6,7] The treatment methods employed are in most cases, not contaminant specific, resulting in excessive reagent use requiring further processing and disposal of spent reagent. Conventional methods are costly and in areas where trace contaminants require removal, these methods remove some valuable drinking water along with the contaminants.[12]

Water treatment technologies that reduce the pathogens present in drinking water are:

- Pre-treatment (roughing filters, storage reservoirs, bank filtration),
- Coagulation, flocculation (addition of polymers which aggregate suspended particles in solution) & sedimentation (conventional clarification, high-rate clarification, dissolved air flotation, lime softening),
- Filtration (granular high-rate filtration, slow sand filtration, pre-coat filtration, membrane filtration (microfiltration, ultrafiltration, nanofiltration & reverse osmosis)).
- Primary disinfection methods (chlorine, chlorine dioxide, ozone & ultraviolet (UV)).[10]

A typical water treatment process is shown in Figure 1.2.

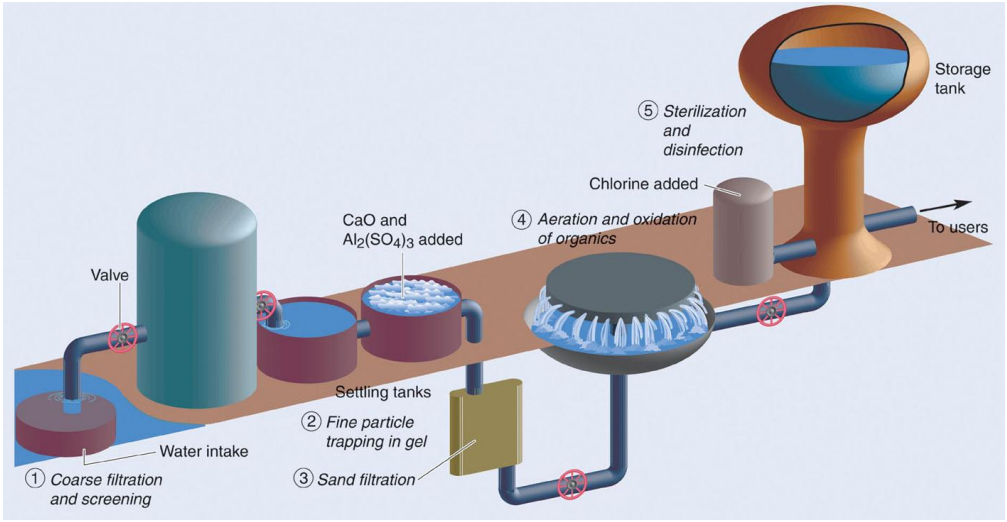


Figure 1.2: Typical steps in the water treatment process.[13]

As a result of the possible presence of pathogens in our drinking water, researchers have developed commercially available antimicrobial water filters which remove or kill bacteria present.

Miller reports the development of an environmentally inert antimicrobial filter media.[14] This media uses a water soluble quaternary amine organosilane (3-trihydroxy silyl propyl dimethyl octadecyl ammonium chloride) which is recognised by the Environmental Protection Agency (EPA) as an antimicrobial agent effective against gram-(+ve) and gram-(-ve) bacteria, yeasts, fungi, spores and viruses. In the filter, this organosilane derivative is covalently attached to perlite which is a chemically inert siliceous rock. This antimicrobial media is non-toxic, leaves no traceable chemical residue, does not rely on physical trapping and no external energy source is needed. This filter was found to be highly effective against *E. coli* and can be applied in the removal of a wide spectrum of waterborne pathogens such as *Legionella*, *Cryptosporidium*, *Staphylococcus*, and *Streptococcus*.[14]

Research in nanotechnology yielded single-walled carbon nanotube (SWNT) filters, a different type of product which removes bacterial and viral pathogens from water. Brady-Estévez *et al.* demonstrated the ability of these filters to remove *E.coli* from water at low pressures.[15] These filters make use of the favourable properties of SWNTs such as their small diameter, high surface area; aggregation tendency, forming highly porous structures that possess inherent antibacterial properties. The bacteria is retained as well as inactivated upon coming into contact with the filter.[15] A review of carbon nanotubes and their application in water treatment has been published by Upadhyayula *et al*.[16] In this review, it is suggested that carbon nanotube filters have the potential to support point of use (POU) treatment in the removal of bacteria, natural organic matter and cyanobacterial toxins present in untreated

water. The proven superior filtration capabilities of these compounds allows for the removal of these macromolecular biomolecules and microorganisms. This study suggested that the use of carbon nanotube based technology in water treatment, is a promising area of research.[16]

Ceramic water filters (CWFs) have been found to be an inexpensive method for the treatment of microbially contaminated water. These filters physically remove larger microorganisms by size exclusion and inactivate bacteria when colloidal silver is impregnated into the filter.[17] However, the bulk use of ceramic filters creates a waste disposal challenge. Hence, the focus of water purification research has shifted to the use of 'green materials' in the remediation of water.

1.3. Natural Polymers in water purification

The use of polymers derived from a natural source in the remediation of water, has been the focus of numerous studies. The key advantage in the use of natural polymers in water treatment is their biodegradability, ease of dissolution and the fact that they are a renewable resource. Due to the inherent biodegradability of polymers from a natural source, recyclability is sometimes limited and needs to be taken into consideration when included in the design of a water treatment plant. Compared to synthetic polymers in water treatment, natural polymers do not always result in a waste disposal challenge. Natural polymers which have been utilized in water treatment include, starch (Figure 1.3, A), guar gum, alginates (Figure 1.3, B) and products based on chitin (Figure 1.3, C) glue and gelatin. These polymers are typically used as flocculants (binder) and retention aids due to their low dosage (1–5 ppm), inertness to pH changes, formation of large cohesive flocs and versatile chemistry.[18]

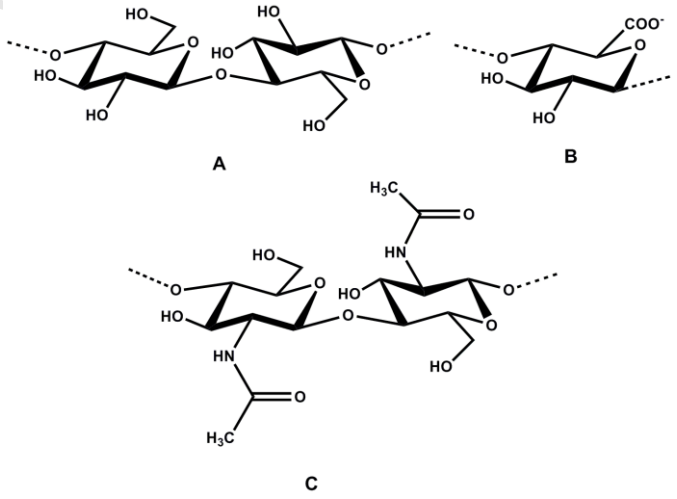


Figure 1.3: Natural polymers starch (A), guar gum (B) and chitin (C).

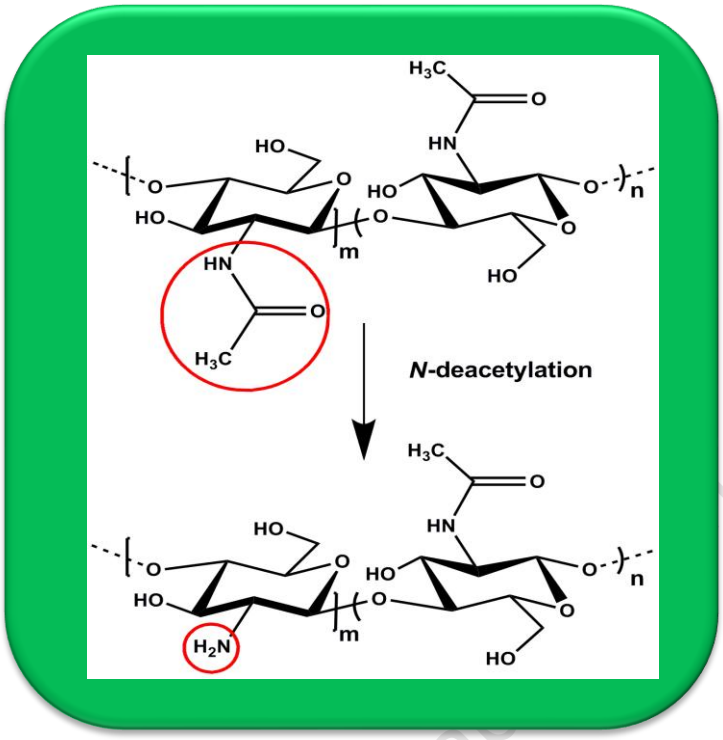
Researchers have investigated the potential of grafting synthetic polymers onto the natural polymer backbone. This association reduces the biodegradability and increases the stability as a result of structural changes producing a more stable polymer. One such example is the amylopectin-g-polyacrylamide co-polymer which is a superior flocculant and reduces the drag associated with water treatment.[18] Starch has been derivatized with a quaternary cation by reacting base treated starch with *N*-(3-chloro-2-hydroxypropyl) trimethylammonium chloride thereby attaching the cation via an ether linkage to the polymer backbone. This modified cationic polyelectrolyte was tested in clarifying clay, as a demulsifier for oil in water emulsions and in the treatment of raw and treated sewage suspensions. Selected starch based polymers have been successfully applied as flocculants. Examples of these copolymers are starch and 2-hydroxy-3-methacryloyloxypropyltrimethylammonium chloride or the combination of dimethylaminoethyl methacrylate and acrylamide. Another natural polymer which has been utilized in water treatment as a flocculating agent is lignin.[19] It was found that a quaternary ammonium derivative of lignin synthesized by chloromethylation and amination was less effective for the removal of colour from pulp mill wastewater compared to aluminium sulfate (alum) which is typically used. However, a weak basic polymer which is the product of the reaction of tannin, formaldehyde and aminoethanol proved to be more effective than alum in the removal of turbidity and colour from river water. An anionic polyelectrolyte lignin sulfonate has been used in the dewatering of sludge where the performance of this compound was found to be similar to that of cationic polyacrylamides.[19,20] A co-polymer of polyacrylamide and Konjac gum had improved flocculation performance and better biodegradable properties compared to the parent polymers.[21] Cellulose acetate was used as a nonwoven membrane for heavy metal ion adsorption and was found to have a high affinity for Hg^{2+}.[22]

In the light of the successful utility of biopolymers in water purification, interest has developed in improving on existing technology and expanding the applications of these biopolymers. Areas of interest include removal of harmful contaminants by ion exchange as well as the development of antimicrobial filters. Therefore, the focus of this review would be to present current literature directly related to these areas of interest. The centre of this study will be a polymer which has received considerable interest in water treatment viz. chitosan.

1.4. Chitosan chemistry

Chitosan, is a linear semi-crystalline polysaccharide which has recently been receiving significant scientific interest, owing to its unique properties including its biocompatibility, chemical versatility, biodegradability and low toxicity.[23] Chitosan may be obtained from the

partial deacetylation of chitin, the second most abundant biopolymer in nature, after cellulose (Scheme 1.1).[23,24]



Scheme 1.1: The deacetylation of chitin.

Sources of chitin include the exoskeletons of arthropods such as crustaceans, fungi, insects, annelids, etc. Approximately 10^{10} tons of chitin is produced annually in nature.[25,26] South Africa in particular, has a rich variety of seafood and the waste generated by the consumption of these crustaceans is a major source of chitin. However, this renewable resource has not been fully exploited. Chitosan is relatively inexpensive and is a cost effective alternative to expensive synthetic polymers which can perform a similar function.[23] It is considered to be an environmentally friendly 'green' polymer which has been classified as a Generally Regarded As Safe (GRAS) material.[27,28] Chitosan is composed of randomly distributed *β*-(1-4)-linked *D*-glucosamine and *N*-acetyl-*D*-glucosamine units (Scheme 1.1). As a result, the polymer does not have a single well-defined molecular structure and may have different molecular weights and sequences.[25,26] The degree of deacetylation (DDA) and depolymerization, determines the molecular weight. The degree of deacetylation ranges between 40 to 98 % with a variation in the molecular weight from 5 x 10^4 to 2 x 10^6 Daltons. As a result of polymers of different molecular weights, different properties of chitosan can be exploited. There are currently four grades of chitosan available, depending on their application these are, agricultural (DA ≥ 85%), industrial (DA > 75%), food & cosmetics (DA 65-90 %, 78-82%) and pharmaceutical grade chitosan (DA 90-95%).[24,29]

Chitosan contains two reactive hydroxyl groups (C-3 & C-6) and an amino group at the C-2 position of the glucosamine residue which is responsible for the unique properties of chitosan. The reactivity of chitosan is largely dependent on pH which affects its charged state and properties. Chitosan is protonated and thus positively charged at a low pH where it is also partially water soluble. In contrast, at a neutral to high pH chitosan is insoluble. Chitosan has an almost neutral pK_a where the soluble-insoluble transition occurs at a pH of ~ 6.0 - 6.5, a range which is favourable for biological applications.[30,31]

Due to the presence of strong intra- and intermolecular hydrogen bonds, the polymer does not dissolve in most organic or aqueous solvents. This poor solubility restricts the possible applications of the polymer. In order to increase polymer solubility, derivatives of chitosan have been synthesized by attaching hydrophilic and hydrophobic groups to the polymer backbone. One particular route to increase solubility, involves the conversion of the C-6 hydroxy group into a carboxy or amino group, thereby increasing solubility in organic and aqueous solvents.[32,33]

1.4.1. General Applications

At present a wide range of industrial applications of chitosan exist. These include water treatment, agriculture, biotechnology, food/health supplements, cosmetic, biomedical, textile and paper.[26,34] Most of these applications require chitosan to be aqueous soluble therefore modifications which enhance solubility are favourable.

In water treatment, chitosan has been used as a flocculent and in the removal of metals from wastewater by chelation. This chelating ability is the result of the great number of hydroxyl groups present in chitosan, the number of primary amino groups which are good absorption sites and the flexible structure of the chitosan chain which allows effective complexation with metal ions.[35] Due to these favourable properties, chitosan has also been applied in ion exchange.

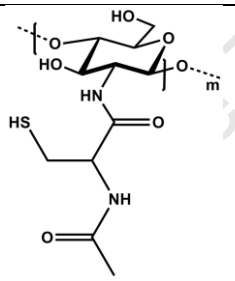
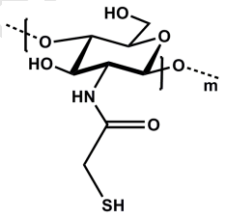
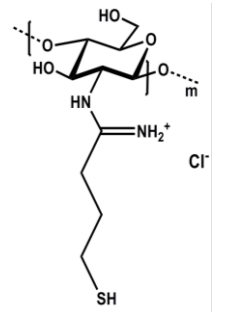
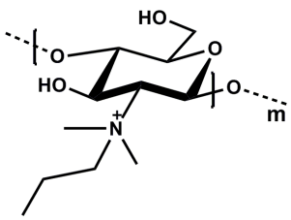
The antimicrobial, antifungal and haemostatic properties of chitosan have found numerous biomedical applications. Since chitosan is fully biodegradable in addition to being non-toxic, the utilization of this polymer in various products will not have a negative effect on humans or the environment. This polymer has proven antimicrobial activity against common waterborne pathogens such as *E. coli* and *Pseudomonas aeruginosa.* [23,24,26]

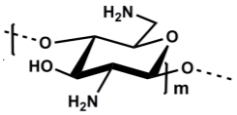
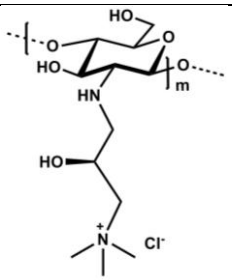
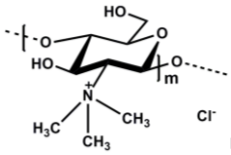
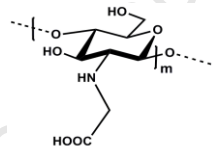
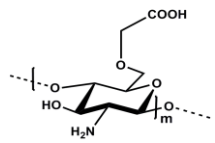
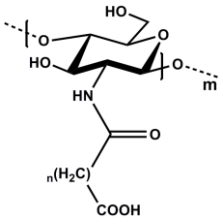
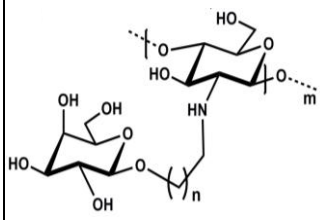
1.4.2. Derivatives

Chitosan has been modified by a variety of methods which include alkylation, acylation, Schiff base formation, nitration, phosphorylation, sulfation, xanthation, hydroxyalkylation, and graft co-polymerization.[36] These modifications have chemical, biological and functional

advantages compared to native chitosan. Some enhanced properties have been reviewed by Sarmento *et al*.[37] These include an increase in solubility, gelling properties and reversion of the net charge from polycationic to polyanionic. In addition, designs for hydrophobic derivatives with amphiphilic character and the capacity to harness self-assembling nanostructures and chemical conjugates with an assortment of bioactive and therapeutic molecules have been evaluated with modified chitosan. Improved biocompatibility (e.g., hemocompatibility) can also be observed as well as an enhancement of properties for complexing pDNA or siRNA.[37] Inamdor *et al.* have compiled a list of the common modified chitosans which have shown enhanced properties such as increased permeability, solubility, chelating abilities, etc.[24] Some of these properties have been highlighted in Table 1.1.

Table 1.1: Common modified chitosan derivatives and their uses.

Chitosan derivative	Examples	General structure	Uses	Solubility
Thiolated chitosan	chitosan–cysteine (**2**)		non-invasive means of delivering peptide and protein drugs, enhancing mucoadhesiveness and swelling.[38]	water soluble
	thioglycolyl chitosan–(**3**)		cross-linked nanoparticles with pDNA improve gene transfer efficiency in Caco-2 cells (model cells), scaffold material for tissue engineering.[39,40]	water soluble
	chitosan–4−thio−butylamidine (**4**)		cosmetic and pharmaceutical uses, controlled drug delivery.[41,42]	water soluble
Alkyl chitosan	*N*-propyl-*N,N*-dimethyl chitosan		antibacterial agents.[43]	water soluble

Diamino chitosan	6-deoxy-6-amino chitosan (**10**)		gene carrier, support for Pd/Rh nanoparticles as catalyst, antibacterial agent. [33,44,45]	water soluble
Quaternized chitosan	3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (**5**)		preservative in personal care products, antimicrobial agent, flocculent.[46,47,48]	water soluble at neutral pH
	trimethylchitosan chloride (**6**)		absorption enhancer for large hydrophilic molecules across mucosal surfaces in neutral and basic environments.[49]	can dissolve in either acidic or basic media
Carboxyalkyl chitosan	*N*-carboxylmethyl chitosan		removal of metal ions.[24]	water soluble
	O-carboxymethyl chitosan			
N-Carboxyacyl-chitosan	from anhydrides such as maleic, succinic, itaconic, glutaric, trimellitic, pyromellitic, thiosuccinic, phthalic, and salicyl.		cosmetic applications.[50]	water soluble
Sugar derivatives	deoxygalactic-1-yl-, 1-deoxyglucit-1-yl-, 1-deoxymelibit-1-yl, 1-deoxylactit-1-yl-,1-deoxylactit-1-yl-4-(2,2,6,6 tetramethylpiperidine-1-oxyl)-, products obtained from ascorbic acid.		drug carriers, recognition component in the targeting of specific cells.[51,52]	water soluble

The chemistries involved in the synthesis of chitosan derivatives include a variety of reaction conditions and purification methods. In the Schiff base reaction between chitosan and aldehydes or ketones, the product obtained is an aldimine or ketimine which is subsequently converted to the *N*-alkyl derivatives by hydride reduction with borohydride, generally known as reductive amination. The thiolated chitosan series is produced by reacting chitosan with coupling reagents bearing thiol moieties. Water soluble carboxymethyl chitosan is obtained by chitosan's reaction with glyoxylic acid while cationic derivative *N*,*N*,*N*-trimethyl chitosan is synthesized *via* reductive methylation under alkaline conditions at an elevated temperature. A cross-linked chitosan marketed as Chitopearl® is produced by the reaction of chitosan with excess of 1,6-diisocyanatohexane which is later exposed to water vapour. This polyurethane-type chitosan is used in chromatography and as an enzyme support. To produce alternative sugar linked chitosan, the polymer undergoes reductive *N*-alkylation with sodium cyanoborohydride and a sugar/sugar-aldehyde derivative. These sugar derivatives are mainly used in targeted drug delivery. Chitosan can also form composites with inorganic compounds such as phosphate where a chitosan-hydroxyapatite scaffold was used in cell growth studies. This range of different chemical reactions possible with chitosan makes this polymer an attractive material for the preparation of many functional polymer products (Figure 1.4).[23]

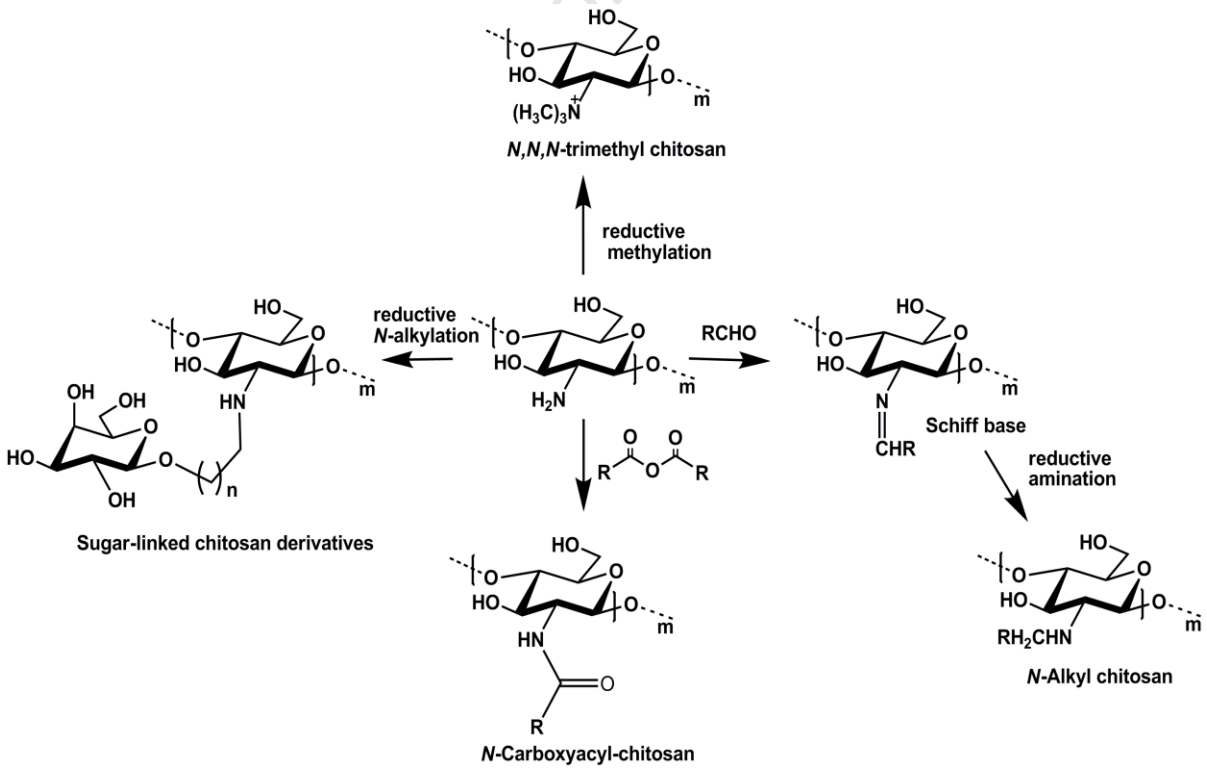


Figure 1.4: Various chitosan chemistries.

Chitosan derivatives of particular importance to this study are those that enhance aqueous solubility and improve antimicrobial activity. Selected examples are discussed below.

1.4.3. Thiolated chitosan

Thiolated chitosan derivatives (Table 1: **2**, **3** & **4**) improve the solubility, mucoadhesiveness, gelling and permeation properties of chitosan. Thiol-containing compounds have previously been immobilized on the polymeric backbone of chitosan; so far this has produced thioglycolyl chitosan (chitosan-TGA), *N*-acetylcysteinyl chitosan conjugates (chitosan-NAC) and chitosan-4-thio-butyl-amidine (chitosan-TBA).[42,53,54] Improved mucoadhesiveness is due to the covalent bonding of the polymer thiol groups to cysteine rich subdomains of glycoproteins, which overall, increases the tensile strength of the thiolated chitosan. The gelling properties are a direct result of disulfide bond formation.[41,53,55]

Wang *et al*. synthesized chitosan-NAC and studied it as a vehicle for nasal insulin administration.[38] In a study conducted by Zhang *et al*., oxidized dextran and the chitosan-NAC were formulated into an interpenetrating double-network hydrogel.[56] This hydrogel resulted from disulfide bonds and Schiff base formations between the oxidized dextran and chitosan-NAC. This gel was found to be mechanically strong with a relatively short time required for gelation to occur. *In vitro* viability tests show that the hydrogel and degradation products thereof are non-cytotoxic. In addition, this conjugate was found to inhibit *E. coli* which is commonly present in contaminated water. In a study by Perez-Giraldo *et al*. it was demonstrated that chitosan-NAC reduced the formation of biofilms of *Staphylococcus epidermidis* on a polystyrene surface.[57] Another study showed that chitosan-NAC reduced adhesion of *Streptococcus pneumoniae* and *Haemophilus influenzae* to oropharyngeal epithelial cells *in vitro*.[58] In a similar trend, this conjugate inhibited attachment of the disease causing bacterium, *Moraxella catarrhalis* to pharyngeal epithelial cells.[59] Olofsson *et al.* demonstrated using 10 different bacterial strains that chitosan-NAC is able to inhibit the formation of bacterial biofilms on solid surfaces.[60]

Chitosan-TGA has been found to exhibit an increase in mucoadhesion by a factor of 10 compared to chitosan. This polymer is biodegradable and has improved swelling properties.[61] Kast *et al.* has reported on the use of chitosan-TGA as a promising new scaffold material for tissue engineering.[40] In an earlier study by Kast *el al*., chitosan-TGA was used as a vehicle for the vaginal application of clotrimazole (1-[(2-chlorophenyl)(diphenyl)methyl]-1*H*-imidazole) in treatment of mycotic infections. In this study it was postulated that the antimycotic effect of clotrimazole may be enhanced due to the inherent antimycotic properties of chitosan and chitosan–TGA.[62]

Bernkop-Schnürch *et al.* showed that chitosan-TBA has improved gelling and mucoadhesive properties compared to the parent chitosan. Prinz *et al*. has reported on the cosmetic as well as the pharmaceutical uses of chitosan-TBA mainly as a preservative due to the inherent antimicrobial properties of the polymer.[41]

Other thiolated derivatives of interest are mercapto chitosan derivatives. Cárdenas *et al*. have studied *N*-(2-hydroxy-3-mercaptopropyl)–chitosan, mercaptoacetate chitosan and *N*-(2-hydroxy-3-methylaminopropyl) chitosan as heavy metal retention agents. These polymers were used for the recovery of copper and mercury.[63] In a separate study, Chang *et al*. used mercaptoacetyl chitosan in the removal of Cu^{2+} and turbidity from wastewater.[64] The antimicrobial attributes of these mercapto derivatives has not been studied. It is possible that the metal scavenging properties of these polymers may make these polymers attractive antimicrobials which would inhibit bacteria by the binding of essential metals necessary for their survival.

1.4.4. Quaternary chitosan derivatives

Quaternary derivatives of chitosan improve the inherent properties of chitosan and increase solubility due to the presence of the quarternized nitrogen. The two better known quaternary chitosan derivatives are 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (CHI-Q188) and *N,N,N*-trimethyl chitosan chloride (TMC) (Table 1.1).[24]

CHI-Q188 was first synthesized by Lang *et al.*, in the reaction of chitosan and glycidyltrimethylammonium chloride. It was tested as a preservative in personal care products such as hair gels and skin cream.[46] Daly *et al*. later synthesized this polymer using commercially available 3-chloro-2-hydroxypropyltrimethylammonium chloride (Quat 188).[47] This alternative method produces the glycidyl reagent *in situ* reducing safety concerns related to the use of epoxides in a large scale synthesis.[65] CHI-Q188 demonstrated antimicrobial properties and could act as a biocide. This polymer has been successfully tested as an antimicrobial agent against *E. coli*, *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa,* exhibiting a biocidal activity of at least an order of magnitude higher than other chitosan derivatives.[47] Ali *et al*. assessed the applicability of CHI-Q188 as a flocculent in water treatment compared to native chitosan. The study concluded that chitosan itself is a good flocculating agent and the modified chitosan with a moderate molecular weight and a moderate charge density showed the best flocculation performance.[48] Numerous other applications of this polymer have also been reported.[66,67]

First prepared by Domard *et al*., TMC is the simplest quaternized derivative of chitosan.[68] It is more soluble than chitosan, and can dissolve in either acidic or basic media. This polymer is typically prepared by reacting chitosan with sodium iodide and methyl iodide (MeI) in the

presence of a base. The *N,N,N*-trimethylammonium iodide formed on this polymer easily exchanges to a *N,N,N*-trimethylammonium chloride ion if available in solution.[69] Repeating the quaternization reaction more than twice yields a higher degree of quaternization (DQ), however, this comes at the cost of decreased water solubility as reported by Sievel *et al*.[70] De Britto *et al*. have synthesized TMC using dimethylsulfate as an alternative methylating agent.[71] This synthetic method produced TMC with varying DQ's where the DQ was found to be time and temperature dependent. Jia *et al*. showed that the antimicrobial activity of quaternized chitosan derivatives including TMC is greater than that of native chitosan when tested against *E.coli*.[43] Kim *et al*. found that TMC inhibited *S. aureus* and that the antimicrobial activity of quaternized chitosan derivatives increases with an increase in the chain length of the alkyl substituent.[72] In addition, TMC was found to inhibit the bacterium *Listeria innocua* to a greater extent compared to chitosan.[73] It is clear that these quaternary derivatives are superior in antimicrobial activity compared to native chitosan.

1.4.5. Carboxyalkylated chitosan derivatives

Carboxyalkylation refers to the introduction of carboxyl groups onto the polymer backbone. The polymer therefore has carboxylic acid and amino groups present which makes the polymer amphoteric. When the pH of the isoelectronic point is reached, the polymer does not dissolve.[65] Therefore these derivatives of chitosan lead to increased solubility in both neutral and basic solutions without affecting the activity of the polymer. Carboxymethyl chitosan (CMC) is synthesized by carboxymethylation of the amine or hydroxyl groups present in chitosan. This anionic chitosan derivative has been used in food and cosmetic applications, in the development of protein drug delivery systems, as a chelating agent for the recovery of metals, for superior adhesion and as an antimicrobial agent.[24,74]

1.4.6. 6-Deoxy-6-amino chitosan

A modification which has greatly improved the solubility of chitosan is the synthesis of 6-deoxy-6-amino chitosan (Table 1.1, **10**).[32,33] This polymer was first prepared by Satoh *et al.* by way of intermediate *N*-phthaloylchitosan synthesis followed by 6-azidation and reduction to the amine.[32] Jardine *et al.* reported an improved synthesis of 6-deoxy-6-amino chitosan using a pathway that did not include halogenated intermediates.[75] Yang *et al*. tested the antimicrobial efficacy of this polymer against *S. aureus*, *E.coli*, *Pseudomonas aeruginosa* (*P. aeruginaos*), and *Aspergillus niger* (*A. niger*).[45] The study found that this modified chitosan derivative showed much higher activity compared to chitosan at pH 5.4 with minimum inhibitory concentrations (MICs) of between 0.025 % and 0.1 % (w/v). In addition, 6-deoxy-6-amino chitosan was an effective antimicrobial at a pH of 6.5 and 7.5 in an acetic acid/sodium acetate medium as well as at pH 6.6-8.45 in distilled water.[76] The trimethylated and triethylated derivatives were synthesized and their antibacterial activity was tested

against *S. aureus.*[77] This study revealed that these polymers had a higher antibacterial activity against *S. aureus* compared to the parent polymers.[77]

1.5. Antimicrobial applications

Waterborne pathogens are clearly distinguished from drinking-water contaminants by the WHO.[10] Waterborne pathogens differ in their characteristics, behaviour and resistance. Common pathogens with high threat levels in contaminated drinking water are shown in Table 1.2.[10]

Table 1.2: Typical pathogens in contaminated water including examples and diseases caused.[10]

Pathogen	Example	Disease
Bacteria	*Burkholderia pseudomallei*	melioidosis
	Campylobacter jejuni	gastroenteritis
	E. coli – Pathogenic	gastroenteritis, urinary tract infections, and neonatal meningitis
	E. coli – Enterohaemorrhagic	acute hemorrhagic diarrhea, hemolytic uremic syndrome
	Salmonella Typhi	typhoid
	Shigella spp.	shigellosis
	Vibrio cholera	cholera
Viruses	Enteroviruses	polio, hand, foot and mouth disease
	Hepatitis A	hepatitis virus
	Hepatitis E	hepatitis E virus
	Noroviruses	viral gastroenteritis
	Rotaviruses	severe diarrhoea, gastroenteritis
Protozoa	*Acanthamoeba* spp.	keratitis, encephalitis
	Cyclospora cayetanensis	gastroenteritis
	Entamoeba histolytica	intestinal infection, liver abscess
Helminths	*Dracunculus medinensis*	dracunculiasis
	Schistosoma spp.	schistosomiasis

Disinfection of water prior to use is an important part of the water treatment process. There is a constant search for safe and affordable means of disinfecting water. Current water treatment processes are successful in the removal of certain infectious agents however; new hazardous agents continue to appear that require innovative new treatment methods. Water is typically treated with chlorine due to its potency and low cost however, chlorine alone does not remove all pathogens present in water. Pathogens resistant to chlorine treatment include

C. parvum and *Mycobacterium avium*. Other means of disinfection which have been tested in water treatment include the use of UV light (photochemical inactivation of pathogens) or ozone (powerful oxidizing agent toxic to waterborne pathogens). Both UV and ozone can be used in combination with chlorine for disinfection since these are effective against *C. parvum*. However, ozone can potentially form carcinogenic disinfection by-products. Therefore, municipalities are hesitant to switch to these disinfection methods. In developing countries, the use of sunlight irradiation for the disinfection of water contained in polyethylene terephthalate bottles has been promoted to kill pathogens. In addition, it has been suggested that sodium hypochlorite be utilized at the point of use for drinking water disinfection.[78]

Activated carbon has also been used in water treatment since 1500 BC, producing water which does not contain undesirable tastes, odours, particulate matter and other impurities. However, it was found that these filters are easily contaminated and therefore water leaving these filters contains more bacteria than before. To remedy this problem, scientists have turned to one of the oldest antimicrobial technologies available, i.e. silver metal.[79]

1.5.1. Antimicrobial Silver

Silver (Ag) is one of the most widely used metals in the world. It has been proven as safe for use in various applications. The use of Ag has been known for centuries, the earliest recorded use for medicinal purposes was in the 8th century. Ag has strong antibacterial properties exhibiting a broad spectrum of action against an estimated 650 disease causing organisms.[80,81] Bacterial resistance to commonly used bactericides is on the rise due to the development of drug resistant strains of bacteria. Since many antimicrobial agents have unwanted side-effects, the search continues for new, non-toxic biocidal agents. Ag and Ag ions (Ag^+) are favoured in the development of health care products as they have been found to be wound-healing agents with low toxicity.[82]

The mechanism by which Ag induces bactericidal activity is not clear, it has been postulated that the ionic Ag interacts with the thiol groups of enzymes and inactivates these important enzymes. This activity of Ag was supported by Feng *et al.* who showed experimentally that bacterial DNA is unable to replicate after it has been exposed to Ag ions.[83] Feng *et al.* and Nover *et al.* have also reported structural changes in the cell membrane in addition to the formation of electron-dense granules formed by Ag and sulfur.[83,84]

When moving to the nanoscale, certain properties of elements are improved due to the higher surface area to volume ratio. Ag nanoparticles have been produced by various methods such as the chemical reduction of Ag ions with or without stabilizing agents,

thermal decomposition in organic solvents, as well as chemical and photoreduction in reverse micelles.[85] Morones *et al.* tested Ag nanoparticles against gram-(-ve) bacteria, the study found that Ag nanoparticles attach to the cell membrane and inhibits proper functioning. Secondly the nanoparticles penetrate the bacteria and prevents DNA replication, and at the same time release Ag ions which add to the bactericidal effect of the nanoparticles.[86]

Furno *et al.* has studied the possibility of coating implantable medical devices with Ag nanoparticles in order to reduce infections.[87] Ag is bacteriostatic as well as bactericidal. In addition, Ag nanoparticles have an effective biocidal concentration at the nanomolar level rather than at the micromolar level as for Ag ions.[88] However, there have been concerns about the safety of nanosilver and its effect on humans. It has been suggested that nanosilver is potentially more toxic compared to its bulk counterpart as it is more chemically reactive and easily ionized. Ag nanoparticles release Ag ions and this "indirect toxicity" is the source of the concerns regarding the use Ag nanoparticles in commercial products.[89] Due to the popularity of silver nanoparticles, there has been significant interest in a method for the 'green synthesis' of these particles. Green synthesis involves three steps, 1) choice of solvent, 2) an environmentally friendly reducing agent and 3) selection of a nontoxic stabilizer.[90]

Recently chitosan has been employed, in the synthesis of nanoparticles to control the formation and dispersion stability of nanoparticles. The primary amines and hydroxyl groups present in chitosan allows the polymer to efficiently coordinate metal ions, therefore producing particles with smaller dimensions either by chelation or an ion exchange route.[28,91] Chitosan and the Ag particles are involved in an electrostatic interaction where the surfactant ions adsorb onto the electrophilic metal surface. This adsorption creates a multi-layer resulting in a coulombic repulsive force between the nanoclusters, thereby preventing aggregation.[92,93] Chitosan−Ag complexes have been reported by Zhan *et al*. and these complexes exhibited antibacterial activity.[94] Due to the enhanced antimicrobial properties of both Ag nanoparticles and chitosan, respectively, the evaluation of silver loaded chitosan derivatives as antimicrobial agents has been explored. Sanpui *et al.* investigated the efficacy of a chitosan−Ag nanoparticle composite against *E. coli* and results indicated that the composite had a higher antimicrobial activity compared to the parent polymers.[95] Chen *et al.* synthesized a thiourea chitosan−Ag^{+} complex which displayed a wide spectrum of antimicrobial activities against *S. aureus*, *E. Coli*, *Bacillus subtilis*, *Aspergillus flavus*, *Mucor bacilliformis* and *Paecilomyces variotii*.[96] MICs were found to be 20 times lower than that reported for chitosan.[145] Wei *et al*. produced Ag-impregnated chitosan films and tested these films together with pure chitosan films for antimicrobial efficacy against *E. coli*. The Ag

loaded films showed both fast and long-lasting antibacterial effectiveness compared to the chitosan films.[97] Recently, Sharma *et al.* has reported the synthesis of alginate-Ag-chitosan blended films which were tested for antibacterial activity. The films demonstrated excellent antibacterial activity against both gram−(-ve) and gram−(+ve) bacteria with higher activity against gram-(+ve) bacteria.[98] Ghosh *et al.* synthesized a hybrid chitosan-Ag film which was applied as an antimicrobial agent against *E.coli*. This film was found to inhibit *E.coli* growth and could be reused with the same result 3 months later. The film showed good mechanical stability and has been proposed as an antibacterial agent for use in water treatment.[82] In addition, Anitha *et al.* have synthesized *O*-carboxymethyl chitosan and *N*,*O*-carboxymethyl chitosan nanoparticles which displayed higher activity compared to chitosan when tested against *S. aureus*.[99] This carboxymethyl chitosan derivative is also an excellent metal chelating agent which forms insoluble metal chelates with transition metals such as Cu^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, Co^{2+} and Cd^{2+} at a neutral pH.[100]

Chitosan which is positively charged under acidic conditions, is attracted to the negatively charged cell wall thereby increasing cell wall permeability which leads to rupturing of the cell and loss of the intracellular contents. However, beyond pH 6 this polymer is ineffective as the amino groups are no longer protonated therefore derivatives containing a quaternary ammonium moiety such as TMC and CHI-Q188 are stronger antimicrobials compared to chitosan. Li *et al.* reported that chitosan and Ag nanoparticles are promising antimicrobial nanomaterial's for the disinfection of water and control of microbial growth.[101]

1.5.2. TB treatment

Tuberculosis (TB) is the second deadliest infectious disease in the world, after AIDS (Acquired Immunodeficiency Syndrome). TB is a bacterial infection caused by the bacterium *Mycobacterium tuberculosis (M.tb)*. The primary target is the lungs, however, TB may also reside in other organs.[102] TB is an airborne disease which is transmitted when an infected individual coughs, sneezes, talks or laughs. Although mycobacteria are not considered water borne, the transmission of TB occurs in water droplets. Once an individual is infected, the disease either ends up in the active form which is contagious or the latent form where the individual may only develop symptoms years later.[103] The map shows that TB incidence is the greatest in Southern African countries for 2010 (Figure 1.5).[104]

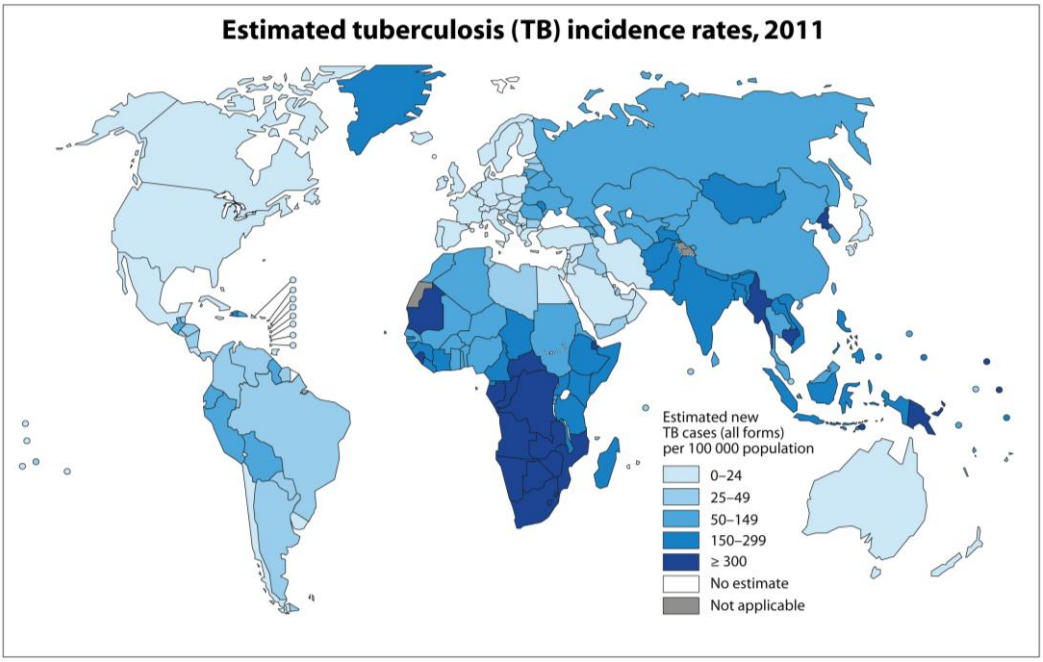


Figure 1.5: The WHO estimated TB incidence worldwide for 2011.[104]

Due to the increase in the development of drug resistance, new anti-TB drugs and delivery methods for existing drugs are highly sought after.

Chitosan's cationic nature allows the polymer to have good mucoadhesive and membrane permeability-enhancing properties.[105] Recently a chitosan-poly(ethylene glycol) polymer network was loaded with the 1st line anti-TB drug isoniazid (INH) and studied as a possible controlled drug delivery agent. In addition due to the inherent anti-bacterial properties of chitosan, the action of INH may be enhanced.[106] Since bacterial cell walls are negatively charged, the positively charged chitosan binds to it and allows for the drug to be released as it slowly degrades.[105] Rafeeq *et al.* synthesized chitosan-tripolyphosphate (TPP) nanoparticles loaded with INH and evaluated these particles for the treatment of TB. The particles showed good encapsulation efficiency and good release profiles following first order kinetics.[107] Chitosan nanoparticles demonstrated great potential for utility in pulmonary delivery of therapeutics, directly to the site of infection thereby, allowing for more efficient treatment of TB.[107]

There are some disadvantages associated with chitosan, such as limited solubility. Quaternized chitosan derivatives have increased water solubility, cationic activity, bioadhesive properties and permeation enhancing and may be a viable alternative.[66] Chitosan and its derivatives have not been extensively studied as anti-mycobacterial agents. However, in a recent study by Vavříková *et al.*, *O*-carboxymethyl chitosan and *N*-succinyl chitosan were synthesized and loaded with INH, PZA and the second-line anti-TB drug

ethionamide (ETA). Results showed that the polymers themselves had better inhibitory activity compared to the drug loaded polymers.[108] The inherent antimycobacterial activity of these chitosan derivatives was interesting and holds potential for the development of anti-tubercular materials.

Chitosan and its derivatives have been found to have many uses that have reached the market. However, its utility in water remediation applications has not yet been fully exploited. A short review of water and the remediation process is given here followed by the role of chitosan in the water treatment process.

1.6. Water treatment

Conventional water treatment methods such as filtration, sedimentation or air-stripping methodologies are not applicable in the removal of certain contaminants. Therefore treatment methods are needed that are ideal for removal of such contaminants and should be endorsed by regulatory agencies. It should be cheap and generate minimal amounts of waste. Such a method should also be ideally unaffected by other constituents present in the water and should not create new problems in other sections of water treatment.[109] Alternative treatment methods available include; ion exchange, bioremediation, adsorption by activated carbon or modified activated carbon, membrane filtration, chemical/catalytic reduction, electrochemical reduction or a combination of integrated technologies.[110] Certain pollutants are not retained by conventional treatment methods and can pose an environmental and health risk. Much effort has been dedicated to remove nitrates and phosphates from water, but not the contaminant perchlorate. The use of ion selective biopolymers for perchlorate removal could be advantageous. Selected methods will be discussed in relation to perchlorate.

1.6.1. Perchlorate

Perchlorates are defined as the salts derived from perchloric acid. Common perchlorate salts include: ammonium perchlorate, lithium perchlorate, magnesium perchlorate, potassium perchlorate and sodium perchlorate. The perchlorate ion (ClO_4^-) consists of Cl in the +7 oxidation state bonded to four O atoms (Figure 1.6).[111]

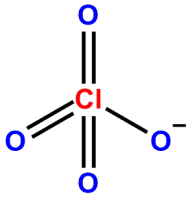


Figure 1.6: The perchlorate ion, ClO_4^-.

Perchlorate occurs naturally in the environment and therefore surface or ground water may contain trace levels of perchlorate. Texas and North Chile are two places with a particularly high concentration of natural ClO_4^-.[111] It is produced atmospherically by oxidation of Cl^- and Cl^- oxyanions by O_3 in arid and semiarid conditions. Due to the natural abundance of Cl^- and O_3, this contributes to the presence of ClO_4^- in the environment, however; the rate at which ClO_4^- forms is very slow.[112] This salt is commercially produced via the electrochemical oxidation of sodium chloride to sodium chlorate, which is further oxidized to sodium perchlorate.[113] It is mainly used in rocket fuel and fireworks, however it has other applications such as components of air bags, leather tanning, bleach and in fertilizers.[9,114] Most countries are not required by law to record the quantity of ClO_4^- manufactured or obtained from other sources.[113]

Perchlorates are highly water soluble and easily leached from soil into groundwater. Both human activity and natural occurrence has led to the presence of ClO_4^- in the environment i.e. in soil and groundwater.[114] In addition, the Food & Drug Administration (FDA) has detected ClO_4^- in food and milk, which may be as a result of crop irrigation using contaminated water or livestock being fed contaminated fodder. Perchlorate enters the human body if the individual has had food or water containing large amounts of this ion. Once in the body, ClO_4^- eventually ends up in the blood stream which carries it to all parts of the body. The body quickly eliminates ClO_4^- via the kidneys although large amounts of ClO_4^- may accumulate in organs such as breast tissue, thyroid and salivary glands.[111] The United States Environmental Protection Agency (EPA) first added ClO_4^- to its Contaminant Candidate List (CCL) in 1998.[114] This is due to the fact that ClO_4^- is a harmful contaminant that met the Safe Drinking Water Act's three requirements for being a regulated contaminant. These requirements are as follows,

1) It inhibits proper functioning of the thyroid gland thereby restricting production of hormones needed for normal growth and development,

2) The ClO_4^- level in drinking water are higher than anticipated and

3) There is the possibility of reducing a potential health risk for millions of people.[111]

The thyroid gland is the organ most affected by ClO_4^- exposure, as it partially inhibits iodine uptake. Iodine is a necessary requirement for the synthesis of thyroid hormone which regulates oxygen consumption and metabolism in the body as a whole. The transport of iodide (I^-) from the bloodstream to thyroid follicle cells is inhibited by the possible competitive blocking of iodide binding to a sodium/iodide symporter. This symporter is responsible for catalyzing the simultaneous transfer of Na^+ and I^- across the membrane of the thyroid follicle

cells. Iodine uptake inhibition limits the amount of iodine available for the synthesis of the thyroid hormones thyroxine (T4) and triiodothyronine (T3).[111,113] T3 is important for the normal development of the nervous system. Lowering the level of available T3 may lead to adverse effects on a number of organs and systems.[115,116]

The most sensitive population in terms of ClO_4^- toxicity are pregnant women and children since the thyroid gland is important for normal growth and development. Up until the 16-20th weeks of gestation, the foetus is entirely dependent on maternal thyroid hormones therefore interference in thyroid hormone production is severely detrimental to unborn babies. Hypothyroidism (insufficient thyroid hormone production) in pregnant and lactating women has been found to cause delayed development and decreased learning capability in infants. In addition, hypothyroidism during childhood negatively affects child growth and cognitive motor functions.[116,117]

As mentioned earlier, there are no regulatory limits on the ClO_4^- levels present in drinking water, however; this issue is under review since ClO_4^- is a known endocrine disrupter. The minimum reported level in the test for ClO_4^- in water is 4 µg/L. The US EPA has found ClO_4^- present in a number of water sources at concentrations ranging from 4-100 µg/L.[113,118] As a result, several states in America have reviewed the legislation around ClO_4^- levels in their drinking water. California and Massachusetts however, are the only states to have adopted drinking water standards of 1 and 2 µg/L respectively.[113,118] A study conducted by Harvey *et al.* tested ClO_4^- levels in Namibian and South African biomes.[119] The study found ClO_4^- levels in water samples ranged from < 0.05 to 5.9 µg/L, soil samples had levels from 0.15 to 179 µg/L while plant samples had levels ranging from 150 to 1800 µg/L.[119] As stated in a recent review by Ye *et al*., ClO_4^- contamination is a worldwide concern in public and private sectors therefore removal methods are of great interest.[112]

1.6.2. Chemical reduction of ClO_4^-

One method used for perchlorate removal is the chemical reduction of the ClO_4^- ion. The reduction of ClO_4^- to the chloride ion is thermodynamically favourable, however; this reduction is not spontaneous.[113] Iron has been used as a reducing agent for other contaminants including chlorinated solvents in anoxic environments (absence of oxygen). The reduction of ClO_4^- to the chloride ion with iron is favourable; however, there is no kinetic evidence to support this. Therefore a catalyst such as UV light is used to facilitate the reaction. Gurol *et al.* successfully reduced ClO_4^- to Cl^- using Fe(0) in the presence of UV in an anoxic environment.[120] In addition, Kim *et al.* successfully removed ClO_4^- at a low pH in the presence of phosphoric acid, since this acid is known to bind strongly to Fe(0) and has been shown to form a complex with ClO_4^-.[120] In a study by Gu *et al*., the absorption affinity

and capacity of strong-base anion-exchange resins was investigated for the removal of uranium and ClO_4^- in the presence of sulfate (SO_4^{2-}). This study found that ClO_4^- absorption in the presence of SO_4^{2-} is adversely affected; in addition the adsorbed ClO_4^- could not be removed from the resin using HCl. Therefore the ClO_4^- was degraded using a $FeCl_3$-HCl solution at an elevated temperature. This method yielded a complete reduction of ClO_4^- in < 1 h at 195 °C in the $FeCl_3$-HCl solution. This method offers a means to economically degrade ClO_4^- which does not affect the properties of the $FeCl_3$-HCl regeneration solution, and can therefore be reused, thus eliminating the production of secondary wastes.[121,122]

These favourable properties of Fe are enhanced on moving to the nanoscale. Nano scale Fe particles display unique characteristics and enhanced magnetic properties. These particles are synthesized usually via co-precipitation of Fe^{2+} and Fe^{3+} in the presence of a base. A number of macromolecules have been utilized as coating agents to protect and stabilize the Fe nanoparticles, examples of these include dextran, starch, arabic gum and chitosan.[123]

Chitosan has successfully been used to remove metals such as arsenic, chromium and lead as well as organic contaminants (e.g. chlorophenols, humic acids, dyes and colours) and certain anions. The removal of these contaminants is due to the presence of amino ($-NH_2$) and hydroxyl (OH^-) groups in chitosan which have high activities as absorption sites.[110] Xie *et al.* have successfully removed ClO_4^- from an aqueous solution using protonated chitosan in an acidic medium.[110]

Bajpai *et al.* have used chitosan-Fe nanoparticles to remove chromium from an aqueous solution.[124] In addition; Kaushik *et al.* have synthesized chitosan-iron oxide nanocomposite films and used these films as a glucose biosensor.[125] Su *et al.* reported the use of superparamagnetic Fe carboxymethyl chitosan nanoparticles in the separation of lysozyme which links to protein separation.[126] The synthesis of CHI-Q188 stabilized Fe nanoparticles was reported by Shen *et al*. and utilized as a potential MRI (Magnetic Resonance Imaging) contrast agent for cell tracking.[127] Belessi *et al.* reported the synthesis of TMC-Fe nanoparticles with well-defined cubic shaped particles ranging in size from 10 to 40 nm.[128]

1.6.3. Ion exchange processes in water purification

Ion exchange involves the exchange of ions across two phases. When an ion exchange resin is used, the resin is the insoluble phase to which an ion is electrostatically bound and this ion is exchanged with another of the same charge from the solution applied to the resin. There are cation and anion exchange resins that has low recyclability. These resins have been used in environmental remediation, wastewater treatment, biomolecular separations, water softening, chromatography, and hydrometallurgy as well as in catalysis. Commonly used resins are phenol-formaldehyde polymers, polyphenols, macroporous and ultrafine

resins to name a few. Subject to the effectiveness and affinity for particular ions, resins are classified as selective and non-selective.[129]

Ion exchange applications with biopolymers, in particular protein purification are the chromatography mainstay of the biotechnology industry. The proteins present in the sample adhere to the stationary phase to a different extent depending on their charge, leading to dissimilar elution times for each protein allowing successful separation of the proteins. These stationary phases may be anion or cation exchange resins or be affinity based. Currently there are conventional media (e.g. polystyrene, Sephadex, etc.) available which are routinely used to separate proteins however, interest in using polymers as alternative ion exchange media has developed.[130]

Cation exchange generally involves the binding of metal ions. Several studies have shown that chitosan and water insoluble derivatives thereof have been successfully used to bind toxic/hazardous metals such as Sn^{2+}, Sn^{4+}, (including organic tins), Hg^{2+}, Pb^{2+}, U^{6+} and transition metal ions such as Cd^{2+},Cu^{2+}, Cr^{3+}, Zn^{2+}, Ni^{2+} and V^{4+}. It has been suggested that the metal absorption capacity of chitosan is related to the particle size and amount of polymer present.[65] This type of exchange forms the basis in the development of flocculants.

The most common method of perchlorate removal is ion exchange where the ClO_4^- ion is exchanged for less harmful ions. Since ClO_4^- is a negatively charged ion, this is classified as anion exchange where ClO_4^- is exchanged for anions such as chlorides (Cl^-) or hydroxides (OH^-).[131] Originally ion exchange resins for perchlorate removal were costly, however, advances in technology, have led to decreased costs. Ion exchange resins are usually utilized in continuous flow processes, whereby water is pumped through the ion exchange resin and the ClO_4^- ion is exchanged with a different anion. After a certain amount of water is treated, the resin reaches its absorption capacity and is saturated with ClO_4^-. This resin is either disposed of or regenerated by ClO_4^- removal. A disadvantage associated with the use of general anion exchange resins, is the presence of competing anions in the water being treated. These anions such as nitrate and sulfate are present in much higher concentrations compared to ClO_4^-, and therefore compete with ClO_4^- for available anion exchange sites. As a result, the development of more selective anion exchange resins is needed. There are two types of ion exchange systems typically used in ClO_4^- removal, a regenerable and a non-regenerable system. With the regenerable system, a saturated resin is regenerated and reused whereas with the non-regenerable system the spent resin is destroyed after use.[131] Purolite® and Applied Research Associates, Inc. have developed a weak base anion resin which is perchlorate selective and regenerable. These resins are pH dependent, where at low pH the functionalized resins are positively charged ($R\text{-}NH_3^+$) facilitating anion exchange.

At a high pH, the functionalized resins lose a proton and are consequently uncharged (R-NH_2), and are therefore regenerated. This resin is less costly and still effective in removing ClO_4^- from contaminated water.[132] The primary method used by the US EPA to determine trace perchlorate in drinking water (EPA Method 314.0 and Method 314.1) makes use of the ion exchange column Dionex IonPac® AS16.[133,134] This column is made up of a highly cross-linked core and a Microbead™ anion exchange layer while the column substrate consists of a macroporous resin comprised of ethylvinylbenzene cross-linked with 55 % divinylbenzene. Quaternary hydrophilic ammonium groups are attached to the anion layer providing the positive charge.[12] Other resins which possess quaternary ammonium groups have been applied in ClO_4^- removal this includes the commercially available resins IRA 400 and IRA 900. These resins are composed of a microporous styrene divinylbenzene co-polymer with a *N*,*N*,*N*-trimethyl quaternary moiety.[135] Kim *et al.* synthesized a mono- or bifunctionalized mesoporous molecular sieve (SBA-15) containing *N* -((trimethoxysilyl)propyl)-*N,N,N*-trimethylammonium chloride (TSPMC) and *N*-((trimethoxysilyl) propyl)-*N,N,N*-tributylammonium chloride (TSPBC). These resins showed fast absorption equilibriums which were reached after 10 minutes compared to the commercially available IRA 900 which only reached equilibris after 5 hours.[136]

Carboxymethyl chitosan (CMC) is one of the few chitosan derivatives which has been investigated as a water purification resin. CMC has superior chelation properties compared to chitosan due to the presence of the carboxylic acid group on the nitrogen of the glucosamine unit of chitosan. This derivative has enhanced Fe, Co, Cu, Ni, Cd, Pb, Pt, Mn, Au, and Zn ion adsorption capacity which is advantageous in the recovery of metals from wastewater. In addition, metal ions can be loaded onto the polymer which may provide complementary properties for uses such as the recovery of organic or inorganic materials.[35,137] Boricha *et al.* have synthesized a *N,O*-carboxymethyl chitosan/cellulose acetate blend nanofiltration membrane which was applied in the removal of copper and chromium from industrial wastewater.[138] Li *et al*. synthesized an *O*-carboxymethyl-*N*-trimethyl chitosan chloride derivative which was used as a flocculent in the treatment of wastewater in sugar refineries.[139] The study found that this derivative was more effective than the parent compounds in the treatment of the wastewater.[139] Another quaternary CMC derivative synthesized was 3-chloro-2-hydroxypropyl trimethyl ammonium chloride (CTA) modified carboxymethyl chitosan. This derivative was tested as an amphoteric flocculant which showed improved solubility and salt-resistance compared to the native polymers chitosan and CHI-Q188.[140] Therefore the use of chitosan as a solid support in water treatment is a promising area of research.

Chitosan has been extensively used in the water purification sector mainly as a flocculent and metal chelator. Various chitosan derivatives have been studied in the remediation of water and have been applied in the prevention of point of source contamination and point of use contamination. Another application of chitosan in the water sector includes the removal of microbial pathogens present in drinking water. Due to chitosans inherent antimicrobial activity and the enhanced activity of its derivatives, the application of chitosan as an antimicrobial water filter is appealing.[137]

1.7. Aims and Objectives

Due to the increasing demand for clean water, interest in the remediation of water using natural polymers has become a major interest. Therefore the broader aim in this project was to synthesize and characterize a range of chitosan derivatives and to investigate the potential applications of these polymers in water remediation.

- To study the applicability of chitosan polymers as ion-exchange resins. The main hypothesis of this study is to determine if modifying chitosan improves the ion-exchange capacity of the polymer, in particular ClO_4^- exchange.

- To investigate the **antimicrobial efficacy** of chitosan derivatives in the presence and absence of Ag nanoparticles. The result will determine if these polymers can be developed into **water filters or membranes** with no, or reduced AgNP levels against bacteria and pathogens present in contaminated water. The **environmental impact** of long term AgNP use has not yet been fully assessed, but is a matter of concern.

- The design of anti-bacterial (including mycobacteria) films or fibres (electrospinned) based on chitosans that can be utilized in respiratory ultrafiltration membranes is the ultimate aim, but outside the scope of this research effort. However, the key objective is to obtain materials that does not only show promise in **antibacterial activity** but has a tendency to form films.

1.7.1. Specific Objectives

In order to evaluate modified chitosan for use as ion-exchange resins and the antimicrobial activity of these polymers, the specific objectives of this study was to:

1. Synthesize and characterize a range of chitosan derivatives namely, thiolated chitosan (*N*-acetylcysteinyl, (2*S*)-2-mercaptosuccinyl & thioglycolyl) and quaternized (CHI-Q188 & TMC) chitosan derivatives.

2. Synthesize and characterize the corresponding 6-deoxy-6-amino chitosan, thiolated and quaternized derivatives.

3. Determine if the 6-deoxy-6-amino chitosan derivatives results in higher loading and improved activity.

4. Explore the applicability of chitosan and quaternized chitosan (CHI-Q188 & TMC) derivatives to act as green ion exchange resins for water purification, including the removal of ClO_4^- from drinking water.

5. Load chitosan and the quaternized chitosan (CHI-Q188 & TMC) derivatives with Fe and apply in the removal of ClO_4^- from drinking water.

6. Test chitosan and the modified derivatives for antimicrobial activity.

7. Load chitosan, 6-deoxy-6-amino chitosan, thiolated chitosans (*N*-acetylcysteinyl, (2S)-2-mercaptosuccinyl &) and quaternized (CHI-Q188 & TMC) chitosan derivatives with silver ions and test the antimicrobial properties of these polymers compared to their native counterparts.

8. Test chitosan, the quaternized and thiolated chitosan derivatives as well as selected Ag loaded versions as potential anti-mycobacterial agents.

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CHAPTER TWO

SYNTHESIS AND CHARACTERIZATION OF CHITOSAN AND 6-DEOXY-6-AMINO CHITOSAN DERIVATIVES

Chitosan and 6-deoxy-6-amino chitosan are natural biopolymers which have shown great potential as biomedical agents due to their inherently favourable properties such as biocompatibility, chemical versatility, biodegradability and low toxicity.[1] This investigation focuses on the synthesis of chitosan derivatives (Figure 2.1) in order to explore their applications in water purification.

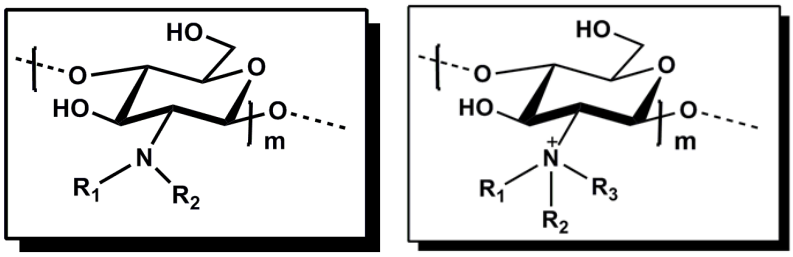


Figure 2.1: General structure of chitosan derivatives.

The corresponding 6-deoxy-6-amino chitosan derivatives were synthesized and characterized (Figure 2.2). Due to the presence of an additional amino group, it is thought that these derivatives will be functionalized at two sites therefore solubility and loading will be significantly increased.

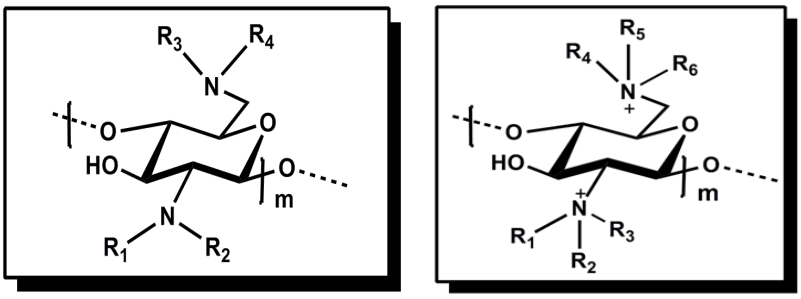


Figure 2.2: General structure of 6-deoxy-6-amino chitosan derivatives.

2.1. Synthesis and characterization of thiolated and quaternary chitosan derivatives.

The thiolated chitosan derivatives which have been synthesized and characterized include *N*-acetylcysteinyl chitosan, (2*S*)-2-mercaptosuccinyl chitosan and thioglycolyl chitosan. The synthesis of the water soluble cysteine and thioglycolic acid derivatives has been previously reported by Schimtz and Kast *et al*.[2,3] The quaternary chitosan derivatives, 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (CHI-Q188) and trimethyl chitosan chloride (TMC) were reported by Domard *et al*. and later by Lang *et al.* respectively.[4,5] These chitosan derivatives were synthesized in order to improve the aqueous solubility of chitosan for the purpose of testing these derivatives for antimicrobial activity and ion-exchange capacity in water treatment applications. The derivatives synthesized are shown below (Figure 2.3). The discussion of the synthesis follows.

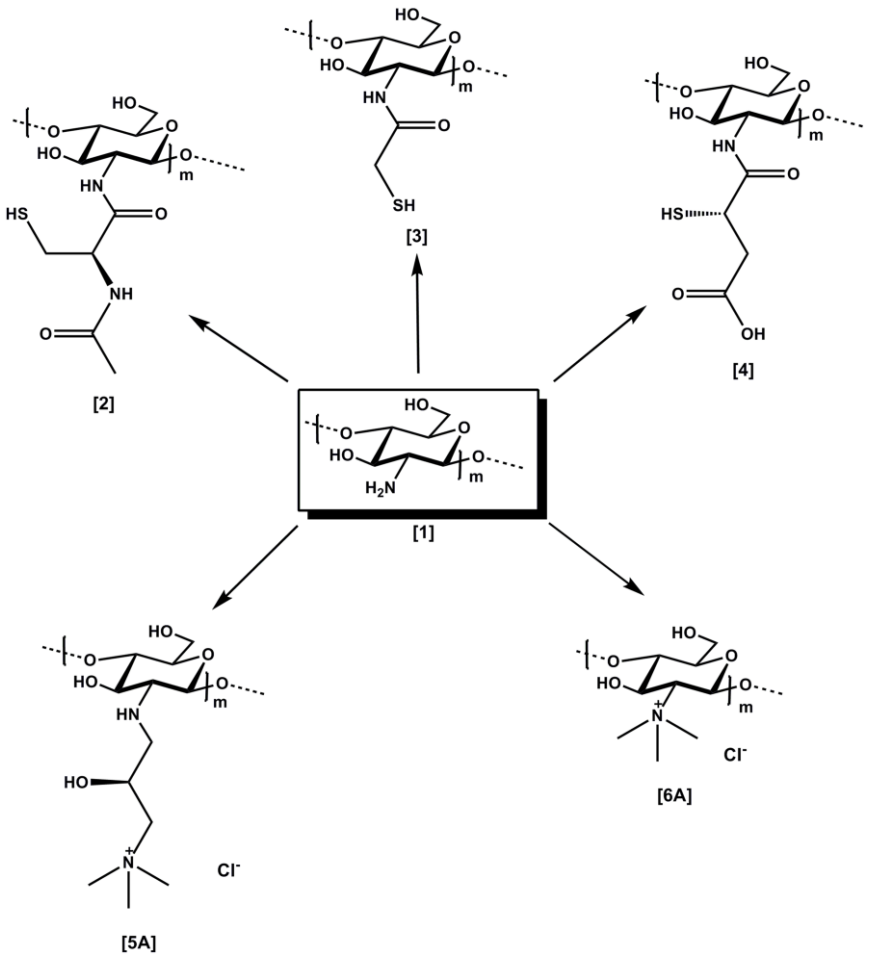
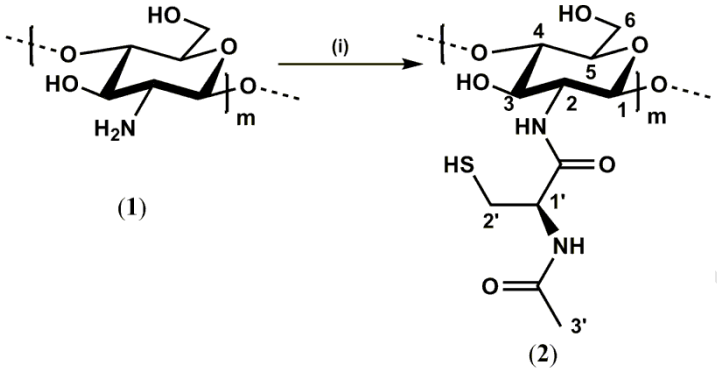


Figure 2.3: General synthetic scheme of thiolated and quaternary chitosan derivatives.

*2.1.1. Synthesis of N-acetylcysteinyl chitosan (**2**)*

The synthesis of *N*-acetylcysteinyl chitosan (**2**) was achieved by reacting chitosan with *N*-acetylcysteine utilizing the water soluble carboxylic acid activating agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Scheme 2.1).[2] The product was obtained as a white film in mass yield of 56 %.



Scheme 2.1. Reagents and conditions: (i) 1 % HCl, pH 5, *N*-acetylcysteine, EDAC, pH 4-5, RT, 6 hrs; 56 %.

The successful synthesis of *N*-acetylcysteinyl chitosan (**2**) was confirmed through analytical and spectroscopic analysis. NMR spectra showed resonances characteristic of modified chitosan (Figure 2.4).[6] NMR spectra were obtained in D_2O since the linking of the cysteine moiety to the chitosan backbone introduced highly hydrophilic –SH groups onto the polymer which led to a disruption of its crystalline structure thereby increasing solubility.[7] All resonances have shifted downfield and appeared at a higher chemical shift compared to native chitosan. The resonance at δ 5.14 ppm was assigned to H-1, at the anomeric carbon. The resonances that lie between δ 4.21 and 3.98 ppm were assigned to H-3 – H-6, while that at δ 3.74 ppm was attributed to H-2. The appearance of a resonance at δ 3.45 ppm corresponded to H-2' while the resonance at δ 2.29 ppm is assigned to H-3' and residual acetyl groups. The ^{1}H NMR spectrum agrees with that reported by Zhang *et al*.[7] The ^{13}C NMR confirmed the synthesis of the polymer, as the correct number of resonances were observed. The appearance of a resonance at δ 170.68 ppm was assigned to the C=O of the cysteine residue on the amide as compared to native chitosan. The resonance at δ 69.07 ppm was assigned to C-1' while that at δ 55.43 ppm corresponded to C-2 & C-3' respectively. The degree of substitution (DS) was calculated from the ^{1}H-NMR spectra using the following equation:

$$DS = \left[\frac{1_{(H\text{-}2)} \text{ x (Signal intensity due to substituent)}}{\text{No. of protons of substituent x (Signal intensity due to H-2)}} \right] \text{ x } 100\ \%$$

where H-2 refers to proton 2 on the polymer backbone.[8] This equation was used to calculate the DS of *N*-acetylcysteinyl chitosan (**2**) where H-2' was used as the reference. The DS was calculated to be 24 %.

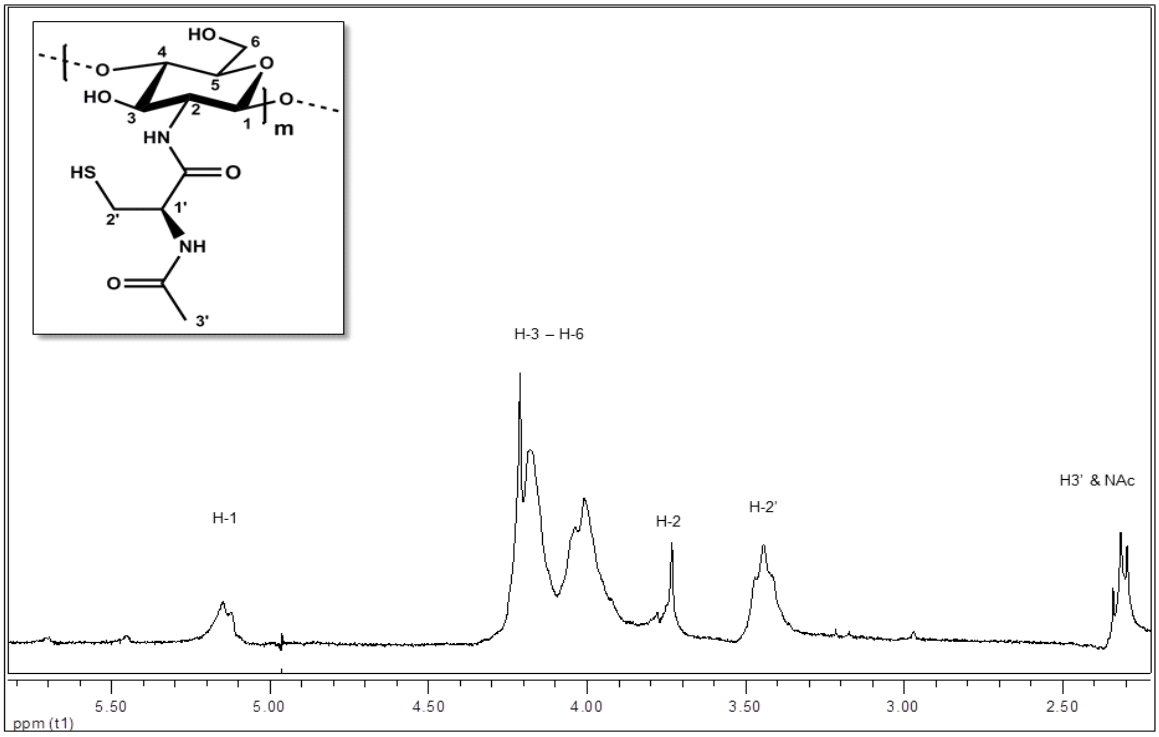


Figure 2.4: ^{1}H NMR of *N*-acetylcysteine chitosan **2** in D_2O.

The elemental analysis agreed with calculated values for the polymer with a DDA of 85 %.

The amount of thiol groups present on the polymer was determined using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent). This colorimetric assay involves the appearance of a yellow colour when Ellman's reagent reacts with sulfhydryl groups present on the polymer. In this study, the amount of thiol groups present on the polymer was determined by quantization of sulfhydryl groups based on molar absorptivity of the reactant. The amount and concentration of sulfhydryls in the sample was calculated from the molar extinction coefficient of DTNB (14 150 M^{-1} cm^{-1}).[10] The thiol content of *N*-acetylcysteinyl chitosan (**2**) was determined to be 18 µmol/g of polymer, which was lower than that reported in literature (62.4 µmol/g of polymer).[2] The effects of the change in reaction conditions such as concentration and reaction time, were studied by Schimtz *et al.* and Rong *et al*. respectively.[2,11] The results of these studies indicated that a higher EDAC concentration and a longer reaction time resulted in higher thiol loading of the polymers.[2,11]

This result is in agreement with the NMR data which suggests a low thiol loading onto the polymer.

The IR spectra (Figure 2.5) showed bands typical of those in chitosan with the introduction of a new absorbance band at 2056 cm^{-1} assigned to –SH. The C=O absorption bands is present at 1632 cm^{-1}. The amide absorption bands at 1520 and 1320 cm^{-1} are visibly stronger. The increase in strength of the amide absorption may be attributed to the presence of the additional amide group from the cysteine residue as reported by Wang *et al.*[9]

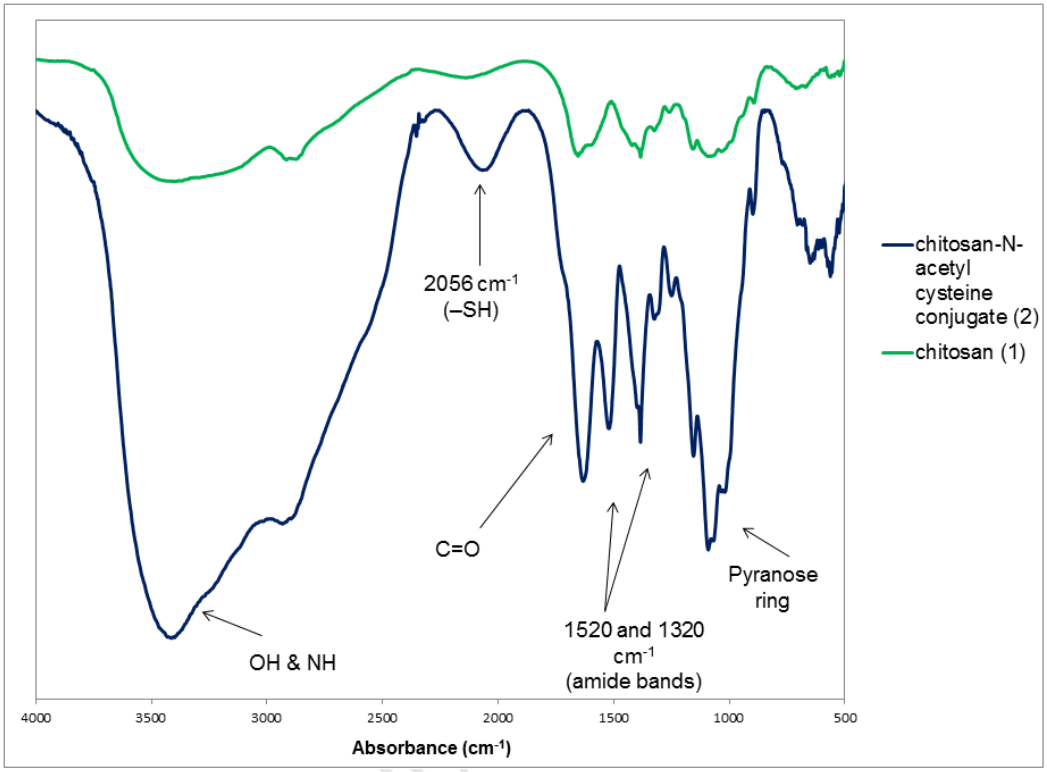
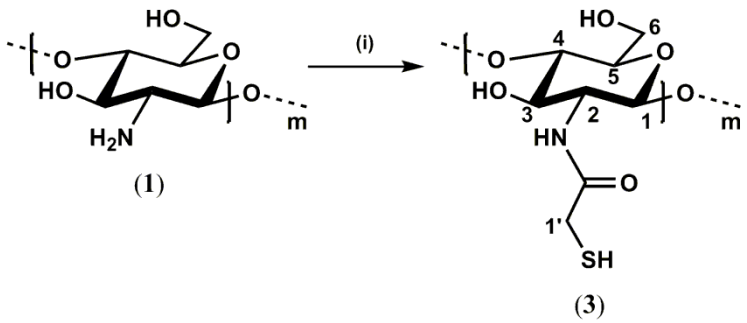


Figure 2.5: IR spectra of chitosan (**1**) and *N*-acetylcysteinyl chitosan **2** (KBr).

*2.1.2. Synthesis of chitosan thioglycolyl (**3**)*

The synthesis of polymer **3** was carried out using chitosan dissolved in an acidic solution which was reacted with thioglycolic acid (TGA) in the presence of EDAC for 3 hrs at room temperature.[3]



Scheme 2.2. Reagents and conditions: (i) 1 M HCl, H_2O, EDAC, thioglycolic acid, pH 5, RT, 3 hrs; 70 %.

The polymer was obtained as a fluffy white solid in a yield of 70 % (Scheme 2.2). The product was characterized using IR, NMR and elemental analysis. The thiol content was determined using the DTNB assay.

The ^{1}H NMR spectrum (Figure 2.6) showed resonances characteristic of modified chitosan with a slight downfield shift of the signals compared to native chitosan.[6] The resonance at δ 4.86 ppm was assigned to H-1 while those that appeared between δ 3.75 and 3.93 ppm were attributed to the ring protons H-3 – H-6. A resonance was observed at δ 3.36 ppm which was assigned to H-1', the signals at δ 3.19 and 2.07 ppm were due to H-2 and residual acetylated chitosan units respectively. The ^{13}C NMR displayed the expected number of resonances with an additional peak at δ 21.51 ppm assigned to C-1'. The DS was found to be 25 % using H-1' as the reference proton.

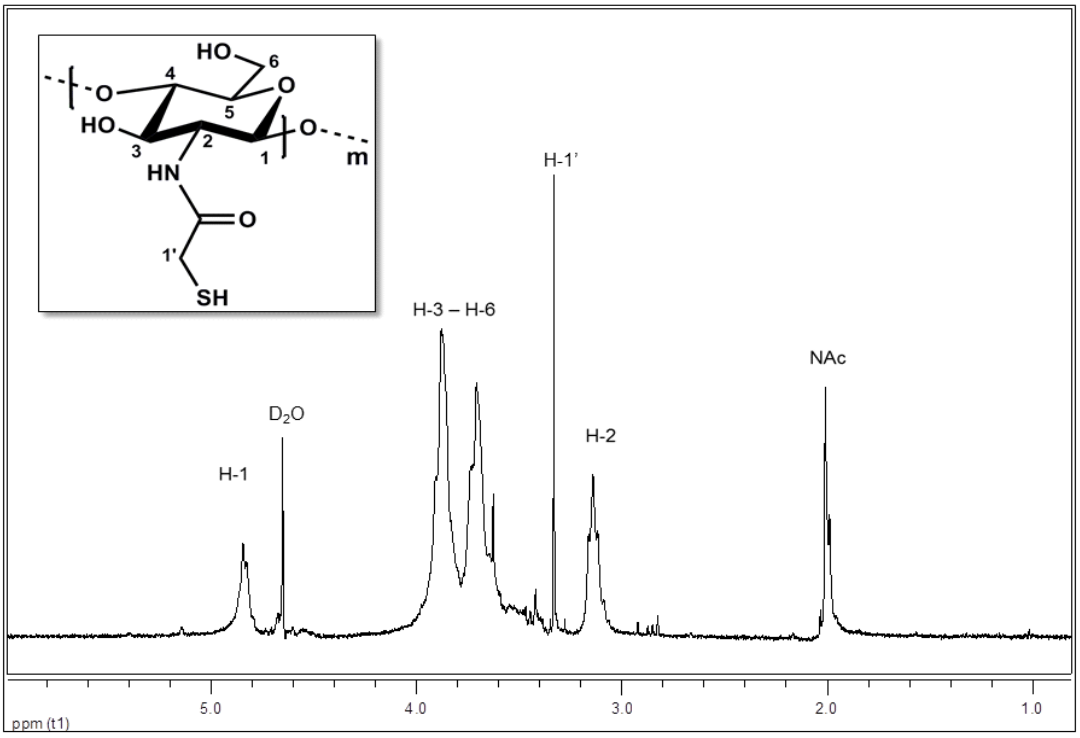


Figure 2.6: ^{1}H NMR of thioglycolyl chitosan (**3**) in D_2O.

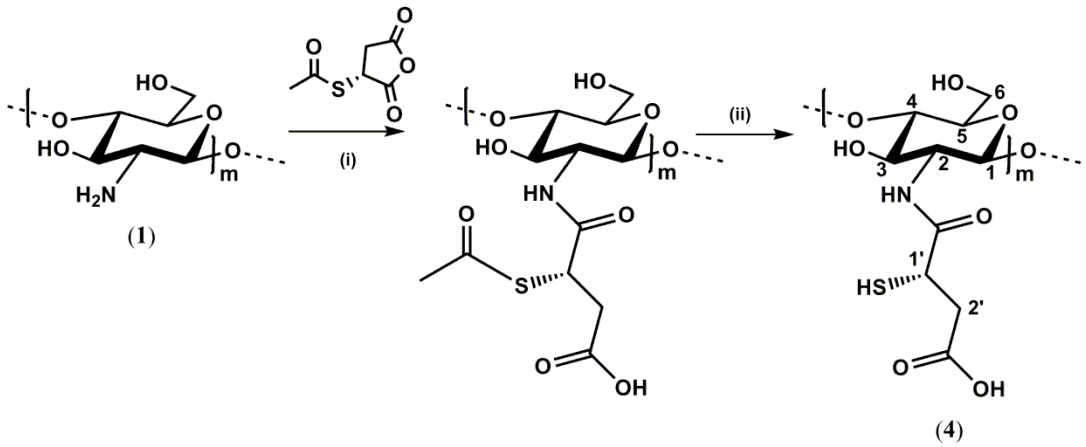
IR analysis revealed an absorption at 3413 cm^{-1} assigned to the –OH stretch. An absorption band at 2064 cm^{-1} corresponded to the –SH stretch, with expected absorption bands at 1628 and 1075 cm^{-1} assigned to the NH and pyranose stretches respectively.

The thiol content of the compound was measured to be 16 µmol/g of polymer using the DTNB assay which was comparable to that reported by Kast *et al.* (~ 19 µmol/g of polymer).[3] This result is in agreement with that obtained from NMR confirming a low thiol loading.

Elemental analysis values for C, N and H agreed with the calculated values for thioglycolyl chitosan.

2.1.3. Synthesis of (2S)-2-mercaptosuccinyl chitosan (***4***)

The (2*S*)-2-mercaptosuccinyl chitosan (**4**) polymer was synthesized according to the procedure reported by Koo *et al*. which involved the reaction of chitosan in DMF with *S*-acetylmercaptosuccinic anhydride (Scheme 2.3).[12]



Scheme 2.3. Reagents and conditions: (i) DMF, *S*-acetylmercaptosuccinic anhydride; RT, 3 hrs (ii) NH_4OH (25 %), RT, 12 hrs; 98 %.

The thiolated chitosan derivative was synthesized by the ring-opening reaction between the amino group of chitosan and *S*-acetylmercaptosuccinic anhydride and subsequent deacetylation. The protection of the thiol moiety by the *S*-acetyl group allows for long-term storage, preventing oxidation.[13]

Polymer **4** was obtained as an off white solid with a yield of 98 %. The product structure was supported by IR, NMR and elemental analysis. The ^{1}H NMR spectrum was obtained using TFA in D_2O as solvent (Figure 2.7). This spectrum revealed resonances characteristic of a modified chitosan, where the bands are now broader and appear overlapped. The anomeric proton H-1 appeared at δ 4.84 ppm while the resonances between δ 3.65 – 3.86 ppm corresponded to the protons of H-3 – H-6. The signal at δ 3.13 ppm was attributed to H-2, while new resonances appeared at δ 2.95 and 2.79 ppm and were assigned to H-1' and H-2' respectively. The signal at δ 2.34 ppm was assigned to the residual *S*-acetylated units of the thiol which were partly removed by the second step of the reaction. The resonance at δ 2.02 ppm corresponded to the residual *N*-acetylated units of the polymer. The ^{13}C NMR spectrum

displayed the expected number of resonances. The appearance of a signal at δ 175.02 ppm confirmed the presence of the amide C=O groups on the alkyl chain. The new resonances at δ 40.01 and 36.86 ppm indicates introduction of C-1' and C-2' respectively. The DS was determined using the NMR spectra, where H-2' was used as the reference. The DS was found to be 50 % for this polymer.

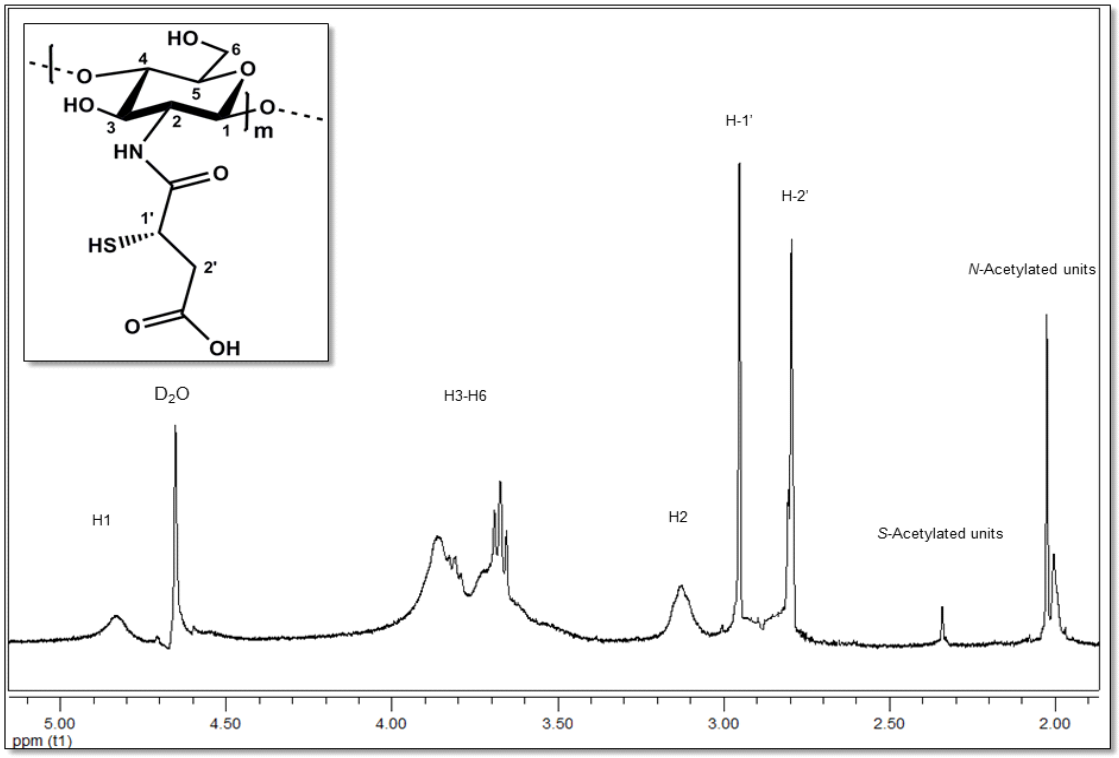


Figure 2.7: ^{1}H NMR spectrum of (2*S*)-2-mercaptosuccinyl chitosan **4** in 2 % TFA/D_2O.

The thiol content of this polymer was determined using the DTNB assay. Results indicated that the polymer had a thiol content of 40 µmol/g of polymer. This confirms the incorporation of a thiol moiety on the polymer backbone as indicated by NMR spectroscopy.

Elemental analysis values were within range of calculated values.

The IR spectrum (Figure 2.8) indicated the characteristic absorption bands of OH at 3413 cm^{-1} and the C-H aliphatic band at 2922 cm^{-1}. A band was observed at 2125 cm^{-1} corresponding to the SH stretch while the band at 1724 cm^{-1} was assigned to the C=O stretch. The absorbances at 1660 and 1572 cm^{-1} have been assigned to N-H stretching vibrations.

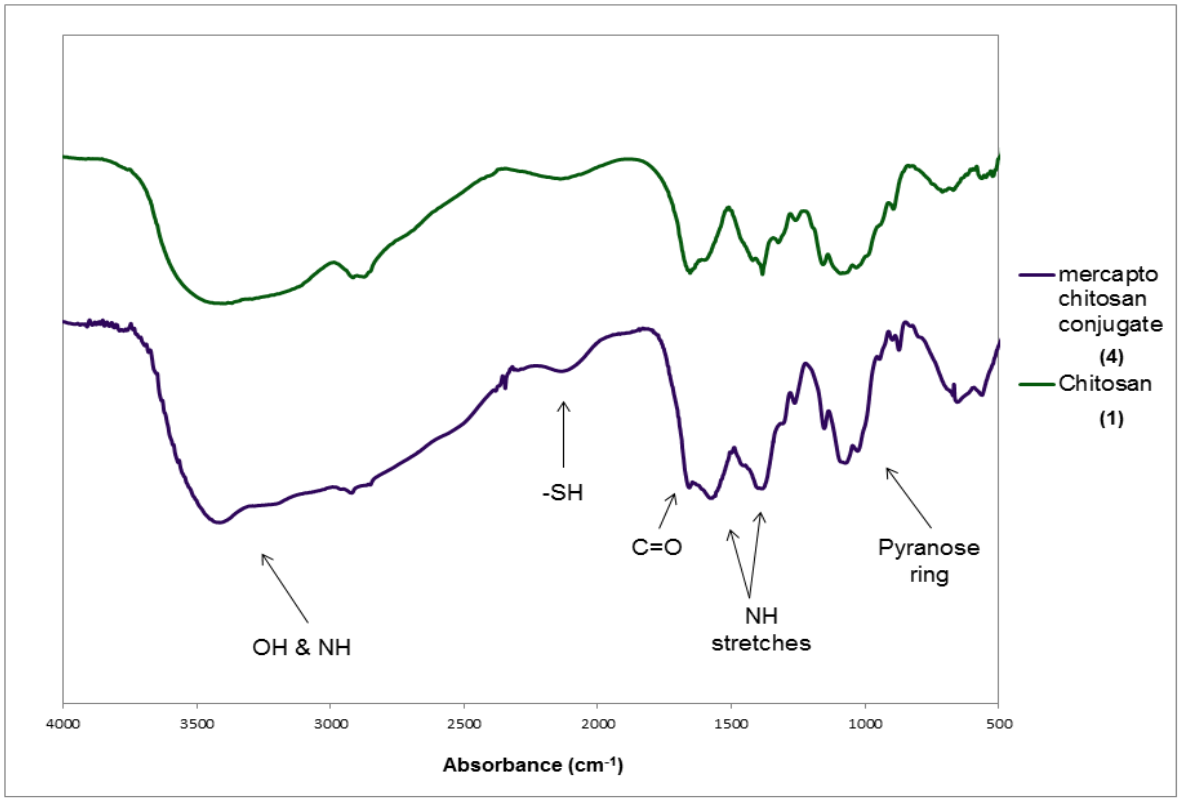
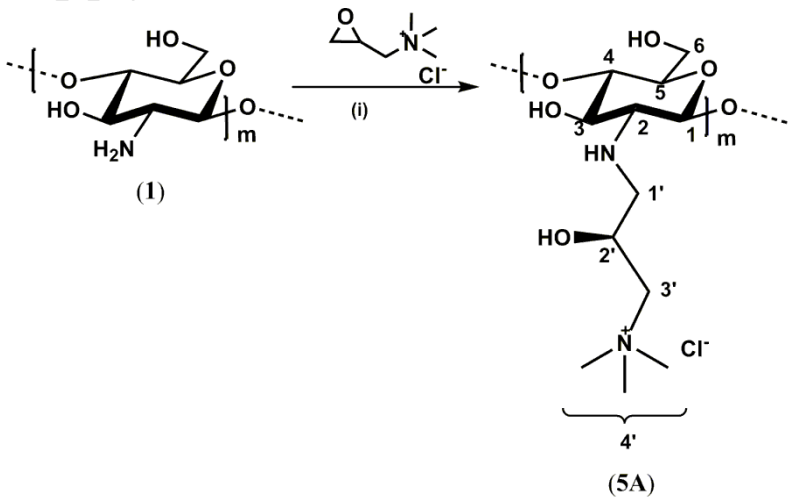


Figure 2.8: The IR spectrum of chitosan (**1**) and (2*S*)-2-mercaptosuccinyl chitosan (**4**) (KBr).

*2.1.4. Synthesis of 3-trimethylammonium-2-hydroxypropyl-N-chitosan chloride (CHI-Q188) (**5A**)*

Following the method described by Lim *et al.,* CHI-Q188 (**5A**) was successfully synthesized where chitosan was reacted with glycidyl trimethylammonium chloride.[14] An off white solid was obtained in 60 % yield (Scheme 2.4).



Scheme 2.4. Reagents and conditions: (i) H_2O, glycidyl trimethylammonium chloride, reflux 60 °C, 24 hrs; 60 %.

The product structure was confirmed *via* ^{1}H NMR and IR spectroscopy. The IR spectrum showed an increase in the strength of the absorption band at 3439 cm^{-1} as a result of the introduction of additional OH groups. The band at 1656 cm^{-1} was assigned to the C=O stretch of the secondary amide. The absorbance at 1481 cm^{-1} was due to the C-H bending of the trimethyl ammonium group as reported by Domard *et al*.[4] The IR spectra agreed with that reported by Lim *et al.* supporting the synthesis of the desired polymer.[14]

The ^{1}H NMR for polymer **5A** is shown in Figure 2.9. A resonance at δ 5.00 ppm was assigned to the anomeric proton H-1, a signal at δ 4.81 ppm was attributed to H-2'. The protons of H-3 to H-6 resonated at δ 3.83 - 3.67 ppm while that of H-2 appeared at δ 3.46 ppm. The resonance at δ 3.27 was assigned to H-3' while that at δ 3.2 ppm corresponded to H-4' respectively. The signal at δ 2.94 ppm corresponded to H-1' and overlaps by the resonance at δ 3.2 ppm due to the high degree of substitution. ^{1}H NMR signals agreed with that reported by Wan *et al.,* although their spectra was obtained in 0.1-0.3 % (v/v) TFA thus explaining the fact that resonances observed here, were further upfield relative to that which was reported.[15] The ^{13}C NMR displayed the expected number of signals with signals appearing at δ 67.32 (C-3'), 61.20 (C-2') 53.86 (C-4'), and 53.68 (C-1') ppm.

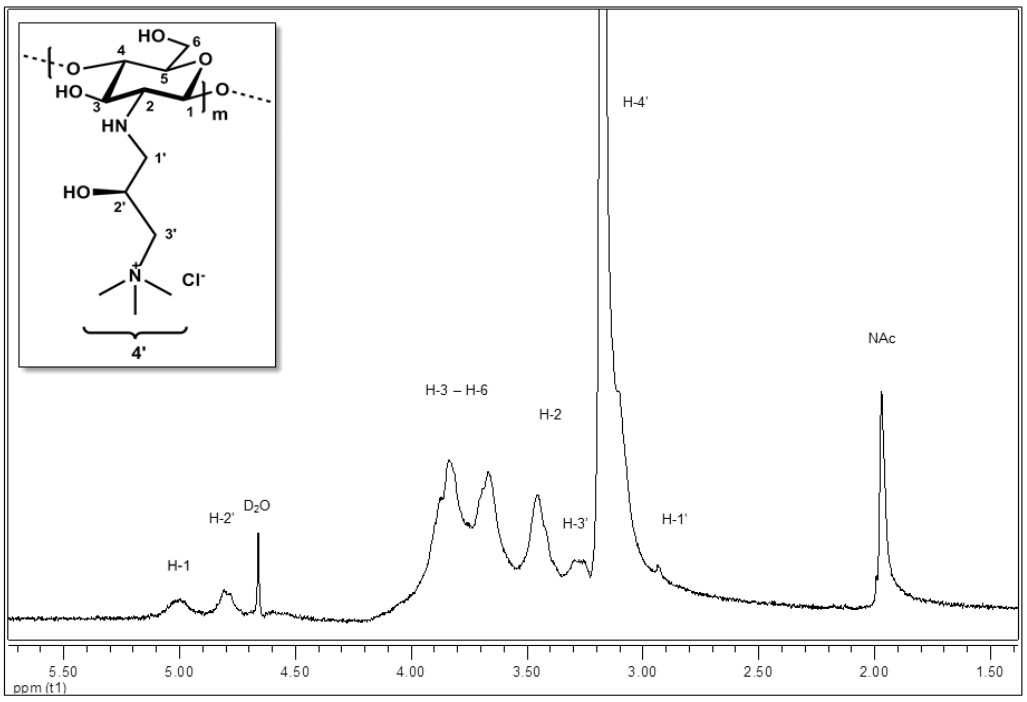


Figure 2.9: ^{1}H NMR of 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (**5A**) (2 % DCl/D_2O).

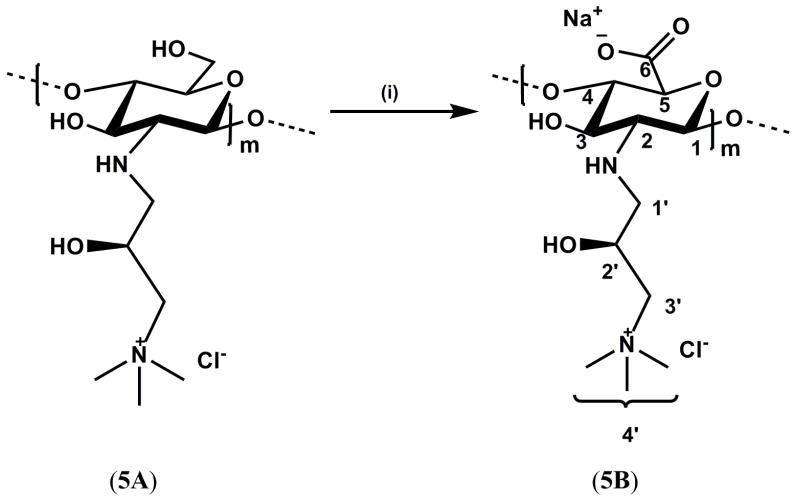
The degree of quaternization (DQ) was determined using the following equation reported by Sajomsang *et al*.: **$DQ = 1 - [(NAc/3)/ N^{+}(CH_3)_3] \times 100$**

where NAc is the integral area of the acetylated protons and $N^+(CH_3)_3$ is the integral area of the methyl protons of the CHI-Q188.[16] The DQ was determined to be 96 % for the polymer synthesized. This DQ was comparable to that obtained by Lim *et al*., who reported a DS of 100 %.[14]

Elemental analysis values indicated that solvent inclusion had occurred. After accounting for inclusion of H_2O, experimental values agreed with those calculated for this polymer.

*2.1.4.1. Selective oxidation of CHI-Q188 (**5A**)*

The C-6 selective oxidation of 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride **5A** has not been reported at the time of this study. The conversion of the primary alcohol of C-6 to an aldehyde and a carboxylic acid would provide polymers that could be modified through amide/ester linkage chemistry.[17] Amphiphilic polymers display electrolytic properties, their charge are usual but pH dependant, potential applications include: electrochemical interfaces, batteries and fast-recharging batteries, fuel cells, sensors, actuators, photo-electrochemical devices, biotechnology and in safety and durability issues of advanced devices. A search of the literature revealed that the selective C-6 oxidation of this quaternary chitosan derivative has not been carried out at the time of this study. This novel polymer may have a variety of potential applications and could be useful in water treatment. The charge associated with this polymer would be functioning pH dependent, thus as an anion or cation exchange resin. The oxidation was carried out using an oxidant specifically for primary alcohols, 2,2,6,6-tetramethylpiperidine-1-oxy radical (TEMPO).



(5A) **(5B)**

Scheme 2.5. Reagents and conditions: (i) H_2O, 5 °C, TEMPO, NaBr, 5 °C, pH 10.75, NaOCl, RT, 30 min; 45 %.

The oxidation was carried out using the method reported by Bordenave *et al.* in the oxidation of trimethyl chitosan chloride.[17] The polymer was reacted with TEMPO, NaBr and NaOCl and the reaction produced a light brown solid in a yield of 45 %.

The IR spectra showed absorption bands at 3435 cm^{-1} assigned to OH, the band at 1757 cm^{-1} can be attributed to COOH while that at 1680 and1651 cm^{-1} corresponded to C=O stretches. The band at 1419 cm^{-1} was attributed to the C-H bending of the trimethyl ammonium group.

The ^{1}H NMR spectrum of the oxidized quaternary derivative (**5B**) (Figure 2.10) revealed that all of the resonances had shifted slightly upfield and appeared broader and less defined relative to polymer **5A**. The resonance at δ 4.48 ppm was assigned to H-1, while the shifts from δ 3.50 to 3.33 were as a result of H-3 to H-5. The proton at H-2 resonated at δ 3.05 ppm while the signal for H-3' could not be properly distinguished. The resonance at δ 2.79 ppm was attributed to the three methyl groups (H-4'). The resonance at δ 2.75 ppm was attributed to H-1' while that at δ 1.61 ppm was assigned to acetylated units of the polymer. A ^{13}C NMR spectrum could not be obtained due to the highly ionic nature of the sample.

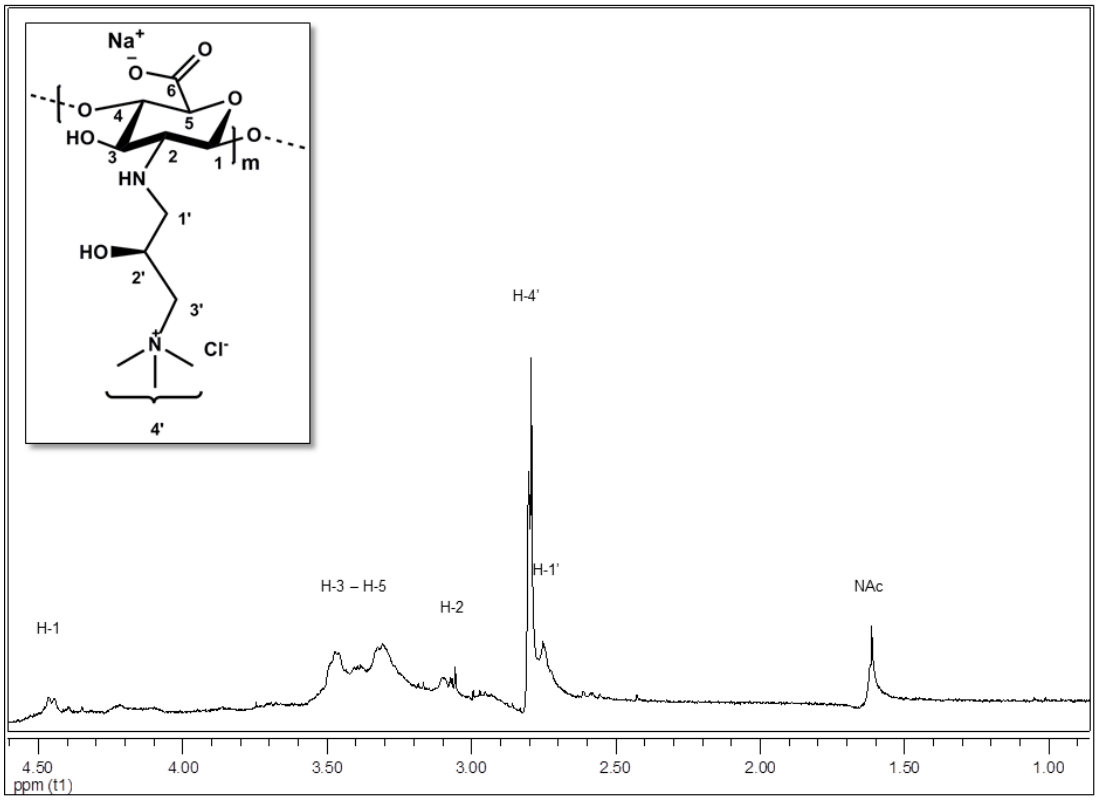
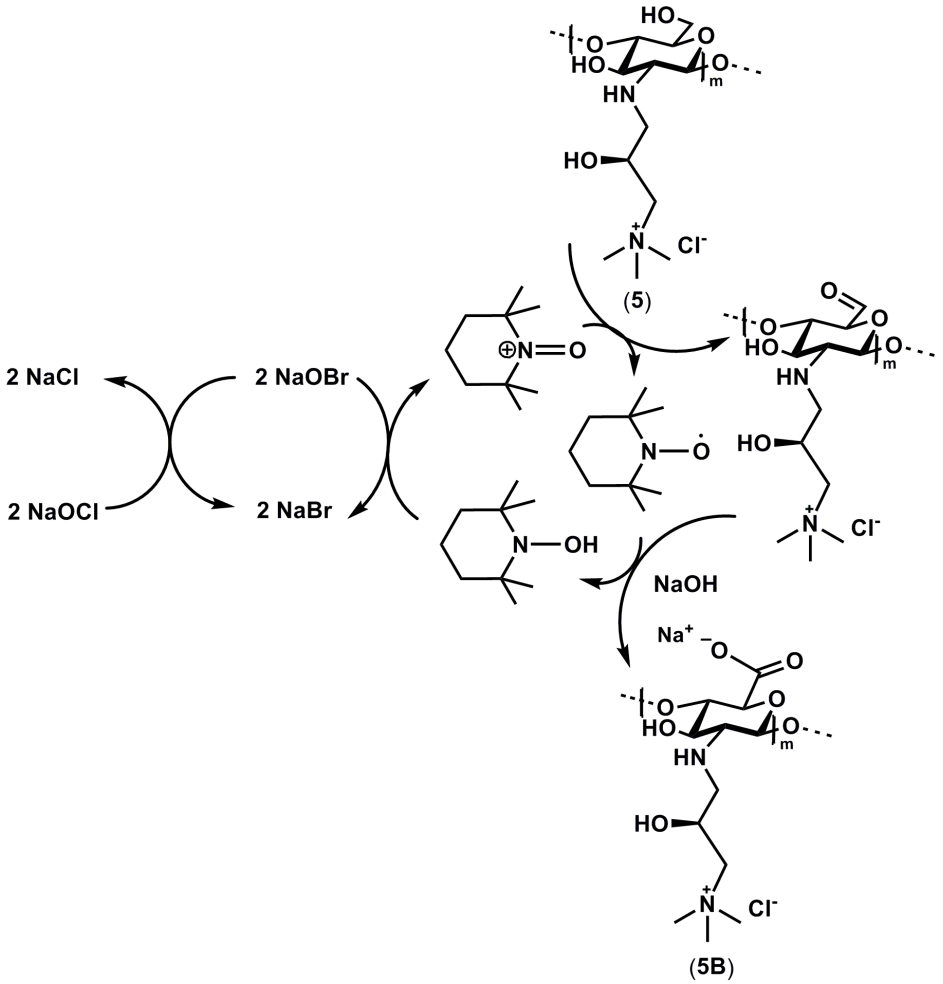


Figure 2.10: ^{1}H NMR of **5B** (2 % DCl/D_2O).

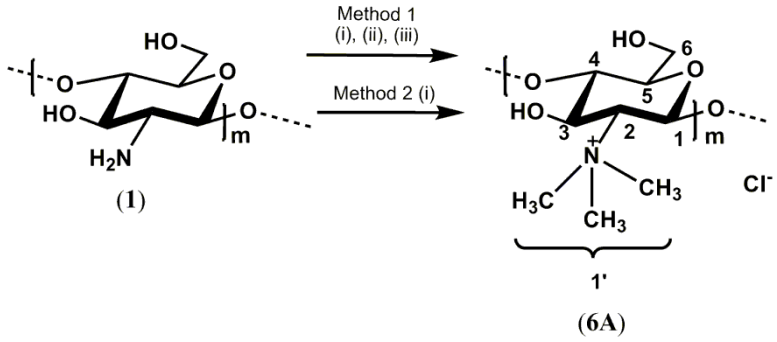
Scheme 2.6 shows the selective oxidation of 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (**5A**). A co-oxidative system was utilized where TEMPO was used in conjunction with NaOCl/NaBr to avoid any unwanted side reactions. The quaternized nitrogen present improved polymer stability, which avoided degradation during the oxidation stage with TEMPO. This is a result of *N*,*N*,*N*-trimethylation which considerably decreases the nucleophilic character of nitrogen in the polymer thereby reducing its reactivity.[17]



Scheme 2.6: Proposed mechanism for the TEMPO mediated oxidation of **5A**.

*2.1.5. Synthesis of trimethyl chitosan chloride (TMC) (**6A**)*

Synthesis of TMC (**6A**) was achieved using two different methods as shown in Scheme 2.7.[18, 19]



Scheme 2.7. Reagents and conditions: Method 1- (i) NMP, NaI, 20 % NaOH, reflux 60 °C, 20 min, MeI, reflux 60 °C, 1 hr; (ii) NMP, NaI, 20 % NaOH, reflux 60 °C, 20 min, MeI, reflux 60 °C, 1 hr, MeI, 20 % NaOH, reflux 60 °C, 1 hr; (iii) 5 % NaCl; 40 %; Method 2- (i) Dimethylsulfate, H_2O, NaOH, NaCl, RT, 6 hrs; 54 %.

In method 1, TMC (**6A**) was synthesized using MeI as the methylating agent in the presence of a base.[18] This reaction yielded the trimethyl chitosan iodide derivative which was exchanged for the more stable chloride ion in the presence of aqueous NaCl. The product was obtained as a fibrous white material in a yield of 40 %.

The IR spectrum supported the synthesis of TMC (**6A**). The characteristic absorption bands of modified chitosan were observed as well the appearance of a band at 1475 cm^{-1} assigned to the C-H bending of the trimethyl ammonium group.[18]

The ^{1}H NMR obtained in D_2O supports the synthesis of TMC (**6A**) (Figure 2.11). Resonances characteristic of a modified chitosan were observed at δ 4.30 ppm (H-1), δ 3.58 - 3.75 ppm (H-3 to H-6) and δ 3.36 ppm (H-2). The resonance at δ 3.45 ppm is due to the formation of alkylated chitosan side products, 3-*O*(CH_3) and 6-*O*(CH_3) as reported by Stepnova *et al*.[20] The resonance at 2.02 ppm corresponded to the acetylated groups present. The two resonances at δ 3.26 and 2.46 ppm were assigned to the protons of H-1' and the dimethylated ($N(CH_3)_2$) polymer respectively. The ^{13}C NMR showed the correct number of resonances with the introduction of two resonances at δ 54.16 and 41.40 ppm assigned to the carbons of $N^{+}(CH_3)_3$ and $N(CH_3)_2$ respectively.

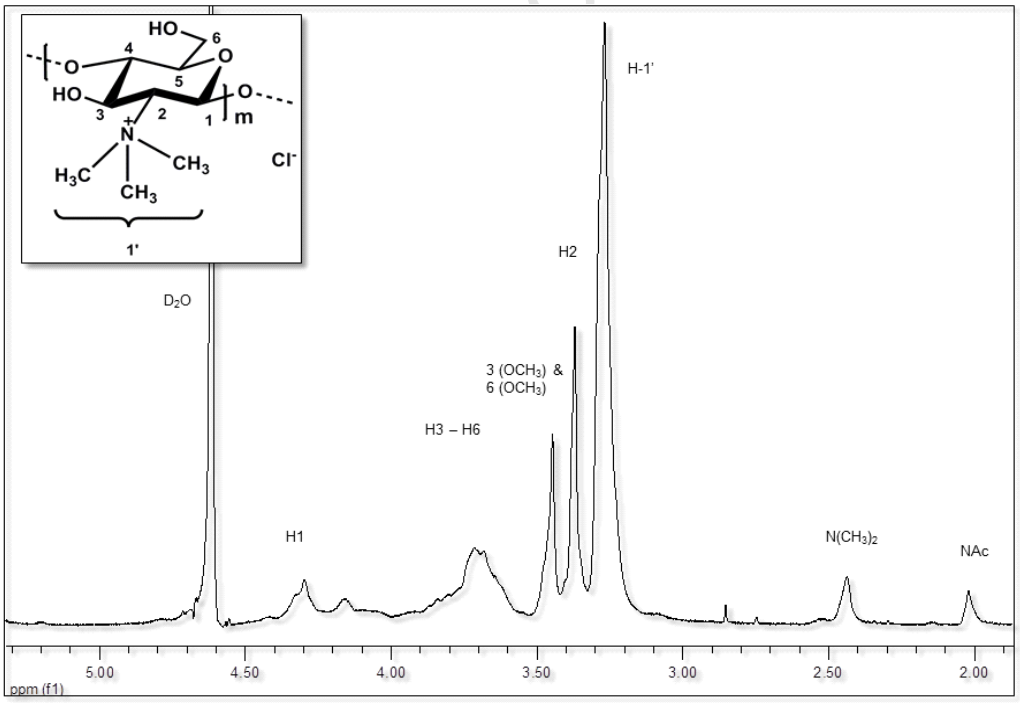


Figure 2.11: ^{1}H NMR spectrum of TMC (**6A**) in D_2O.

The degree of quaternization (DQ) was determined using the following formula as reported by Hamman *et al*.:

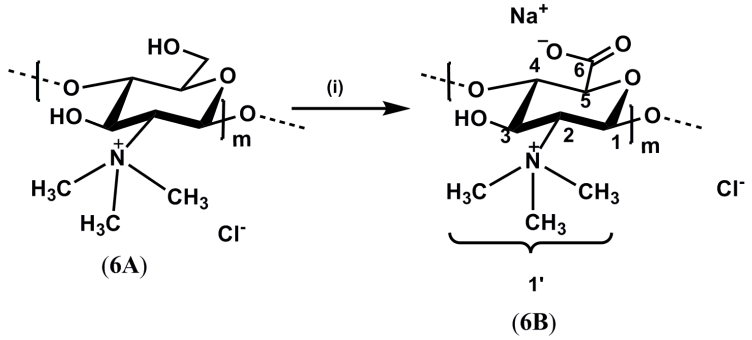
DQ (%) = [(∫ TM / ∫ H) X 1/9] X 100

where ∫TM is the integral of the trimethyl amino group (quaternary amino) resonance in the ^{1}H-NMR spectrum, and ∫H is the integral of the resonances on the polymer backbone.[21] The quaternary chitosan derivative produced, using MeI, yielded a DQ of 62 %. This DQ was lower compared to that reported by Polnok *et al.* which was 78 %.[18]

Elemental analysis agreed with calculated values after accounting for the inclusion of a single H_2O molecule.

In the second method, dimethylsulfate was used as the methylating agent in the presence of a base, this reaction consisted of one step to produce TMC (**6A**).[19] The polymer was characterized by ^{1}H NMR spectroscopy which was recorded in D_2O. A similar spectrum was observed as for the TMC which was synthesized using the previous method. This alternative methylating agent produced TMC with a DQ of 20 %. This DQ was much lower compared to that reported by de Britto *et al*. (53 %) under the reported synthesis conditions.[19] A possible explanation for this is that a higher degree of *O*-methylation and *N*-methylation occurred.

*2.1.5.1. Selective oxidation of TMC (**6A**)*



Scheme 2.8. Reagents and conditions: (i) H_2O, 5 °C, TEMPO, NaBr, 5 °C, pH 10.75, NaOCl, RT, 30 min; 47 %.

TMC (**6A**) was oxidized by the addition of TEMPO, NaBr and NaOCl to give the desired product (**6B**) as a cream fibrous solid with a yield of 47 %. The obtained yield was slightly higher than that reported by Bordenave *et al.* (30 %) due to a modification in the procedure. Bordenave *et al.* isolated only the precipitated polymer and lost some product in the supernatant whereas in this study, the product was not isolated by centrifugation after the addition of acetone.[17]

The ^{1}H NMR spectrum obtained, agreed with the spectrum reported by Bordenave *et al.*, with a slight upfield shift of all the resonances observed relative to that of chitosan (Figure 2.12).[17] The resonances at δ 3.32 and 2.47 ppm were assigned to the protons of H-1' and

$N(CH_3)_2$, these resonances did not have noticeable upfield shifts. The ^{13}C NMR spectrum contained the correct number of resonances with the appearance of resonance at δ 162.11 ppm assigned to the amide C=O group.

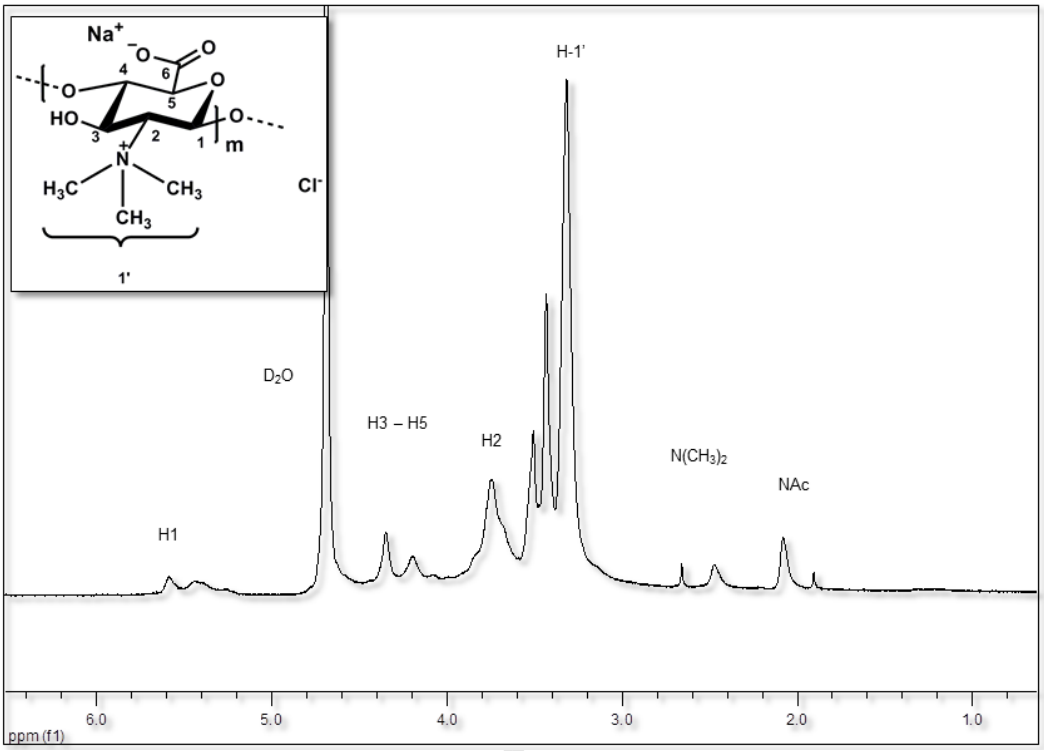


Figure 2.12: ^{1}H NMR of the 6-carboxy-TMC (**6B**) in D_2O.

2.2. Synthesis and characterization of 6-deoxy-6-amino chitosan and derivatives.

The 6-deoxy-6-amino chitosan derivative of chitosan (**10**) was synthesized via intermediates **7** – **10**. This derivatives was used to synthesize the corresponding thiolated and quaternary derivatives. The thiolated and quaternary derivatives was expected to have significantly higher loading compared to the chitosan derivatives as a result of the presence of an additional amine group. In addition, these derivatives are predicted to be more soluble in water.

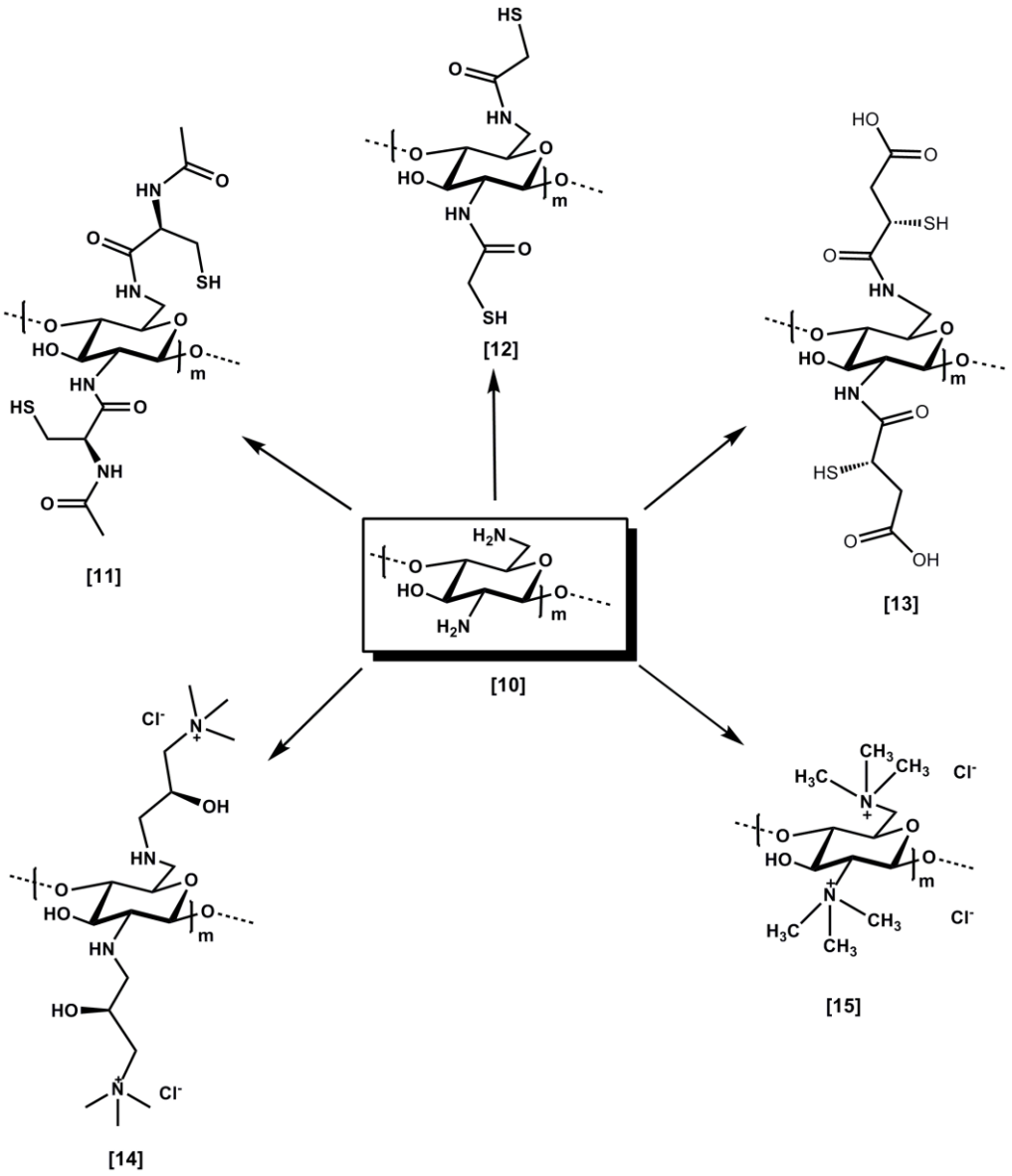
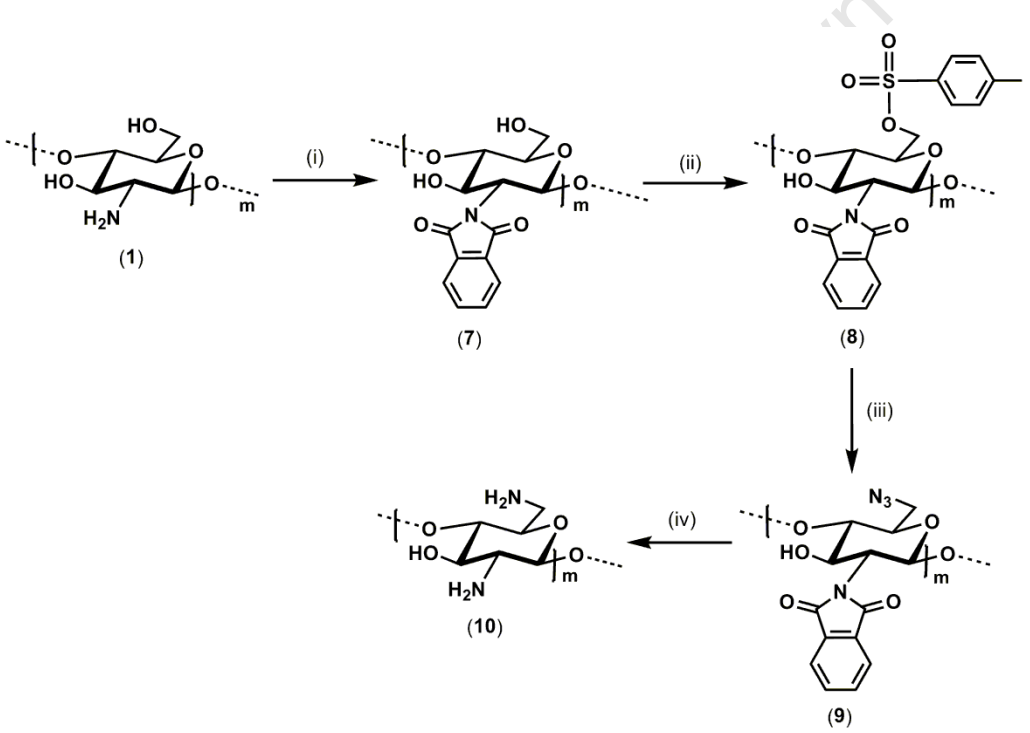


Figure 2.13: General scheme of synthesis of 6-deoxy-6-amino chitosan derivatives **11**, **12**, **13**, **14** and **15**.

Certain polymers (**11**, **12**, **13** and **14**) have not been found in literature at the time of this study. These novel polymers have many potential applications as they possess the inherent properties of chitosan and certain derivatives have double the thiol content of the chitosan derivative.

*2.2.1. Synthesis of 6-deoxy-6-amino chitosan (**10**)*

The synthesis of 6-deoxy-6-amino chitosan, previously reported by our group, was accomplished in four steps (Scheme 2.9).[22] The pathway involved the synthesis of *N*-phthaloyl chitosan (**7**), which in turn was converted to 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**8**). The third intermediate is 6-deoxy-6-azido *N*-phthaloyl chitosan **9** which was finally reduced to 6-deoxy-6-amino chitosan (**10**).



Scheme 2.9. Reagents and conditions: (i) phthalic anhydride, 5 % DMF/H_2O, reflux 120 °C, 8 hrs, 97 %; (ii) pyridine, 0 °C, *p*-toluenesulfonyl chloride, 17 hrs, RT, 97 %; (iii) NMP, NaN_3, 80 °C, 4 hrs, N_2, 96 %; (iv) NMP, TPP, 15 hrs, RT, N_2; $H_2NNH_2.H_2O$, H_2O, 100 °C, 4hrs, N_2, 81 %.

The first step in this synthesis was the protection of the amino group on chitosan. *N*-phthaloyl was chosen as the protecting group as it provides chemoselective protection of the functional groups present in chitosan for further modification. In addition, the phthaloyl protecting group is easily removed using hydrazine hydrate.[23] Ifuku *et al.* has recently reported the chemoselective synthesis of *N*-phthaloyl chitosan in aqueous acetic acid media, making the synthesis of this intermediate much more environmentally friendly.[24]

N-phthaloyl chitosan (**7**) was synthesized by the addition of phthalic anhydride in DMF to chitosan in H_2O. Water was used as a co-solvent to prevent *O*-phthaloylation.[23] The product was obtained as a pale tan powder in a yield of 97 %.

N-Phthaloyl chitosan (**7**) was found to be poorly soluble in common polar solvents (DMSO, NMP, etc.). This could be due to the introduction of the sterically bulky phthaloyl group whose uniform structure and phthalimide C=O groups could contribute to the formation of a more crystalline structure.[23,24] Due to these solubility problems, some of the derivatives synthesized in this study required a low pH (DCl or TFA) in order to obtain an NMR spectrum.[25]

IR spectroscopy supported the synthesis of the product with the characteristic bands for the modified chitosan clearly visible (Figure 2.14).[23] These characteristic bands included 3422 cm^{-1} which was assigned to the OH and NH vibrations. The band at 2909 cm^{-1} is due to the C-H aliphatic present while that at 2133 cm^{-1} corresponded to the C-NH_2 stretch. Typical absorption bands at 1656 and 1421 cm^{-1} resulted from the NH bending frequencies. The band between 1155 and 1092 cm^{-1} is a result of the pyranose ring while that at 1032 cm^{-1} corresponded to the C-N vibration. The presence of two phthalimido absorbance bands at 1774 and 1711 cm^{-1} as well as a band at 724 cm^{-1} was due to the phthaloyl-aromatic ring, thus supporting the synthesis of *N*-phthaloyl chitosan (**7**) as reported by Jardine *et al*.[22]

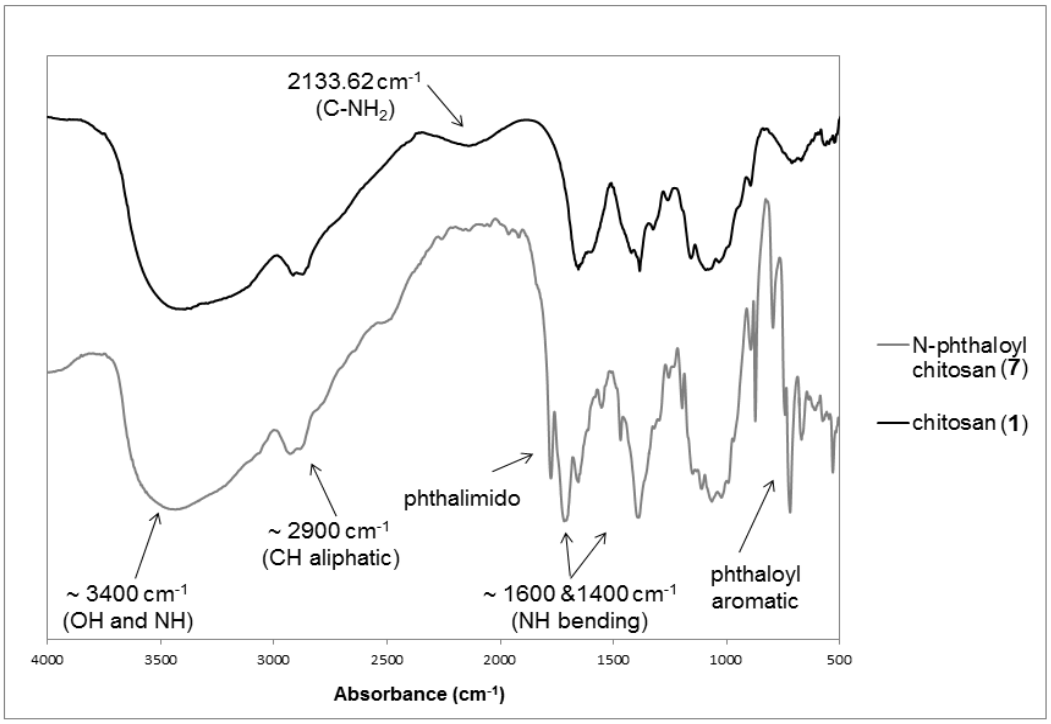


Figure 2.14: IR spectrum of *N*-phthaloyl chitosan (**7**) and chitosan (**1**) (KBr).

Elemental analysis was used to support the synthesis of *N*-phthaloyl chitosan (**7**), and the analysis was found to correlate with calculated results.

After the successful synthesis of *N*-phthaloyl chitosan (**7**), the compound was tosylated by reacting with *p*-toluoylsulfonyl-chloride in pyridine to produce the desired product (**8**). This product was isolated as a light brown powder with a yield of 97 %. The regioselective tosylation of the -OH group at C6 of *N*-phthaloyl chitosan (**7**) occurs due to the bulky tosyl group and the steric hindrance at the –OH group at C3. In addition, the –OH at C6 is a primary hydroxyl group whereas that at C3 is a secondary hydroxyl, favouring attack at C6.[26]

The IR spectrum of the polymer **8** (Figure 2.15) confirmed the presence of the S=O group indicated by the absorbance band at 1174 cm^{-1} and the symmetrical C-O-S stretch at 815 cm^{-1}. In addition, an absorbance due to the *p*-toluoyl group was noted at 718 cm^{-1}. These bands agreed with those reported by Jardine *et al.*[22] Elemental analysis agreed with calculated values.

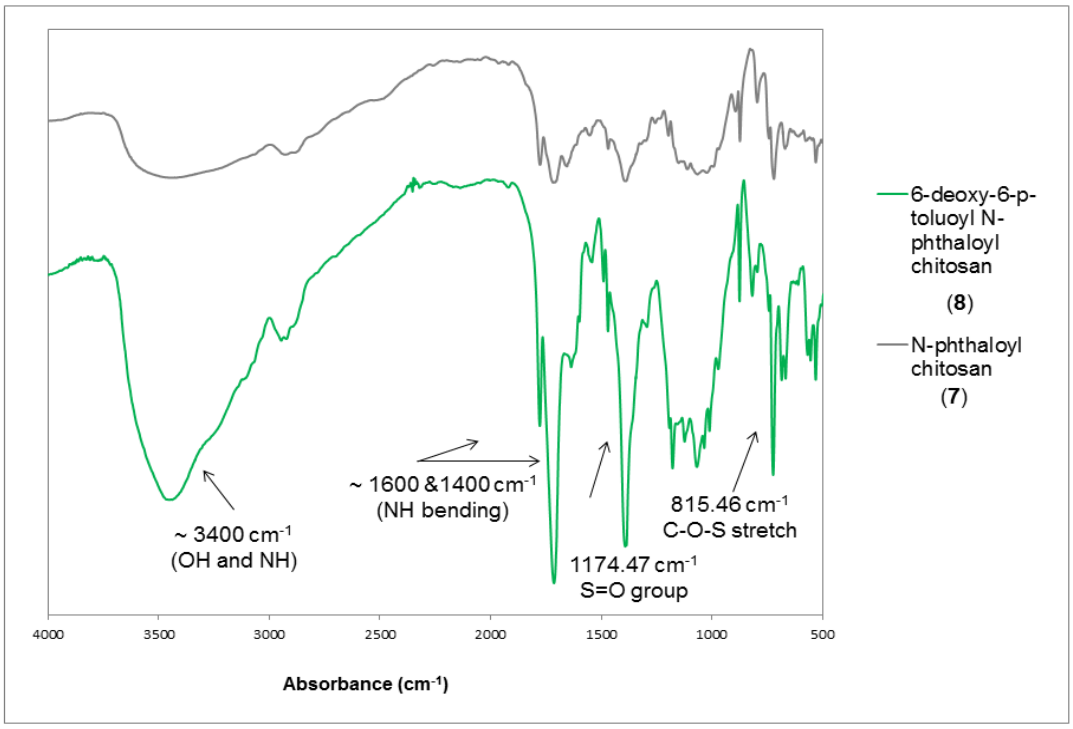


Figure 2.15: IR spectrum of 6-deoxy-6-*p*- toluenesulfonyl *N*-phthaloyl chitosan (**8**) and *N*-phthaloyl chitosan (**7**) (KBr).

With polymer **8** in hand, the 6-deoxy-6-azido *N*-phthaloyl chitosan (**9**) intermediate was synthesized, via a nucleophilic displacement of the tosyl group. 6-Deoxy-6-*p*- toluenesulfonyl *N*-phthaloyl chitosan (**8**) was refluxed in the presence of sodium azide in NMP under a nitrogen atmosphere. This reaction produced a brown solid in a yield of 96 %.

The IR spectrum (Figure 2.16) confirmed the presence of an azide group with a strong absorption band at 2103 cm^{-1}. The weak signal at 1174 cm^{-1} was attributed to the S=O absorption band and that at 817 cm^{-1} assigned to the C-O-S stretch, showed incomplete substitution of the tosyl group. Therefore longer reaction times may be necessary for complete substitution. However, the degree of substitution was acceptable for our study. Elemental analysis supports the synthesis of the product.

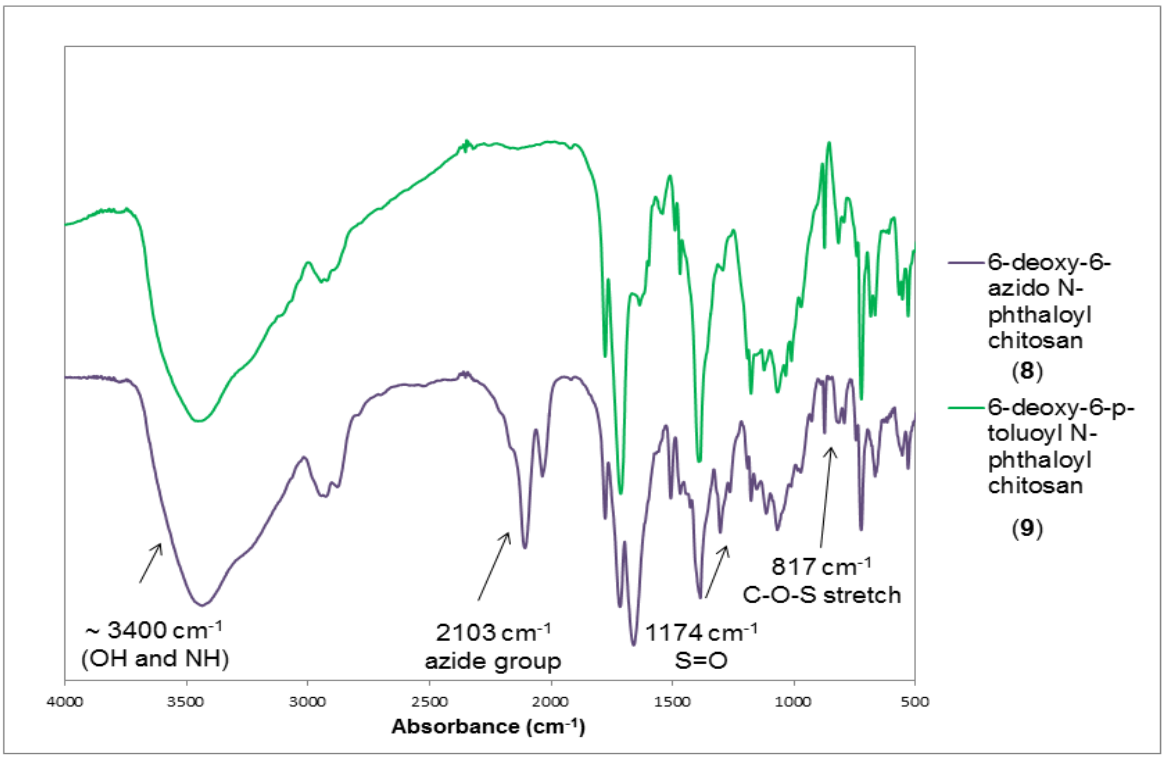


Figure 2.16: IR spectrum of 6-deoxy-6-azido *N*-phthaloyl chitosan (**9**) and polymer **8** (KBr).

Finally, the azide derivative was simultaneously reduced and deprotected in one pot to yield 6-deoxy-6-amino chitosan (**10**). 6-Deoxy-6-azido *N*-phthaloyl chitosan (**9**) was allowed to stir in NMP in the presence of TPP under a nitrogen atmosphere. The mixture was subsequently refluxed in the presence of hydrazine monohydrate and H_2O. The formation of 6-deoxy-6-amino chitosan proceeded *via* a Staudinger type mechanism involving the formation of an azo-ylide intermediate.[26]

The product structure was supported by ^1H NMR analysis which was obtained in 2 % DCl/D_2O (Figure 2.17). A multiplet was observed in the region of δ 7.84 ppm which was assigned to the aromatic protons of the phthaloyl group indicating incomplete deprotection. This is typical of this reaction as reported by Yang *et al*., however, a longer reaction time may result in complete deprotection. The degree of deprotection was sufficient for our

purposes. The anomeric proton resonated at δ 4.52 ppm while the signals between δ 3.22 and 3.57 were assigned to H-3 to H-6. The resonance at δ 2.84 ppm was assigned to the proton H-2. The signal at δ 2.17 ppm was assigned to that of the residual *N*-acetyl groups present after deacetylation. The resonance at δ 1.89 ppm was assigned to CH_3COO^- (as suggested by Kurita *et al.*), which is present as a counter ion due to the fact that the amino groups existed partially as $R\text{-}NH_3^+$.[25]

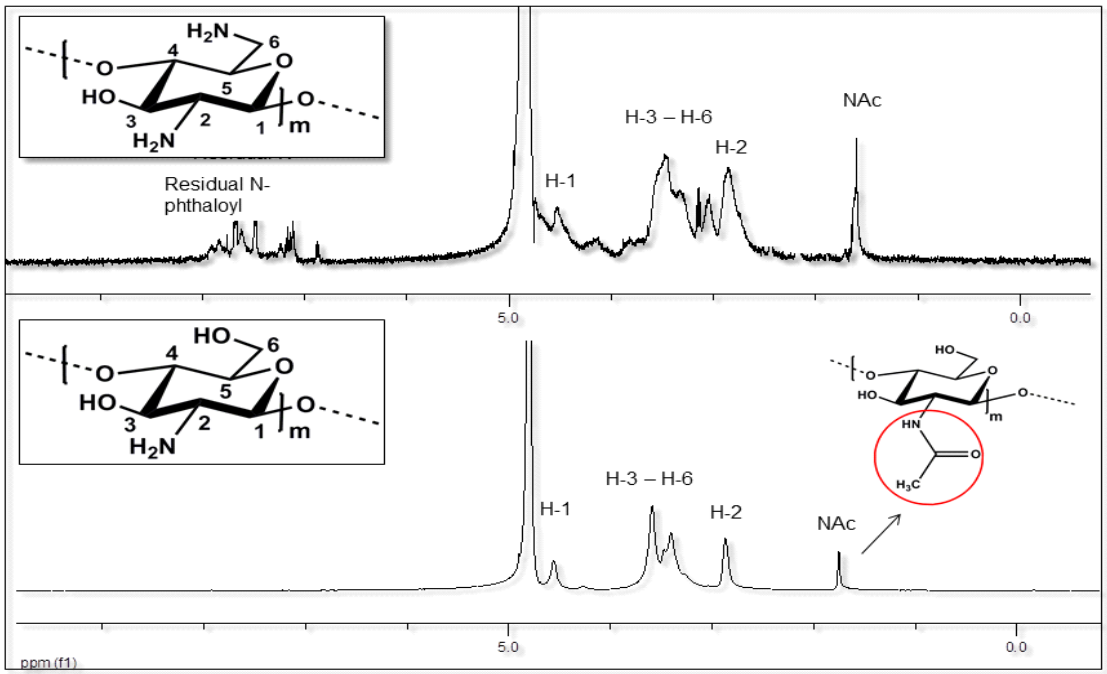


Figure 2.17: ^{1}H NMR of chitosan (**1**) and 6-deoxy-6-amino chitosan (**10**) in 2 % DCl/D_2O.

The IR spectrum showed a broader band at 3379 cm^{-1} assigned to the free amino groups (Figure 2.18). The azido band decreased in intensity while the phthaloyl group absorption bands were still present but much weaker, confirming incomplete deprotection. The intense band at 1591 cm^{-1} was assigned to the bending vibration of the amino groups showing that deprotection had occurred to great extent. It has been reported by Yang *et al*. that partial deprotection may have occurred due to incomplete cyclization of the amino group in the first reaction, or the hydrolytic cleavage of the imide ring in the hydrazinolysis reaction.[27]

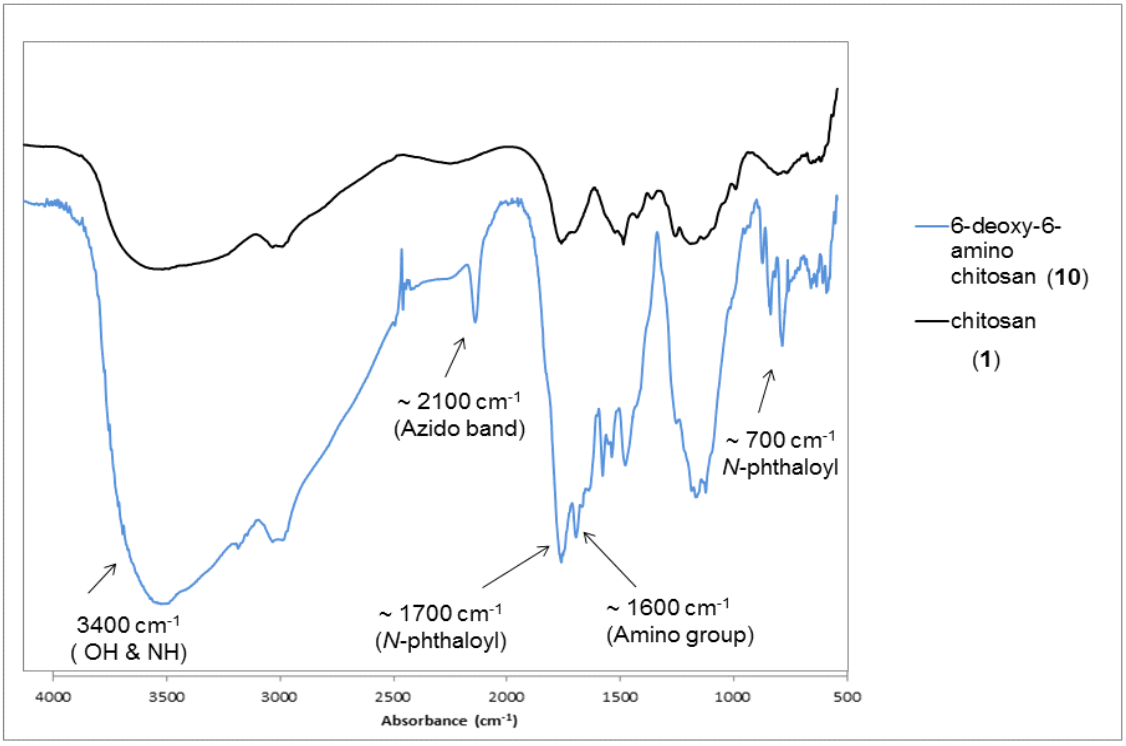
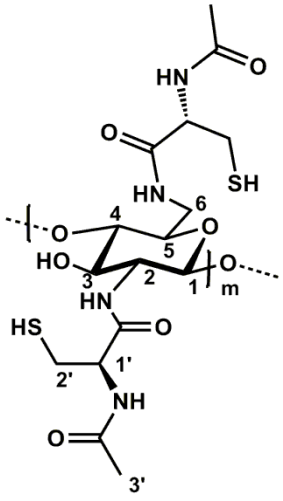


Figure 2.18: IR spectrum of 6-deoxy-6-amino chitosan (**10**) and chitosan (**1**) (KBr).

Elemental analysis confirms the synthesis of the expected product.

After having successfully synthesized 6-deoxy-6-amino chitosan (**10**), this polymer was used as a precursor in the synthesis of thiolated and quaternary polymers (**11**), (**12**), (**13**), (**14**) and (**15**).

*2.2.2. Synthesis of 6-deoxy-2,6-bis[N-acetylcysteinyl] chitosan (**11**)*



The synthesis of 6-deoxy-2,6-bis[*N*-acetylcysteinyl] chitosan (**11**) was carried out using the same procedure used to synthesize *N*-acetylcysteinyl chitosan (Scheme 2.1).[2] The product was recovered as a brown powder in a yield of 36 %.

Due to the high degree of crystallinity of the compound, it was found to be insoluble in a number of solvents (e.g. DMSO, D_2O, etc.). An NMR spectrum could only be obtained in 1 M DCl/D_2O after brief heating. The ^{1}H NMR spectrum (Figure 2.19) of the desired polymer (**11**) showed characteristic resonances associated with the *N*-acetylcysteine moiety. Signals observed between δ 7.26-8.19 ppm were assigned to residual *N*-phthaloyl aromatic protons. The protons attributed to H-3 to H-6 resonated between δ 3.41 and 3.79 ppm. The signal corresponding to H-2 appeared at δ 3.21 ppm while resonance at δ 2.87 ppm was assigned to H-2'. The sharp singlet at δ 1.78 ppm was assigned to H-3' and the residual acetylated units of the polymer. The anomeric proton was not observed. These sharp resonances are possibly due to the cysteine, which could suggest greater flexibility in this part of the polymer. Due to the brief acid treatment, some depolymerisation may have occurred giving rise to sharper resonances observed in the spectrum. The DS of this polymer was calculated as for the chitosan derivative and was found to be 51 %.

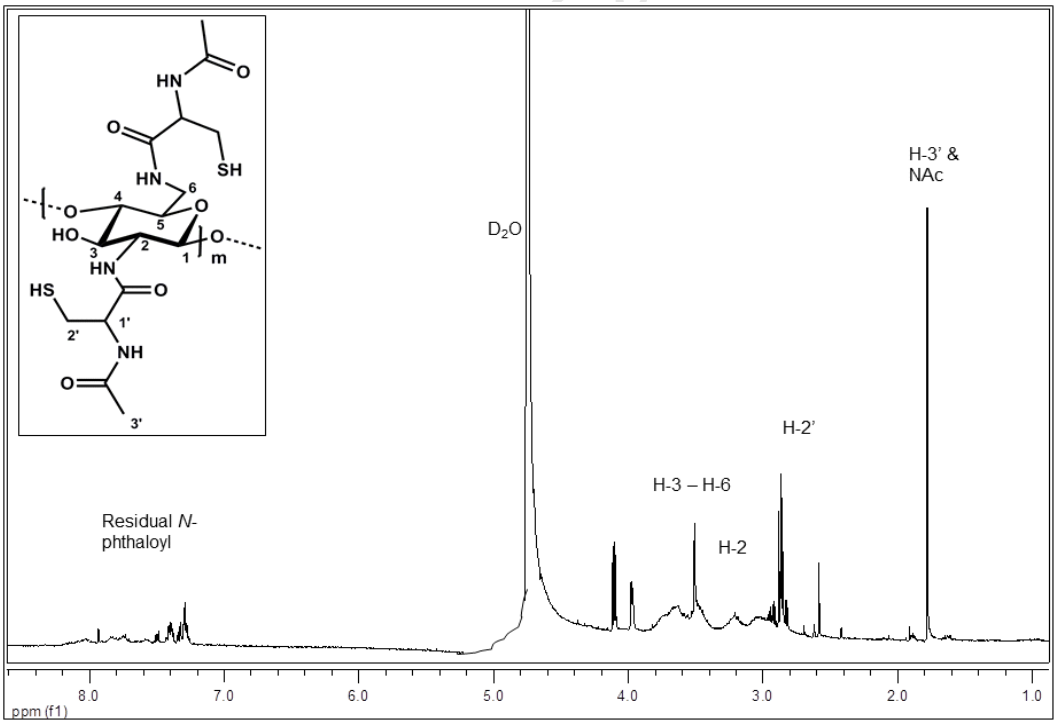


Figure 2.19: ^{1}H NMR of 6-deoxy-2,6-bis[*N*-acetylcysteinyl] chitosan (**11**) in 1 M DCl/D_2O.

Elemental analysis values are comparable to calculated values.

The IR spectrum (Figure 2.20) showed the characteristic absorption bands at 3418 cm^{-1} attributed to the OH group. The bands at 1625 and 1514 cm^{-1} correspond to C=O and NH stretching vibrations while the absorption band of –SH at ~ 2000 cm^{-1} is hidden by the broad -OH band.

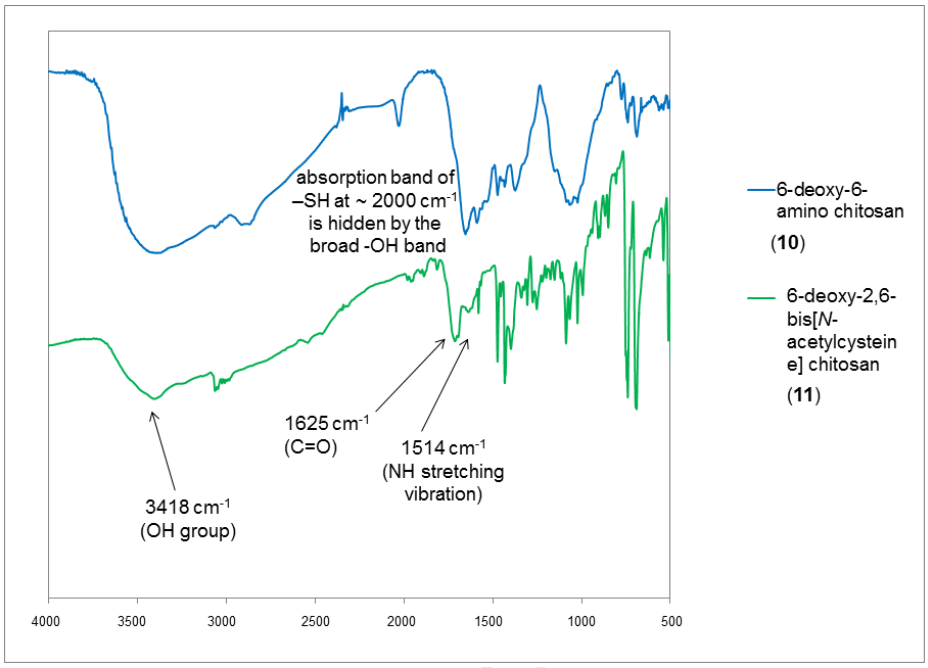
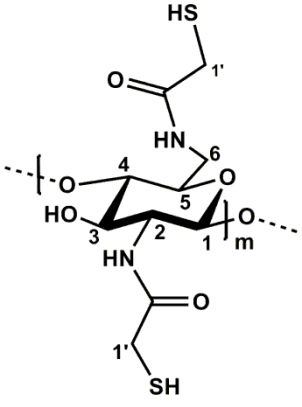


Figure 2.20: IR spectrum of 6-deoxy-2,6-bis[*N*-acetylcysteinyl] chitosan (**11**) vs. 6-deoxy-6-amino chitosan (**10**) (KBr).

The thiol content of the diamino derivative **11** was determined to be 82 µmol/g of polymer. This loading is almost five times higher compared to that of the chitosan derivative, as there is now an additional amine site which is open to functionalization. This result agrees with the DS calculated from the ^{1}H NMR spectra which is double that of *N*-acetylcysteinyl chitosan (**2**).

*2.2.3. Synthesis of 6-deoxy-2,6-bis[thioglycolyl] chitosan (**12**)*



(**12**)

6-Deoxy-2,6-bis[thioglycolyl] chitosan (**12**), was synthesized by reacting polymer (**10**) with thioglycolic acid.[3] A pale tan solid was recovered in a yield of 52 %.

The thiol content of the polymer (**12**) was determined to be 37 µmol/g of polymer. This is double the loading relative to the chitosan derivative as expected, due to the extra site of functionalization available.

A ^{1}H NMR spectrum for the polymer (**12**) could only be obtained in 1 M DCl/D_2O after brief heating. The spectrum (Figure 2.21) was similar to that obtained for 6-deoxy-6-amino chitosan. Residual *N*-phthaloyl aromatic resonances were observed in the region δ 7.25 to 8.20 ppm. The anomeric proton H-1, was assigned to the resonance at δ 5.15 ppm while the protons H-3 to H-6 resonated in the region of δ 3.38 to 3.84 ppm. The resonance at δ 3.22 ppm was assigned to H-2 while the resonance at δ 3.09 ppm was attributed to H-1'. The distinct, sharp singlet at δ 1.78 was due to the acetylated units of the polymer. The DS was determined to be 51 % for this polymer. This result is in agreement with the thiol loading calculated from the DTNB assay.

Elemental analysis agrees with calculated values for C, H and N.

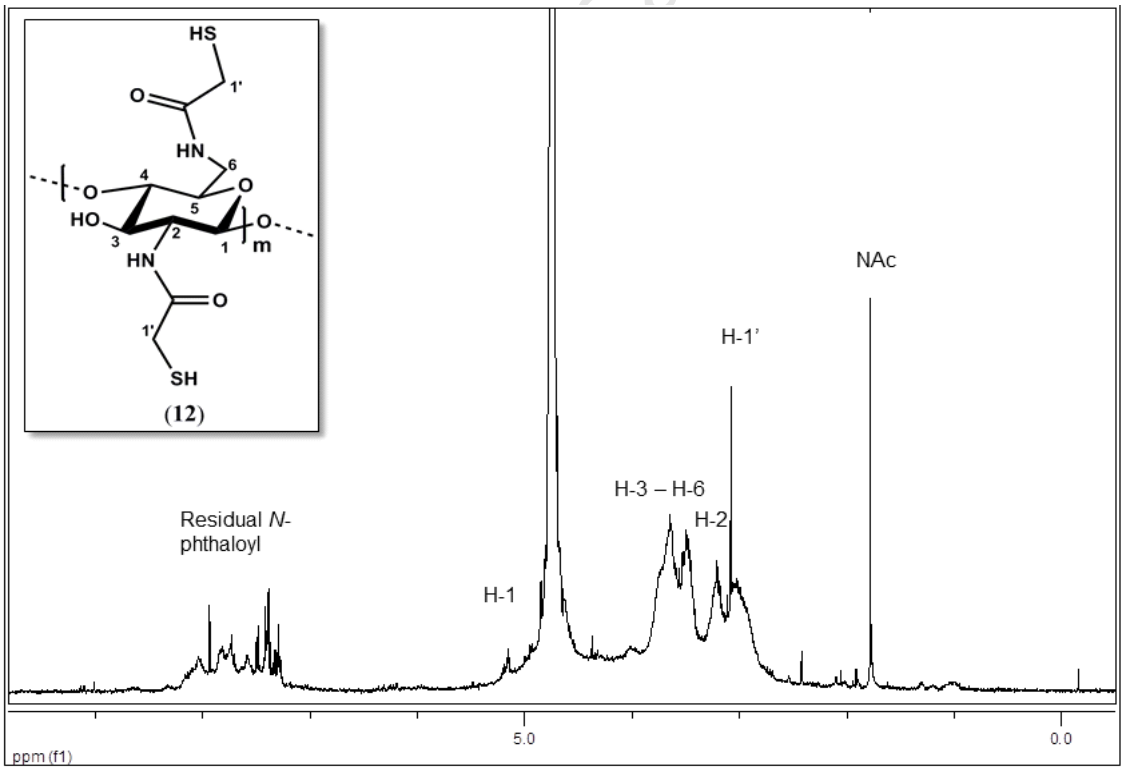


Figure 2.21: ^{1}H NMR of 6-deoxy-2,6-bis[thioglycolyl] chitosan (**12**).

IR analysis (Figure 2.22) revealed the characteristic absorption band at 3400 cm^{-1} belonging to the OH stretch. The band at 2080 cm^{-1} corresponded to the –SH stretch while bands at 1634 and 1090 cm^{-1} confirmed the presence of C=O groups and the pyranose ring.

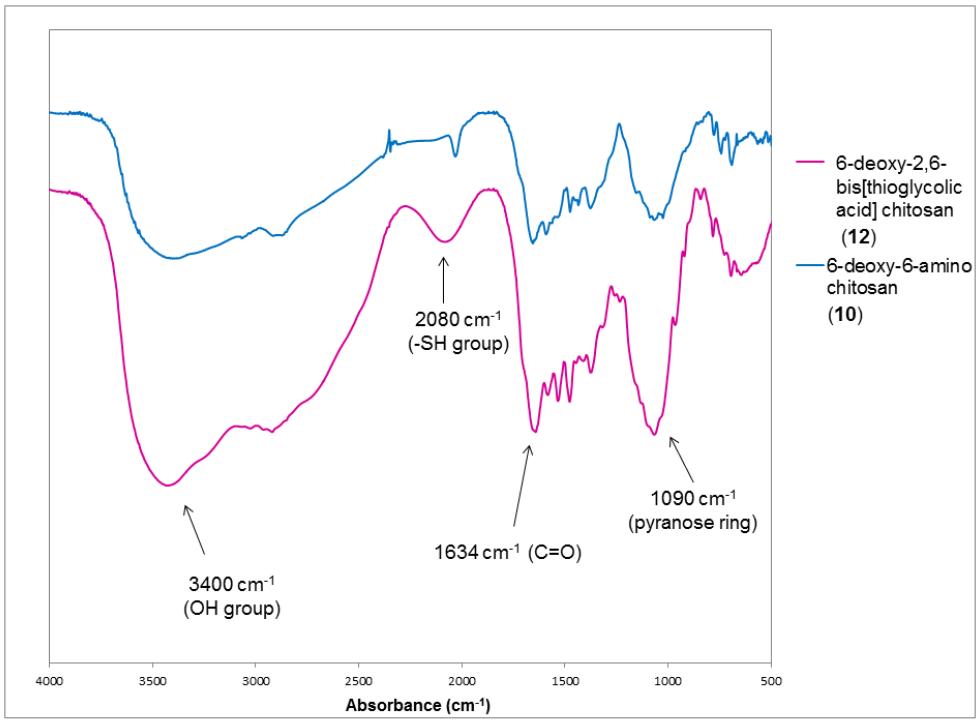
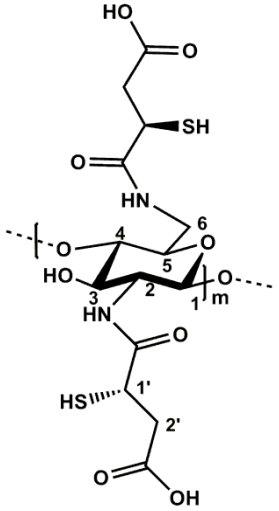


Figure 2.22: IR analysis of 6-deoxy-2,6-bis[thioglycolyl] chitosan (**12**) vs. 6-deoxy-6-amino chitosan (**10**) (KBr).

*2.2.4. Synthesis of 6-deoxy-2,6-bis[2S'-mercaptosuccinyl] chitosan (**13**)*



In this study, 6-deoxy-2,6-bis[2'-mercaptosuccinyl] chitosan (**13**) was synthesized by reacting **10** with *S*-acetylmercaptosuccinic anhydride (Scheme 2.3).[14] The compound obtained was a pale tan powder in a yield of 96 %.

The ^{1}H NMR revealed some residual *N*-phthaloyl aromatics protons resonating from δ 6.75 to 7.40 ppm. The protons of the pyranose ring, H-3 to H-6 resonated between δ 2.40 and 3.23 ppm. The resonance at δ 2.11 ppm was due to H-2 while the resonances at δ 1.88 and 1.83 ppm was assigned to H-1' & H-2' respectively. The sharp singlet at δ 1.24 ppm was attributed to acetylated units of the polymer. The DS based on ^{1}H NMR was determined to be 19 % which was relatively and can be attributed to the low solubility of this compound.

The thiol content determined was 21 µmol/g of polymer, which is lower than expected since there was an additional site available for functionalization. This observation could be as a result of the insolubility of the polymer in the buffer, and therefore little reaction occurred with the Ellman's reagent present. Due to the poor solubility of this polymer, the reaction was not further optimised. This poor loading as determined by the DTNB assay supports the NMR data obtained.

The elemental analysis values were within the range of the calculated values.

The IR spectrum in Figure 2.23 showed an absorption band at 3413 cm^{-1} assigned to OH while a weak band was observed for SH in the region of 2100 cm^{-1}. The band at 1662 and 1570 cm^{-1} correspond to C=O and NH stretching vibrations while that at 1101 cm^{-1} was assigned to the pyranose ring.

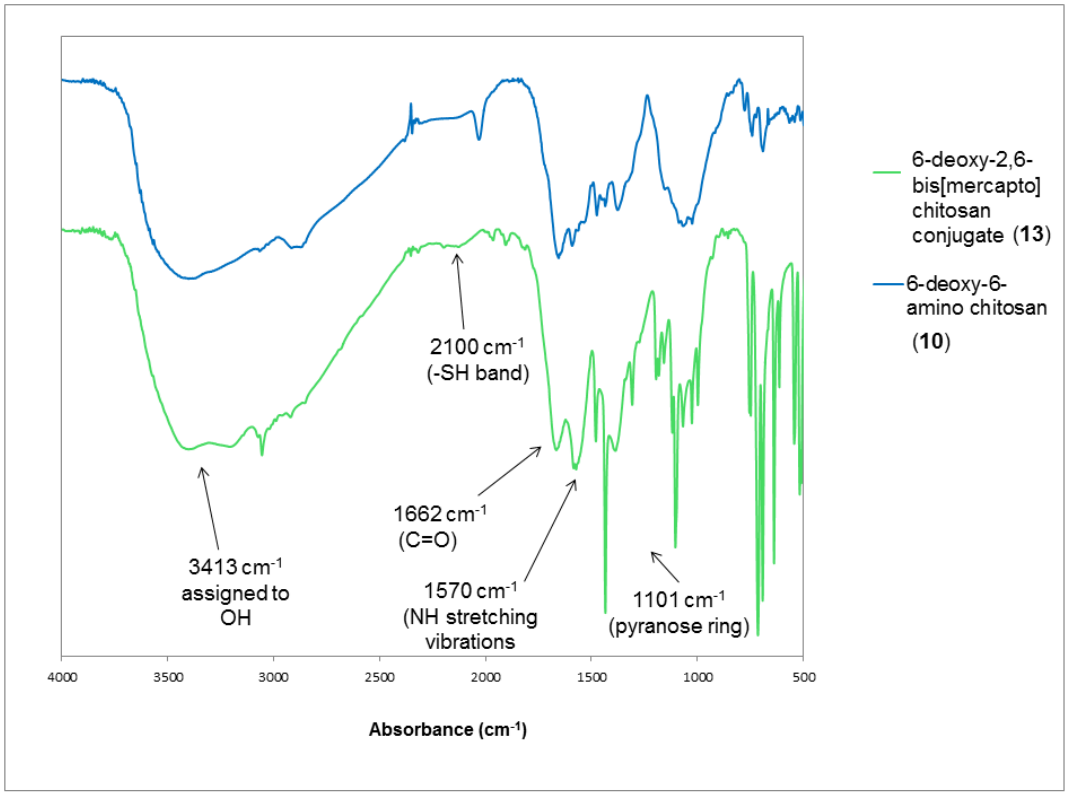
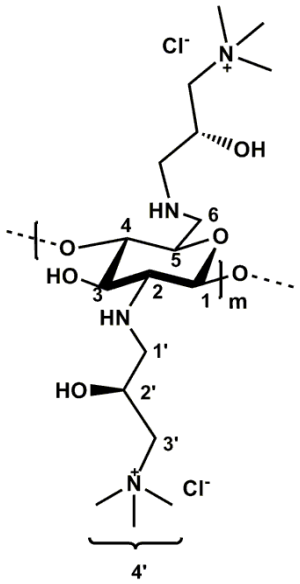


Figure 2.23: IR spectrum of 6-deoxy-2,6-bis[2*S*'-mercaptosuccinyl] chitosan conjugate (**13**) vs. 6-deoxy-amino chitosan (**10**) (KBr).

*2.2.5. Synthesis of 6-deoxy-2,6-bis[3-trimethylammonium-2-hydroxypropyl-N-chitosan chloride] (**14**)*



The polymer 6-deoxy-2,6-bis[3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride] (**14**) was synthesized by reacting 10 with glycidyl trimethylammonium chloride under heat (Scheme 2.4).[14] A brown powder was obtained with a yield of 25 %.

The degree of quaternization (DQ) was determined using the equation reported by Sajomsang *et al*.[16] The DQ for this polymer was found to be 98 %.

The ^{1}H NMR spectrum contained *N*-phthaloyl aromatic protons which were attributed to resonances from δ 7.39 to 8.17 ppm. A resonance observed at δ 4.45 ppm was assigned to H-2' while the polymer backbone protons resonated at δ 3.45 to 3.97 ppm. The resonance at δ 3.29 ppm was assigned to H-2 while the resonance observed at δ 3.01 ppm was assigned to the trimethyl moiety (H-4'). The resonances at δ 2.90 and 2.71 ppm were attributed to H-3' and H-1' respectively with acetylated units resonating at δ 1.81 ppm.

The elemental analysis indicates the inclusion of a water molecule.

The IR spectrum in Figure 2.24 showed absorption bands at 3435 cm^{-1} corresponding to the OH stretch. The band at 1643 cm^{-1} was assigned to the C=O stretch of the residual NAc while the stretch at 1477 cm^{-1} is due to the C-H bending of trimethyl ammonium group.

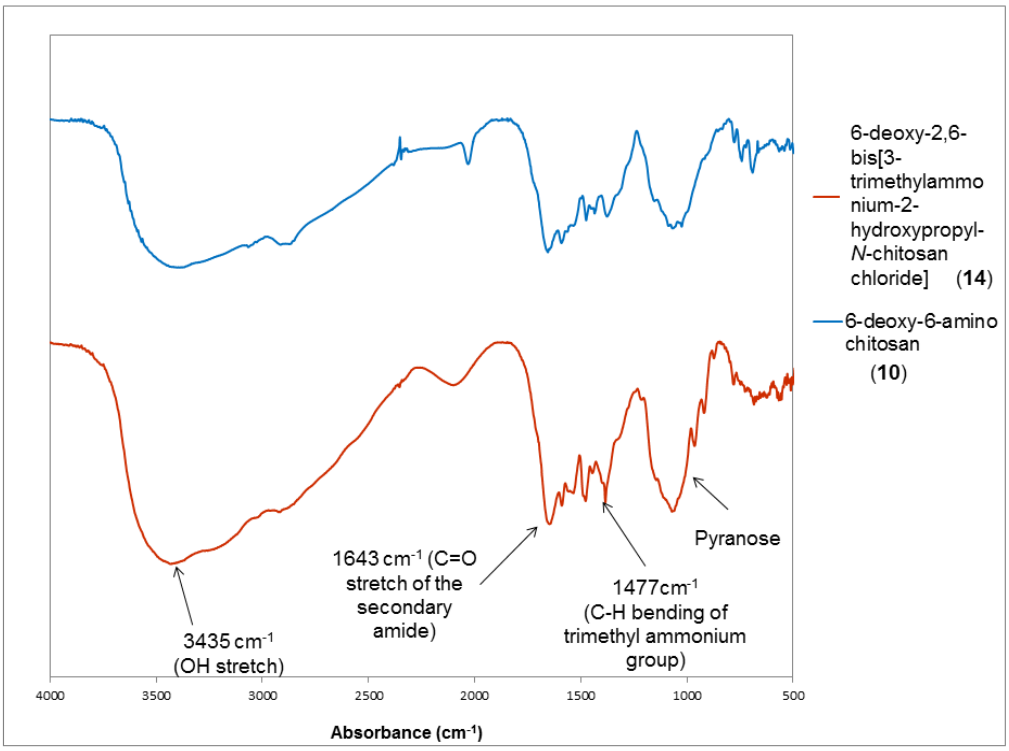
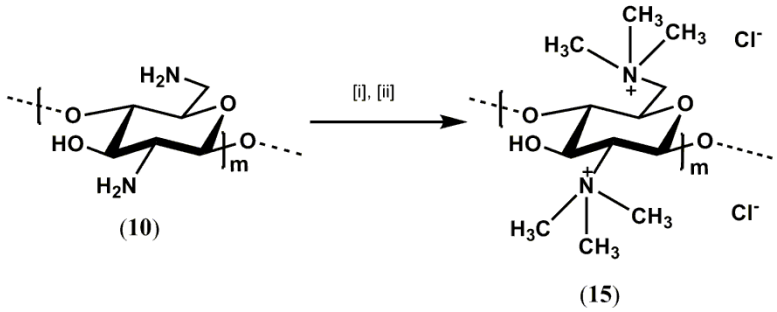


Figure 2.24: IR spectrum of 6-deoxy-2,6-bis[3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride] (**14**) vs 6-deoxy-6-amino chitosan (**10**) (KBr).

*2.2.6. Synthesis of 6-deoxy-2,6-bis[trimethyl] chitosan chloride (**15**)*

The synthesis of polymer **15** was achieved using the method reported by Sadeghi *et al*.[6] 6-Deoxy-6-amino chitosan **10** was reacted with NaI and MeI, to produce the iodide derivative. This iodide derivative naturally converted to the more stable chloride derivative by ion exchange in the presence of Cl^- ions (Scheme 2.11). The product was recovered as a brown powder with a yield of 71 %.



Scheme 2.11. Reagents and conditions: (i) NMP, RT, 4 hrs; NaI, MeI, NaOH, 65 °C, 6 hrs; (ii) acetone, 5 % NaCl, 71 %.

The polymer obtained in this study was insoluble in H_2O, possibly due to the high degree of crystallinity present in the polymer. An NMR spectrum was obtained in a 1 M DCl/D_2O solution. Residual *N*-phthaloyl aromatic protons resonated in the region of δ 7.35 to 8.10 ppm while the protons of the polymer backbone (H-3 to H-6) were observed between δ 3.41 and 4.07 ppm. The resonance at δ 3.05 ppm was assigned to H-2 while the resonances at δ 2.78 and 2.53 ppm assigned to H-1' and $(NCH_3)_2$ respectively. The acetylated units of the polymer appeared at δ 1.79 ppm.

The IR spectrum (Figure 2.25) confirmed synthesis of the polymer with the appearance of the characteristic bands of 6-deoxy-6-amino chitosan, and the introduction of a new band at 1477 cm^{-1} assigned to the C-H bending of the trimethyl ammonium group.

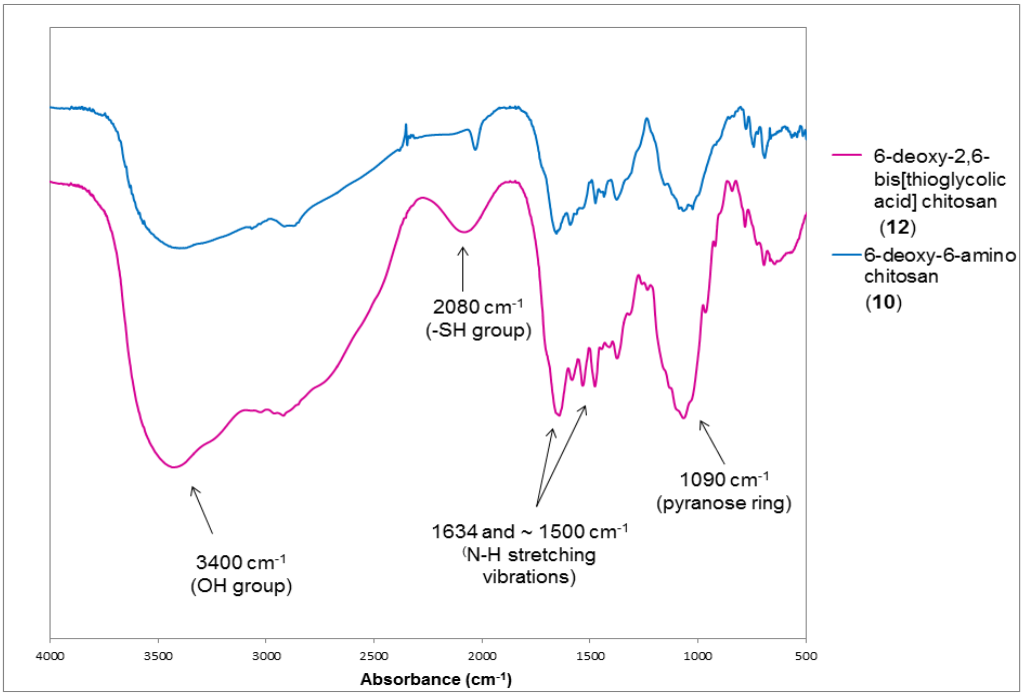


Figure 2.25: IR spectrum of 6-deoxy-2,6-bis[trimethyl] chitosan chloride **15** vs. 6-deoxy-6-amino chitosan **10** (KBr).

The DQ was determined to be 24 % which is considerably lower compared to that reported by Sadeghi *et al*. which was 65 %.[6] This could be due to the fact that the polymer synthesized in this study is not as soluble as the one reported by Sadeghi *et al*.[6] This polymer was sufficient for our purpose and was not further treated to obtain higher loading.

Upon successful completion of the above synthetic reactions, selected polymers were subsequently tested for their potential utility as ion exchange resins. In addition, the antimicrobial activity of selected derivatives was evaluated together with their silver-loaded counterparts. Details of those studies are described in the chapters that follow.

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CHAPTER THREE

APPLICATION OF MODIFIED CHITOSAN IN WATER TREATMENT

3.1. Water remediation

The remediation of water as a means of water conservation is important and key to meeting the ever growing needs of the world's population. Therefore methods for the purification of contaminated water and the re-use of wastewater are highly sought after. The majority of contaminants enter water supplies from human activity. These include heavy metals, distillates, endocrine disrupters and nitrosamines.[1] The focus of the current water remediation study is to remove the contaminant, perchlorate.

3.2. Perchlorate

Metal perchlorate (ClO_4^-) salts are commonly used in propellants and occur naturally to some extent. Its use is currently unregulated in South Africa and perhaps across Africa.[2,3,4] Since ClO_4^- is water soluble and passes into groundwater, it can easily enter into drinking water resources.[3] In the U.S. the Environmental Protection Agency (EPA) added ClO_4^- to its contaminant candidate list, due to studies providing evidence that it inhibits proper functioning of the thyroid gland in humans.[4] The EPA has set a limit of 1 µg/L for perchlorate in drinking water. Inhibition of proper thyroid function occurs *via* ClO_4^- blocking iodine uptake by the thyroid gland, which interferes in the production of thyroid hormone. This can lead to altered neurodevelopment as well as thyroid hyperplasia and tumours. The most susceptible population being pregnant females and foetuses.[2,4] It has been shown that hypothyroidism in breast-feeding and expecting mothers have led to delayed development and decreased learning capability in infants. In addition, hypothyroidism during childhood negatively affects child growth and cognitive motor functions.[5,6]

The most commonly used process for ClO_4^- detection is EPA method 314, where ClO_4^- present in drinking water is determined using ion chromatography. However, this method has drawbacks, such as the fact that only a non-specific response is recorded at a specific retention time which is compared to a ClO_4^- standard. This method therefore does not account for any ionic interference present. Alternative methods which have been proposed for ClO_4^- detection includes, ion selective electrodes, capillary electrophoresis, ion chromatography with mass spectrometry, flow injection colorimetric analysis and ion-pair extraction.[9] The diagram in Figure 3.1 shows the available detection and removal methods for ClO_4^-.

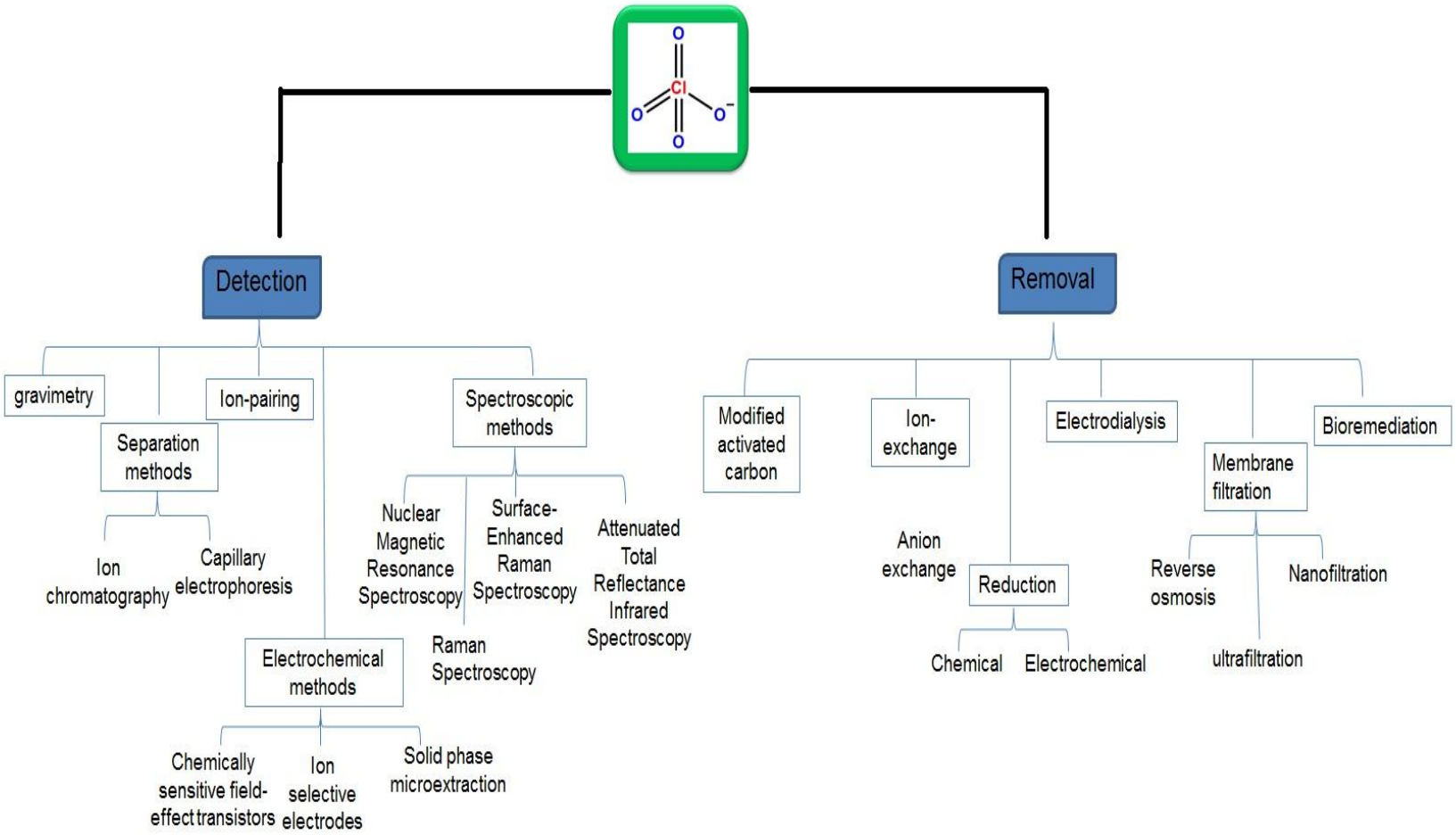


Figure 3.1: Perchlorate detection and removal methods.[9,10]

Burns *et al.* used an ion-pairing reagent to determine the presence of ClO_4^- spectrophotometrically.[6] The cation used in this study was Brilliant Green (BG) dye, forming an ion-pair with ClO_4^- which was extracted into benzene.[6] BG dye contains a triarylmethane moiety with a quaternary nitrogen making the structure inherently stable (Figure 3.2).

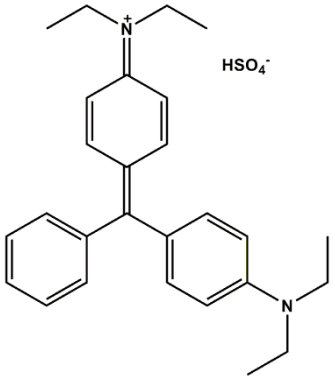


Figure 3.2: Brilliant Green dye.

Thorne *et al*. later used a modified version of this method, where the less toxic xylene was used as the extraction solvent and acetone was used as the solvent for the dye.[7] In the latter method, a commercially available solid-phase extraction cartridge pre-conditioned with a ClO_4^- selective ion-pairing reagent (decyltrimethylammonium bromide) was used. Promising results were reported for the selective retention and detection of ClO_4^-.[7] This result indicates that it is possible to devise a cartridge that will undergo a colour change upon the presence of a low amount of ClO_4^-. These cartridges would serve as a perfect calorimetric test for use in the field. Therefore compounds containing the quaternary alkyl moiety are perfect for ClO_4^- extraction due to the high affinity of the ClO_4^- for the quaternary nitrogen.

3.3. Perchlorate removal

As a result of the detrimental health effects of ClO_4^-, and the high levels present in the environment, the removal of this contaminant is of great interest. Treatment methods available for ClO_4^- removal are ion exchange, biological treatment, adsorption by activated carbon or modified activated carbon, membrane filtration, chemical/catalytic reduction and electrochemical reduction, where ion exchange is the most common method of removal currently used.[11] The negatively charged ClO_4^- anion is exchanged for a harmless ion (e.g. Cl^- or OH^-). Most resins are modified to selectively bind the ClO_4^- ion when competing anions such as sulfate and nitrate are present e.g. quaternary alkyl resins.[12]

The other common method for ClO_4^- removal is the chemical reduction of ClO_4^- to chloride.[4] Fe metal favourably reduces ClO_4^- to the chloride ion and the reduction proceeds at a faster

rate in the presence of UV light, which acts as a catalyst.[13] Nano-scale Fe particles may act as better reducing agents compared to normal Fe particles. To keep the Fe nanoparticle sizes to a minimum, a coating agent such as chitosan is used.[14] Chitosan-coated iron oxide nanoparticles have been investigated for use in molecular imaging and drug delivery.[15] Chitosan-Fe nanoparticles have been successfully used to remove chromium from aqueous solutions. The removal of the chromium was based on the adhesion of the metal to magnetic chitosan-Fe sorbent material.[16] Gupta *et al.* has used iron–chitosan composites to remove arsenic from groundwater.[17] In addition, a protonated form of chitosan has been used to remove ClO_4^- from solution. This process involved the formation of a quaternary ammonium ion of chitosan which bound ClO_4^- in an electrostatic attraction. However, ClO_4^- adsorption was decreased due to the presence of competing ions such as sulfate which was preferentially adsorbed.[11] Therefore, the use of chitosan-Fe nanoparticles in the removal of ClO_4^- seems encouraging.

Modifications of chitosan to render it more selective for ClO_4^- binding seemed promising. One such modification that was considered included the introduction of quaternary ammonium groups onto the polymer backbone. It is known that trialkylammonium quats stabilizes the ClO_4^- anion.[9] Two common quaternized chitosan derivatives are trimethyl chitosan chloride (TMC) and 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (CHI-Q188).[18,19] These modified chitosans are expected to have improved ClO_4^- affinity and additionally, Fe may be loaded on these polymers to potentially reduce the complexed ClO_4^-. Fe-loaded versions of these quaternary chitosan derivatives have been synthesized.[20,21]

In this study, chitosan and its derivatives, CHI-Q188 (**5A**) and TMC (**6A**) (Figure 3.3), were tested for their potential to act as ion exchange resins for the selective removal of ClO_4^- from water.

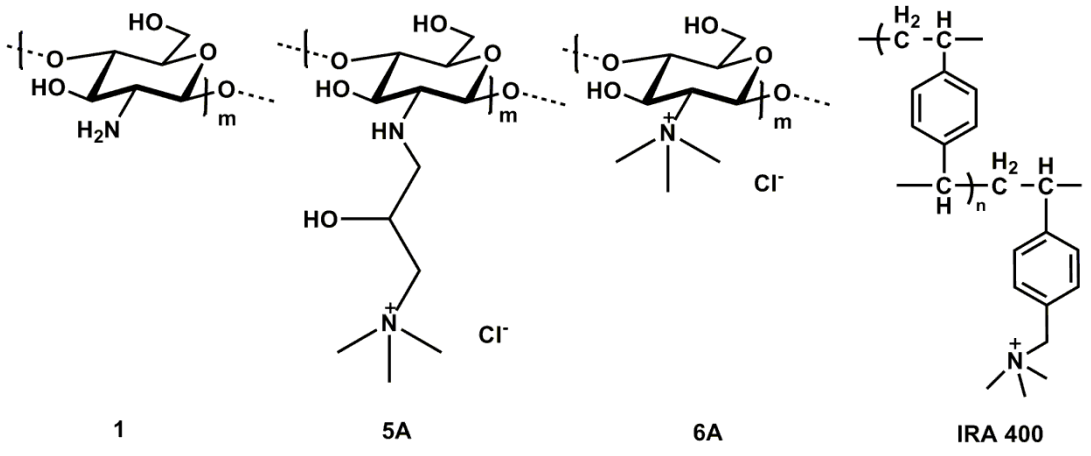


Figure 3.3: The structures of chitosan (**1**), CHI-Q188 (**5A**), TMC (**6A**) and IRA 400.

Selected polymers were loaded with Fe nanoparticles which previously demonstrated ability to reduce ClO_4^-.[4] The polymers were packed into solid phase extraction (SPE) cartridges and evaluated for their ClO_4^- removal ability. A commercially available synthetic resin, IRA 400 containing a similar quaternary ammonium group (Figure 3.3) was used as a benchmark/standard. IRA 400 has been previously tested for its ClO_4^- removal capability in a study conducted by Tripp *et al*.[22]

3.4. Fe-loaded polymers

Chitosan (**1**) and its derivatives, CHI-Q188 (**5A**) and TMC (**6A**), were used to synthesize encapsulated Fe nanoparticles. The method followed was a modified version of the method reported by Tsai *et al.*[14] Co-precipitation from a solution of ferrous/ferric salts by the addition of a base is the standard way to prepare iron oxide nanoparticles. However, in the current study, iron oxide nanoparticles were produced by precipitation of iron(III) chloride hexahydrate by ammonium hydroxide. All compounds were obtained as red/brown solids. These solids were characterized by IR and TEM analysis where the sizes were calculated from an average of 100 particles. The iron concentration of the samples was determined calorimetrically using *O*-phenanthroline and comparing the samples' absorbance (at 508 nm) to known standards.[23]

*Chitosan-Fe (**16**)*

Fe loaded chitosan has been previously reported by a number of authors for a variety of uses.[14,16] The Fe is complexed to the polymer by an electrostatic attraction. A number of methods have been used to prepare these chitosan-Fe particles, Bajpai *et al.* prepared magnetite particles by co-precipitation of Fe(II) and Fe(III) in an alkaline medium at an elevated temperature.[12] These particles were subsequently stirred with an acid solution of chitosan to yield a magnetite-chitosan nanocomposite. This nanocomposite was thereafter loaded with Fe(III) and the average diameter of these particle was found to be 29 nm.[16] The Fe content of the polymer synthesized in this study was determined by spectroscopic analysis comparing the absorbance of the polymer to that of a Fe standard. The Fe content of this polymer was determined to be 0.0475 mmol/g of polymer. The ferrofluid synthesized by Tsai *et al*. had a Fe concentration of 10.4 mg Fe/ml (0.051 mmol/g), which is comparable to the result obtained in this study.[14]

TEM was used to examine the particles at a higher magnification, enabling the determination of particle size, shape and dispersion. The chitosan-Fe particles appeared to be spherical as shown in the TEM image. The particle size distribution was very broad, with an average size

of 9 ± 3 nm (Figure 3.4). The sizes of these particles ranged from 2 – 17 nm. These sizes were slightly smaller than those reported by Bajpai *et al.,* indicating that agglomeration had occurred to a lesser extent.[16]

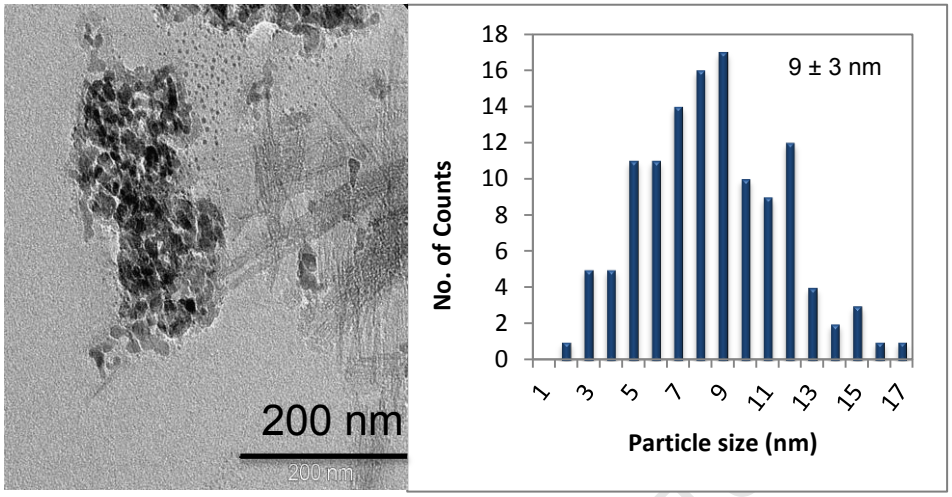


Figure 3.4: TEM micrograph of chitosan-stabilized Fe nanoparticles and corresponding size distributions.

The IR spectrum of chitosan vs. chitosan-Fe is shown below (Figure 3.5). The chitosan-Fe spectrum shows the characteristic bands of chitosan with no major shift in the peaks. The peaks in the chitosan-Fe complex are sharper than those observed in native chitosan. There is a reduction in the absorbance observed at ~ 3400 cm^{-1}, which points to the participation of the amine group in the formation of the chitosan-Fe complex as reported by Kaushik *et al*.[24] In addition, a new absorbance is observed at 672 cm^{-1} which corresponds to β-FeOOH.[25]

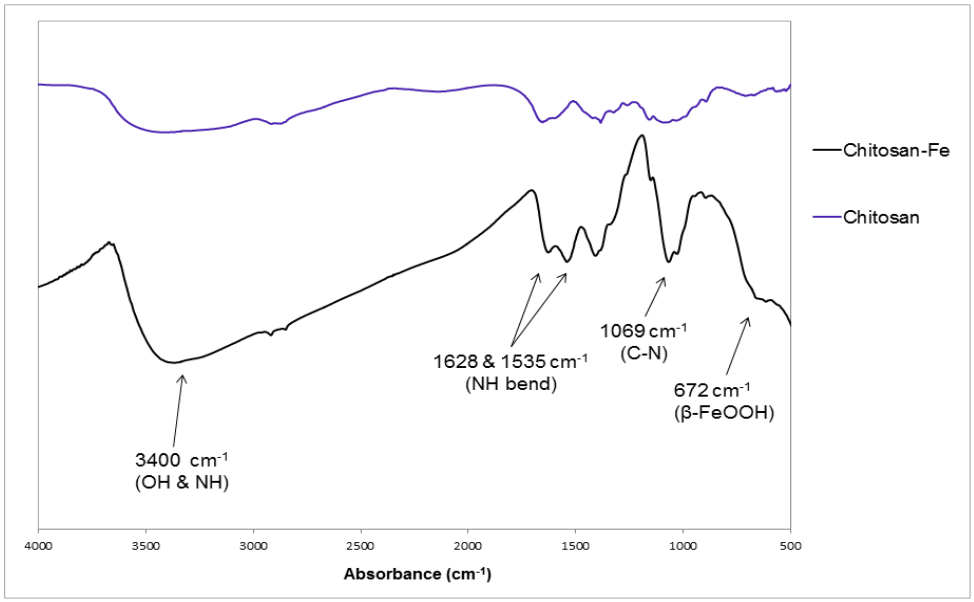


Figure 3.5: IR spectrum of chitosan vs. chitosan-Fe.

*CHI-Q188-Fe (**17**)*

Shen *et al.* have previously reported the synthesis of Fe-loaded CHI-Q188.[20] This compound was tested as a novel potential magnetic resonance imaging contrast agent for cell tracking.

The IR spectrum of CHI-Q188-Fe is similar to that observed for chitosan-Fe. The Fe loaded polymer showed the characteristic bands of CHI-Q188, however, the bands have shifted to a lower wavenumber. It has been reported that in the formation of a complexed species, functional group absorption can be displaced below or above the original region of absorption. The decrease in wavenumber of the OH & NH bands in the region of ~ 3100 cm^{-1} provides evidence for the participation of the amine group in the coordination with the metal ion. This agrees with the observations reported by Hernàndez *et al.,* who have proposed structures for possible chitosan–Fe^{3+} complexes.[26] However, techniques such as Atomic absorption spectroscopy or solid state ^{15}N-NMR would provide further evidence for Fe-NH_2 coordination.

The Fe content of this polymer was determined to be 0.4089 mmol/g of polymer using UV spectroscopy.

The samples were analyzed using TEM; the particles appeared spherical in shape. The particles size distribution was very broad with an average size of 9 ± 2 nm as shown in the bar graph (Figure 3.6). The sizes of these particles ranged from 3 – 17 nm.

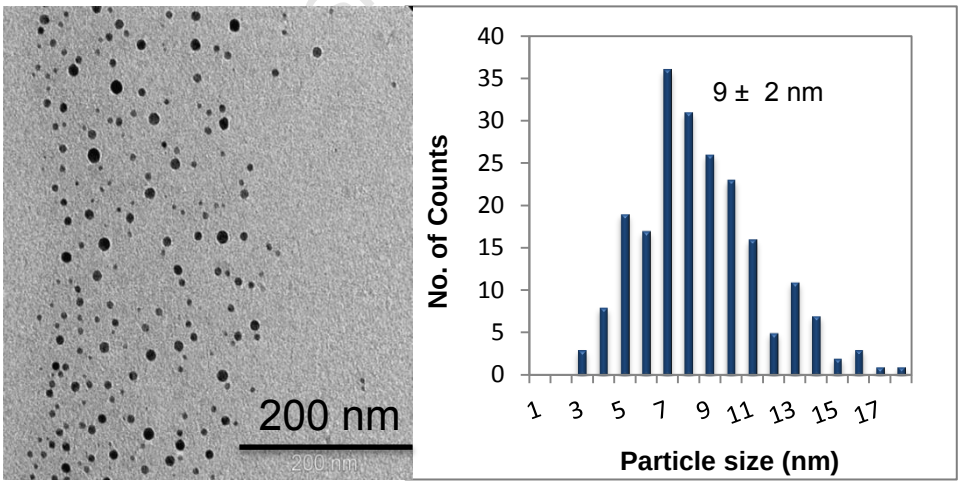


Figure 3.6: TEM micrograph of CHI-Q188-stabilized Fe nanoparticles and corresponding size distributions.

*TMC-Fe (**18**)*

This Fe-loaded polymer has been previously reported by Belessi *et al.,* following a different synthetic method.[21] The Fe content of this polymer was determined to be 0.3232 mmol/g of polymer. The IR spectrum of TMC and its Fe-loaded counterpart is similar. The absorption bands of the TMC-Fe-loaded spectrum appeared broader when compared to that of the native TMC. However, the decrease of the absorption band of OH & NH to the region of ~ 3100 cm^{-1} and the intensity of the amine at ~1600 cm^{-1} points to the participation of the amine group in the coordination with the metal ion. In addition, the intensity of the CH bending band of the trimethyl ammonium group is more pronounced. The band at 668 cm^{-1} was assigned to β-FeOOH.

Analysis of the sample using TEM revealed that the particles appeared as a mixture of spherical and triangular shapes. The particle size distribution was very broad with an average size of 10 ± 3 nm (Figure 3.7). The sizes of these particles ranged from 3 – 23 nm. The larger particles are most likely due to agglomeration (clustering) of smaller particles. However, these particles were slightly smaller compared to those reported by Belessi *et al*. (20 – 40 nm).[21]

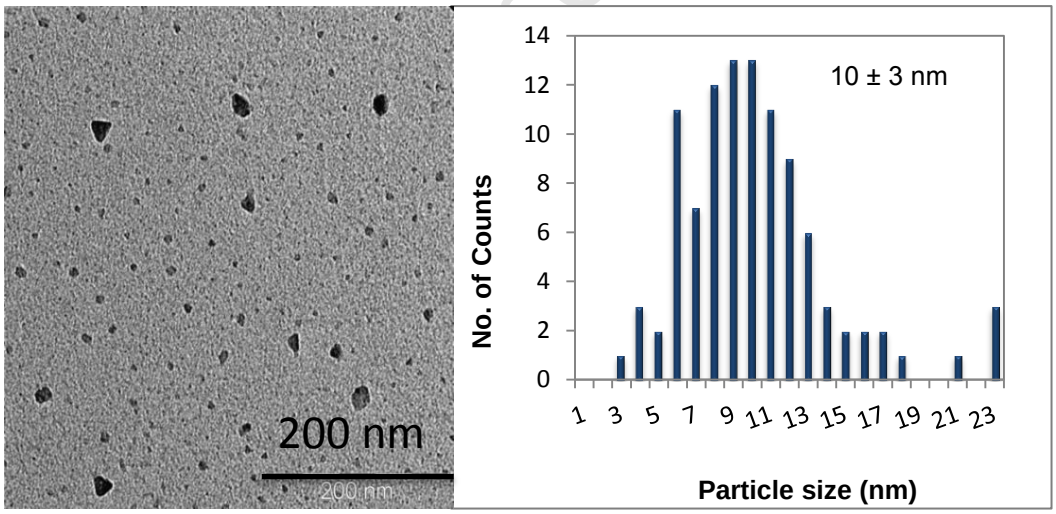


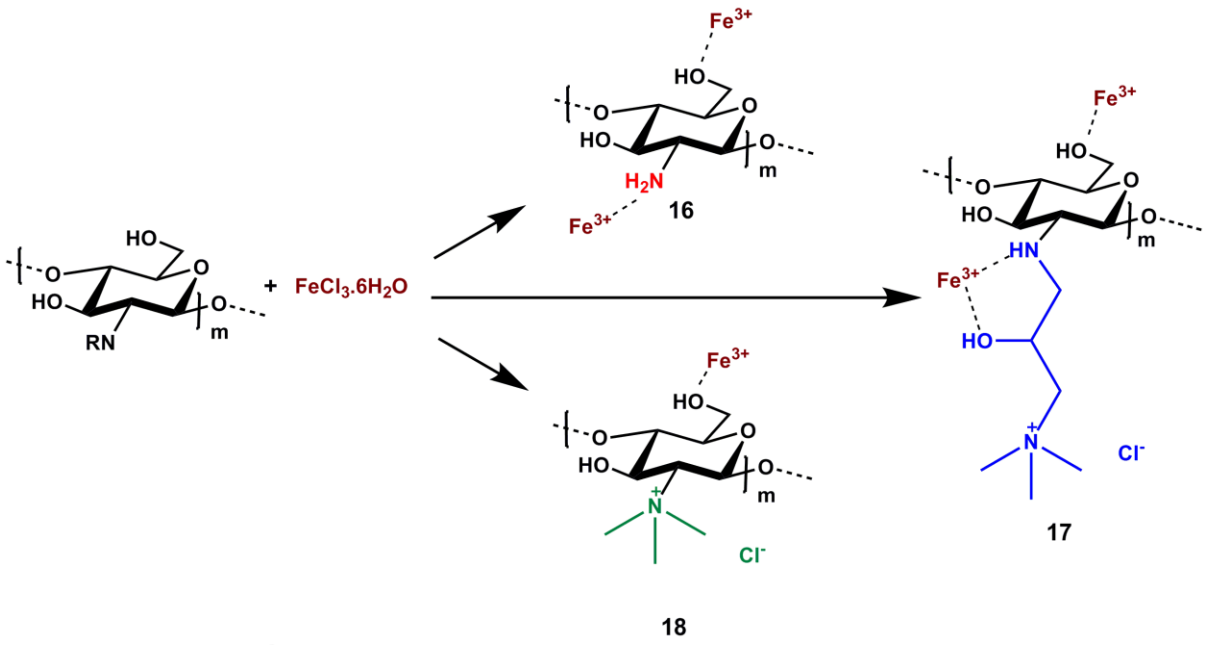
Figure 3.7: TEM micrograph of TMC-stabilized Fe nanoparticles and corresponding size distributions.

The polymers synthesized here have been loaded with Fe and subsequently characterized. Results indicate that chitosan and its derivatives have formed encapsulated Fe nanoparticles with sizes less than 50 nm. The Fe content of these polymers has been determined and the quaternary derivatives have displayed significantly higher Fe content compared to those of native chitosan. This result points to the possible participation of the quaternary nitrogen in

the formation and stabilization of the Fe nanoparticles. Another explanation could be that these quaternary polymers are simply more soluble which results in a higher loading. At the time of this study, these polymers had not been utilized for the removal of ClO_4^-.

3.5. Perchlorate (ClO_4^-) removal

Chitosan (**1**) and its quaternary derivatives CHI-Q188 (**5A**) were tested as potential stationary phases in the removal of ClO_4^-. In addition, these compounds were loaded with Fe and the ClO_4^- removal abilities of these Fe loaded derivatives was assessed (Scheme 3.1). The ClO_4^- removal abilities of all polymers were compared to that of IRA 400. The commercially available anion exchange resin Amberlite IRA 400 was used as a control. TMC (**6A**) was not tested as a ClO_4^- retention resin as the anion could not be applied to the polymer without dissolving the stationary phase.



Scheme 3.1: The Fe loaded polymers tested in this study with most likely points of interaction with the polymer.

3.5.1. Testing Protocol

The testing protocol used in this study is a modified version of that used by Thorne *et al*.[7] ClO_4^- standards (1-6 µg/L) were prepared and subsequently spiked with Brilliant Green (BG) dye. This dye is an ion-pairing agent which binds to the ClO_4^- ion which can then be extracted with an organic solvent. Common organic solvents which have been used in ClO_4^- extraction with BG include benzene, xylene and toluene.[8,27] Toluene was used in the current study as it is more environmentally friendly compared to benzene and xylene. The

absorbance (arbitrary units (AU)) of the ClO_4^- standards was read at 640 nm and a calibration curve was plotted (Figure 3.9).

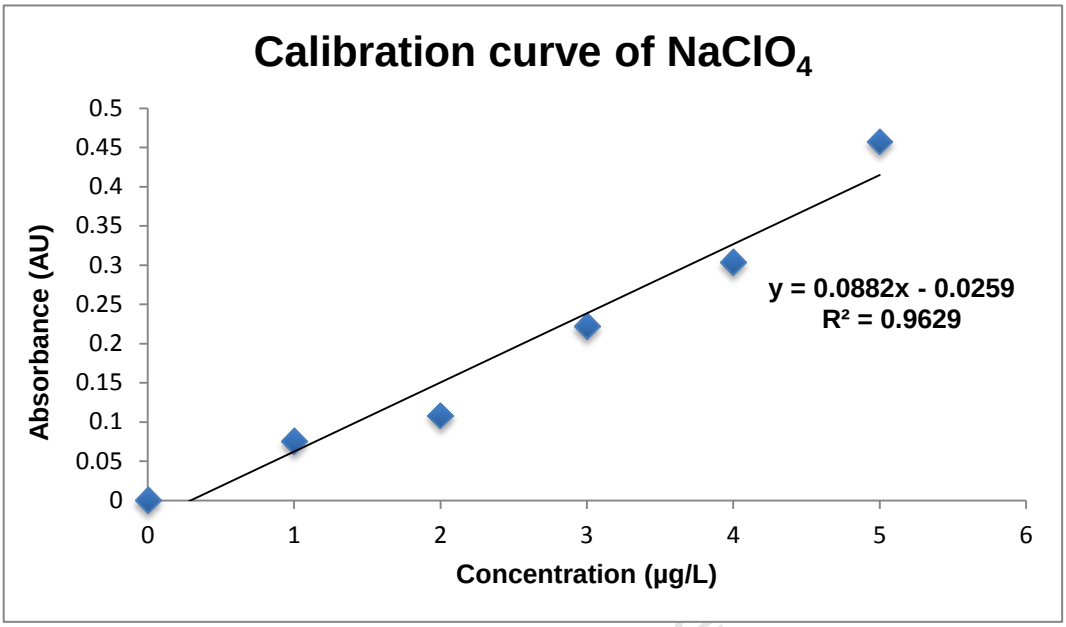


Figure 3.9: Calibration curve of $NaClO_4$ standards.

The polymer was packed into an SPE cartridge, pre-conditioned with acetone and H_2O, and thereafter a solution of $NaClO_4$ (5 µg/L ClO_4^-, 100 mL) was passed through the column. A total of 0.5 µg of ClO_4^- was applied to the column. The eluent was spiked with BG dye and extracted with toluene. The absorbance of the sample was read at 640 nm and the ClO_4^- concentration was extrapolated from the graph. The addition of acetone and extraction with toluene was repeated until the level of ClO_4^- was below the calibration minimum.

The above mentioned procedure was followed for the majority of samples tested. However, a modified version of this method was used when testing with polymer **5A**, as the polymer formed a gel when pre-conditioned. The polymer was allowed to stir in a 5 µg/L ClO_4^- solution (100 mL) for 1 hr and the subsequent viscous solution was centrifuged, the supernatant removed and the absorbance measured and the pellet dried by lyophilisation. The dry powder was packed into an SPE cartridge and the retained ClO_4^- was eluted using acetone followed by water. Polymer (**17**), the Fe loaded derivative of polymer (**5A**) was preconditioned, dried and run using the same procedure as the other polymers tested.

Each polymer was tested three times in order to ensure reproducibility of results. All columns were treated with the same sample elution program. New batches of dye were prepared for each column and the concentration of the ClO_4^- solution was checked just before running of

the columns. The graph in Figure 3.10 shows the percentage ClO_4^- retention of the polymers tested, together with IRA 400 for comparative purposes. These values were obtained by calculating the difference of the total amount of ClO_4^- applied to the column *vs.* that which was detected in the collected fractions (see section 6.6). This is a batch process whereby retention of perchlorate on the column will result in less perchlorate detected in the collective fractions over the test period

IRA 400 is a resin composed of 8 % cross-linked styrene/divinylbenzene with a quaternary trimethyl ammonium functional group. IRA-400 is a strong basic quaternary ammonium ion exchange resin which has a positive charge, and can form an ionic bond with the ClO_4^- ion. This ion exchange resin has previously been used for the recovery of L-(+)-lactic acid from a fermentation broth at a pH above and below the p*K*a of lactic acid.[28] Chromate has also been removed using IRA 400 where it was found that the ion sorption increases with a decrease in pH and an increase in temperature.[29] Chabani *et al.* used this resin to remove nitrates from water with good retention.[30] The commercially available Amberlite IRA 400 anion exchange resin was tested using the procedure as outlined above and used as the benchmark for all other resins tested. The absorbance values recorded at 640 nm indicated if any ClO_4^- was present in the eluted fractions.

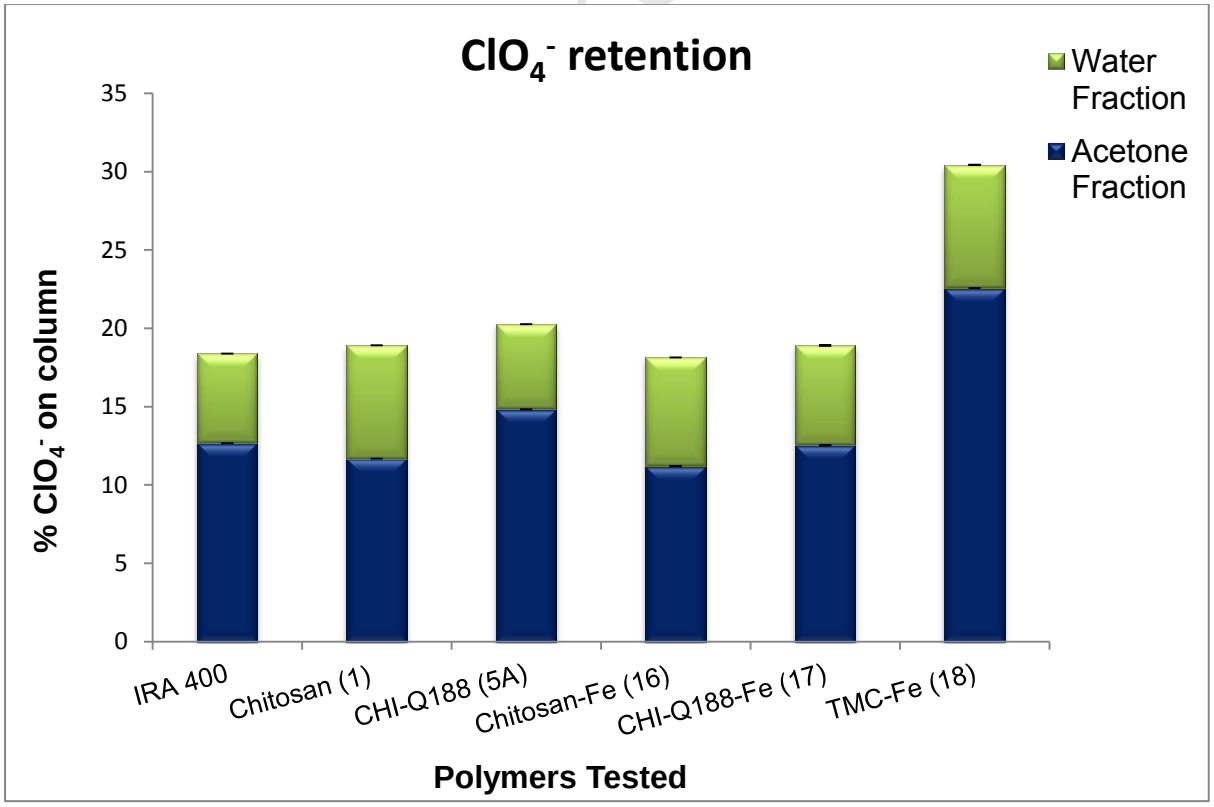


Figure 3.10: The % ClO_4^- retention of IRA 400 and the polymers tested [chitosan (**1**), CHI-Q188 (**5A**), chitosan-Fe (**16**), CHI-Q188-Fe (**17**) and TMC-FE (**18**)] eluted using acetone followed by water together with error bars.

The graph shows that IRA 400 is capable of binding the ClO_4^- anion as reported by Tripp *et al*.[22] This affinity for the ClO_4^- anion is due to the presence of the positively charged quaternary ammonium functional group. A higher percentage of ClO_4^- is eluted with acetone compared to the small amount recovered in the water fraction. Acetone may therefore be considered if it is deemed necessary to recycle the column. This can be attributed to the hydrophobic nature of the polymer favouring release of the ClO_4^- ions in the acetone fraction. Accordingly, IRA 400 retained approximately 63 % of the ClO_4^- applied to the column.

*Chitosan (**1**)*

Chitosan has been shown to chelate heavy metals and transition metals which may be present in contaminated water.[31] Protonated chitosan in an acidic medium has been used to successfully remove ClO_4^- from solution. Chitosan was tested as a potential water treatment resin for the removal of ClO_4^- using the procedure reported by Thorne *et al*.[7] Chitosan is positively charged as the amino group present has a pK_a of ~6.5, therefore the polymer can bind to negatively charged ions such as ClO_4^-.[32]

As with the IRA column, the ClO_4^- solution was applied to the column after pre-conditioning with acetone and water. Any ClO_4^- bound to the resin was eluted using acetone, spiked with BG and extracted with toluene. The absorbance values recorded at 640 nm indicated if any ClO_4^- was present. Chitosan performed similarly to IRA 400 confirming that this polymer does retain ClO_4^- which agrees with that reported by Xie *et al*. when they used protonated chitosan in ClO_4^- removal.[11] A slightly higher percentage of ClO_4^- was obtained in the water layer compared to IRA 400 retention. Chitosans retains on average 62 % of the intial ClO_4^- applied to the cartridge. This data suggests that chitosan can be used as an alternative resin in the removal of ClO_4^- from contaminated water.

*CHI-Q188 (**5A**)*

This chitosan derivative was chosen for this study due to the presence of a quaternary ammonium functional group on the polymer backbone. The positive charge allows this polymer to function as an anion exchange resin. The study by Tripp *et al*. showed that the presence of quaternary ammonium functional groups improves retention of ClO_4^-.[22] This chitosan derivative has been used as a flocculating agent in a study by Ali *et al*., who found that polymers with a moderate molecular weight and charge density displayed the best flocculation performance in the model suspensions tested.[33] A slightly different method was used when this polymer was tested for ClO_4^- retention, since the polymer formed a gel when preconditioned. After stirring the polymer in a ClO_4^- solution for 1 hr, the mixture was freeze

dried, followed by elution of any retained ClO_4^- using acetone followed by water. The graph shows that this polymer performed slightly better than IRA 400 and chitosan in terms of ClO_4^- retention. A higher fraction of ClO_4^- was obtained in the acetone layer compared to the water layer which is indicative of theincrease in hydrophobicity in relation to chitosan. It is also noteworthy that the polymer synthesized in this study is not as water soluble as previously reported but forms a gel when it comes into contact with water.[19] CHI-Q188 (**5A**) bound approximately 59 % of the ClO_4^- solution which the polymer was exposed to.

*Chitosan-Fe (**16**)*

Chitosan was used as a surfactant in the production of Fe nanoparticles. In a previous study by Geng *et al*., chitosan-stabilized Fe^0 nanoparticles were used to remove hexavalent chromium from water.[34] In addition; Gupta *et al*. used a Fe-chitosan composite to remove arsenic (III and V) from contaminated water.[17] In this study, the compound was obtained by the precipitation of $FeCl_3 \cdot 6H_2O$. The nanoparticles produced had an average size of 9 ± 3 nm and the Fe content of the sample was found to be 0.0475 mmol/g of polymer. The Fe-coated particles were packed into a SPE column, preconditioned with acetone and water and a 5 µg/L ClO_4^- solution was passed through the column which was subsequently eluted using acetone and water respectively. The graph shows that the results for this polymer are similar to those obtained for chitosan, however, the reduction of ClO_4^- by Fe has not been determined in this study. Therefore a lower percentage recovery does not necessarily indicate that the polymer is not as or more effective than chitosan itself as reduction on the column has not been established. Retention of 63 % of all ClO_4^- applied to the stationary phase was observed for this polymer.

*CHI-Q188-Fe (**17**)*

The elution of ClO_4^- from this polymer with encapsulated Fe nanoparticles was monitored. This polymer did not form a gel when water was applied to the column unlike the native polymer. Results indicate that a lower percentage of ClO_4^- was obtained in the acetone layer compared to the native polymer, however, a higher concentration of ClO_4^- was recovered in the water fraction. As mentioned previously, this study did not include the reducing effects of Fe on the ClO_4^- anion. Kim *et al*. reported the reduction of ClO_4^- in the presence of Fe.[13] The Fe concentration on this polymer was found to be 0.4089 mmol/g which is considerably

higher compared to the amount of ClO_4^- applied to the column (0.5 µg) therefore we expect to see perchlorate-Fe complexations as well.

CHI-Q188-Fe nanoparticles for the use as ClO_4^- reducing agents has not been previously reported however, protonated chitosan has been used to remove ClO_4^-.[11] CHI-Q188-Fe retained 62 % of the initial ClO_4^- solution which was very similar to those reported above.

*TMC-Fe (**18**)*

The TMC-Fe (**18**) showed a significantly higher ClO_4^- retention, which was almost double that of the other polymers for the acetone fraction. There is also a high recovery of the anion in the water fraction. This can be attributed to the high solubility of the parent polymer which makes ClO_4^- leaching into the resin easier for this polymer compared to the other polymers tested. The TMC-Fe (**18**) polymer will therefore be a good candidate to study the reduction potential for ClO_4^- on the column.

Summary

Overall, the columns tested appeared to retain ClO_4^-, some better than others. The commercially available IRA 400 absorbs ClO_4^- while chitosan (**1**) had a similar affinity for the ClO_4^- anion. In comparison, CHI-Q188 (**5A**) had a slightly better average ClO_4^- retention, possibly due to the presence of the quaternary ammonium group as well as the fact that this polymer swelled when exposed to the ClO_4^- solution, effectively trapping a greater amount of ClO_4^-. The chitosan-Fe (**16**) loaded polymer displayed similar retention to that of chitosan, however, the ClO_4^- reduction capabilities of this resin has yet to be investigated. The CHI-Q188 (**5A**) polymer displayed a slightly improved retention compared to that of CHI-Q188-Fe (**17**). This is a possible result of reduction of ClO_4^- on the Fe-loaded column. However, the latter finding will need to be proven. The TMC-Fe (**18**) polymer displayed the best ClO_4^- retention capabilities of all the polymers tested thus far and the presence of the Fe may facilitate the reduction of ClO_4^- to the chloride ion. This investigation of the effect of Fe on ClO_4^- reduction will form a part of further studies. The reduction of ClO_4^- to Cl^- can be improved with the use of a catalyst such as UV light. The reduction can be confirmed using ion chromatography analysis of the eluent where an anion trap column can be modified to distinguish between ClO_4^- and Cl^-. The pH of the solution can also be used as an indicator of reduction as the pH drops when reduction occurs. It has been reported that, ClO_4^- removal correlates with an increased concentration of Fe metal.[13] The incorporation of Fe on these polymers presents a dual functionality where the ClO_4^- anion is retained and degraded with

minimum waste generation and the biodegradability of these polymers is also an added advantage.

In the pursuit of multi-functional polymers, the chitosan derivatives were also tested for their applicability as potential antimicrobials. Polymers which displayed antimicrobial activity may be applied as water filters in the treatment of contaminated water.

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CHAPTER FOUR

ANTIMICROBIAL APPLICATIONS OF CHITOSAN IN WATER TREATMENT

4.1. Introduction

Microorganisms are present in all environments, where they play a role in food and biogeochemical cycles in nature. Potentially harmful microorganisms may also be present; and are typically associated with communicable diseases that in many cases are transmitted through drinking water. The WHO has published guidelines which distinguish waterborne pathogens from drinking-water contaminants.[1] As a result of the potential presence of these pathogens in our drinking water, it is necessary to develop water treatment technologies to eliminate them. In households, it is common to guard against point of use water borne pathogens by using water filters which are attached directly to the tap or sink (point-of-use filters) or at the domestic water supply (point-of-entry filter). These filters cannot guard against all pathogens present in the water.[2] Water filters available include ceramic filters, membrane filters (microfilter, ultrafilter, nanofilter and reverse osmosis), carbon filters, granular media filters etc. Filters which are effective against bacteria are those which have a pore size less than 1 μm since the majority of bacteria and viruses lie in that size range. In addition, microfilters do not remove all viruses, therefore added treatment such as chemical disinfection, boiling/pasteurization or the use of UV light can be used to reduce the threat of water borne pathogens.[2] On a large scale, water is purified by treatment options which include pre-treatment, coagulation, flocculation, sedimentation, filtration and primary disinfection methods.[1]

To meet the ever present threat of water contamination, researchers have developed commercially available antimicrobial water filters.[3] There are a variety of water filters in the market with many claiming to remove all microbes present. Currently twelve common types of filters or water treatment options are available, these include charcoal filters (low cost, commonly used), KDF filter media (Kinetic Degradation Fluxion, redox filtration media which converts free chlorine to a harmless chloride), sediment filters (mostly for well water), water distillers (removes some pathogens and minerals), ceramic filters, reverse osmosis, atmospheric water generators, ultraviolet water filters, magnetic water filters, infrared water filters, catalytic water filters (targets specific toxins) and alkaline water ionizers.[3] In today's water treatment industry, silver and nanosilver has generated considerable interest as potential water filters with inherent antibacterial properties.[4]

4.2. Silver Studies

Silver (Ag) metal has proven antibacterial properties, and has been shown to inhibit a range of disease causing organisms.[5,6]

Drug resistance has forced scientists to re-evaluate potential novel, non-toxic biocidal agents. Since Ag and Ag ions have been found to be wound-healing agents with low toxicity, attention has been focused on the development of Ag antimicrobial agents.[7] The mechanism by which Ag induces bactericidal activity is unclear. It has been postulated that the ionic Ag inactivates important enzymes in the bacteria.[8] It has been reported that the inherent antibacterial properties of Ag can be enhanced upon reducing its size to the nanoscale.[9]

The higher surface area to volume ratio at the nanoscale may improve certain properties of Ag such as the inherent antimicrobial properties. Nanoparticles are synthesized as follows: by the chemical reduction of Ag ions with or without stabilizing agents, thermal decomposition in organic solvents as well as chemical and photoreduction in reverse micelles.[10] Current nanoparticle synthetic techniques involve the use of strong reducing agents which generates hazardous waste. Therefore, in an effort to minimize the production of these hazardous materials, alternative methods of producing nanoparticles are being sought. A green synthetic method for the production of Ag nanoparticles is an area of great interest. Green synthetic methods in contrast to normal synthetic methods, takes into consideration the solvent, reducing agent and a nontoxic stabilizer.[11] A nontoxic stabilizer which has generated considerable interest is the biopolymer chitosan. As mentioned earlier, chitosan has excellent metal chelating abilities. This chelation ability stems from the presence of hydroxyl and primary amino groups on the polymer backbone, these groups are good metal chelating sites. Chitosan-metal nanoparticles have been previously synthesized where the primary amines and hydroxyl groups present allow efficient coordination to metal ions. This leads to the production of particles with smaller dimensions either by chelating or an ion exchange route.[12,13] Chitosan itself possesses inherent antimicrobial properties, however, the mechanism of this interaction is yet to be proven. Common theories postulate that the positively charged chitosan binds to the negatively charged cell membrane of the bacteria *via* an electrostatic attraction. Low concentrations of chitosan (0.2 mg/mL) have been shown to cause aggregation of bacterial cells and degradation of the cell walls has been confirmed by TEM analysis upon application of chitosan. In addition, it was found that the cytoplasmic membrane of the bacterial cell detached from the cell wall when exposed to chitosan. Chitosan essentially affects cell membrane permeability leading to the loss of intracellular components of the bacterial cell which in turn leads to cell death. Also as a result of chitosan's metal chelating ability, the polymer is able to bind metal ions from the

bacterial intracellular fluid which leads to disruption of proper cell functioning. The degree of deacetylation (DDA) of chitosan is another factor which affects the antimicrobial activity of this polymer. A higher DDA results in a greater number of potentially cationic sites available on the polymer backbone therefore chitosan is more active against bacteria at a lower pH. Temperature also plays a role in chitosan activity where the optimum temperature for activity of chitosan is 37 °C.[14] Chitosan-Ag nanoparticles have exhibited antibacterial activity as reported by Zhan *et al*.[15] Chitosan stabilizes the particles and prevents further growth keeping particle size to a minimum.[15] Due to the greatly enhanced antimicrobial properties of silver nanoparticles and the inherent antimicrobial properties of chitosan, the synthesis of silver loaded chitosan derivatives and the antimicrobial activities thereof is of great interest.

4.2.1 Silver loading of chitosan and its derivatives

The method used to load Ag onto chitosan and its derivatives was adapted from a report by Wei *et al*.[16] Fourteen chitosan derivatives were loaded with Ag. Chitosan and its derivatives **1-6** & **10-15** in H_2O were allowed to stir in a solution of 52 mM $AgNO_3$ (molar ratio of Ag:chitosan = 1.5) for 16 hours. The resulting solutions were centrifuged and the supernatant removed. The pellet was washed with H_2O, centrifuged, isolated and freeze-dried. In cases where the silver loaded polymer did not precipitate, the whole sample was dialyzed and thereafter freeze dried. The presence of Ag(I) was confirmed through ICP-MS analysis, IR spectroscopy and TEM analysis. The modified chitosan and 6-deoxy-6-amino chitosan derivatives that were loaded with Ag are shown in Figure 4.1.

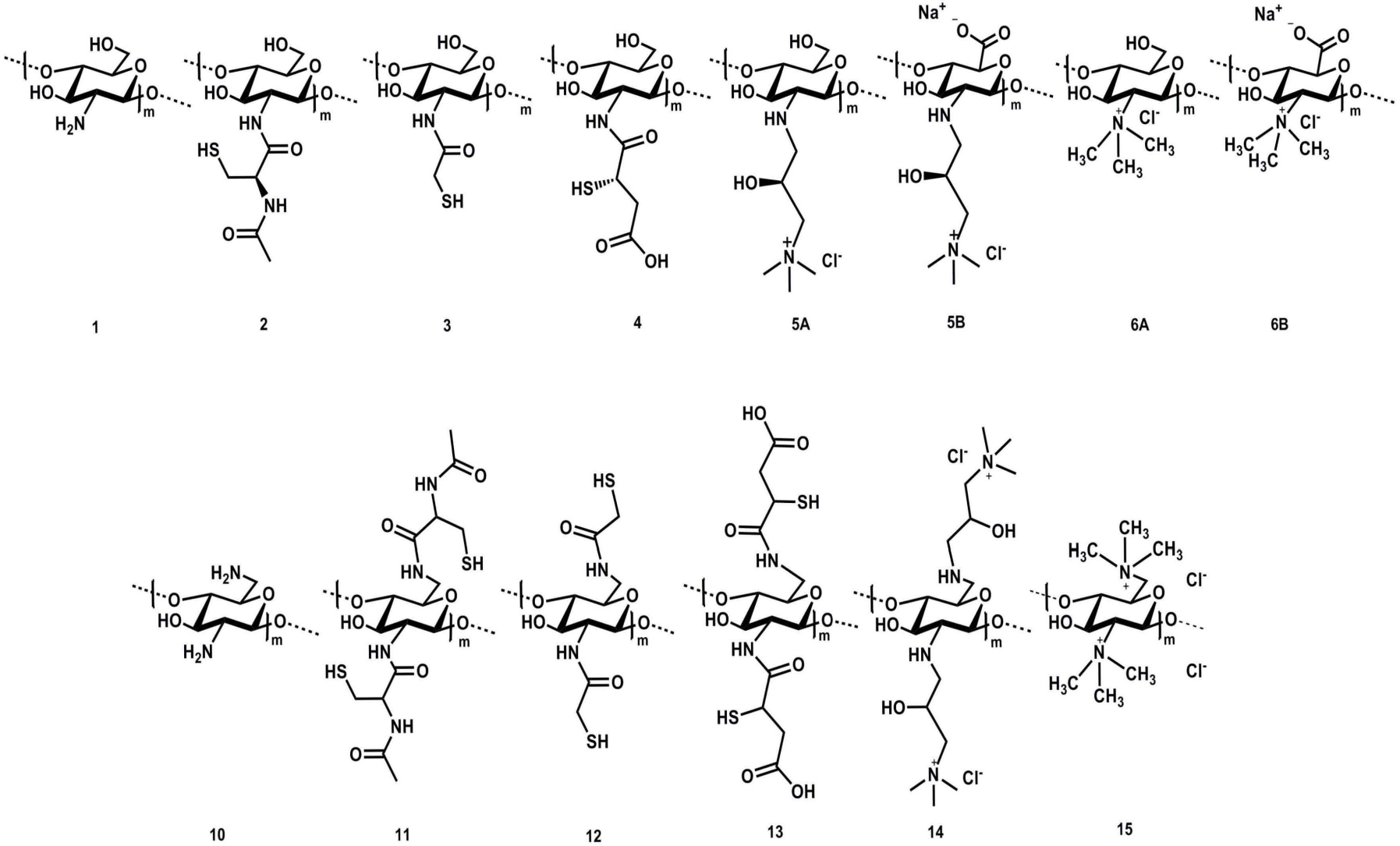

Figure 4.1: Chitosan and 6-deoxy-6-amino chitosan derivatives which were loaded with Ag.

4.2.2. Confirmation of Ag loading

Chitosan and its derivatives were submitted for ICP-MS analysis, the data obtained is shown in Table 4.1. Ag loading ranged from 0.01 to 6.38 mmol/g of polymer. Chitosan (**1**) and polymer **6A** were amongst the polymers which had the highest loading. The polymers **5A** and **6A** display higher loading due to the quaternary nitrogen present, however, the expected increased loading of Ag was not observed for polymers **14** and **15**. This is possibly due to the low solubility as a result of the high degree of crystallinity found in these polymers. Polymers **2** and **11** displayed very low Ag loading this may be a result of the extra nitrogen atom of the cysteine moiety present in the polymer. In addition, the Ag ions may have been tightly bound to the incompletely digested polymer leading to retention of Ag by the polymer and under estimation in ICP-MS analysis. However, this trend does not apply to polymers **12** or **13** as these derivatives contain a thiol group which may be responsible for the higher loading. These inconsistent trends are most likely due to initial low polymer solubility when loading with Ag or incomplete digestion of the polymers prior to ICP-MS analysis.

Table 4.1: ICP-MS analysis of silver loaded modified chitosans and their associated solubility.

Polymer	Solubility in H_2O	Ag^+ Loading (mmol/g)	Polymer	Solubility in H_2O	Ag^+ Loading (mmol/g)
1-Ag	++	5.41	**10-Ag**	++	3.12
2-Ag	+++	0.80	**11-Ag**	+	0.07
3-Ag	+++	1.91	**12-Ag**	+	1.78
4-Ag	++	4.28	**13-Ag**	+	4.27
5A-Ag	++	3.37	**14-Ag**	+	0.05
6A-Ag	+++	6.38	**15-Ag**	+	0.01

Solubility (soluble, +++; partially soluble, ++; insoluble, +).

It has been reported that in quaternary ammonium chitosan derivatives (**5A** and **6A**), the chloride ions (Cl^-) are located between the $-N^+(CH_3)_3$ groups and -Ag nanoparticles bridging the metallic core and the polymer shell.[17] The Cl^- ions form electrostatic interactions with the $-N^+(CH_3)_3$ groups and surface ion pairs on the Ag nanoparticles. This bridged structure and the long chain framework of the quaternary polymers forms the polymer shell around the metallic nanoparticles. The repulsion effect between the charged polymers, is what prevents possible aggregation of the metallic nanoparticle core, thus making these polymers more stable.[17]

IR spectra of the silver loaded chitosan derivatives showed characteristic absorbances, which appeared narrower and sharper compared to derivatives without silver. In the case of chitosan, when the polymer was treated with Ag^+, the broad absorbance at 3434 cm^{-1}

became narrow (Figure 4.2). This points to the participation of OH groups in the reduction of Ag^{+} to Ag^{0}, resulting in the weakening of the intensity of these bands. Another shift was observed from 2878 cm^{-1} to 2917 cm^{-1} for the C–H aliphatic peak due to the electrostatic interaction of the Ag with the chitosan functional groups. The amine and hydroxyl groups in chitosan support nucleation and stabilization of Ag nanoparticles while preventing further aggregation.[12]

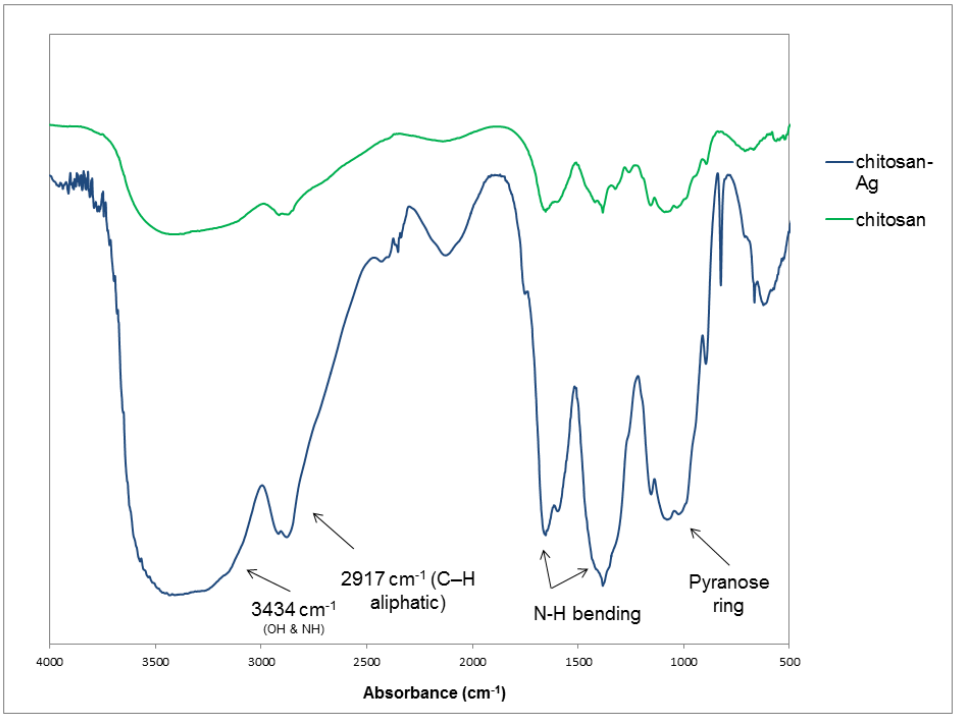

Figure 4.2: IR spectra of chitosan and the Ag loaded chitosan (KBr).

Solids recovered after Ag loading had different colours compared to the native polymer, ranging from tan to purple/black. This is a clear indication that Ag is present in sufficient quantities to alter the colour of the polymers and that variable degree of Ag loading occured. These samples were analyzed using TEM to confirm Ag loading and to determine particle sizes. The sizes of selected chitosan derivatives are reported in Table 4.2.

Table 4.2: Particle sizes of the chitosan derivatives.

Polymer	Size (nm)	Polymer	Size (nm)
1-Ag	14 ± 6	**5A-Ag**	12 ± 6
2-Ag	14 ± 6	**5B-Ag**	13 ± 4
3-Ag	16 ± 5	**6A-Ag**	11 ± 4
4-Ag	12 ± 5	**6B-Ag**	11 ± 4

The size range of the Ag particles are roughly the same for the majority of the compounds with very broad size distributions. Wei *et al*. reported spherical chitosan encapsulated Ag

nanoparticles with sizes of 6-8 nm and a narrow size distribution.[16] Particles appear spherical and well dispersed, the image in Figure 4.3 shows chitosan coated Ag particles and the associated size distribution. All Ag-loaded polymers displayed similar particles in terms of size and dispersity. The resultant nanoparticles are coated with chitosan due to the electrostatic attraction between the polymer and the metal where different preparation methods produce particles with different shapes and sizes.

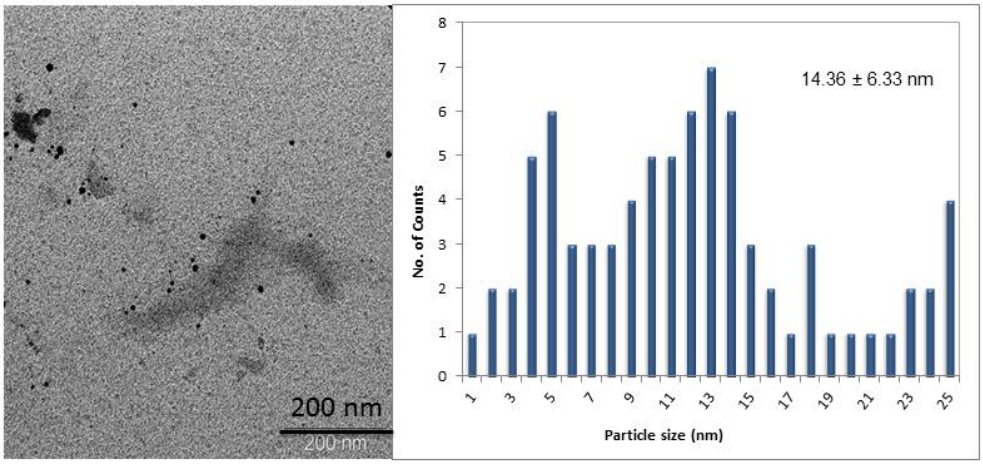

Figure 4.3: Chitosan-Ag loaded particles and associated size distribution.

The effect of the presence of the Ag ions on the antimicrobial activity of the polymers was thereafter studied.

4.2.3. Antimicrobial studies of chitosan derivatives

The antimicrobial activities of the chitosan derivatives were evaluated against two different microorganisms. The minimum inhibitory concentrations (MICs) of the chitosan derivatives against the gram-(+ve) bacterium *Staphylococcus aureus* (*S. aureus*) and gram-(-ve) bacteria *Escherichia coli* (*E. coli*) were evaluated. These bacteria were chosen as they are common water borne pathogens and can be used as model bacteria in faecal coliform antimicrobial studies. The gram-(-ve) bacteria, possess an anionic bacterial surface to which the cationic chitosan derivatives interact electrostatically. To improve this electrostatic interaction, many chitosan derivatives have cationic moieties including ammonium, pyridinium or piperazinium. The gram-(+ve) bacterium are hindered by the binding of chitosan to DNA or RNA.[18] The antibacterial activity of chitosan is most likely due to the amino group which forms an ionic bond with negative charges on the cell walls of both gram-(+ve) and gram-(-ve) bacteria thereby inhibiting growth.[19] The antioxidant scavenging activity of chitosan has been attributed to its strong hydrogen-donating ability.[18] In addition, drug or biocide resistance is a major problem as evidenced by the

occurrence of methicillin-resistant *S. aureus*.[20] Therefore the development of antimicrobial materials is of great interest. The antimicrobial assessments were performed by a test well method using liquid cultured bacteria. In the case of the silver loaded compounds, some samples were tested as films on agar plates to determine contact kill times as it is a well-known fact that Ag is bactericidal.[5,6]

Chitosan (**1**) and the derivatives **2**, **3**, **4**, **5A**, **5B**, **6A, 6B** and **15** were tested for their activity against *E. coli* and *S. aureus*. The 6-deoxy-6-amino chitosan derivatives (**10**-**14**) (Figure 4.1) could not be tested due to low solubility. Efforts were made to increase the solubility by heating however, the polymers did not dissolve. Antibacterial assessments were performed in sterile 96 well plates. The testing protocol followed was reported by Thatte *et al*.[14] Each test polymer was diluted in sterile phosphate buffer (pH 7) to make stock test solutions (10, 50,100, 250 and 500 µg/mL). Bacteria were inoculated at 37 °C in a culture tube. The resulting bacterial culture was diluted to obtain appropriate optical densities (OD) for bacteria in mid log phase growth, *E. coli* (0.400) and *S. aureus* (0.800) was measured at 600 nm. Samples were tested in triplicate. Control tests were performed for each sample to ensure proper bacterial growth in the absence of any agent (cell control). In addition, certain wells only contained the samples to ensure no growth (agent control). Plates were incubated at 37 °C for 14 hrs, after which they were visually assessed. Wells where growth occurred turned visibly turbid while the MIC was defined as the first clear well of lowest concentration of the antimicrobial agent. Higher values of MIC indicate lower antibacterial activity while low MIC concentrations indicate higher activity.[14] The MIC values of the polymers tested are shown in Table 4.3 together with the literature reported values, where available, in brackets.

Table 4.3: MIC values for selected polymers. (*NA - No activity)

Polymer	MIC (µg/mL)	
	E. coli	***S. aureus***
1	100 (100)	500 (100, >800)
2	50 (20)	NA
3	NA	50
4	250	100
5A	250 (16)	10 (16)
5B	NA	10
6A	NA (1000)	10 (125)
6B	250	10
15	10	100

In the case of chitosan (**1**), the MIC value obtained for *E. coli* agreed with that reported by Tsai *et al*.[21] The MIC value for *S. aureus* is somewhat higher compared to that reported by

Devlieghere *et al*. but lower than that reported by Omura *et al*.[22,23] This result confirms the inherent antimicrobial activity of chitosan which can potentially be enhanced by specific functionalization of the polymer.

Polymer **2**, is the *N*-acetyl cysteine conjugate of chitosan. *N*-acetyl cysteine itself is a strong antioxidant which disrupts disulfide bonds in mucus hence its pharmaceutical use as a mucolytic drug as well as an inhibitor of cysteine utilization. It has also been shown to be able to detach bacterial biofilms which have adhered to surfaces as well as inhibit bacterial growth *in vitro*.[24,25] Mansouri *et al*. reported the MIC of *N*-acetylcysteine against *E. coli* as 20 µg/mL.[24] In the current study, the MIC of chitosan-*N*-acetyl cysteine was 50 µg/mL. The enhanced activity of this polymer against *E. coli* compared to chitosan is possibly due to the presence of the thiol groups which adhere more strongly to the bacterial cell wall.[25] This polymer displayed no inhibitory activity against the *S. aureus* strain tested and at the time of this study, a literature value could not be found.

The chitosan-thioglycolyl conjugate **3** showed no inhibitory activity when tested against *E. coli.* On the other hand, the MIC of this compound when tested against *S. aureus* was found to be 50 µg/mL which is 10-times lower than that of chitosan. The presence of the thiol groups possibly increased adhesion to the bacterial cell wall by forming disulfide bonds with thiol groups on the surface of adjacent cells and the polymers inherent antibacterial properties inhibited cell growth.[26] No data has been found in the literature with regards to the MIC of this polymer against either *E. coli* or *S. aureus.*

For the novel polymer, (2*S*)-2-mercaptosuccinyl chitosan (**4**), the MIC value against *E. coli* was 250 µg/mL which was higher than that observed for the parent compound chitosan. However, the MIC value against *S. aureus* was an order of five times lower than that observed for chitosan. A possible explanation for this is that the mercapto groups increase the permeability of the cell wall thereby allowing the inherent antibacterial properties of the polymer to inhibit bacterial growth. In addition, thiols are strong metal chelators therefore the polymer potentially bound essential metals necessary for cell survival leading to cell death. However, this theory does not account for the decrease in activity against *E.coli*. This increase in mucoadhesivity in the presence of thiolated chitosan compounds was demonstrated by Millotti *et al.* in a study on chitosan-4-mercaptobenzoic acid (chitosan-4-MBA) (Figure 4.4).[27] This chitosan derivative had a total thiol content of 176 µmoles/g of polymer and displayed a 60-fold increase in mucoadhesiveness compared to chitosan.[27] The compound used in this study had a thiol content of 40 µmoles/g of polymer which was determined spectrophotometrically. This result implies that there is a correlation between the thiol content and the mucoadhesive properties of polymers. Therefore since the compound

in this study had a higher thiol content, a greater degree of binding with certain bacterial cells was observed. This increased binding allows the inherent antimicrobial properties of chitosan to inhibit bacteria.

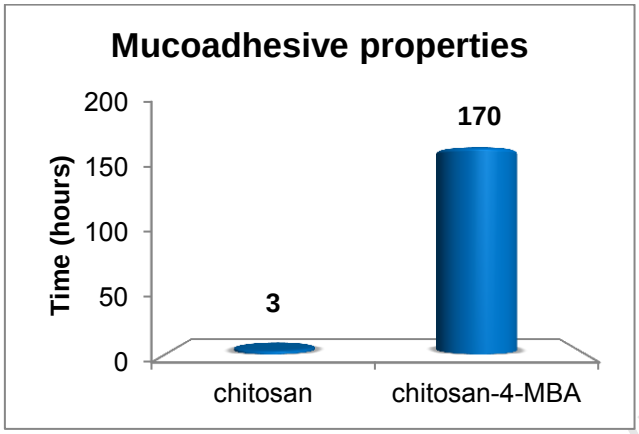

Figure 4.4: Mucoadhesive properties of chitosan and chitosan-4-MBA.[27]

The quaternary derivative 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (**5A**) exhibited an MIC of 250 µg/mL against *E. coli.* This value is much higher than the 16 µg/mL reported by Daly *et al*.[28] The MIC of this polymer against *S. aureus* agrees with that reported by Daly *et al.* which was 16 µg/mL.[28] This result is counterintuitive as the MIC obtained for *E. coli* was much higher compared to literature. However, in this study the experiment was only repeated thrice whereas Daly *et al.* achieved reproducible results with a total of twenty one experiments.[28] In addition, the DDA of the chitosan used in the study by Daly *et al*. was not given therefore a direct comparison between these results cannot be made.[28] The bactericidal properties of this polymer arise due to the presence of the quaternary nitrogen which is believed to adsorb onto the cell surface, thereby increasing lipid membrane permeability. This leads to cell death due to loss of essential cellular material.[28]

When 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (**5A**) was oxidized, to polymer (**5B**), antimicrobial activity against *E. coli* was not observed. Reasons for this lack of activity are unknown, this result may point to the involvement of the hydroxyl group on C6 in the attachment to the gram-(–ve) bacterial cell wall and subsequent inhibitory activity. In addition, when this polymer was obtained from the oxidation of polymer (**5A**), depolymerization may have occurred during the reaction. In a study by Qin *et al*. it was shown that the antimicrobial activity of chitosan is dependent on the molecular weight of the polymer.[29] The antimicrobial activity of chitosan against *E.coli* decreased with a decrease in molecular weight which can potentially explain the result obtained for the oxidized polymers in this study.[29] When tested against gram-(+ve) *S. aureus,* the MIC was the same as the original polymer **5A**.

It was observed that trimethyl chitosan chloride (TMC) (**6A**) exhibited no antimicrobial activity against *E. coli*. The MIC against *S.aureus* was determined to be 10 µg/mL which is lower than that reported by Sadeghi *et al.* (125 µg/mL).[30] It was noted in a study by Sajomsang *et al*. that *S.aureus* was more sensitive to TMC compared to *E. coli* which is possibly due to the different components of the bacterial cell walls.[31] In this study it was reported that the MIC with regards to *E. coli* was 1000 µg/ml for a polymer with a degree of quaternization (DQ) of 28 % and 250 µg/mL for a DQ of 64 % respectively.[31] Therefore the MIC decreased with an increase in DQ. However, this does not explain the lack of activity of the polymer tested in this study against *E. coli* which had a DQ of 62 %.

The oxidized form of TMC (**6B**) exhibited better antimicrobial activity against *E. coli* (MIC of 250 µg/mL) compared to the original polymer (**6A**) that gave no activity. This is a possible result of the introduction of a carboxylic moiety onto the polymeric backbone. The activity against *S.aureus* remained the same as for trimethyl chitosan chloride. These observations are opposite to what was recorded for polymer (**5A**), also a quaternary derivative. This is possibly related to the aqueous solubility of the polymers, where polymers **6A** and **6B** are more soluble than **5A** and **5B**.

The 6-deoxy-2,6-bis[trimethyl] chitosan chloride (**15**) derivative was found to have an MIC of 10 µg/mL against *E. coli*. This was 10 fold lower than that reported for chitosan. Yang *et al*. reported that the antibacterial activity of 6-deoxy-6-amino chitosan is higher than that of chitosan in an acidic solution.[32] This is due to the fact that 6-deoxy-6-amino chitosan has higher positive charge densities in an acidic solution. While in basic solution, the antimicrobial action could be attributed to the polymer acting as a chelating agent thereby rendering metals, trace elements, or essential nutrients unavailable for organism growth.[30] The MIC value for 6-deoxy-6-amino chitosan against *E. coli* was between 500 and 1000 µg/mL as reported by Yang *et al*.[32] Therefore the addition of the trimethyl moiety appeared to lower the MIC of native 6-deoxy-6-amino chitosan (**10**). Polymer (**15**) was found to have an MIC of 100 µg/mL against *S.aureus.* This result was similar to that reported by Sadhegi *et al*. who found the MIC to be 62.5 µg/mL.[30] Sadhegi *et al.* reported that the 6-deoxy-2,6-bis[trimethyl] chitosan chloride (**15**) derivative has a very high zeta potential which suggests that this derivative binds strongly to the negative peptidoglycans on the bacterial cell wall which may lead to severe morphological alterations in gram-(+ve) bacteria.[30]

From the results of the chitosan and 6-deoxy-6-amino chitosan derivatives, it is clear that the majority of these polymers do possess antimicrobial activity. This activity is most likely due to the inherent antimicrobial properties of chitosan and modified chitosan.[14] However, certain derivatives displayed enhanced activity against the bacteria tested which is possibly due to

the incorporation of the thiol/quaternary moiety onto the polymer backbone. These moieties are reported to have increased mucoadhesivity in the case of the thiols and display increased absorption across membranes in the case of the quaternary derivatives.[33] Comparing all the polymers tested, polymer **15** had the best activity against *E.coli* while the quaternary derivatives (**5A**), (**5B**), (**6A**) & (**6B**) had the highest activity against *S. aureus*. This indicates that the quaternary chitosan and 6-deoxy-6-amino chitosan derivatives are the best in terms of antimicrobial activity. This result may be attributed to the charged quaternary nitrogen present which is absent in native chitosan. A proposed mechanism for the chitosan-bacteria interaction involves an initial binding which occurs due to the electrostatic interactions between the positively charged chitosan macromolecule and negatively charged bacterial cell wall which is more pronounced in the case of the quaternary derivatives.[14]

4.2.4. Antimicrobial studies of silver loaded chitosan derivatives

Selected derivatives (**1-Ag**, **2-Ag**, **3-Ag**, **4-Ag**, **5A-Ag**, **5B-Ag**, **6A-Ag** and **6B-Ag**) were tested for Ag leaching by means of a disk diffusion method. Disks were soaked in a 1 % (w/v) solution of the polymer/ Ag loaded polymer in H_2O. The disks were then applied to agar plates which had been spread with 100 μL of a suspension of bacterial cells (*S. aureus, E. coli* and *Pseudomonas aeruginosa*). The plates were incubated at 37 °C overnight. Below is an example of chitosan, its Ag loaded derivative and the antimicrobial activities of these polymers against the three bacteria tested. This image shows the effect of the polymer itself against the bacteria and the effect of the Ag loaded derivative. From these images, it is clear that native chitosan has very little effect against these bacteria at the chosen concentration. However, when Ag is loaded onto this polymer a clear zone of inhibition (ZOI) is observed. This ZOI is clearly due to Ag leaching from the polymer. This is a common incorrect assessment in the literature with regards to the evaluation of the activity of Ag loaded polymers.

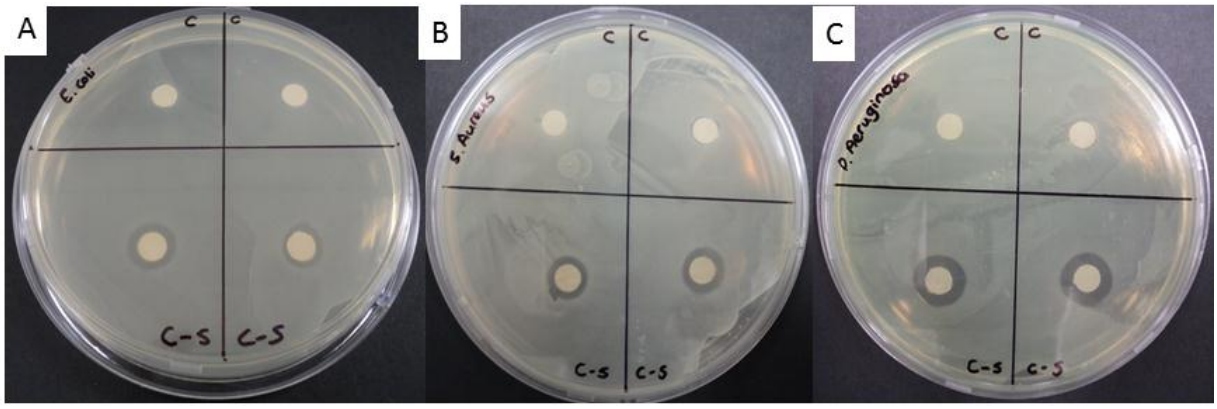

Figure 4.5: chitosan (C), Ag loaded chitosan (C-S) and the antimicrobial activity against *E. coli* (A), *S. aureus* (B) and *P. aeruginosa* (C).

This same leaching was observed for all samples tested. The chitosan derivative most likely remained on the filter paper which is composed of cellulose, another biopolymer. As a result of this, it was not possible to ascribe antimicrobial activity observed to that of the polymers themselves. Therefore an alternative method was needed to determine the antimicrobial activities of the Ag loaded polymers. The antimicrobial activities of selected silver loaded chitosan derivatives were evaluated against gram-(-ve) *E. coli*. Viability of cells was assessed by counting colony-forming units (CFUs) in a contact kill time study. *E. coli* was cultured in a nutrient broth solution at 37 °C overnight and the optical density was thereafter adjusted to 0.300 - 0.400 using nutrient broth at a wavelength of 600 nm. Approximately 100 *µ*L of the selected silver loaded chitosan derivative, at a concentration of 50 µg/mL Ag was incubated together with the bacteria at 37 °C. At time intervals of 0, 1, 2, 3 & 4 hrs, 100 *µ*L of the sample was pipetted, diluted consecutively with 9.9 mL of sterile saline buffer solution and 100 *µ*L of the final dilution was plated on nutrient agar. After overnight incubation at 37 °C, the viable colonies visible to the naked eye were counted manually and compared against a positive control.[34]

These silver loaded polymer derivatives were selected based on their solubility in water. A test concentration of 50 µg/mL Ag was chosen in this study as the MIC of chitosan without Ag loading is 48 µg/mL as reported by Wei *et al*.[16] Due to the well-known antimicrobial activity of Ag and established bactericidal concentration, a control using Ag was not included as part of this study.

Table 4.4: Contact killing times for selected silver loaded polymers against *E. coli*.

Compound	CFU at time (hrs)				
	0	1	2	3	4
Control	+++	+++	+++	+++	+++
1-Ag	+++	+++	+++	+++	+++
3-Ag	+++	++	+	+	+
5A-Ag	+++	+++	+++	+++	+++
5B-Ag	+++	+++	+++	+++	+++
6A-Ag	+++	++	+	-	-
6B-Ag	+++	+++	+++	+++	+++

Growth (strong, +++; moderate, ++; low, +; none, -).

The antimicrobial activity data with contact times is shown in Table 4.4. In most cases, the bacteria grew densely and the colonies were too numerous to count, however inhibition was observed in certain cases. Inhibition was confirmed by comparing the sample plates to that of the blank. At time 0 hr, no inhibition was observed as the silver loaded polymers had just

been added to the bacterial suspension. After 1 hr, polymer **3-Ag** displayed some inhibition with only 551 colonies observed however; this is still outside of the range of acceptable numbers of colonies (~250 colonies). The oxidized polymer **5B-Ag** also showed signs of inhibition as the plate was not as densely populated as the blank, this was also the case for polymer **6A-Ag** (299 colonies) as shown in Figure 4.6.

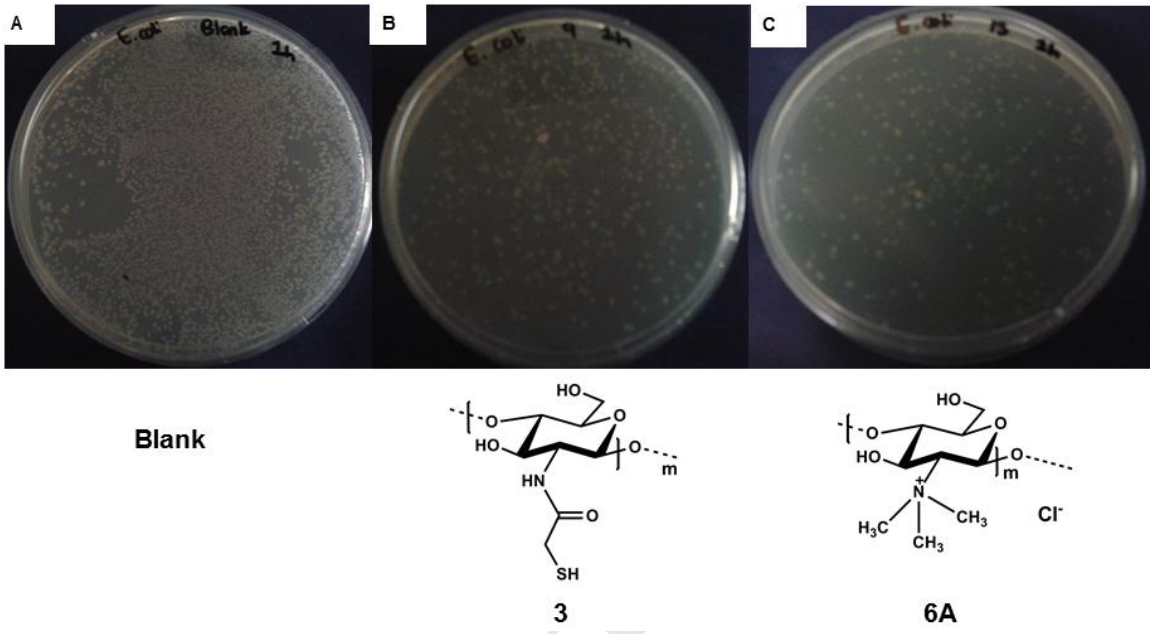

Figure 4.6: The antimicrobial activity of polymers **3-Ag** & **6A-Ag** (B & C) compared to the blank (A) after 1 hr of incubation.

After 2 hrs, polymers **3-Ag** and **6A-Ag** showed almost complete inhibition with only 2 colonies observed. The remaining polymers (**1-Ag**, **5A-Ag**, **5B-Ag**, **6B-Ag**) did not show noticeable reduction in growth at 2 hrs except for **5B-Ag** which showed slight inhibition of bacterial growth. The plates in Figure 4.7 show the difference in inhibition between polymer **6A-Ag** and the oxidized version of this polymer **6B-Ag**.

At 3 hrs, **3-Ag** displayed less inhibition with 163 colonies visible while **6A-Ag** showed complete inhibition. Polymer **5B-Ag** once again was less populated compared to the blank.

After 4 hrs, **1-Ag** showed slight inhibitory activity as the number of colonies observed was less dense compared to the blank. Polymer **3** showed better activity with only 47 colonies visible while **6A-Ag** once again showed complete inhibition. The oxidized polymers **5B-** & **6B-Ag** also showed some inhibitory activity in that their plates had fewer colonies compared to the blank.

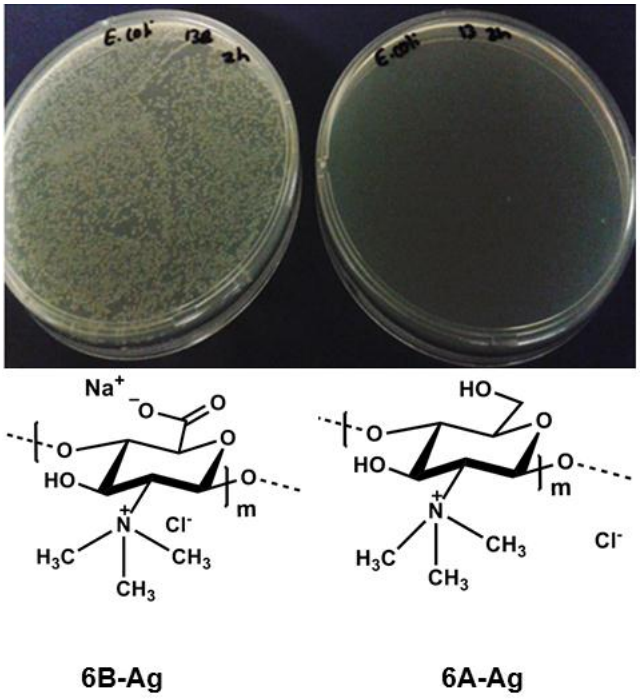

Figure 4.7: Inhibition activity of polymers 6A-Ag (right) and 6B-Ag (left) after 2 hrs.

The contact kill time study shows that chitosan-Ag (**1-Ag**) is not highly active against *E.coli* after 4 hrs indicating that a longer time period is necessary as in the study of native chitosan the MIC was found to be 100 µg/mL after 24 hrs.

The loading of Ag onto polymer **3** boosted the antimicrobial activity of this polymer which did not inhibit *E. coli* in the absence of Ag. The antimicrobial activity peaked at 2 hrs, this activity can therefore be attributed to the antibacterial action of Ag.

As with chitosan-Ag, compound **5A-Ag** did not display any antibacterial activity after 4 hrs where an MIC of 250 µg/mL was obtained for the polymer alone after 24 hrs.

In the case of the oxidized compound **5B-Ag**, no inhibitory activity against *E. coli* was observed at the end of 4 hrs. This result echoes that obtained for the polymer itself, the loading of Ag onto the polymer did not have an effect on the antimicrobial activity. However, a longer contact time may be necessary as Ag nanoparticles have an MIC of between 50 & 60 µg/mL after 24 hrs as reported by Sondi *et al*.[35]

Polymer **6A-Ag** which had the highest Ag loading, exhibited excellent activity against *E.coli*. After 1 hr, the number of cfu's had reduced significantly and complete inhibition was observed after 3 hrs. This shows an interaction between the polymer and Ag which increased the antimicrobial efficacy of the polymer which was not observed in testing with

the polymer alone, even with concentrations as high as 1000 µg/mL. This may either be the result of synergy between the polymer and Ag or the antimicrobial activity of Ag alone. Silver is bactericidal at a concentration of 50 – 60 µg/mL.[35]

The oxidized Ag loaded polymer **6B-Ag** did not show any antimicrobial activity at the end of 4 hrs possibly indicating the need for a higher concentration as this polymer showed efficacy against *E. coli* after 24 hrs when not loaded with Ag.

Generally good antibacterial activity was observed as fewer colonies were observed on the polymer treated plates compared to the blank. As seen from the results, Ag loading does increase the antimicrobial activity of selected polymers; however, a more in-depth study with shorter contact kill times and possibly a higher polymer concentration is needed to confirm this. The majority of the polymers both deliver Ag and kill bacteria inherently, while other polymers only deliver the bactericidal Ag.

These polymers show promising antimicrobial activity therefore the application of these chitosan derivatives as water filters is of interest. Further studies would need to be carried out to evaluate the suitability of these polymers as antimicrobial water filters.

4.3. Anti-mycobacterial studies

Tuberculosis is a global health concern, which has been the focus of intense research for decades. *Mycobacterium tuberculosis* (*M. tuberculosis*) is the bacterium responsible for TB. This disease can be spread in a number of ways these include transmission from inhaling infected droplets when an infected individual is talking, sneezing or coughing. Another transmission route is through contact with infected water. Greenberg *et al*. reviewed the transmission of TB by wastewater from institutions treating TB patients as well as diaries and slaughter houses treating TB infected animals.[36] This study found that the wastes from these institutions and industries contained tubercle bacilli, the organism which causes TB.[36] It has been shown that aerosol dispersal of infectious agents in wastewater spray sites can occur. Therefore it is necessary to treat the waters discharged from these sources with conventional sewage treatment as well as chlorination to ensure that these waters do not transmit any diseases.[36]

Researchers have begun to look at the pulmonary route of drug delivery in relation to the treatment of TB since the lungs are the primary site of infection of the bacterium.[37] Recently, attention has shifted to biopolymers as drug delivery agents. A well-known example is chitosan, a biopolymer which has previously been studied as a pulmonary delivery agent.[38,39]

A chitosan-poly(ethylene glycol) polymer network was synthesized and loaded with the 1st line anti-TB drug isoniazid (INH). This compound was studied as a possible controlled drug delivery agent. The study found that by varying the degree of cross-linking and the pH of the solution, desired drug release rates could be achieved. In addition due to chitosan's inherent anti-bacterial properties, the action of INH may be enhanced.[38] In a study by Rafeeq *et al*., chitosan-tripolyphosphate nanoparticles loaded with INH were synthesized and evaluated for the treatment of TB.[39] In a recent study by Vavříková *et al*., *O*-carboxymethyl chitosan and *N*-succinyl chitosan were synthesized and loaded with INH, pyrazinamide (PZA) and second-line anti-TB drug ethionamide (ETA). The native compounds including those which were loaded with the chosen drugs were tested against *M. tuberculosis*. Results showed that the polymers themselves had MIC values of 62.5 and > 500 µg/mL for *O*-carboxymethyl chitosan and *N*-succinyl chitosan respectively after 14 and 21 days of growth. The drug loaded polymers had the same MIC value of 125 µg/mL which is higher than that reported for the polymers themselves. The authors postulated that the amino group on chitosan is the active functional group which is necessary for antibacterial activity.[40]

Chitosan is a unique and versatile polymer and due to its inherent antimicrobial activity, the synthesis of derivatives with increased activity would be of interest.[41]

In the current study, thiolated and quaternized chitosan derivatives were synthesized and their efficacy against *Mycobacterium smegmatis* (*M. smegmatis*) was tested. This particular bacterium was chosen for this study as it has been suggested that screening compounds against *M. smegmatis* is a good non-pathogenic model organism for TB drug discovery.[42] In addition, the Ag loaded counterparts of these polymers were also evaluated. Apart from being utilized as drug delivery agents, these polymers may be utilized as water filters due to their inherent antimicrobial activity which can remove pathogens including TB from contaminated water.

The chitosan derivatives including selected Ag loaded versions (**1**, **2**, **3**, **4**, **5A**, **5B**, **6A**, **6B**, **1-Ag**, **5A-Ag**, **6A-Ag** and **6B-Ag**) were applied to agar plates and allowed to dry, thus forming transparent films. Thereafter, these plates were inoculated with 6 dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of *M. smegmatis* and incubated for 4 days. The growth of the bacterium was visually assessed on the 4th day.

These polymers were chosen for their improved aqueous solubility. The Ag loaded versions tested (**1-Ag**, **5A-Ag**, **6A-Ag** and **6B-Ag**) were selected as the quaternary derivatives displayed good antimicrobial activity and had some of the highest Ag loading. In addition, the antimicrobial efficacy of the Ag-loaded oxidized quaternary derivative **6B** has not been reported. Chitosan was studied for comparative purposes.

The control plate showed growth at all 6 dilutions. No inhibition was observed for compounds **1**, **4**, **5A**, **6A**, **6B**, **1-Ag**, **5A-Ag**, **6A-Ag** and **6B-Ag**. Some inhibition was observed for polymers (**2**) and (**3**) at a dilution of 10^{-4} and polymer (**5B**) displayed activity at a dilution of 10^{-5}. This activity can be attributed to the presence of the thiol groups in polymers (**2**) and (**3**) which display increased adhesion to the bacterial cell wall allowing the antimicrobial properties of chitosan to inhibit the bacteria.[25] The activity of polymer (**5B**) can be attributed to the quaternary moiety on the polymer backbone which allows for increased adsorption to the cell surface, increasing lipid membrane permeability which can lead to cell death as a result of the loss of essential cellular material.[28] Inhibition of growth is shown in Figure 4.8 which shows polymer **2** and the blank control plate.

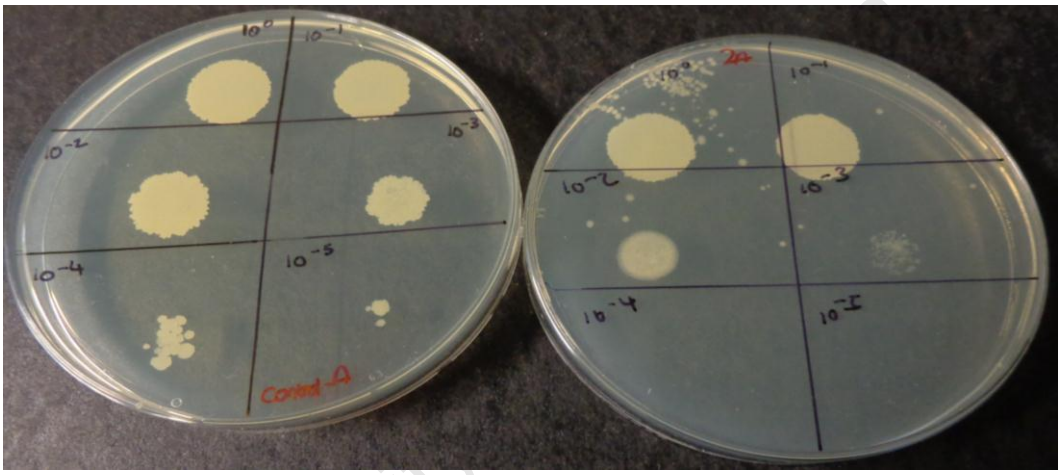

Figure 4.8: Inhibition of *M. Smegmatis* by polymer **2**.

Therefore these active polymers can be potentially developed as anti-TB fibres or as water filters to help prevent the spread or transmission of this disease. Further studies are needed for the polymers which were less active, but showed some potential.

The majority of the polymers synthesized in this study display antimicrobial activity against either gram-(+) or gram-(-) bacteria. This is most likely due to the inherent antimicrobial activity of chitosan where improved activity is attributed to the presence of the side chain in the modified chitosan derivatives. The addition of Ag onto these modified derivatives showed some enhanced activity, however, further studies are needed to optimize the activities of these encapsulated nanoparticles. Certain polymers displayed anti-mycobacterial activity, in particular the thiolated chitosan derivatives. This activity can be improved upon by optimizing the polymers thereby increasing the substitution of chitosan possibly leading to an increase in the activity of these polymers.

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CHAPTER FIVE

CONCLUSIONS AND FUTURE WORK

5.1 Overall Summary

Synthetic non-biodegradable polymers are well established in water purification as ion exchange solid supports.[1] As a biodegradable alternative, chitosan has found commercial application in the flocculation of suspended biomatter.[2,3] However, its applications in water purification and remediation as ion exchange resins have not been fully exploited. This research effort has addressed this technology gap by research of both ion exchange capacity and antimicrobial activity of selected modified chitosans. Low molecular weight chitosan (**1**) and 6-deoxy-6-amino chitosan (**10**) was used to synthesize a range of thiolated and quaternary chitosan derivatives (**2 – 15**) (Figure 5.1). Numerous available synthetic strategies have been utilized for the synthesis of these chitosan polymers. Where applicable, literature procedures were followed and applied on both chitosan (**1**) and 6-deoxy-6-amino chitosan (**10**) to synthesize these derivatives.[4-11] All target polymers were characterized using analytical and spectroscopic methods, followed by evaluation of their intended applications.

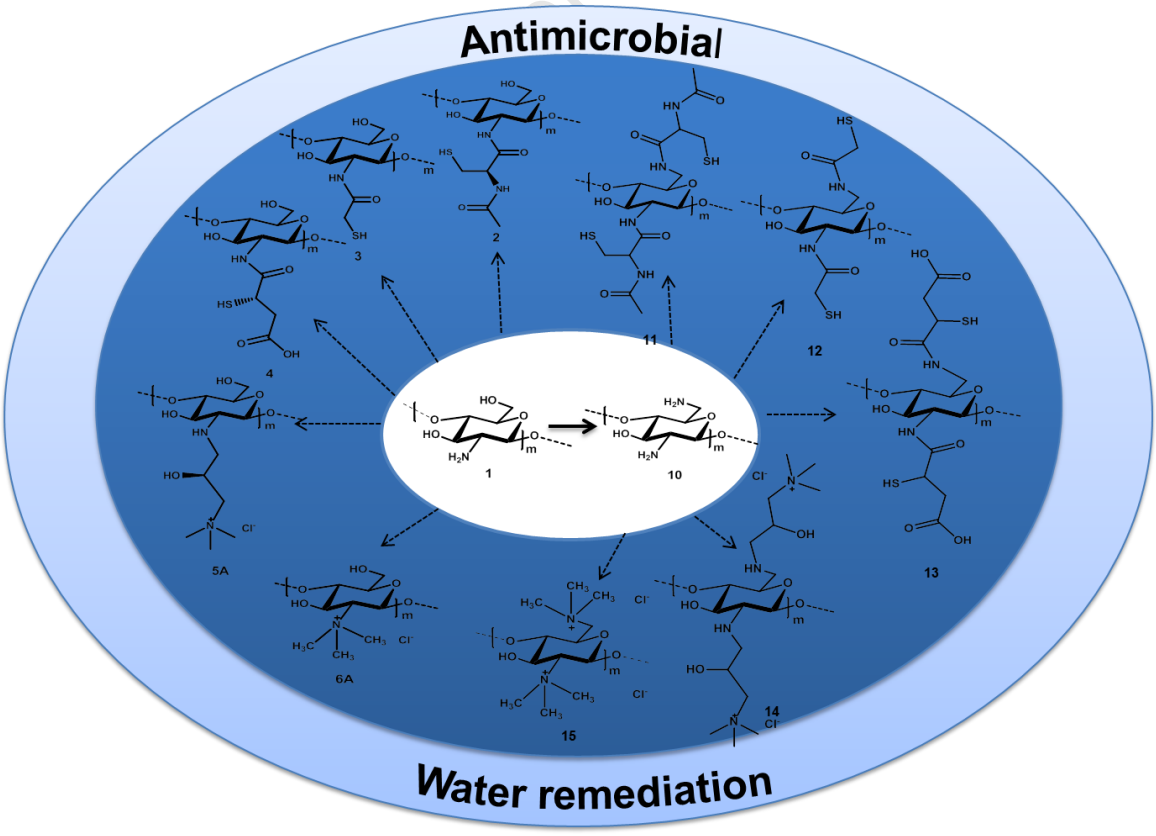

Figure 5.1: Chitosan, modified derivatives and their applications.

Of these polymers, **11** - **14** are novel polymers whose utility has not been fully explored. It was found that the 6-deoxy-6-amino chitosan derivatives displayed higher loading in the case of 6-deoxy-2,6-bis[*N*-acetylcysteinyl] chitosan (**11**) and 6-deoxy-2,6-bis[thioglycolyl] chitosan (**12**). The 6-deoxy-2,6-bis[2*S*'-mercaptosuccinyl] chitosan (**13**) and 6-deoxy-2,6-bis[trimethyl] chitosan chloride (**15**) displayed a lower loading, however, this could possibly be attributed to the insolubility of these polymers. The 6-deoxy-2,6-bis[3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride] (**14**) derivative displayed a similar degree of quaternization as the chitosan derivative. The loading of these modified polymers was improved, however, due to solubility problems, the true loading could not be determined.

In some of the water treatment applications, complexed metals were important functional features. Technologies for the introduction of nanosized metal particles on solid supports are currently sought after.

One of the goals was to prepare a polymer that has both cationic and anionic sites that can simultaneously complex iron and perchlorate. The intended utility of these Fe-loaded derivatives is the removal and simultaneous decomposition of toxic ClO_4^-. Chitosan and their quaternized derivatives were loaded with iron(III) by following reported methods.[12] The loading of iron was confirmed by IR, UV and TEM analysis. Synthesized polymers were compared with the commercially available IRA 400 (strong basic anion exchanger) for its ability to retain perchlorate.[13] Overall, the modified chitosan polymers performed similarly compared to the commercial benchmark in perchlorate retention. Where the quaternary polymer **5A** had a slightly better retention profile. The Fe-loaded quaternary chitosan polymers generally displayed moderate ClO_4^- exchange when compared to IRA 400, where the quaternary polymer (**18**) displayed the highest ClO_4^- retention capability. However, at this stage the reduction of ClO_4^- on these Fe-loaded polymers has yet to be explored. Thus, modified chitosan does present an alternative to synthetic resins for ClO_4^- ion-exchange. Further exploration of this technology is underway.

The antimicrobial activity of the polymers (**1** – **5A**, **5B**, **6A**, **6B** and **15**) were tested against the gram-(+ve) bacteria *S. aureus* and gram-(-ve) bacteria *E. coli*. It was established that chitosan (**1**) and 6-deoxy-6-amino chitosan (**10**) derivatives possesses inherent antimicrobial activity.[14] While the exact mechanism of action is unknown, activity is most likely attributed to the added functionality. Selected thiolated and quaternary derivatives **3**, **5A**, **5B**, **6A**, **6B** and **15** displayed superior activity amongst others against *S. aureus*. This enhanced activity is attributed to the addition of a thiol/quaternary moiety onto the polymer backbone, where mucoadhesivity is improved in the case of the thiols and absorption across membranes is

increased in the case of the quaternary derivatives.[15] The 6-deoxy-2,6-bis[trimethyl] chitosan chloride (**15**) derivative had better activity against *E.coli* and lower activity against *S. aureus* compared to the chitosan derivative. Therefore in some respects, increased loading may have contributed to the increased activity of this polymer against certain bacteria. Hence, these resins have the potential to be applied as antimicrobial water filters.

After establishing the antimicrobial activity of selected polymers, their silver loaded versions were evaluated to investigate potential synergy. It is well established that silver is antimicrobial at low concentrations.[16] The aim was to achieve similar antimicrobial activity for our polymers at lower silver loading reducing toxicity concerns related to the use of Ag nanoparticles. Silver was loaded onto polymers (**1** – **6A** and **10** - **15**) by stirring in an $AgNO_3$ solution. Silver loading was confirmed by IR, ICP-MS and TEM analysis. The water-soluble Ag-loaded compounds were tested for antimicrobial activity against *E. coli.* In general, good antibacterial activity was observed as fewer colonies were noted on the polymer-treated agar plates compared to the untreated blank. Therefore, Ag loading does increase the antimicrobial activity of selected polymers at sufficiently high concentrations. This result is in agreement with that reported in the literature.[17,18] Thus, a potential application of these silver loaded polymers could be in the coating of equipment such as medical devices where prevention of infection is a top priority.[19] These polymers and their Ag loaded counterparts have demonstrated antimicrobial efficacy and can therefore be considered for application as water filters or membranes.

In a separate study, chitosan derivatives and selected Ag loaded polymers (**1**, **2**, **3**, **4**, **5A**, **5B**, **6A**, **6B**, **1-Ag**, **5A-Ag**, **6A-Ag** and **6B-Ag**) were evaluated for activity against *Mycobacterium smegmatis* as a model organism for *M.tb*. The polymers were applied to agar plates and dried to form films. Inhibition was observed for thiolated polymers (**2**) and (**3**) at a cell culture dilution of 10^{-4} and polymer (**5B**) displayed activity at a cell culture dilution of 10^{-5}. The activity of these polymers can be attributed to the presence of the thiol groups in polymers **2** and **3** and the quaternary moiety in polymer **5B**. A search of the literature revealed almost no chitosan derivatives have been studied against *M.tb*.[20] Therefore these derivatives have the potential to be developed as anti-tb fibers to act as filter in the prevention of the transmission of this deadly disease.

5.2. Future work

This project has demonstrated that a variety of chitosan polymers can be synthesized by different synthetic methods. Many synthetic procedures gave good yields and are environmentally friendly. In addition the 6-deoxy-6-amino chitosan derivatives were also

synthesized. Green synthetic procedures can now be developed for the resins which displayed the best performance and can be scaled up to pilot sized applications in various stages of water treatment. Potential uses for the novel polymers (**11** – **14**) may be further explored and synthesis of these polymers may be optimized.

For the water treatment application, the calorimetric protocol used for perchlorate analysis can be optimized by using mass spectrometry to produce more accurate results. In addition, the reduction of ClO_4^- to Cl^- on Fe loaded columns may be studied. The dual functionality of these columns is a promising area of research which has not been thoroughly studied. Other ion exchange resins (cation and anion) can be used for comparative purposes such as those with a higher selectivity for ClO_4^-. Ultimately, the goal to identify chitosan derivatives which can remove ClO_4^- from solutions was achieved and a filtration system incorporating this polymer as an ion exchange resin may be designed. The removal of other contaminants or heavy metals using these polymers is another area that can also be investigated. The quaternary polymers absorbed more Fe compared to chitosan, therefore, they can be potentially applied as filters used to remove excess Fe typically present in borehole water causing discolouration of walls.

The antimicrobial activity of the polymers synthesized in this study can be tested against a larger variety of microorganisms relevant to water purification including *Mycobacterium tuberculosis*. The activity of the Ag-loaded polymers can be retested with a higher concentration of Ag. These polymers can also be evaluated for their potential to produce nanofibers through electrospinning. The latter types of synthetic polymers have found commercial utility as antimicrobial water membranes or filters.

5.3. References

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CHAPTER SIX

EXPERIMENTAL

6.1. General Remarks

All reagents and solvents used were purchased from commercial suppliers (Sigma-Aldrich, Fluka, Merck, Kimix) and used as received. Distilled/Milli-Q H_2O was used in all reactions, unless otherwise stated. Low molecular weight (LMW) chitosan was used in all reactions. All calculations were based on one unit of chitosan with an 85 % DDA.

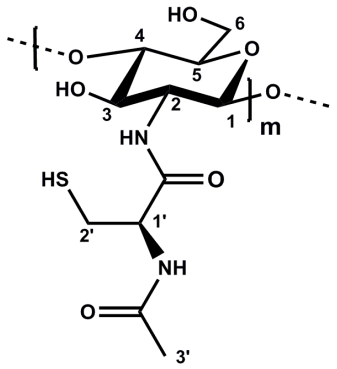
Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Unity XR400 MHz (^{1}H at 399.95 MHz, ^{13}C at 100.58 MHz), Varian Unity XR300 MHz (^{1}H at 300.08 MHz, ^{13}C at 75.46 MHz) or a Bruker Ultrashield 400 Plus spectrometer (^{1}H at 400.20 MHz, ^{13}C at 100.60 MHz). ^{1}H and ^{13}C NMR chemical shifts were reported using tetramethylsilane (TMS) as the internal standard. Degree of substitution (DS)/ Degree of quaternization (DQ) was calculated from ^{1}H NMR analysis. In the case of certain insoluble polymers, ^{1}H NMR spectra was obtained at low pH and in some cases required brief heating. Microwave digestions were performed in a CEM Discover® microwave synthesizer at a temperature of 100 °C at 300 W for varying times.

Infrared absorptions were measured on a Perkin-Elmer Spectrum One FT-IR Spectrometer using KBr discs. Microanalyses for C, H and N were carried out on a Thermo Flash 1112 Series CHN Analyser and the EA Euro 3000. UV-Vis spectroscopic analyses were carried out on a Varian Cary 50 UV-Visible spectrophotometer using cuvettes with a 1 cm path length quartz cell.

Transmission electron microscopy (TEM) measurements were performed on a LEO EM 912 operating at 120 kV. Inductively coupled plasma mass spectroscopy (ICP-MS) was performed on a Perkin-Elmer Elan 600 quadrupole with a Cetax LSX-200 UV laser module.

6.2. Synthesis and Characterization of Thiolated and Quaternary Chitosan derivatives

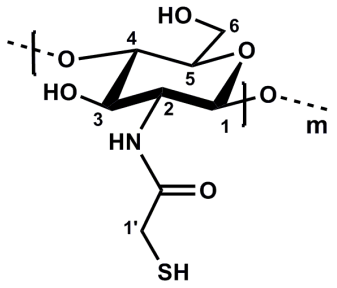
*6.2.1. N-Acetylcysteinyl chitosan (**2**)*[1]



Chitosan (1.00 g, 6.20 mmol) was dissolved in 1 % HCl (v/v) and adjusted to pH 5 with 1 M NaOH. Separately, *N*-acetylcysteine (8.00 g, 49 mmol) was dissolved in H_2O (100 mL). The carboxylic group of the *N*-acetylcysteine was activated by the addition of EDAC (50 mM) whilst stirring for 20 min. The pH was adjusted within the range of 4 – 5 and maintained during the reaction. The reaction mixture containing chitosan and activated *N*-acetylcysteine were allowed to stir for 6 hrs at RT. Thereafter, the reaction mixture was dialysed against a solution of 1 mM HCl containing 2 µM EDTA. The dialysis buffer was later changed to a mixture of 1 mM HCl, 2 µM EDTA and 1 % NaCl. The polymer was finally dialysed against a 0.5 mM HCl solution. The clear solution was freeze dried to yield the product as a white film (1.06 g, 56 %).

IR (KBr): *v* (cm^{-1}) 3409 (broad strong band, OH), 2926 (sharp band, CH aliphatic), 2056 (SH), 1632 (sharp band, C=O), 1520 & 1320 (strong bands, NH), and 1092 (broad band, pyranose); ^{1}H NMR (300 MHz, D_2O) δ 5.14 (1H, s, H-1), 4.21-3.98 (5H, m, H-3 – H-6), 3.74 (1H, s, H-2), 3.45 (2H, s, H-2'), 2.29 (m, acetylated units & H3'); ^{13}C NMR (75 MHz, D_2O) δ 170.68 (amide C=O), 96.88 (C1), 76.07 (C5), 74.32 (C4), 69.60 (C3), 69.07 (C1'), 59.63 (C6), 55.43 (C2 & C3'); DS: 24 % (from ^{1}H NMR); Thiol content: 18 µmol/g (from DTNB assay); Elemental Analysis (%): Calc. For $[C_{11}H_{18}N_2O_6S]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 39.85; H, 5.92; N, 6.91; Found: C, 39.27; H, 5.99; N, 6.53.

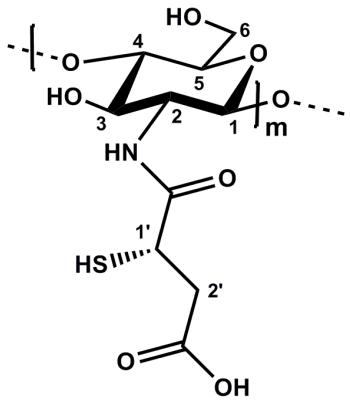
*6.2.2. Thioglycolyl chitosan (**3**)*[2]



Chitosan (1.03 g, 6.4 mmol) was hydrated in 1 M HCl (8 mL) and dissolved by the addition of H_2O in order to obtain a 1 % solution (w/v) of chitosan hydrochloride. Thereafter EDAC was added at a concentration of 50 mM. After the reaction mixture became homogeneous, TGA (1.00 g, 10.9 mmol) was added and the pH adjusted to 5 using 1 M NaOH. The reaction mixtures were incubated for 3 hrs at RT under continuous stirring. In order to eliminate unbound TGA and to isolate the polymer conjugates, the reaction mixture was dialysed for 3 days against 5 mM HCl. Thereafter the dialysing solution was changed to 5 mM HCl and 1 % NaCl. The compound was freeze dried after which a fluffy white solid was obtained (1.06 g, 70 %).

IR (KBr): *v* (cm^{-1}) 3413 (broad strong band, OH), 2064 (SH), 1628 (sharp band, C=O), 1075 (broad band, pyranose); ^{1}H NMR (300 MHz, D_2O) δ 4.86 (1H, s, H-1), 3.75-3.93 (5H, m, H-3 – H-6), 3.36 (2H, s, H-1'), 3.19 (1H, s, H-2), 2.07 (3H, s, acetylated units); ^{13}C NMR (75 MHz, D_2O) δ 97.06 (C1), 76.07 (C5), 74.50 (C4), 69.42 (C3), 59.46 (C2), 55.43 (C6), 21.51 (C1'); DS: 25 % (from ^{1}H NMR); Thiol content: 16 µmol/g (from DTNB assay); Elemental Analysis (%): Calc. For $[C_8H_{13}NO_5S]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}.2H_2O$: C, 32.01; H, 5.57; N, 5.25; Found: C, 32.83; H, 5.77; N, 5.41.

*6.2.3. (2S)-2-Mercaptosuccinyl chitosan (**4**)*[3]

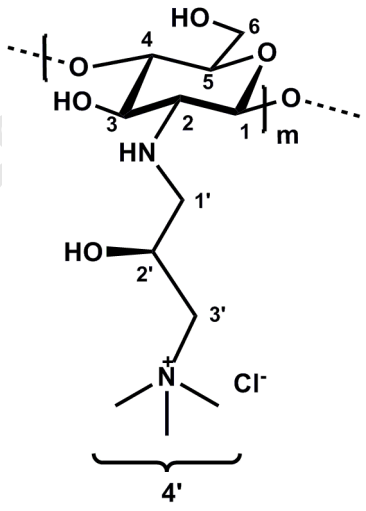


Chitosan (0.926 g, 5.7 mmol) in DMF (30 mL) was added to *S*-acetylmercapto-succinic anhydride (5.06 g, 29.1 mmol) whilst stirring in DMF (5 mL). The mixture was allowed to stir

for a further 3 hrs at RT. The yellow solution was extracted numerous times with EtOAc and excess of DMF was removed by vacuum distillation. The polymer was dissolved in H_2O, dialysed against distilled H_2O and freeze dried. The product was recovered as a white film (1.65 g). A portion of the resulting white film (0.240 g) was allowed to stir in a 25 % ammonia solution (30 mL) at RT for 12 hrs. The solution was subsequently concentrated *in vacuo* and freeze dried to yield (**4**) as an off white solid (0.206 g, 98 %).

IR (KBr): *v* (cm^{-1}) 3413 (broad strong band, OH), 2922 (sharp band, C-H aliphatic), 2125 (SH), 1724 (C=O stretch), 1660 & 1572 (strong sharp bands, N-H), 1066 (broad band, pyranose); ^{1}H NMR (300 MHz, 2 % TFA/D_2O) δ 4.34 (1H, s, H1), 3.65 – 3.86 (5H, m, H3 – H6), 3.13 (1H, s, H2), 2.95 (1H, m, H1'), 2.79 (2H, m, H2'), 2.43 (3H, s, acetylated groups from the initial reaction, CH_3COS-), 2.02 (3H, s, acetylated units); ^{13}C NMR (75 MHz, 2 % TFA/D_2O) δ 175.02 (amide C=O), 96.85 (C1), 75.86 (C5), 74.29 (C4), 69.22 (C3), 59.42 (C6), 55.05 (C2), 40.01 (C1'), 36.86 (C2'); DS: 50 % (from ^{1}H NMR); Thiol content: 40 µmol/g (from DTNB assay); Elemental Analysis (%): Calc. For $[C_{10}H_{15}NO_7S]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 37.78; H, 5.16; N, 5.01; Found: C, 36.97; H, 5.55; N, 6.01.

*6.2.4. 3-Trimethylammonium-2-hydroxypropyl-N-chitosan chloride (CHI-Q188) (**5A**)[4]*

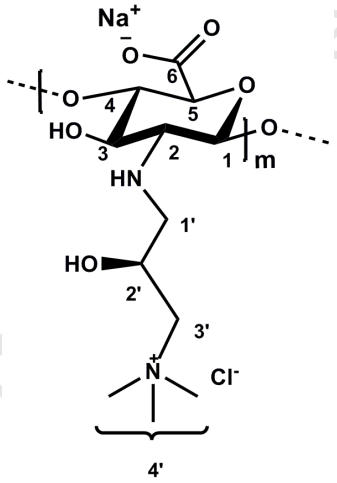


Chitosan (5.04 g, 31.3 mmol) was suspended in H_2O (100 mL) followed by the addition of glycidyl trimethylammonium chloride (GTMAC) (11.4 g, 75.2 mmol) in 3 portions over a period of 8 hrs. The reaction mixture was allowed to stir at 60 °C for 24 hrs. The solution was subsequently adjusted to approximately pH 5 using HCl, followed by addition to cold acetone and then kept at 4 °C overnight. The acetone was decanted and the remaining white gel-like polymer was dissolved in MeOH (100 mL). The methanolic solution was precipitated in 4:1

acetone:EtOH (~ 250 mL). The precipitate was filtered, washed with EtOH and dried under vacuum to afford the desired product as an off white solid (5.88 g, 60 %).

IR (KBr): *v* (cm^{-1}) 3439 (strong broad band, OH), 1656 (sharp band, C=O stretch of secondary amide), 1481 (sharp band, C-H bending of trimethyl ammonium group), 1079 (broad band, pyranose); ^{1}H NMR (300 MHz, 2 % DCl/D_2O) δ 5.00 (1H, s, H-1), 4.81 (1H, s, H-2'), 3.83-3.67 (5H, m, H-3 – H-6), 3.46 (1H, s, H-2), 3.27 (2H, s, H-3'), 3.2 (9H, s, H-4'), 2.94 (2H, s, H-1'), 2.00 (s, acetylated units); ^{13}C NMR (75 MHz, 2 % DCl/D_2O) δ 96.71 (C1), 75.72 (C5),73.97 (C4), 69.07 (C3), 67.32 (C3'), 61.20 (2'), 59.46 (C6), 55.08 (C2), 53.86 (C4'), 53.68 (C1'), 21.51 (acetylated units); DQ: 96 % (from ^{1}H NMR); Elemental Analysis (%): Calc. For $[C_{12}H_{25}N_2O_5Cl]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}.2H_2O$: C, 37.94; H, 7.35; N, 7.80; Found: C, 37.90; H, 7.07; N, 6.5.

*6.2.4.1 6-Carboxy-(3-trimethylammonium-2-hydroxypropy)l-N-chitosan chloride (**5B**)*

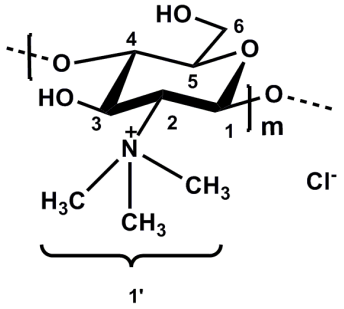


CHI-Q188 (1.02 g, 3.3 mmol) was suspended in H_2O (50 mL) at a temperature of 5 °C. In a separate reaction, TEMPO (0.0150 g, 0.0960 mmol) and NaBr (0.214 g, 2.1 mmol) were dissolved in H_2O (3.3 mL) at 5 °C. The reaction mixtures were combined and the pH adjusted to 10.75 using 0.5 M NaOH. Sodium hypochlorite (24.3 mL, 3.3 % solution, 362 mmol) was added gradually over 30 min while maintaining the pH at 10.75. Thereafter, EtOH (1 mL) was added to quench the oxidation. A H_2O/acetone (1:7 w/w) solution was added and the mixture concentrated by rotary evaporation. The crude product was freeze-dried and then dialysed against H_2O. Subsequent freeze- drying yielded (**5B**) as a light brown solid (0.486 g, 45 %).

IR (KBr): *v* (cm^{-1}) 3435 (OH), 1757 (C=O), 1651 (C=O stretch of secondary amide), 1419 (C-H bending of trimethyl ammonium group), 1075 (pyranose); ^{1}H NMR (300 MHz, 2 %

DCl/D_2O) δ 4.48 (1H, s, H-1), 3.50 - 3.33 (3H, m, H-3 – H-5), 3.05 (1H, s, H-2), 2.79 (9H, s, H-4'), 2.75 (s, H-1'), 1.61 (s, acetylated units); Elemental Analysis (%): Calc. For $[C_{12}H_{22}N_2O_6Cl]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}.2H_2O$: C, 36.73; H, 6.28; N, 7.55; Found: C, 36.84; H, 6.24; N, 6.64.

*6.2.5 Trimethyl chitosan chloride (TMC) (**6A**)*[6,7]



Method 1[6]

It must be noted that methyl iodide is an extremely toxic agent and care must be taking when working with this reagent. (MeI) Chitosan (1.09 g, 6.76 mmol), NaI (2.41 g, 16.1 mmol) and 20 % NaOH (5 mL) were added to *N*-methyl-2-pyrrolidone (NMP) (30 mL). The reaction mixture was allowed to stir at 60 °C for 20 min, thereafter MeI (6 mL, 96.4 mmol) was added and the solution was heated under reflux for 1 hr. A solid precipitated after cooling and the addition of a mixture of EtOH and diethyl ether (100 ml, 1:1). The resulting precipitate was filtered and dried under vacuum. NaI (2.41 g, 16.1 mmol), 20 % NaOH (5 mL) and NMP were added to the dry solid and allowed to stir at 60 °C for 20 min, after which MeI (7 mL, 112 mmol) was added. The reaction mixture was heated under reflux for 1 hr. Subsequently MeI (3 mL, 48.2 mol) and 20 % NaOH (4ml) were added and the mixture was allowed to stir at 60 °C for 1 hr. A solid precipitated after cooling and the addition of EtOH and diethyl ether (100 ml, 1:1). The cream-coloured material obtained was dried under vacuum, suspended in 5 % (w/v) NaCl (40 mL) and dialysed against H_2O. The product was subsequently freeze dried and obtained as a fibrous white material (0.659 g, 40 %).

IR (KBr): *v* (cm^{-1}) 3439 (strong broad band, OH), 1935 (sharp band, CH aliphatic), 1656 (sharp band, NH), 1475 (sharp band, C-H bending of trimethyl ammonium group), 1077 (broad band, pyranose); ^{1}H NMR (300 MHz, D_2O) δ 4.30 (1H, s, H-1), 3.58 - 3.75 (5H, m, H-3 – H-6), 3.45 (3H, s, 3 (OCH_3)/6 (OCH_3)), 3.36 (1H, s, H-2) 3.26 (9H, s, H-1'), 2.46 (6H, s, $N(CH_3)_2$), 2.02 (s, acetylated units); ^{13}C NMR (75 MHz, D_2O) δ 98.11(C1), 77.51 (C5), 72.74 (C4), 68.20 (C3), 58.65 (C2), 57.82 (C6), 54.16 (C1'), 41.40 ($N(CH_3)_2$); DQ: 62 % (from ^{1}H NMR); Elemental Analysis (%): Calc. For

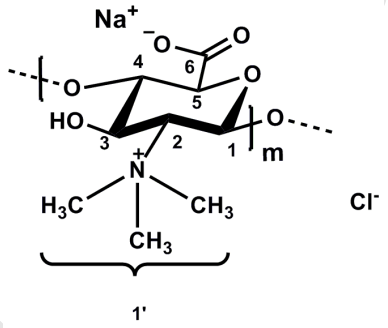
$[C_9H_{18}NO_4Cl]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}.1H_2O$: C, 37.14; H, 6.78; N, 4.72; Found: C, 37.16; H, 6.66 ; N, 3.84.

Method 2[7]

Chitosan (2.08 g, 12.9 mmol) was added to dimethyl sulfate (32 mL, 337 mmol) and H_2O (8 mL). NaOH (2.38 g, 59.5 mmol) and NaCl (1.65 g, 28.2 mmol) were added to the solution and the mixture was allowed to stir for 6 hrs at RT. The mixture was subsequently dialysed against H_2O for 3 days. A precipitate formed upon the addition of acetone. The solution was concentrated and freeze dried, giving the product as a white film (1.66 g, 54 %).

^{1}H NMR (300 MHz, D_2O) δ (ppm) 5.01 (1H, s, H1), 4.04 – 4.14 (3H, m, 3(OCH_3) & 6(OCH_3)), 3.69 (9H, s, H1'), 3.26 - 3.37 (5H, m, H3 – H6), 3.01 (6H, s, $N(CH_3)_2$), 2.81 (1H, s, H2), 2.01 (s, NAc); DQ: 20 % (from ^{1}H NMR).

*6.2.5.1 6-Carboxy-trimethyl chitosan chloride (**6B**)*[5]



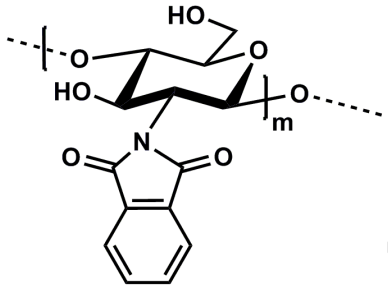
TMC (0.504 g, 2.10 mmol) was suspended in H_2O (50 mL) at a temperature of 5 °C. In a separate reaction, TEMPO (0.01 g, 0.045 mmol) and NaBr (0.137 g, 1.33 mmol) were dissolved in H_2O (3.3 mL) at 5 °C. The reaction mixtures were combined and the pH adjusted to 10.75 using 0.5 M NaOH. Sodium hypochlorite (12.2 mL of a 3.3 % solution, 182 mmol) was added gradually over 30 minutes while the pH was maintained. Thereafter, EtOH (1 mL) was added to quench the oxidation. A H_2O/acetone (1:7 w/w) solution was added and the solution concentrated by rotary evaporation. The subsequent product was frozen and freeze dried. A brown solid was recovered and dialysed against H_2O. Subsequent freeze drying yielded **6B** as a cream fibrous solid (0.249 g, 44 %).

IR (KBr): *v* (cm^{-1}) 3431 (strong broad band, OH), 2918 (sharp band, C-H symmetric stretch of methyl groups), 1658 (strong band, C=O), 1481 (sharp band, C-H asymmetric stretch of methyl groups); ^{1}H NMR (300 MHz, D_2O) δ 5.58 (1H, s, H-1), 4.34 - 4.19 (3H, m, H-3 – H-5), 3.75 (1H, s, H-2), 3.32 (9H, s,H-1'), 2.47 (6H, s, $N(CH_3)_2$), 2.06 (3H, s, acetylated units); ^{13}C

NMR (75 MHz, D_2O) δ 162.11 (amide C=O), 98.28 (C1), 77.64 (C5), 72.57 (C4), 68.60 (C3), 58.63 (C2), 57.86 (C6), 53.83 (C1'), 41.19 ($N(CH_3)_2$), 22.14 (acetylated unit); Elemental Analysis (%): Calc. For $[C_9H_{15}O_5NCl]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 42.78; H, 5.98; N, 4.52; Found: C, 42.73; H, 6.98; N, 3.64.

6.3. Synthesis and Characterization of 6-deoxy-6-amino chitosan and derivatives thereof

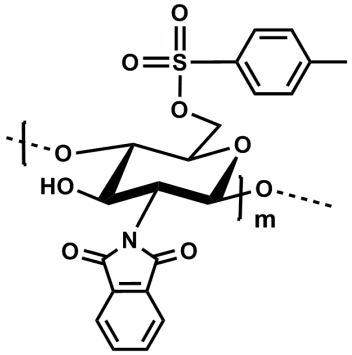
*6.3.1. N-Phthaloyl Chitosan (**7**)*[8]



LMW chitosan (6.01 g, 37.3 mmol) was added to a solution of phthalic anhydride (18.8 g, 127 mmol) in DMF/H_2O (5 % v/v, 120 mL) and heated under reflux (120 °C) for 8 hrs. The reaction mixture was poured into ice water (200 mL) and the resulting precipitate filtered under suction. The filtered precipitate was washed with methanol (300 mL) and dried under suction to a constant mass, to yield the product as a pale tan powder (10.5 g, 97 %).

IR (KBr): *v* (cm^{-1}) 3422 (broad band, OH), 2909 (sharp peak, CH aliphatic), 2133 (C-NH_2 stretch), 1774 & 1711 (phthalimido), 1656 & 1421 (NH bending frequencies), 1155-1092 (broad band, pyranose), 1032 (C-N vibration) and 724 (sharp peak, phthaloyl-aromatic); Elemental Analysis (%): Calc. For $[C_{14}H_{13}NO_6]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 52.69; H, 4.71; N, 5.04; Found: C, 51.63; H, 5.16; N, 4.76.

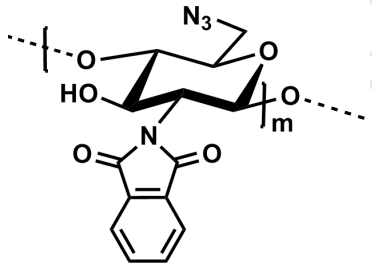
*6.3.2. 6-Deoxy 6-p-toluenesulfonyl N-phthaloyl chitosan (**8**)*[8]



N-phthaloyl chitosan (**7**) (5.78 g, 19.8 mmol) was suspended in pyridine (120 mL) and cooled to 0 °C, followed by the addition of *p*-toluenesulfonyl chloride, (40.5 g, 212 mmol). The mixture was allowed to stir for 17 hr at RT. The red/brown viscous solution was added to ice water (400 mL) and the resulting precipitate was filtered and washed with copious amounts (~ 500 mL) of ethanol and diethyl ether followed by drying under suction and vacuum to constant mass to obtain (**8**) as a light brown powder (8.63 g , 97 %).

IR (KBr): *v* (cm^{-1}) 3448 (broad band, OH), 2948 (sharp band, CH aliphatic),1774 & 1716 (sharp band, imide C=O), 1174 (sharp band, S=O), 1069 – 1006 (broad band, pyranose), 815 (sharp band, C-O-S) and 718 (sharp band, phthaloyl-aromatic); Elemental Analysis (%): Calc. For $[C_{21}H_{19}NO_8S]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 53.29; H, 4.46; N, 3.42; Found: C, 53.75; H, 4.50; N, 2.76.

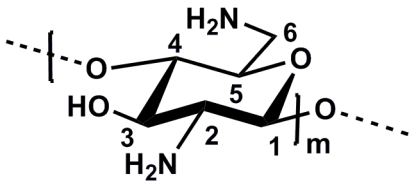
*6.3.3. 6-Deoxy 6-azido N-phthaloyl chitosan (**9**)*[8]



6-Deoxy 6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**8**) (8.63 g, 19.4 mmol) was suspended in NMP (500 mL) followed by the addition of NaN_3 (16.5 g, 254 mmol). The resulting mixture was allowed to stir at 80 °C for 4 hr under nitrogen. Excess of NMP was removed after cooling to RT, and the solution poured into ethanol (200 mL) to form the precipitate. The mixture was then left overnight for the precipitate to settle. The supernatant was removed and ethanol-water 40 % (v/v) (200 mL) was added and the resulting solution was centrifuged. The supernatant was discarded and the precipitate washed with acetone, collected and dried under suction. The resulting brown powder was dried under vacuum to a constant mass, resulting in the desired product (**9**) (5.87 g, 96 %).

IR (KBr): *v* (cm^{-1}) 3435 (broad band, OH), 2939 (sharp band, CH aliphatic), 2103 (sharp band, azido), 1772 & 1718 (sharp bands, N-C=O), 1660 (sharp band, C=O stretch of secondary amide), 1174 (weak band, residual S=O), 1107 – 1011 (broad band, pyranose) and 718 (phthaloyl-aromatic); Elemental Analysis (%): Calc. For $[C_{14}H_{12}N_4O_5]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 48.95; H, 3.99; N, 15.91; Found: C, 49.87; H, 4.76; N, 15.08.

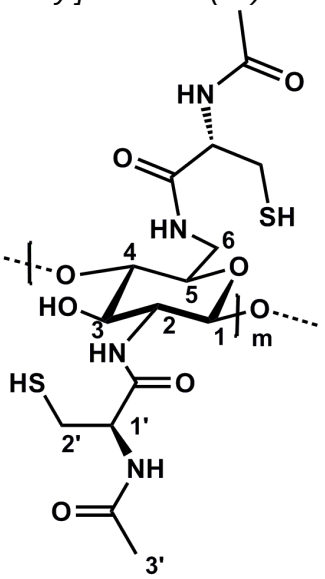
*6.3.4. 6-Deoxy-6-amino chitosan (**10**)*[8]



TPP (8.25 g, 31.5 mmol) was added to a solution of 6-deoxy 6-azido *N*-phthaloyl chitosan (**9**) (5.16 g, 16.3 mmol) in NMP (40 mL), and the reaction was allowed to stir for 15 hr under nitrogen. Subsequent treatment with hydrazine monohydrate (5 mL, 161 mmol) and water (35 mL) followed by stirring under nitrogen at 100 °C for an additional 4 hrs. Excess of water in the reaction mixture was evaporated and the remaining mixture added to ethanol (100 mL). The precipitate was collected by centrifugation, washed with ethanol and collected by suction filtration. The resulting precipitate was suspended in deionised water and purified by ultra-filtration using a dialysis bag with a cut off molecular weight of 10 kDa. The product was obtained and lyophilized to yield a tan powder (2.10 g, 80 %).

IR (KBr): *v* (cm^{-1}) 3379 (broad stong band, OH & NH), 2922 (sharp band, CH aliphatic), 1653 and 1591 (sharp, strong bands, NH_2), and 1060 (broad band, pyranose); ^1H NMR (400 MHz, 2 % DCl/D_2O) δ 6.87-7.84 (5H, m, residual *N*-phthaloyl), 4.52 (1H, s, H-1), 3.22 - 3.57 (5H, m, H-3 – H-6), 2.84 (1H, s, H-2), 2.17 (3H, s, acetylated units); Elemental Analysis (%): Calc. For $[C_6H_8O_3(NH_2)_2]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 38.92; H, 7.35; N, 12.9; Found: C, 39.54; H, 6.70; N,12.87.

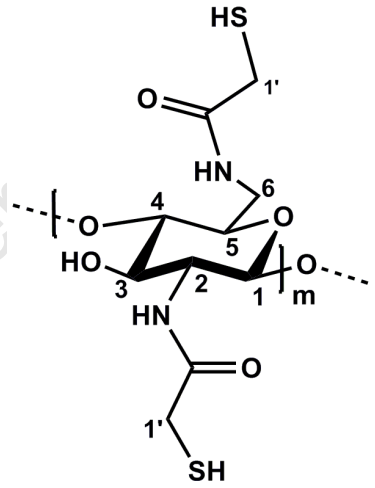
*6.3.5. 6-Deoxy-2,6-bis[N-acetylcysteinyl] chitosan (**11**)*



6-Deoxy-6-amino chitosan (**10**) (0.539 g, 3.4 mmol) was suspended in 1 % HCl (v/v) and adjusted to pH 5. In a separate vessel, *N*-acetylcysteine (4.38 g, 26.8 mmol) was dissolved in H_2O (50 mL) and EDAC (50 mM) was added. After 20 minutes, the pH of this solution was adjusted to 4 - 5 and the two reaction mixtures were combined and allowed to stir for 6 hr while maintaining a constant pH. The polymer was subsequently dialysed against 1 mM HCl + 2 µM EDTA in H_2O. The dialysis buffer was later changed to a mixture of 1 mM HCl, 2 µM EDTA and 1 % NaCl. Lastly the compound was dialysed against a 0.5 mM HCl solution and freeze dried to yield (**11**) as a tan solid (0.545 g, 36 %).

IR (KBr): *v* (cm^{-1}) 3418 (broad strong band, OH), 1625 (sharp band, C=O), 1514 (sharp band, NH) and 1086 (broad band, pyranose); ^1H NMR (300 MHz, 1 M DCl in D_2O (100 °C, 300 W, 2 min) δ 7.26-8.19 (5H, m, residual *N*-phthaloyl), 3.41-3.79 (5H, m, H-3 – H-6), 3.21 (1H, s, H-2), 2.87 (2H, m, H-2'), 1.78 (s, acetylated units & H3'); DS: 51 % (from ^1H NMR); Thiol content: 82 µmol/g (from DTNB assay); Elemental Analysis (%): Calc. For $[C_{16}H_{26}N_4O_7S_2]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 40.39; H, 5.49; N, 12.03; Found: C, 40.87; H, 5.36; N, 11.38.

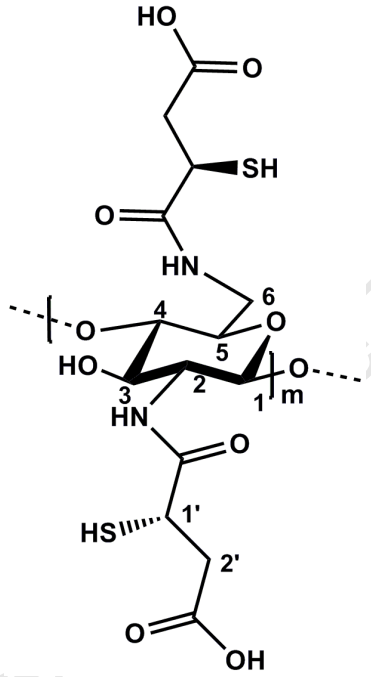
*6.3.6. 6-Deoxy-2,6-bis[thioglycolyl] chitosan (**12**)*



6-Deoxy-6-amino chitosan (**10**) (0.502 g, 3.1 mmol) was hydrated in 1 M HCl (4 mL) and distilled water was added in order to obtain a 1 % solution. EDAC (50 mM) was subsequently added, after EDAC was completely dissolved in the solution, TGA (0.502 g, 5.4 mmol) was added and the pH adjusted to 5. The reaction mixture was allowed to stir for 3 hr at RT. The reaction mixtures were thereafter dialysed for 3 days against 5 mM HCl. The dialysis buffer was later changed to a mixture of 5 mM HCl and 1 % NaCl. The compound was freeze dried and the product was recovered as a pale tan solid (0.507 g, 52 %).

IR (KBr): *v* (cm^{-1}) 3400 (strong broad band, OH), 1634 (sharp band, C=O), 1090 (broad band, pyranose); ^{1}H NMR (300 MHz, 1 M DCl in D_2O (100 °C, 300 W, 3 min) δ 7.25-8.20 (5H, m, residual *N*-phthaloyl), 5.15 (1H, s, H-1), 3.38-3.84 (5H, m, H-3 – H-6), 3.22 (1H, s, H-2), 3.09 (2H, s, H-1'), 1.78 (3H, s, acetylated units); DS: 51 % (from ^{1}H NMR); Thiol content: 37 µmol/g (from DTNB assay); Elemental Analysis (%): Calc. For $[C_{10}H_{16}N_2O_5S_2]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 40.34; H, 5.40; N, 9.09; Found: C, 40.60; H, 5.77; N, 9.05.

*6.3.7. 6-Deoxy-2,6-bis[2S'-mercaptosuccinyl] chitosan (**13**)*

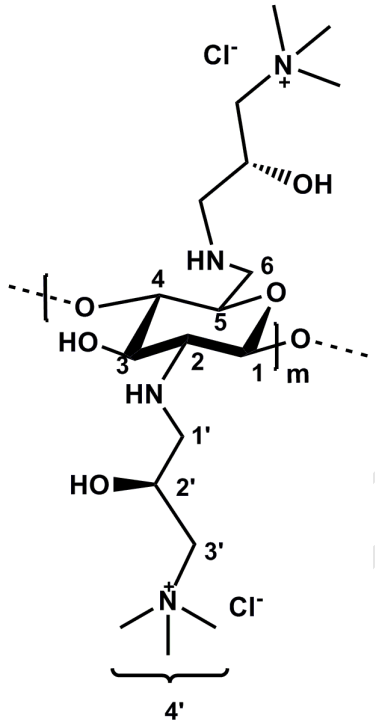


6-Deoxy-6-amino chitosan (**10**) (0.846 g, 5.3 mmol) was suspended in DMF (30 mL). In a separate reaction, *S*-acetylmercaptosuccinic anhydride (5.24 g, 30.1 mmol) was dissolved in DMF (5 mL). The mixtures were combined and allowed to stir for 3 hrs at RT. The yellow/brown solution was extracted with an excess of EtOAc. DMF was removed via vacuum distillation. The resulting polymer was suspended in water, dialysed and thereafter freeze dried. This yielded a pale tan solid of which 0.213 g was stirred in 25 % ammonia solution (30 mL) overnight at RT. The solution was concentrated *in vacuo* and freeze dried. The product (**8**) was recovered as a pale tan powder (0.170 g, 96 %).

IR (KBr): *v* (cm^{-1}) 3413 (broad strong band, OH), 1662 (sharp band, C=O), 1570 (sharp band, N-H), 1101 (broad band, pyranose); ^{1}H NMR (300 MHz, 1 M DCl in D_2O (100 °C, 300 W, 6 min) δ 6.75-7.40 (5H, m, residual *N*-phthaloyl), 2.40 – 3.23 (5H, m, H3 – H6), 2.11 (1H, m, H2), 1.88 (1H, s, H1'), 1.83 (2H, s, H2'), 1.24 (acetylated units); DS: 19 % (from ^{1}H NMR); Thiol content: 21 µmol/g (from DTNB assay); Elemental Analysis (%): Calc. For

$[C_{14}H_{20}N_2O_9S_2]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 40.23; H, 5.48; N, 6.60; Found: C, 40.68; H, 5.68; N, 6.64.

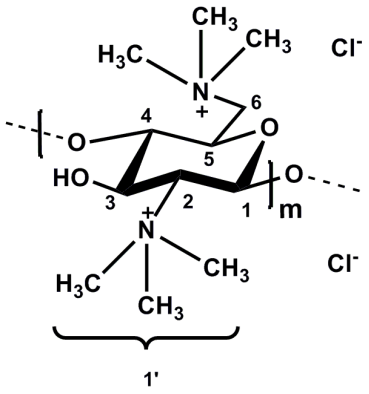
*6.3.8. 6-Deoxy-2,6-bis[3-trimethylammonium-2-hydroxypropyl-N-chitosan chloride] (**14**)*



6-Deoxy-6-amino chitosan (**10**) (0.501 g, 3.13 mmol) was suspended in water (100 mL) followed by the addition of GTMAC (1.14 g, 7.52 mmol) in 3 portions, 2.5 hrs apart. The reaction was allowed to stir at 60 °C for 24 hr. The pH of the solution was adjusted to ~ 5 and the mixture was added to cold acetone and kept at 4 °C overnight. The acetone was decanted and the remaining brown compound was suspended in MeOH (100 mL). The polymer was precipitated in 4:1 acetone: EtOH (~ 250 mL). The precipitate was filtered, washed with EtOH and dried under vacuum to yield (**14**) as a brown powder (0.359 g, 25 %).

IR (KBr): *v* (cm^{-1}) 3435 (strong broad band, OH), 1643 (sharp band, C=O stretch of secondary amide), 1477 (sharp band, C-H bending of trimethyl ammonium group), 1064 (broad band, pyranose); ^{1}H NMR (300 MHz, 1 M DCl in D_2O (100 °C, 300 W, 2 min) δ 7.39-8.17 (5H, m, residual *N*-phthaloyl), 4.45 (1H, s, H-2'), 3.45-3.97 (5H, m, H-3 – H-6), 3.29 (1H, s, H-2), 3.01 (9H, s, H-4'), 2.90 (2H, s, H-3'), 2.71 (2H, s, H-1'), 1.81 (s, acetylated units); DQ: 98 % (from ^{1}H NMR); Elemental Analysis (%): Calc. For $[C_{18}H_{40}N_4O_5Cl_2]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}.1H_2O$: C, 42.35; H, 7.85; N, 10.76; Found: C, 41.28; H, 7.20; N, 9.64.

*6.3.9. 6-Deoxy-2,6-bis[trimethyl] chitosan chloride (**15**)*[9]



6-Deoxy-6-amino chitosan (**10**) (0.406 g, 2.53 mol) was dispersed in NMP (15 mL) and allowed to stir at RT for 4 hr. Thereafter 2.5 mL NaOH (1 M), NaI (0.927 g, 6.2 mmol) and MeI (7 mL, 112 mmol) were added and the solution was allowed to stir at 65 °C for 6 hr. Subsequently, acetone (100 mL) was added, the solvent removed and the product dissolved in 5 % NaCl (10 mL). After the solid was completely dissolved, the compound was freeze dried. The desired product was obtained as a brown powder (0.571 g, yield: 71 %).

IR (KBr): *v* (cm^{-1}) 3425 (strong broad band, OH), 2073 (sharp band, CH aliphatic), 1643 (sharp band, NH), 1477 (sharp band, C-H bending of trimethyl ammonium group), 1062 (broad band, pyranose). ^{1}H NMR (300 MHz, 1 M DCl in D_2O (100 °C, 300 W, 2 min) δ 7.35-8.10 (5H, m, residual *N*-phthaloyl), 3.41 – 4.07 (5H, m, H-3 – H-6), 3.05 (1H, s,H-2), 2.78 (9H, s, H-1'), 2.53 (6H, s, $(NCH_3)_2$ dimethylated version), 1.79 (s, acetylated units); DQ: 24 % (from ^{1}H NMR); Elemental Analysis (%): Calc. For $[C_{12}H_{26}O_3N_2Cl_2]_{0.85}[C_6H_9O_4(HNCOCH_3)]_{0.15}$: C, 42.02; H, 7.57; N, 8.64; Found: C, 41.37; H, 7.20; N, 8.72.

6.4. DTNB Assay to determine Thiol content[10]

This procedure for quantifying sulfhydryl groups is based on molar absorptivity. The reaction buffer used is a solution of 0.1 M sodium phosphate, containing 1 mM EDTA at pH 8.0. The Ellman's reagent solution used in the measurement was made by dissolving 4 mg Ellman's Reagent in 1 mL of Reaction Buffer.

The blank used in the assay contained 50 µL of Ellman's reagent solution and 2.5 mL of reaction buffer. For each sample assayed, the tube contained 50 µL of Ellman's reagent solution, 2.5 mL of reaction buffer and 250 µL of the sample.

Note: For the samples, sulfhydryl concentration must be less than 1.0 mM as concentrations exceeding 1 mM free sulfhydryl will result in high absorbance values and less accurate estimation of the concentration based on the extinction coefficient.

The contents of the tubes were mixed and incubated at RT for 15 min. The absorbance was read using a spectrophotometer set to 412 nm. From the absorbance obtained, the amount and concentration of sulfhydryl's in the sample was calculated from the molar extinction coefficient of DTNB (14,150 $M^{-1}cm^{-1}$).

Sample Calculation:

Calculations for sulfhydryl amount and concentration:

$$E = A/bc$$

Molar extinction coefficient (E) of DTNB = 14.150 M^{-1}, A = absorbance, b = path length (cm), c = concentration (M).

- Cysteine
  Absorbance: 0.050

  C = A/bc

  = 0.050/(1)(14.150)

  = 3.53×10^{-3} M

This value represents the concentration of the solution in the cuvette, therefore to calculate the concentration of the original sample all dilution factors must be accounted for.

Total volume of solution being measured:

2.50 mL reaction buffer

0.25 mL sample

0.05 mL Ellman's reagent solution

2.80 mL of solution

If the concentration of the assay solution is 3.53×10^{-3} M, then 2.80 mL of that solution contains:

$$2.80 \text{ mL} \times 1 \text{ L}/1000 \text{ ml} \times 3.53 \times 10^{-3} \text{ M}$$

$$= 9.88 \times 10^{-6} \text{ moles}$$

These 9.88×10^{-6} moles of sulfhydryl in the assay solution were contributed by the original 2.50 mL sample. Therefore the concentration of free sulfhydryl groups in the original sample is:

$$9.88 \times 10^{-6} \text{ moles}/0.25 \text{ mL} \times 1000 \text{ mL}/1 \text{ L}$$

$$= 3.95 \times 10^{-2} \text{ M}$$

6.5. Synthesis of polymer encapsulated Iron (Fe) nanoparticles

*6.5.1. Chitosan-Fe (**16**)*[11]

Chitosan (0.430 g, 2.7 mmol) was dissolved in 0.5 % (v/v) acetic acid (200 mL) followed by the addition of $FeCl_3.6H_2O$ (2.95 g, 10.9 mmol). Subsequently 25 % ammonium hydroxide solution (10 mL) was rapidly added to the brown solution under sonication at 50 °C. The mixture was then treated for a further 40 min with sonication. The brown precipitate was left to settle and filtered while washing with H_2O. The polymer was freeze dried and obtained as a brown powder (1.40 g).

IR: v (cm^{-1}) = 3400 (strong sharp band, OH & NH), 2913 (weak band, CH aliphatic), 1628 (sharp band, C=O) 1535 (sharp bands, NH bend), 1030 (C-N), 1069 (sharp band, pyranose), 672 (medium band, β-FeOOH).

For TEM analysis, the samples were prepared by suspending a minimum amount of polymer in methanol with sonication. Samples were subsequently placed on a copper grid and allowed to dry under a sun lamp for ten minutes. The particle size was determined by using 2-3 random images of the sample where the average particle size and the standard deviation were obtained using Microsoft Excel.

*6.5.2. CHI-Q188-Fe (**17**)*[11]

$FeCl_3.6H_2O$ (2.92 g, 10.8 mmol) was added to a suspension of CHI-Q188 (0.413 g, 1.32 mmol) in 0.5 % acetic acid (v/v, 200 mL) followed by the rapid addition of 25 % ammonium hydroxide solution (v/v, 10 mL) under sonication at 50 °C. The mixture was sonicated for 40 min and the resulting brown precipitate was left to settle. The precipitate was filtered while washing with water. The polymer was subsequently freeze dried and obtained as a brown powder (1.70 g).

IR: *v* (cm^{-1}) 3194 (sharp band, OH), 1559 (sharp band, C=O stretch of secondary amide), 1400 (strong band, C-H bending of trimethyl ammonium group), 1017 (broad band, pyranose), 674 (medium band, β-FeOOH).

*6.5.3. TMC-Fe (**18**)*[11]

TMC (0.113 g, 0.471 mmol) was dissolved in 0.5 % acetic acid (v/v, 50 mL) followed by the addition of $FeCl_3.6H_2O$ (0.726 g, 2.7 mmol). Thereafter 25 % ammonium hydroxide solution (2.5 mL) was rapidly added to the brown solution under sonication at 50 °C. The mixture was then treated for a further 40 min with sonication. The brown precipitate was left to settle and

filtered while washing with water. The solid was subsequently freeze dried and obtained as a brown powder (0.302 g).

IR: *v* (cm^{-1}) 3133 (strong band, OH), 1400 (strong sharp band, C-H bending of trimethyl ammonium group), 1062 (broad band, pyranose), 668 (medium band, β-FeOOH).

Fe Loading

The iron content of these polymers was determined calorimetrically.[12] A stock Fe solution was prepared by adding ferric chloride hexahydrate (0.0048 g) and H_2SO_4 (0.25 mL) to a 100 mL volumetric flask and filling to the graduated mark with H_2O. Standard solutions of the following were prepared: sodium acetate buffer (1.2 M), hydroxylamine sulfate (100 g/L) and 1,10-phenanthroline (1 g/L). To generate a standard curve for the absorbance of Fe, the following standards were made:

Table 6.1: Standards prepared (diluted to 100 mL, added in the order shown).

Standard	Concentration [x 10^{-6}] ($mol.dm^{-3}$)	Fe stock soln (mL)	NH_2OH (mL)	Phenanthroline soln (mL)	Sodium acetate buffer (mL)
1	1.79	1	1	10	8
2	8.95	5	1	10	8
3	17.9	10	1	10	8
4	35.8	20	1	10	8
5	62.7	35	1	10	8

The absorbance was read at 508 nm and the calibration curve obtained was used for the subsequent determination of Fe loading on the selected polymers.

Preparation of the polymers for determination of Fe loading:

Fe loaded polymer (0.5 g) was allowed to stir in HNO_3 (1 mL) for 24 hr. The resulting solution was added to a volumetric flask (100 mL). Hydroxylamine (1 mL), phenanthroline soln (10 mL) and sodium acetate buffer (8 mL) were added systematically and the solution was diluted to the graduated mark with H_2O. The absorbance was measured at 508 nm and the Fe concentration extrapolated from the standard curve.

6.6. Perchlorate (ClO_4^-) removal

Chitosan and selected derivatives, as well as a commercially available anion exchange resin (Amberlite IRA 400), were tested as potential stationary phases in the removal of ClO_4^-. The compounds tested as stationary phases were: chitosan (**1**), CHI-Q188 (**5A**), TMC (**6A**), chitosan-Fe (**16**), CHI-Q188-Fe (**17**) and TMC-Fe (**18**).

- **Testing Protocol**[13]

1. Standard solutions of ClO_4^- (1-6 µg/L) were prepared.
2. Solutions were spiked with Brilliant green (BG) dye.
3. Extracted with toluene and absorbance measured at 640 nm.
4. Calibration curve was plotted.

- **Column Preparation**

1. 1 g of polymer/stationary phase was packed into a 5 mL SPE cartridge.
2. The column was pre-conditioned with 3 mL acetone and 20 mL H_2O, thereafter air was pushed through the column.
3. Approximately 100 mL 5 µg/L ClO_4^- was passed through the column. Elution took place under gravity.
4. Air was pushed through the column to remove any eluent.
5. 1 mL acetone was added and forced through the column.
6. Eluted samples were spiked with BG, 1 mL H_2O was added and allowed to stand for 15 min.
7. Samples were extracted using 1 mL toluene, adding a few drops of acetone to remove any cloudiness.
8. Steps 5-7 were repeated to obtain a total of five fractions.
   From the 6th fraction to the 10th fraction, 2 mL of acetone was used, from fractions 11 – 13, 3 mL of acetone was used. For fractions 14 – 20, 5 mL of water was passed through the column to remove the remaining ClO_4^-.
9. Absorbance was read at 640 nm.
10. Concentration of ClO_4^- was extrapolated from the calibration curve.

The above procedure was modified when testing with **5A** as a solid support, as the polymer formed a gel when pre-conditioned. This gel retained most of the water applied to the

column, therefore the procedure was modified. The polymer was allowed to stir in a 5 µg/L ClO_4^- solution for 1 hr at RT. The subsequent viscous solution was centrifuged, the supernatant removed and the residue dried. The dry powder was packed into a SPE cartridge and the retained ClO_4^- was eluted using acetone. Steps 5 – 10 in the above procedure were subsequently followed.

Polymer **17**, the Fe loaded derivative of **5A**, was pre-conditioned, dried and run using the same procedure as all other compounds tested.

The concentration values obtained from the graph were converted to % ClO_4^- retention using the following formula:

$$\% ClO_4^- \text{ on column} = (1 - \text{total eluted}/0.5) \times 100$$

Where total eluted refers to the acetone or water fractions collected.

6.7. Silver Loading

Approximately 0.5 g of chitosan and its derivatives **2-6** and **10-15** were mixed with H_2O (25 mL) and a solution of 52 mM $AgNO_3$ in Milli-Q H_2O (molar ratio = 1.5) was added. The flask was covered with aluminium foil to exclude light. The solution was allowed to stir at RT for 16 hr and the resulting solutions were centrifuged and the supernatant removed. The precipitate was washed with H_2O, centrifuged and freeze-dried.[14] The presence of Ag(I) was confirmed through ICP-MS, TEM and IR analysis.

ICP-MS analysis preparation

Chitosan and its derivatives were prepared for ICP-MS analysis by the addition of 5 M HNO_3 (5 mL) to 20 mg of each polymer. The solutions were stirred overnight and filtered; the resulting filtrate was submitted for ICP-MS analysis.

Table 6.2: ICP-MS data of the Ag-loaded polymers.

Polymer	ppm	Ag^+ Loading (mmol/g)	Polymer	ppm	Ag^+ Loading (mmol/g)
1	584	5.41	**10**	337	3.12
2	86.1	0.80	**11**	7.98	0.07
3	206	1.91	**12**	192	1.78
4	462	4.28	**13**	461	4.27
5A	364	3.37	**14**	5.71	0.05
6A	689	6.38	**15**	1.32	0.01

TEM analysis

Samples were prepared for TEM analysis as described in section 6.5.1.

6.8. Evaluation of Antimicrobial activity of selected polymers

6.8.1. Microorganisms and culture conditions

Selected polymers were tested for antimicrobial activity. The polymers were evaluated against two microorganisms, the gram-(+ve) bacteria *S. aureus* and two gram-(-ve) bacterial species, *E. coli* and *Pseudomonas aeruginosa (P. aeruginosa)*. These bacterial cultures were provided by the Molecular and Cell Biology Department of the University of Cape Town. The cultures were obtained by plating the bacteria on luria agar by streaking, or in a luria broth by inoculating a single colony from an overnight plate.

6.8.2. Evaluation of antibacterial activity of chitosan derivatives

Antibacterial activity of various chitosan derivatives was established using a Minimum Inhibitory Concentration (MIC) method. The assessments were performed by test well method using liquid culture of bacteria. In the case of the silver loaded compounds, certain samples were tested by plating on agar plates.

Phosphate buffer used in the assessments was prepared from potassium phosphate monobasic. Milli-Q H_2O was used for all solutions. Antibacterial assessments were performed in sterile 96 well plates. Selected polymers tested were: chitosan (**1**) and the derivatives **2**, **3**, **4**, **5A**, **5B**, **6A, 6B** and **15**.

6.8.3. Testing Protocol

The method followed was that reported by Thatte *et al*.[15] Each antibacterial agent was made up as a 1 % (w/v) solution in Milli-Q H_2O. In some cases, the solution was acidified to allow the polymer to dissolve. The pH of these solutions was subsequently adjusted to 5.4. These solutions were further diluted in sterile phosphate buffer to make stock test solutions with concentrations of 10, 50,100, 250 and 500 µg/mL. Phosphate buffer was prepared by diluting 1.37 g KH_2PO_4 to 100 mL with H_2O. This 0.1 M solution was further diluted to a final concentration of 50 mM and the pH adjusted to 7.

Bacteria were inoculated at 37 °C in a culture tube containing sterile NB (~ 5 mL) for 12 hrs. The resulting culture (~ 1 mL) was transferred into a culture flask containing sterile

NB (25 mL) and incubated at 37 °C with shaking for 3.5 hr. A portion of the resulting bacterial culture (~ 2 mL) was diluted with NB to obtain appropriate optical densities (OD). The OD of *E. coli* and *S. aureus* in mid-log phase growth is approximately 0.400 and 0.800 respectively when compared to sterile NB as the blank. After the desired OD was obtained, the sample was further diluted with 4X its volume of NB. The bacterial cell density in this solution was calculated to be 4 x 10^7 cells/mL.

96-Well plate assessment: The plate was divided into three sections (cell control, Agent control and test well). Each well contained the following:

Cell Control: PBS (50 μL) + Cell solution (50 μL) + Milli-Q H_2O (100 μL)

Agent Control: PBS (50 μL) + NB (50 μL) + Antibacterial agent (100 μL)

Test Well: PBS (50 μL) + Cell solution (50 μL) + Antibacterial agent (100 μL)

The Figure below shows the assessment plate. Polymers were tested in triplicate. Cells A1 – H1 contained only bacteria and buffer while all even numbered cells contained only the antimicrobial agent and buffer. The odd numbered wells contained both bacteria and antimicrobial agent. The chosen concentrations of the antimicrobial agent were 10, 50,100, 250 and 500 μg/mL. Concentrations increased from left to right along each row.

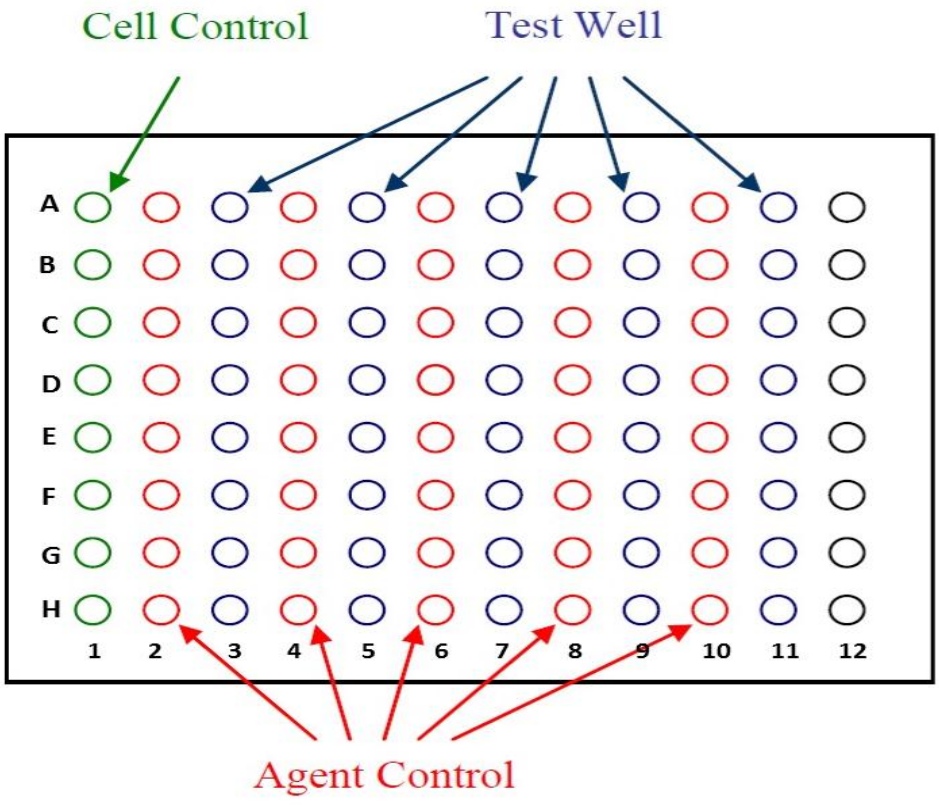


Figure 6.1: The layout used for this protocol.

These plates were incubated at 37 °C for 14 hrs. After this time, plates were assessed visually. Wells where growth occurred turned visibly turbid while the MIC was defined as the first clear well of lowest concentration of the antimicrobial agent.[15]

6.8.4. Evaluation of antibacterial activity of silver loaded chitosan derivatives

Selected Ag loaded polymers tested for their antimicrobial properties are: chitosan (**1**), **3**, **5A**, **5B**, **6A** and **6B**, these polymers were chosen as these were the only polymers which readily dissolved in water.

In an initial test to observe Ag leaching the following method was used. Each antibacterial agent was made up as a 1 % (w/v) solution in Milli-Q H_2O. In some cases, the solution was acidified to allow the polymer to dissolve. The pH of these solutions was subsequently adjusted to 5.4. A disk diffusion method was used, where disks were soaked in the polymer solutions and applied to agar plates which had been spread with 100 µL of bacteria (*S. aureus, E. coli* and *P. aeruginosa*). The plates were incubated at 37 °C overnight.

In the test to observe the antimicrobial properties of these Ag loaded polymers as a function of time, the following method was followed. The polymers were tested at a concentration of 50 µg/mL. Antimicrobial activity was assessed by counting colony-forming units (CFU). *E. coli* was cultured in a nutrient broth solution at 37 °C overnight with shaking. The OD of the bacterial culture was adjusted to 0.300 - 0.400 using NB. The sample (100 µL) was added to the culture and incubated with shaking at 37 °C. Prior to incubation, 100 µL of the solution was taken and diluted consecutively with 9.9 mL of sterile saline solution from which an aliquot of 100 µL was taken for plating in nutrient agar plates. The solution was incubated for a total of 4 hr and at every 1 hr interval, 100 µL of the samples was removed, diluted as stated above and plated. After overnight incubation at 37 °C, viable colonies were visible to the naked eye and thus were counted manually and compared with the positive control.[16]

6.8.5. Evaluation of anti-mycobacterial activity of chitosan derivatives and their Ag loaded counter parts

Anti-mycobacterial testing was performed using *Mycobacterium smegmatis* (*M. smegmatis*) as a model organism. *M. smegmatis* was grown up in Difco™ middebrook 7H9 broth + Glucose salt (5 % tween, 8.5 % NaCl, 20 % glucose) at 37 °C to an OD_{600} of 1.5. A serial dilution was performed by adding 100 µL of the culture to 900 µL of 7H9 broth (10^{-1}). This procedure was repeated until a total of 6 dilutions were obtained (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}). Separately, the polymers and their Ag-loaded counterparts (**1**, **2**, **3**, **4**, **5A**, **5B**, **6A**,

6B, **1-Ag**, **5A-Ag**, **6A-Ag** and **6B-Ag**) were dissolved and made up to a concentration of 100 µg/L. Each sample (1 mL) was applied to a luria broth (Miller, Merck) agar plate, and allowed to dry overnight. The plates were inoculated with 10 µL of the bacterial dilutions where each plate contained 6 dilutions. The plates were incubated at 37 °C for 4 days and thereafter inhibition was determined visually. Testing was performed in duplicate.

6.9. References

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