

Synthesis of 6-Deoxy-6-Amino Chitosan and Applications thereof

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UNIVERSITY OF CAPE TOWN

2018

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Synthesis of 6-Deoxy-6-Amino Chitosan and Applications thereof

Thesis presented for the degree of

Doctor of Philosophy

by

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2018

Declaration

I declare that “**Synthesis of 6-Deoxy-6-Amino Chitosan and Applications thereof**” 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.

..

Signed by candidate

 ..

Shakeela Sayed

17/05/2018

This thesis is dedicated to the memory of my father Rashid Sayed whose presence is greatly missed and to my mother Aysha Sayed who gives me strength to this day by showing me that it is possible to be completely shattered and then rebuild yourself stronger than ever.

“You must read, you must persevere, you must sit up nights, you must inquire, and exert the utmost power of your mind. If one way does not lead to the desired meaning, take another; if obstacles arise, then still another; until, if your strength holds out, you will find that clear which at first looked dark.”

Giovanni Boccaccio

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Publications

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Book Chapters:

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S. Sayed and M.A. Jardine, *Antimicrobial Biopolymers* in *Advanced Functional Materials*, A. Tiwari and L. Uzun eds., Scrivener Publishing, ch 12, 493 – 533, 2015.

Patent (In the process of being filed):

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A. Jardine and **S. Sayed**, Challenges in the valorisation of chitinous biomass within the biorefinery concept. *Current Opinion in Green and Sustainable Chemistry*, 2016, **2**, 34 – 39.

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Abstract

Chitin and chitosan play an important role in the context of a biorefinery, where waste is converted into useful products. The introduction of an amine onto chitosan at the C-6 position has led to the synthesis of a highly water soluble chitosan derivative, 6-deoxy-6-amino chitosan. However, all known synthetic methods for the synthesis of 6-deoxy-6-amino chitosan have some limitations, which acts as impediments to the scale up process. The introduction of an additional amine functionality allows for the further modification of the polymer, which can potentially enhance favourable properties such as solubility and biological activity. The solubility of the polymer in water is advantageous and opens up many different avenues of application. A shorter, greener and scalable synthetic pathway was sought due to the prior use of hazardous solvents and the generation of large quantities of waste. To aid the modification of chitin and chitosan, the solubility of these polymers was investigated using theoretical and computational modelling. Innovative synthetic means of introducing an amine at the C-6 position of the biopolymer was investigated. Ultimately, an expedient synthesis of 6-deoxy-6-amino chitosan was developed resulting in a much-improved yield and quality of 6-deoxy-6-amino chitin and chitosan.

The improved 6-deoxy-6-amino chitosan was utilised in various applications such as antimicrobials, solid supported catalysts and electrospun nanofibers. 6-Deoxy-6-amino chitosan was co-spun with poly(ethylene oxide) and the resulting nanofibres were subsequently coated with silver nanoparticles which improved antimicrobial applications. Nanoparticles were produced using 6-deoxy-6-amino chitosan as the reducing and capping agent. The antimicrobial activity of these silver coated nanofibres was evaluated against *E.coli* and the antifungal activity of 6-deoxy-6-amino chitosan was studied against *Fusarium verticillioides*. Studies indicated that the 6-deoxy-6-amino chitosan and oligomers thereof exhibited an inhibitory effect on the growth of the fungus. Further work on the use of 6-deoxy-6-amino chitosan as an edible food coating in the treatment of strawberries was conducted. Initial results indicated that the onset of decay was delayed in the samples treated with the polymer.

The previous successful application of recyclable 6-deoxy-6-amino chitosan as a water-soluble platinum group metal catalyst support was extended to a biomimetic pyridoxal 5'-phosphate (PLP) based support. Immobilised pyridoxal 5'-phosphate (PLP) was investigated as an enzyme mimic in the deamination of L-phenylalanine to phenylpyruvate.

Abbreviations

AcOH	acetic acid
ADH	alcohol dehydrogenase
α	alpha
Ar	aromatic
Ar-q	aromatic quaternary
ATR	Attenuated Total Reflection
β	beta
BMIMCl	1-butyl-3-methylimidazolium chloride
δ	chemical shift
C=O	carbonyl
^{13}C NMR	carbon nuclear magnetic resonance
cm	centimetre
CPMAS	cross-polarisation magic angle spinning
$^{\circ}$	degree(s)
$^{\circ}\text{C}$	degree(s) Celsius
DDA	degree of deacetylation
CDCl_3	deuterated chloroform
DCl	deuterated hydrochloric acid
CD_3OD	deuterated methanol
D_2O	deuterated water
DCM	dichloromethane
DSC	differential scanning calorimetry
DMAc	<i>N,N</i> -dimethylacetamide
DMC	dimethylcarbonate
DMF	<i>N,N</i> -dimethylformamide
Da	dalton
d	doublet
dd	doublet of doublets

dt	doublet of triplets
EtOH	ethanol
EtOAc	ethyl acetate
Et ₃ N	triethylamine
e.g.	example
Fig.	figure
FT-IR	fourier transform infrared spectroscopy
γ	Gamma
g	gram(s)
HSP	Hansen solubility parameters
HSQC	Heteronuclear Single Quantum Coherence
Hz	hertz
HMPA	hexamethylphosphoramide
HSM	hot stage microscopy
hr	hour(s)
HCl	hydrochloric acid
IR	infrared
IL	ionic liquid
LMW	low molecular weight
<i>J</i>	coupling constant
KivD	2-keto acid decarboxylase
kDa	kilo-daltons
Lit.	literature
MgSO ₄	magnesium sulfate
w/v	mass concentration
MHz	megahertz
M.p.	melting point
MeOH	methanol
Me	methyl

NMP	<i>N</i> -Methyl-2-pyrrolidone
µg	micrograms
µL	microlitres
µM	micromolar
mg	milligram(s)
mL	millilitre(s)
mmol	millimole
min	minute
M	molar
mol	mole(s)
MW	molecular weight
mol.dm ⁻³	moles per litre
m	multiplet
NAc	<i>N</i> -acetyl
nm	nanometre
NMR	nuclear magnetic resonance
OH	alcohol/hydroxyl group
ppm	parts per million
%	percentage
PAMAM	polyamidoamine
PEO	poly(ethylene oxide)
PXRD	powder X-ray diffraction
¹ H NMR	proton nuclear magnetic resonance
PLP	pyridoxal 5'-phosphate
RDF	Radial Distribution Functions
cm ⁻¹	reciprocal centimetres
RT	room temperature
SEM	scanning electron microscopy
s	singlet

NaHCO ₃	sodium bicarbonate
NaCl	sodium chloride
NaOH	sodium hydroxide
TMS	tetramethylsilane
TGA	thermogravimetric analysis
TLC	thin layer chromatography
G3	third generation
TEM	transmission electron microscopy
TPP	triphenylphosphine
t	triplet
UV-Vis	ultraviolet-visible spectroscopy
ZOI	zone of inhibition

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Appendix

Chapter One

Chitin, chitosan, their derivatives and selected applications thereof

Chitin (1.1, Figure 1.1) and its deacetylated form chitosan (1.2, Figure 1.1), are well known biopolymers which have attracted significant attention due to their inherently appealing properties, since they are biocompatible, chemically versatile (due to reactive groups present), biodegradable and exhibit low toxicity. In addition, these polymers can be modified or applied in different forms to improve their properties. As a result, chitin, chitosan and their derivatives have found utility in a wide variety of applications.¹

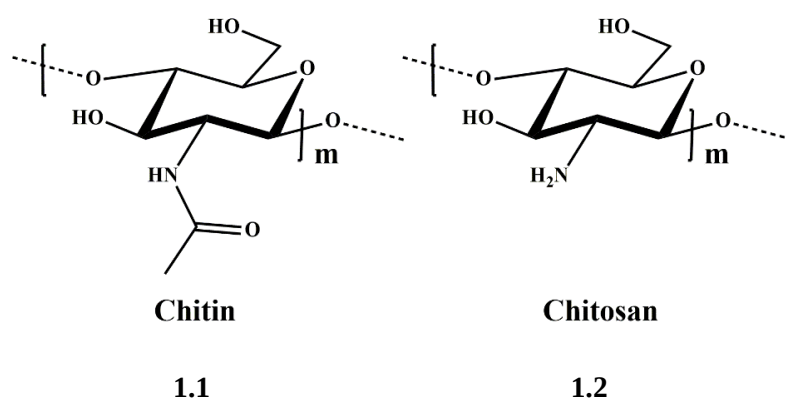


Figure 1.1: Chitin (1.1) and the deacetylated form of the polymer, chitosan (1.2).

1.1 Chitin and chitosan

Energy, chemicals and materials have traditionally been derived from petroleum based resources.² With dwindling non-renewable petroleum based resources becoming a cause for increasing concern, alternative sources of environmentally friendly and sustainable materials has become an area of great interest. Recently, researchers have begun to look to biomass (organic matter) including food waste, as sources of value-added products.

Biomass which does not compete with food or feed is ideal (Figure 1.2).³ The feedstocks which have been investigated as possible sources of alternative materials includes that obtained from the forestry, agricultural and food waste sectors such as cellulose, chitin, corn stover, pectin, starch, straw, vegetable oil, waxes, wood, and zein.⁴ The waste and value add hierarchy is given in Figure 1.2 and Figure 1.3.

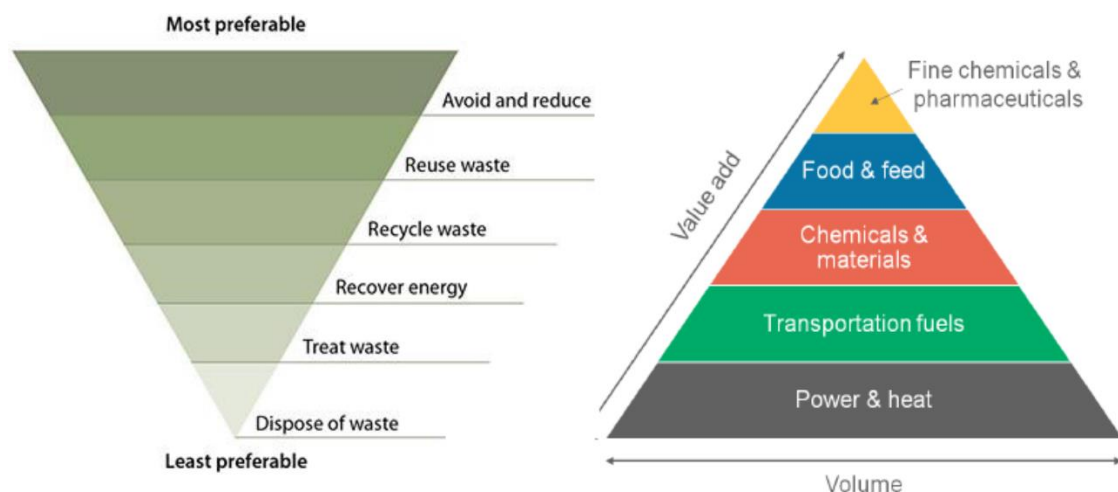


Figure 1.2: The waste hierarchy.⁵

The majority of these materials come from agricultural waste; however, there has been increasing interest in marine biomass. Terrestrial biorefineries are limited by competition with food production, since water and land are needed. However, these aquatic biorefineries avoid problems faced by terrestrial biorefineries such as change in land usage or interference in the food chain.^{3, 6} In addition, aquatic biomass have been shown to yield higher organic content compared to terrestrial biomass making it an attractive resource.⁷

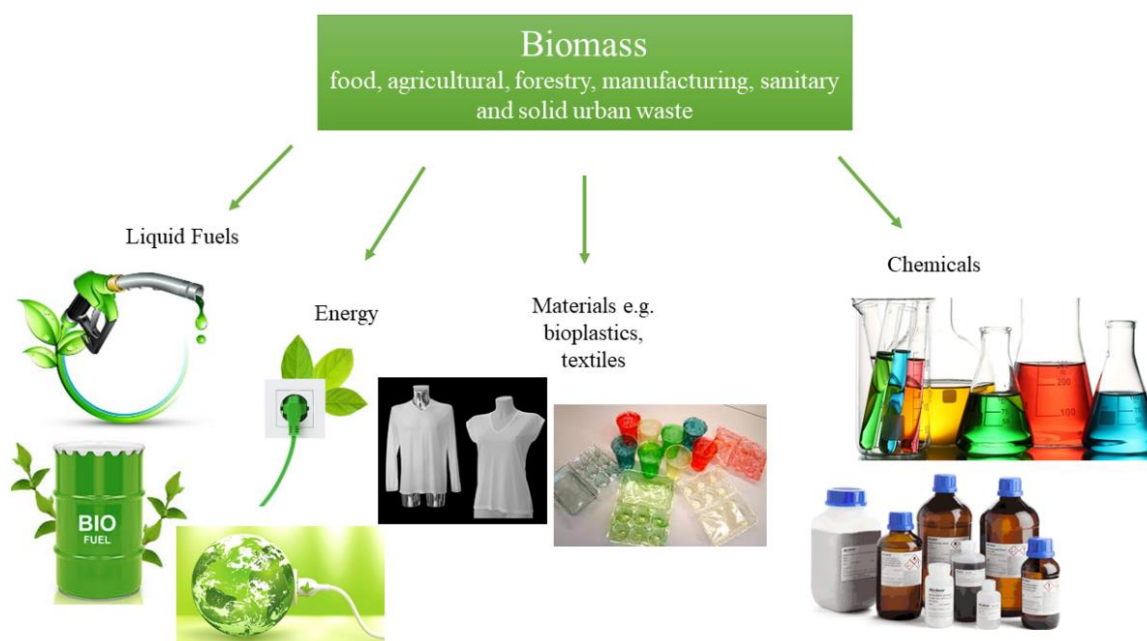


Figure 1.3: Examples of biomass and the final products obtained from their processing.

Chitin is the major material obtained from marine biomass. It was first reported by Braconnot in 1811 when he isolated the polymer from mushrooms.⁸ Chitin was later found to be present in large quantities in other organisms such as microorganisms and in particular the exoskeletons

of arthropods such as crustaceans, fungi (e.g. mushrooms), insects (e.g. silkworms, honeybees) and annelids (Figure 1.4). Approximately 10^{11} tons of chitin is produced annually in nature, making it the largest source of nitrogen available to living organisms. The majority of the chitin obtained through indirect methods (e.g. fishing, cultivation), is generally discarded as commercial waste such as seafood and fungal waste.⁸

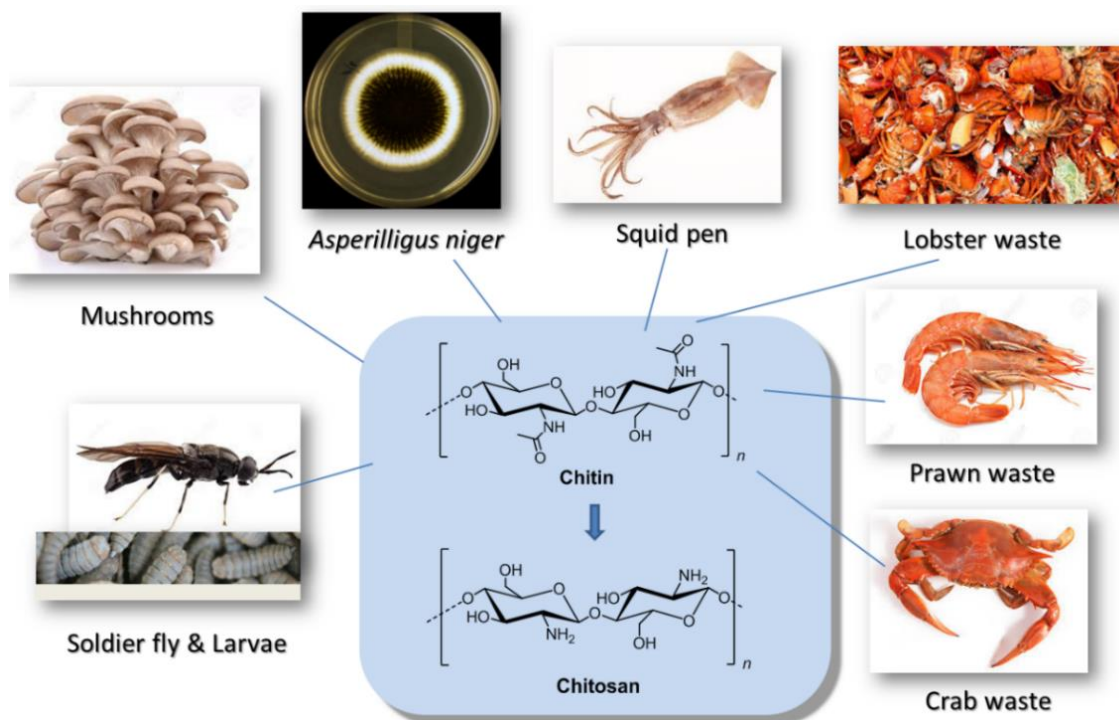


Figure 1.4: The various sources of the biopolymers chitin and chitosan.⁸

In nature, chitin synthases are responsible for chitin production where UDP- N -acetylglucosamine is utilised as the starting material. Chitin is a cationic polysaccharide consisting of long chains of N -acetylglucosamine linked through β (1 $\rightarrow$ 4) glycosidic bonds. The repeating unit present is a chitobiose dimer as every sugar residue is a 180° reflection of its neighbour, where the chair is held in a rigid low energy conformation (Figure 1.4). Chitin is found in nature in three different forms, α , β (Figure 1.5) and γ . The forms vary in terms of their degree of hydration, the size of the unit cell and the quantity of chitin chains per unit cell. α -Chitin consists of antiparallel sheets with extensive hydrogen bonding, β -chitin consists of parallel chains and γ -chitin is a combination of α and β -chitin. The form most widely available in nature is α -chitin, where β - and γ -chitin can be converted to α -chitin by acid treatment. It has been reported that β - and γ -chitin can provide flexibility and toughness whereas α -chitin provides robust rigidity (e.g. in the cuticle). The squid *Loligo* has been found to contain all three polymorphs.

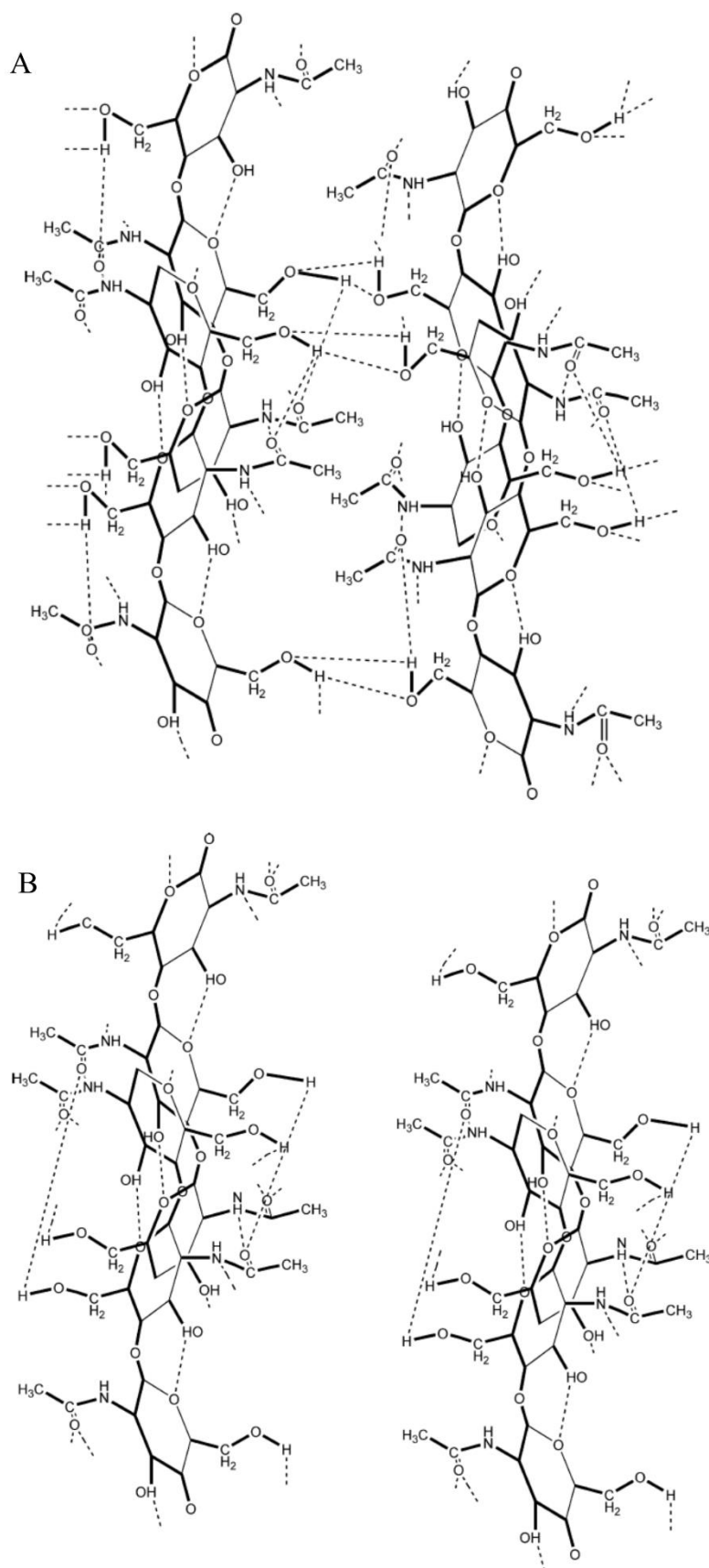


Figure 1.5: The molecular structure and hydrogen bonding present in (A) α -chitin and (B) β -chitin.⁹

Fungal chitin is typically present with other polysaccharides compared to exoskeletal chitin which is normally conjugated with proteins. The main function of chitin is as a structural supporting material in the organisms from which it is isolated.¹⁰

Commercial chitin is primarily derived from marine food waste (crab, krill, lobster, prawn and shrimp shells). This waste may be obtained from commercial fishing as well as aquaculture. Countries which have well developed fishing industries such as China are the major producers of chitin, primarily from shellfish waste for high volume, low-value applications. In industry, the use of chitin and chitosan is not widespread due to the inconsistent properties obtained and solubility issues; however, this is slowly changing as the usefulness of these polymers is being recognised.¹¹ In a recent review by Hamed *et al.* the industrial applications of chitin, chitosan and oligosaccharides (COS) (low molecular polymers of chitin and chitosan) was investigated. This review looked at the applications in the food sector (antioxidant activity, food preservative, antimicrobial packaging, dietary fibre), in the pharmaceutical industry, in the biomedical sector, in the cosmetic industry and finally in the textile industry. This study found that despite the vast amount of literature present, these polymers are still underutilised and industries need to be encouraged to benefit from the various applications of these polymers.¹²

Despite the successful application of crustacean derived chitin and chitosan, it is preferable to have a non-animal based source of the polymer especially for applications in areas such as the biomedical sector due to allergens present in shellfish. Market demands for non-shellfish derived chitin have led to the cultivation of mushrooms and insects for the sole purpose of chitin manufacturing. The biopolymers derived from mushrooms have a narrower molecular weight range compared to that derived from shellfish waste. The physical characteristics of the extracted polymer are dependent on the growth medium composition.¹³ As a result of the increasing demand for alternative sources of chitin and chitosan, numerous companies have been formed to meet this need. The non-animal based chitin and chitosan manufacturer KitoZyme[®], has patented a new process for obtaining medical grade chitosan from edible mushrooms and more recently, from the mycelium of the fungus *Aspergillus niger*. There have also been numerous publications reporting the extraction of chitin from many different insects.⁸

The shells from marine food waste used for extraction of chitin, generally consists of 30 – 40% protein, 30 – 50% calcium carbonate and 20 – 30% chitin with the remainder being attributed to pigments. Chitin is extracted from the shells *via* deproteinisation using harsh basic conditions, demineralisation with strong acids and decolourisation using organic solvents.

There are both chemical and enzymatic processes used for the recovery of chitin. Chemical extraction is often a harsh process generating large quantities of waste and the isolated chitin has inconsistent physiological properties due to uncontrolled hydrolysis. Enzymatic methods on the other hand, are lengthy processes which are costly and in some cases residual protein still remains after isolation of the polymer. The most commonly used enzymes in the isolation of chitin are alcalase, bromelain, chymotrypsin and papain.^{12, 14, 15}

The key factor in determining the type of extraction used in the isolation of chitin, is the end application of the polymer. Furthermore, the energy requirements for the extraction, the environmental impact and the production of waste by-products have to also be taken into consideration. In order to promote the use of chitin and chitosan products, it is imperative that improved extraction processes are developed.

The quality and properties of the chitinous polymer obtained after recovery, are dependent on the source (e.g. crab versus shrimp or mushrooms) and the processes involved in extraction. The extraction method chosen also depends on the chemical composition of the chitin being processed. For example, α -chitin possesses a network of many hydrogen bonds which require harsher extraction conditions compared to the β - and γ - forms of chitin. Various properties of the isolated polymer are affected by the recovery method used, such as the degree of deacetylation, molecular weight, polydispersity (distribution of molecular mass) and the crystallinity of the polymer.^{10, 14}

Chitin has a number of beneficial properties including and not limited to its non-toxic nature, it is biodegradable and biocompatible as well as possessing enhanced adsorption properties. In addition, chitin has displayed antimicrobial activity and the ability to chelate metals. Another favourable quality of chitin, is its characterisation as a Generally Regarded As Safe (GRAS) material which clears the way for its application in many sectors such as biomedical applications. It has previously been applied as a cosmetic preservative, as a drug carrier, in the artificial production of skin, as a plant defence mechanism elicitor and in the production of edible films. However, due to its poor aqueous solubility its potential application in a wide variety of sectors is limited.^{16, 17}

The insolubility of chitin can be attributed to extensive intra- and intermolecular hydrogen bonding between polymer chains. Many studies have looked at the solubility of the different forms of chitin in a variety of solvents. Selected solvents which have been used for the dissolution of chitin includes inorganic chemicals such as calcium bromide, calcium chloride

calcium dithiocyanate, calcium iodide and lithium thiocyanate. Strong acids and polar solvents such as dichloroacetic acid, trichloroacetic acid (TCA), TCA/dichloromethane (DCM), TCA/chloral hydrate/DCM and highly polar solvents for example hexafluoroisopropyl alcohol, hexafluoracetone sesquihydrate and methane sulfonic acid have been used. Included in this list are amide–lithium chloride systems of *N,N*-dimethylacetamide (DMAc) and *N*-methyl-2-pyrrolidone (NMP), CaCl_2 -MeOH/chloroethanol and sulfuric acid.^{17, 18}

Therefore, interest has also been directed towards the most widely known derivative of chitin, chitosan which is produced by removing the acetyl groups present on chitin until it becomes soluble in dilute acids. Chitin is typically converted to chitosan by thermochemical deacetylation using a base treatment. Chitosan itself is not found commonly in nature, only the fungi *Mucoraceae* has been known to produce chitosan naturally.

Owing to its free amino group, enhanced solubility and lower degree of crystallinity, chitosan possesses more desirable properties when compared to chitin.¹⁹ Due to random deacetylation, chitosan is composed of irregularly distributed β -(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units. This results in a polymer which does not have a single well-defined molecular structure, thus leading to chitosan of varying molecular weights and sequences.²⁰ The properties of chitosan are dependent on the source of the polymer, where the molecular weight of the polymer is determined based on the degree of deacetylation (DDA) and depolymerisation (DP). Chitosan is preferred over chitin due to its improved solubility, easily modifiable reactive groups as well as its enhanced antimicrobial activity and antifungal activity. The DDA of commercially available chitosan ranges between 40 – 98% leading to a variation in the molecular weight from 5×10^4 – 2×10^6 Daltons. As a result of the requirements of different industries, there are typically four chitosan grades available:

- Agricultural (DDA $\geq$ 85%)
- Food and cosmetic (DDA 65–90%, 78–82%, application dependent)
- Industrial (DDA $>$ 75%)
- Pharmaceutical (DDA 90–95%).

Currently, milder and more efficient methods of chitosan processing are being studied to avoid excessive waste generation, lengthy reaction times and the use of high reaction temperatures. Chitin (α - and β -) can be successfully converted to chitosan in aqueous sodium hydroxide faster and at a lower temperature with the use of high intensity ultrasound irradiation, compared to

conventional procedures. The chitosan produced using high intensity ultrasound irradiation was acid soluble, and possessed a higher degree of deacetylation compared to that produced under the standard thermochemical method using similar reaction conditions.²¹ Another improved method of chitosan production involves the use of microwave technology, where the reaction is complete in seconds producing chitosan with a high DDA and a low molecular weight. However, limitations of these techniques include cost of equipment and issues related to the scaling up of such methods.²² These issues are being addressed by organisations such as the Biorenewables Development Centre in the United Kingdom.²³ It has been found that in some cases the benefit of the technique outweighs the cost. One such example of this is the increased interest in the conversion of biomass into useful materials. Food waste, forestry waste, agricultural waste and marine resources containing hemicellulose, cellulose and lignin have been converted with the aid of microwave processing into useful products such as bio-fuel (bio-char, liquid fuels and syngas), chemicals (furans, phenols, sugars, etc.) and materials (activated carbons, charcoal, mesoporous polysaccharides, etc.). The advantages of using microwave technology in the processing of materials includes: the use of lower temperatures, mobility of the processors which can be located in the vicinity of the biomass, the flexibility in processing allowing for the production of liquid fuels, solid fuels and chemical products, the reduction of the carbon footprint as well as continuous processing and high energy efficiency.²⁴ An example of the successful application of this technology from bench scale to industrial scale is the production of pectin from citrus waste. The research involved the use of a low temperature hydrothermal microwave process which separated pectin from cellulose in the cell wall, without the aid of acid or any other additive. During this process pectin, D-limonene, flavonoids, sugars, furans and cellulose are obtained.²⁵ This technology is ideal for application in the extraction of chitin from waste material and in the conversion of chitin to chitosan.

As a result of the widespread issues related to the difference in properties observed for chitin and chitosan, the global standardisation of chitin and chitosan grades is necessary.^{20, 26} It would be beneficial if the supplier would provide these details in the case of commercially sourced chitosan or the researcher, in the case of chitin being directly isolated in the reported study. Many authors have found it difficult to compare experimental data to that cited in the literature, since the majority of studies do not state the source and properties of the polymers used. It is important to emphasise the full characterisation of the polymers that are used so that an accurate comparisons may be drawn. This issue of global standardisation was raised by Heppe Medical Chitosan GmbH (HMC⁺) who are front runners in the development and supply of medical

grade chitin and chitosan based materials. The company has stated that “A *standardized description and consistent measuring methods are especially important in the pharmaceutical and medical sectors where they are essential for their acceptance by regulatory authorities. This approval would ease the breakthrough of this magnificent polymer particularly in the pharmaceutical industry*” emphasising the importance of this issue. As a result of this call for standardisation ten parameters were proposed for the full characterisation of chitosan, these are:

- Identity - for example IR spectrum
- Appearance of powder
- Degree of deacetylation
- Viscosity
- Loss on drying/dry matter content
- Ash content
- Protein content
- Heavy metal content (Pb, Hg, Cd)
- Microbiological quality (total viable count, fungi)
- Endotoxin level.²⁷

The review by Verlee *et al.* discussed the barriers to the use of chitosan and chief among the issues was that related to the diversity of the chitin and chitosan used and the variability associated with their properties.²⁸ In addition, the definition and use of various terms such as oligo-chitosan, low, medium and high molecular weight chitosan.²⁸

The following section details the various methods available for the characterisation of chitin and chitosan, and the information that can be extracted from these analyses.

1.2 Characterisation of chitin and chitosan

As a result of the varying sources and properties of chitin and chitosan, it is difficult to find reproducible and consistent batch-to-batch analytical data.²⁹ Strict experimental control of reactions involving synthetic polymers produces consistent results, whereas with chitosan this is challenging due to the variation of polymer chains present in the starting material. This is one of the factors that limits the full application of chitin and chitosan in industry, where consistent results is a key issue. Therefore, when reporting on the activity of chitin or chitosan

and derivatives thereof, it is important that all aspects of the polymer be analysed and reported. Typically, these polymers are characterised using a variety of analytical and spectroscopic techniques which are described below. It is important that the DDA of the polymer being analysed is known as the properties displayed by the polymer such as solubility and reactivity, are directly affected. The most common techniques used to analyse chitin, chitosan and their derivatives have been listed below, together with a short description of their utility.

- Size exclusion chromatography (SEC) – used to determine the average molecular weight number (Mn) and the weight average molecular weight (Mw). These two values are then used to calculate the polydispersity index (Mw/Mn) of the polymer, which gives an indication of the molecular weight range.
- Nuclear Magnetic Resonance Spectroscopy (NMR) - ^1H , ^{13}C and ^{15}N -NMR are used for structural analysis of samples in solution, whereby solid samples can be analysed using solid state ^{13}C NMR. This is one of the most commonly used methods as it is quick, cost effective and accurate. Reports show that NMR analysis of chitin and chitosan is the best choice for use in DDA determination. In the case of chitin or chitosan which has been modified through the introduction of various functional groups, NMR spectroscopy can help indicate the degree of substitution of the polymers.^{30, 31} Sample analysis of chitin and chitosan using solid state ^{13}C NMR can be expensive in the case of insoluble samples; therefore, other methods are being developed for structural analysis of these samples. An emerging NMR technique, Time Domain Nuclear Magnetic Resonance (TD-NMR) has recently been used in the analysis of chitosan based samples. A recent publication used TD-NMR to study chitosan coagulation as a function of pH.³² A continuation of this study investigated the use of TD-NMR as an alternative method for the analysis of chitosan-paramagnetic ion interactions in solution and the effect of pH on the polymer.³³
- Viscometry – the viscosity of a polymer in a given solution can be used to calculate the molecular weight of said polymer. The equation used to relate the molecular weight of chitosan to its viscosity in a given solvent system is described by the Mark-Houwink-Sakurada (MHS) equation,

$$[\eta] = K(M_v)^a$$

Where $[\eta]$ is the intrinsic viscosity, M_v the viscosity-average molecular weight, K and a are constant for a given solute-solvent system.³⁴

- Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) – thermal techniques which have been used to describe the change in polymers upon modification. Chitin and chitosan which have been derivatised would potentially have a different thermal profile, attributed to the changes in the chemical structure of the polymers. These are useful techniques to assess the effect of any modification on the polymer.³¹
- Infrared spectroscopy (IR) – the easiest technique and most convenient method of chitin and chitosan analysis. It can be utilised as a qualitative and quantitative method in the determination of DDA. The DDA of chitosan can be determined by looking at the ratio of specific bands, where various authors have used different characteristic bands for this purpose for example absorbance bands previously used have been A1560/A2875, A1655/A2875 and A1655/A3450. The changes in structure can be observed for substitution reactions; however, peak broadening and overlapping of signals can hamper its diagnostic use.^{31, 35}
- Elemental Analysis (EA) – this technique allows for the determination of the elemental composition of chitin and chitosan derivatives. The C/N ratio obtained from this analysis can also be used to determine the degree of substitution of chitin and chitosan derivatives. However, destruction of the sample during analysis and inaccurate estimations due to varying molecular weights and DDA's as a result of the nature of the polymer, are limiting factors.³⁶
- X-ray diffraction (PXRD) – is used to determine the crystallinity of a polymer. PXRD can also be used to determine the effect of a modification on the polymer, e.g. metal loading. This analytical method is rarely used due to the high cost of the instrument.³⁷

The techniques discussed above are a fraction of the methods available for the full characterisation of these polymers. Other common techniques available for polymer analysis includes UV-Vis spectroscopy, mass spectrometry, matrix assisted laser desorption ionisation

(MALDI), gas chromatography mass spectrometry (GC-MS), time-of-flight secondary ion mass spectrometry (TOF-SIMS), high-performance liquid chromatography (HPLC), scanning electron microscopy (SEM), atomic force microscopy (AFM), isothermal titration calorimetry (ITC) and zeta potential.²⁰ With the development of improved analysis methods, more and more information is being contributed to the field of chitin and chitosan research. Once all the parameters of the starting material are known, it can potentially guide modification of the polymer to improve the inherent properties or introduce new properties *via* modification. In addition, with modification of the polymers new properties can be tested and different methods of analysis applied.

1.3 General Applications of Chitin and Chitosan derivatives

Chitin and chitosan have been used across all sectors ranging from biomedical applications to textiles and agriculture. The different areas of application of these versatile polymers are shown in Figure 1.6. Since chitin and chitosan are fully biodegradable in addition to being non-toxic, the utilisation of these polymers in various products will not have a negative effect on humans or the environment.⁸ A short review of specific applications is given below.

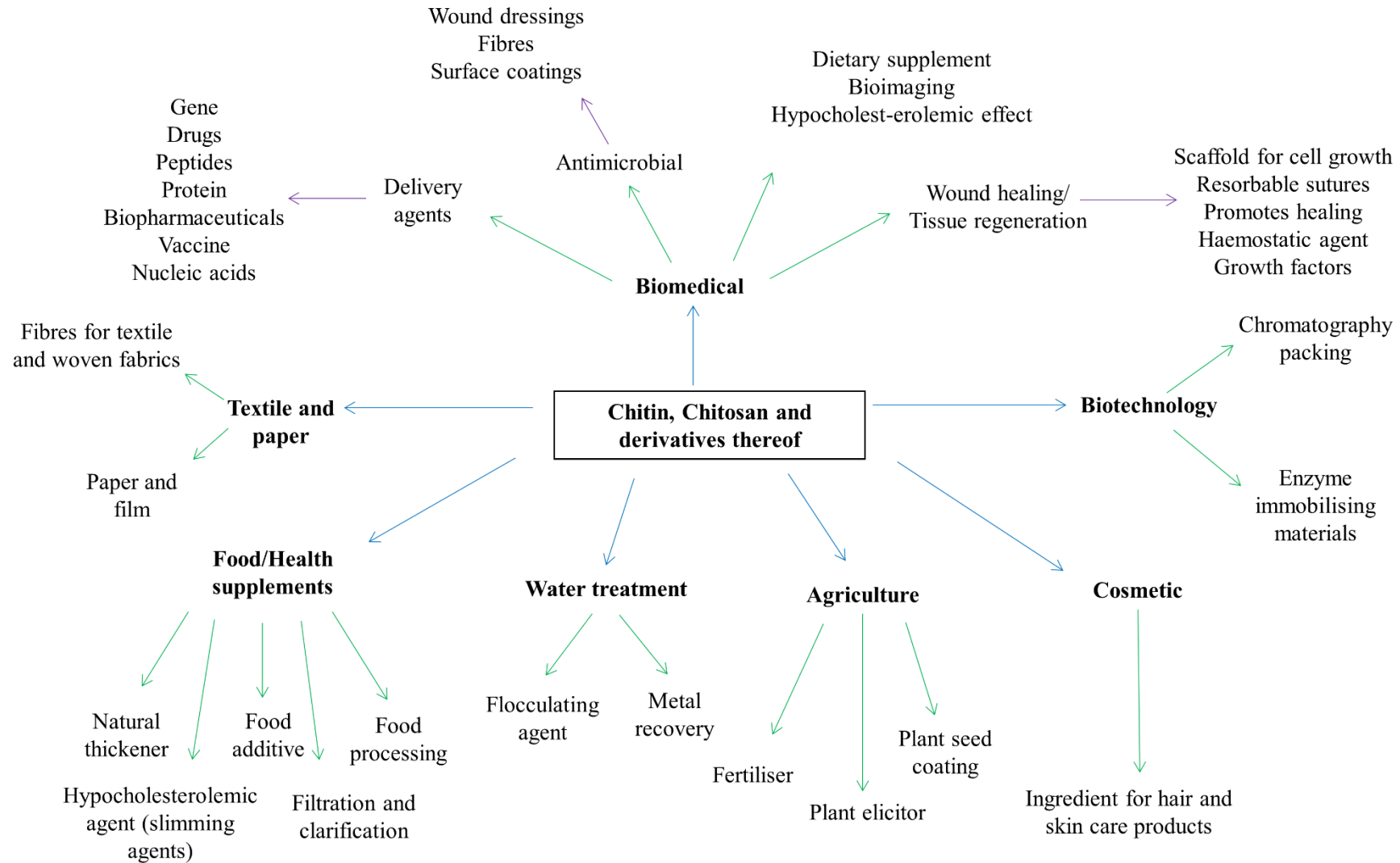


Figure 1.6: General applications of chitin, chitosan and derivatives thereof.

Agriculture

Chitin and chitosan have been extensively used in the agriculture sector. The polymers may be applied as a part of soil enrichment, foliar spraying, seed coating to stimulate germination, supplements in hydroponics, as an antimicrobial and antifungal agent or as a supplement in plant tissue culture medium. Furthermore, studies have shown that chitosan and chitosan oligomers improve the mineral nutrient uptake of plants, which in turn leads to a greater crop yield. The majority of these applications require a water soluble polymer which would make it highly desirable by the agriculture industry.³⁸

General Industry/water purification

In industry, chitin and chitosan have been used in the water treatment sector as a flocculating agent or as a chelator of metal ions to treat polluted water. A recent study described the use of quaternised chitosan derivatives (Figure 1.7) in both treatment and chelation. Whereby the derivatives assisted in the removal of perchlorate from water, where the polymer functioned as an anion exchange column.³⁹ In addition, the polymers were loaded with iron nanoparticles to aid remediation of the water samples showing promising results which compared favourably to an industry standard.³⁹ Chitosan and its derivatives have also been reported to remove certain pesticides, organic matter as well as dyes present in textile water waste. Nechita *et al.* recently published an in depth review on the application of chitosan in the treatment of waste water.⁴⁰

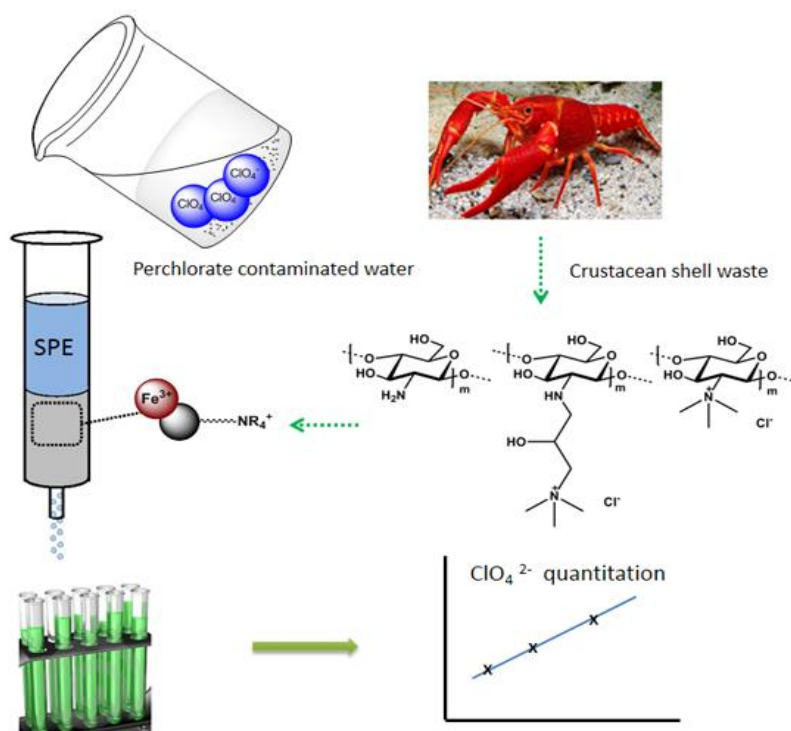


Figure 1.7: The perchlorate ion exchange resin reported.³⁹

Food and Health

These polymers have also been used in the food and health supplement sectors as additives to thicken and stabilise food such as emulsions (e.g. salad dressings), to filter and clarify liquids and as hypocholesterolemic agents (slimming agents). In addition, chitin, chitosan and their derivatives have been used in the production of edible biodegradable films, which can potentially extend the shelf life of produce while acting as an antioxidative, antimicrobial and antifungal agent. Fruits and edible items which have been coated with these polymers or derivatives thereof includes, berries, oranges, fish, meat, etc. The majority of polymers tested, have also shown antimicrobial activity against the common food pathogens responsible for food spoilage. Chitosan based oligomers have also been used as prebiotics (stimulates growth of beneficial bacteria in the gut) and as calcium absorption enhancers.⁴¹

Textile

The textile industry has used these polymers in the production of fibres, paper and film. Islam *et al.* reviewed environmentally friendly or ‘green’ approaches to develop antimicrobial textiles based on sustainable biopolymers.⁴² This review noted that chitosan and its derivatives have been used in the finishing of wool, cotton, cellulose and polyester; however, these polymers do not bind well to the textile fibres. To avoid this issue, methods such as cross-linking were employed. Chitosan-metal complexes have also been used to impart multifunctional properties onto treated fabrics such as antimicrobial properties.⁴²

Cosmetic Industry

The cosmetic industry utilises chitin, chitosan and their derivatives; in hair, oral and skin care. In hair care, the positively charged chitosan is attracted to the negatively charged hair forming a surface coating, which acts as an antistatic agent and a conditioner. In skin care, chitosan helps in keeping the skin hydrated and in the treatment of acne. In oral health care, these polymers have been used as dental fillers and to prevent plaque formation. Typical examples of cosmetic products which contain these polymers includes selected creams, lotions, toothpastes, chewing gum (for dental care), make-up (foundation, eye shadow, lipstick), etc.⁴³ Carboxymethyl chitosan is a water soluble derivative of chitosan that is stable over a broad pH range and is used in moisture absorption–retention, for its antimicrobial properties, its antioxidant activity and as an emulsion stabiliser.⁴⁴ Quaternary derivatives of chitin and chitosan have also found extensive application in the cosmetic sector due to the positively charged nature of the polymers. Huang *et al.* capitalised on the improved properties of carboxymethyl chitosan by incorporating a quaternary ammonium group onto the polymer

backbone and incorporating silver nanoparticles.⁴⁵ This study found that the silver particles were formed with the polymer acting as a chemical reducer and stabiliser. The silver loaded polymer displayed enhanced antibacterial activity compared to carboxymethyl chitosan and silver nanoparticles.⁴⁵

Biotechnology Industry

The biotechnology industry has produced separation membranes and ion-exchange media based on chitin and chitosan. An example of this, was reported by Jardine *et al.*, who produced a variety of modified chitosan derivatives, which were used as chromatographic resins in the separation of saturated and unsaturated fatty acids.⁴⁶ There have also been recent reports on the production of biodegradable plastic based on chitosan. The study by Fernandez *et al.* on the development of a scalable manufacturing strategy for the fabrication of three-dimensional (3D) objects based on chitosan showed that the production of bio-based plastics is feasible.⁴⁷ The bioplastics produced were fully biodegradable, making this process a promising alternative to the use of conventional plastics which accounts for a large part of the current pollution problem.⁴⁷

Biomedical Sector

Chitin and chitosan have attracted a considerable amount of attention especially in the biomedical sector due to their favourable properties and classification as a GRAS material. The book edited by Sarmiento *et al.* provides a good overview of the biomedical applications of these polymers.⁴⁸ The topics addressed includes the general aspects of chitosan such as the biocompatibility and biodegradation of chitosan and derivatives thereof, the formulation of biopharmaceuticals and delivery thereof using chitosan and the advanced application of chitosan and its derivatives as biopharmaceuticals themselves.⁴⁸ As a result of the beneficial properties of these polymers, commercialisation of certain products based on chitosan has been reported. The uses of these products range from antibacterial fibres to bandages and dietary supplements. Due to this interest in commercialising the applications of chitosan there has been a significant number of patents filed relating to the use of chitin, chitosan and their derivatives in the biomedical field.²⁰

The majority of these applications involve soluble polymers, which necessitates the modification of chitin and chitosan to enhance solubility. Chitin and chitosan can be modified due to the presence of reactive functional groups which opens up a whole new range of properties for various applications.

1.4 Selective Modifications and Noteworthy Derivatives

As previously mentioned, chitin and chitosan have strong intra- and intermolecular hydrogen bonds, making dissolution of these polymers quite difficult in organic or aqueous solvents. This poor solubility has been one of the key factors restricting the use of these biopolymers. The most common method of increasing the polymer solubility lies in the derivatisation of chitin and chitosan by attaching hydrophilic or hydrophobic groups to the polymer backbone.⁴⁹ Modification of chitin and chitosan is possible due to the presence of reactive hydroxyl groups (C-3 and C-6) and an amino group at the C-2 position (chitosan only) of the glucosamine residue (Figure 1.8). The modification of the polymers should not affect the structural backbone to the point where it cannot be classified as either chitin or chitosan. The reactivity of chitosan is affected to a great degree by pH which dictates its charged state and properties. At a lower pH chitosan is protonated and thus positively charged making the polymer partially water soluble. Whereas, at a neutral to high pH chitosan is insoluble in water and most solvents. The soluble-insoluble transition of chitosan occurs at a pH of $\sim 6.0 - 6.5$, which makes the application of this biopolymer in biomedical applications quite appealing.⁴⁹ In addition, the intended application may dictate the site at which the polymer is modified. An example of this, is in gene transfection where the presence of the primary amine is advantageous.¹³

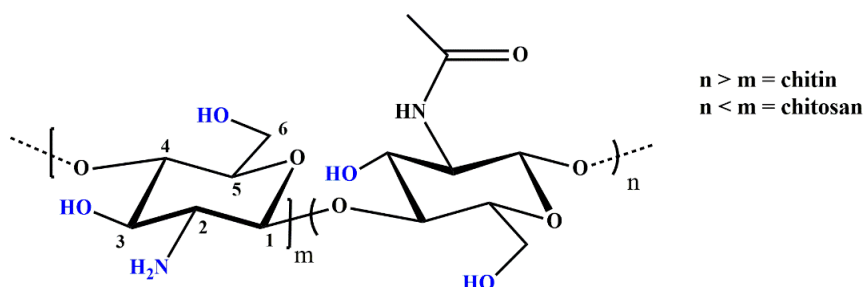


Figure 1.8: The functional groups open to modification on chitin and chitosan shown in blue.

Due to the versatility of chitin and chitosan, and the presence of reactive functional groups, modifications through different chemical reactions has been employed. This includes and is not limited to acylation, alkylation, carboxyalkylation, hydrolysis to oligomers, hydroxyalkylation, phosphorylation, quaternisation, sulfation, Schiff base formation, thiolation, and through grafting of sugars, cyclodextrins and other molecules. The literature dealing with the modification of chitin and chitosan is extensive, where more applications are being published as the development of technology progresses.^{20, 50}

Modifications to chitin and chitosan have been shown to have chemical, biological and functional advantages when compared to the native polymers. These enhanced properties have

been extensively reviewed by numerous authors, one review that stands out is that by Ravi and Muzzarelli *et al.* who are leaders in the field of chitin and chitosan research.¹⁶ Properties which are most commonly altered includes, solubility, charge, gelation ability, mucoadhesivity, affinity for metal ions, biocompatibility, etc.¹⁶ Selected examples of common transformations are shown in Figure 1.9.²⁰

Depending on the reagents and reaction conditions there are three types of derivatives possible these are *N*-modified chitosan, *O*-modified chitosan or *N,O*-modified chitosan. Typically the amino group reacts first due to the higher nucleophilicity compared to the hydroxyl; however, both groups react with acids, alkyl halides or iso(thio)cyanides resulting in nonselective *N,O*-substituted chitosan derivatives. Selectively substituted derivatives can be obtained through manipulation of reaction conditions. Substitution at the hydroxyl can be obtained through the use of acids as solvents or additives due to protonation of the amine although degradation of the polymer may be observed. Other methods employ the use of protecting groups to selectively modify the polymer.²⁸

Selected derivatives of chitin and chitosan containing desirable attributes which have been applied in a variety of applications are discussed below.

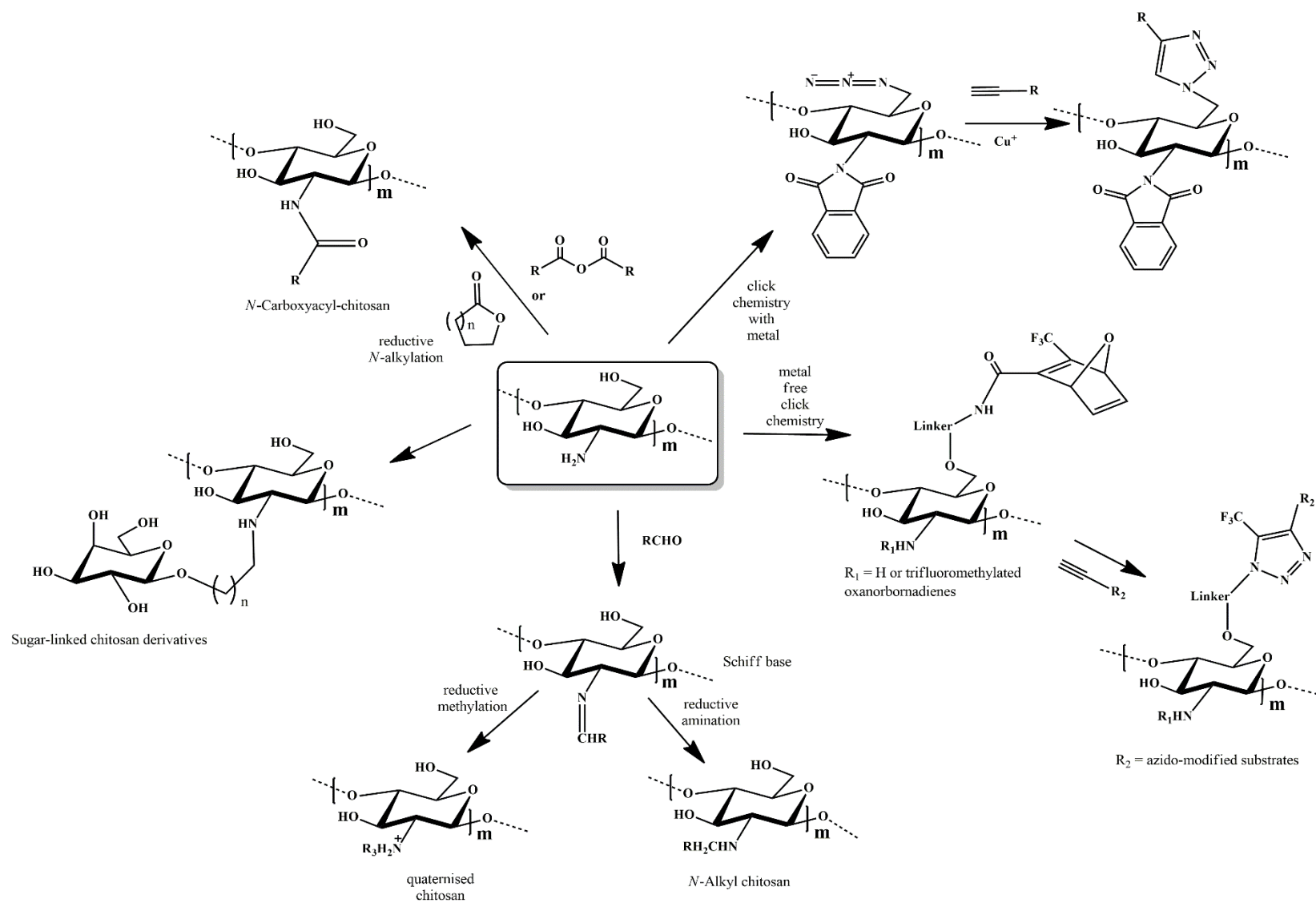


Figure 1.9: Selected chemical modifications which have been carried out on chitin and chitosan.²⁰

Alkylation

Alkylation is one of the routine methods used in the modification of chitin and chitosan. *N*-alkylation and carboxyalkylation have produced some interesting chitin and chitosan derivatives. A particularly useful set of polymers which were synthesised by reductive alkylation or direct alkylation; is carboxymethyl chitin and carboxymethyl chitosan (CMC). There are four different derivatives, these are: *N*-carboxymethyl chitosan (NCMC) (**1.3**, Figure 1.10), *O*-carboxymethyl chitosan (OCMC) (**1.4**, Figure 1.10), *N,O*-carboxymethyl chitosan (NOCMC) (**1.5**, Figure 1.10) and *N,N*-carboxymethyl chitosan (**1.6**, Figure 1.10).⁵¹

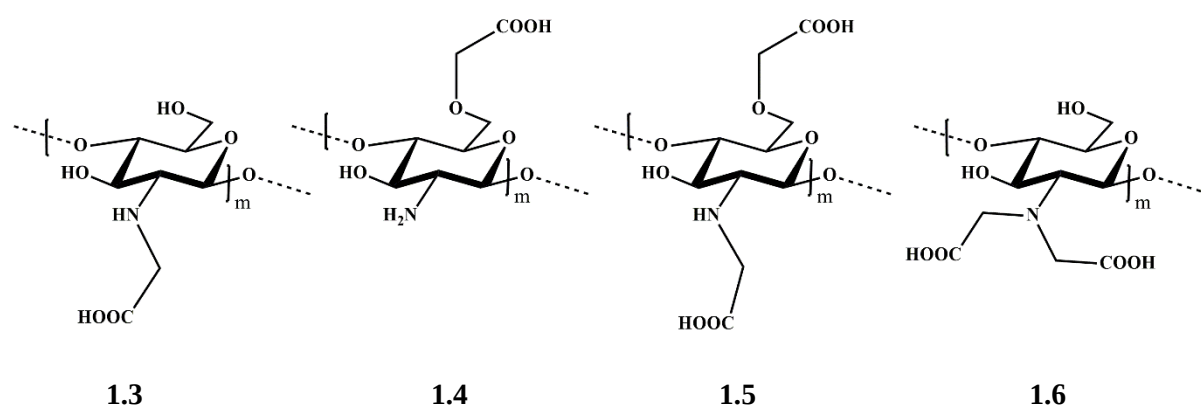


Figure 1.10: *N*-carboxymethyl chitosan (**1.3**), *O*-carboxymethyl chitosan (**1.4**), *N,O*-carboxymethyl chitosan (**1.5**) and *N,N*-carboxymethyl chitosan (**1.6**).

To selectively obtain *O*-carboxymethyl chitosan, chitosan is reacted with monochloroacetic acid in the presence of sodium hydroxide at room temperature or lower, typically in an isopropanol/water suspension. When the reaction is carried out at a higher temperature, *N*-carboxymethyl chitosan and *N,O*-carboxymethyl chitosan are obtained. Alternatively *N*-carboxymethyl chitosan is prepared by reacting chitosan with glyoxylic acid followed by reduction with sodium cyanoborohydride; however, this reaction also produces *N,N*-carboxymethyl chitosan.⁵² Therefore, selectively producing polymers with fine-tuned properties is possible by utilising different methods or reagents.

These polymers are reported to be water soluble and display low toxicity. Carboxymethylation based on chitin and chitosan has been applied in metal ion capture, drug delivery, as an antimicrobial, in wound healing, gene delivery, as a permeation enhancer, in cosmetics, as a cotton fabric finish, in cell imaging and as a food additive. In addition, nanoparticles based on these polymers have been prepared by ionic gelation. The use of CMC based nanoparticles was reviewed in depth by Narayanan *et al.* who noted that the addition of acetate groups onto chitin and chitosan gave the polymers “brilliant biological properties”.⁵¹ CMC has also been

fabricated into hydrogels, films and fibres. The improved properties which these polymers display compared to the parent polymers, can be attributed to their zwitterionic character. CMC based polymers have also been further modified by acylation, alkylation and through grafting. Mourya *et al.* reviewed CMC extensively where the authors investigated the synthesis, characterisation, physicochemical properties, biological properties, applications, further modifications, analogous derivatives and the commercial exploitation of these chitin and chitosan based derivatives.⁵³ More recently, Verlee and co-workers studied the developments in antibacterial and antifungal chitosan and its derivatives, where a number of derivatives of CMC were discussed with regard to their improved properties.²⁸

Quaternisation

A quaternary derivative of chitosan, *N,N,N*-trimethyl chitosan chloride (TMC) (**1.7**, Figure 1.11) has emerged as one of the most applied derivatives of chitosan. Properties of TMC which are improved compared to chitosan includes, solubility, antimicrobial activity, antifungal activity and mucoadhesivity.⁵⁴

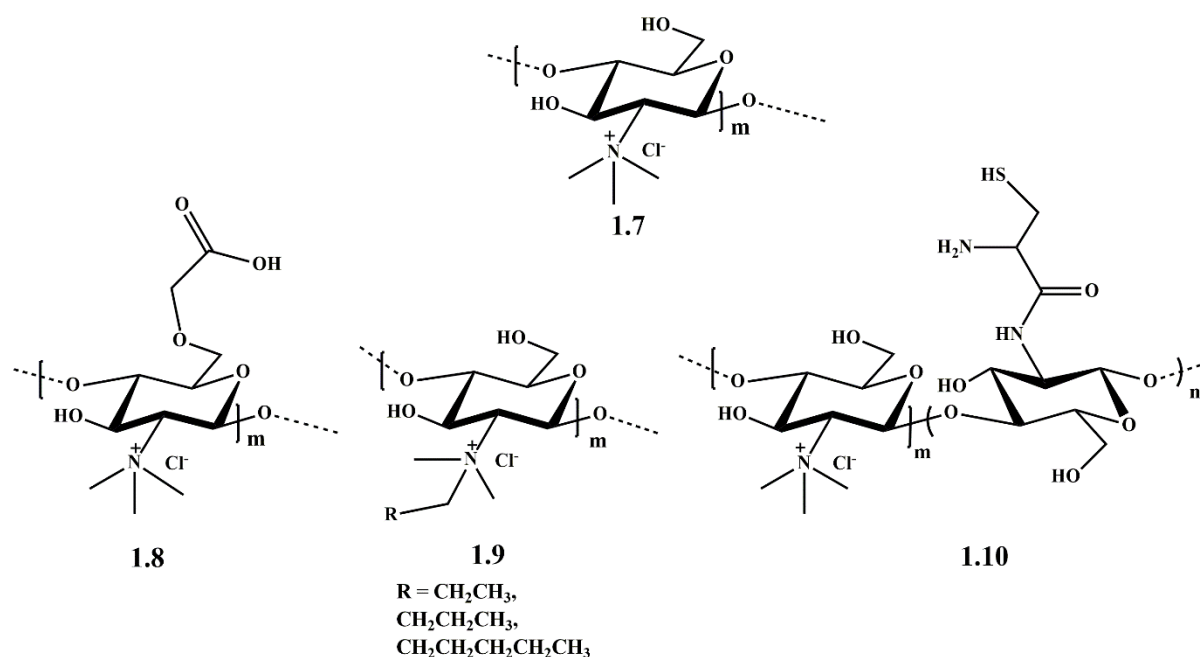


Figure 1.11: TMC (**1.7**), *O*-carboxymethyl-*N,N,N*-trimethyl chitosan (**1.8**), *N*-alkyl-*N,N*-dimethyl chitosan derivatives (**1.9**) and *N,N,N*-trimethyl chitosan-cysteine conjugate (**1.10**).

TMC has a permanent positive charge due to quaternisation of the amine moiety and the polymer is soluble over a wide pH range (acidic, neutral and basic). This polymer is typically synthesised by reacting chitosan with methyl iodide in the presence of a base and is performed in two cycles in order to obtain a highly substituted polymer. One drawback associated with

this method, is the methylation which occurs at C-6 in addition to C-2. Other methods of synthesis includes protection of C-6 and subsequent methylation at C-2. A third method involves synthesising *N,N*-dimethyl chitosan in a formic acid-formaldehyde solution, and thereafter quaternising the polymer with methyl iodide in the absence of a base.⁵⁴ Rúnarsson *et al.* also reported on the one-pot synthesis of highly substituted TMC; however, this method was time consuming and required the addition of more reagents over time to increase the degree of quaternisation (DQ).⁵⁵ This polymer can also be produced by reacting chitosan with dimethylsulfate; however, a much lower DQ is observed compared to the methyl iodide reaction with additional toxicity issues associated with dimethylsulfate to consider. A recent study reported on the synthesis of TMC using dimethyl carbonate. Chitosan was trimethylated by dimethyl carbonate using an ionic liquid, 1-butyl-3-methylimidazolium chloride as a solvent and catalyst. Results indicated that no *O*-methylation occurred and a higher DQ can be obtained by increasing the concentration of dimethyl carbonate used.⁵⁶

The key area of application of this polymer lies in the delivery of actives where TMC has been applied for oral, nasal, pulmonary, buccal, ocular and intestinal applications as well as in gene delivery. In addition, this polymer can be formulated in many different ways including microparticles, nanoparticles, films, polyelectrolyte complexes, hydrogels, beads, etc. It has been reported that TMC is active against both Gram-positive and Gram-negative bacteria where it was shown that the counterion-ion present affects the activity of the polymer. Facchi *et al.* recently looked at the current aspects and technological applications of TMC highlighting the importance of this polymer as a versatile biomaterial with a variety of applications.⁵⁴ TMC has also been modified (**1.8 – 1.10**, Figure 1.11) in an attempt to improve the properties of the polymer. A study by Yin *et al.* reported the synthesis of a thiolated trimethyl chitosan as nanoparticles, which displayed superior transport of insulin through rat intestines compared to the parent polymers due to the increase in the mucoadhesion and permeation enhancing effect of this new polymer.⁵⁷ Mourya *et al.* and a later review by Verlee *et al.* covered the synthesis and major applications of this polymer as well as derivatives thereof.^{28, 58}

Oligosaccharides

Oligosaccharides (carbohydrates whose molecules are composed of a relatively small number of monosaccharide units) based on chitin and chitosan (Figure 1.12) have been well researched and are generally referred to as chitooligosaccharides (COS). Generally, oligosaccharides have different degrees of polymerisation and acetylation giving them altered properties compared to the parent polymers. However, there is some debate over the classification of monomers where

some studies use the term low molecular weight chitosan and chitosan oligomers interchangeably. Oligomers are generally defined as chains consisting of two to ten monomers; however, this rule is not often followed by manufacturers when supplying information on chitosan oligomers. The review by Verlee *et al.* noted this discrepancy and in an attempt to avoid incorrect classification referred to all chitosan with a weight less than 16 kDa as oligomers.²⁸ This weight corresponds to roughly 100 monomeric units of 100% deacetylated chitosan.²⁸

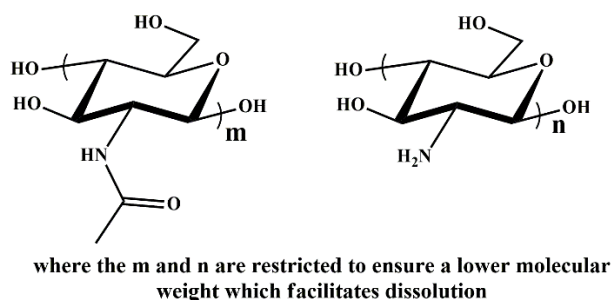


Figure 1.12: COS based on chitin and chitosan.

Chitoooligosaccharides are typically water soluble and have a lower viscosity compared to chitin and chitosan making them attractive research materials. COS can be produced through a variety of methods including physical (hydrothermal conditions, microwave irradiation, ultrasonication, γ -rays), chemical (acid treatment, H_2O_2 oxidation) and enzymatic (chitinase, chitosanase, lysozyme, cellulase, pectinase, protease, lipase, pepsin) treatment.⁵⁹ Chemical hydrolysis is the most common method to produce COS; however, due to the hazardous waste produced and the low yields, it is not an ideal method. Enzymatic COS production is preferred; however, the cost of the enzymes makes the large scale synthesis of COS limited. It is also difficult to control the outcome of these reactions. Properties of COS which have been studied includes their antioxidant, antimicrobial, antifungal, anticancer, and immunostimulant properties. The activity of the COS was found to be dependent on the molecular weight, DP, the DDA and the charge distribution. From the literature, it can be noted that COS has been researched extensively in the biomedical sector; although there are other sectors such as the food sector which have recognised the potential of these oligomers. The development of edible films and coatings based on COS which are designed to preserve fruit and enhance its shelf life has been a subject of major interest. COS may be further modified to improve their inherent activities, therefore allowing for a broader range of applications as is the case with chitin and chitosan.⁶⁰

As mentioned previously, the poor aqueous solubility of natural chitin and more so, chitosan necessitates the modification of these polymers to produce water soluble derivatives. Numerous strategies have been proposed for conjugating polar groups to the 6-hydroxy and/or the 2-amino functional groups of chitosan. The substitution of the 6-hydroxy group with a functional group that could provide additional useful properties would be advantageous. However, it has been shown that attempts to directly substitute at C-3 and C-6 have been unsuccessful or non-specific. This substitution has been explored by a number of researchers, where the most common route involves a step wise approach to the synthesis of the desired polymer. The first step in the substitution of the C-3 or C-6 position in chitosan, involves the protection of the amine functionality at C-2 with the most common precursor being *N*-phthaloyl chitosan. The frequent use of this precursor for further modification, can be attributed to its solubility in polar aprotic solvents and the simple deprotection using hydrazine hydrate to regenerate the amine.⁶¹ Thereafter, modification of C-6 by various groups is possible. Modifications which have been reported includes 6-*O*-tritylation, 6-*O*-tosylation, and 6-*O*-trimethylsilylation. The 6-hydroxy group has also been converted to halogenated intermediates (Br, Cl and I), which are then used for subsequent modification of the polymer.⁶²

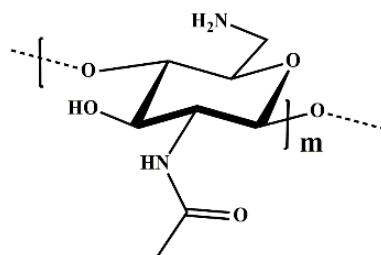
One such modification of chitosan which has been receiving increased interest in recent times, is the introduction of an amine functionality at C-6. The advantage that the modification holds is that a sparingly soluble polymer now becomes completely soluble in aqueous media. Solubility in aqueous media broadens the scope of applications of the polymer. In addition, the amine group on chitosan is protonated when in solution. This in turn leads to a strong cationic character as a result of the original and introduced amino groups.⁶³ The diamino derivatives of chitin and chitosan forms the basis of the current research.

1.5 The introduction of an amine at C-6 of the biopolymer backbone

Below is a short overview on the introduction of an additional amine functionality at position C-6 on the polymer backbone, with specific focus on the diamino derivatives of chitin and chitosan.

Furuhata *et al.* had published a study on the synthesis and reduction of azidodeoxy derivatives of chitin.⁶⁴ Halogenated chitin (Br and Cl) intermediates were reacted homogenously with sodium azide at various temperatures and over different time periods. Reaction kinetics were studied to find the optimum reaction conditions to form the azido derivatives. These derivatives

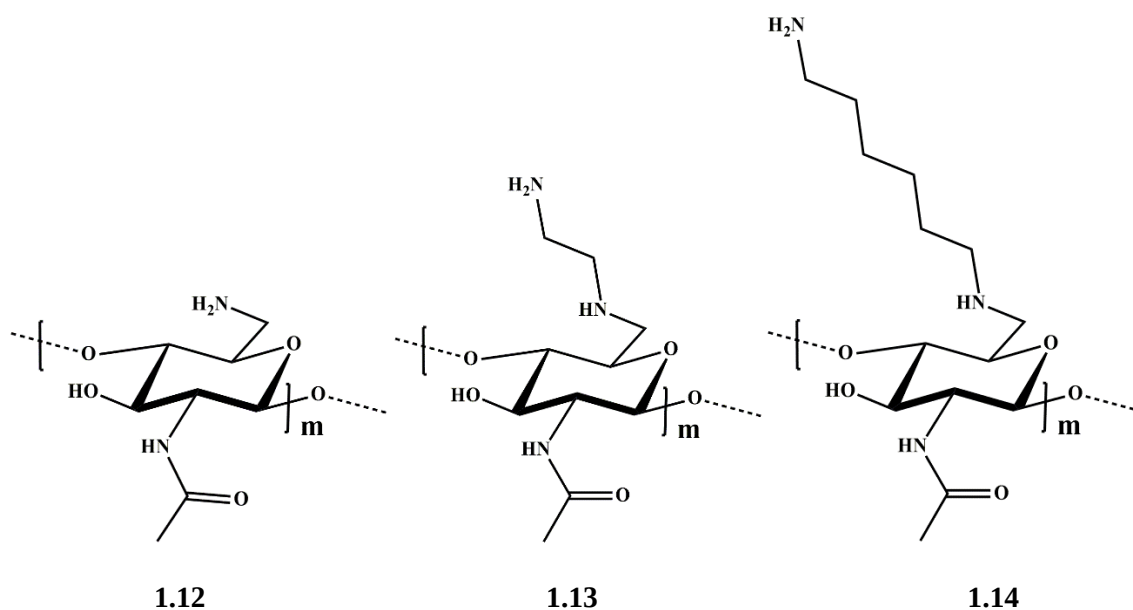
were subsequently reduced using sodium borohydride to yield 6-deoxy-6-amino chitin (**1.11**, Figure 1.13).⁶⁴



1.11

Figure 1.13: 6-Deoxy-6-amino chitin (**1.11**).

A later study reported on the synthesis of amino(alkylamino)deoxychitins (**1.12** – **1.14**, Figure 1.14). Tosylchitin was reacted with ammonia, ethylenediamine and hexamethylenediamine, respectively. The synthesised polymers were tested for their adsorption abilities for acidic dyes and the Cu^{2+} ion. The study showed that the aminodeoxychitins adsorbed close to 10 times the amount of Orange 7 (a representative dye) compared to chitin and that the Cu^{2+} adsorption was comparable to that shown by crosslinked chitosan. The pH of the solution was also found to affect the activity of the polymers where a lower pH led to a higher adsorption value. This study also determined the amine content at C-2 and C-6 by measuring the saturation values of the dyes tested.⁶⁵



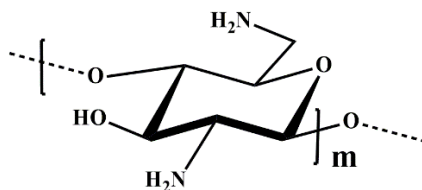
1.12

1.13

1.14

Figure 1.14: Amino(alkylamino)deoxychitins synthesised by the reaction of chitin with ammonia (**1.12**), ethylenediamine (**1.13**) and hexamethylenediamine (**1.14**).

Satoh *et al.* reported the preparation of 6-deoxy-6-amino chitosan, which was used as a gene carrier (**1.15**, Figure 1.15).⁶³



1.15

Figure 1.15: The structure of 6-deoxy-6-amino chitosan (**1.15**).

This method involved the synthesis of a 2-amino protected form of the polymer as *N*-phthaloyl-chitosan. Having protected the amino group, the 6-hydroxy group could selectively be converted to the 6-bromo, 6-chloro or 6-iodomethylene polymer derivative. Substitution of the halogen with an azido group, followed by reduction thereof, gave the protected form of the polymer. Deprotection yielded the water soluble 6-deoxy-6-amino chitosan. This is advantageous as the protonated chitosan with its positive charge is able to interact with negatively charged sites on all membranes and tight junctions. As a result, this polymer was used as a gene carrier and the transfection efficiency and cytotoxicity in COS-1 cells compared to that of chitosan. The transfection efficiency was found to be higher while the cytotoxicity was comparable to chitosan. The polymer was later modified with galactose to enhance cell specificity through receptor mediated recognition, and to improve stability of the polymer as compared to peptides and proteins.⁶⁶

Introduction of an additional amine functionality allows for the further modification of the polymer, which can potentially enhance favourable properties such as solubility and biological activity. Sadeghi and colleagues synthesised the trimethylated (**1.16**, Figure 1.16) and triethylated versions of 6-deoxy-6-amino chitosan (**1.17**, Figure 1.16).⁶⁷ The diamino polymers were synthesised *via* a tosylated intermediate which was reacted with ammonia under pressure to introduce the additional amine functionality. These polymers were evaluated against *Staphylococcus aureus* (*S.aureus*) and reportedly displayed higher activity compared to chitosan as well as the trimethylated and triethylated chitosan derivatives. The trimethylated diamino polymer displayed the highest antibacterial activity as well as the highest positive charge as determined by zeta potential analysis. It was suggested that the high positive charge allowed the polymer to bind to a greater degree to the negatively-charged bacterial cell wall. The lower activity displayed by the triethylated version, may possibly be due to steric hindrance

caused by the bulky ethyl group. These polymers show great potential as drug delivery agents with an added antibacterial effect.⁶⁷

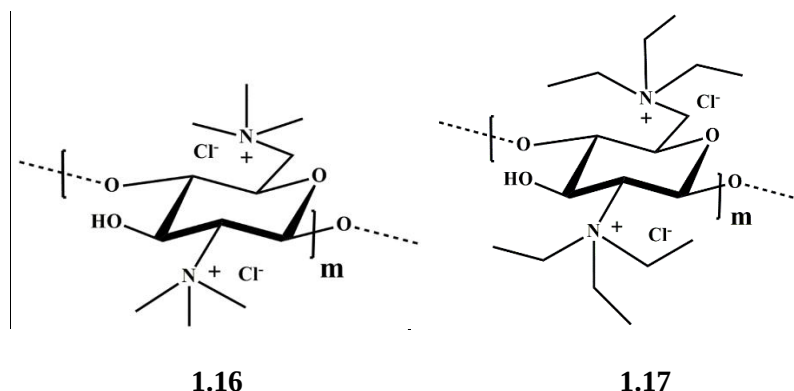
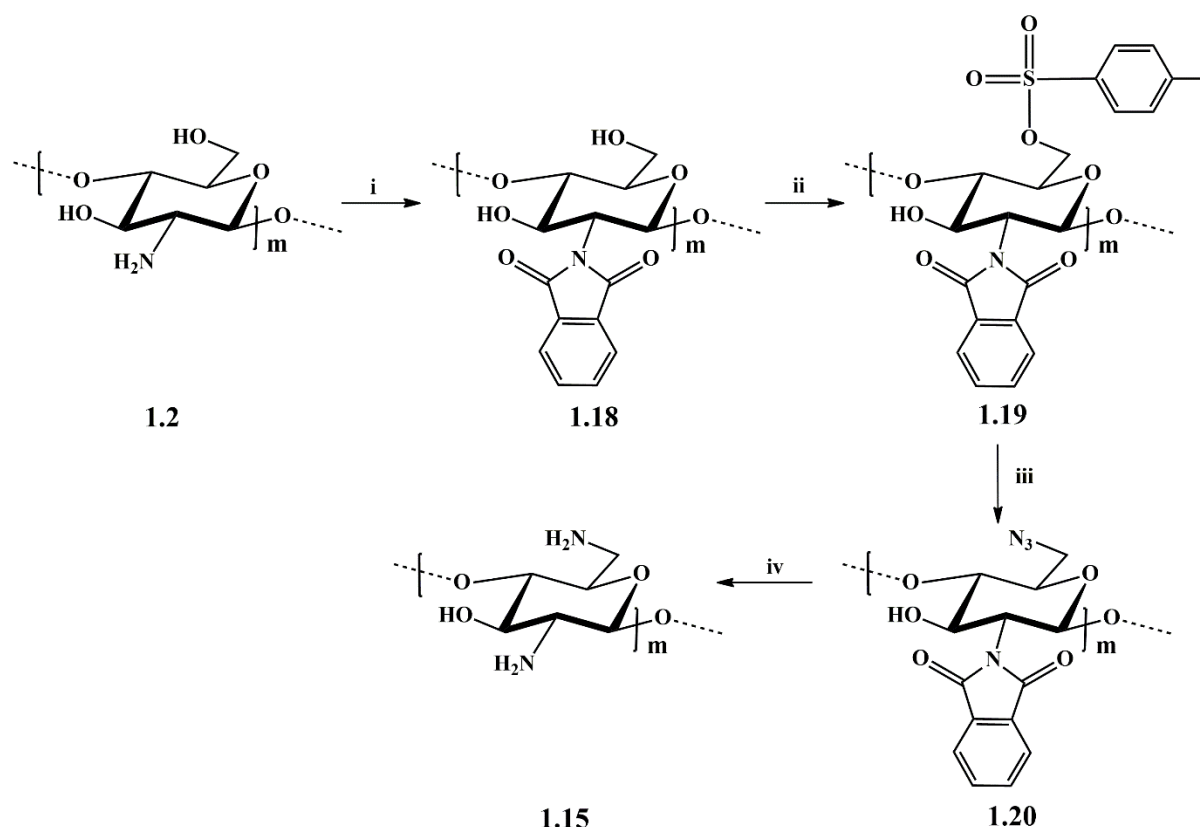


Figure 1.16: The trimethylated (**1.16**) and triethylated (**1.17**) derivatives of 6-deoxy-6-amino chitosan.

A synthetic method which avoided the use of halogenated intermediates was later reported by Jardine *et al.*⁶⁸ The use of halogenated intermediates are not favourable especially in terms of large scale synthesis and the production of large quantities of hazardous waste. The synthesis of 6-deoxy-6-amino chitosan, was accomplished in four steps. The pathway involved the synthesis of *N*-phthaloyl chitosan (**1.18**, Scheme 1.1), which in turn was converted to 6-deoxy-6-*p*-toluoyl-*N*-phthaloyl chitosan (**1.19**, Scheme 1.1). The OTs substituent was displaced to form 6-deoxy-6-azido-*N*-phthaloyl chitosan (**1.20**, Scheme 1.1) which was finally reduced to 6-deoxy-6-amino chitosan (**1.15**, Scheme 1.1) using triphenylphosphine and hydrazine hydrate.⁶⁸



Scheme 1.1: Synthesis of 6-deoxy-6-amino chitosan from chitosan reported by Jardine *et al.* Reagents and conditions: (i) phthalic anhydride, 5 % DMF/H₂O, 120 °C, 8 hr, 74 %; (ii) pyridine, 0 °C, *p*-toluenesulfonyl chloride, 17 hrs, RT, 112 %; (iii) NMP, NaN₃, 80 °C, 4 hr, 66 %; (iv) NMP, TPP, 15 hr, RT; H₂NNH₂·H₂O, H₂O, 100 °C, 4hr, 218 %.

The polymer was then further functionalised with iminopyridyl and iminophosphine moieties and subsequently complexed with rhodium or palladium (Figure 1.17). The metal immobilised polymer was used as a catalyst support for Suzuki-Miyaura and Heck cross-coupling reactions. The study found that the catalysts showed high activity for both reactions in organic and aqueous media and could be reused up to five times without significant loss of activity.^{68, 69}

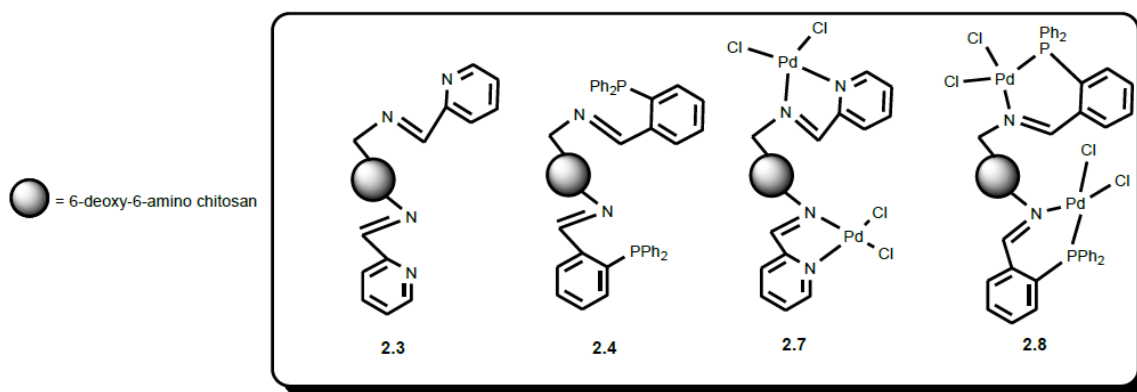


Figure 1.17: Iminopyridyl and iminophosphine derivatives of 6-deoxy-6-amino chitosan.^{68, 69}

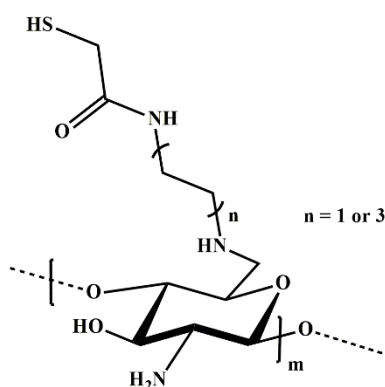
In a later study, Yang *et al.* looked at the antimicrobial activity of 6-deoxy-6-amino chitosan on a wider range of bacteria.⁷⁰ The antimicrobial efficacy of the polymer on *S.aureus*, *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Aspergillus niger* (*A. niger*) was determined and it was found that the diamino derivative displayed superior activity relative to chitosan with lower minimum inhibitory concentration (MIC) values. In addition, the polymer was found to be active against bacteria and fungus at an acidic and neutral pH. In an extension of the antimicrobial study, 6-deoxy-6-amino chitosan was dissolved in distilled water and the activity of the polymer was investigated against the bacteria and fungus. MIC values were similar to those reported for the acidic and neutral media, indicating that 6-deoxy-6-amino chitosan has great potential as an antimicrobial and antifungal agent.⁷⁰

Singh *et al.* synthesised 6-deoxy-6-amino chitosan using a combination of the methods reported by Satoh, Sadeghi and Kurita *et al.*⁷¹ This polymer was modified, through graft copolymerisation of acrylic acid to form a novel hydrogel. The graft reaction was initiated in an aqueous medium by a combination of hydrogen peroxide and ascorbic acid. These reaction conditions minimised the degradation of the chitosan backbone and the grafted polymer was reportedly more stable compared to chitosan and 6-deoxy-6-amino chitosan as determined by thermal analysis. The grafted polymer was found to absorb the most water at a neutral pH and could hold up to 140 times its own weight. The porosity of the polymer is thought to be responsible for its water absorbing ability, therefore this hydrogel can potentially be applied in a variety of biomedical applications.⁷¹

Kim and colleagues synthesised an oligomer of 6-deoxy-6-amino chitosan which was produced with the intent of applying them as carbohydrate solid supports; for the immobilisation of protein and epidermal growth factor conjugates.⁷² The oligomers produced had a molecular weight between 3 and 10 kDa and were conjugated to epidermal growth factor using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as activator. The conjugates were tested for cytotoxicity and its effects on cell proliferation, where no detrimental effects due to the polymer was observed.⁷²

These results have generated increased interest in the introduction of an additional amine at the C-6 position of chitin or chitosan. Derivatives with varying amine moieties have also been evaluated for their improved properties. The amino groups have a higher nucleophilicity compared to the hydroxyl group, thus providing another site for further selective functionalisation of the polymer.

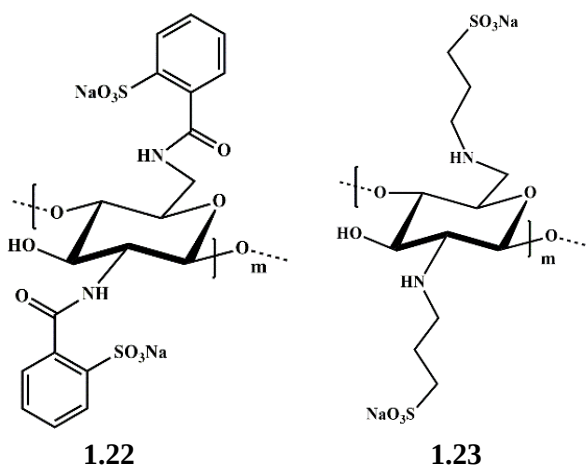
In an extension of the study on chitin, Shimizu *et al.* synthesised amino(alkylamino)deoxychitosans.⁷³ The polymers were used as acid dye adsorbents and later modified by the introduction of a thiol moiety (**1.21**, Figure 1.18). The thiolated polymers were tested for their ability to bind Hg^{2+} ions. The study reported that the polymers displayed superior adsorption properties for both the dyes and the Hg^{2+} ions, making them attractive as solid phase extraction materials.⁷³



1.21

Figure 1.18: Thiol functionalised amino(alkylamino)deoxychitosans (**1.21**).

Sulfonated diamino derivatives, 6-deoxy-2,6-bis[sodium sulfobenzamido] chitosan (**1.22**, Figure 1.19) and 6-deoxy-2,6-bis[sodium sulfopropyl] chitosan (**1.23**, Figure 1.19) were synthesised by Manuel *et al.*^{46, 74} These polymers were loaded with Ag^+ ions and applied in the separation of saturated and unsaturated fatty acid methyl esters. The retention capabilities of these diamino polymers were superior to that of the chitosan derivatives and comparable to the synthetic industry benchmark tested.⁷⁴



1.22

1.23

Figure 1.19: The structure of 6-deoxy-2,6-bis[sodium sulfobenzamido] chitosan (**1.22**) and 6-deoxy-2,6-bis[sodium sulfopropyl] chitosan (**1.23**).

A novel aminated polymer was obtained when *p*-benzoquinone was conjugated to the C-6 position of chitin, followed by reaction with ethylenediamine. Deacetylation produced the aminated chitosan derivative (**1.24**, Figure 1.20). Various effects such as pH, temperature and reaction time were altered to produce a highly substituted polymer. The synthesised polymer was evaluated for its antimicrobial activity against *E. coli*, *Bacillus cereus* (*B. cereus*), *P. aeruginosa*, and *S. aureus*. The study concluded that the activity is dependent on the amine content, the DDA, the molecular weight and the pH of the media during testing. It was also noted that the polymer displayed higher activity against Gram-negative bacteria compared to Gram-positive bacteria, potentially due to the difference in the cell walls of the bacteria. The cytotoxicity of this polymer against the Caco-2 cell line was low and comparable to that of chitosan, making it a promising polymer in the field of biomedical applications.⁷⁵

In a continuation of this research, Kenawy *et al.* produced aminated Schiff bases by reacting the ethylenediamine modified polymer with *p*-hydroxy benzaldehyde and vanillin respectively (**1.25**, Figure 1.20).⁷⁶ The antimicrobial activity of these polymers was tested against Gram-negative bacteria (*E. coli*, *Salmonella typhi*, *P. aeruginosa*), Gram-positive bacteria (*S. aureus*) and fungi (*Candida albicans*, *Cryptococcus neoformans* (*C. neoformans*), *Aspergillus flavus* and *A. niger*). The Schiff base derivatives display activity against Gram-positive and Gram-negative bacteria. The vanillin conjugated derivative was the only derivative to display activity against fungi, however, only *C. neoformans* and *A. niger* were affected. Activity of the modified Schiff bases versus the original polymer varied with the bacteria and fungus tested where in some cases enhanced activity was observed.⁷⁶

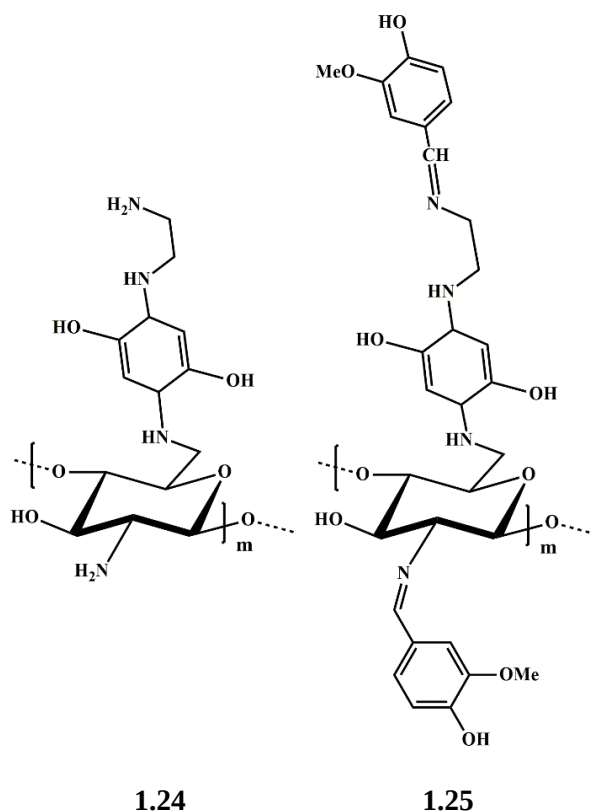
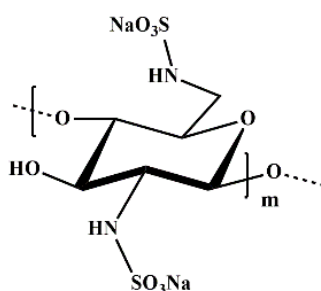


Figure 1.20: The structure of *p*-benzoquinone (1.24) and vanillin (1.25) modified diamino chitosan.

Hu and colleagues have proposed that the antimicrobial activity of chitosan can be attributed to the electrostatic interaction between the amine group of chitosan and the phosphoryl groups of phospholipids present in cell membranes.⁷⁷ To test this hypothesis, three diamino derivatives of chitosan (6-aminoethylamino-6-deoxy chitosan, 6-butylamino-6-deoxy chitosan and 6-pyridyl-6-deoxy chitosan) were synthesised and subjected to antimicrobial testing. The polymers were tested against Gram-negative (*E. coli* and *S. typhi*) and Gram-positive bacteria (*S. aureus*, *Bacillus subtilis* and *Bacillus anthracis*). In addition, *in vitro* antifungal activity was evaluated against four plant pathogens (*Fusarium graminearum*, *Rhizoctonia cerealis*, *Fusarium oxysporum* and *Botrytis cinerea*). Results indicated that all derivatives displayed antimicrobial and antifungal activity. The ethylamino and butylamino derivatives displayed superior activity compared to chitosan against *S.aureus* and *E.coli*. In addition, all derivatives displayed a greater degree of inhibition of fungi compared to the parent polymer. These results supported the theory that the amino group contributes to the enhancement of the antimicrobial and antifungal activity of chitosan.⁷⁷

The antioxidant activity of chitosan is well known and has been linked to the free amino and hydroxyl groups on the polymer backbone. The antioxidant activity of 6-deoxy-6-amino

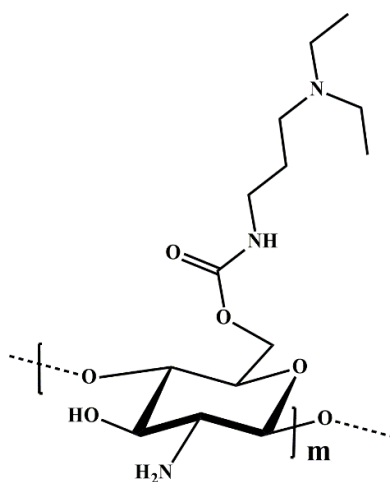
chitosan has recently been studied. This study included the synthesis of a 2,6-di-*N*-sulfonated derivative (**1.26**, Figure 1.21) and the antioxidant evaluation thereof. The reducing power and scavenging capacities for superoxide and hydroxyl radicals were studied together with factors influencing these activities. It was found that the diamino chitosan displayed a strong reducing power as well as a superoxide anion and hydroxyl radical scavenging ability. It was suggested by the authors, that the factors affecting the activity of the polymers were dependent on the polymer and the radicals tested. This theory was subsequently tested, and it was found that the activity was affected by polymer structure, degree of substitution of both amino and sulfonate groups, charge and chain conformations of the polymer.⁷⁸



1.26

Figure 1.21: The structure of a 2,6-di-*N*-sulfonated derivative (**1.26**) of 6-deoxy-6-amino chitosan.

The applicability of diamino chitosan as a thin film composite in the separation of carbon dioxide (CO₂) was reported by Seidi *et al.*⁷⁹ In this study *N,N*-diethylaminopropylcarbamate chitosan (**1.27**, Figure 1.22) was synthesised, having both primary and tertiary amines which are particularly useful in the transport of CO₂. The polymer was blended with different ratios of poly(vinyl alcohol) and used to cast thin film composite membranes to facilitate transport of CO₂ from a CO₂/CH₄ gas mixture. The membranes demonstrated high CO₂ permeance as well as a high CO₂/CH₄ selectivity under optimal conditions of a lower feed temperature and pressure.⁷⁹



1.27

Figure 1.22: The structure of *N,N*-diethylaminopropylcarbamate chitosan (**1.27**).

From the literature available on diamino chitin and chitosan, it is clear that the potential of these polymers has yet to be fully realised. Due the growing interest in diamino chitosan, the bulk scale synthesis of this polymer by a ‘greener’ and more efficient route is needed.

1.6 Review of solvents utilised in the production of improved chitin and chitosan derivatives.

As mentioned, the modification of chitin and chitosan is necessary to exploit the utility of these biopolymers. The majority of these chemical transformations are substitution reactions involving *O*- and/or *N*-alkylations of chitin and chitosan to render them soluble. These reactions are usually performed in dipolar aprotic solvents such as *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), *N*-methyl pyrrolidinone (NMP) and *N,N*-dimethylacetamide (DMAc). In some cases a combination of organic and inorganic reagents can be used to aid dissolution such as the amide-LiCl solvent systems. However, due to the development of new legislation in Europe, the Registration, Evaluation, Authorisation & restriction of Chemicals (REACH), the use of these hazardous solvents will be restricted.

This development necessitates the research and application of alternative, safer solvents. Farrán *et al.* recently reviewed the literature to report on the latest developments in the use of green solvents for carbohydrate chemistry.⁸⁰ This review highlighted the use of ionic Liquids (ILs), supercritical fluids (SCFs), fluoruous solvents, deep eutectic solvents (DES) and solvent-free processes. In addition, biomass derived solvents such as, γ -valerolactone (GVL) (**1.28**, Figure 1.23) and 2-methyltetrahydrofuran (MeTHF) (**1.29**, Figure 1.23) were reviewed.⁸⁰ Other

emerging bio-based solvents are also being investigated for their application in the synthesis and modification of a variety of compounds. Soh *et al.* investigated the use of green solvents in biomass processing highlighting green solvent metrics with respect to functionality and environmental impacts.⁸¹ Sherwood and colleagues have offered an alternative to conventional dipolar aprotic solvents in the form of dihydrolevoglucosenone (Cyrene™) (**1.30**, Figure 1.23).⁸² Cyrene™ is produced from cellulose *via* a two-step process and can potentially be applied as an alternative to the solvents used in conventional polymer synthesis.⁸²

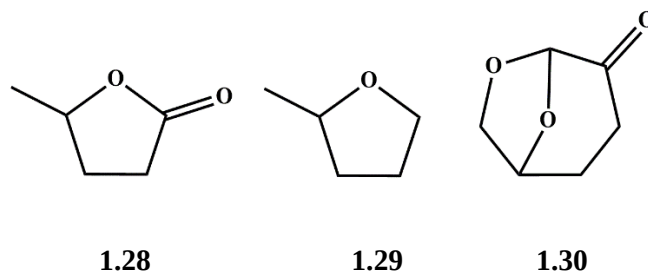


Figure 1.23: Proposed alternative solvents γ -valerolactone (**1.28**), 2-methyltetrahydrofuran (**1.29**) and dihydrolevoglucosenone (Cyrene™)(**1.30**).

There has been a gradual shift in chitin and chitosan chemistry, to find alternatives for the processing and modifications of these polymers. A few selected examples to demonstrate this are reviewed below. DES were used in the dissolution of α -chitin through three methods namely, conventional heating, microwave irradiation and ultra-sonication.⁸³ Chitin nanofibres were also produced using a DES based on a mixture of choline chloride-thiourea and the fibres produced were compared to that produced in ILs.⁸⁴ SCFs have also been used in the treatment of chitin and chitosan where sub and supercritical water was used as an alternative for decalcification and deproteinisation of crab shells.⁸⁵ Supercritical CO_2 has also been applied in the presence of an organic acid (acetic, malic or citric acid) for the extraction of chitin and chitosan.⁸⁶

Apart from being used in the processing of chitin and chitosan, green solvents have also been investigated in the chemical transformation of these polymers. A good example of this, is the production of carboxymethyl and quaternised chitin where a dilute NaOH -urea solvent system was applied.^{87, 88} Fang *et al.* recently reported on the production of chitin and chitosan based hydrogels obtained from LiOH/KOH /urea aqueous solutions, these hydrogels were also tagged with a fluorescent molecule to quantify the amine content of the gels.⁸⁹ The wastewater obtained as a by-product of hydrogel generation was recycled without further processing.⁸⁹ A review by Barber *et al.* stated that a “green” industrial revolution will occur with transformative

technologies forming the core of this shift, the example they cited was the use of chitin together with green solvents such as ILs.⁹⁰ It was also stated that the development of “better” materials designed with the principles of green chemistry in mind would provide sustainable industries.⁹⁰

1.7 Aims and objectives

1.7.1 General aims

This work deals with the synthesis and application of 6-deoxy-6-amino chitosan. The existing synthesis of 6-deoxy-6-amino chitosan uses hazardous materials which results in the generation of large quantities of waste and is not amenable to large scale synthesis. Therefore, improved methods are being sought to synthesise this polymer. Additionally, due to the limited applications for this polymer, the second aim of this study is to research novel applications for 6-deoxy-6-amino chitosan.

1.7.2 Specific objectives

Objective 1: A scalable production process for 6-deoxy-6-amino chitosan.

1. A process, starting with chitin is ideal with a minimum of steps.

Objective 2: A study of the dissolution behaviour of chitin and chitosan

1. Modelling of the solubility of chitin and chitosan
2. The best solvents selected for specific reactions and compared to the currently used solvent.

Objective 3: Using glucosamine as a model for polymer substitution reactions.

1. Model reactions using glucosamine (the monomer of chitosan) to develop an alternative route for the synthesis of diamino polymers.
2. Using the results from the model monomer reactions to introduce an additional amine group onto chitosan.
3. The synthesis of alkylamino chitosan derivatives using different nitrogen containing reagents to introduce the amine moiety.
4. Characterise all polymers using a variety of spectroscopic and analytical techniques including, NMR, IR, EA, TGA, DSC, PXRD, etc.

Objective 4: Applications of 6-deoxy-6-amino chitosan.

1. Electrospinning of 6-deoxy-6-amino chitosan to form nanofibres

- Characterisation of the nanofibres formed
- Loading of the nanofibres formed with silver nanoparticles
- Evaluation of the nanofibres and the silver loaded nanofibres as antimicrobial agents

2. Assessment of 6-deoxy-6-amino chitosan as an antifungal agent in crop protection

- Evaluate the activity of the polymer against *Fusarium verticillioides*
- Determine the applicability of 6-deoxy-6-amino chitosan as an edible food coating

3. Immobilisation of pyridoxal phosphate (PLP)

- Covalent and ionic binding of PLP using chitosan, 6-deoxy-6-amino chitosan and 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride, respectively.
- Characterise the resulting PLP immobilised polymers.
- Apply the PLP bound polymers as enzyme mimics in the deamination of amino acids to provide α -keto acids, specifically in the conversion of phenylalanine to phenylpyruvate
- Determine if the 6-deoxy-6-amino chitosan bound PLP is recyclable

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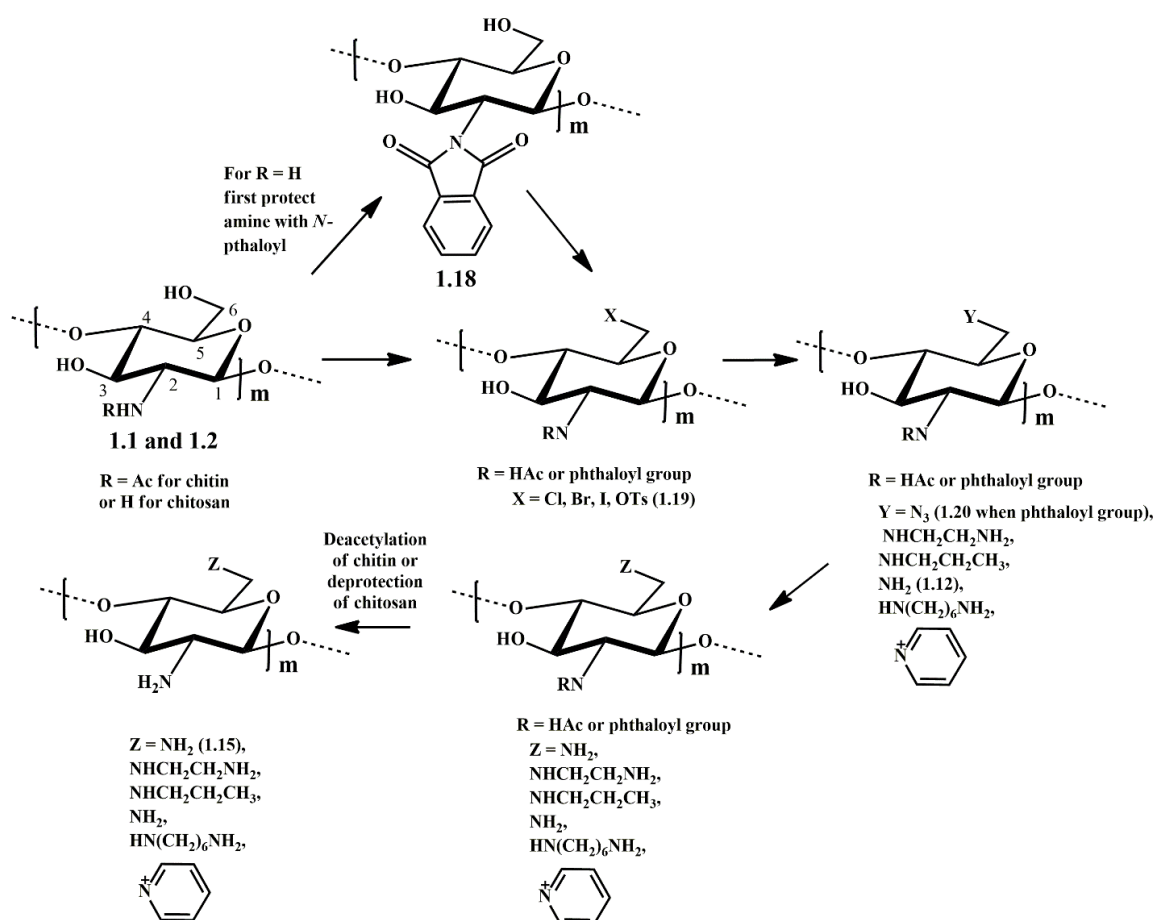
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Chapter Two

Synthesis of 6-deoxy-6-amino chitosan and the challenges thereof

2.1 Background

Chitin and chitosan have been used in various applications producing a diverse range of products, some of which are commercially available. However, the potential of these polymers is limited due to their poor aqueous solubility. Numerous strategies have been proposed for conjugating polar groups to the 6-hydroxy and/ or the 2-amino functional groups of chitosan to enhance solubility.¹ A modification on chitosan which holds promising value is the addition of an amine group onto C-6 of the polymer backbone. The introduction of an amine at C-6 changes the inherent properties of the polymer, including imparting improved solubility due to the increased positive charge. Modifications to introduce an amine at C-6 have been reported with a variety of reagents being used as the amine source (Scheme 2.1).²⁻⁵



Scheme 2.1: Previously reported methods for the introduction of an amine onto C-6 of chitin or chitosan.

The synthesis of these functionalised chitosan derivatives begins with protection at C-2 and subsequent modification at C-6. The most common chitosan derivative used for modification of C-6 is *N*-phthaloyl chitosan, since it is soluble in most polar aprotic solvents. This facilitates nucleophilic substitution (S_N2) reactions, making this derivative useful in regioselective modification.⁶ There are a variety of methods available for the modification of the C-6 position of chitin or chitosan, here follows a short overview of selected methods.

One of the most common methods for the synthesis of modified derivatives of chitin and chitosan, is to introduce a good leaving group at C-6 which can be substituted with the functional group of interest. Substitution of the hydroxyl at C-6 with a halogenated substituent has been reported and widely utilised due to its increased reactivity and homogeneity in subsequent substitution reactions. The bromo, chloro and iodo derivatives of *N*-phthaloyl protected chitosan were synthesised by reaction with *N*-halosuccinimides in the presence of triphenylphosphine. It was found that the halogens substituted in the order, chlorine > bromine > iodine, reflecting the nucleophilicities of the halide ions.³

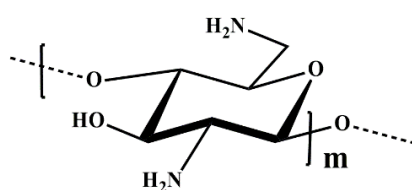
Tosylation of chitin or *N*-phthaloyl chitosan is another popular route in order to increase the solubility of the polymer in polar solvents by the introduction of a bulky group. This strategy was employed after cellulose was successfully tosylated and this derivative used as a useful intermediate in the synthesis of a variety of materials. Tosylation was generally performed after activation of cellulose with an alkali followed by treatment with *p*-toluenesulfonyl chloride in the presence of a base. Tosylated chitin and chitosan intermediates were reported to have a high reactivity which would allow for controlled modification of the polymer under mild conditions.⁷

Chitin and chitosan may be reacted with various reagents to introduce protecting groups at C-6 in order to selectively modify C-2 or C-3. One example of this strategy is the tritylation at C-6 by reaction with trityl chloride and subsequent substitution, followed by acid treatment to remove the trityl group. There are certain C-6 derivatives of chitin and chitosan which can be synthesised directly without an intermediate step.⁸

Regioselective alkylation of C-2 or C-6 is an area of interest due to the production of useful materials such as the commercially available glycol chitin used to assay chitinolytic enzymes. In the synthesis of hydroxyalkyl chitosan, the polymer is reacted with epoxides and depending on the reaction conditions (solvent, temperature and catalyst) the reaction may occur at the amino or alcohol group or at both sites. In the reaction of chitosan with propylene oxide, attack

of the hydroxyl group occurs preferentially when the reaction medium is basic, where *N*-alkylation occurs in an acidic medium only.⁸ Another method of introducing an alkyl group onto the polymer backbone is the reaction of chitosan with an aldehyde or ketone to produce an imine *via* Schiff base formation. Reduction with reagents such as sodium borohydride or sodium cyanoborohydride converts the imine into an *N*-alkylated derivative.⁹

The introduction of an amine at C-6 of the chitosan backbone is known to improve the inherent solubility of the polymer and imparts new properties due to the enhanced charge present. 6-Deoxy-6-amino chitosan (**1.15**, Figure 2.1) was first reported by Satoh and co-workers and has since begun receiving more attention.³



1.15

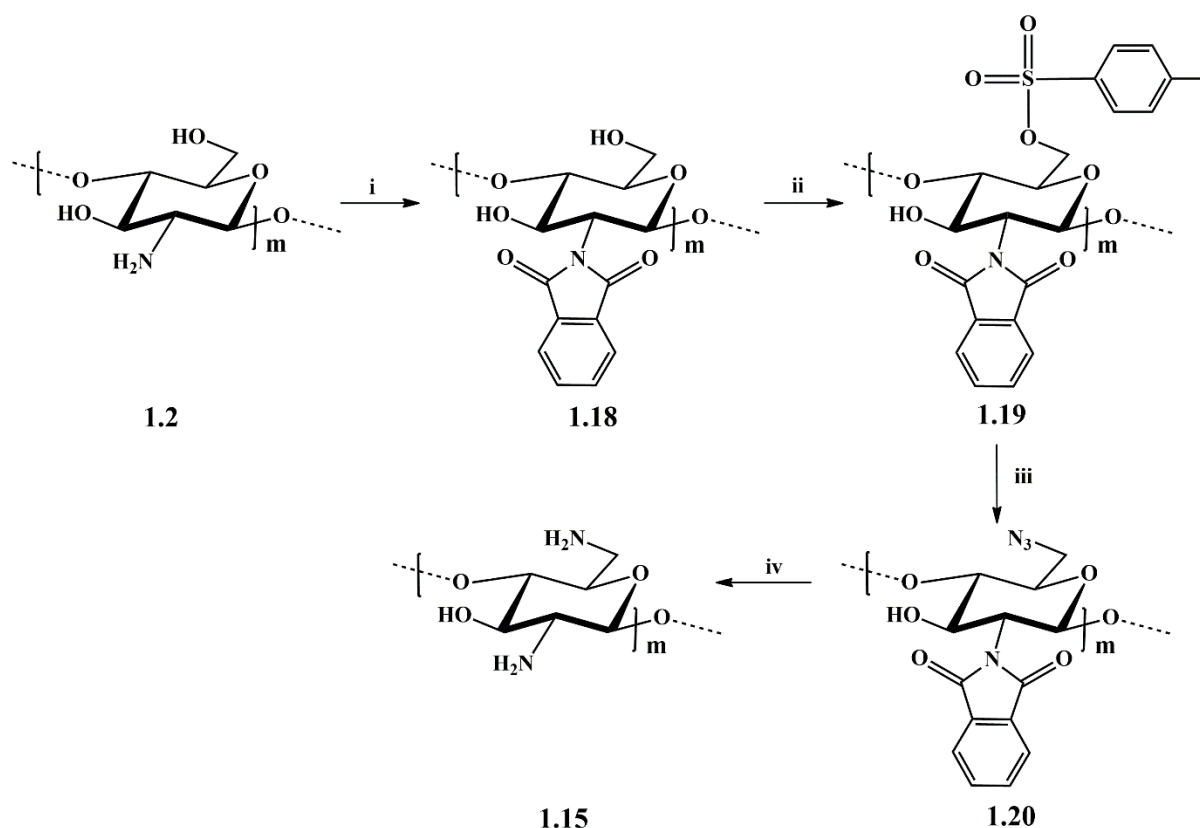
Figure 2.1: The structure of 6-deoxy-6-amino chitosan (**1.15**).

The most common method to synthesise 6-deoxy-6-amino chitosan was reported by Satoh *et al.* which involved the protection of the amine at C-2 in the form of *N*-phthaloyl-chitosan.³ Having protected the amino group, selective deoxyhalogenation of the C-6 position was used to obtain the 6-bromo, 6-chloro or 6-iodomethylene derivatives, respectively. Substitution of the halogen with an azido group, followed by reduction and phthaloyl deprotection following the Staudinger reaction gives diamino chitosan.³ However, since halogenated intermediates are not particularly green, an alternative method was needed. Jardine *et al.* reported an additional route to the 6-deoxy-6-amino chitosan derivative using a tosylated intermediate instead of the less favourable halogenated intermediates.¹⁰ Although, the method reported by Jardine *et al.* produced a poorly soluble 6-deoxy-6-amino chitosan where certain individual steps provided poor yields potentially indicating the incorporation of impurities which were carried through to the final product. Additionally, purification generated large quantities of waste.¹⁰

The majority of synthetic methods reported for the production of diamino derivatives of chitosan are impractical for large scale synthesis as they require relatively large reagent mass transfers and generally result in poor quality of the product. In addition, the variability in the degree of substitution obtained in the various steps of the pathway due to reaction conditions and the source of the chitosan is a concern. Scalability of these reactions is also quite

problematic, sometimes due to the nature of the reaction. This includes the use of large amounts of hazardous chemicals and the generation of large quantities of waste. Until these drawbacks are resolved, applications of diamino chitosan derivatives will remain limited. It is therefore desirable to find greener methods to produce water soluble diamino chitosan derivatives in order to broaden the scope of the applications of these polymers.

In recent year's incremental improvements in the synthesis of 6-deoxy-6-amino chitosan has appeared. As part of the current study, the existing synthetic procedure as reported by Jardine *et al.* was adapted to reduce the generation of hazardous waste and improve the quality of polymer produced.¹⁰ The synthesis of 6-deoxy-6-amino chitosan began by following the pathway outlined by Jardine *et al.* and was accomplished in four steps starting with chitosan (Scheme 2.2).¹⁰ The route involved the synthesis of *N*-phthaloyl chitosan (**1.18**), which was then converted to 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**). Substitution with an azide produced 6-deoxy-6-azido *N*-phthaloyl chitosan (**1.20**) which was then finally reduced to 6-deoxy-6-amino chitosan (**1.15**).¹⁰



Scheme 2.2: The pathway followed by Jardine *et al.* for the synthesis of 6-deoxy-6-amino chitosan. Reagents and conditions: (i) Phthalic anhydride, 5% DMF/ H_2O , 120 °C, 8 hr, 74%; (ii) *p*-Toluenesulfonyl chloride, Pyridine 17 hr, RT, 112%; (iii) NaN_3 , NMP, 80 °C, 4 hr, 66%; (iv) (a) PPh_3 , 15 hrs, 25 °C; (b) $\text{H}_2\text{NNH}_2 \cdot \text{H}_2\text{O}$, H_2O , NMP, 100 °C, 4 hr, 218%.¹⁰

2.2 General synthesis of 6-deoxy-6-amino chitosan (1.15)

C-2 protection strategies

Chitosan has reactive groups at C-2, C-3 and C-6, where reactions mainly occur at C-2 and C-6 due to steric constraints at C-3. In order to selectively modify the hydroxyl at C-6, an amino protecting group was utilised at C-2. Protecting groups often serve a dual function in that they can also help aid the solubility of the polymer in organic solvents. An ideal protecting group would be introduced under mild conditions, stable in various environments and be easily removed. Very few protecting groups have been reported relating to chitosan chemistry. Gaware *et al.* reports that the most common protecting groups includes phthaloyl protection of the amine at C-2 or Schiff base formation (aldehyde), trityl protection of the C-6 hydroxyl group and silyl ether protection of the C-3 and C-6 hydroxyl groups.¹¹ The aldehyde reacts with the amine at C-2 to form a Schiff base where the imine linkage formed is stable in neutral and/or basic media. This bond is rapidly hydrolysed under acidic conditions which regenerates the free amino group. The Schiff base can also be converted into an *N*-alkyl derivative through treatment with a reducing agent.⁹

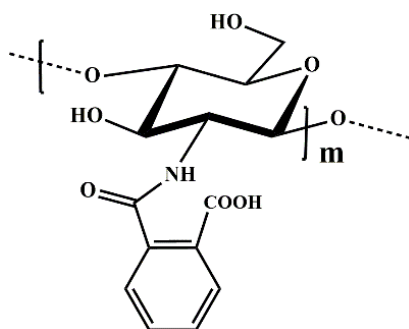
N-phthaloyl chitosan (1.18)

As mentioned above, the first step in this synthesis was the protection of the amino group on chitosan. The phthaloyl group was chosen for the protection of the C-2 amine, as it provides simple protection of the functional groups present in chitosan. In a review of the controlled functionalisation of chitin, Kurita *et al.* stated that *N*-phthaloyl chitosan has proved to be one of the best options for chitosan modification with excellent regioselectivity under mild conditions.⁹ The bulky phthaloyl group interferes with the hydrogen bonding network in the polymer promoting dissolution in various organic solvents such as dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and *N*-methyl-2-pyrrolidone (NMP), allowing for further modification. In addition, the *N*-phthaloyl protecting group can easily be removed using the readily available hydrazine hydrate. It must be noted that hydrazine hydrate is toxic however, the benefits of its use outweighs the risks and other conventional bases do not compare very well.⁹

Previously, the *N*-phthaloylation of chitosan was typically performed in DMF; however, this reaction results in partial *O*-phthaloylation. The reaction conditions were later modified using a DMF:H₂O mixture as the solvent system.¹⁰ The presence of water in the reaction medium prevents *O*-phthaloylation from occurring, due to the hydrolysis of any *O*-phthaloylation

products.¹² Anhydrous DMF has been used to promote *O*-phthaloylation since substitution at C-3 with a phthaloyl group has been shown to enhance solubility, as a result of the suppression of the crystallinity of the polymer. *O*-Phthaloyl groups can also be removed by transesterification with methoxide or methanol to produce the *N*-phthaloyl polymer exclusively. Although, this involves an extended reaction time of up to four days or repetition of the reaction in order to remove all *O*-phthaloyl groups due to the heterogeneous nature of the reaction.¹³ An electron withdrawing phthaloyl derivative, such as the 4,5-dichlorophthaloyl group has been used in the protection of chitosan in order to facilitate a milder deprotection. However, this strategy did not significantly improve the deprotection of chitosan as it required harsher conditions where the polymer was treated with hydrazine and subsequently with 6 M NaOH (aq) at 50 °C for complete removal.¹¹

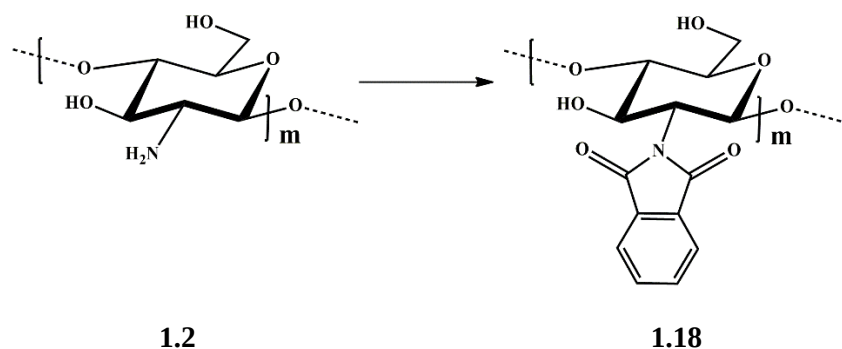
Ifuku *et al.* recently reported the chemoselective homogeneous synthesis of *N*-phthaloyl chitosan in aqueous acetic acid media, making the synthesis of this intermediate much more environmentally friendly.¹⁴ The study also reported the synthesis of the desired polymer in water, as *N*-phthaloylation disrupted the hydrogen bonding interactions of the chitosan chains allowing the polymer to dissolve after a certain period of time facilitating further substitution. However, the polymer obtained showed traces of the formation of the *N*-(2-carboxy)benzoyl glucosamine unit (**2.1**, Figure 2.2) which were removed by thermal dehydration.¹⁴



2.1

Figure 2.2: *N*-(2-carboxy)benzoyl chitosan derivative (**2.1**).

The literature reports helped guide the synthesis of *N*-phthaloyl chitosan in this work. In the current study *N*-phthaloyl chitosan (**1.18**) was synthesised by the addition of phthalic anhydride to chitosan in 10% v/v AcOH as opposed to the DMF/H₂O solvent system typically used (Scheme 2.3).¹⁰



Scheme 2.3: The general synthesis of *N*-phthaloyl chitosan (**1.18**). Reagents and conditions: 10% v/v AcOH, 120 °C, 24 hr, 81%.

A distinct advantage of this method, is that the introduction of hydrophobic phthaloyl groups causes the product to precipitate from the solution as a result of the hydrophobicity of the substituted polymer making isolation and purification simpler. The reported work up method was slightly modified in that acetone was used in the final washing step to remove excess phthalic anhydride present as opposed to just water and ethanol. ^1H and ^{13}C NMR spectral data supported the synthesis of this polymer where the spectra was comparable to that reported in the literature.¹⁵ The ^1H NMR spectra (Figure 2.3) showed the characteristic peaks assigned to the pyranose protons between 3.5 and 5.2 ppm. The distinct peaks attributed to the *N*-phthaloyl aromatic groups were present between 7.49 and 8.04 ppm. In addition, these peaks confirm that *N*-phthaloylation occurred selectively since peaks assigned to *O*-phthaloylation are not seen resonating in the region 7.3 to 7.5 ppm.¹⁵

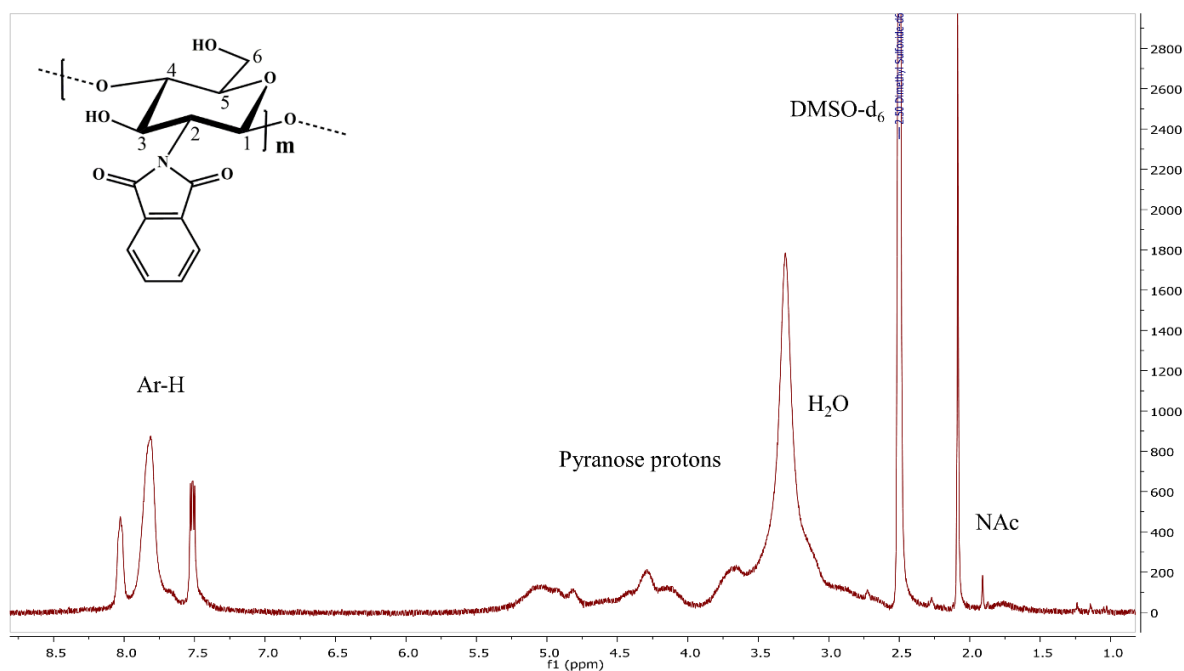


Figure 2.3: ^1H NMR spectrum of *N*-phthaloyl chitosan (**1.18**) in $\text{DMSO-}d_6$.

IR spectroscopy supported the synthesis of the product with characteristic bands for the modified chitosan clearly visible (Figure 2.4). The presence of two phthalimido (C=O) absorbance bands at 1775 and 1704 cm^{-1} as well as a band at 715 cm^{-1} are due to the phthaloyl-aromatic ring.¹⁴ Figure 2.4 also showed *N*-phthaloyl chitosan synthesised in a DMF/ H_2O solution versus that synthesised in aqueous acetic acid. From the spectra it can be seen that the characteristic absorption bands of the C=O phthalimide groups are much sharper for the reaction in $\text{AcOH}/\text{H}_2\text{O}$ with no stretching bands observed for the *N*-(2-carboxy)benzoyl glucosamine unit, which can potentially be formed in the course of the reaction.¹⁴ Since the reaction in $\text{AcOH}/\text{H}_2\text{O}$ is homogeneous, a greater degree of substitution was observed as evidenced by the well resolved peaks of the C=O of the phthalimide groups. The synthesised polymer was insoluble in typical polar aprotic solvents such as DMF, NMP and pyridine. However, contrary to the result by Ifuku *et al.*, the polymer was soluble in DMSO after sonication possibly due to the difference in the degree of substitution and the quality of chitosan used.¹⁴ The quality of the chitosan used affects the behaviour of the polymer significantly, therefore, it is imperative that a good quality chitosan with known characteristics be used.¹⁶

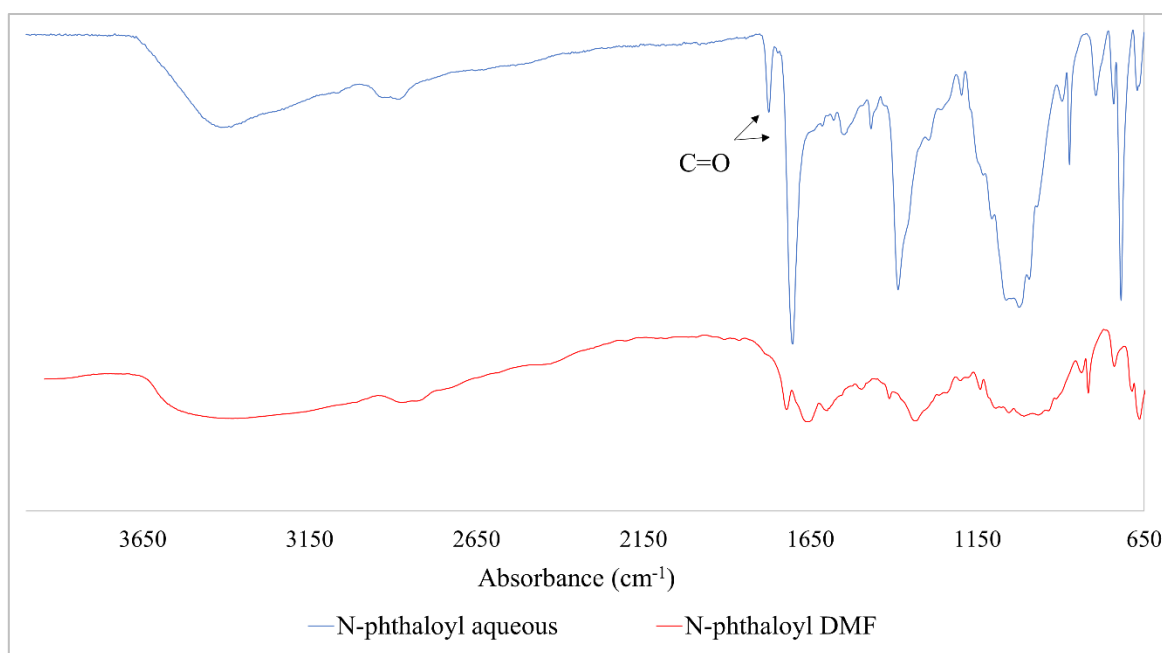
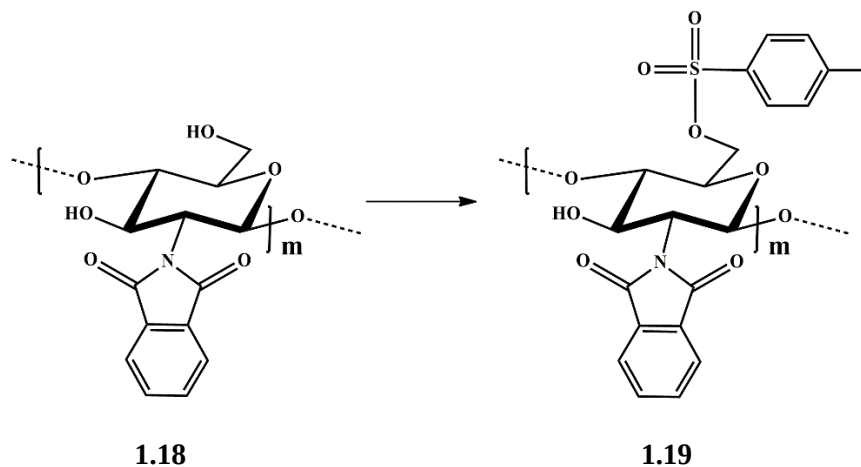


Figure 2.4: The IR spectra of *N*-phthaloyl chitosan (**1.18**) synthesised in 10% acetic acid and DMF.

C-6 tosylation of *N*-phthaloyl chitosan

After the successful synthesis of *N*-phthaloyl chitosan (**1.18**), the polymer could now be modified at C-6. Substituting *N*-phthaloyl chitosan requires activation of the hydroxyl at C-6 with a suitable intermediate which will allow substitution. One of the more popular chitosan

intermediates is formed by the reaction of C-2 protected chitosan with *p*-toluenesulfonyl chloride in the presence of a base (Scheme 2.4).



Scheme 2.4: The general synthesis of 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**). Reagents and conditions: *p*-toluenesulfonyl chloride, pyridine, RT, 48 hr, 99%.

Generally, the base used is typically triethylamine or pyridine. Substitution occurred at C-6 regioselectivity, due to the bulky tosyl group and the steric hindrance around the hydroxyl group at C-3 not allowing for attachment there. In addition, the hydroxyl at C-6 is a primary hydroxyl group whereas that at C-3 is a secondary hydroxyl, thus favouring attack at C-6 as primary hydroxyls are more reactive.⁹ The tosylation is preferred for C-6 modification over substitution with a halide as the tosylation method is milder, improves solubility and the tosyl group itself is a better leaving group. Substitution with a halide generally requires harsh conditions such as prolonged heating which can lead to partial destruction of the polymer.¹⁷ However, Yang and co-workers have stated concerns that the tosylation of chitosan leads to degradation of the polymer which can possibly be attributed to β -elimination in a basic medium or acid degradation in the tosylation process.¹⁸ There has not been any follow up reports investigating the possibility of polymer degradation during tosylation, therefore this useful intermediate cannot be overlooked.

Tosylation of chitosan is generally carried out neat in anhydrous pyridine or in a mixture of *N,N*-dimethylacetamide and lithium chloride using triethylamine (Et_3N) as the base. The addition of the base is necessary due to the production of HCl as a by-product of the tosylation step. The synthesis of tosylated chitosan in pyridine with the addition of Et_3N has been reported, which is counterintuitive.¹⁸ The reaction is generally conducted at low temperatures ($< 10\text{ }^\circ\text{C}$) to avoid the formation of undesirable side products. Varying ratios of tosyl chloride

to sugar residue have been reported, where the lowest reported = 1:3 and the highest = 1:15, the degree of substitution (DS) is highly dependent on the ratio used.¹⁰

Recently, there have been numerous studies which report on the green tosylation of polymers particularly using cellulose. Elchlinger *et al.* reported the tosylation of cellulose in NaOH/H₂O and NaOH/NaCl solutions, with varying ratios of tosyl chloride and Et₃N.¹⁹ Cellulose has previously been tosylated in a NaOH/urea solvent system, in the presence of a non-ionic surfactant and separately at ambient temperature in the ionic liquids 1-butyl-3-methylimidazolium chloride (BMIMCl) and 1-allyl-3-methylimidazolium chloride with pyridine acting as a co-solvent.^{20, 21} Kurita *et al.* reported the tosylation of chitin in a biphasic aqueous alkali solution, which was later applied to chitosan by Munro *et al.*^{7, 22} However, there are drawbacks associated with these eco-friendly synthesis methods, such as saponification of tosyl chloride in the presence of NaOH (therefore requiring excess reagent), the potential polymer hydrolysis due to the basicity of the reaction medium, as well as the control of the pH of the reaction. This was demonstrated in the study by Morita and co-workers, where they tosylated primary alcohols in water in the presence of catalytic amines (such as *N,N*-dimethylbenzylamine and Et₃N) and potassium hydroxide, where the pH is maintained at 10 for the duration of the reaction.²³

In the current study, *N*-phthaloyl chitosan synthesised in the acetic acid medium was reacted with *p*-toluenesulfonyl chloride in anhydrous pyridine as reported by Jardine *et al.*¹⁰ After several optimisation reactions to improve the yield and quality of the product, the final procedure involved a ratio of 1:4 for anhydroglucosamine unit to tosyl chloride. This was half the amount compared to the original synthesis and extending the reaction time to 48 hours from 17 hours. Due to the insolubility of the polymer obtained in DMSO, an NMR spectrum could not be obtained. This insolubility can potentially be attributed to the difference in the quality of chitosan used or the inclusion of impurities in the isolated polymer.

The IR spectrum of polymer (**1.19**) confirmed the presence of the S=O group, which was seen in the absorbance band at 1174 cm⁻¹ and the symmetrical C-O-S stretch at 815 cm⁻¹ (Figure 2.5). 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.* confirming successful synthesis of the tosylated product.¹⁰ The IR spectra reported by Munro *et al.* obtained for the tosylation of β-chitosan in mixture of sodium hydroxide and chloroform with a large molar excess of tosyl chloride appeared similar to that reported here.²² This study did not protect the amine at C-2 and reported

a tosylation degree of 30% for the polymer, calculated from elemental analysis.²² It is important to note that primary amines react with tosyl chloride to form primary sulfonamides. However, depending on which base is used in the reaction, selective *O*-tosylation can occur in the presence of a free amine. The study in question did not discuss the absence of a protecting group at C-2 and further did not comment on the hydrolysis of *p*-toluenesulfonyl chloride in the presence of the hydroxide ion in water.²² Elemental analysis further confirmed the incorporation of a sulfur moiety.

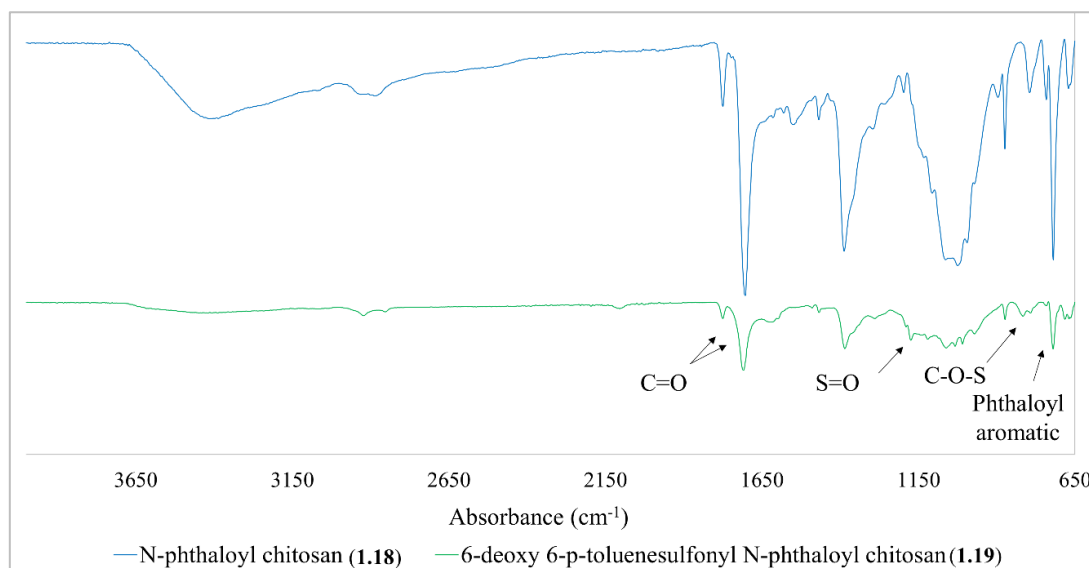


Figure 2.5: The IR spectra of 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) versus *N*-phthaloyl chitosan (**1.18**).

Since 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) was successfully synthesised, the substitution of the tosyl moiety was the next step.

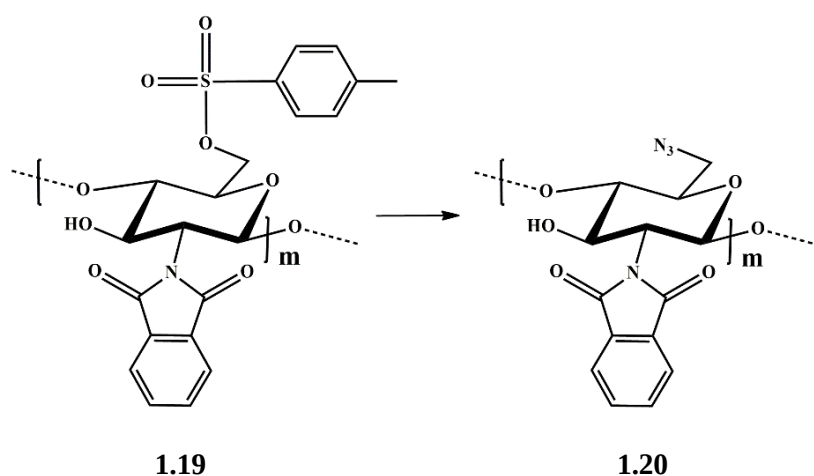
C-6 azidation of 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan

Substitution of the tosyl group with an azide moiety was carried out to form 6-deoxy-6-azido *N*-phthaloyl chitosan (**1.20**).¹⁰ The azido derivative of chitosan has been synthesised by a number of authors as a valuable intermediate in the synthesis of modified chitosan derivatives with varying properties. This derivative is notably used in click chemistry reactions. Those specific to chitosan include copper-catalysed azide – alkyne dipolar cycloadditions, metal-free cycloadditions of azides to highly dipolarophilic systems and metal-free thiolene additions. In these click chemistry reactions, the functional groups used are azides, alkynes, highly dipolarophilic fragments, and thiol groups or double bonds. The review by Kritchenkov *et al.* gave a broad overview of click reactions in chitosan chemistry, where special emphasis was

placed on the application of these polymers.¹⁷ This type of reaction is quite useful for the generation of grafted co-polymers. Hydrogels are common products of these reactions, and have found many applications due to their favourable properties conferred upon them by linking different polymers.¹⁷

Substituted *N*-phthaloyl chitosan is generally dissolved or dispersed in the chosen solvent (DMF, DMSO or NMP) followed by the addition of various ratios of NaN₃ under an inert atmosphere to avoid any side reactions leading to the formation of hazardous products (e.g. hydrogen azide). Ratios vary from 3 to up to 10 equivalents of the azide to a singular polymer unit. The solution was heated, where reaction times varied from four hours to thirty six hours depending on the reaction temperature, where the lower the temperature the longer reaction time was needed.¹⁷ The polymer is generally isolated by the addition of water or an alcohol where the product is washed thoroughly to remove any unreacted reagent (NaN₃). Some authors employ multiple solvents to remove the excess reagents creating excessive waste. One such example is where the isolated polymer was washed with water, ethanol, acetone and diethyl ether. Azido functionalised chitosan has also been synthesised *via* a one-pot process where *N*-phthaloyl chitosan was brominated at C-6 and subsequently reacted with NaN₃.²⁴

In the current study, 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan **1.19** was heated in the presence of sodium azide in NMP for four hours as reported by Jardine *et al.* (Scheme 2.5). The polymer was subsequently isolated by the addition of ethanol and an ethanol/water mixture, followed by recovery through centrifugation to produce 6-deoxy-6-azido *N*-phthaloyl chitosan (**1.20**) in 99% yield.¹⁰



Scheme 2.5: The general synthesis of 6-deoxy-6-azido *N*-phthaloyl chitosan (**1.20**). Reagents and conditions: NaN₃, NMP, 80 °C, 4 hr, 99%.

The IR spectrum (Figure 2.6) confirmed the presence of an azide group with a strong absorption band at 2103 cm^{-1} . In addition, the weak signal at 1174 cm^{-1} was attributed to the $\text{S}=\text{O}$ absorption band and that at 817 cm^{-1} assigned to the $\text{C}-\text{O}-\text{S}$ stretch, showed incomplete substitution of the tosyl group. The IR spectra obtained agreed with that reported by Zampano *et al.* including the insolubility of the polymer obtained *via* tosylation of chitosan.²⁵ This insolubility was potentially attributed to possible crosslinking either from nitrene formation or during the substitution reaction where the residual hydroxyl groups present could possibly react with the tosyl moieties.²⁵ In addition, the elemental analysis noted an increase in the nitrogen content of the polymer supporting the substitution of the tosyl group with the azide moiety.

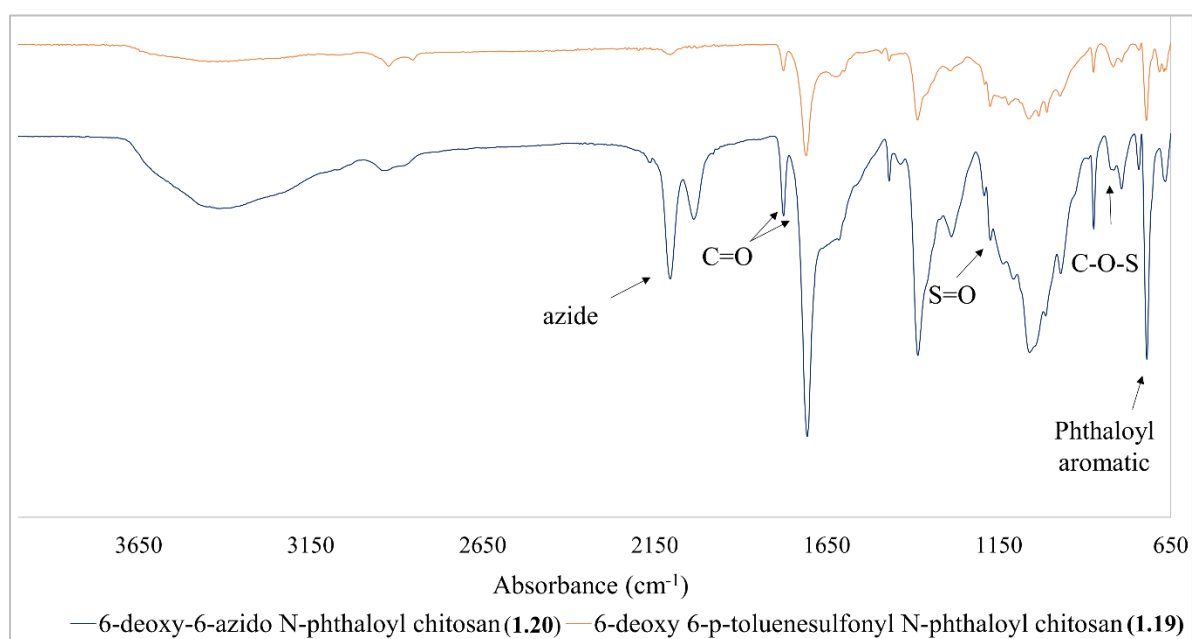
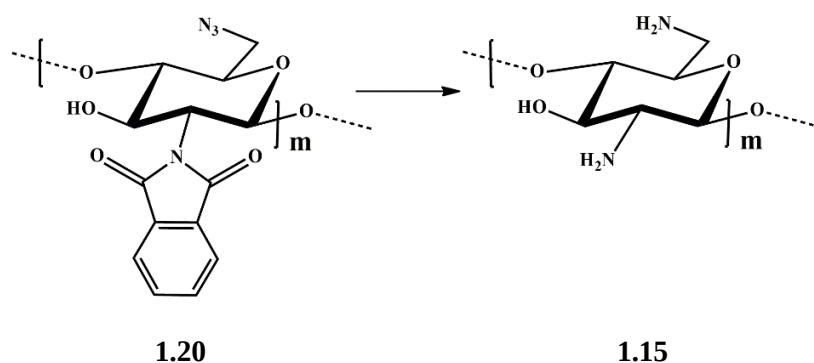


Figure 2.6: The IR spectra of 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) and 6-deoxy 6-azido *N*-phthaloyl chitosan (**1.20**).

Once 6-deoxy-6-azido *N*-phthaloyl chitosan (**1.20**) was obtained, the amine could now be formed by reduction of the azide and deprotection of the amine at C-2.

Reduction of C-6 azido chitosan

In order to produce 6-deoxy-6-amino chitosan (**1.15**) from 6-deoxy-6-azido *N*-phthaloyl chitosan (**1.20**), the azide was reduced and the phthaloyl group removed (Scheme 2.6).



Scheme 2.6: The general synthesis of 6-deoxy-6-amino chitosan (**1.15**). Reagents and conditions: (i) PPh_3 , 15 hrs, 25 °C; (ii) $\text{H}_2\text{NNH}_2 \cdot \text{H}_2\text{O}$, H_2O , NMP, 100 °C, 4 hr, 100%.

The azide group can be reduced to the amine using a variety of methods. These include treatment with sodium borohydride, lithium aluminium hydride or by catalytic hydrogenation using palladium over carbon. Other methods include the Staudinger reaction where triphenylphosphine (PPh_3) is used to form the corresponding phosphinimine, which is thereafter base hydrolysed to produce the amine.^{3, 26} The *N*-phthaloyl protecting group is generally removed using a strong base at an elevated temperature. Bases which are commonly used for the removal of the *N*-phthaloyl group are, butylamine, ethylenediamine, hydrazine hydrate, hydroxylamine and sodium borohydride. Other less common routes used alcoholic methylamine, ammonia in methanol and hydrazine acetate.²⁷

In the current synthetic scheme, the most efficient method to convert the azide to an amine would be treatment with PPh_3 alone; however, due to possible partial hydrolysis of the *N*-phthaloyl group, this is not recommended. A base such as hydrazine hydrate used for the removal of the phthaloyl group is a good option since this reagent also promotes the hydrolysis of the phosphinimine intermediate. Therefore, the azide derivative was simultaneously reduced and deprotected in a one pot reaction to yield 6-deoxy-6-amino chitosan (**1.15**).¹⁰ 6-Deoxy-6-azido *N*-phthaloyl chitosan (**1.20**) was stirred in the presence of PPh_3 in NMP for fifteen hours and subsequently heated (100 °C) in the presence of hydrazine monohydrate and water for a further four hours. The solid was isolated by filtration and washed with ethanol followed by dialysis against water after which it was lyophilised.¹⁰ The formation of 6-deoxy-6-amino chitosan proceeded *via* a Staudinger type mechanism involving the formation of an azo-ylide intermediate.²⁸

The product obtained after dialysis was not soluble in water as previously reported. This insolubility of the product can possibly be related to the presence of impurities in the polymer such as triphenylphosphine oxide which is a by-product of the reduction of the azide. Another

factor can possibly be due to the incomplete removal of the phthalimide protecting group whose presence negatively affects solubility in polar solvents. In addition, a higher degree of hydrogen bonding between the polymer chains can render the material insoluble in water. Further characterisation of 6-deoxy-6-amino chitosan (**1.15**) was used to support this hypothesis.

The product structure was supported by ^1H NMR spectral analysis which was obtained in 2% DCl/D₂O (Figure 2.7).

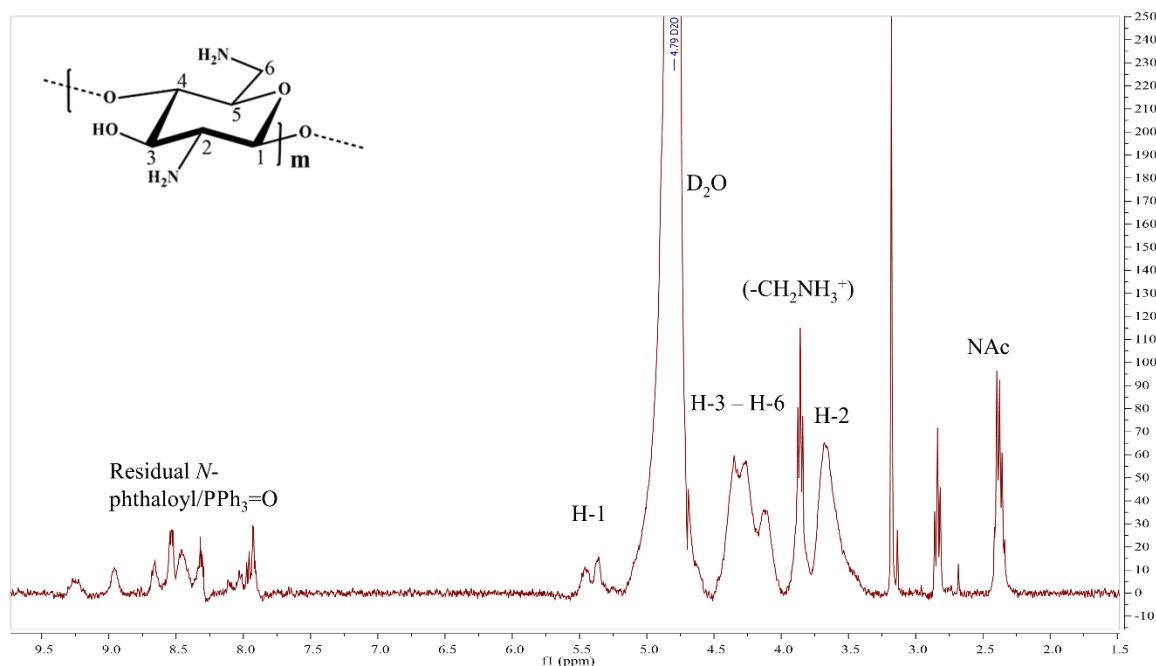


Figure 2.7: ^1H NMR spectrum of 6-deoxy-6-amino chitosan (**1.15**) in 2% DCl/D₂O.

A multiplet was observed in the ^1H NMR spectrum of (**1.15**) in the region of δ 7.86 – 8.71 ppm which was assigned to the aromatic protons of the phthaloyl group, which indicated incomplete deprotection and possible traces of the by-product triphenylphosphine oxide. This incomplete deprotection is not uncommon in the field of chitosan work, as described by Yang and co-workers who also reported the incomplete deprotection using similar reagents and conditions.¹⁸ The report by Lebouc *et al.* estimated the amount of protected groups remaining after deprotection with hydrazine hydrate to be 20% by the method of colloidal titration.²⁹ Kritchenkov *et al.* has stated that the phthalimide group requires drastic conditions for its removal typically at temperatures of 130 °C for seven hours.¹⁷ However, most studies treat protected polymers for shorter periods at lower temperatures which leads to incomplete deprotection. It has been reported by Yang *et al.* that partial deprotection may have occurred

due to incomplete cyclisation of the amino group in the reaction with triphenylphosphine, or the hydrolytic cleavage of the imide ring in the hydrazinolysis reaction.³⁰

In the ^1H NMR spectrum, the anomeric proton resonated at δ 5.36 ppm while the signals between δ 4.03 and 4.51 were assigned to protons H-3 to H-6. In solution the polymer is typically protonated, therefore, the resonance at δ 3.86 ppm was assigned to the proton of the protonated amine ($-\text{CH}_2\text{NH}_3^+$).³⁰ The signal at δ 3.67 ppm was assigned to that of H-2. For the ^1H NMR spectrum reported by Yang *et al.* the peaks are shifted higher upfield.^{10, 18} This difference can be attributed to the solubility of the polymer, where that reported by Yang *et al.* was fully water soluble versus the polymer synthesised here which required acid treatment to dissolve.¹⁸ One difference in the synthesis of these two polymers, is that the study by Yang *et al.* first removed the phthaloyl protecting group then treated the deprotected polymer with sodium borohydride to reduce the azide moiety.¹⁸ In addition, the polymer was treated with acetic acid, dialysed against dilute sodium hydroxide (pH 9), treated with acid once again and lastly dialysed against water, concentrated and lyophilised. This extended isolation procedure was used to remove the excess salt present. However, drawbacks associated with the route used by Yang *et al.* was the range of polymers synthesised were found to have low molecular weights, possibly due to degradation of the polymer during the different transformations to obtain the amine at C-6.¹⁸ Therefore, this method is not scalable due to the amount of waste generated from the synthesis of the polymer.

The IR spectrum of 6-deoxy-6-amino chitosan (**1.15**) (Figure 2.8) showed a broader band at 3379 cm^{-1} which was assigned to the free amino groups, while the azido band decreased considerably in intensity. The intense band at 1591 cm^{-1} was assigned to the bending vibration of the amino groups showing that deprotection had occurred.

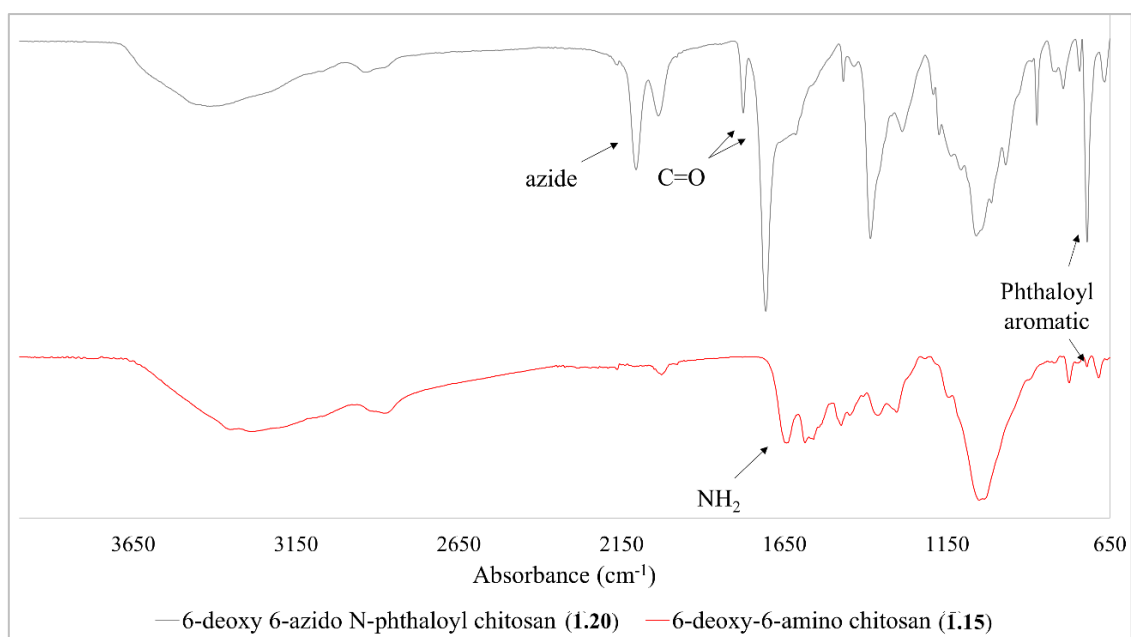


Figure 2.8: IR spectra of 6-deoxy-6-azido *N*-phthaloyl chitosan (**1.20**) and 6-deoxy-6-amino chitosan (**1.15**).

This reaction was also performed in DMAc as a possible alternative to the more hazardous NMP, with similar results obtained.

The degree of substitution (DS) of any chitosan derivative can be determined using a number of methods. These include the difference in the C/N ratio in elemental analysis or using specific peaks or bands in the ^1H NMR and IR spectra. Other less common methods include potentiometric titration or conductivity measurements. However, a major issue with reporting of the DS is the inconsistencies of the methods used, making it difficult to compare derivatives across the literature. Looking at each derivative in the current synthetic pathway, the DS was calculated here using results obtained from the elemental analysis as follows:

$$DS = \left[\frac{\left(\frac{C}{N} \right)_{\text{derivative}}}{\left(\frac{C}{N} \right)_{\text{chitosan}}} \right] * DDA (96\%)$$

The values obtained for the derivatives are reported in Table 2.1. Due to the difference in the method used for calculating the DS in different studies, these values were not compared to any literature reports. However, literature values are given in the table to show the wide range of DS obtained in different studies. Where a DS of greater than one is observed, this could indicate the presence of impurities or substitution at the unprotected hydroxyls at C-2 and/ or C-3.

Table 2.1: The DS of polymers synthesised in the current study.

Polymer	%C*	%N*	C/N	DS	Literature DS
Chitosan (1.2)	44.04	6.65	6.62	-	
<i>N</i> -Phthaloyl chitosan (1.18)	52.14	4.99	10.04	1.42	0.65 – 1.54 ^{12, 14}
6-Deoxy-6- <i>p</i> -toluenesulfonyl <i>N</i> -phthaloyl chitosan (1.19)	43.92	4.43	9.91	1.46	0.30, > 0.99 ^{22, 31}
6-Deoxy-6-azido <i>N</i> -phthaloyl chitosan (1.20)	50.20	12.78	3.93	0.57	0.28 - 0.95 ^{3, 24}
6-Deoxy-6-amino chitosan (1.15)	41.50	13.57	3.06	0.44	0.7 – 0.94 ^{3, 18}

*determined by elemental analysis

The solubility of 6-deoxy-6-amino chitosan (**1.15**) was tested in common solvents such as water, NMP, DMAc, etc. where it was found to behave similarly to chitosan. Swelling was observed in the majority of these solutions rather than dissolution. This insolubility can possibly be attributed to the high degree of crystallinity of the polymer formed at the end of the reaction sequence or the presence of impurities.³² The structure of 6-deoxy-6-amino chitosan (**1.15**) was further investigated by X-ray diffraction analysis to determine whether the polymer underwent any major changes to its crystalline structure due to the modifications on chitosan. XRD data for the polymer showed two peaks at 2θ of 9° and 20° which corresponded to the hydrated and anhydrous forms of chitosan (Figure 2.9).³³ The X-ray diffraction spectrum here was different to the one reported by Yang *et al.* where peaks were clearly distinguishable.³⁰ Their study reported that the width of the peaks generally indicated the size of the crystallites where broad peaks indicated smaller crystallites.³⁰ The polymer synthesised in the present work was highly amorphous confirming the high degree of crystallinity present which was confirmed by the PXRD pattern obtained.

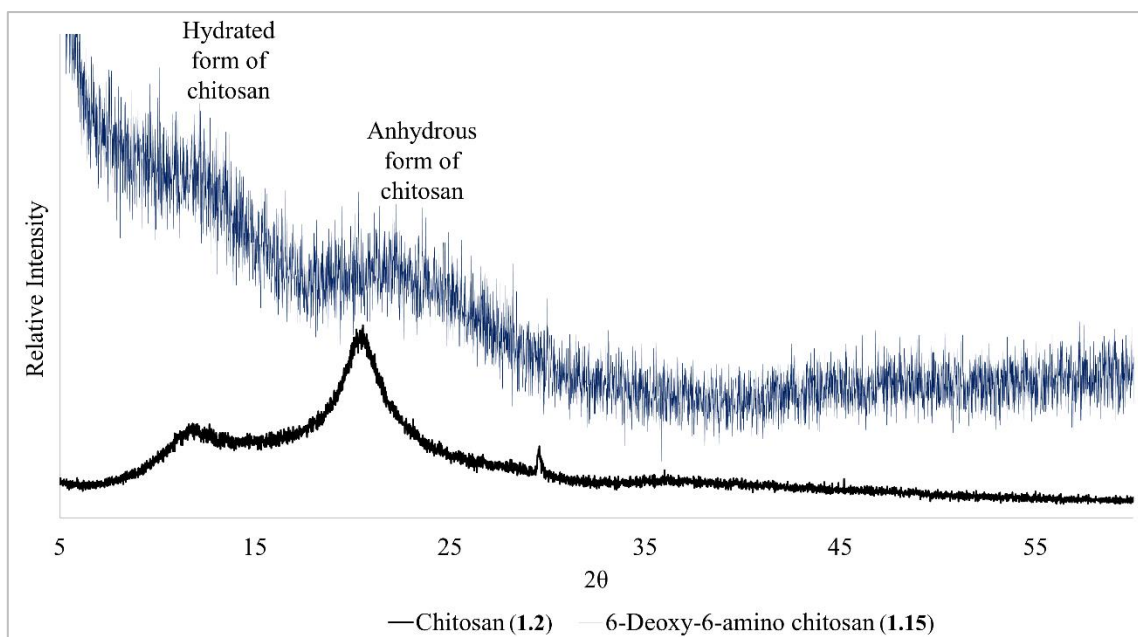


Figure 2.9: PXRD spectrum of chitosan (**1.2**) versus 6-deoxy-6-amino chitosan (**1.15**).

In addition, the polymer was studied by TGA and DSC to determine whether the incorporation of an amine at C-6 affected the thermal properties of the original polymer (Figure 2.10). Thermal methods, such as TGA and DSC are powerful thermoanalytical techniques to monitor characteristic physical and chemical changes in both natural and synthetic polymers. Modified chitosan would yield curves that are unique for a particular composition where slight changes in chemical structure will bring about discrete and reproducible variations in the thermograms. The loss of structural water in volatilisation and subsequent decomposition can be quantitatively measured by TGA. Thus, TGA is a useful method to compare the thermal stability of chitosan versus chitosan derivatives. For chitosan, there were two characteristic degradations, at 50 – 100 °C which were attributed to the loss of water from the polysaccharide chains and at 250 – 400 °C which resulted from the depolymerisation of the polysaccharide with the loss of volatile compounds. DSC was also used to confirm the results obtained from TGA and NMR data.³⁴ By comparing the TGA data with that of the DSC, it can be seen that polymer (**1.15**) displayed two stages of weight loss. The first stage took place in the range 24 – 81 °C, attributed to the loss of residual moisture accounting for a 2.00 wt% mass loss. The second stage occurred in the range of 109 – 316 °C with a mass loss of 86.67 wt%, this can be attributed to the decomposition stage of the polysaccharide. This result was similar to that observed for chitosan itself, where the high thermal stability of the polymer is an advantage when it comes to utilising this polymer in various applications.

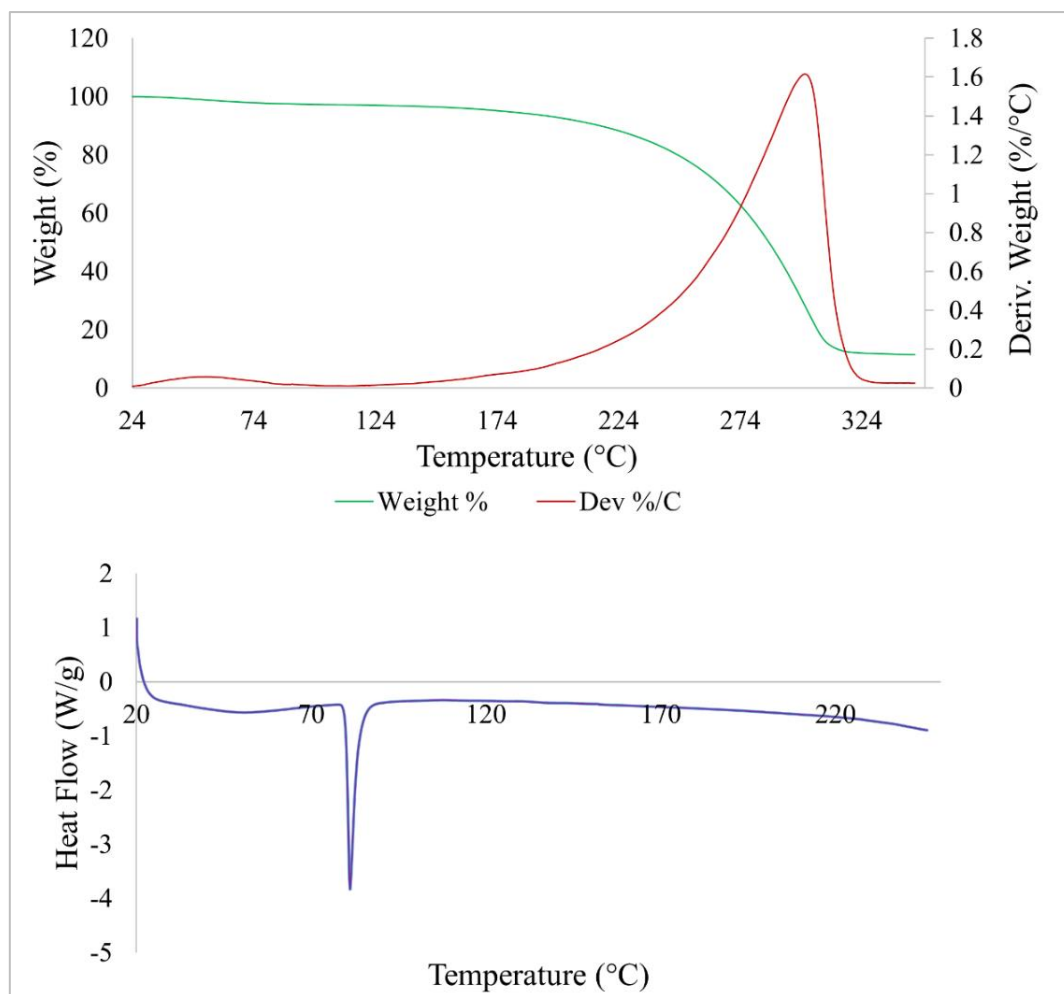


Figure 2.10: TGA (top) and DSC (bottom) data for 6-deoxy-6-amino chitosan (**1.15**).

2.3 Synthesis of 6-deoxy-6-amino chitosan (**1.15**) starting with chitin

Repeated synthesis of 6-deoxy-6-amino chitosan (**1.15**) from commercial chitosan showed that the polymer was not produced uniformly and therefore, its properties were not consistent. Batch to batch inconsistencies occurred, and the degree of substitution as well as the appearance and solubility differed across the reported literature. One of the aims of this study was to look at other options to achieve the desired polymer while minimising the production of large quantities of waste and attempting to report a scalable process which provides batch to batch consistency. Therefore, it is necessary to take a critical look at the synthetic pathway and find alternative synthetic strategies to minimise waste and impurities.

Attempts were made to improve the original synthesis by effecting small changes. The disadvantage associated for the synthesis of a tosylated polymer, was the use of a hazardous solvent such as pyridine. Additionally, large volumes of ethanol and diethyl ether were required to remove pyridine from the polymer making this method highly undesirable. In order to

decrease the use of the unfavourable pyridine, the tosylation was also performed in *N,N*-dimethylacetamide (DMAc) using Et_3N as the base. The tosylated polymer was successfully synthesised; however, the elemental analysis and IR spectrum indicated a slightly lower sulfur content compared to the reaction in straight pyridine. This was a better alternative to prior procedures, since a smaller quantity of base was used and therefore less hazardous waste produced.

Additionally, the tosylation of *N*-phthaloyl chitosan was also attempted in the ionic liquids 1-butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium acetate as per the co-solvent method reported by Gericke *et al.*²¹ This reaction involved stirring of the polymer at an elevated temperature (80 °C) in the ionic liquid to aid dissolution. Pyridine was then added and the mixture stirred for a further one hour at 80 °C. Thereafter, the reaction was allowed to cool to room temperature overnight. A gel formed which was subsequently treated with tosyl chloride dissolved in pyridine, and stirred for a further eight hours. The solid obtained was washed with ethanol and characterised. The IR spectra of the polymer did not indicate any tosylation in either ionic liquid used.

In the substitution of the tosyl moiety for the azide, alternative solvents were investigated. In an attempt to avoid the use of NMP, acetonitrile (MeCN) and ethanol were tested as alternative solvents for this reaction. The reaction mixtures were refluxed overnight and worked up as per the NMP reaction. IR analysis of the polymers obtained (Figure 2.11) indicated that substitution had occurred; however, the absorbance bands attributed to the tosyl group were still present to a greater degree when compared to the polymer synthesised in NMP. Moreover, DMAc was tested and found to be a suitable alternative, yet the increase in the nitrogen content was lower than the reaction reported in NMP, suggesting a lower degree of substitution.

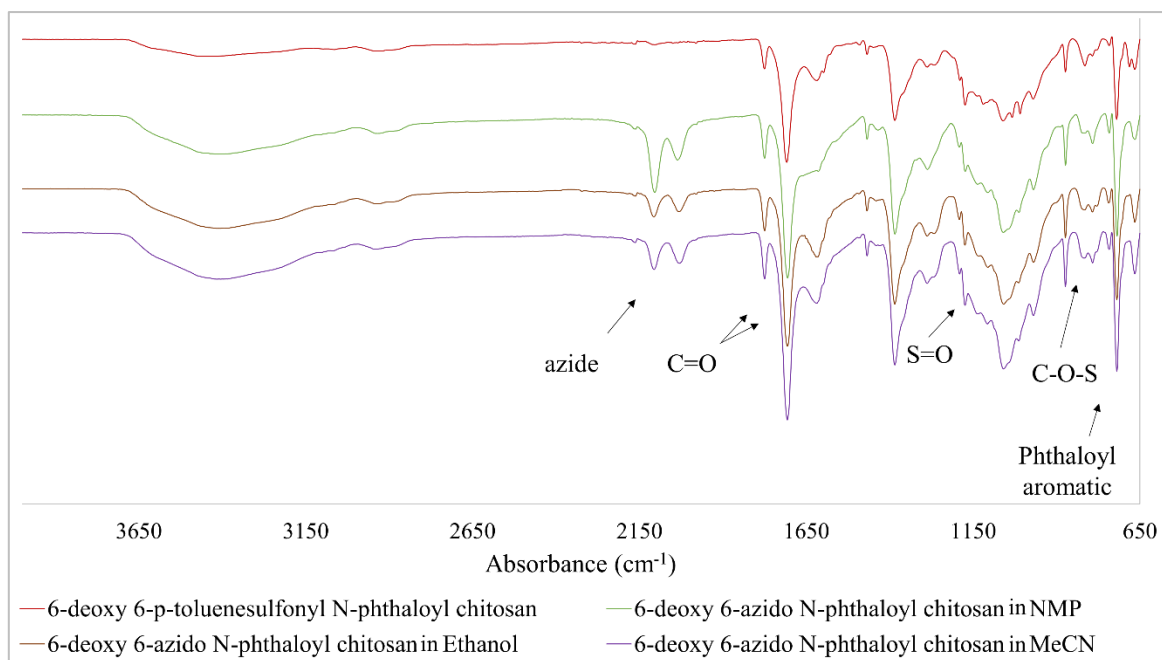
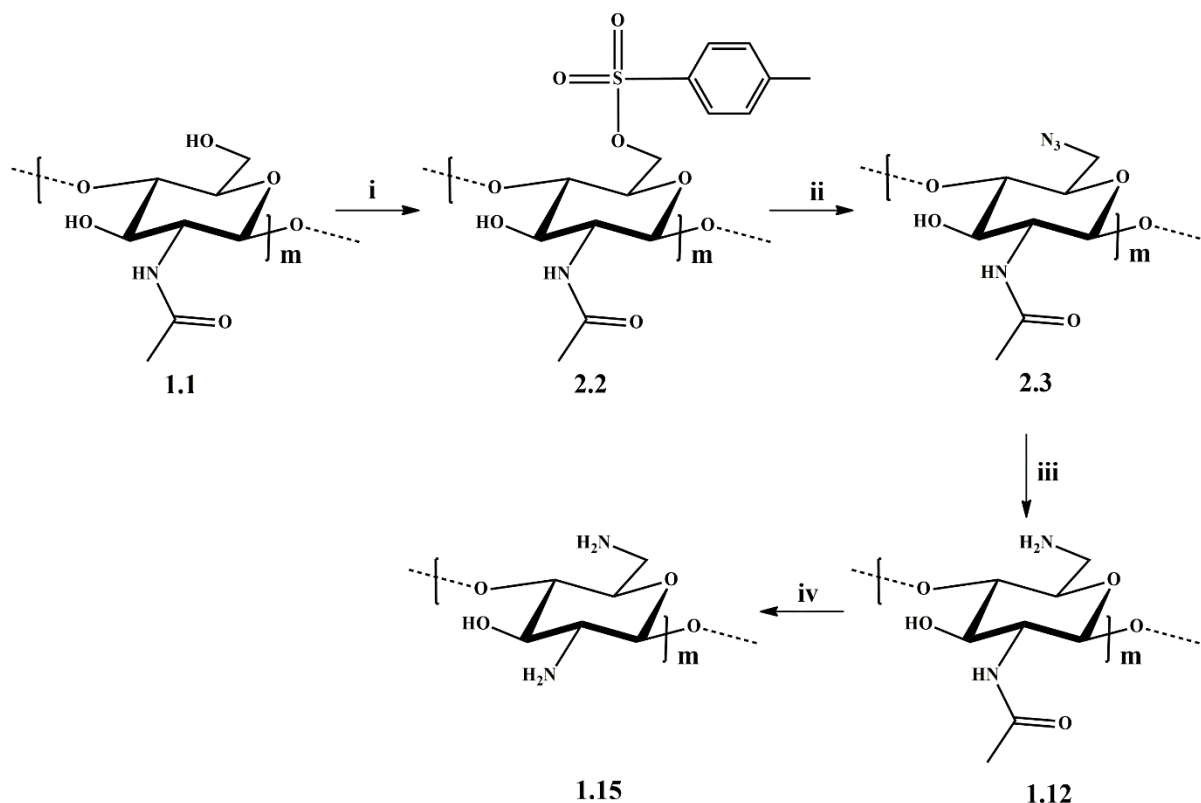


Figure 2.11: The IR spectra of 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) and 6-deoxy-6-azido *N*-phthaloyl chitosan (**1.20**).

An alternative method starting from chitin

The key issues in the modification of chitosan was the use of hazardous solvents and the production of excess waste, which increases the cost of the synthesis and is also considered undesirable in the context of a green chemical process. One approach to minimise these issues and shorten the synthesis of the polymer, involves starting with chitin. Tosylation of chitin followed by C-6 substitution, eliminated the phthaloylation step since the 2-amino is already protected by an acetyl group. Thereafter, an amine functionality was introduced by displacement of the tosyl group and deacetylation to produce diamino chitosan. As illustrated in Scheme 2.7, a synthetic route based on the work by Jardine and co-workers using chitin as the starting material was attempted.¹⁰ Since most modifications are performed on chitosan due to solubility issues associated with chitin, it is advantageous to find a means to solubilise chitin itself. Heterogeneous reactions of chitin have certain disadvantages associated with them. These disadvantages include polymer degradation, deacetylating the chitin to an unknown extent making a reacetylation step necessary and allowing for inconsistency in the product obtained in a reaction. Whereas, homogeneous reactions minimise these disadvantages to a certain extent.³⁵ The solvent system chosen here was the combination of *N,N*-dimethylacetamide (DMAc) and lithium chloride (LiCl).



Scheme 2.7: Synthesis of 6-deoxy-6-amino chitosan starting with chitin. Reagents and conditions: (i) *p*-Toluenesulfonyl chloride, Et₃N, DMAc/LiCl, 5 °C, 48 hr, 99%; (ii) NaN₃, DMAc, 80 °C, 4 hr, 86%; (iii) PPh₃, DMAc, 25 °C, 15 hr, H₂O, 17 hr, 95%; (iv) NaOH (45% w/v), 5 hr, 100 °C, 61%.

C-6 tosylation of chitin

Chitin was stirred in DMAc/LiCl until a homogeneous gel was obtained after approximately 12 hours at room temperature. It was noted that the rate of stirring was an important factor in the dissolution and subsequent reaction of the homogeneous chitin solution.³⁵ Since a gel was formed, insufficient stirring would lead to different degrees of substitution within the same reaction mixture. In the current study, the reaction was performed in a Reactor-Ready™ Lab Reactor which enabled uniform stirring of the reaction mixture as well as efficient jacketed cooling. In addition, the work up (given below) was performed directly in the reaction vessel allowing for quick and easy isolation and purification of the polymer.

The chitin solution was treated with *p*-toluenesulfonyl chloride in the presence of triethylamine at 5 °C to give 6-deoxy-6-*p*-toluenesulfonyl chitin (**2.2**).³⁵ Half the amount of tosyl chloride was used and the reaction time was doubled achieving similar results to the original conditions used. Furthermore, the work-up procedure was changed from precipitating in acetone and washing with methanol, water and then acetone to precipitating in water and washing with ethanol. These modifications were chosen based on the results of the synthesis of the tosyl derivative of chitosan as mentioned above. This minimised the generation of hazardous waste

making the procedure greener in comparison. The polymer was isolated as a uniform brown solid in quantitative yield (99%) after drying. This yield was comparable to that obtained by Yuquan *et al.* which was above 80%. However, the increased yield potentially indicated chlorination at C-3 or C-6 had occurred although the low temperature of the reaction should have avoided this.³⁵ The IR spectrum of the polymer (Figure 2.12) indicated the introduction of two bands at 1160 and 812 cm^{-1} which were assigned to S=O and C-O-S, respectively. The elemental analysis indicated incorporation of sulfur onto the polymer backbone, with 5.75% of sulfur recorded.

In addition, 6-deoxy-6-*p*-toluenesulfonyl chitin (**2.2**) was also synthesised using the method reported by Kurita *et al.* for comparative purposes.⁷ The tosylation of chitin was attempted in a biphasic mixture of NaOH (42% w/v) and chloroform.⁷ The reaction was allowed to proceed for four hours before being transferred into water and the solid collected by filtration while washing with methanol and diethyl ether. The solid obtained appeared to be similar to chitin, however IR and EA analyses indicated that tosylation had occurred to some extent. A second reaction using this method was performed using an extended time of 18 hours where the IR and EA analyses indicated a higher degree of tosylation therefore, suggesting that the reaction was also time dependent.

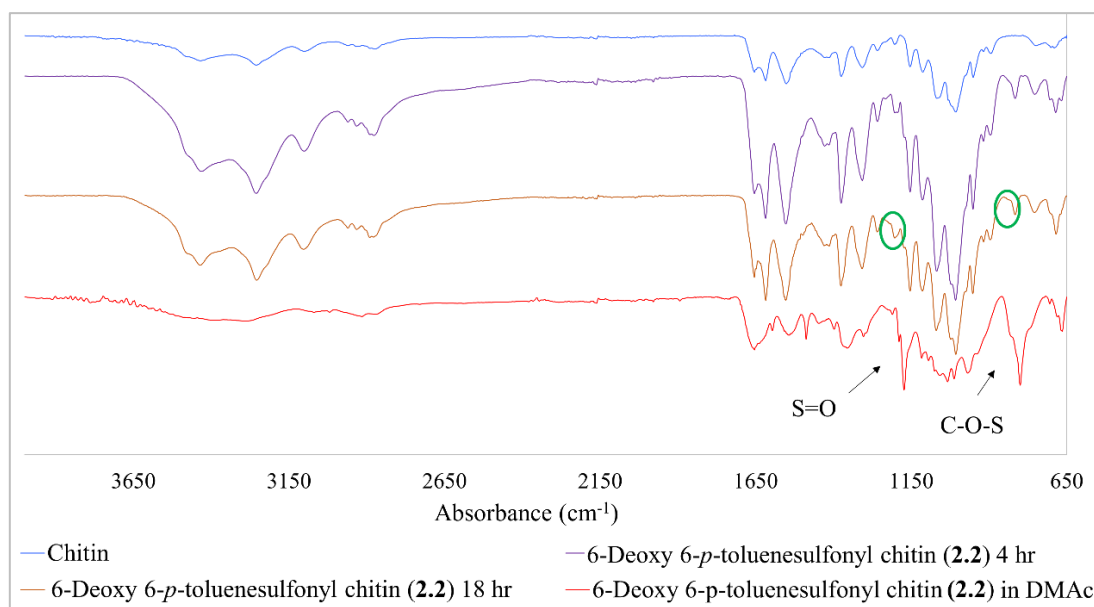


Figure 2.12: IR spectra of 6-deoxy-6-*p*-toluenesulfonyl chitin (**2.2**) synthesised in DMac/LiCl vs that synthesised in aq. NaOH for 4 or 18 hr.

The bands observed in the IR spectrum were much weaker (Figure 2.12, highlighted in green) when compared to that of the reaction performed in DMac and the EA indicated a sulfur

percentage of 1.50% (4 hr) and 2.87% (18 hr) respectively. The drawbacks associated with this method for the production of tosyl chitin, was the hydrolysis or saponification of tosyl chloride leading to the use of excess reagent as well as uncontrolled deacetylation of chitin making an additional reacetylation step necessary to allow for further modification at C-6.

It is clear that the reaction in DMAc/LiCl facilitated the substitution at C-6 as evidenced by the clear absorption bands of the tosyl moiety as compared to the polymer synthesised in the aqueous NaOH solution by the method reported by Kurita *et al.*⁷

Azidation of chitin

The synthesis of the azido substituted chitin (2.3) has previously been reported by Furuhashi *et al.* where halogenated derivatives of chitin were reacted with sodium azide in polar aprotic solvents.³⁶ Conditions which were varied include the solvent, reaction time and temperature. The general procedure involved the halogenated chitin derivative (where C-6 substituent is Br or Cl) being dissolved in a DMAc/LiBr or LiCl solution depending on the halide on chitin, and reacted with sodium azide at 50 °C for twenty four hours. Upon completion of the reaction, water was added and the mixture dialysed against water and thereafter lyophilised. Various degrees of substitution were reported based on the different conditions used.³⁶

In the current study, the substitution of the tosyl moiety for an azide was based on the analogous reaction of chitosan. 6-Deoxy-6-*p*-toluenesulfonyl chitin (2.2) was reacted with sodium azide in DMAc, leading to the nucleophilic displacement of the tosyl group. In the reaction of chitosan with sodium azide, NMP was used as the solvent as reported by Jardine *et al.*¹⁰ Changing the solvent to DMAc gave similar results as that obtained for NMP. The IR spectrum of the synthesised polymer confirmed the introduction of an azide group with a strong absorption band at 2099 cm⁻¹ (Figure 2.13). This result agreed with that reported in the literature.³⁶ Elemental analysis indicated an increase in the nitrogen content of the polymer further supporting the successful formation of the azide, in the synthesis of 6-deoxy 6-azido chitin (2.3).

Reduction of C-6 azido chitin

The reduction of the azidodeoxy derivative of chitin has previously been reported.³⁶ This reduction in literature, was accomplished by dissolving the polymer in DMSO followed by treatment with sodium borohydride at 60 °C for twenty four hours. The reduced derivative was recovered by treatment with 1 N HCl, dialysed against water and finally recovered by lyophilisation.³⁶ This method is not appealing due to the use of sodium borohydride which is

dangerous and the added production of inorganic impurities which is challenging to remove from the polymer. In the current study, 6-deoxy 6-amino chitin (**1.12**) was synthesised by the reduction of the azide using triphenylphosphine *via* the Staudinger mechanism. The IR spectrum of (**1.12**) (Figure 2.13) indicated the disappearance of the azide band and the increase in the intensity of the amine bands. The elemental analysis supported the synthesis of the polymer. To confirm the presence of the amine, a ninhydrin assay was conducted. Ninhydrin is known to react with free amines, either primary or secondary to produce a deep purple or blue colour. The ninhydrin test performed here was positive, indicating the presence of an amine.

6-Deoxy-6-amino chitosan (**1.15**)

6-Deoxy-6-amino chitin (**1.12**) was subsequently deacetylated using aqueous NaOH (45%) to obtain 6-deoxy-6-amino chitosan (**1.15**). A ninhydrin test gave a deep purple intensity when this polymer was tested. The IR spectra of the polymers used in this route for the synthesis of 6-deoxy 6-amino chitosan (**1.15**) are given in Figure 2.13.

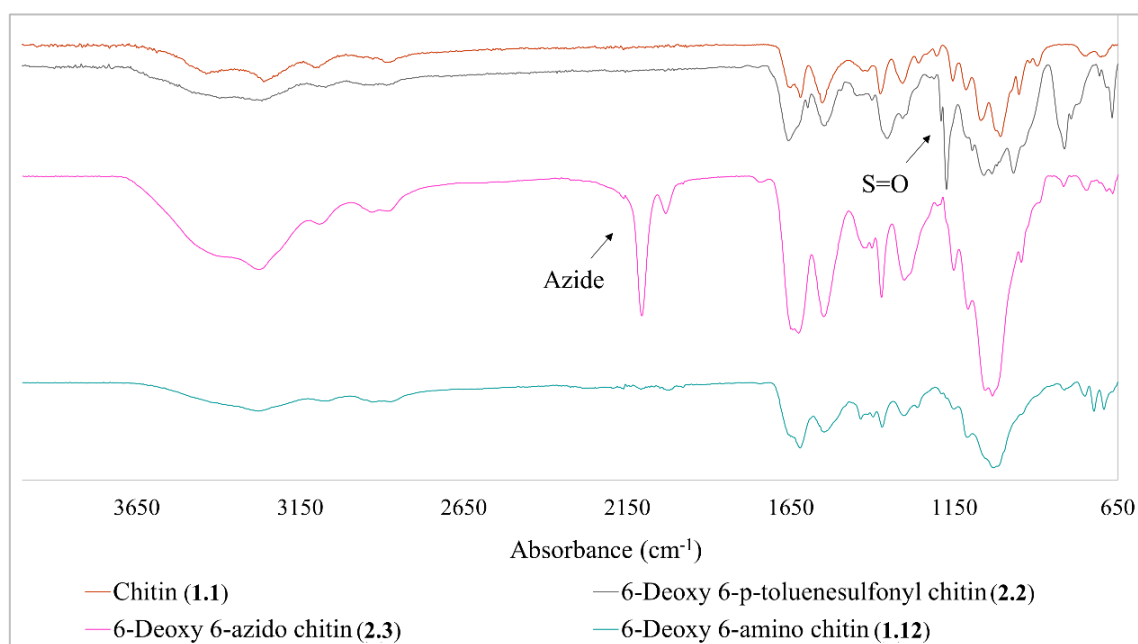


Figure 2.13: The IR spectra of the intermediates in the synthesis of the diamino polymer.

As mentioned previously, comparison of the degree of substitution of derivatised chitosan polymers is quite challenging due to the broad variety of techniques used. The DS for each polymer synthesised in the route using chitin was calculated using the equation below:

$$DS = \left[\frac{\left(\frac{C}{N}\right)_{\text{derivative}}}{\left(\frac{C}{N}\right)_{\text{chitin}}} \right]$$

Since chitin is assumed to be fully acetylated, the degree of deacetylation is not accounted for. The values obtained for the derivatives are reported in Table 2.2. As mentioned above, these values were not compared to literature reports due to the inconsistency in reporting of DS values, although, literature values are given in the table. Where a DS of greater than one is observed, this could indicate the presence of impurities or substitution at unprotected sites such as C-2 or C-3.

Table 2.2: The DS of polymers synthesised in the current study.

Polymer	%C*	%N*	C/N	DS	Literature DS
Chitin (1.1)	43.31	6.98	6.20	-	
6-Deoxy-6- <i>p</i> -toluenesulfonyl chitin (2.2)	44.70	5.30	8.43	1.36	0.25 – 3.0 ^{22, 35}
6-Deoxy-6-azido chitin (2.3)	41.88	16.87	2.48	0.40	0.47 – 0.57 ³⁶
6-Deoxy-6-amino chitin (1.12)	47.39	8.14	5.82	0.94	0.48 ³⁶
6-Deoxy-6-amino chitosan (1.15)	44.24	9.40	4.71	0.76	0.7 - 0.94 ^{3, 18}

*determined by elemental analysis

Following the introduction of the additional amine onto the chitosan backbone, it was important to determine whether the methods used were efficient and if they can be used to produce large scale quantities of the desired polymers. The routes reported by Satoh and Jardine *et al.* were not scalable due to their large reagent mass transfers and use of highly toxic solvents such as DMF, NMP and pyridine.^{3, 10} These solvents are highly undesirable in the synthesis of biopolymers, which may have a number of potential applications especially in the biomedical field requiring large scale synthesis. Other synthetic routes use hazardous chemicals and extra steps in addition to hazardous solvents. As mentioned previously, Yang *et al.* in the tosylation of *N*-phthaloyl chitosan used triethylamine as a base in the presence of pyridine.¹⁸ The same study used NaBH₄ in DMSO for the reduction of the azide to the amine where this reduction reaction was allowed to proceed for 70 hours. Overall, two separate steps were used in deprotection and reduction of the polymer to yield the final product.¹⁸ Therefore, this synthetic method is not amenable to the scaling up due to the reagents and conditions used. The synthesis of low molecular weight 6-deoxy-6-amino chitosan by Kim *et al.* did not involve the protection of the amine at C-2 and instead the polymer was tosylated directly.³⁷ In this study, the azido chitosan derivative was reduced using lithium aluminium hydride (LiAlH₄) in dioxane, making the scale up synthesis of this polymer quite challenging due to the use of hazardous reagents.³⁷

The synthetic conditions reported in the current study while not exactly 'green' are marginally better and generally have similar yields compared to previous synthetic methods. However, the polymers produced were not completely water soluble, which does limit their application in certain fields. Therefore, other synthetic routes need to be investigated for the synthesis of 6-deoxy-6-amino chitosan using a scalable, more environmentally friendly process.

2.4 Conclusion

The current study investigated the synthesis of 6-deoxy-6-amino chitosan with a focus on the improvement of the previously reported literature methods. The polymer 6-deoxy-6-amino chitosan (**1.15**) was synthesised using the method reported by Jardine *et al.*¹⁰ This method was modified in an attempt to produce a uniform polymer which could be synthesised on a large scale. Solubility was optimised at each step where the *N*-phthaloylation of chitosan was performed in 10% AcOH versus the reported 95% DMF/H₂O. Tosylation was carried out in anhydrous pyridine, DMAc/Et₃N and attempted in the ionic liquids 1-butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium acetate (with pyridine as co-solvent). In the reaction to introduce the azide moiety, DMAc, acetonitrile and ethanol were tested as alternatives to NMP with some success.

In addition to this method, chitin was also used as the starting polymer following similar modifications; however a protection step was not necessary and the final polymer had to be deacetylated to produce the chitosan derivative. The tosylation of chitin was performed in DMAc/LiCl where the method was improved by a reduction in the amount of reagent used as well a decrease in the waste generated during polymer processing by altering work up conditions. Tosylation was attempted in NaOH solution with limited success, while azidation and subsequent reduction produced the amino chitin derivative which was deacetylated to obtain 6-deoxy-6-amino chitosan. The 6-deoxy-6-amino chitosan synthesised using this methods was reproducible and the overall synthesis was slightly improved compared to the original reported methods. However, there is still a need to improve on the introduction chemistry of an amine at C-6 of chitosan.

Further investigation into an improved synthesis method as well as the application of these modified polymers of chitosan are discussed in the following chapters.

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Chapter Three

A study of the solubility of chitin and chitosan

3.1 Background

Hitherto, an expedient synthesis of 6-deoxy-6-amino chitosan has been demonstrated. However, one major drawback was the use of toxic solvents in its synthesis and the generation of large quantities of waste.¹ Due to the poor solubility profile of chitin and chitosan in most solvents, the use of conventional toxic solvents needs to be re-evaluated. The synthetic routes which utilises hazardous solvents such as DMF, NMP and pyridine will soon be restricted, due to the recent change in European chemicals legislation, REACH—the Registration, Evaluation and Authorisation of Chemicals.^{1, 2} As a result, a significant number of chemical applications will become very expensive or even be prohibited. It is therefore, necessary to investigate alternative synthetic methods to avoid the use of highly toxic and environmentally unfriendly reagents and solvents.² It is understood that the majority of common organic synthetic methods/reagents cannot be changed immediately and there needs to be a gradual shift to encourage the development of more environmentally friendly processes. In the case where hazardous solvents, reagents or processes cannot be avoided, it is preferable that a less harmful alternative be employed.² With the rise in bio-based solvents which do not exhibit pronounced toxicity, the potential applications related to chitin and chitosan increases.

When defining solubility it is generally understood that the solute dissolves in the solvent. A point to note with chitin and chitosan, these polymers dissolve in a limited number of solvents as discussed in Chapter One. The majority of solvents used for the modification of chitin and chitosan do not fully dissolve the polymers; however, they allow the polymers to swell and disperse facilitating modification. Therefore, when referring to the solubility of the polymers here it is generally referring to the dispersibility as well.

In silico methods have been used to study the behaviour in solution and aid in the modification of polymers. The lack of solubility of chitin and chitosan is a key factor in the underutilisation of these polymers. Solubility parameters of polymers can be used to predict their behaviour in certain solvents. One of the most commonly employed methods to predict a polymer's expected solubility behaviour, is the Hansen solubility parameters (HSP). In the case of chitin and chitosan there has been very little work in this area where only one citation of the application of a similar method was found.³ A comment on this study was recently published showing

revised calculations for the solubility parameters of chitin and chitosan.⁴ Gharagheizi *et al.* and a later study by Vebber *et al.* reported on improved methods of determining the HSP for polymers based on intrinsic viscosities and genetic algorithms respectively.^{5, 6} These methods suffer from drawbacks and incorrect predictions due to the lack of good data for most solvents. Therefore, the majority of research with regard to chitin and chitosan solubility relies on computational modelling of the polymers in specific solvents. One such example is the study of the dissolution of chitosan in the ionic liquid 1-butyl-3-methylimidazolium acetate, where density functional theory (DFT) was used to predict the polymer behaviour.⁷

The solubility of polymers can be predicted using HSP and computational modelling. The results can be used to potentially guide the experimental aspect of the research. Solubility studies would also provide valuable data on the use of certain solvents for the modification of chitin and chitosan. In addition, the influence of different co-factors such as inorganic salts and co-solvents needs to be considered.

In this chapter, the behaviour of chitin and chitosan in different solvents including ionic liquids and bio-based solvents which can be modelled and compared to conventional solvents were studied. Building on these results, a solubility study using a variety of solvents and the application of selected solvents to a model reaction was conducted and these are discussed below. The use of various co-factors to replace the inorganic salt utilised in the solubilisation of chitin was investigated.

3.2 Hansen solubility studies of chitin and chitosan

Hansen solubility parameters (HSP) are typically used to find a suitable solvent for polymer processing. These parameters are numerical values related to the intermolecular forces in a solvent.⁶ The three major forces considered are:

δ_d - energy related to the dispersion forces between molecules

δ_p - energy related to the dipolar intermolecular forces between molecules

δ_h - energy related to the hydrogen bonds between molecules

These parameters are combined to give the solubility parameter δ , where

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2$$

The HSP distance between two molecules is referred to as R_a and gives a measure of how alike the molecules are. This distance can be used to plot a sphere which then gives a zone in which the solvents lie depending on their HSP parameters.⁵ The equation determining the radius (R) of the sphere is given by:

$$(R_a)^2 = 4(\delta_{d1} - \delta_{d2})^2 + (\delta_{p1} - \delta_{p2})^2 + (\delta_{h1} - \delta_{h2})^2$$

To determine if two molecules, in this case the polymer and the solvent are within range of each other in the Hansen space, a value known as the interaction radius is used given by R_0 . The radius of the sphere and the interaction radius is used to obtain the relative energy difference (RED) between the two molecules. This difference indicates the solubility of the sample in the solvent where:

$$RED = R_a/R_0$$

$RED < 1$ the molecules are alike and will therefore dissolve

$RED = 1$ the sample will partially dissolve or swell

$RED > 1$ the sample will not dissolve

An example of a typical Hansen solubility sphere is given in Figure 3.1.⁵

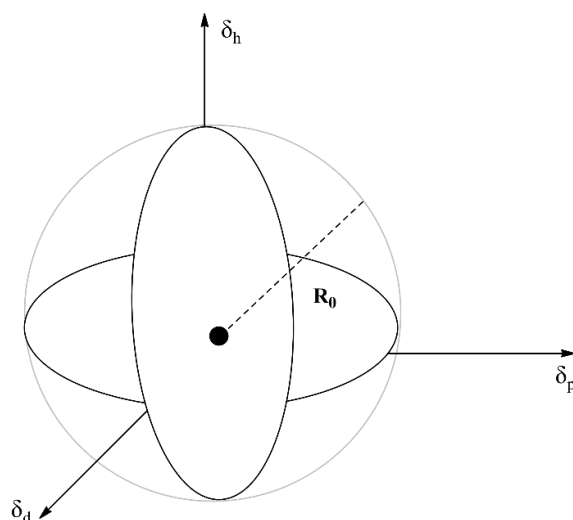


Figure 3.1: A typical Hansen solubility sphere.

In order to study the solubility of chitin and chitosan, various solvents were chosen and evaluated. The selected solvents are shown in Table 3.1 together with their average partial solubility parameters obtained from literature.^{6, 8}

Table 3.1: Hansen solubility parameters of selected solvents.^{6, 8}

Solvent	δ_d	δ_p	δ_h
Polar Aprotic Solvents			
Acetone	15.5	10.4	7.0
Acetonitrile	15.3	18.0	6.1
Cyclopentanone	17.9	11.9	5.2
Cyrene TM	18.8	10.6	6.9
DMAc	17.8	14.1	11.8
Dimethyl carbonate	15.5	8.6	9.7
Dimethylformamide	17.4	13.7	11.3
DMSO	18.4	16.4	10.2
γ -Valerolactone	16.9	11.5	6.3
NMP	18.0	12.3	7.2
Propylene carbonate	20.0	18.0	4.1
Sulfolane	20.3	18.2	10.9
Tamisolve [®]	17.8	8.2	5.9
Hydrocarbons			
Cyclohexane	17.8	6.3	5.1
<i>p</i> -Cymene	17.3	2.3	2.4
Toluene	18.0	1.4	2.0
Medium Polarity Solvents			
DCM	18.2	6.3	6.1
1,3-Dioxolane	18.1	6.6	9.3
Ethyl acetate	15.8	5.3	7.2
THF	16.8	5.7	8.0
Polar protic Solvents			
Acetic acid	14.5	8.0	13.5
Ethanol	15.8	8.8	19.4
Glycerol	17.4	12.1	29.3
Methanol	15.1	12.3	22.3
Propionic acid	14.7	5.3	12.4
Universal Solvent			
Water	15.6	16.0	42.3

In addition, the Hansen solubility parameters for chitin and chitosan were obtained from literature. The original parameters were reported by Khan *et al.*; however, a recent communication by Lehnert and co-worker pointed out discrepancies with the calculations relating to the determination of the HSP parameters.^{3, 4} This communication reported that a number of misprints, incorrect calculations and data evaluations were reported in the determination of the solubility parameters.⁴ These parameters were subsequently recalculated and are shown in Table 3.2 together with the original reported values in brackets. Additionally, the HSP values for selected synthetic polymers are shown to highlight the difference to chitin and chitosan where it is noted that there is a significant difference in the δ_p and δ_h related to the

dipolar intermolecular forces between molecules and the hydrogen bonding between molecules.

Table 3.2: HSP parameters for chitin and chitosan as well as selected synthetic polymers.^{4, 9}

Polymer	δ_d	δ_p	δ_h
Chitin	23.3 (23.9)	14.9 (17.9)	22.5 (22.2)
Chitosan	22.9 (22.8)	16.6 (17.1)	26.6 (26.6)
Polycaprolactone	15.9	1.4	2.0
Polyethylene glycol	20.3	9.6	6.0
Poly lactide	19.8	4.0	6.7

However, HSP parameters for certain solvents of interest have not yet been reported. These include the ionic liquid 1-butyl-3-methylimidazolium acetate.

Comparing the values for the different solvents to that of chitin and chitosan, it is evident that finding a solvent for the solubilisation of these polymers is a challenge. The HSP parameters of most solvents reviewed here do not fall in the same region of chitin and chitosan. However, these parameters do not account for any other factors such as the presence of an inorganic ion, where one such example is the use of a DMAc/LiCl combination in the complete dissolution of chitin. The solvents which present parameters that are somewhat similar to chitin and chitosan are CyreneTM, DMAc, DMF, DMSO, glycerol, propylene carbonate, NMP and sulfolane. These solvents can potentially be used in the dissolution/swelling and modification of chitin and chitosan. Computational modelling was further used to assess the behaviour of these polymers using selected solvents.

3.3 Computational modelling for the dissolution of chitosan in alternative solvents

Computational modelling can be utilised to simulate and study the behaviour of complex systems by a combination of mathematics, physics and computer science. In a computational model there are numerous variables which characterise the system being studied. These variables are adjusted individually or in combination and the effect of the changes are observed. Model simulations can help researchers predict outcomes in the real system that is being studied in response to changing conditions. Modelling can expedite research by allowing scientists to conduct thousands of simulated experiments. These simulations can help identify the experiments that are most likely to benefit the researcher. Many researchers in the field of chitin and chitosan research have realised that computational analysis of these polymers can provide a better understanding at the molecular level which saves time spent on research in the

laboratory.^{10, 11} Therefore, materials can be designed on truly rational design criteria instead of using trial and error.¹²

In the current study density functional theory (DFT) has been used to assess the strength and nature of the interactions between chitosan and selected solvent molecules. The strength and nature of the hydrogen bonding present in the polymer was characterised using the Quantum Theory of Atoms In Molecules (QTAIM) and Natural Bond Orbital (NBO) analyses. Whereby, QTAIM was used to confirm the existence of hydrogen bonding and to obtain electron densities. NBO analysis was used to study the second order perturbation stabilisation energies ($E(2)$) associated with hydrogen bonding interactions. Molecular Dynamics (MD) simulations were conducted to investigate the solvent structure and average interaction energies in an explicit solvent environment.

Modelling of chitin and chitosan presents its own set of challenges. In previous molecular dynamics calculations, a chitin/chitosan membrane was modelled using fifteen monomer unit chains with three different types of monomers (*N*-acetyl glucosamine, glucosamine and protonated glucosamine). This was done in order to simulate a 66% degree of deacetylation of the sample.¹¹ Owing to the size and complexity of chitosan chains, it was not possible to directly use the polymer in these computational models therefore, in the current study 1,4-dimethoxyglucosamine (**3.1**, Figure 3.2) was used instead. In the current study, the Molecular Dynamics calculations were performed using a 1,4-dimethoxychitobiose dimer (**3.2**, Figure 3.2), while other theoretical studies determining the dissolution of chitosan in a chosen solvent used chitobiose, a dimer of β -1,4-linked glucosamine units.⁷ The hydroxyl groups of 1,4-dimethoxychitobiose at C-1 and C-4 are bonded to another carbon atom, thereby making it a better representation of the polymer.

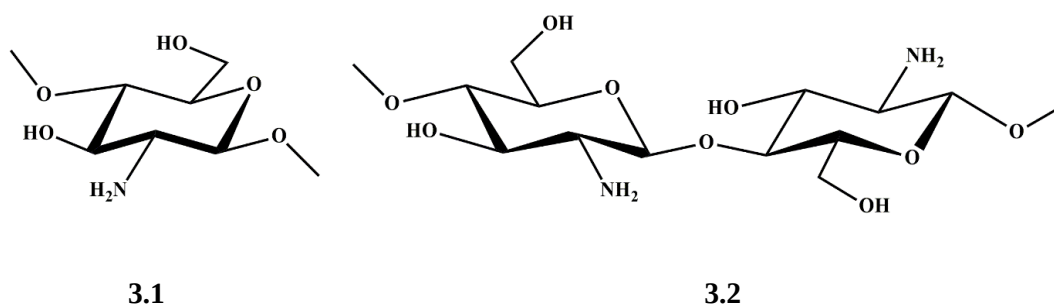


Figure 3.2: 1,4-Dimethoxyglucosamine (**3.1**) and 1,4-dimethoxychitobiose (**3.2**).

The level of theory behind these computational methods will not be discussed here; for a comprehensive overview of the underlying principles refer to the works reported by Cunha,

Tian and Coa *et al.*^{7, 13, 14} These studies focused on chitin and chitosan from a computational aspect and its molecular structure, conformational dynamics and behaviour in solution.^{7, 13, 14}

The solvents selected for assessment included the ionic liquid, 1-butyl-3-methylimidazolium acetate (BMIMAc) (**3.3**, Figure 3.3), the new bio-based solvent Cyrene[™] (**1.30**, Figure 3.3), and the dipolar aprotic solvents, *N,N*-dimethylformamide (DMF) (**3.4**, Figure 3.3), dimethyl sulfoxide (DMSO) (**3.5**, Figure 3.3), and *N*-methyl-2-pyrrolidone (NMP) (**3.6**, Figure 3.3). These particular solvents were chosen due to the similarity in their HSP parameters to that of chitin and chitosan. Although DMAc showed similar HSP parameters to the solvents chosen, it was not assessed through computational modelling. This was due to the presence of LiCl which was required for complete dissolution of the polymer. Studies have been performed on the dissolution of cellulose in a DMAc/LiCl mixture where it was found that the relatively weak solvation of Li⁺, Cl⁻, and the polysaccharide chains by DMAc results in strong effective interactions of Li⁺ and Cl⁻ ions with the polysaccharide, leading to cellulose dissolution. In addition, the small size of the Li⁺ cation facilitates its binding to multiple interaction sites including spatially restricted regions around the ether linkages connecting neighbouring polysaccharide residues. Due to the similarity of cellulose to chitin and chitosan, it can be inferred that the dissolution mechanism is similar.

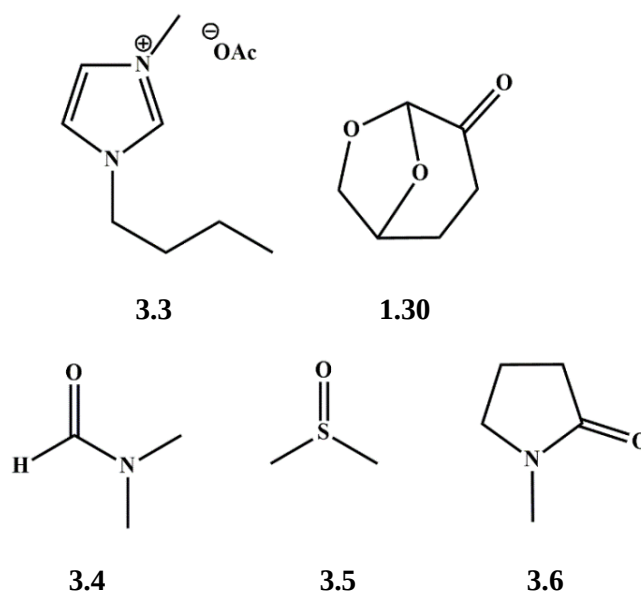


Figure 3.3: Solvents used in the current computational modelling study BMIMAc (**3.3**), Cyrene[™] (**1.30**), DMF (**3.4**), DMSO (**3.5**) and NMP (**3.6**).

The above mentioned solvents will now be discussed for their suitability in the dissolution of chitosan. As previously mentioned, the move towards greener, more bio-based chemicals is being encouraged to promote sustainability. As a result of this, new solvents are being

investigated for their potential to replace conventional hazardous solvents without compromising the desired outcome.¹⁵ Among the new solvents reported is the bio-based CyreneTM and ionic liquids.

CyreneTM

One such bio-based solvent which has been gaining recognition, is dihydrolevoglucosenone (trade name: CyreneTM). It is obtained from the hydrogenation of levoglucosenone, which is a chiral bicyclic molecule obtained from the pyrolysis and acid treatment of cellulose. Sherwood *et al.* has suggested CyreneTM as a bio-based alternative to dipolar aprotic solvents, as computational modelling has shown that it has similar solvating properties to NMP, DMF and sulfolane.⁸ CyreneTM has been used as an alternative solvent for heteroatom alkylations, nucleophilic fluorinations, and in metal-organic frameworks (MOFs) synthesis and as well as in the synthesis of ureas from isocyanates and secondary amines.^{8, 16, 17} A recent paper reported on the use of CyreneTM in Sonogashira cross-coupling and Cacchi-type annulation together with its scope and limitations in terms of thermal and chemical (base) stabilities.¹⁸ The increasing amount of literature relating to the use of CyreneTM indicates that the interest in bio-based solvents is rapidly expanding to conventional organic synthesis.

Ionic liquids

Ionic liquids (ILs) have been particularly well researched in the last decade, where they have been referred to as ‘green and designer media’ for chemical reactions.¹⁹ These liquids typically consist of an organic component and an ion with a delocalised charge, where most ILs consist of an organic cation and an inorganic anion. Typical anions and cations are given in Figure 3.4.

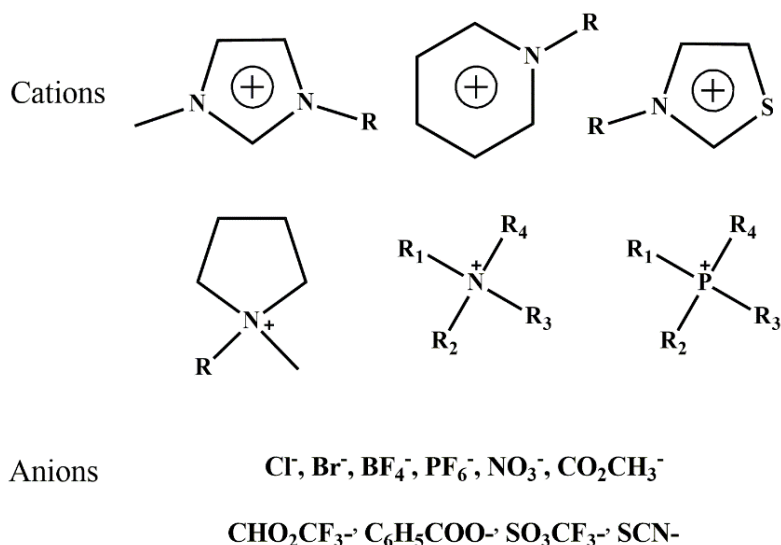


Figure 3.4: Common anions and cations used in the synthesis of ionic liquids.

Ionic liquids have been classified as environmentally friendly solvents due to their negligible vapour pressure and high thermal stability. These characteristics plus its recyclability, allow ionic liquids to be considered as cost effective alternatives to volatile conventional organic solvents. It is generally accepted that the melting points of ILs are typically less than 100 °C. In addition, the properties of these liquids can be tailored for specific purposes. Properties which are generally manipulated include, conductivity, density, melting point, hydrophobicity, polarity and viscosity. Ionic liquids, in addition to their use as solvents, has found other uses as organocatalysts, organometallic catalysts, extraction media, in the synthesis of nanomaterials, in polymerisation reactions and in electrochemistry as the solvent or part of the reaction.²⁰ Chitin and chitosan processing and modification has been previously investigated using ILs. Chitin has been successfully extracted from crustacean shells using ILs under microwave irradiation and subsequently electrospun.²¹ Bocheck and co-workers investigated the solubility of cellulose, chitin and chitosan in various ionic liquids and the structural organisation of the regenerated polysaccharides.²² They found that factors affecting solubility includes pressure, temperature, the presence of an aprotic solvent and the anion of the IL. In the case of chitosan, solubility was also dependent on the degree of deacetylation of the polymer. It is important to note that the structure of the ionic liquid affects the solubility of the biopolymers.²² Chen *et al.* studied the influence of the anion on chitosan solubility in 1-butyl-3-methylimidazolium based ILs.²³ It was suggested that the IL disrupts the hydrogen bonds in the polymer rendering it soluble, making the hydrogen bond accepting/donating ability of the IL key to solubilisation. This study reported that polymer solubility is directly proportional to the hydrogen bond accepting ability of anions present in the IL, as well as the temperature of the solution.²³ The dissolution mechanism of chitosan was studied by Sun and colleagues, and it was reported that in acetate based ILs the cations and anions affect the dissolution of the polymer.²⁴ The dissolution, processability, and functionalisation of chitin and chitosan in ILs has been recently reviewed by Silva *et al.* who pointed out that many chemical reactions including acetylation, amidation and cross-linking among others have been reported.²⁵

Dipolar aprotic solvents

Dipolar aprotic solvents such as DMF, DMSO and NMP were used for comparative purposes. DMAc, which is commonly used for the dissolution of chitin and chitosan, was not modelled due to the polymer dissolution generally requiring the presence of lithium chloride. DMAc/LiCl modelling was not attempted due to the complexity of the interactions involved.

DFT studies

As mentioned, 1,4-dimethoxyglucosamine will be used as a template for chitosan, due to its smaller structure. Both 1,4-dimethoxyglucosamine and the selected solvent were modelled using DFT after complexation and optimisation. The complexed structures are shown in Figure 3.5 and a list of the calculated interaction energies is given in Table A1 in the Appendix (Cyrene[™] has three hydrogen bond accepting oxygens which can potentially interact with the hydroxyl or amine group present on the 1,4-dimethoxyglucosamine moiety; therefore, three different orientations were modelled).

The results indicated that the total interaction of the solvents with 1,4-dimethoxyglucosamine decreases in the order of BMIMAc > DMSO > DMF > NMP > Cyrene[™], suggesting that the strongest interaction of 1,4-dimethoxyglucosamine occurs with the ionic liquid BMIMAc. It is proposed that this interaction between the acetate anion and 1,4-dimethoxyglucosamine is due to the anions in the IL breaking the hydrogen bonds present. The hydrogen bond accepting and donating abilities of an IL are directly related to the anion present. The study by Chen *et al.* reported that the solubility of chitosan increased with an increase in the hydrogen bond accepting ability of the anion, where it was found that BMIMAc dissolved chitosan to a greater extent compared to the other anions tested.²³ This study postulated that hydrogen bonds are formed between the proton on the hydroxyl and/or amine functional groups on chitosan and the oxygen of the acetate anion. These hydrogen bonds subsequently disrupts the inter- and intra-molecular hydrogen bonds present in the polymer allowing for dissolution.²³

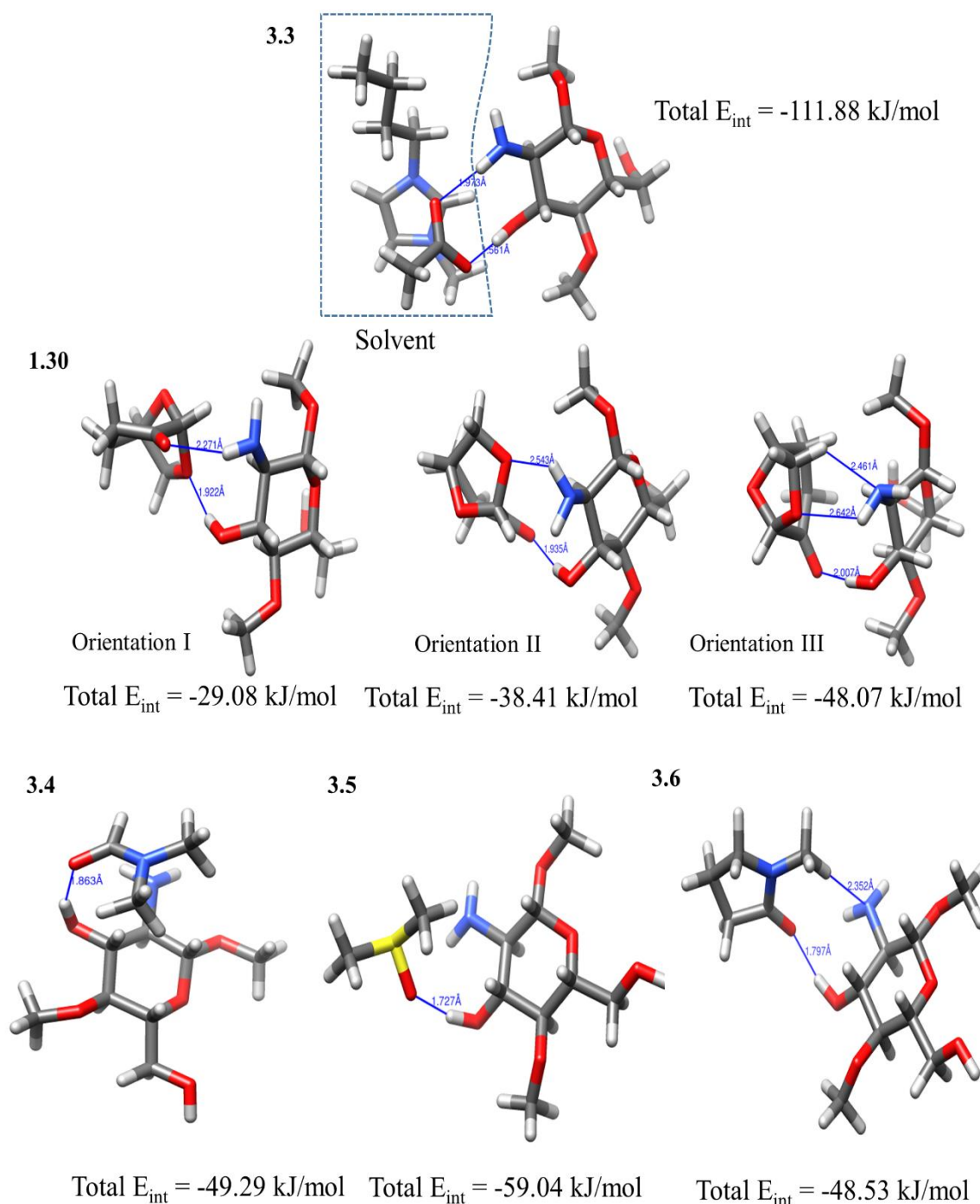


Figure 3.5: Optimised complexes of 1,4-dimethoxyglucosamine with BMIMAc (3.3), CyreneTM (1.30), DMF (3.4), DMSO (3.5) and NMP (3.6). Bond lengths are given in Angströms (Å).

Even though 1,4-dimethoxyglucosamine forms only one hydrogen bond with each of DMSO and DMF, they have the greatest interaction of the neutral solvents. DMSO was found to be the stronger of the two which can be attributed to the fact that it has a higher dipole moment compared to DMF. NMP and CyreneTM show similar behaviour with lower interaction energies. As mentioned above, CyreneTM possesses three hydrogen bond accepting oxygens;

therefore, three different orientations were modelled. These hydrogen bond accepting oxygens can interact with the hydroxyl and/or amine group on the 1,4-dimethoxyglucosamine moiety. Of the three orientations given, orientation III possesses the highest energy as it has the strongest interaction. Therefore, it was suggested that orientation III would have a stronger hydrogen bond disrupting ability. Following on from this result, a further calculation was used to determine the dipole moment and charge of orientation III which was calculated and compared to that of DMF and NMP. The results showed that Cyrene™ had a higher dipole moment and similar charge values on the oxygen atoms compared to DMF and NMP. Contrary to this result, the interaction energy of Cyrene™ with 1,4-dimethoxyglucosamine is lower than DMF or NMP. A possible explanation for this result, is that the Cyrene™ molecule being a bulky and ring-fused structure initially forms one hydrogen bond with the 1,4-dimethoxyglucosamine molecule and this bond restricts further hydrogen bond formation.

QTAIM analysis

QTAIM analysis of 1,4-dimethoxyglucosamine and the selected solvent was performed to identify hydrogen bonds present. The electron densities of the hydrogen bonds formed and their corresponding energies are given in Table A2 in the Appendix. The results obtained agreed with that reported by the DFT analysis where the largest interaction energy is between the IL BMIMAc and the model compound. This result further confirmed that the IL with the acetate ion was the best solvent predicted for the dissolution of chitosan. The analysis supported the order of interaction between the solvents and 1,4-dimethoxyglucosamine, with DMSO displaying a greater energy compared to DMF, NMP and Cyrene™. Therefore, DMF, NMP and Cyrene™ were not the top choices for reactions of chitosan which are required to be mostly homogenous.

Natural bond orbital analyses

Natural bond orbital (NBO) analyses on 1,4-dimethoxyglucosamine and the selected solvents was used to obtain second order perturbation stabilisation energies, $E(2)$, which is related to the strength of the hydrogen bonding present. The energies obtained are given in Table A2 in the Appendix. From the results obtained, it was noted that the greatest stabilisation energy occurs in the BMIMAc...1,4-dimethoxyglucosamine complex. This energy is due to the interaction of the lone pairs of electrons of O1_{AC} on the acetate anion with the $\sigma^*(\text{O3} - \text{H3})$ antibonding orbital of 1,4-dimethoxyglucosamine. This finding further confirmed the results obtained by QTAIM analysis, in that the O1_{AC}...H3 – O3 hydrogen bond was the strongest compared to the hydrogen bonds formed when 1,4-dimethoxyglucosamine interacts with

Cyrene™, DMF, DMSO and NMP. The stabilisation energy is 249.52 kJ/mol which is significantly greater than the value which is obtained when the BMIMAc cation is the donor and the glucosamine hydroxyl the acceptor (31.34 kJ/mol). These results support literature reports that have found that the anion plays an important role in polymer dissolution.²³ For the complexes formed by 1,4-dimethoxyglucosamine with BMIMAc, Cyrene™, DMF, DMSO, and NMP, the largest $E(2)$ values for each complex correspond to the $O_{\text{solv}} \cdots H3 - O3$ hydrogen bond (where O_{solv} is the hydrogen bond accepting oxygen of the respective solvent molecules), and were found to be 249.53, 37.40, 29.79, 46.74 and 51.46 kJ/mol, respectively (Table A2). These values decrease in the order BMIMAc > NMP > DMSO > DMF > Cyrene™. This trend gives an indication of the hydrogen bonding present in the solvents systems investigated which can determine the solvent which would interact the greatest with the polymers.

Molecular dynamics calculations

Molecular dynamics calculations (Table A3 in the Appendix) were performed in cubic boxes containing (a) 150 BMIMAc ion pairs (150 1-butyl-3-methylimidazolium cations and 150 acetate anions) (b) 400 Cyrene™ (c) 600 DMF, (d) 500 DMSO, and (e) 400 NMP solvent molecules using the 1,4-dimethoxychitobiose molecule (3.2, Figure 3.6).

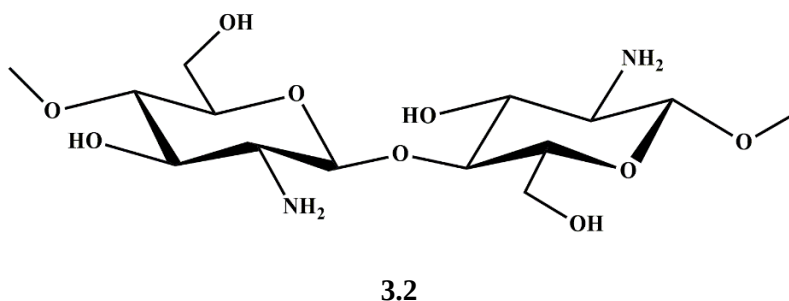


Figure 3.6: The 1,4-dimethoxychitobiose molecule (3.2).

Molecular dynamics simulations using 1,4-dimethoxychitobiose yielded the average interaction energies (with their corresponding standard deviations) shown in Table A3 in the Appendix. The total interaction energies were normalised to a value per solvent molecule as the various simulated systems contained a different number of solvent molecules.

Results indicated that the total interaction energy per solvent molecule (E_{total}/N) decreased in the order BMIMAc > NMP > Cyrene™ > DMSO > DMF. This pattern was not consistent with the results obtained by DFT calculations; therefore, a clear conclusion could not be drawn. However, as seen with the DFT calculations, the interaction energies for the dimer in Cyrene™,

DMF, DMSO and NMP were relatively close; therefore, differentiating between the results was not possible. QM calculations indicated that DMSO had the second strongest interaction with the solute molecule, whereas MD calculations showed that NMP was the second best solvent and DMSO interacts relatively weakly. This can be attributed to the O_{DMSO} partial charge in the force field which was low compared to the partial charge obtained by NBO analysis. These results suggested that optimisation of the force field parameters used in these calculations may be required. Another factor may be the use of different box sizes for the different solvents in the MD calculations. Although the results from the MD simulations were not consistent with that obtained from the DFT calculations, all the analyses agreed that BMIMAc has the strongest interaction with 1,4-dimethoxyglucosamine and 1,4-dimethoxychitobiose.

Radial Distribution Functions

The radial distribution function is a convenient tool to describe the structure of a system, particularly of liquids. The Radial Distribution Functions (RDFs) between each of the hydrogen bond accepting oxygen atoms of the solvent molecules and the hydrogen bond donating atoms of the dimer (N2, O3 and O6) were obtained (Figure 3.7). The RDFs indicated that the solvent molecules tended to be closer to O3 than to the other hydrogen bond donating atoms in all the solvent systems modelled. A shorter distance between interacting atoms suggests a stronger hydrogen bond. This observation is in agreement with the results obtained by QTAIM and NBO analyses. The dimer in BMIMAc results in RDFs that have peaks at shorter distances compared to the other systems. These results indicated that BMIMAc interacts more strongly with 1,4'-dimethoxychitobiose than CyreneTM, DMF, DMSO and NMP. Both the height ($g(r)$) and the position (r) of the peaks of the $O_{\text{solv}} \cdots O3$ RDFs indicate that the strength of the hydrogen bond interaction between 1,4'-dimethoxychitobiose and the different solvents decreases in the order BMIMAc > NMP > CyreneTM > DMF > DMSO. This result was also supported by the charges of the hydrogen bond accepting oxygen atoms of the different solvent molecules which were calculated in the plotting of the RDFs. The RDF results were in agreement with the total interaction energies (per molecule) obtained from MD simulations.

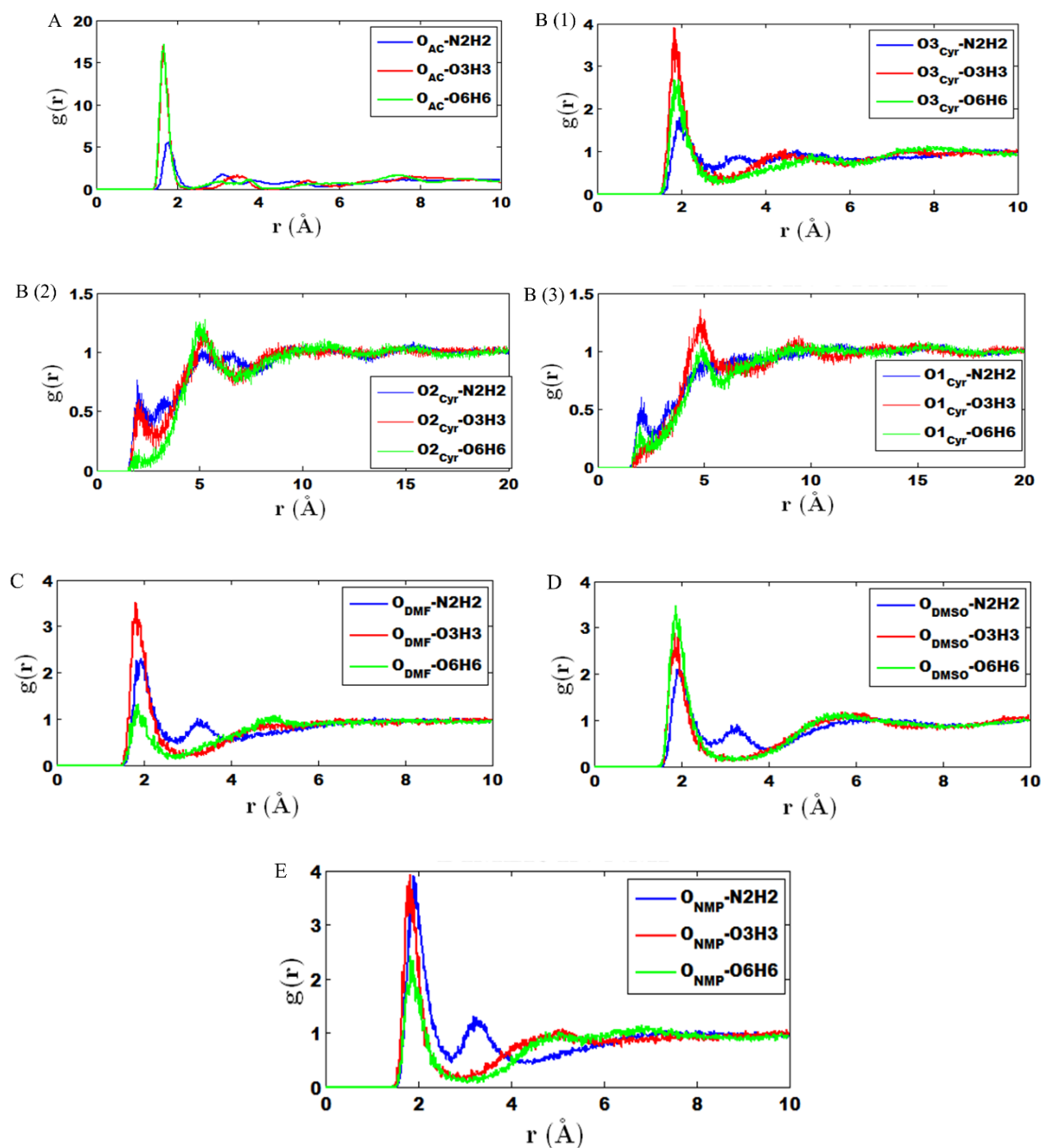


Figure 3.7: Atom-atom radial distribution functions between the hydrogen bond donating atoms of 1,4-dimethoxychitobiose and the hydrogen bond accepting oxygens of (A) BMIMAc, (B) Cyrene™, (C) DMF, (D) DMSO and (E) NMP.

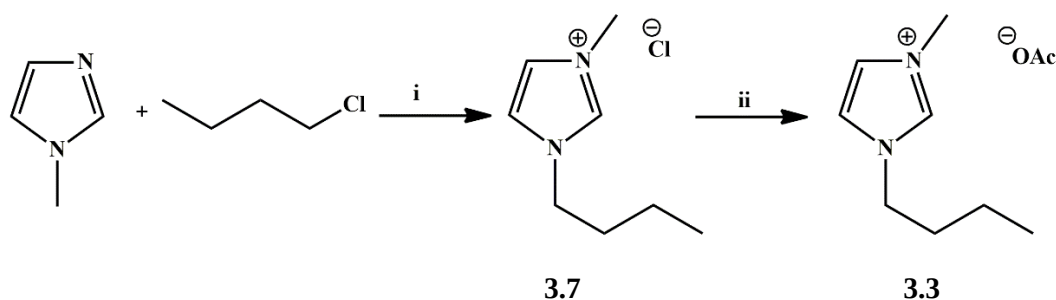
Computational versus experimental comparisons

In order to determine whether the computational study reflected the real world behaviour of chitosan in solution, solubility tests were performed using the selected solvents. The dissolution was compared to that of chitosan in a DMAc/LiCl mixture as a point of reference. Chitosan

was mixed with the solvents and stirred at 60 °C for two hours and the solubility was thereafter visually assessed.

Synthesis of the ionic liquid BMIMAc

All solvents for this study were available except for BMIMAc which had to be synthesised *via* the production of BMIMCl. The ionic liquids were synthesised by the procedures reported by Carson and Liu *et al.* *via* the reaction of 1-chlorobutane and 1-methylimidazole (Scheme 3.1).^{26, 27} The compounds obtained were characterised and compared to the literature reports. The ¹H NMR spectra of BMIMCl (3.7, Scheme 3.1) and BMIMAc (3.3, Scheme 3.1) are shown in Figure 3.8 and agree with that reported in the literature.^{26, 27}



Scheme 3.1: Synthesis of the ILs, BMIMCl (3.7) and BMIMAc (3.3). Reagents and conditions: (i) 100 °C, 7 days, 86%; (ii) potassium acetate, 40 °C, 6 hr, 80%.

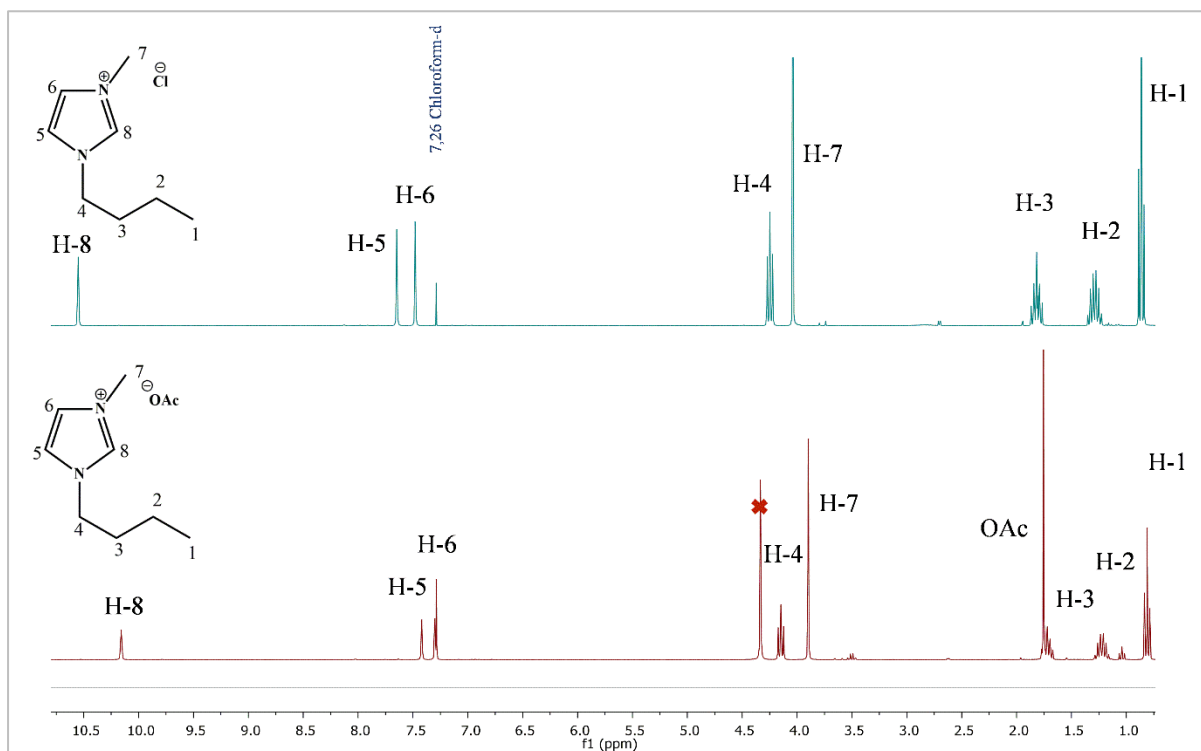


Figure 3.8: ¹H NMR spectra of 1-butyl-3-methylimidazolium chloride (top) and 1-butyl-3-methylimidazolium acetate (bottom) in CDCl₃.

Solubility studies of Chitosan

Optical density analysis could not be utilised to quantify the dissolution of the polymer in the different solvents. Chitosan only swells in the solvents being studied and thus the polymer was not uniformly dispersed. The values obtained from optical density measurements would therefore, not be a true representation of the ability of each solvent to dissolve/disperse chitosan. The polymer dissolved completely in the DMAc/LiCl mixture and this solution was used as the benchmark. Solubility was rated on a scale from 1 to 6, where 1 represented complete dissolution or dispersion and 6 represented a completely heterogeneous mixture (Table 3.3). After visual assessment the samples were centrifuged and the supernatant quantified (Table 3.3) in order to determine the quantity of solvent that the polymer retained. The amount of solvent retained was correlated to the degree of swelling in the polymer.

Table 3.3: Solubility of chitosan in the different solvents.

Solvent	Solubility*	Swelling	Mass solvent retained (g)	Mols of solvent retained (mmol)
DMAc/LiCl	1	—	—	—
DMAc	5	yes	0.15	1.82
BMIMAc	3	yes	2.62	13.2
Cyrene™	6	yes	0.068	0.53
DMF	6	yes	0.638	8.16
DMSO	5	yes	0.352	4.82
NMP	5	yes	0.109	1.10

*1 = complete dissolution and 6 = completely heterogeneous

From the information in Table 3.3 it can be seen that a greater amount of BMIMAc is retained by the polymer compared to the other solvents. This suggests that BMIMAc is a better solvent for the dissolution of chitosan in comparison to the other solvents studied. This is in agreement with the results obtained for the computational modelling of chitosan. Cyrene™ was shown to have been retained the least, thus suggesting that it was not as effective in the dissolution of chitosan. DMAc alone was also tested to emphasize the role of the LiCl in the dissolution of the polymer. A visual representation of this data is given in Figure 3.9.

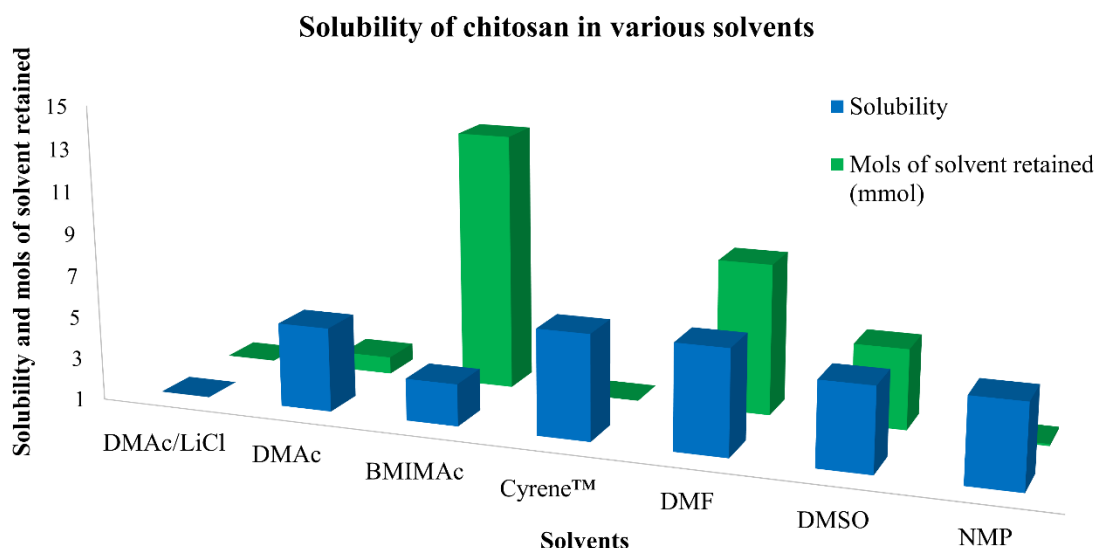


Figure 3.9: The solubility of chitosan in various solvents and the amount of solvent retained by the polymer.

However, since some studies have reported promising results using Cyrene™ as an alternative to conventional dipolar aprotic solvents, it is worth further evaluation for the dissolution of other bio-based polymers. This is due to the fact that Cyrene™ is a relatively new solvent and has not been studied with relation to chitin and chitosan; thus contributing to the understanding of the behaviour of chitin and chitosan in a bio-based solvent. When comparing the results obtained through the various computational methods and the experimental results, it was found that the DFT results correlated the best to experimental results. This indicated that the model and the pair geometries (solvent-model complex) were appropriate for describing the swelling of the polymer in the selected solvents.

Chitin is less soluble than chitosan due to extensive hydrogen bonding; therefore, its behaviour in a variety of solvents including new bio-based solvents is of interest. In addition, reactions involving chitin do not require protection at C-2 making modification at C-6 simpler compared to chitosan. The results obtained for the dissolution of chitin, are discussed below.

3.4 Solvents for the dissolution of chitin

Biopolymer chemistry often involves the use of classical chemistry and reagents, typically environmentally unfriendly and toxic. The review of chemical technology for the valorisation of biomass is currently under examination and solvent replacement is central to this challenge.²⁸ Solvents which have been utilised for the dissolution of these polymers has been discussed in Chapter One. In the current study the solubility of chitin was assessed in various

solvents. It has been reported that inorganic salts assist in the disruption of the hydrogen bonds present in the polymer; therefore, the chosen solvents were assessed in the presence of LiCl. This inorganic salt was chosen as the solubility of cellulose and chitin in a DMAc/LiCl solution is well documented. The purpose of this study was to find a greener alternative to conventionally used solvents.²⁹ To begin with, a solubility study involving a range of solvents including polar aprotics, hydrocarbons, medium polarity and polar protic solvents were conducted for the dissolution of chitin in the presence of lithium chloride (Table 3.4). In total, 23 solvents were tested for their solubilisation of chitin in the presence of LiCl. The samples were ranked according to visual inspection on a scale from 1 to 6 where 1 represented completely dispersed samples and 6 represented a heterogeneous mixture.

In general, similar solubility was observed for the polar protic solvents. NMP was the only other solvent which displayed solubility comparable to that of DMAc; however, due to toxicity issues and problems with the development of new legislation, this solvent could not be used.²⁹ From the results, four potentially applicable solvents were identified, these were BMIMAc, CyreneTM, sulfolane, and Tamisolve[®]. When comparing these results to that obtained in the absence of LiCl (Table 3.3), it is clear that the inorganic salt aids solubility. Dipolar aprotic solvents generally induce swelling in polymers such as chitin and chitosan; however, complete dissolution is not achieved. In the amide-LiCl solvents system, the hydrogen bonds present in the polymer are disrupted by strong electrolytes, here LiCl is employed in polar aprotic solvents. The model for chitin dissolution in LiCl is given in Figure 3.10.

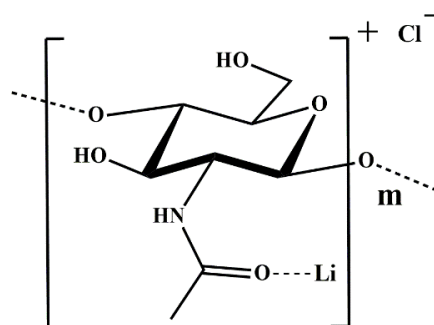
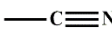
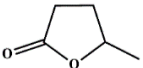
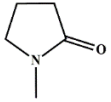
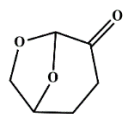
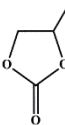
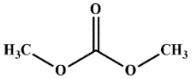
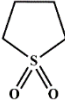
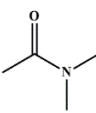
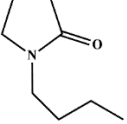
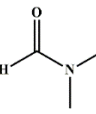
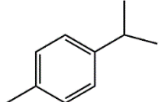
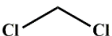
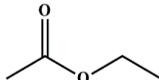
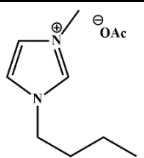
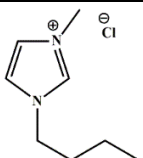


Figure 3.10: The proposed model for chitosan dissolution in the presence of LiCl.

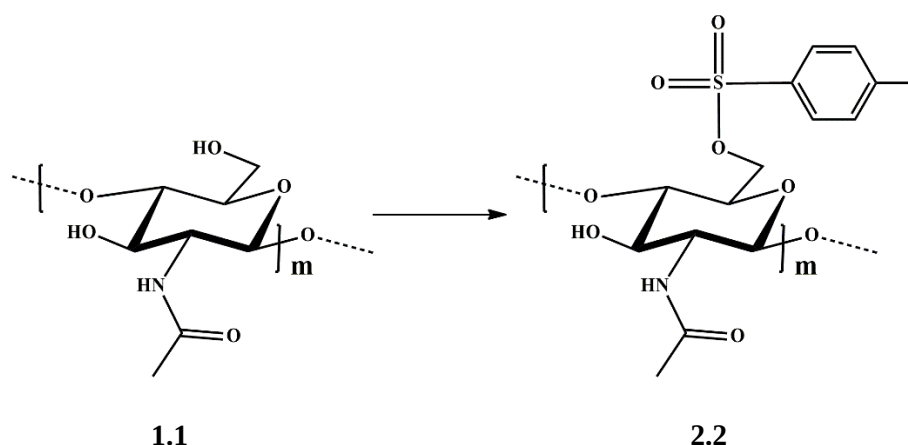
The polymer was swollen and more dispersed in these solvents compared to the other solvents being assessed, therefore modification could be attempted.

Table 3.4: Selected solvents tested for their applicability for chitin dissolution with LiCl (3.07 mmol).

Polar Aprotic Solvents	Solvent	Structure	Solubility	Solvent	Structure	Solubility
	Acetone		6	DMSO		3
	Acetonitrile		5	γ -Valerolactone		5
	Cyclopentanone		5	NMP		1
	Cyrene™		4	Propylene carbonate		6
	Dimethyl carbonate		5	Sulfolane		3
	DMAc		1	Tamisolve®		4
Hydrocarbons	DMF		4			
	Cyclohexane		6	Toluene		6
Medium polarity	<i>p</i> -Cymene		6			
	DCM		5	Ethyl acetate		6
Ionic liquids	1,3-Dioxolane		5	THF		6
	BMIMAc		2	BMIMCl		4
Universal Solvent	Water		6			

* 1 – soluble, 6 – insoluble

The focus of the current study is the modification of chitin and chitosan. One of the key steps to consider is the tosylation of chitin (Scheme 3.2). Therefore, in the search of ‘greener’ methods for the modification of chitin and chitosan, the tosylation of chitin was chosen as a model reaction in order to assess the use of other greener solvents. Tosylation is the first step on the path to the modification of chitin, as the tosyl group is a good leaving and activating group making substitution with various nucleophiles easier.³⁰ Starting with chitin avoids the protection of the amine which is required when chitosan is used. Scheme 3.2 shows the model reaction that was chosen for this study. Here, *N,N*-dimethylacetamide (DMAc) in the presence of lithium chloride (LiCl) was used for the solubilisation of chitin for the reaction. The polymer is subsequently reacted with tosyl chloride in the presence of triethylamine to produce 6-deoxy-6-*p*-toluenesulfonyl chitin (2.2, Scheme 3.2)



Scheme 3.2: Synthesis of the tosyl ester of chitin. Reagents and conditions: *p*-Toluenesulfonyl chloride, Et₃N, DMAc/LiCl, 5 °C, 48 hr, 99%.

The remaining four solvents BMIMAc, Cyrene™, sulfolane, and Tamisolve® were used in the tosylation of chitin with DMAc being used as the reference solution. The procedure that was followed for the tosylation of chitin was that reported by Zou *et al.* with certain modifications. These modifications include the use of half the amount of tosyl chloride and an extended reaction time from 12 hours to 24 hours.³¹

With the protocol in hand, 1.23 mmol of chitin was reacted with LiCl and *p*-toluenesulfonyl chloride in the presence of triethylamine (26 mmol) in the selected solvent at 0 °C. After 24 hours, the polymer was isolated by the addition of acetone and characterised. From the IR spectra (Figure 3.11), it was found that tosylation occurred in DMAc only.

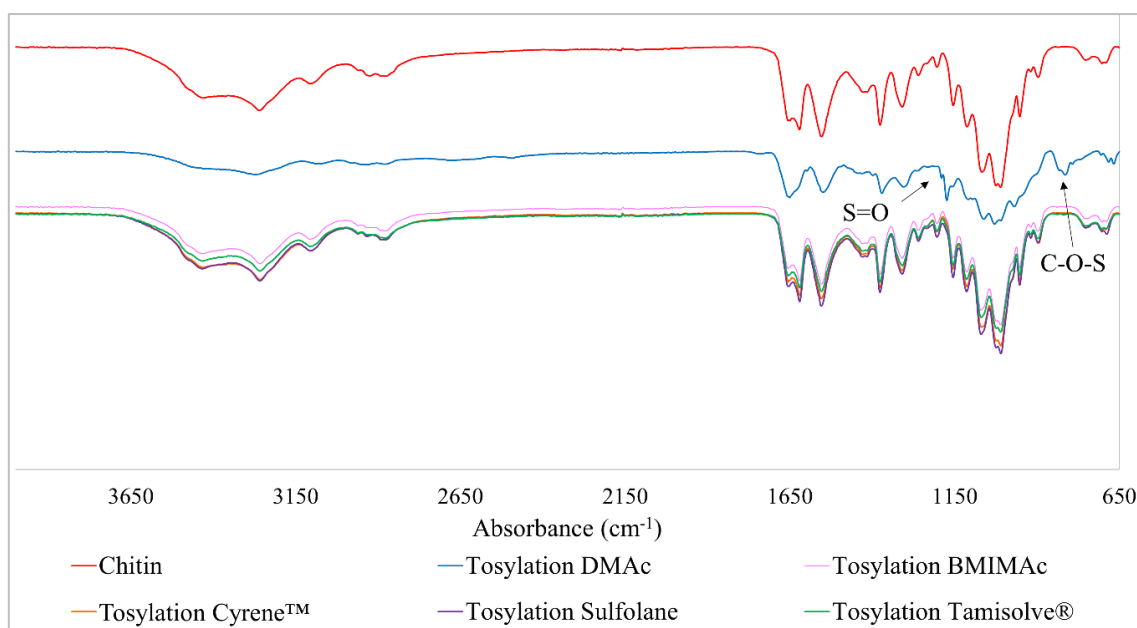


Figure 3.11: The IR spectra of chitin versus the tosylation of chitin in selected solvents.

The characteristic bands (1180 and 740 cm^{-1}) for the tosylation of chitin were absent in all reactions except that of the DMAc reaction. This could be due to possible side reactions of the solvent with tosyl chloride or incomplete dissolution of the polymer in the solvent making the reaction mixture heterogeneous, which does not favour tosylate formation. BMIMAc has previously been used in the modification of chitin and chitosan and tosylation had been achieved on cellulose using this IL. Therefore, it is of interest to note that tosylation did not occur in this case, a potentially stronger base can be utilised as in the case where cellulose was tosylated in an IL using pyridine as a co-solvent.³² In the case of the reaction involving Tamisolve®, a change in the colour of the reaction mixture occurred, from off-white to brown in colour potentially indicating the occurrence of side reactions. The general side reactions that occur during tosylations as reported in literature, are cyclisation by attack of a secondary hydroxyl group, the formation of a C-6 pyridinium salt or the substitution of C-6 by a chloride atom. These side reactions are commonly observed when long reaction times together with increased temperatures are used or when the reactions are predominantly heterogeneous.³³ The results are counterintuitive as one would expect tosylation to occur in polar aprotic solvents. However, an older report by Kurita *et al.* pointed out that tosylation of chitin did not occur under heterogeneous conditions.³⁴ In addition, due to the insolubility of chitin in common inert solvents typically used for tosylation, the probability of finding another solvent is low.³⁴

There have been recent literature reports as noted in Chapter Two on the tosylation of various materials using greener reaction media. Cellulose for example has been tosylated in a mixture of NaOH/H₂O, NaOH/NaCl and in a NaOH/urea solvent systems in the presence of a non-ionic surfactant (polyethylene glycol alkyl-(C11–C15) ether).^{30, 35}

The dissolution of chitin and chitosan in a mixture of urea and NaOH using freeze-thaw treatments has received significant interest. One of the first studies to report on the dissolution of chitin in NaOH/urea solutions were published by Hu *et al.*³⁶ They reported that the optimal conditions for chitin dissolution, is a solution containing 8 wt% NaOH and 4 wt% urea at a temperature of -20 °C.³⁶ Fang and colleagues studied the intermolecular interactions and the extended conformation of the chitin chain in this solvent system, where the authors found that NaOH formed a hydrogen-bonded complex with chitin which was surrounded by hydrates of urea thus enabling dissolution.³⁷ Some researchers have gone a step further and attempted the modification of chitin and chitosan in NaOH/urea solutions. Liu *et al.* synthesised carboxymethyl chitin whose thermo- and pH-sensitive behaviour was studied and found to be different compared to that previously reported.³⁸ The polymer was able to form a gel at body temperature (37 °C) making it a good candidate for biomedical applications.³⁸ A recent study by Peng *et al.* synthesised quaternised chitin in NaOH/urea solutions where the polymer was used to obtain nanoparticles of DNA, which exhibited low cytotoxicity and an effective transfection ability.³⁹

In the current study, the tosylation of chitin in a mixture of NaOH and urea was attempted. Chitin was dissolved in a mixture of 8 wt% NaOH and 4 wt% urea at -20 °C with five freeze-thaw cycles. This homogenous solution was subsequently reacted with *p*-toluenesulfonyl chloride at 0 °C for 24 hours. The polymer was precipitated using 80% ethanol and isolated as a white solid. The analysis of the material recovered did not indicate that tosylation had occurred as evidenced by the lack of characteristic bands in the IR spectra (Figure 3.12).

In addition, the IR spectra did not display the characteristic bands of the polymer pointing to possible degradation of the polymer backbone. Isolation of chitin from a mixture of NaOH/urea did not indicate any major difference in the characteristic polymer peaks, except in the increase in intensity of the C-H bending and C-CH₃ deformation modes which can be attributed to the breaking of the inter- and intramolecular-hydrogen bonds. Literature reports do not indicate polymer degradation in the cases where NaOH/urea have been used as a solvent system for tosylation on polymers such as cellulose.³⁵ In addition, Li *et al.* pointed out that NaOH/urea is

not an ideal solvent system as their study showed that the solution formed from chitosan in the presence of NaOH/urea was not stable and turned into a gel upon reaching room temperature.⁴⁰ Therefore, the use of this solvent system was not feasible in the tosylation of chitin.

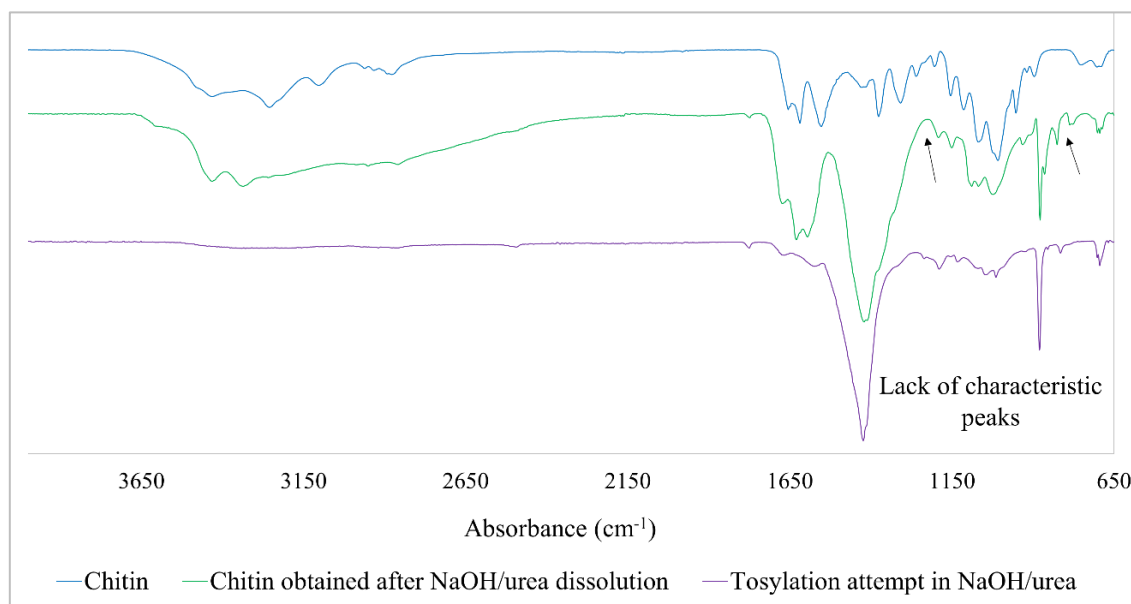


Figure 3.12: The IR spectra of chitin versus the attempted tosylation of chitin in NaOH/urea.

As mentioned above, there has been increasing interest in the use of ionic liquids as alternative solvents for a variety of reactions including those involving chitin and chitosan. In the current study two ionic liquids, 1-butyl-3-methylimidazolium chloride (BMIMCl) and 1-butyl-3-methylimidazolium acetate (BMIMAc) were studied.

The tosylation of chitin and *N*-phthaloyl chitosan was attempted using the co-solvent method reported by Gericke *et al*, which used pyridine as a co-solvent in the presence of the ionic liquid.³² These reactions were not conducted in the presence of LiCl as above. Tosylation was not observed in either case and literature reports of tosylation in ionic liquids is limited to cellulose and agarose.^{32, 41} A possible explanation for the absence of a reaction lies in the solubility of the polymers in the ionic liquids chosen. The polymers were not fully soluble in the ionic liquids therefore limiting the reaction. Since chitin and chitosan has previously been modified through different chemical reactions using ILs as the reaction media, adjustment of the reaction conditions could potentially push the reaction in the forward direction. However, considering that there has not been any literature reports on the tosylation of chitin and chitosan in ILs further studies are needed.

Since substituting the solvent in the tosylation of chitin was not possible, the replacement of LiCl for a cost effective, greener alternative was investigated. The salts chosen to substitute LiCl were sodium chloride (NaCl), 1-butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium acetate. Chitin was dispersed in the IL and thereafter treated with Et₃N and tosyl chloride as per the procedure for the LiCl reaction. After the set reaction time acetone was added and the solid isolated. The materials recovered from the reactions were analysed and the data compared to the LiCl standard reaction. The IR analyses did not indicate tosylation as seen in the spectra in Figure 3.13. The reaction mixtures were heterogeneous in the case of NaCl, BMIMCl and BMIMAc, in comparison to the homogeneous LiCl reaction, indicating that LiCl assists in improving the solubility of chitin thereby promoting the reaction.³⁰

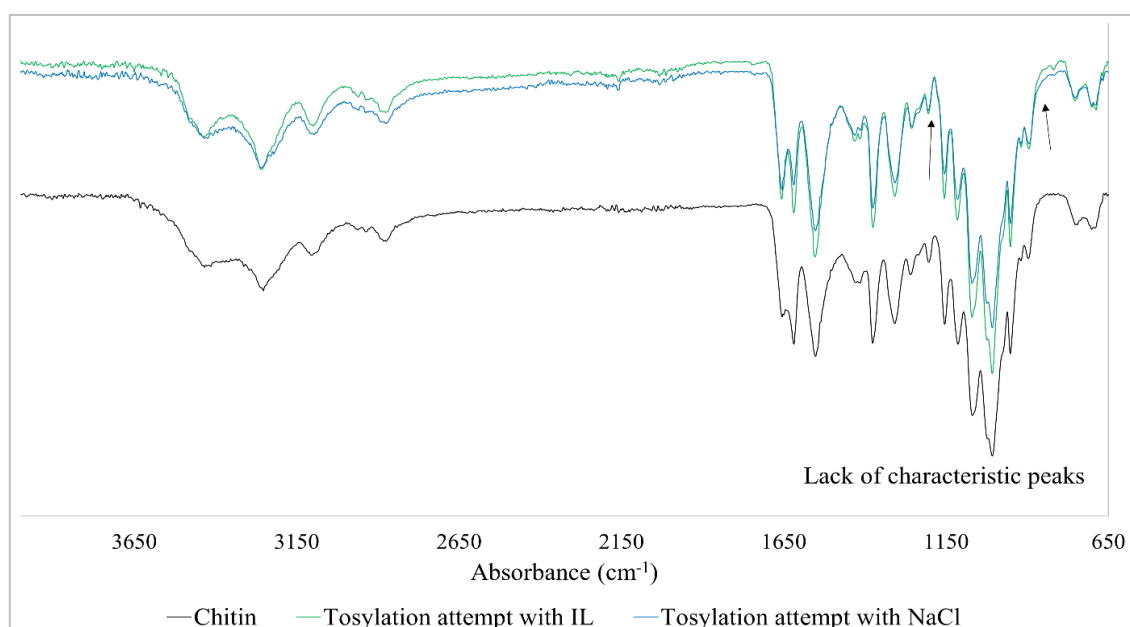


Figure 3.13: The IR spectra of the attempted tosylation of chitin using NaCl and BMIMAc.

The solubility of the tosylate in the chosen solvent can also affect the rate of the forward reaction. Another possibility toward the synthesis of an intermediate which may be substituted to produce useful derivatives would be to consider alternative tosyl ester intermediates as leaving groups. Other tosyl ester intermediates which can be considered includes the *O*-mesylate. Mesylation of chitosan has previously been reported by the reaction of chitosan with mesyl chloride under mild conditions, where the degree of substitution can be increased by repeating the reaction several times.⁴² Sherwood *et al.* recommended the use of the bio-based *p*-cymenesulfonyl chloride as an alternative to the use of the tosylate leaving group.⁴³ However, the application of an alternative leaving group is out of the scope of this study. The aim of the current study is to report on a more efficient and scalable synthesis of a water soluble

6-deoxy-6-amino chitosan. Since modification of the tosylation step proved to be complex, alternative methods for the addition of an amine moiety were studied.

3.5 Conclusion

The solubility of chitin and chitosan in various solvents was assessed by comparing their HSP parameters to that of the selected solvents obtained from literature. Results indicated that Cyrene™, DMAc, DMF, DMSO, glycerol, propylene carbonate, NMP and sulfolane could potentially serve as solvents for these polymers. Computational studies using a variety of simulations were thereafter performed on BMIMAc, Cyrene™, DMF, DMSO and NMP to further investigate polymer solubility. This included DFT calculations, MD simulations, QTAIM analysis, NBO analysis and RDFs calculations. From the results obtained, it has been shown that BMIMAc interacts more strongly with the modelled molecule and therefore, this solvent could potentially be useful in the modification of chitin and chitosan. However, experimentally this solvent was found not to facilitate tosylation despite being used in other modification reactions of chitin and chitosan. Results also indicated that the new solvent Cyrene™ displayed similar results to that of the other dipolar aprotic solvents tested. Although not quantitatively in agreement, experimental studies agreed with the theoretical studies that BMIMAc is of interest with regards to chitin and chitosan and that Cyrene™ is not any better than the other toxic dipolar aprotic solvents studied.

Further studies involved the interaction of chitin with different solvents in the presence of the salt LiCl. A total of 26 solvents were tested and those that displayed slightly better dispersibility, BMIMAc, Cyrene™, sulfolane, and Tamisolve® were used in a model reaction. The model reaction chosen was that of the tosylation of chitin, which is usually performed in solvents such as NMP, DMF or pyridine. The tosylation of chitin in DMAc/LiCl was used as a point of reference. The tosylation of chitin was only observed in the case of the DMAc/LiCl reaction due to the homogeneous nature of the reaction. Tosylation in a homogenous mixture of chitin in NaOH/urea was attempted where results were inconclusive pointing to possible degradation of the polymer backbone in the reaction mixture as evidenced by IR analysis of the recovered material. The tosylation of chitin and *N*-phthaloyl chitosan in mixtures of the ILs BMIMCl and BMIMAc in the presence of a co-solvent, pyridine was attempted where analysis of the recovered polymers indicated no reaction. Switching the co-salt to NaCl, BMIMCl or BMIMAc was also investigated without success with no evidence of tosylation occurring.

From the studies related to the solubility of chitin and chitosan in various solvents and in the presence of various salts it is evident that the most suitable solvent for dissolution is the mixture of DMAc/LiCl. However, it must be stated that DMAc is also on the list of solvents with toxicity concerns, although not as severe as solvents such as DMF and NMP. Therefore, this solvent cannot be seen as a proper alternative to other toxic solvents despite the fact that it is somewhat less hazardous.² With the continuous development of new, environmentally friendly solvents, this challenge can potentially be addressed in the near future. Additionally solvents can be designed specifically for certain reactions such as tailored ILs.

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Chapter Four

The synthesis of 6-deoxy-6-amino chitosan using phthalimide as a source of the 6-amino group

4.1 Background

Reports of the chemical modifications and applications of chitin and chitosan have increased greatly in recent years. Typical reactions of these polymers require protection and deprotection steps toward the synthesis of modified derivatives. Owing to the poor water solubility associated with chitin and chitosan, these reactions often involve the use of toxic solvents such as DMF, NMP and pyridine. Various toxic waste streams are released, which may lead to disposal restrictions and environmental damage. Researchers are well aware of this mounting need for alternative solvents as evidenced by the numerous publications on alternative reaction media and the development of bio-based solvents.¹

The introduction of an amine at the C-6 position of chitosan has led to the synthesis of a highly water soluble chitosan derivative, 6-deoxy-6-amino chitosan (**1.15**, Figure 4.1).² Generally, this polymer is synthesised with protection of the amine at C-2, substitution of the hydroxyl at C-6 for a better leaving group, followed by displacement with an aminating reagent which is finally reduced to the amine.

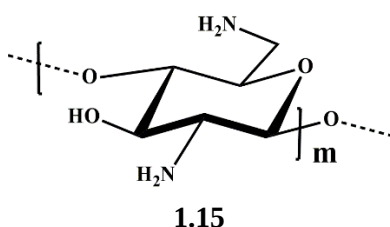


Figure 4.1: The structure of 6-deoxy6-amino chitosan (**1.15**).

The problems associated with the known synthetic methods of 6-deoxy-6-amino chitosan were fully discussed in Chapter Two. The use of different reagents to introduce the C-6 amino group is an area of interest, since the current method employs sodium azide which is undesirable due to toxicity, increased waste due to the reduction step as well as combustibility issues.³ Other reported methods have used ammonia to introduce the amine at C-6 after activation, however, this reaction requires a pressurised vessel which has safety concerns for a large scale synthesis.⁴ The current study employed the use of solvents such as DMF and NMP with various limitations such as their associated toxicity, production of excessive waste and the use of hazardous

reagents. Furthermore, in the reported methods for the synthesis of 6-deoxy-6-amino chitosan, the last step produces a substantial amount of triphenylphosphine oxide as a by-product of the Staudinger reaction. Triphenylphosphine oxide ($\text{PPh}_3=\text{O}$) is known to be trapped inside the polymer and difficult to separate leading to a large amount of waste.⁵ This, in addition to the toxic reagents and REACH incompliant solvents further complicates the synthesis of 6-deoxy-6-amino chitosan. It was therefore necessary to investigate alternative solvents and methods for the introduction of the amino group at C-6 of chitosan.

The use of glucosamine (**4.1**, Figure 4.2) as a model for chitosan C-6 substitution reactions has not been extensively studied. There have been very few reports which deal with the functionalisation of glucosamine to act as a model or monomer for polymer synthesis reported in the literature. By studying the functionalisation of the monomer, the various substitutions can be monitored *via* thin layer chromatography (TLC) and solution state NMR. In addition, conditions for synthesis may be optimised on the monomer before being applied to the polymer.

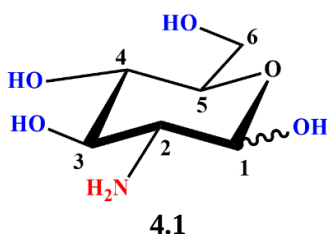
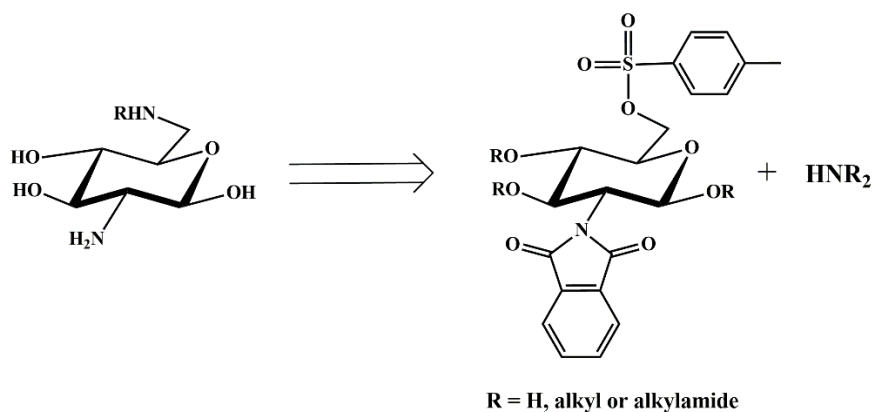


Figure 4.2: The structure of glucosamine with reactive groups highlighted in red and blue.

A study by Johannesen *et al.* reports on the use of glucosamine mono- and disaccharides to understand the structure of chitosan-Pt (II) metal complexes.⁶ Whereas the study by Wu *et al.* studied the antioxidant and antimicrobial activity of Maillard reaction products as well as the associated reaction kinetics based on chitosan, chitooligomer, glucosamine hydrochloride and taurine model systems.⁷ This study reported that the steric hindrance present in the polymer significantly influenced the formation of intermediate compounds.⁷ The latest report for the use of glucosamine as a model for chitosan substitution was reported by Toeri *et al.* who used glucosamine to study the reaction between the amine group and the allyl groups of azacrown ether.⁸ The results obtained were used to confirm the interpretation of the solid state NMR spectra of the chitosan azacrown ether derivative.⁸

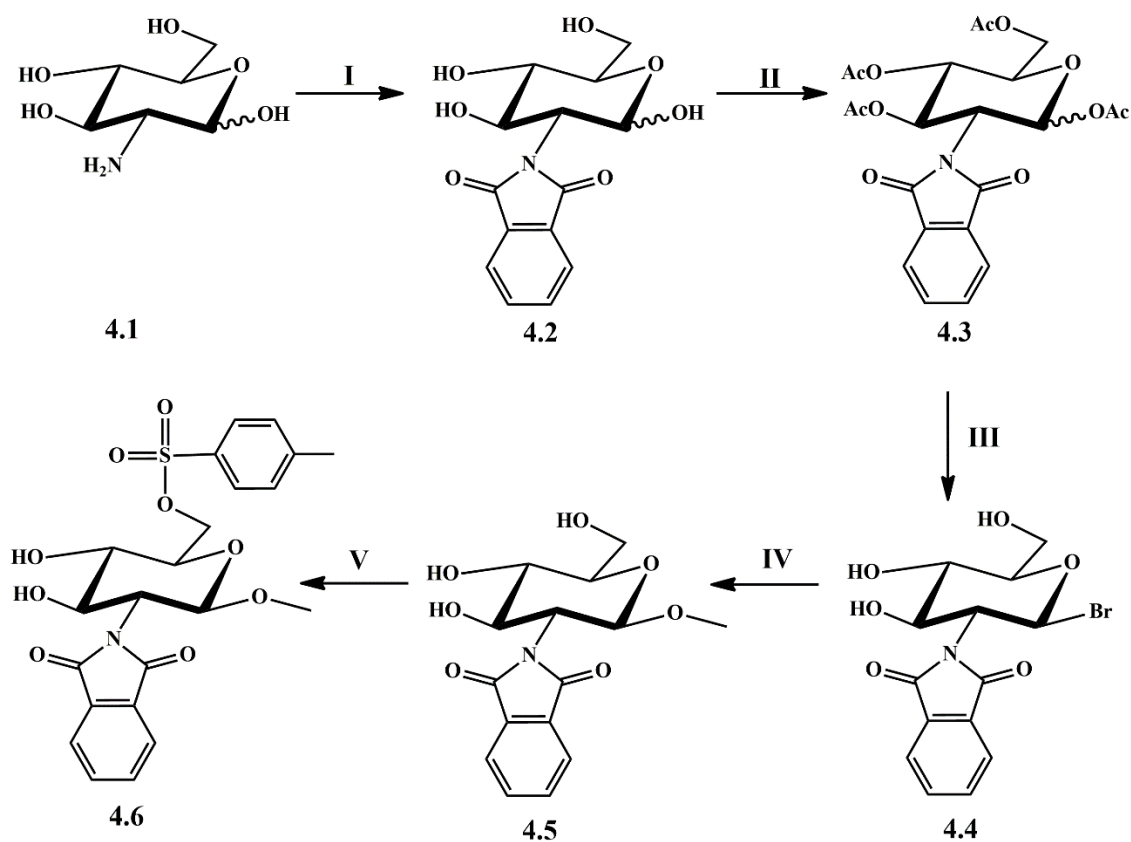
The presence of reactive hydroxyl groups at C-1, C-3, C-4 and C-6 as well as the amine at C-2 warranted strategic protection-deprotection steps. A general retrosynthesis is given in Scheme 4.1.



4.1

Scheme 4.1: Retrosynthetic analysis of the functionalisation of glucosamine (4.1).**4.2.1 Synthesis of C-6 substituted amino glucosamine as a model compound**

To achieve substitution of C-6 with an amine functionality, the amine at C-2 of glucosamine needed to be protected and the anomeric proton selectively methylated (Scheme 4.2). Thereafter, the C-6 hydroxyl had to be selectively converted to a tosyl ester which potentiates further functionalisation.



Scheme 4.2: C-6 tosylation of glucosamine. Reagents and conditions: (i) Phthalic anhydride, NaHCO_3 , H_2O , 25°C , 18 hr, 45%; (ii) Acetic anhydride, pyridine, 25°C , 18 hr, 86%; (iii) 33% HBr in acetic acid, acetic anhydride, 25°C , 90 min, (iv) methanol, 17 hr, Dowex[®] 1X2 HCO_3^- ion exchange resin, 71%; (v) *p*-Toluenesulfonyl chloride, pyridine, -60°C , 6 hr, 80%.

2-Deoxy-2-phthalimido- α,β -D-glucopyranoside (**4.2**) was synthesised according to the method reported by McGeary *et al.* by the reaction of glucosamine with phthalic anhydride in the presence of sodium bicarbonate.⁹ Spectroscopic analysis via ^1H and ^{13}C NMR supported the introduction of a phthaloyl moiety as evidenced by the presence of peaks in the aromatic region. The protected glucose was isolated as a mixture of α and β isomers. IR analysis of the compound confirmed the introduction of the *N*-phthaloyl protecting group as evidenced by the absorbance band at 706 cm^{-1} . The melting point of the material ($191 - 193\text{ }^\circ\text{C}$) was within range of that reported by McGeary *et al.*⁹

The protected glucosamine intermediate (**4.2**), was subsequently reacted with acetic anhydride in pyridine to protect the free hydroxyl groups at C-1, C-3, C-4 and C-6. The synthetic method followed was that reported by McGeary *et al.*; however, the workup was slightly modified.⁹ The polymer was isolated by precipitation over ice and washed with ethanol as opposed to an organic extraction. 2-Deoxy-2-phthalimido- α,β -D-glucopyranoside tetraacetate (**4.3**) was recovered in 86% yield, as a mixture of anomers ($\alpha:\beta$, 1:0.4). This was evident in the ^{13}C NMR spectrum, where the smaller peaks represent the minor isomer (Figure 4.3). The ^{13}C NMR spectrum obtained agreed with that reported by Bednarczyk *et al.* where the introduction of the acetyl groups was evidenced by the appearance of new peaks in the region of 20 ppm and the associated carbonyl groups in the region of 170 ppm.¹⁰

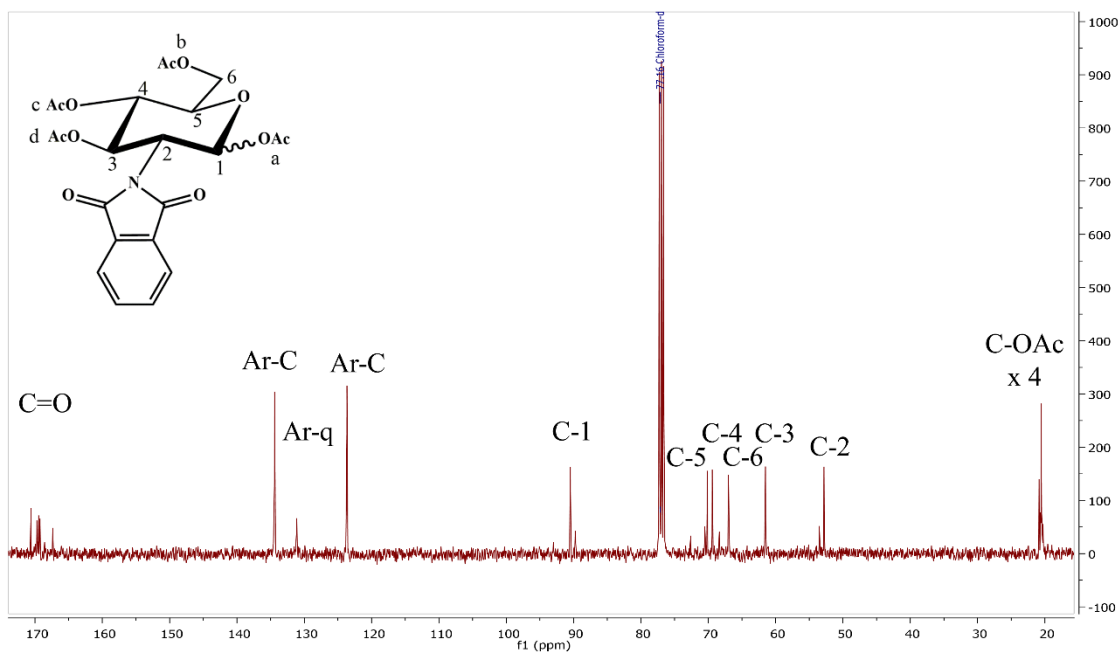


Figure 4.3: ^{13}C NMR spectrum of 2-deoxy-2-phthalimido- α,β -D-glucopyranoside tetraacetate (**4.3**) in CDCl_3 .

There have been previous reports of the direct conversion of glucosamine to 2-deoxy-2-phthalimido- α,β -D-glucopyranoside tetraacetate (**4.3**) by treatment with sodium methoxide, phthalic anhydride and triethylamine, followed by overnight treatment with acetic anhydride and pyridine.¹¹ To obtain the desired intermediate (**4.3**), intermediate (**4.2**) is typically treated with methanol in anhydrous dichloromethane in the presence of a Lewis acid such as tin tetrachloride (SnCl_4) over two steps.¹² In this work, a one-pot method was used where the tetraacetate was treated with a 33% solution of HBr in acetic acid to form the anomeric bromide intermediate (**4.4**). In a second step the material was treated with methanol and a HCO_3^- functionalised ion exchange resin.⁹ This reaction yielded methyl 2-deoxy-2-phthalimido- β -D-glucopyranoside (**4.5**) selectively. The absence of the Lewis acid in the formation of the desired material is of interest. The study by Simon investigated the stereoselective synthesis of aminoglycosides where glycosylation was studied with various conditions and reagents.¹³ This study proposed a mechanism whereby neighbouring group participation of the *N*-allyloxycarbonyl protecting group plays a major role in the outcome of the reaction. The importance of the Lewis acid was stressed where Lewis acid metal salts studied included tert-butyl-di-methyl silyltrifluoromethane sulfonate (TBDMSOTf), boron trifluoride diethyl etherate and titanium tetrachloride.¹³ However, there have been recent reports of metal-free glycosylations which were discussed in the review by Das *et al.* covering all aspects of chemical *O*-glycosylations.¹⁴ In the current study, the presence of the phthalimide protecting group at C-2 potentially stabilises the oxocarbenium intermediate formed which is then substituted to produce the methoxy glucosamine derivative.

NMR and IR analysis supported the synthesis of the β -isomer where the selectivity is influenced by factors such as the temperature, the nature of the protecting groups, the solvent used, the anomeric leaving group, and the promoter. Due to the influence of the phthaloyl group at C-2, delocalisation at the anomeric centre results in the formation of an intermediate which favours the formation of a 1,2-*trans* linkage favouring the β -isomer.¹⁵ The spectra obtained was in agreement with that reported by Kendra *et al.* The ^1H NMR is shown in Figure 4.4 with corresponding assignments, where the absence of the acetyl peaks confirmed their removal. Additionally, no peaks attributed to the α -isomer was observed making further purification by chromatography unnecessary.

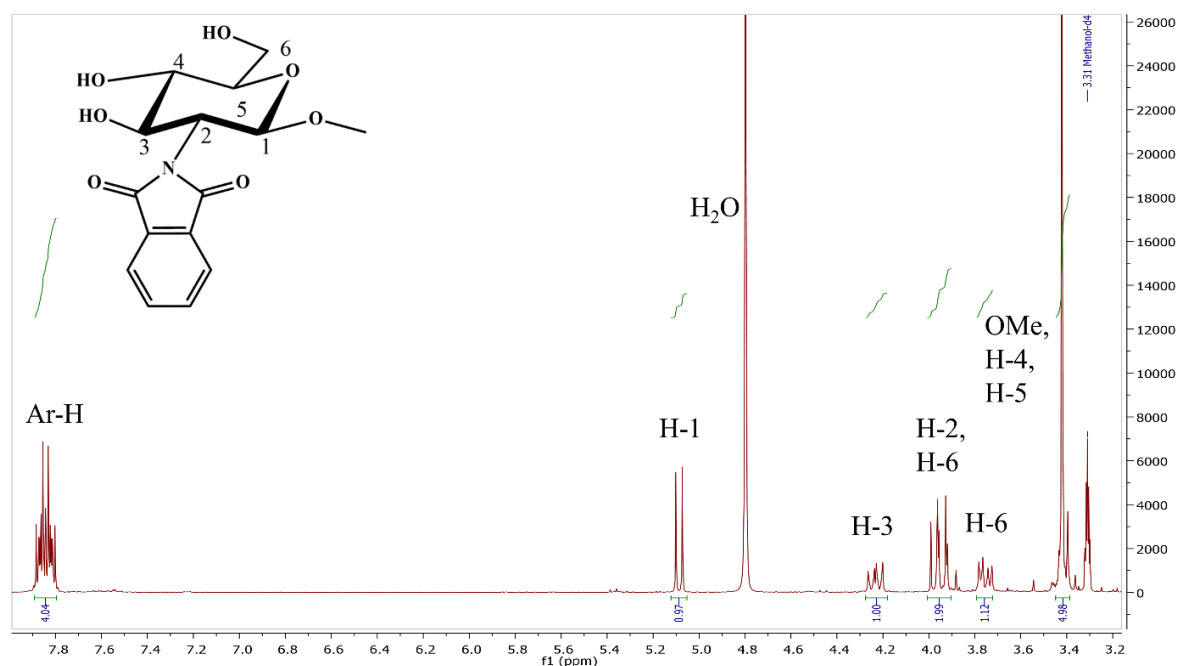


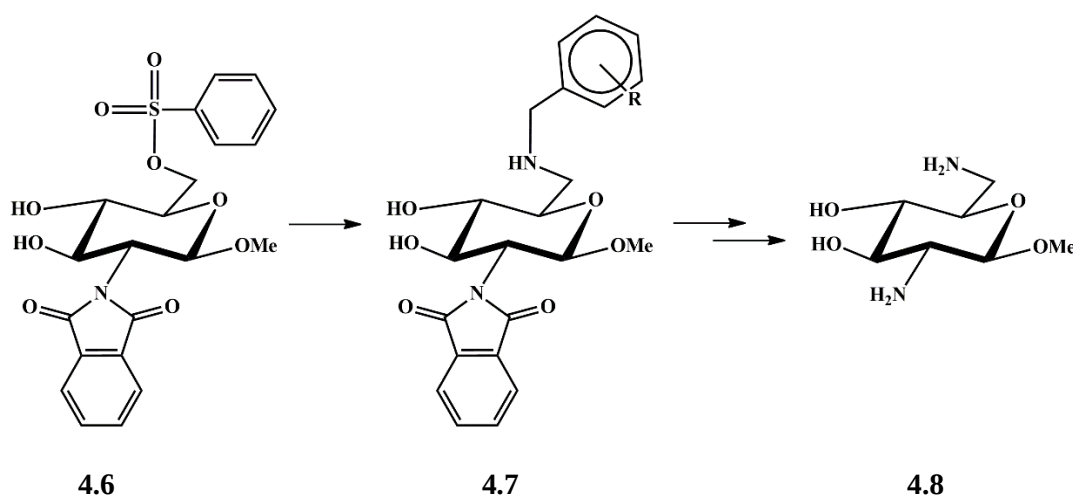
Figure 4.4: ^1H NMR spectrum of methyl 2-deoxy-2-phthalimido- β -D-glucopyranoside (**4.5**) in $\text{CD}_3\text{OD}-d^4$.

Regioselective tosylation at C-6 was achieved by the reaction of intermediate (**4.5**) with *p*-toluenesulfonyl chloride in the presence of pyridine. The procedure followed was that of Kitahara *et al.* where the reaction was allowed to stir at $-60\text{ }^\circ\text{C}$ as opposed to the reported $0 - 5\text{ }^\circ\text{C}$.¹⁶ This lower temperature promoted substitution at C-6 only, while avoiding the generation of unwanted side products such as C-3 tosylation. Methyl 2-deoxy-2-phthalimido-6-*O-p*-toluenesulfonyl- β -D-glucopyranoside (**4.6**), was isolated as an off-white solid in a yield of 80%. Tosylation occurred regioselectively at C-6, which was supported by ^1H and ^{13}C NMR spectra where the data correlated with that reported by van den Bos *et al.*¹⁷ The melting point recorded was in the range of $152 - 155\text{ }^\circ\text{C}$ which was out of the range reported by Kitahara *et al.*; however the literature melting point ($97 - 100\text{ }^\circ\text{C}$) was that of a mixture of anomers.¹⁶

Synthesis of C-6 amino glucosamine

An investigation of reports on the introduction of an amine at C-6 of glucosamine yielded limited results. Bundle and co-worker synthesised methyl 2-acetamido-6-amino-2,6-dideoxy- β -D-glucopyranoside via C-6 chlorination and subsequent substitution with an azide which was reduced by hydrogenation.¹⁸ This halogenation followed by reaction with an azide and reduction thereof is a popular method of introducing an amine. Another report which uses the azide to introduce an amine uses a tosylated intermediate in preference to the halogenated one. In this manner, novel, non-natural, carbohydrate-based macrolide analogues with antimicrobial properties were produced.¹⁹

In a broader search of the literature with a focus on reagents used in the amination of sugars benzylamine was identified. Corsi *et al.* investigated *N*-benzyl intermediates as an alternative to sodium azide in the synthesis of primary amino sugars.²⁰ The free hydroxyls on isopropylidene acetals were substituted with a tosylate ester which was then reacted with benzylamine to produce a masked amine. The benzyl group was thereafter removed by metal catalysed hydrogenolysis (Pd/C) to produce the free amine.²⁰ Therefore, masked amino groups in the form of benzylamine were investigated to introduce an amine functionality at C-6 of glucosamine. Benzylamine and derivatives thereof were considered as potential aminating agents as shown in Scheme 4.3. Once substitution was achieved debenzoylation would be carried out followed by dephthaloylation to produce the diamino monomer (**4.8**).

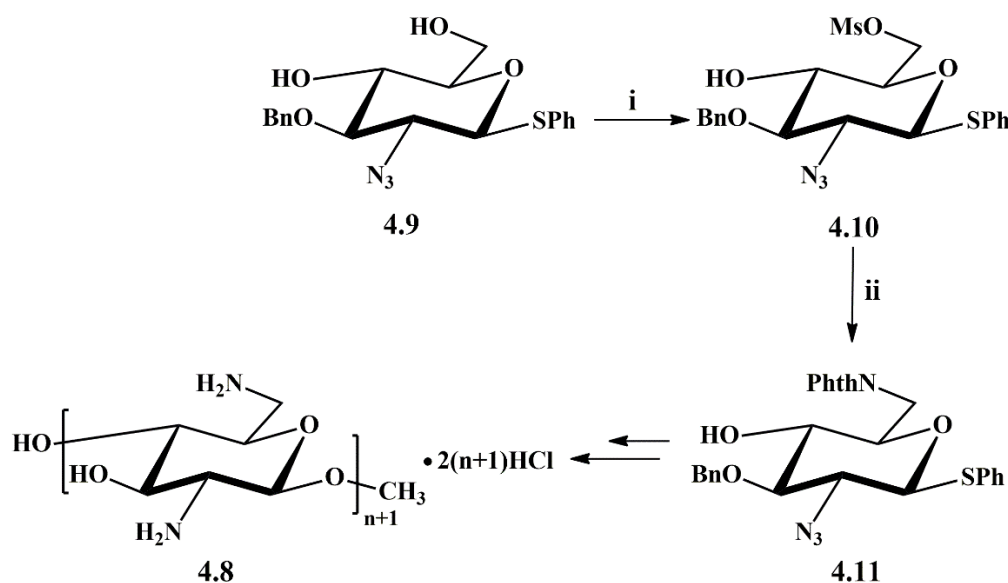


Scheme 4.3: Proposed use of benzylamine derivatives in the synthesis of diamino glucosamine.

Various attempts to substitute the tosyl group at C-6 with benzylamine were performed. Different solvents and work up conditions were attempted with no success. Other benzylamine based reagents (2-nitrobenzylamine, 2-bromobenzylamine and 2-chlorobenzylamine) with various reaction conditions were tried with no success as mixture of side products was obtained making separation difficult. Therefore, due to issues related to the functionalisation of glucosamine with benzylamine and substituted benzylamine, this pathway was abandoned and other reagents considered.

After an extensive literature search for other potential aminating agents, potassium phthalimide was chosen for further evaluation. Potassium phthalimide has previously been used as a mild, inexpensive, green organocatalyst for the preparation of important pharmaceutical precursors.^{21, 22} The phthalimide is widely used in the synthesis of amino acids *via* the Gabriel synthesis for the transformation of primary alkyl halides into primary amines. A previous study

reports the use of potassium phthalimide in the facile synthesis of chiral glycine from D-ribose.²³ This reagent was also used in the synthesis of hepta-6-thioaminoalkyl and hepta-6-thioguanidinoalkyl derivatives of β -cyclodextrin using phthalimide as a nitrogen source.²⁴ The study by Iwata *et al.* synthesised novel oligodiaminosaccharides, *via* a chain elongation cycle including glycosylation reactions of a 6-phthalimide glycosyl donor was used in the synthesis of α -(1-4)-linked-2,6-diamino-2,6-dideoxy-D-glucopyranose oligomers (**4.8**, Scheme 4.4).²⁵ A rational assumption was made that potassium phthalimide would be a good source of an amine for attachment to the polymer.

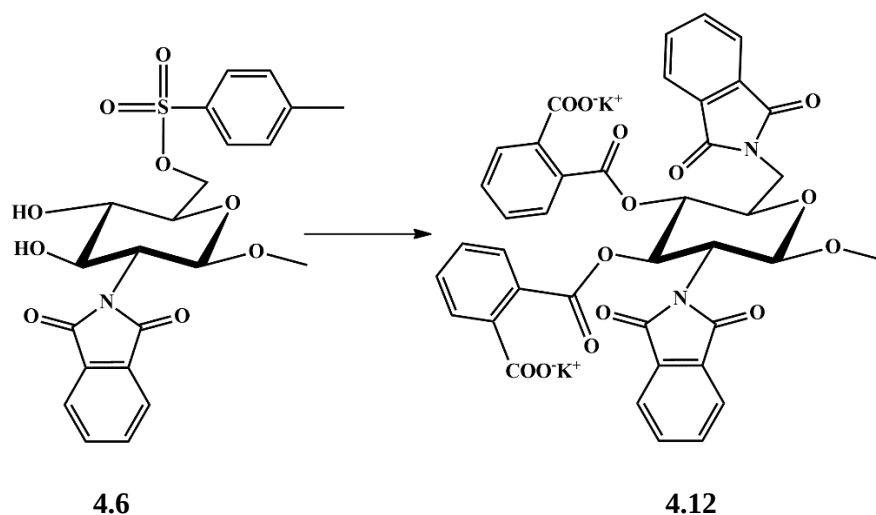


Scheme 4.4: Synthesis of a glycosyl donor by Iwata *et al.* Reagents and conditions: i) methanesulfonyl chloride, pyridine, -15 °C; ii) potassium phthalimide, DMF, 100 °C, 13 hr, 73%.²⁵

Hence, a model study of phthalimide substitution on the chitosan monomer, glucosamine was considered.

Synthesis of Methyl 3,4-diphthalate-2,6-diphthalimido- β -D-glucopyranoside (**4.12**) using potassium phthalimide as a source of nitrogen

Methyl 2-deoxy-2-phthalimido-6-O-*p*-toluenesulfonyl- β -D-glucopyranoside (**4.6**) was thereafter reacted with 2.60 mmol of potassium phthalimide in DMAc following the method reported by Ohru *et al.* (Scheme 4.5).²³ The reaction was complete after four hours as visualised by the disappearance of the starting material on TLC. The product was isolated by the addition of water followed by extraction with diethyl ether, to give a white solid in a 49% yield. The low yield can be attributed to the formation of the potassium salt which was not recovered from the water layer.



Scheme 4.5: C-6 substitution with potassium phthalimide. Reagents and conditions: potassium phthalimide, DMAc, 110 °C, 4.5 hrs, 49%.

NMR and IR analysis confirmed the structure of the product, since the hydroxyls at C-3 and C-4 were unprotected, esterification occurred at these positions in addition to C-6. The esterifications at C-3 and C-4 was not unprecedented as the phthalimide displayed behaviour similar to phthalic anhydride. The ^1H NMR spectrum of methyl 3,4-diphthalate-2,6-diphthalimido- β -D-glucopyranoside (Figure 4.5) clearly showed the presence of four aromatic rings (expanded region shown) and ^{13}C NMR analysis corroborated the introduction of additional phthalimide groups.

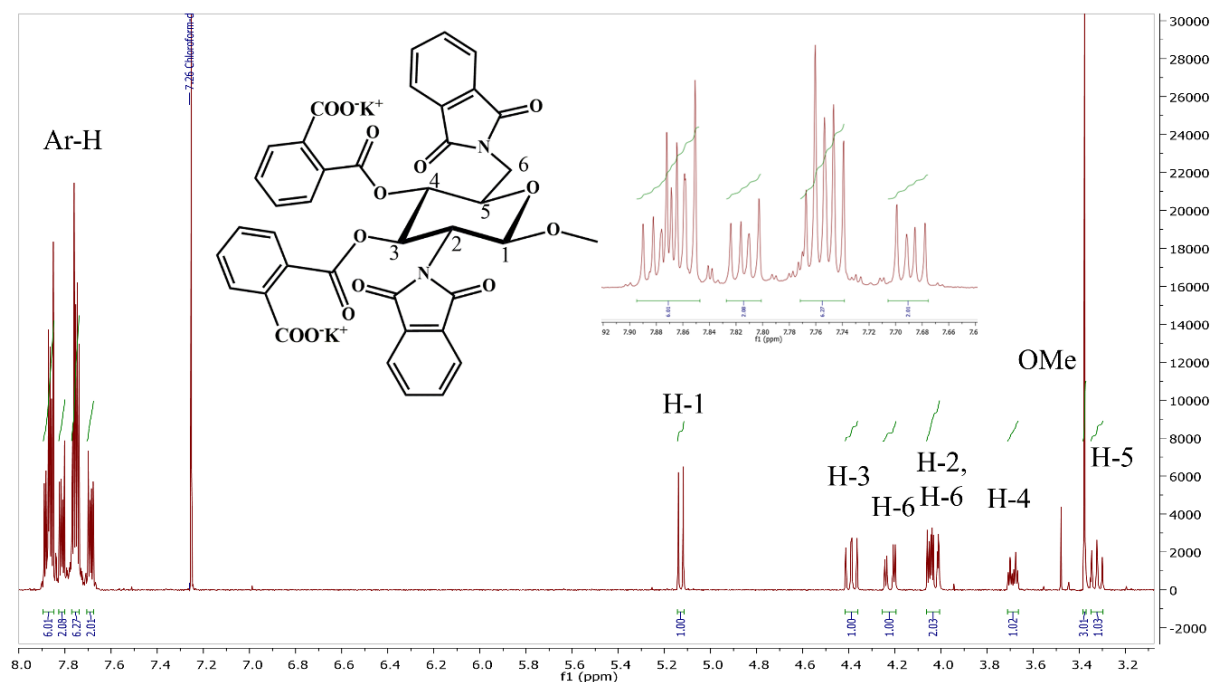
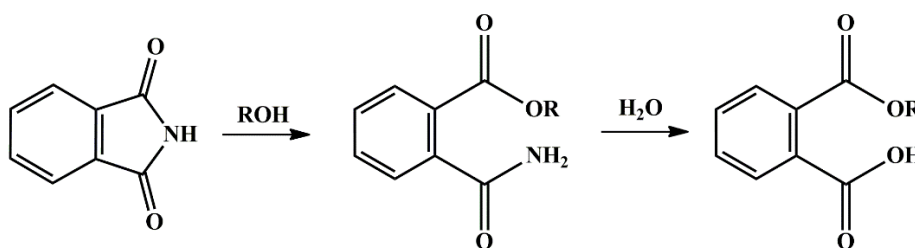


Figure 4.5: ^1H NMR spectrum of methyl 3,4-diphthalate-2,6-diphthalimido- β -D-glucopyranoside (4.12) in CDCl_3 .

High resolution mass spectrometry displayed a peak corresponding to m/z 475.1114 which was attributed to the $[M - 2(C_8H_5O_3 + Na)]^+$ ion, indicative of the labile C-3 and C-4 esters. The low yield can potentially be attributed to a fraction of the product entering the water layer due to the introduction of the phthaloyl groups. In future, the recovery procedure would need to be optimised to avoid loss of any material.

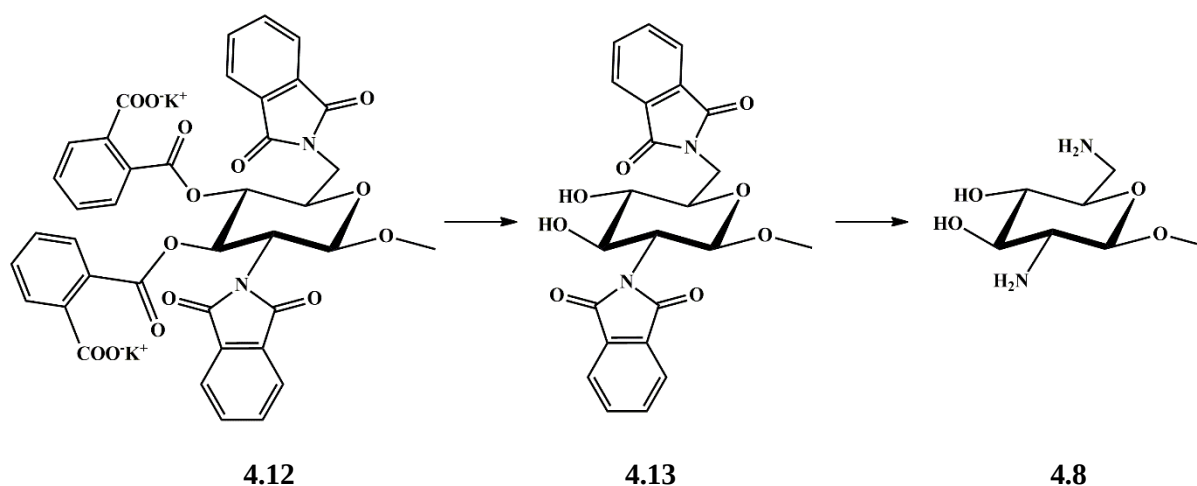
The presence of the free hydroxyls in the structure makes this esterification possible. A further explanation is the long reaction time that would allow for substitution to occur at those positions. This esterification occurs in the synthesis of *N*-phthaloyl chitosan however, a solvent system which includes water is used to cause its hydrolysis. Additionally, at the required reaction temperature (110 °C) it is possible to open the phthalimide ring with the reactive oxygen from an alcohol to give the ester. This is illustrated by the general representation in Scheme 4.6.



Scheme 4.6: Proposed hydrolysis of the phthalimide.

A brief explanation on the stereoelectronic effects affecting carbohydrate chemistry can be found in the book by Miljkovic who extensively reviewed the electrostatic and stereoelectronic effects in carbohydrate chemistry.²⁶ With chitin and chitosan, esterification of C-4 is highly unlikely due to steric strain and the presence of intermolecular links between the monomeric units of the polymer. This reaction was not further optimised as the required substitution was achieved.

The next step was the removal of the esters and the phthaloyl protecting group by treatment of methyl 3,4-diphthalate-2,6-diphthalimido- β -D-glucopyranoside with excess hydrazine hydrate in absolute ethanol, which selectively removed the phthaloyl groups at C-3 and C-4 only, while leaving C-2 and C-6 protected (Scheme 4.7). The reaction was allowed to proceed for longer in an attempt to remove the phthaloyl groups at C-2 and C-6; however, TLC analysis indicated no change even after excess hydrazine was added and the reaction time extended up to 3 days.



Scheme 4.7: Deprotection of 3,4-diphthalate-2,6-diphthalimido-β-D-glucopyranoside (**4.12**).

Complete deprotection was attempted with the standard methods and was unsuccessful from TLC analysis; therefore, this warranted investigation into alternative deprotection methods. Ethylenediamine was used as a deprotecting agent directly or by binding to Merrifields resin; however, this yielded the same result, as seen by the presence of phthaloyl groups in the ^1H NMR spectrum (Figure 4.6). ^1H and ^{13}C NMR spectra confirmed removal of the phthalimide groups at C-3 and C-4 and the retention of the groups at C-2 and C-6. In addition, after removal of the esters at C-3 and C-4, the monomer became soluble in water.

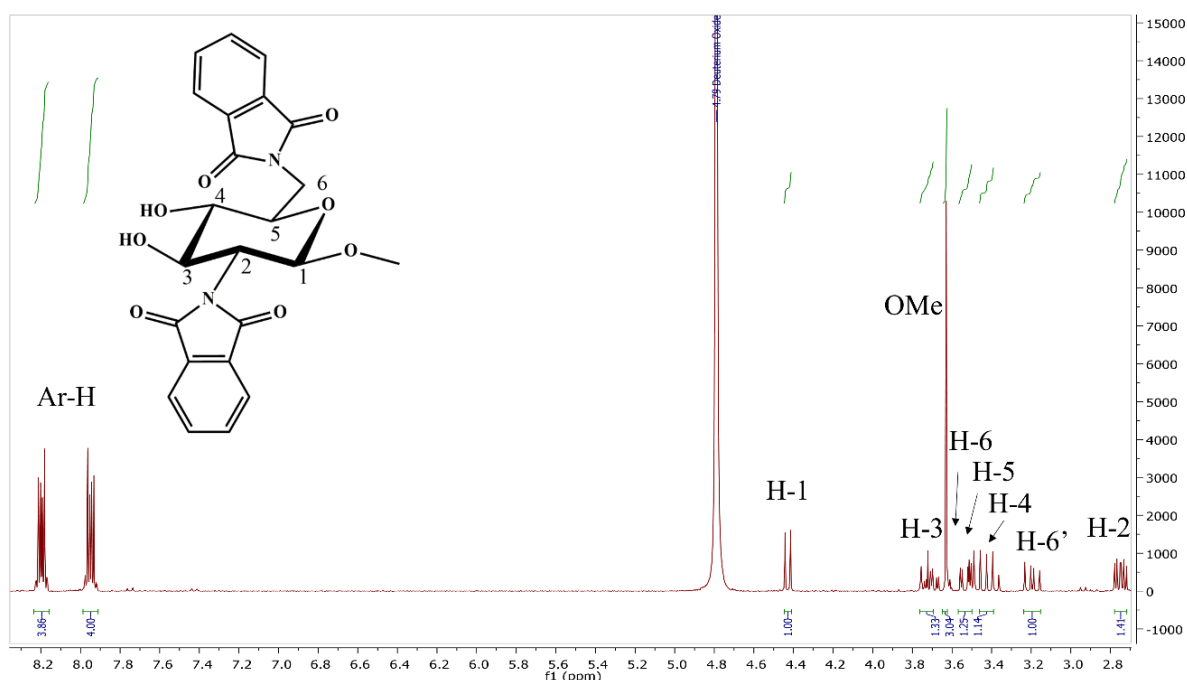


Figure 4.6: ^1H NMR spectrum of methyl 2,6-dideoxy-2,6-diphthalimido-β-D-glucopyranoside (**4.13**) in D_2O .

The stability of the 2,6-dideoxy-2,6-diphtalimido- β -D-glucopyranoside (**4.13**) toward hydrolysis was intriguing. Phthaloyl protecting groups are generally removed using nucleophiles such as hydrazine, *n*-butylamine, methylamine and sodium borohydride. However, as stated by Shimizu *et al.* these reactions often require high temperatures and extended reaction times which can lead to degradation of the starting material.²⁷ The course of dephthaloylation is difficult to monitor and extensive investigation is required to find the optimal conditions for different substrates.²⁷ The study by Rakotondramasy and co-workers reported similar issues in the removal of a phthaloyl protecting group, hydrazine was initially employed which resulted in the production of unwanted side products.²⁸ Thereafter, lithium hydroxide was applied which resulted in opening of the phthalimide residues. Acidic and basic reaction conditions were unsuccessful and a two-step deprotection strategy was adopted to completely remove all protecting groups present.²⁸ Du *et al.* who synthesised cationic glycopolymers through controlled polymerisation of a glucosamine-based monomer, abandoned the phthaloyl route of amine protection as the protecting group could not be removed after treatment with both hydrazine hydrate and sodium borohydride.²⁹

In an effort to understand the stability of the 2,6-dideoxy-2,6-diphtalimido- β -D-glucopyranoside (**4.13**) toward hydrolysis, this compound was modelled *in silico* using Materials Studio 2016.³⁰ The compound was drawn in Chemdraw 12.0.2 and imported into Material Studios after which the structure was cleaned and hydrogens were added.^{30, 31} A geometry optimisation calculation was performed using these parameters; quality: ultrafine and forcefield: Dreiding. All energy calculations were done using the Dreiding force field, which is a generic force field for molecular simulations. This force field has previously been used in the modelling of *N,N'*-diacetylchitobiose.³² This molecule is a chitooligosaccharides containing two *N*-acetyl-D-glucosamine units which is the repeating unit of chitin.³² The geometry optimised structure was manipulated to form a hydrogen bond (between the phthalimide at C-2 and the hydroxyl at C-3) and the geometry optimisation calculation was performed again. The hydrogen bonding distance measured for this constrained bond was 2.4 Å where hydrogen bonds are categorised as short, mostly covalent (2.2 – 2.5 Å), moderate, mostly electrostatic (2.5 - 3.2 Å) and as weak, electrostatic (3.2 - 4.0 Å). As a representative of the polymer, a trisaccharide diamine was modelled where each amine was protected with a phthalimide group. The optimised structure of 2,6-dideoxy-2,6-diphtalimido- β -D-glucopyranoside (**4.13**) and the constrained monomer together with a space filling model are given in Figure 4.7 A, B, C and D respectively.

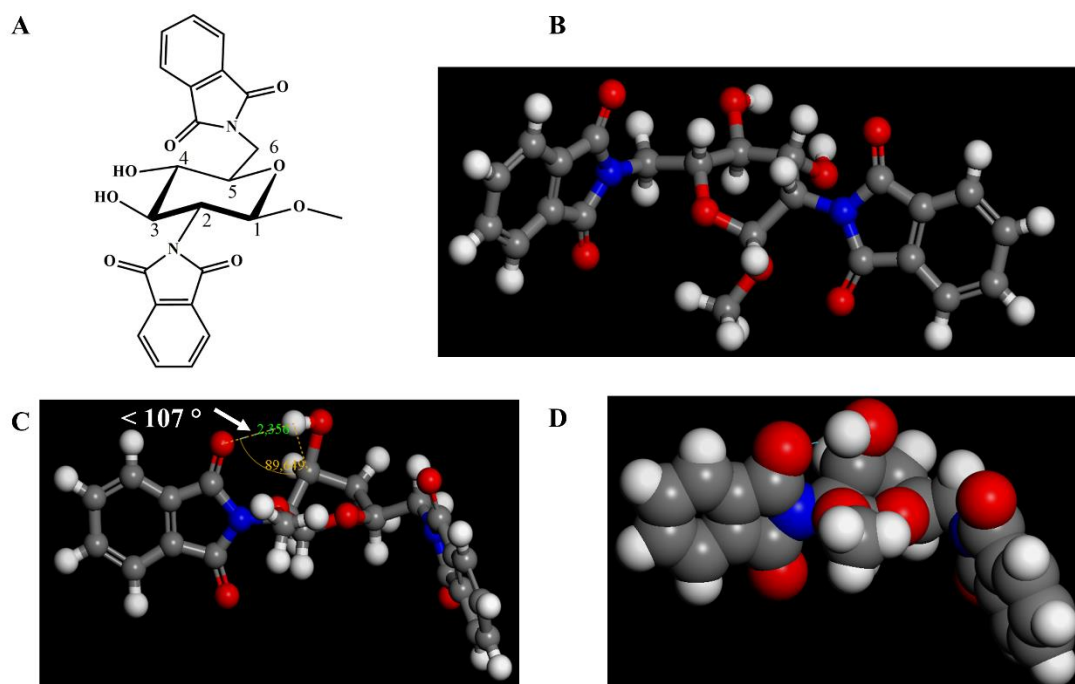
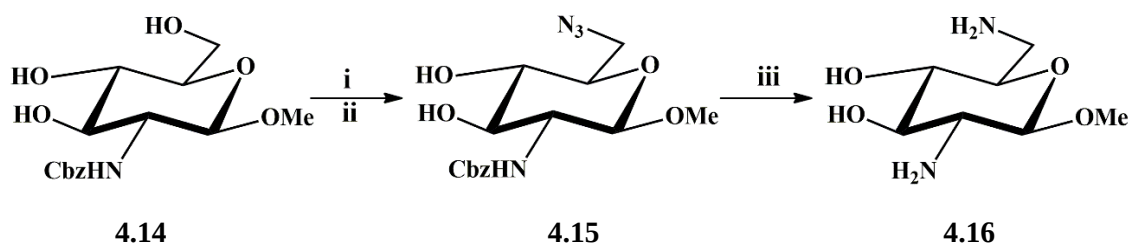


Figure 4.7: The optimised structure of (A) and (B) 2,6-dideoxy-2,6-diphthalimido- β -D-glucopyranoside (**4.13**), (C) the constrained monomer and (D) a space filling model of the constrained monomer.

Energy minimisation calculations of these three molecules yielded the following values: 2,6-dideoxy-2,6-diphthalimido- β -D-glucopyranoside (**4.13**) (96.87 kcal/mol), 2,6-dideoxy-2,6-diphthalimido- β -D-glucopyranoside constrained molecule containing a hydrogen bond (78.29 kcal/mol) and the trisaccharide (250.19 kcal/mol). These results demonstrate that the 2,6-dideoxy-2,6-diphthalimido- β -D-glucopyranoside (**4.13**) is stabilised through hydrogen bonding and the base is therefore unable to hydrolyse the protecting group due to the closed geometry of the molecule. The nucleophile in this case, hydrazine, typically requires an angle of 107° to the C=O bond known as the Bürgi–Dunitz trajectory which is difficult in the hydrogen bond stabilised monomer.³³ Furthermore, any nucleophile will require a trajectory along the axial plane of the carbohydrate ring which is known to be difficult. The studies by Iwata *et al.* on using mannosamine in the formation of oligodiaminomannoses reported ring opening of the phthalimide protecting group in several steps which was attributed to the lower stability of the phthalimide group at the 2-axial position in D-mannosamine derivatives compared to the equatorial 2-phthalimide groups in glucosamine and galactosamine derivatives.^{34, 35} This result further confirms the stability of the phthalimide group in glucosamine toward hydrolysis.^{34, 35}

Since the goal of this study was to introduce an amine at C-6 using a different aminating source as opposed to sodium azide, there was no need to fully deprotect the monomer. Methods for

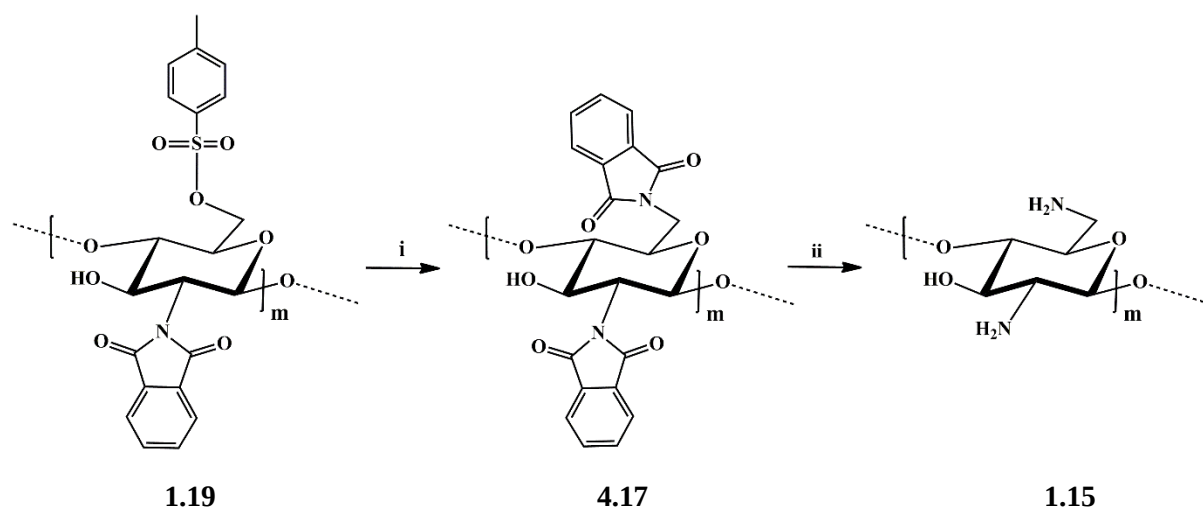
the removal of the phthaloyl group are given in the review by Enugala *et al.*¹⁵ In addition, methyl 2,6-diamino-2,6-dideoxy-D-glucopyranoside was recently reported by Kato *et al.*³⁶ This monomer was synthesised by the reaction of a C-2 protected methyl 2-*N*-carboxybenzyl glucosaminide with sodium azide *via* the C-6 alkyl halide and subsequent hydrogenation of the azide to produce the amine. The reaction conditions are given in Scheme 4.8.³⁶



Scheme 4.8: Synthesis of methyl 2,6-diamino-2,6-dideoxy-D-glucopyranoside (**4.16**). Reagents and conditions: i) 2,4,6-triisopropylbenzenesulfonyl chloride, pyridine, RT, 9 hr, 68%; ii) NaN₃, DMF, 80 °C, 6 hr, 78%; iii) H₂, Pd(OH)₂, dioxane, 12 hr, 88%.³⁶

4.2.2 Synthesis of 6-deoxy-2,6-bis(phthalimido) chitosan (**4.17**)

The monomer route was one step short of the fully deprotected amine; however, since the study was only a proof of concept for the substitution of the phthalimide at C-6, the diamine monomer itself was not required due to the publication of the spectra by Kato *et al.* (Scheme 4.8).³⁶ The conditions of substitution established in the monomer pathway could now be applied in the synthesis of 6-deoxy-6-amino chitosan (**1.15**). Additionally, it was useful to have the di-phthaloyl protected monomer for comparison in the solid state NMR analysis. Chitosan was used as the starting material (Scheme 4.9) for the synthesis of 6-deoxy-6-amino chitosan using potassium phthalimide as the aminating agent (**1.15**).



Scheme 4.9: Synthesis of 6-deoxy-6-amino chitosan starting with chitosan. Reagents and conditions: (i) Potassium phthalimide, DMAc, 120 °C, 4.5 hr, 86%; (ii) NMP, H₂NNH₂.H₂O, 100 °C, 4 hr, 98%.

The amine at C-2 required protection by a phthaloyl group, therefore chitosan was reacted with phthalic anhydride and the hydroxyl was thereafter functionalised with tosyl chloride. 6-Deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) was reacted with potassium phthalimide and the resulting polymer 6-deoxy-2,6-bis(phthalimido) chitosan (**4.17**) isolated with an 86% yield. The product was characterised using IR spectroscopy and elemental analysis, which confirmed the displacement of the tosyl group and subsequent substitution with the phthalimide moiety. In the IR spectra, the peaks assigned to the tosyl moiety visibly weakened and there was a slight upfield shift of the absorbance band assigned to the phthaloyl moiety. This indicated substitution at C-6 and the introduction of the additional phthalimide moiety.

This 2,6-protected version of the polymer (**4.17**) was treated with hydrazine hydrate in ethanol to remove the phthalimide groups present. 6-Deoxy-6-amino chitosan (**1.15**) was successfully isolated from the ethanol mixture in excellent yield (98%). Previously, this reaction was performed in NMP; however, after investigation of alternative solvents, it was found that absolute ethanol could successfully be used for this step, making the process comparatively greener. The replacement of the solvent with ethanol also led to a reduction in the amount of waste produced as typically reactions that are performed in NMP require copious amounts washing with organic solvents and the ethanol can be recovered after use.

The ^1H NMR spectrum shown in Figure 4.8 shows the characteristic peaks as those observed for the synthesis of 6-deoxy-6-amino chitosan (**1.15**) *via* sodium azide mediated amination.³⁷

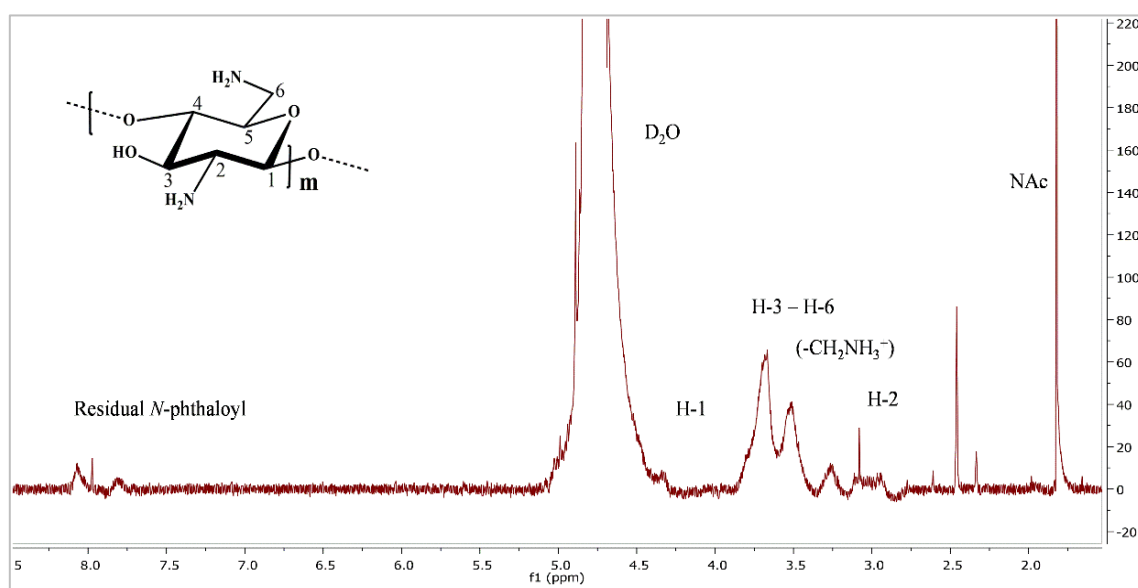


Figure 4.8: ^1H NMR spectrum of 6-deoxy-6-amino chitosan (**1.15**) in 2% DCl/ D_2O .

The peaks were shifted upfield compared to that reported, where H-1 resonated at 4.35 ppm versus the 5.36 ppm in the reported pathway.³⁸ The protons assigned to H-3 – H-6 appeared between 3.44 and 3.84 ppm versus 4.51 and 4.03 ppm originally. The signal for H-2 also appeared upfield at 3.05 ppm compared to the originally reported 3.67 ppm. This shift may be due to a difference in the pH of the solution when analysed. The peak assigned to residual *N*-acetyl from the acetylated units of chitosan was visible at 2.46 ppm while that assigned to the proton of the protonated amine ($-\text{CH}_2\text{NH}_3^+$) was present at 3.20 ppm. Minor peaks observed in the aromatic region (7 – 8 ppm) of the ^1H NMR spectrum were assigned to residual phthaloyl groups. However, these bands were considerably weaker compared to those observed when using the Staudinger reduction method.³⁸ This points to a noticeable difference in the spectra when the $\text{PPh}_3=\text{O}$ impurity is present.

The IR spectra for the polymers obtained from this synthetic route is shown in Figure 4.9. The introduction of the phthaloyl moiety in the synthesis of *N*-phthaloyl chitosan (**1.18**) was clearly visible by the peak in the region of 1700 cm^{-1} . Substitution by the tosyl group at C-6 was evident by the peaks at 815 and 1174 cm^{-1} , where these bands became less visible after substitution with the phthalimide. Deprotection with hydrazine yielded 6-deoxy-6-amino chitosan (**1.15**) which was evident from the appearance of bands relating to the amine groups in the region $1500 - 1650\text{ cm}^{-1}$ and the increase in the absorption band in the region of 3000 cm^{-1} .

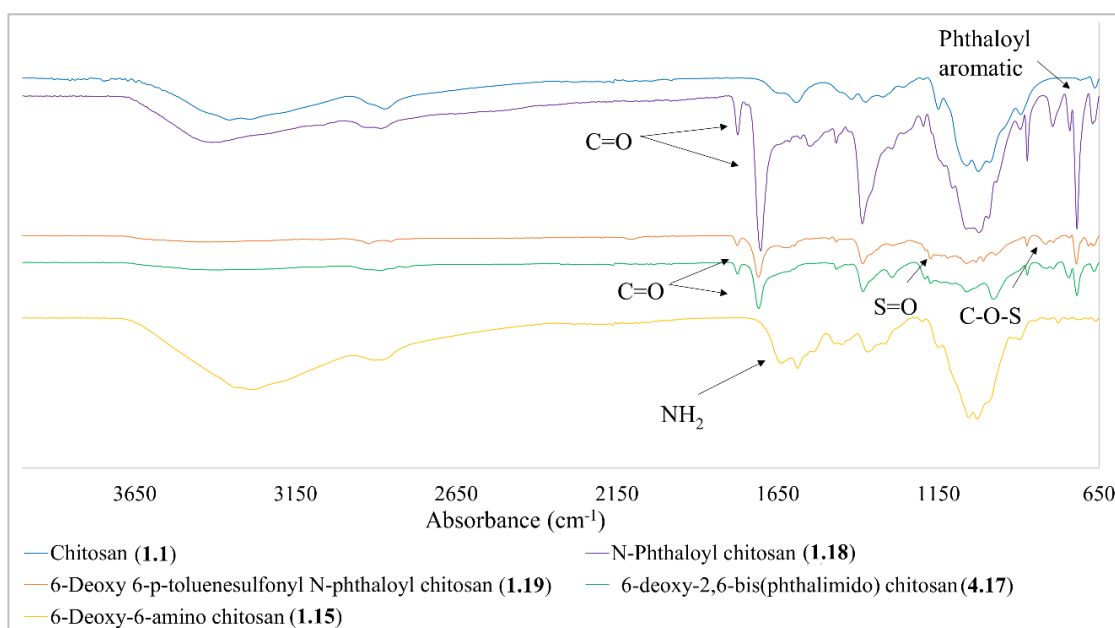


Figure 4.9: IR spectrum of (**1.15**) synthesised *via* the intermediates (**1.18**), (**1.19**) and (**4.17**).

The degree of substitution (DS) of the polymers in this pathway was calculated using results obtained from the elemental analysis as follows:

$$\text{For chitosan: } DS = \left[\frac{\left(\frac{C}{N}\right)_{\text{derivative}}}{\left(\frac{C}{N}\right)_{\text{chitosan}}} \right] * DDA (96\%)$$

The values obtained for the DS are given in Table 4.1. The changes in the percentages of carbon and nitrogen clearly demonstrated substitution and ultimately the introduction of an additional amine group onto the polymer backbone. The DS cannot be directly compared to those reported in the literature due to the inconsistencies associated with the reporting of the DS *via* different analytical methods. However, literature values are given to show the range of substitution that has been reported.

Table 4.1: The DS of polymers synthesised in the current study.

Polymer	%C*	%N*	C/N	DS	Lit. values
Chitosan (1.2)	44.04	6.65	6.62	-	
<i>N</i> -Phthaloyl chitosan (1.18)	52.14	4.99	10.04	1.42	0.65 – 1.54 ^{39, 40}
6-Deoxy-6- <i>p</i> -toluenesulfonyl <i>N</i> -phthaloyl chitosan (1.19)	43.92	4.43	9.91	1.46	0.30, > 0.99 ^{41, 42}
6-deoxy-2,6-bis(phthalimido) chitosan (4.17)	50.88	6.02	8.45	1.22	
6-Deoxy-6-amino chitosan (1.15)	40.71	12.02	3.39	0.49	0.7 – 0.94 ^{2, 43}

*determined by elemental analysis

A ninhydrin assay was used here to confirm the introduction of the amine moiety as evidenced by the increase in the purple colour (ninhydrin positive) from the reaction of the free amine with ninhydrin. The same amount of each polymer in this synthetic pathway was tested with the ninhydrin assay and compared to a blank and the starting material chitosan (Figure 4.10). This assay also confirmed the substitution of the phthaloyl group onto the free amine as evidenced by the lack of colour in tubes C and D. Deprotection of the polymer was clearly evident by the appearance of an intense purple colour in tube E confirming the presence of an additional amine.

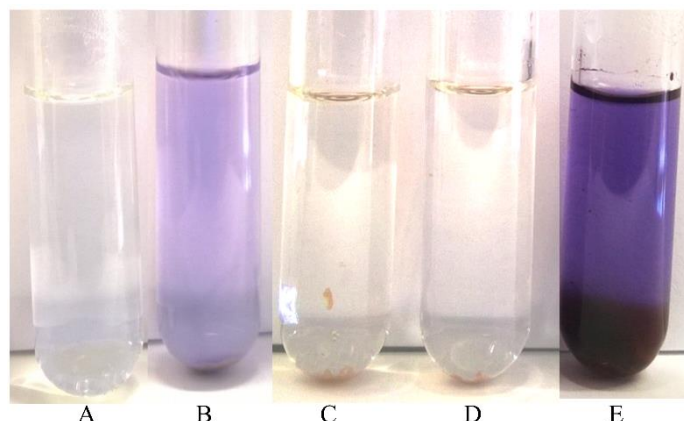
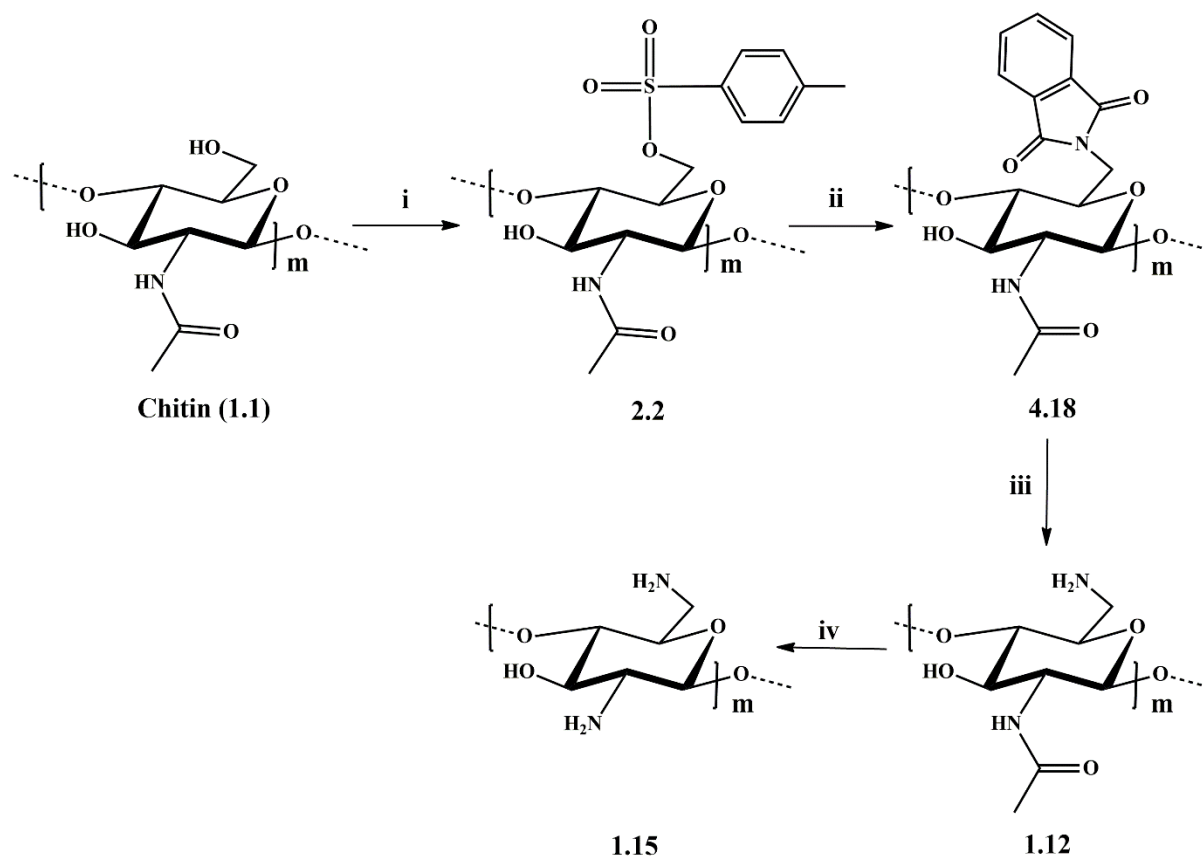


Figure 4.10: Ninhydrin assay of polymers (A) blank, (B) chitosan (**1.2**), (C) 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**), (D) 6-deoxy-2,6-bis(phthalimido) chitosan (**4.17**) and (E) 6-deoxy-6-amino chitosan (**1.15**).

The ninhydrin assay together with the IR spectra supported the synthesis of a diamino chitosan polymer, making the addition of an amine at C-6 much easier compared to previous reported methods.

4.2.3 Synthesis of 6-deoxy-6-amino chitosan (**1.15**) via 6-deoxy-6-amino chitin (**1.12**)

Owing to the greater availability of chitin over chitosan and to avoid the protection of the amine moiety of chitosan in step one, chitin was used as the starting material following the synthetic method in Scheme 4.10.



Scheme 4.10: Synthesis of 6-deoxy-6-amino chitosan. Reagents and conditions: (i) *p*-Toluenesulfonyl chloride, Et₃N, DMAc/LiCl, 48 hr, 5 °C, 99%; (ii) Potassium phthalimide, DMAc, 120 °C, 4.5 hr, 79%; (iii) EtOH, H₂NNH₂·H₂O, 95 °C, 4 hr, 99%; (iv) NaOH (45% w/v), 1 hr, 100 °C, 99%.

6-Deoxy-6-*p*-toluenesulfonyl chitin (**2.2**) was synthesised by reacting a homogenous solution of chitin in DMAc/LiCl with *p*-toluenesulfonyl chloride in the presence of Et₃N. Analysis of the IR spectra confirmed the introduction of the tosyl group where the characteristic peaks of S=O and C-O-S were evident. Thereafter, the tosylated chitin was reacted with potassium phthalimide and the obtained polymer was analysed by solid state ¹³C NMR spectroscopy (Figure 4.11). The ¹³C NMR spectrum of the polymer in the solid state is shown together with the monomer containing two phthalimide groups for comparative purposes. It is noted that the spectra obtained are relatively similar, making the monomer study useful in terms of confirmation of the introduction of the phthaloyl moiety onto the polymer as well as assignment of characteristic NMR signals.

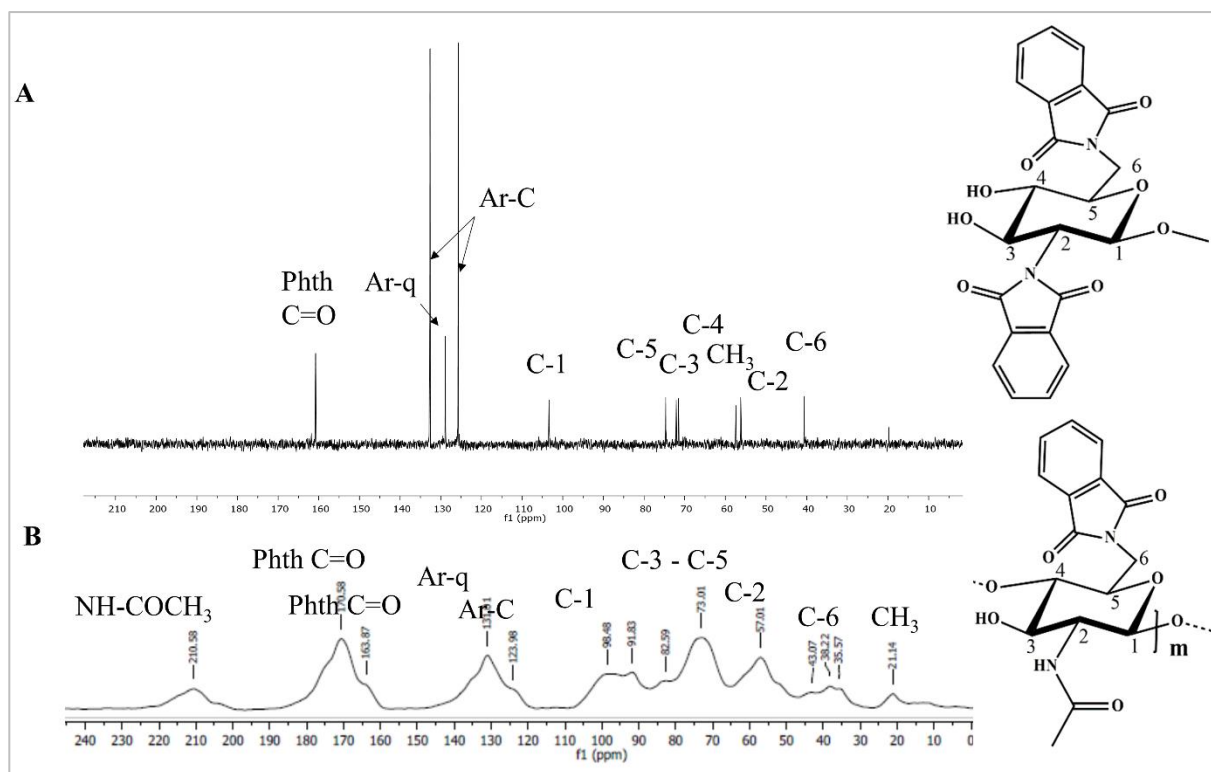


Figure 4.11: Solution state ^{13}C NMR spectra of (A) methyl 2,6-dideoxy-2,6-diphthalimido- β -D-glucopyranoside (4.13) in D_2O and (B) solid state ^{13}C NMR spectra of 6-deoxy-6-phthalimido chitin (4.18).

The characteristic peak assigned to the C=O of the phthaloyl group can be seen at 160.8 ppm and 163.9 ppm for the monomer and polymer respectively. In addition, the peak at 210.6 ppm in the polymer NMR spectrum was attributed to the *N*-acetyl group of chitin. 6-Deoxy-6-phthalimido chitin (4.18) was further characterised using IR spectroscopy and elemental analysis both of which confirmed the displacement of the tosyl moiety and subsequent substitution with the phthalimide group.

After confirming the incorporation of the phthalimide moiety at C-6 of chitin, the polymer was treated with hydrazine hydrate in absolute ethanol to remove the phthaloyl group thereby deprotecting the amine. The IR spectra of 6-deoxy-6-amino chitin (1.12) produced here indicated the loss of the phthaloyl aromatic absorbance at 721 cm^{-1} and an increase in the intensity of the bands at 1647 and 1585 cm^{-1} were seen for the amine. The elemental analysis showed a clear increase in the nitrogen content of the polymer and a positive ninhydrin test indicated the presence of a free amine.

The last step in the synthesis of 6-deoxy-6-amino chitosan (1.15) was deacetylation by treatment with a 45% solution of NaOH. The homogenous solution was thereafter neutralised

and dialysed against water to remove excess salt present. 6-Deoxy-6-amino chitosan (**1.15**) was subsequently lyophilised to produce the polymer in an excellent yield of 99%. Analysis of the solid recovered revealed that it was fully water soluble at a neutral pH, compared to previous methods which required lowering of the pH to obtain a soluble polymer, thus making this method the most effective in producing water soluble 6-deoxy-6-amino chitosan.

To further confirm the synthesis, the HSQC of 6-deoxy-6-amino chitosan (**1.15**) synthesised here versus that obtained by Yang *et al.* is given in Figure 4.12.³⁷ The HSQC obtained was compared to that of Yang *et al.* as the synthetic route used was quite similar and previous studies did not report an HSQC.³⁷ Additionally, the effect of substitution with potassium phthalimide versus sodium azide on the spectra of the final product was highlighted. The spectra of the polymer synthesised here showed the characteristic peaks of the polymer backbone. The peak at 2.08 ppm in the ^1H NMR spectrum and at 21.6 ppm in the ^{13}C NMR spectrum were assigned to the remaining acetyl groups. In the ^1H NMR spectrum the peak at 2.84 ppm was assigned to H-2 and correlated with the peak at 56.4 ppm in the ^{13}C NMR spectrum. The peaks at 3.28 and 3.49 ppm in the ^1H NMR spectrum were assigned to $-\text{CH}_2\text{NH}_2$ and $-\text{CH}_2\text{NH}_3^+$ at C-6 where this peak overlaps with any remaining C-6 linked to a hydroxyl group. The overlapping peaks at 3.52 and 4.75 ppm corresponded to the polymer protons H-3 to H-6 with their carbons signals resonating between 68.7 and 78.4 ppm in the ^{13}C NMR. The signal for the anomeric proton resonated at 5.05 ppm with its corresponding carbon signal appearing at 102.1 ppm. The removal of the phthaloyl group appeared to be almost complete as seen by the barely visible peaks in the range of 7.50 – 8.00 ppm in the ^1H NMR spectrum and at 131 ppm in the ^{13}C NMR spectrum. Exhaustive/repeated treatment with hydrazine would provide complete deprotection. Comparing the polymer obtained here to that analysed by Yang *et al.* it was noted that there was a shift of the peaks upfield, although the signal for H-1 was in generally the same position.³⁷ The most significant difference was in the residual phthaloyl groups observed in the aromatic region (7 – 8.5 ppm). The signals recorded by Yang *et al.* were stronger compared to the signals observed in the spectra for the polymer obtained *via* the new synthetic route.³⁷ Furthermore, the spectra obtained for 6-deoxy-6-amino chitosan using the original method reported by Jardine *et al.* was similar to that obtained here; however, with a greater intensity of the peaks related to the phthaloyl moiety in the aromatic region (this was fully discussed in Chapter Two).³⁸

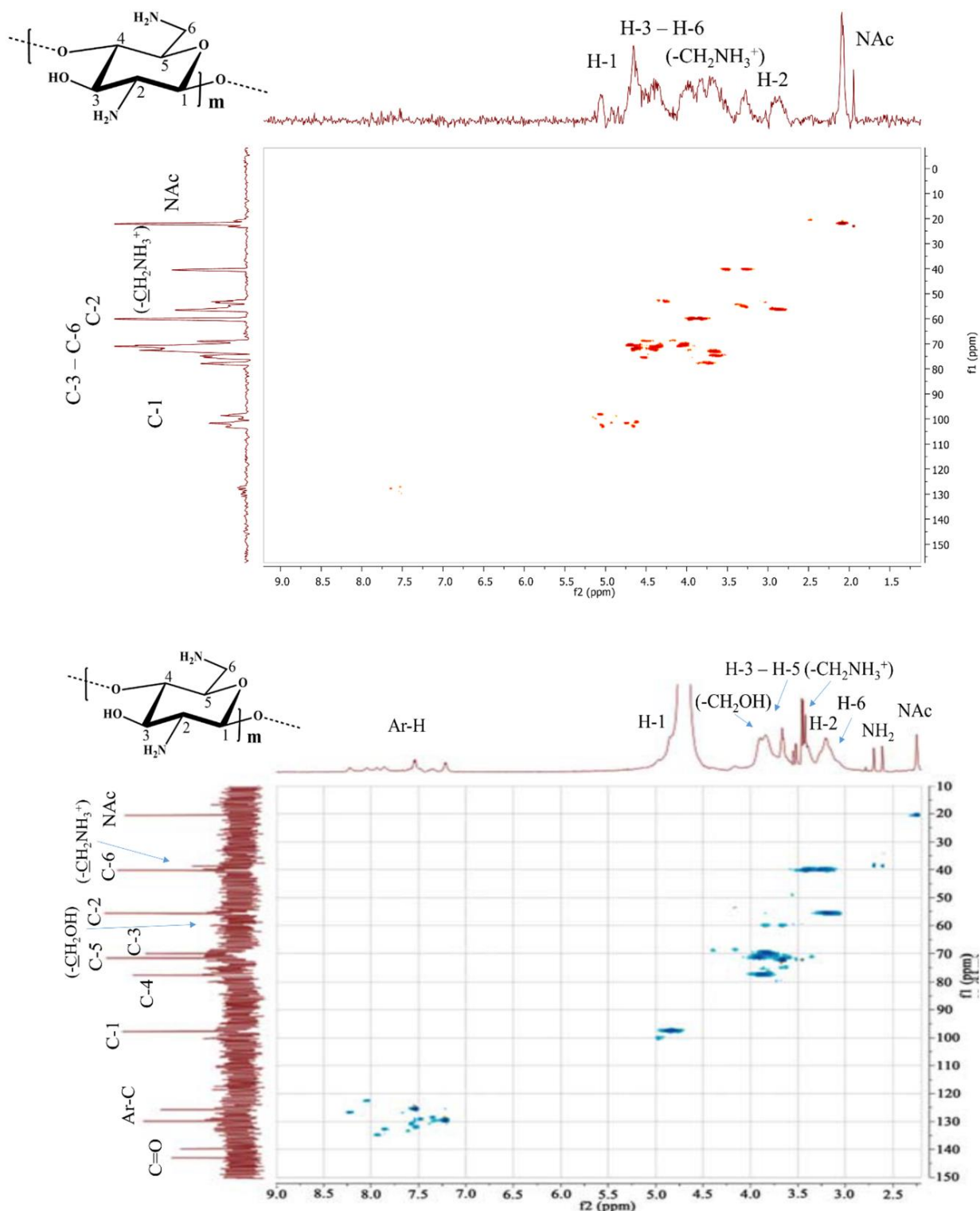


Figure 4.12: HSQC NMR spectrum of 6-deoxy-6-amino chitosan (**1.15**) in D₂O synthesised in the current study via the novel route (top) and by Yang *et al.* (bottom).³⁵

Considering the deprotection methods used, both routes use hydrazine hydrate in water (Yang *et al.*) and ethanol respectively.³⁷ Whereas Yang *et al.* treated the polymer with a huge excess of hydrazine, the ratio of polymer to hydrazine used was 1:106 as opposed to 1:24 used in the current study.³⁷ In addition, Yang *et al.* removed the phthaloyl protecting group at C-2 prior to

the reduction of the azide at C-6 using sodium borohydride.³⁷ The peaks obtained are significantly sharper for the polymer obtained through borohydride reduction possibly due to the low molecular weight as a result of degradation during the synthetic pathway. The peaks obtained for the polymer synthesised here are much broader indicating a higher degree of crystallinity which were confirmed using other techniques such as DSC and PXRD.

Infrared analysis of the polymer indicated an increase in the bands attributed to the NH₂ moiety in the 1500 – 1600 region of the spectrum. The IR spectra of the polymers in this particular pathway are shown in Figure 4.13. The characteristic bands for the tosylation and subsequent displacement with the phthalimide moiety are clearly visible by the labelled bands. Removal of the phthalimide groups and subsequent deacetylation increased the absorbance bands related to the amine group in the region of 1600 cm⁻¹.

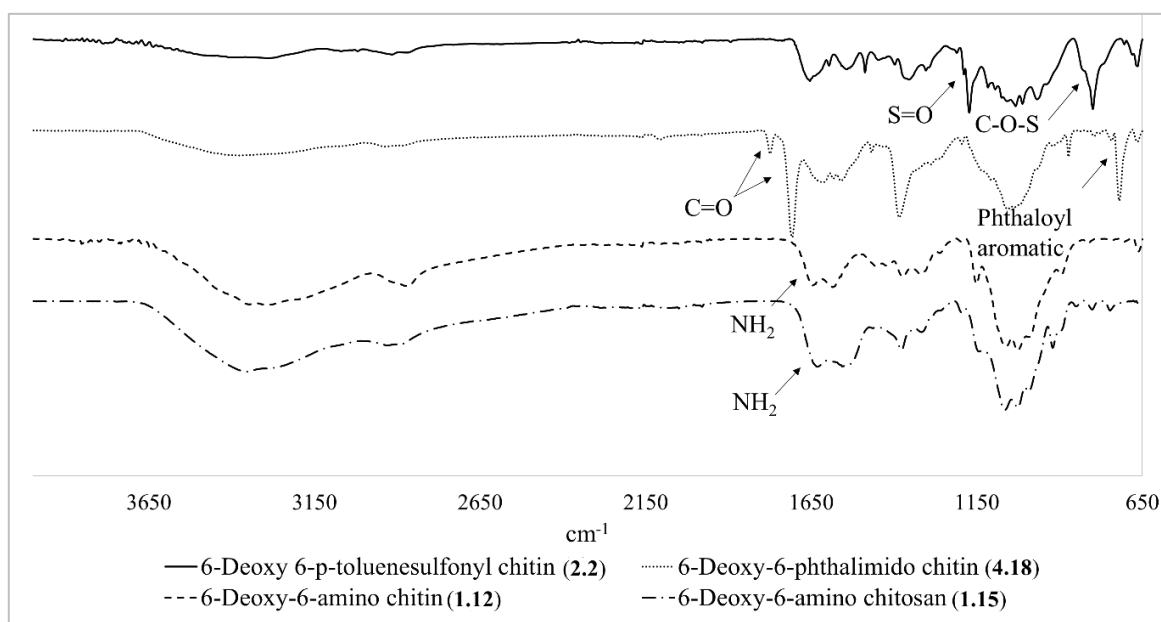


Figure 4.13: IR spectrum of (1.15) synthesised *via* the intermediates (2.2), (4.18) and (1.12).

The elemental analysis of the polymers synthesised using this pathway together with the degree of substitution (DS) calculated is given in Table 4.2. The equation used is given as follows:

$$\text{For chitin: } DS = \left[\frac{\left(\frac{C}{N} \right)_{\text{derivative}}}{\left(\frac{C}{N} \right)_{\text{chitin}}} \right]$$

The changes in the percentages of carbon and nitrogen clearly demonstrated substitution and ultimately the introduction of an additional amine group onto the polymer backbone. The DS cannot be directly compared to those reported in the literature due to the inconsistencies associated with the reporting of the DS *via* different analytical methods.

Table 4.2: The DS of polymers synthesised in the current study.

Polymer	%C*	%N*	C/N	DS†	Lit. values
Chitin	43.31	6.98	6.20	-	
6-Deoxy-6- <i>p</i> -toluenesulfonyl chitin (2.2)	44.70	5.30	8.43	1.36	0.25 – 3.0 ^{41, 43}
6-Deoxy-6-phthalimido chitin (4.18)	49.79	7.19	6.92	1.11	-
6-Deoxy-6-amino chitin (1.12)	46.69	8.22	5.68	0.92	0.48 ⁴⁵
6-Deoxy-6-amino chitosan (1.15)	40.55	12.52	3.24	0.52	0.7 - 0.94 ^{2, 43}

*determined by elemental analysis

A ninhydrin assay was again performed in each step of the sequence in order to confirm the presence of an amine moiety, where Figure 4.14 shows chitosan with a faint purple colour (B), as the positive control. For the route involving chitin, the substitution with a tosyl moiety at C-6 did not produce a reaction with ninhydrin and the solution remained clear (F). The same result was observed when the tosyl was substituted with the phthalimide and no change was observed (G). When the polymer was treated hydrazine hydrate to remove the phthaloyl group, the ninhydrin test was positive (H) and upon deacetylation the reaction of the polymer with ninhydrin gave a deep purple colour (I) indicating the presence of an additional amine.

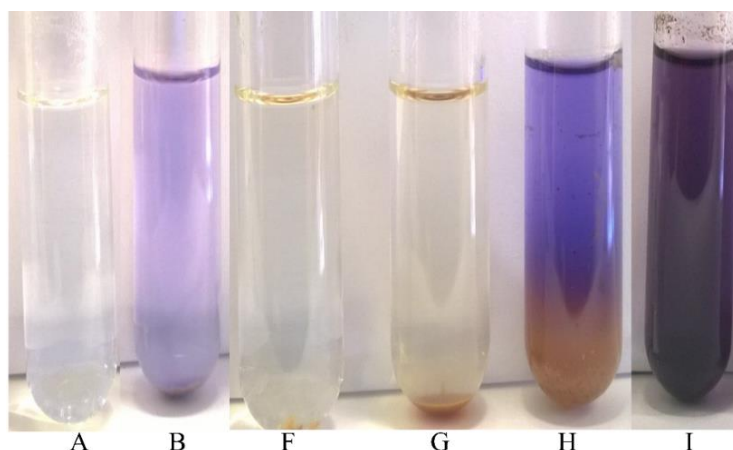


Figure 4.14: Ninhydrin assay of polymers (A) blank, (B) chitosan (**1.2**), (F) 6-deoxy-6-*p*-toluenesulfonyl chitin (**2.2**), (G) 6-deoxy-6-phthalimido chitin (**4.18**), (H) 6-deoxy-6-amino chitin (**1.12**) and (I) 6-deoxy-6-amino chitosan (**1.15**).

6-Deoxy-6-amino chitosan (**1.15**) synthesised *via* phthalimide substitution of tosyl chitin was then analysed by PXRD to examine the crystallinity of the polymer. The diffraction patterns of chitosan and that of 6-deoxy-6-amino chitosan (**1.15**) are shown in Figure 4.15. The polymer displayed two peaks characteristic of the hydrated and anhydrous forms of chitosan at a 2θ of 9° and 20° ; however, the peaks are quite small which can be attributed to the formation of an

amorphous polymer where the crystal structure is disordered. Additional peaks were observed at a 2θ of 32° , 45° and 56° , these peaks indicated a higher degree of crystallinity and could be due to contaminants in the sample.

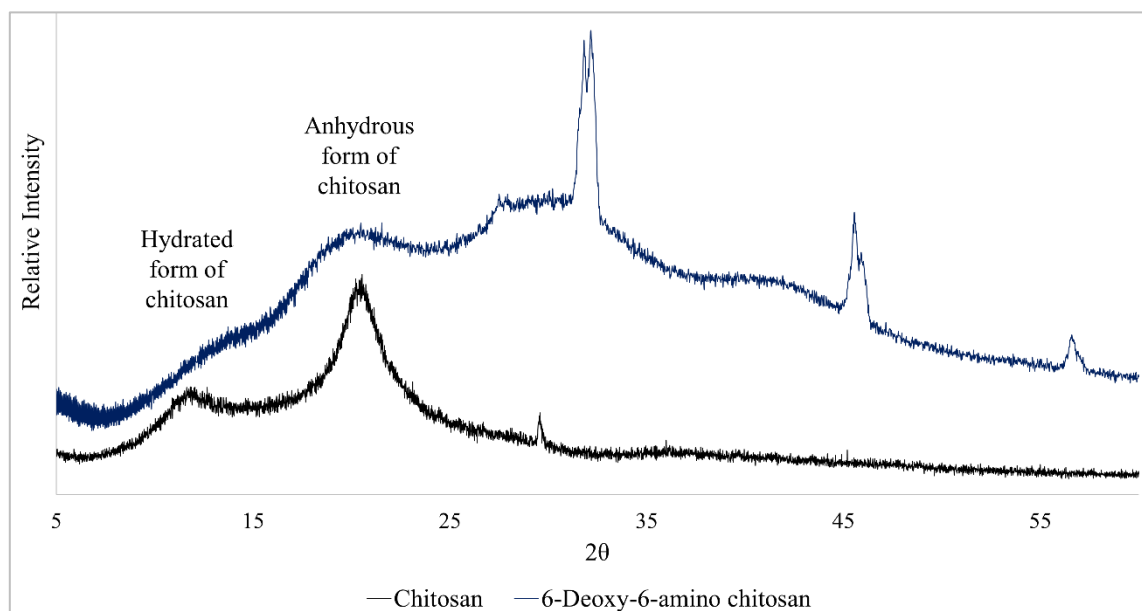


Figure 4.15: PXRD spectrum of chitosan and 6-deoxy-6-amino chitosan (**1.15**).

Thermal analysis of chitosan and 6-deoxy-6-amino chitosan (**1.15**) is shown in Figure 4.16 together with the corresponding derivative-grams (DTG). For chitosan, there were two characteristic degradations, the first one between 43.8 and 89.8°C was due to the loss of water from the polysaccharide chains. The second degradation between 292.6 and 338.6°C (DTG_{max} at 314°C) corresponding to a mass loss of 56.1% resulted from the depolymerisation of the polymer with the loss of any volatile compounds present. For 6-deoxy-6-amino chitosan (**1.15**), two stages of mass were observed. In the first stage, between 44 and 95.8°C a mass loss of 15% was observed and during the second stage between 226.1 and 274.0°C (DTG_{max} at 242°C) a 38.7% mass loss was observed. The onset temperature of the second degradation was higher for chitosan compared to 6-deoxy-6-amino chitosan (**1.15**), which indicated a lower degree of hydrogen bonding which in turn supports the better solubility observed for this polymer. In total, the mass loss for chitosan and 6-deoxy-6-amino chitosan (**1.15**) was 65.8% and 53.7% respectively, supporting the thermal stability of these polymers.

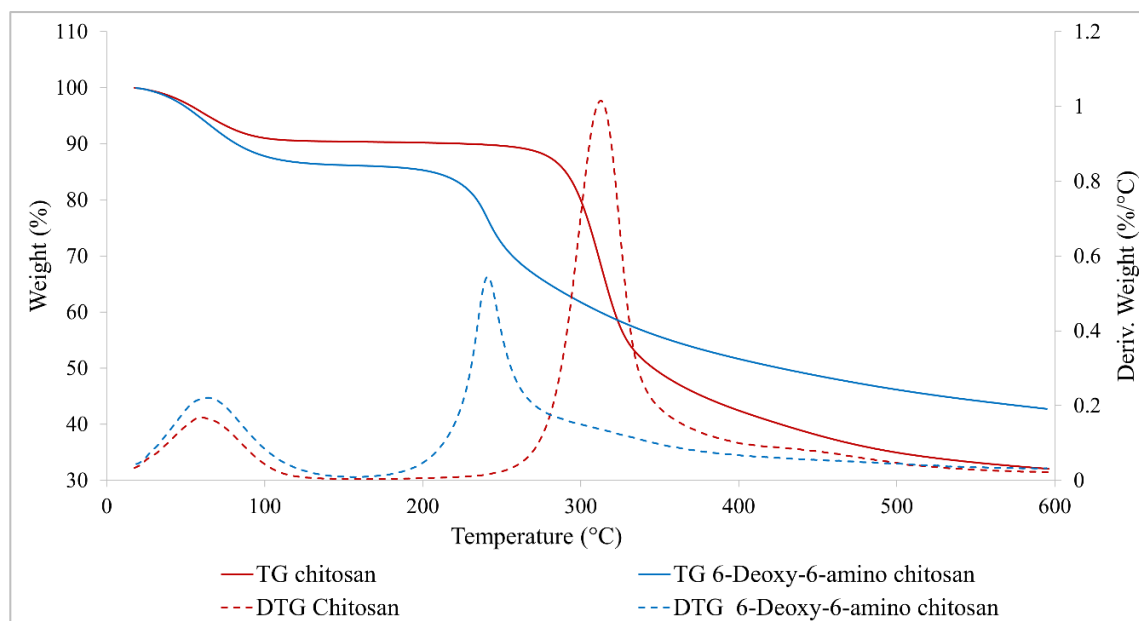


Figure 4.16: TGA of chitosan (1.2) and 6-deoxy-6-amino chitosan (1.15).

As discussed in Chapter Two, there are various concerns surrounding the complete removal of phthalimide groups; yet, the use of potassium phthalimide versus sodium azide outweighs the removal concerns. This is due to the difference in the safety concerns of the two reagents where potassium phthalimide is greener, more cost effective and the disposal of any waste produced from the reaction is not as concerning as that of the waste stream associated with sodium azide usage (requires separate disposal). In addition, since this route avoids the generation of triphenylphosphine oxide as a by-product, the solubility and quality of the polymer were greatly improved. Owing to the principle that impurities present affect polymer properties, the added benefit of the use of a phthalic anhydride derived reagent is the renewability of the material. This is a result of various reports for the production of phthalic anhydride from renewable resources such as agricultural residue (e.g. corn stover). The anhydride is produced by the reaction of biomass-derived furan and maleic anhydride. A recent techno-economic assessment of the production of phthalic anhydride from corn stover demonstrated that an economically viable process which is cost-competitive compared to the traditional synthetic route is possible with the proper designing of a biorefinery system.⁴⁶

Reagents which introduce an alkylamine can also be considered for modification of chitin and chitosan. After the development and synthesis of a water soluble chitosan derivative, the next step in this work was the introduction of an additional amine using an alkyl amine.

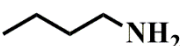
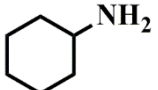
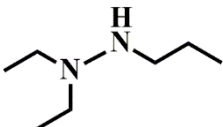
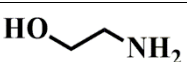
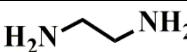
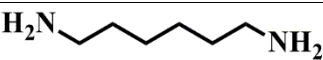
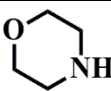
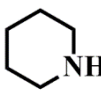
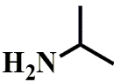
4.3 Synthesis of 6-deoxy-6-amino-alkylamino chitin and chitosan

Alkylamines have previously been used for the introduction of an amine functionality onto a variety of molecules. These alkylamines confer advantageous properties onto the modified material. Additionally, the length of the alkyl chain can affect properties observed therefore, researchers tend to choose chains of different lengths when studying the effect of modifications on the properties of the material synthesised. Owing to the similarity in the structure of cellulose, chitin and chitosan, reactions which have been performed on cellulose can typically be transferred to chitin and chitosan. The modification of cellulose with various amines is a topic of interest as the addition of amines onto the polymer provides for interesting properties, such as solubility, mucoadhesivity, and increase in the charge on the polymer. Heinze *et al.* has done numerous studies on the addition of an amine onto the cellulose backbone.⁴⁷ One of the first studies involved the reaction of tosyl cellulose with an excess of di- or trifunctional amines namely; tris(2-aminoethyl)amine, 1,2-diaminoethane and 4,7,10-trioxa-1,13-tridecandiamine. These compounds were solubilised in DMSO to produce water soluble materials.⁴⁸ 6-Deoxy-6-amino cellulose sulfate was synthesised and further derivatised.⁴⁹ These derivatives were studied for their physicochemical properties and bioactivity and subsequently used in the modification of viscose fibres.^{50, 51} 6-Deoxy-6-aminoethyleneamino cellulose was also studied for its hemocompatibility using various assays which demonstrated that this polymer showed concentration- and DS-dependent influences on hemocompatibility *in vitro*, making it a promising material for biomedical applications.⁵²

Aminocellulose nanospheres were recently successfully used in conjunction with hyaluronic acid as a bacteria responsive multilayer coating for the prevention of biofilms on urinary catheters, demonstrating the potential of these polymers in the biomedical sector.⁵³ As a result of their successful application, the attention was shifted to improving the synthesis of those polymers. Heinze *et al.* recently reported a solvent free synthesis of 6-deoxy-6-(ω -aminoalkyl)amino cellulose.⁴⁷ Tosyl cellulose was reacted with ethylene diamine or tris(2-aminoethyl)amine where the amines themselves were used as the reaction medium to yield water soluble products. In addition, the recycling of the unreacted amine and the precipitant in this case 2-propanol was successfully achieved.⁴⁷

The modification of chitin and chitosan using alkylamines has previously been reported. Examples of the reagents used are given in Table 4.3.

Table 4.3: Reagents utilised for the introduction of an alkylamino group onto chitin or chitosan.

Reagent	Structure	Toxicity/Hazard	Reference
Butylamine		Skin and respiratory irritant	Hu <i>et al.</i> ⁵⁴
Cyclohexanamine		Toxic and flammable	Pourjavadi <i>et al.</i> ⁴
<i>N,N</i> -diethylaminopropylamine		Toxic	Seidi <i>et al.</i> ⁵⁵
Dimethylamine		Flammable gas	Pourjavadi <i>et al.</i> ⁴
Ethanolamine		Skin and respiratory irritant	Pourjavadi <i>et al.</i> ⁴
Ethylenediamine		Skin and respiratory irritant	Hu <i>et al.</i> ⁵⁴
Hexamethylenediamine		Toxic, skin and respiratory irritant	Shimizu <i>et al.</i> ⁵⁶
Morpholine		Flammable, skin and respiratory irritant	Pourjavadi <i>et al.</i> ⁴
Piperidine		Skin and respiratory irritant	Pourjavadi <i>et al.</i> ⁴
Propan-2-amine		Flammable and respiratory irritant	Pourjavadi <i>et al.</i> ⁴

Some examples include the reaction of tosylchitin with ethylenediamine or hexamethylenediamine to produce aminoalkylaminodeoxychitins in DMSO, which were applied as adsorbents for acid dyes (**1.13**, **1.14**, Figure 4.17).⁵⁷ A further study by the same authors synthesised thiolated aminoalkylaminodeoxychitins which were tested as acid dye adsorbents and for their adsorption of Hg^{2+} ions.⁵⁶ These studies highlighted the difference in the adsorption activity between amino substituted and alkylamino substituted polymers where it was found that the derivatives containing additional amine groups have a higher adsorption ability for an acid dye.⁵⁶

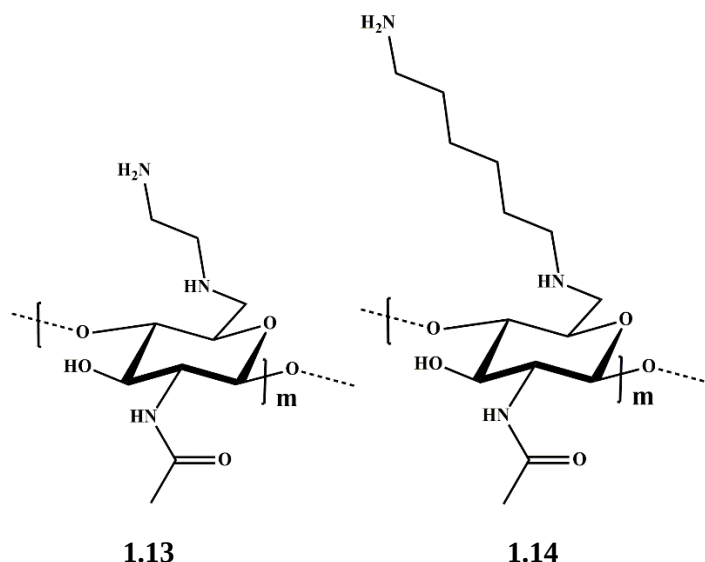


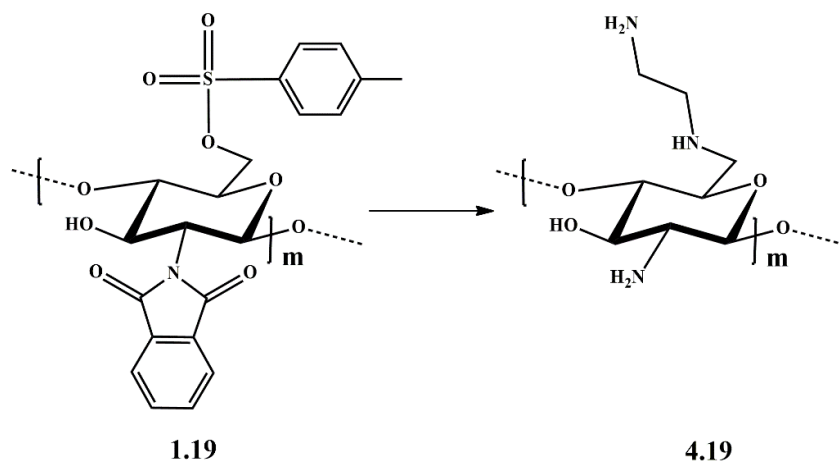
Figure 4.17: Aminoalkylaminodeoxychitins synthesised by Shimizu *et al.*⁵⁷

Chethan *et al.* reported the synthesis of an ethylenediamine modified chitosan which was used in the removal of divalent metal ions from water.⁵⁸ This study utilised *N*-phthaloyl chitosan and 6-deoxy-6-chloro-*N*-phthaloyl chitosan as precursors in the synthesis of the ethylenediamine modified polymer.^{58, 59} The first study reported on the use of the polymer for the removal of Cu(II), Pb(II) and Zn(II) where a later study looked at the adsorption of Cu(II), Pb(II), Zn(II) and Cr(IV). A recent study by Zhou and colleagues reported the synthesis of 6-deoxy-6-(2-aminoethyl) amino chitosan and its aminoethyl derivative.⁶⁰ The synthesis of these polymers followed a similar pathway to that reported previously where the 6-deoxy-6-(2-aminoethyl) amino chitosan polymer was further reacted with 2-chloroethylamine hydrochloride. The red blood agglutination properties of these polymers were tested over a wide pH and concentration range.⁶⁰ The use of halogenated intermediates and solvents such as DMSO are not favourable especially in terms of large scale synthesis, therefore alternatives needed to be investigated. In the current study, chitin and chitosan were used as the starting materials for the synthesis of an ethylenediamine modified derivative.

Solvent free synthesis of 6-deoxy-6-(2-aminoethyl) amino chitosan (4.19)

6-Deoxy-6-(2-aminoethyl) amino chitosan (**4.19**) was synthesised by reacting 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) with ethylenediamine, under reflux (Scheme 4.11). Tosylated chitosan (**1.19**) was refluxed in ethylenediamine leading to the introduction of the alkyl amine at C-6 as well as the removal of the phthaloyl protecting group at C-2. The removal of a phthaloyl protecting group using ethylenediamine has been reported previously in numerous sources.⁶¹ However, these deprotection reactions were typically performed in

alcohols whereas in the current reaction the ethylenediamine serves as deprotecting agent and solvent.



Scheme 4.11: Synthesis of 6-deoxy-6-(2-aminoethyl) amino chitosan (**4.19**). Reagents and conditions: ethylenediamine, 6 hr, 120 °C, 93%.

This one-pot substitution and deprotection was advantageous and convenient in the synthesis of a highly substituted polymer as evidenced by the IR (Figure 4.18) and EA data, which showed a distinct increase in the nitrogen content of the polymer. The IR spectra confirmed the substitution and deprotection of the polymer with a clear absence of the peaks related to the phthaloyl protecting group and an increase in the intensity of the peaks attributed to the amine moiety when compared to the starting material.

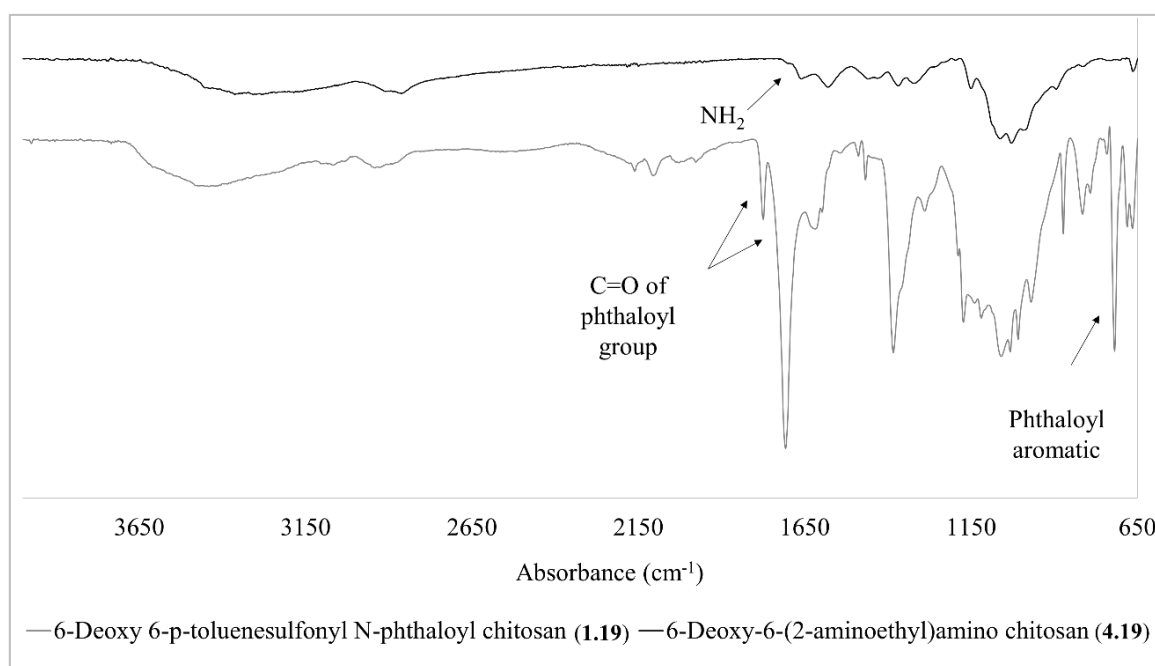
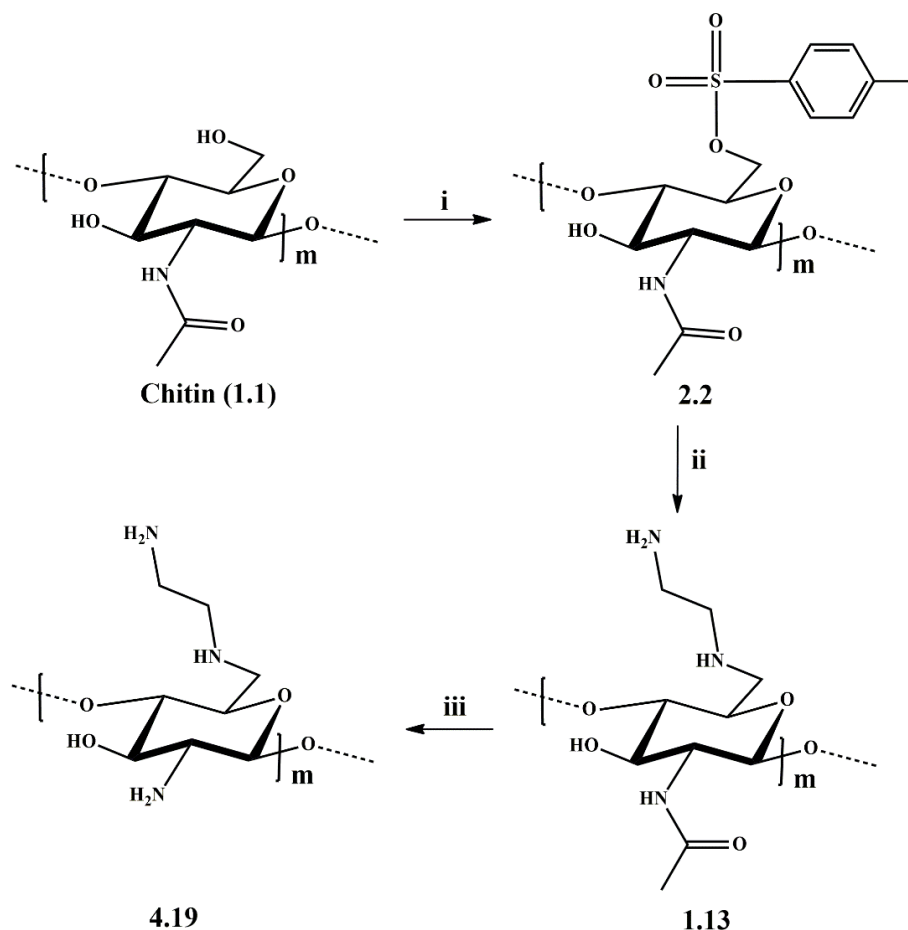


Figure 4.18: The IR spectra of the ethylenediamine modified chitosan derivative.

As mentioned previously, starting with chitin avoids the use of a C-2 protection-deprotection strategy which is advantageous in order to minimise the waste produced from these modification reactions. Scheme 4.12 shows the pathway using chitin as the starting material.



Scheme 4.12: Synthesis of ethylenediamine modified chitosan derivative starting with chitin. Reagents and conditions: (i) *p*-Toluenesulfonyl chloride, DMAc/LiCl, 48 hr, 5 °C, 99%; (ii) ethylenediamine, 6 hr, 120 °C, 83%; (iii) NaOH (45% w/v), 5 hr, 100 °C, 79%.

Starting with chitin, 6-deoxy-6-(2-aminoethyl) amino chitin (**1.13**) was synthesised by reacting tosylated chitin (**2.2**) with ethylenediamine at 120 °C for 6 hours. The reaction was homogenous, utilising the reagent as the solvent thus favouring an increased degree of substitution of the polymer. The polymer was precipitated with isopropanol and isolated with a good yield of 83%. The IR spectrum supported the successful synthesis of polymer (**1.13**) as indicated by the increase of the intensity of the NH₂ band at 1661 cm⁻¹ and a shift to a lower frequency for the acetylated amine band. The elemental analysis supported incorporation of the ethylenediamine moiety with a substantial increase in the nitrogen content as compared to its precursor.

6-Deoxy-6-(2-aminoethyl) amino chitin (**1.13**) was then deacetylated to form the chitosan derivative (**4.19**), by refluxing in concentrated sodium hydroxide. A brown solid was obtained with a yield of 79% after neutralisation and dialysis of the solution against water followed by lyophilisation. A 2% DCl/D₂O solution was added to a sample of the isolated polymer in order to obtain a ¹H NMR spectrum (Figure 4.19). The ¹H NMR spectrum indicated incomplete substitution with signals for residual tosyl groups still present in the region 7 – 7.5 ppm after optimisation. The introduction of new peaks between 2.82 – 2.88 ppm were assigned to the methylene protons in the side chain, while the IR spectrum indicated the presence of a free NH₂ and a decrease in the acetylated amine band. The combination of the above analysis, further confirmed the synthesis of polymer (**4.19**). In addition, the ¹H NMR spectra agreed with that reported by Zhou *et al.* further confirming successful modification of the polymer.⁶⁰

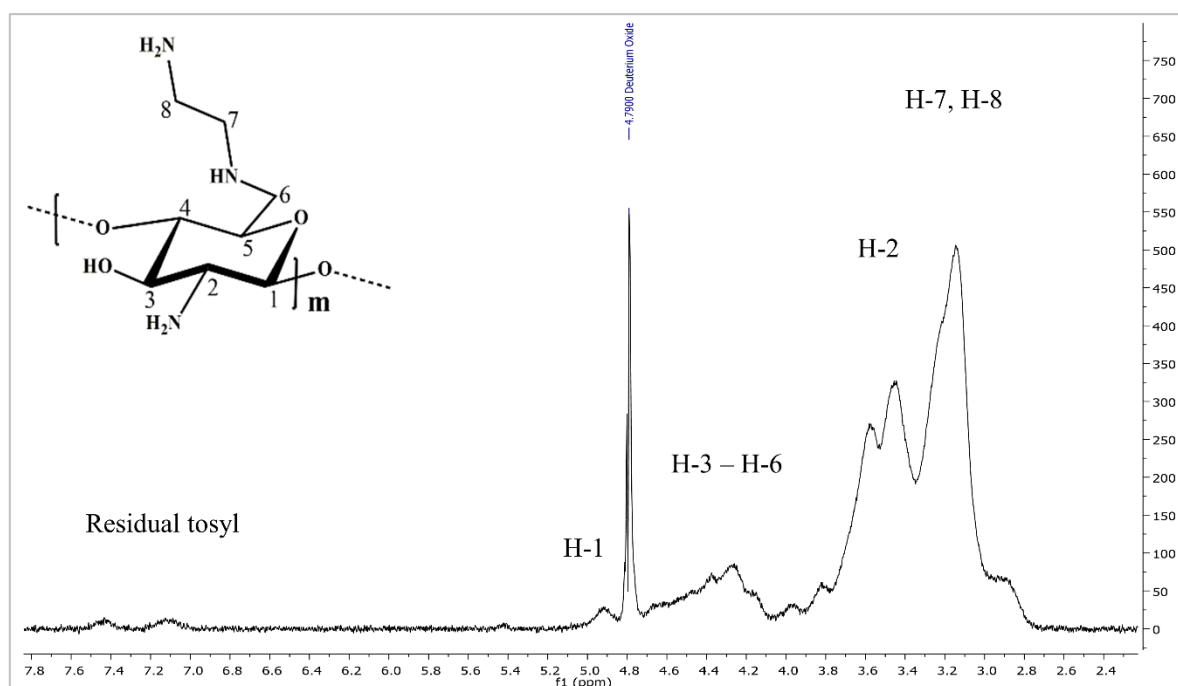


Figure 4.19: ¹H NMR spectrum of 6-deoxy-6-(2-aminoethyl) amino chitosan **4.19** in 2% DCl/D₂O.

The DS of the polymers in this pathway was calculated using results obtained from the elemental analysis as follows:

$$\text{For chitosan: } DS = \left[\frac{\left(\frac{C}{N}\right)_{\text{derivative}}}{\left(\frac{C}{N}\right)_{\text{chitosan}}} \right] * DDA (96\%)$$

$$\text{For chitin: } DS = \left[\frac{\left(\frac{C}{N}\right)_{\text{derivative}}}{\left(\frac{C}{N}\right)_{\text{chitin}}} \right]$$

The values obtained for the derivatives are reported in Table 4.4. Due to the difference in the method used for calculating the DS in different studies, these values were not comparable to any literature reports. Where a DS of greater than one is observed, this could indicate the presence of impurities or substitution at unprotected sites such as C-2 or C-3.

Table 4.4: The DS of polymers synthesised in the current study. (*determined by elemental analysis)

Polymer	%C*	%N*	C/N	DS	Literature values
Chitosan (1.2)	44.04	6.65	6.62	-	-
<i>N</i> -Phthaloyl chitosan (1.18)	52.14	4.71	11.07	1.60	0.65 – 1.54 ^{39, 40}
6-Deoxy-6- <i>p</i> -toluenesulfonyl <i>N</i> -phthaloyl chitosan (1.19)	43.92	4.43	9.91	1.46	0.30, > 0.99 ^{41, 42}
6-Deoxy-6-(2-aminoethyl) amino chitosan (4.19)	44.85	20.29	2.21	0.35	0.34 ⁶⁰
Chitin (1.1)	43.31	6.98	6.20	-	-
6-Deoxy-6- <i>p</i> -toluenesulfonyl chitin (2.2)	44.70	5.30	8.43	1.36	0.25 – 3.0 ^{41, 44}
6-Deoxy-6-(2-aminoethyl) amino chitin (1.12)	42.44	11.85	3.58	0.58	-
6-Deoxy-6-(2-aminoethyl) amino chitosan (4.19)	45.77	13.54	3.38	0.54	0.34 ⁶⁰

6-Deoxy-6-(2-aminoethyl) amino chitosan (**4.19**) was analysed by X-ray diffractometry to further evaluate its physical properties (Figure 4.20).

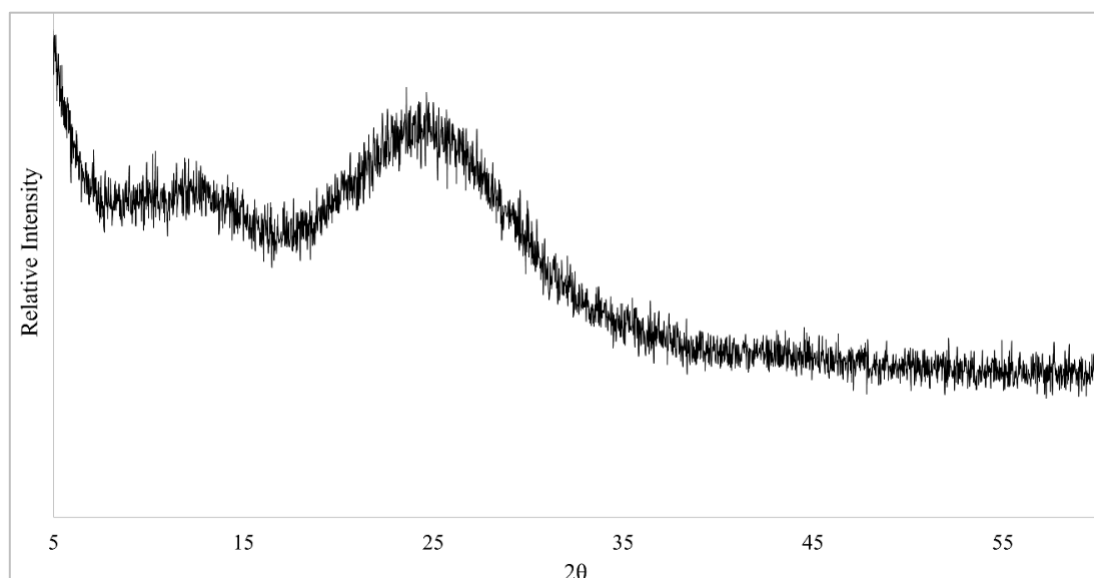


Figure 4.20: XRD pattern of 6-deoxy-6-(2-aminoethyl) amino chitosan (**4.19**).

The XRD pattern observed was very similar to that obtained for 6-deoxy-6-amino chitosan synthesised *via* the method reported by Jardine *et al.*; however, the peak at 2θ of 9° was not very well defined potentially due to the increased crystallinity of the polymer.³⁸

The thermal properties of (**4.19**) were analysed using TGA and DSC (Figure 4.21).

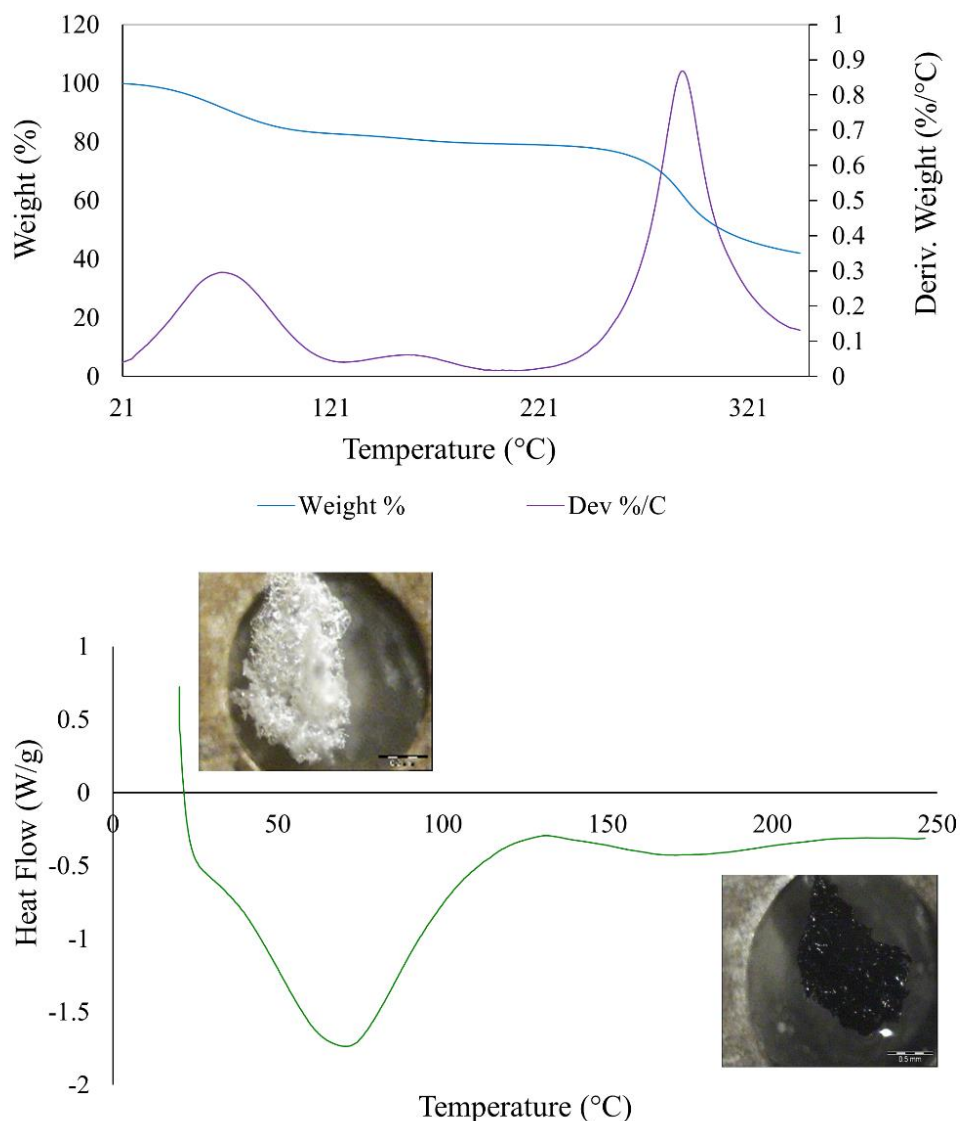


Figure 4.21: TGA (top) and DSC (bottom) of 6-deoxy-6-(2-aminoethyl) amino chitosan (**4.19**).

The TGA of **4.19** was similar to that reported by Chethan *et al.* for this modified polymer.⁵⁸ The first weight loss was observed between 23 – 126 °C followed by a sharp loss between 126 – 202 °C, while the final weight loss occurred between 202 – 345 °C. These mass losses can be attributed to the removal of residual water, both free and unbound, as well as the decomposition of the polymer chain. The DSC confirmed the thermal stability of the polymer over the range 0 – 250 °C where it only displayed one endothermic peak at 70 °C, resulting

from the dehydration of the polymer. The hot stage microscopy (HSM) pictures are shown as inserts in the DSC obtained, to illustrate the appearance of the polymer before and after analysis once dehydration and decomposition have occurred.

When comparing the methods reported for the synthesis of the current polymer, it was noted, that the method by Chethan *et al.* involved four steps whereas that reported here involved three steps.⁵⁸ The study by Chethan *et al.* included an extra step for the deprotection of the *N*-phthaloyl group by hydrazine hydrate in NMP, due to the low amount of ethylenediamine used.⁵⁸ Furthermore, in the study by Shimizu *et al.* tosylated chitosan was produced directly without the protection of the amine at C-2, which can potentially produce side products.⁵⁶ Zhou *et al.* used hydrazine hydrate for the removal of the phthaloyl group at C-2; however from the IR spectra it can be seen that partial removal of the phthaloyl group was already achieved in the previous step due to the treatment of the polymer with ethylenediamine.⁶⁰ Overall, the novel synthetic pathway was found to be greener than previously reported methods, as it does not contain any halogenated intermediates and was synthesised using relatively milder, greener solvents in excellent yields.

4.4 Conclusion

In summary, 6-deoxy-6-amino chitosan (**1.15**) was synthesised *via* the introduction of an amine at C-6 using potassium phthalimide as a nitrogen source. A model study involving the functionalisation of glucosamine was also carried out to monitor substitution of the phthalimide moiety when potassium phthalimide was used as a nitrogen source. The tosylated monomer was reacted with the phthalimide and substitution was observed at C-6. Additionally, esters were formed at C-3 and C-4. The monomer was treated with hydrazine hydrate to remove the phthaloyl protecting; however, only the esters at C-3 and C-4 were removed. The stability of the phthaloyl groups at C-2 and C-6 was investigated using computational modelling which showed that this monomer was conformationally rigid due to hydrogen bond mediated stabilisation.

After comparing all the methods used to synthesise 6-deoxy-6-amino chitosan (**1.15**) in this study, it is clear that the method starting with chitin involving the reaction of the 6-OTs polymer with a phthalimide salt is the most effective means of introducing the amine. This method reduces the number of steps required for the overall synthesis and minimises the production of by-products and waste. In addition, this synthetic route completely avoids the production of triphenylphosphine oxide as a by-product which cuts down significantly on the amount of

waste produced compared to the original method reported by Jardine *et al.*³⁸ The replacement of sodium azide, known to be toxic and combustible with potassium phthalimide as the aminating agent is advantageous both from a safety as well as an economic point of view, as the phthalimide salt is considerably cheaper.⁶²

The solvent used for the removal of the phthaloyl group was substituted for absolute ethanol thereby minimising the use of toxic solvents such as DMAc and NMP. The ethanol can also potentially be recycled giving this process an added advantage. The overall yields compare favourably to the previously reported methods making this synthetic method feasible in the production of a water soluble 6-deoxy-6-amino chitosan (**1.15**) derivative. While the new methods reported here are not completely 'green' they are a step in the right direction to produce a polymer using less hazardous reagents and solvents which can be scaled up to produce large quantities of the polymer.

Other means of introducing an amine at C-6 were investigated, using an alkylamine as the nitrogen source due to the varying properties obtained when different chain lengths are introduced. In the current study, ethylenediamine served as the amine source where both chitin and chitosan were successfully modified. The synthesised polymer compared well with that reported in literature and the synthesis was shorter compared to previous literature methods. In this study, ethylenediamine was used as the solvent, reagent and deprotection agent in the case of chitosan, making this synthesis simple, efficient and more importantly cost effective for the introduction of an additional amine.

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Chapter Five

Applications of 6-Deoxy-6-Amino Chitosan

5.1 Overview

The derivatives of chitin, chitosan and 6-deoxy-6-amino chitosan have been reviewed in Chapter One together with the novel properties that the introduced 6-amino group imparts. The synthesis of 6-deoxy-6-amino chitosan (**1.15**), has been improved to a stage where the scale-up of polymer production is feasible. From an economic point of view, the cost of producing this polymer is lower compared to the originally reported synthetic pathway. This cost saving comes from the reduction in the synthetic steps needed by utilising chitin instead of chitosan as the starting material, which makes a protection-deprotection strategy redundant. Additionally, potassium phthalimide is cheaper compared to sodium azide and produces waste which is not as hazardous as that generated by the use of azides. Replacement of certain solvents has further lowered the cost making this pathway overall more feasible for scale up. The difference in the cost between chitosan and 6-deoxy-6-amino chitosan should be negligible considering the opportunity that the additional amine at C-6 presents in broader applications. An in depth costing of the synthesis of 6-deoxy-6-amino chitosan (**1.15**) should be performed; however, this is out of the scope of the current study.

The solubility of the polymer in water is advantageous and opens up many different avenues of application. From a review of the literature available on 6-deoxy-6-amino chitosan, a number of applications have been reported (Figure 5.1). The existing applications which have been reported includes gene transfection, antioxidant ability, cell growth, as a catalyst support and in argentation chromatography.

This chapter is divided into two distinct sections where in the first section, the application of 6-deoxy-6-amino chitosan chosen to be investigated, is the antimicrobial activity. This section investigated the

- production of nanofibres loaded with antibacterial silver nanoparticles
- use of the polymer as an antifungal edible food coating and as a plant defence elicitor.

In the second section the application of 6-deoxy-6-amino chitosan (**1.15**) as an enzyme mimic/polymer support for pyridoxal mediated reactions was investigated (Figure 5.1).

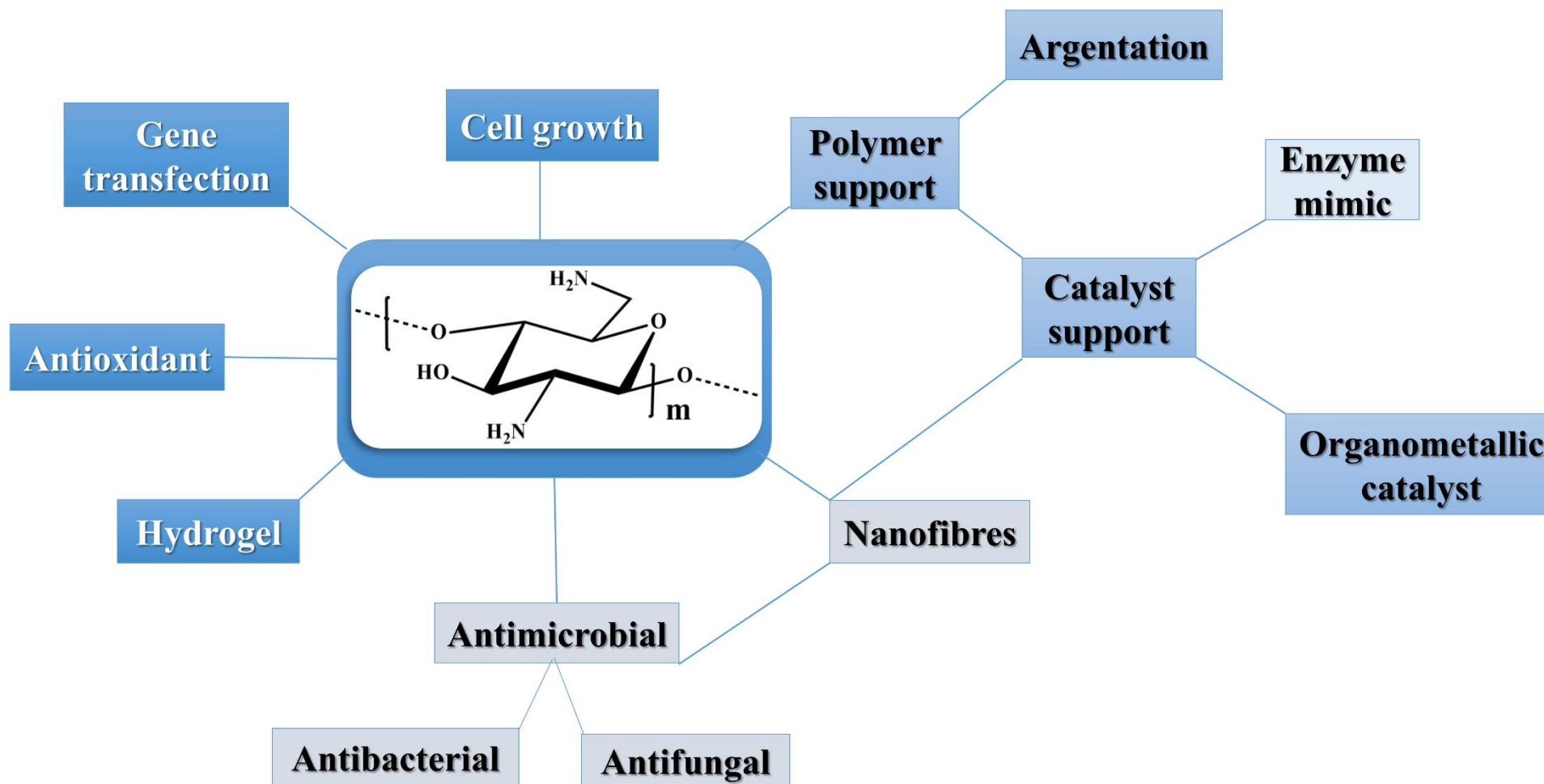


Figure 5.1: Existing and intended applications of 6-deoxy-6-amino chitosan (1.15).

5.2 The antimicrobial applications of 6-deoxy-6-amino chitosan

5.2.1 Background to the application

There has been extensive research related to the antimicrobial properties of chitin, chitosan and various derivatives thereof. A review of the antimicrobial applications is given by Jardine *et al.* which focused on valorisation of chitinous biomass.¹ The antimicrobial activity of 6-deoxy-6-amino chitosan has also been investigated with a focus on the effect of the introduction of the amine at C-6 on the activity of the polymer against various pathogens. In the study by Yang *et al.* the antimicrobial efficacy of the polymer against *S. aureus*, *E. coli*, *P. aeruginosa*, and *A. niger* was determined.² The diamino derivative displayed superior activity to chitosan with lower minimum inhibitory concentration (MIC) values reported and the polymer was active against bacteria and fungi at an acidic and neutral pH (pH 7).²

The specific applications that were investigated are as follows;

- 1) Electrospinning of chitosan and 6-deoxy-6-amino chitosan (**1.15**) for the production of nanofibres. The loading of these nanofibres with silver nanoparticles and the evaluation of the antibacterial activity thereof.
- 2) The evaluation of 6-deoxy-6-amino chitosan (**1.15**) as an antifungal agent, specifically against the fungus *Fusarium verticillioides*. Furthermore, the effect of the polymer and oligomers thereof on maize plants exposed to *Fusarium verticillioides* was studied. Another application of 6-deoxy-6-amino chitosan **1.15** as an edible food coating for the treatment of strawberries was investigated.

5.2.2 Production of nanofibres and loading of silver nanoparticles for antibacterial assessment

Ultrafine non-woven fibres with advantageous properties can be produced using electrospinning, where macromolecules are spun into fibres in the nanometre range. The appeal of these nanofibres lies in their favourable properties which include large surface area to volume ratios, high porosity, high gas permeability and small pore sizes.³ These properties have found the best application in the biomedical sector as wound dressings, drug delivery systems, and as tissue engineering scaffolds.^{4, 5} Other uses include filtration products (permeable membranes), fibre reinforcement composites and in the manufacture of protective clothing.⁵ Nanofibres (NFs) may be produced from almost any soluble polymer, which has a sufficiently high molecular weight. Electrospinning relies on the application of an electric field

to a polymer fluid. The application of an electric field to the polymer solution induces an electric charge on the polymer surface, and when the repulsive electrical forces overcome the surface tension nanofibres are ejected. The non-woven fibre mat produced is deposited onto a collector plate.⁶ Figure 5.2 shows the general set-up of a simple electrospinning apparatus. The set-up may be vertical or horizontal and generally consists of a high voltage electric field generator, a spinneret and a collecting electrode. By adjusting the parameters such as tip to collector distance, voltage, spinning solvent and the addition of co-polymers, fibres with specific properties can be obtained.⁷

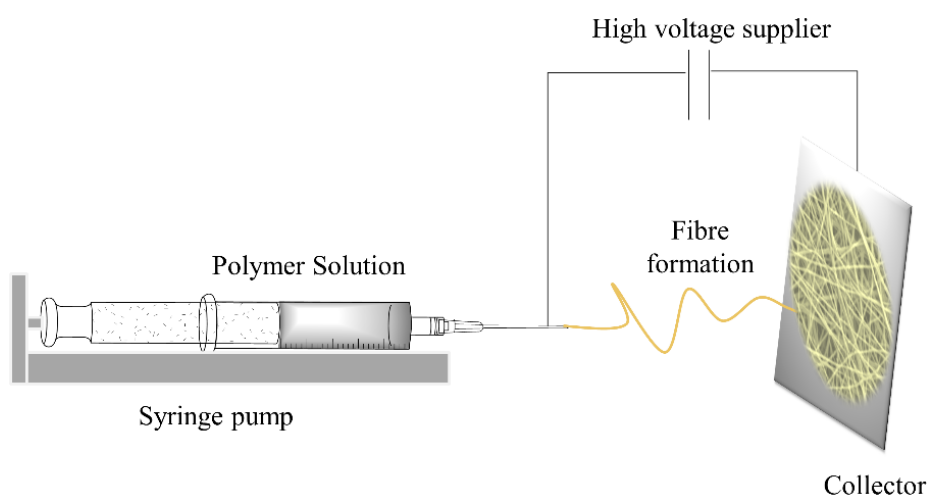


Figure 5.2: The typical set-up for electrospinning.⁷

The interest in the production of NFs from biopolymers has increased in recent years as these biopolymers are biodegradable and biocompatible, making their use attractive as opposed to their synthetic counterparts. However, due to the inconsistencies associated with the source and processing of biopolymers, each material may require unique processing conditions. A drawback associated with the production of NFs from biopolymers, is that these polymers typically require polar solvents. These polar solvents are generally harder to spin. Biopolymers which have been electrospun include alginate, cellulose, chitin and chitosan, collagen, fibrinogen and silk.^{8, 9} Parameters which have been shown to affect the morphology and properties of NFs are:

- concentration of the polymer in solution,
- electrical conductivity and viscosity of the polymer-solvent system,
- the glass-transition temperature, molecular weight, solubility of the polymer and the vapour pressure of the solvent used.⁸

In addition, there is no standardisation of the electrospinning parameters for biopolymer NFs as the properties of the material/biopolymer vary in purity as well as origin. Therefore, it is difficult to directly compare NFs reported in literature due to the differences in the biopolymer source and electrospinning conditions used.

Electrospinning of biopolymers

Chitin has been electrospun to produce nanofibres where the most commonly used solvent was 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). However, HFIP has been reported to pose health and safety concerns.⁸ Recent reports have shown that chitin can be electrospun directly from the treatment of shells with the ionic liquid 1-ethyl-3-methylimidazolium acetate, where the parameters affecting the NFs obtained were primarily viscosity and polymer concentration (Figure 5.3).⁹ Electrospun chitin NFs have further been deacetylated to produce chitosan NFs.⁴

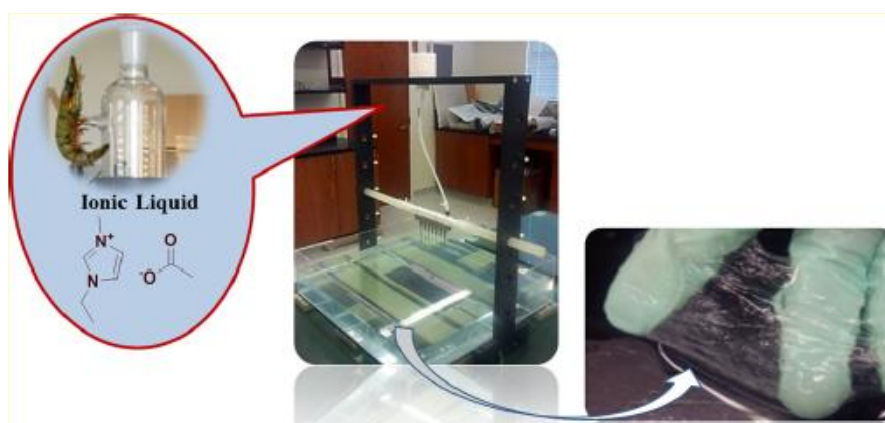


Figure 5.3: Chitin NFs reported by Zavgorodnya *et al.*, spun directly from an ionic liquid solution.⁹

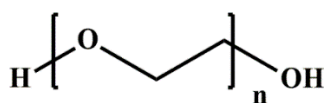
Chitosan itself has been successfully spun into nanofibres using dry and wet spinning methods. Dry methods require the polymer to be dissolved in the solvent and the NFs were obtained through evaporation of the solvent, wet spinning also involved a dissolved polymer; however, the NF was precipitated by placement into a chemical bath. Solvents which have been used in the fabrication of chitosan NFs include acetic acid, trifluoroacetic acid (TFA) and TFA/dichloromethane mixtures. The fibres produced from the solvents mentioned, typically have poor mechanical strength and hydrolysis may occur in water or tissue fluid. The NFs produced may therefore be cross-linked to increase stability with cross-linking agents such as epichlorohydrin or glutaraldehyde.⁸

However, there are still various problems related to the electrospinning of chitosan, which stems from its polycationic nature in solution and the rigidity of the chemical structure due to

its inter- and intra-molecular interactions. Hydrogen bonding is a major factor, where the fluid jet formed during electrospinning can be broken due to the formation of hydrogen bonds in the polymer chain. Stretching, whipping and bending observed during the electrospinning of chitosan can be attributed to the repulsive forces present between the ionic groups on the polymer back bone which makes continuous fibre formation almost impossible.¹⁰ Another major challenge is the wide range of molecular weights of chitin and chitosan from various sources. In order to overcome these difficulties, chitosan may be spun together with other synthetic or natural polymers to produce NFs. In addition, the co-spinning of chitosan with these bioactive substances may confer favourable properties to the nanofibres. Polymers which have been successfully spun together with chitosan are collagen, polyethylene glycol (PEG), polyethylene oxide, polyvinyl alcohol, poly(lactic acid) and silk fibroin.⁸

Electrospinning of chitosan and 6-deoxy-6-amino chitosan

The experimental details for the electrospinning conditions of chitosan and 6-deoxy-6-amino chitosan (**1.15**) are given in Chapter Seven section 7.6. Different solvents were considered in the initial attempts to electrospin the desired polymers. The solvent found to have the ideal viscosity, was a 3% solution of acetic acid (AA) ($c = 0.5$ M) which dissolved both chitosan and 6-deoxy-6-amino chitosan (**1.15**) at the chosen concentration. Electrospinning of the solution was attempted by manipulating various parameters such as the applied voltage, the tip-to-collector distance and the flowrate. After numerous optimisation steps, NFs were not obtained as the solution could not be spun by itself. It was concluded that a co-polymer was required to act as a carrier in the formation of chitosan-NFs. Poly(ethylene oxide) (PEO, **5.1**, Figure 5.4) with an average molecular weight of 600,000 g/mol was chosen to act as a carrier.



5.1

Figure 5.4: The structure of poly(ethylene oxide).

PEO (**5.1**) is a non-toxic and biocompatible synthetic polymer that has previously been successfully electrospun in the presence of chitosan. PEO in particular was chosen as it has excellent fibre forming ability and can act as a carrier in the electrospinning process by forming entanglements and physical bonds with chitosan.¹¹ One such example is the production of chitosan(PEO)/silica hybrid nanofibres produced by Toskas *et al.* which were investigated for

their applicability as a biomaterial for use in bone regeneration.¹² These fibres were found to be cytocompatible and promoted attachment of cells, as well as the enhancement of the formation of apatite when Ca^{2+} ions were incorporated.¹² Other than their use in the biomedical sector, chitosan/PEO nanofibrous membranes have been applied in the adsorption of divalent metal ions and was found to be recyclable without any significant loss of activity.¹³

In the current study, chitosan (**1.2**) and 6-deoxy-6-amino chitosan (**1.15**) were blended separately with PEO, which had been dissolved in a 0.5 M acetic acid solution and thereafter electrospun. The spinning was performed at room temperature with a relative humidity of 50%. The nanofibrous mats were collected on the surface of aluminium foil fixed on a grounded copper current collector and dried at room temperature for 24 hours. The surface morphology of the electrospun fibres was characterised by scanning electron microscopy (SEM). The samples were observed to visually evaluate the electrospinnability and for the existence of defects such as beads and droplets. The SEM image of the fibres obtained is shown in Figure 5.5.

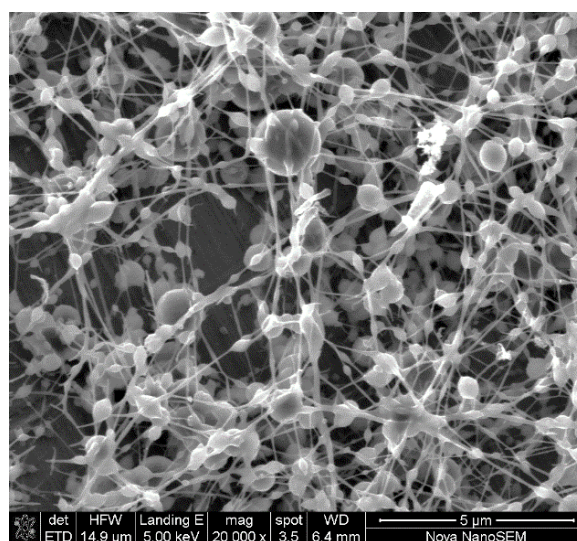


Figure 5.5: SEM image of the NFs formed from 6-deoxy-6-amino chitosan (**1.15**) after blending with PEO (magnification 20 000x).

The presence of beads, which are a known defect of some electrospun fibres, were more prominent on the chitosan based fibres compared to that of the 6-deoxy-6-amino chitosan (**1.15**) based fibres. Therefore, attempts were made to minimise beading, by varying the ratios of polymer to PEO. A final weight ratio of 1:1:1, polymer:PEO:0.5 M AA was chosen and NFs produced. The final fibres were not bead free; however, any further adjustments to the spinning conditions did not improve the NFs produced. The beading can most likely be attributed to the

surface tension of the chitosan in the spinning solution. Differences in the surface tension of the solution causes beading when the fibres were being spun. In a study by Fahnestock, a quaternary derivative of chitosan was electrospun. Initial attempts of this produced electrospinning and beading as a result of the charge repulsion effects which led to a higher surface tension.¹⁴ In the current study, the protonation of chitosan and 6-deoxy-6-amino chitosan in the acetic acid solution may lead to the same charge repulsion effects being observed. However, depending on the intended application, beading of the fibres was not necessarily an issue. For applications such as water filtration it may not be necessary to optimise spinning conditions to remove beading. The nanofibres obtained for 6-deoxy-6-amino chitosan, from the optimised spinning conditions are shown in Figure 5.6 together with a SEM micrograph. The NFs range in size from 54.4 to 89.6 nm for chitosan and 77.5 to 131.0 nm for 6-deoxy-6-amino chitosan as determined from an average measurement of 25 NFs. Fibres appeared cylindrical, continuous, and were randomly oriented.

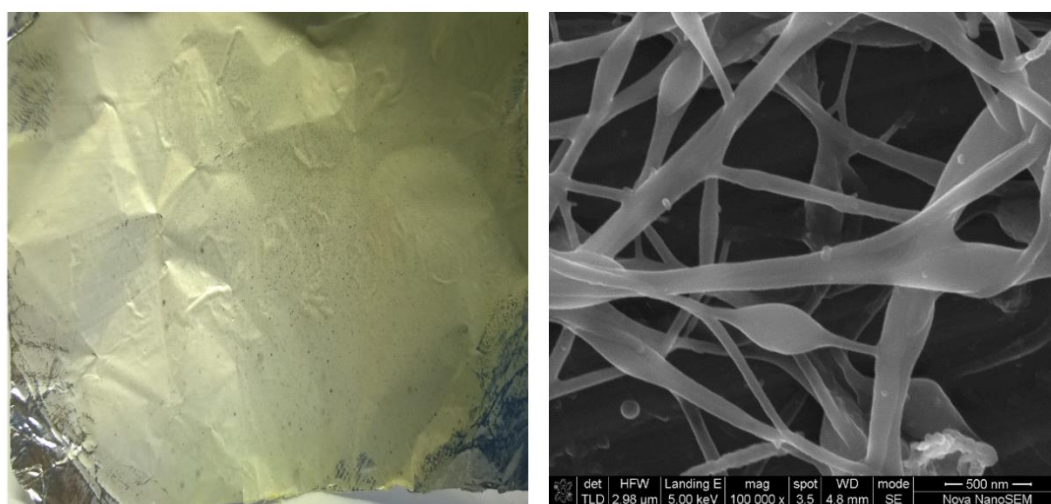


Figure 5.6: The NFs of 6-deoxy-6-amino chitosan (1.15) blended with PEO after optimisation (right-SEM image magnification 100 000X).

Comparing the size of the NFs obtained in the current study to that reported in literature is challenging, due to the difference in the spinning conditions as well as the starting materials used. However, from the increased amount of publications relating to the spinning of chitosan in aqueous acetic acid it is clear that these polymers have great potential for use in a variety of sectors.^{15,16}

The NFs were characterised by IR analysis to determine if the polymer structures remained intact. The IR spectrum recorded is shown in Figure 5.7. The characteristic peaks of 6-deoxy-6-amino chitosan and PEO are both visible in the spectra recorded indicating that both

polymers are still intact after spinning. There are shifts in the band positions related to the NH/OH, C-H and amide bands, in addition, the bands assigned to the hydroxide, amino and amide groups appear reduced in the electrospun mat. This peak reduction can be attributed to the protonation of these functional groups by the solvent or can potentially indicate the formation of strong hydrogen bonds or electrostatic interactions between the polymers as pointed out by Kohsari *et al.*¹⁷

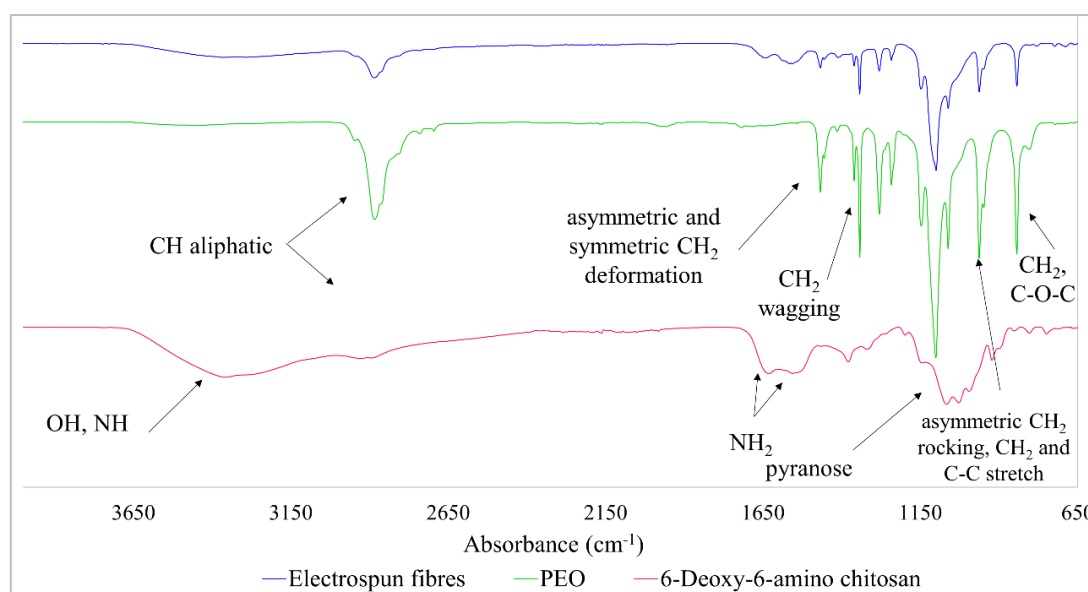


Figure 5.7: IR spectra of the electrospun fibres of 6-deoxy-6-amino chitosan and PEO.

Cross-linking was performed to enhance the mechanical integrity of the fibres as the native fibres were found to be water soluble. Chitosan has previously been cross-linked with glutaraldehyde, epichlorohydrin, genipin and oxidised glucose among others. In this work, glutaraldehyde was chosen as the cross-linking agent. Polymer cross-linking occurs through the formation of imine bonds *via* Schiff base formation between the polymer and glutaraldehyde.¹⁸ The fibres were cross-linked by vapour deposition of glutaraldehyde, where the NFs were placed in a vapour chamber in the presence of glutaraldehyde. A vacuum was applied and the glutaraldehyde was vaporised due to its low boiling point, thereby coating the NFs. The fibres were removed from the chamber and tested for their solubility in water.

The fibres were insoluble after cross-linking which was further confirmed by IR analysis of the sample. The IR spectrum (Figure 5.8) of the cross-linked 6-deoxy-6-amino chitosan–PEO nanofibres showed an absorbance band in the region of 1587 cm^{-1} attributed to C=N, further confirming the successful formation of the Schiff base. For the 6-deoxy-6-amino chitosan NFs, there are two possible sites for Schiff base formation, C-2 and C-6.

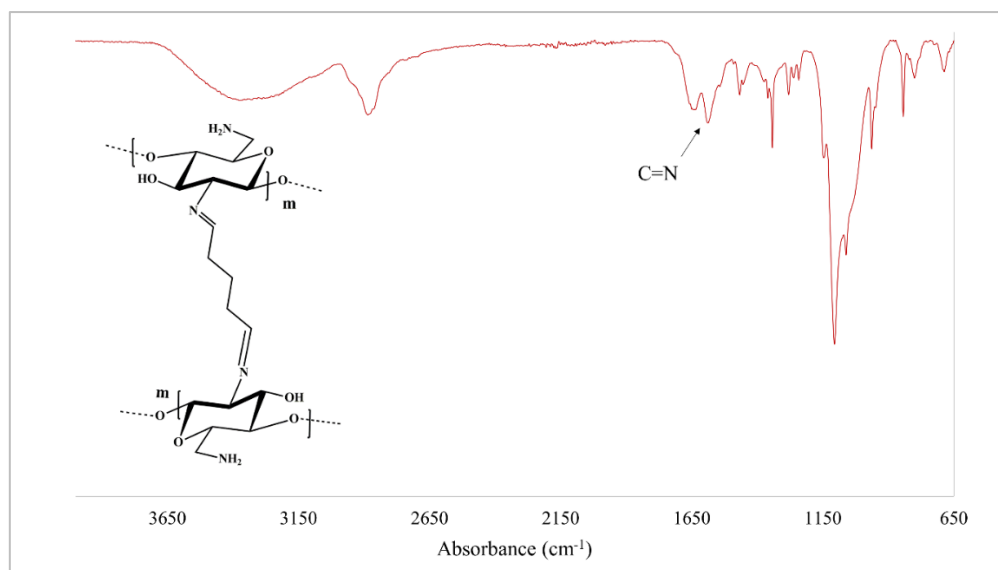


Figure 5.8: IR spectrum of cross-linked electrospun fibres of 6-deoxy-6-amino chitosan.

Loading of the nanofibres with silver nanoparticles

Metal ions have been previously incorporated into the production of NFs and have been shown to impart improved properties onto the electrospun fibres. One of the most common examples of this is the incorporation of silver nanoparticles onto electrospun fibres.¹⁹ Elemental silver (Ag) and silver ions have been reported to be wound-healing agents with low toxicity.²⁰ The mechanism by which silver induces bactericidal activity is unclear. It has been postulated that the ionic silver inactivates various enzymes in the bacterium. Studies have shown that the inherent antibacterial properties of Ag can be enhanced upon reducing its size to the nanoscale.²⁰

Nanoparticles of silver have been synthesised using a variety of methods such as chemical reduction of Ag^0 ions with or without stabilising agents, thermal decomposition in organic solvents as well as chemical and photo-reduction in reverse micelles. Generally, current nanoparticle synthetic techniques involve the use of strong reducing agents which generates hazardous waste. Therefore, in an effort to minimise the production of hazardous waste, green synthetic methods for the production of Ag nanoparticles have been investigated. Chitosan has previously been used as a non-toxic stabiliser in the production of Ag nanoparticles and therefore was used in this work as the stabilising agent.²¹

The NFs produced here were subsequently loaded with silver nanoparticles as per the procedure reported by Wei *et al.* Silver nitrate (2% w/v) was reduced using chitosan or 6-deoxy-6-amino chitosan as the reducing agent. In a typical preparation of these nanoparticles, a silver nitrate

solution (52 mM) was stirred together with the polymer until a homogenous solution was obtained. Thereafter, the solution was incubated at 95 °C overnight for chitosan and at room temperature for 6-deoxy-6-amino chitosan (**1.15**). The UV-Vis spectrum is given in Figure 5.9, which showed the characteristic absorbance for the surface plasmon resonance (SPR) band of silver nanoparticles, which was observed ~ 420 nm. Samples were withdrawn at six and eighteen hours respectively. As the reaction time increases, the SPR band becomes more intense pointing to a greater amount of reduced silver in the solution.¹⁷

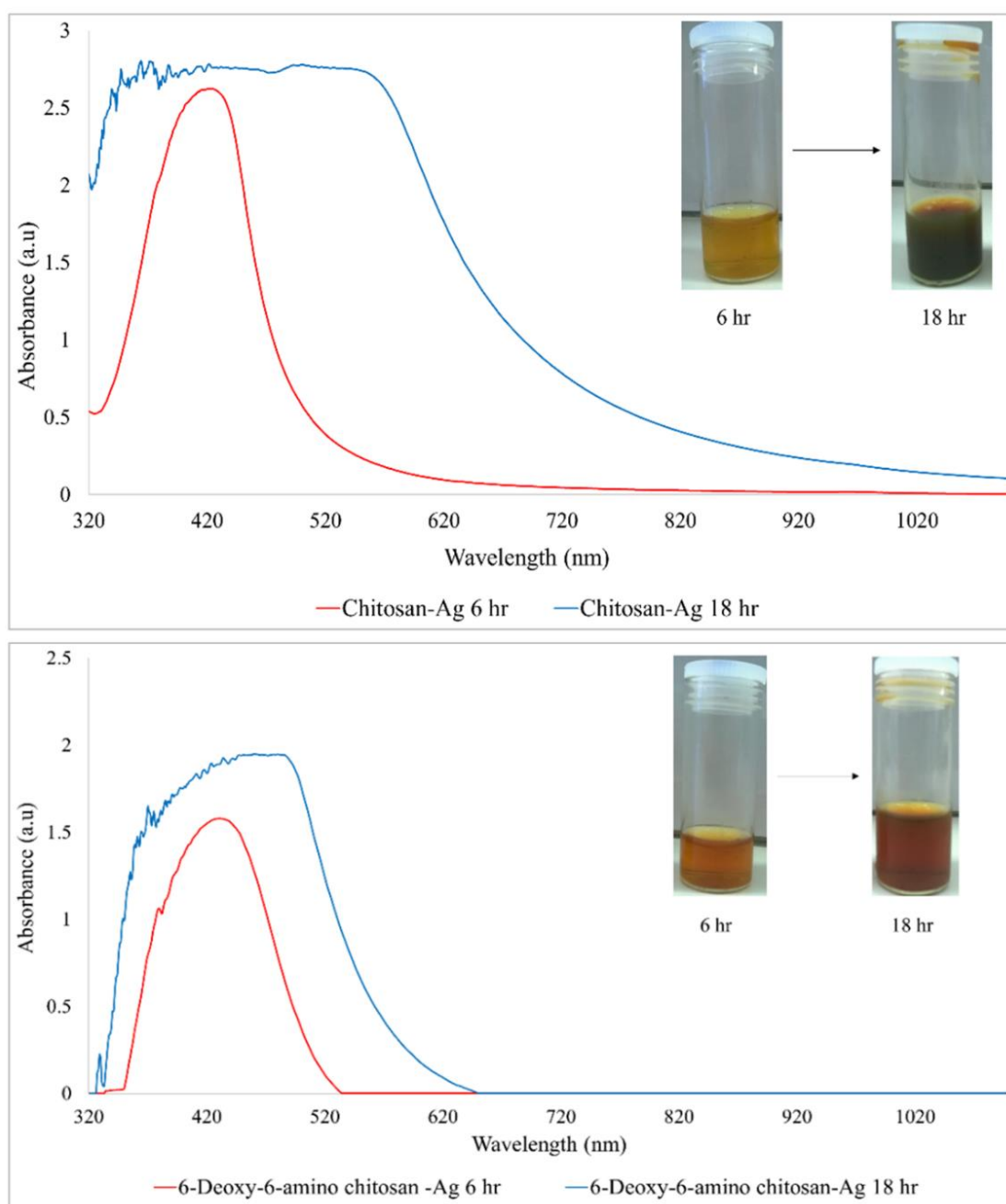


Figure 5.9: UV-Vis absorption spectra of chitosan (**1.2-Ag**, top) and 6-deoxy-6-amino chitosan (**1.15-Ag**, bottom) based silver nanoparticles.

Transmission electron microscopy (TEM) was used to examine the particles, enabling the determination of particle size, shape and dispersity. The TEM images of (A) chitosan and (B) 6-deoxy-6-amino chitosan based silver nanoparticles are shown in Figure 5.10.

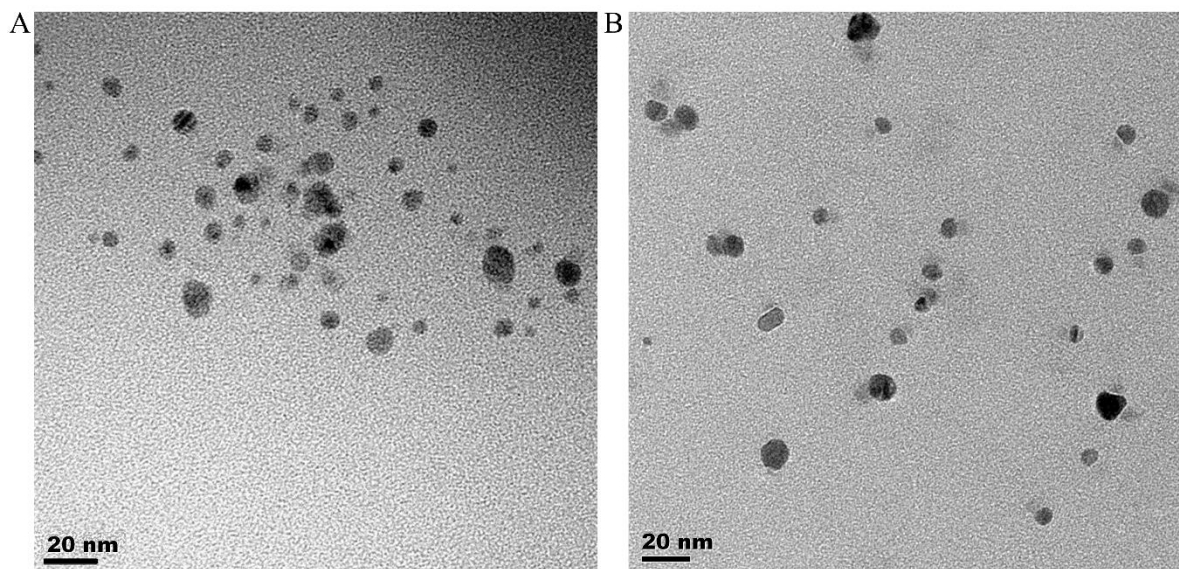


Figure 5.10: TEM images of (A) chitosan and (B) 6-deoxy-6-amino chitosan stabilised Ag NPs.

The Ag NPs obtained were spherical in the case of both chitosan and 6-deoxy-6-amino chitosan. Silver capped chitosan NPs ranged from 3.7 to 12.1 nm with an average size of 6.5 ± 1.8 nm, while the 6-deoxy-6-amino chitosan capped particles ranged from 5.5 to 11.3 nm with an average size of 7.6 ± 1.5 nm. The size of the nanoparticles were determined from an average measurement of 25 particles. The sizes of the particles produced were comparable to that reported by Wei *et al.* (6 – 8 nm).²⁰ The preparation of nanoparticles using 6-deoxy-6-amino chitosan was quite interesting in that the polymer did not need to be heated in order to promote the formation of nanoparticles. This synthesis of nanoparticles under 20 nm in the absence of both a chemical reducing agent as well as heat was advantageous. In the literature, it has been reported that the temperature used in the formation of nanoparticles is a key factor to obtaining smaller particles. Akmaz *et al.* demonstrated that the optimal synthesis temperature for chitosan capped nanoparticles was 95 °C, where the high temperature is necessary for the reduction of silver ions.²² It is possible that the presence of the two amines increased the basicity of the solution to a point where the silver ions were reduced to silver nanoparticles. In the production of other metal nanoparticles, ammonia is often used to adjust the pH of solutions to between 9 and 12 in order to form the desired nanoparticles from metal salts.²³ Additionally, 6-deoxy-6-amino chitosan may function as a better dispersing agent compared to chitosan

which further contributed to the formation of uniform silver nanoparticles with a smaller diameter. There have been previous reports in literature which state that the stability and dispersion of silver nanoparticles is crucial and the amine and hydroxyl groups present in chitosan contribute to this by chelating the nanoparticles.²⁴ The prepared nanoparticles were applied to the NFs and thereafter allowed to dry. In previous studies, silver has been reduced and then added to the polymer solution to be spun together. The study by Wang and co-workers reported silver NPs with an average of 3.5 ± 0.6 nm formed through reduction with UV light in the presence of chitosan and PEO where this solution was subsequently electrospun thereby incorporating the NPs directly.²⁵ However, in order to avoid having to re-optimize spinning conditions the NPs were applied post spinning.

Evaluation of the nanofibres and the silver loaded nanofibres as antimicrobial agents

The silver nanoparticles loaded onto the NFs were assessed for their antimicrobial activity against *E. coli*. The nanoparticles were in the ideal size range for evaluation of their antibacterial efficacy.

The fibres themselves could not be removed from either the foil or filter paper. Therefore, filter paper which had been coated with electrospun NFs, with and without silver nanoparticles were assessed using an agar diffusion test. The Agar Disk Diffusion Test method is the standard method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. There are many accepted and approved standards published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts testing. It is a qualitative test to check the action of an antimicrobial agent and is read as the zone of inhibition exerted on an agar medium plate. The test is typically used for screening the antimicrobial activity for a variety of samples such as pharmaceuticals, drug intermediates, new chemicals, etc.^{26, 27} In this test the *E. coli* bacterial culture with a concentration of 10^5 colony forming units per ml (CFU/ml) was spread uniformly onto the surface of an agar plate. This was followed by the placement of the NF loaded filter paper with and without silver as 25 mm² sections in duplicate. The plates were incubated at 37 °C for 24 hours. After incubation the plates were observed for growth and zone of inhibition (ZOI). The diameter of the ZOI is considered proportional to the activity of the antimicrobial substance present in the sample or product. The *E. coli* cell concentration was determined by staining with trypan blue and counting using the Countess Cell Counter. The plates testing the electrospun fibres only, showed no marked inhibition of the growth of *E. coli* potentially due to the low concentration of polymer present. For the nanofibres loaded with silver (Figure 5.11) there were clear and moderate zones of inhibition,

which suggested that silver loaded NFs could inhibit the growth of bacteria. The ZOI of both the chitosan spun nanofibres and the 6-deoxy-6-amino chitosan fibres impregnated with silver were similar overall.

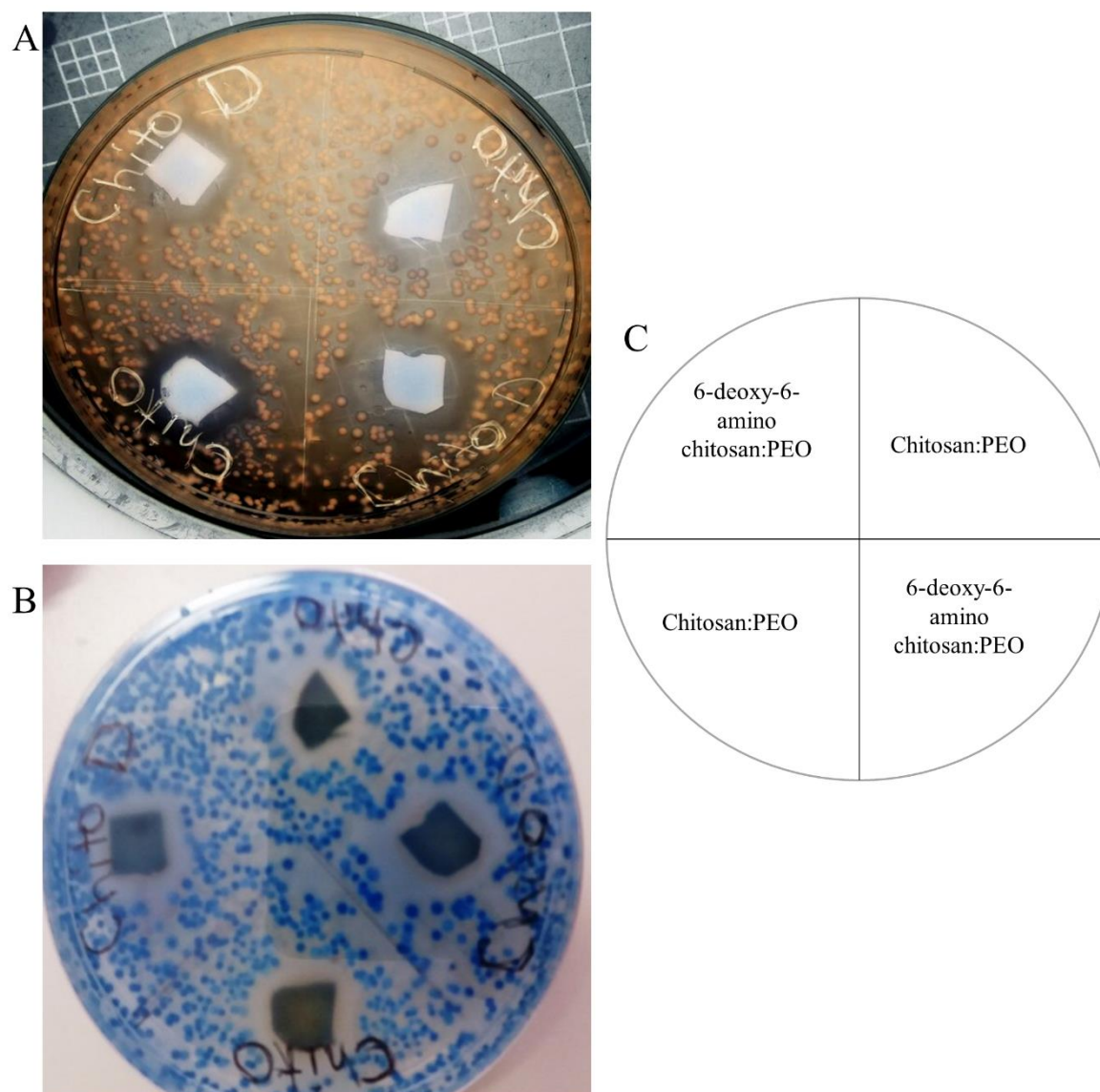


Figure 5.11: The inhibitory activity of silver loaded chitosan and 6-deoxy-6-amino chitosan NFs against *E. coli*. (A) the plate after incubation for 24 hours, (B) the plate after tryptan staining and (C) an illustration to show how the plate was divided.

The demonstrated inhibition was in line with what has previously been reported in the literature. One such example is the study by Wang *et al.* where chitosan/PEO nanofibre mats were imbedded with silver nanoparticles.²⁵ These mats showed antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Additionally results indicated

that a sustained release of nanoparticles was achieved for up to 72 hours and that the silver loaded nanofibres exhibited excellent cytocompatibility.²⁵

The use of the biopolymers as the reducing agents allowed for a faster, simpler and more economical process than conventional methods in the synthesis of silver nanoparticles. Furthermore, 6-deoxy-6-amino chitosan did not require heat for the formation of nanoparticles making this synthetic method particularly appealing. The antimicrobial activity of these silver loaded nanofibres allows for the further application of these biopolymers in fields such as the biomedical sector or in the development of antimicrobial packaging in the food industry.

5.2.3 Assessment of 6-deoxy-6-amino chitosan as an antifungal agent

Globally, maize is one of the three major cereal grains together with rice and wheat. It is an essential agricultural product as it can be used for human and animal consumption, where it is considered a dietary staple in many regions of the world. Maize is produced throughout the world in diverse environments, where both white and yellow maize is cultivated for human consumption and animal feed production.²⁸ The global corn production for 2017/2018 is estimated to be 1031.86 million metric tons.²⁹ In addition, maize is further processed into a number of useful materials such as fuel (ethanol) and starch which in turn is found in many household items such as batteries, ink and cosmetics (Figure 5.12).³⁰

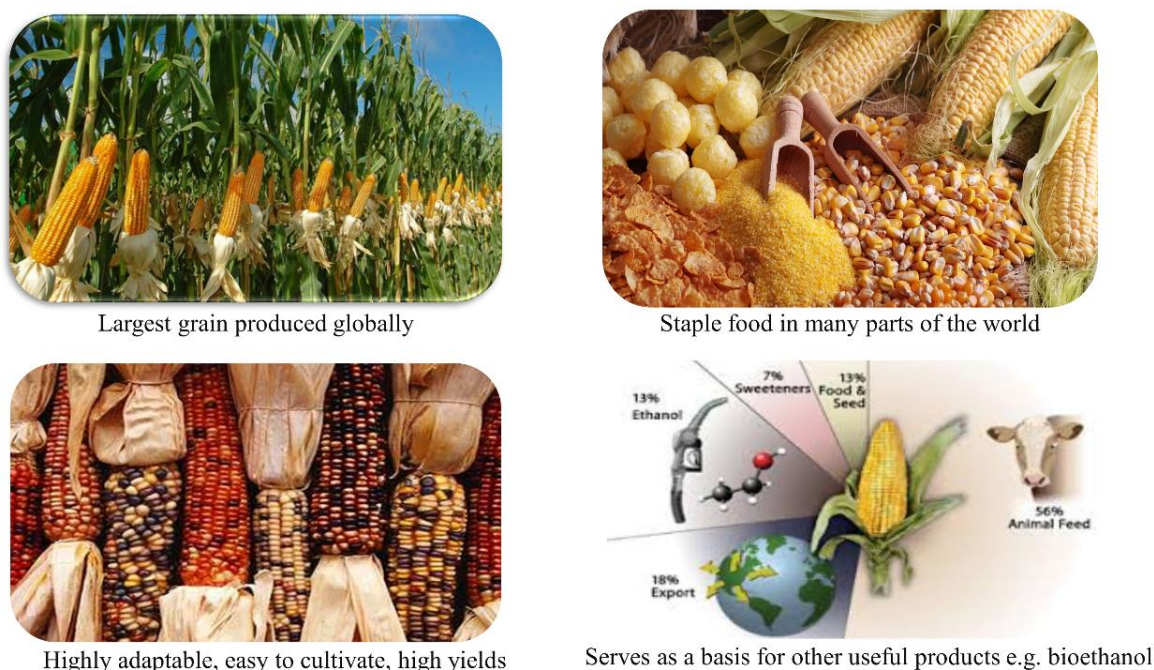


Figure 5.12: An overview of the usefulness of maize.

Maize production can be affected by a number of factors including climate, water availability, soil quality, agricultural pests and diseases. The use of agrochemicals for plant growth and protection has become increasingly challenging due to the development of resistance in pathogenic microbes, the adverse effects on other organisms and the negative impact on soil quality. Therefore, there has been a call for the development of bio-based biodegradable alternatives to synthetic agrochemicals in the treatment of various agricultural crops.³¹

This part of the study focused on the effect of chitosan (**1.2**) and its derivative, 6-deoxy-6-amino chitosan (**1.15**) as an antifungal agent and plant immune stimulant in crop protection.

Maize generally suffers from ear, seed, stalk and root rot due to fungal infection (Figure 5.13). In addition, certain fungi are toxigenic due to the production of mycotoxins which affect human and animal health after consumption of infected crops. The toxigenic fungus *Fusarium verticillioides* has been responsible for ear rot in maize crops and the production of fumonisins (a class of mycotoxins). *Fusarium* affects 80% of economically important crops and can lead to major issues in food chain security.³²

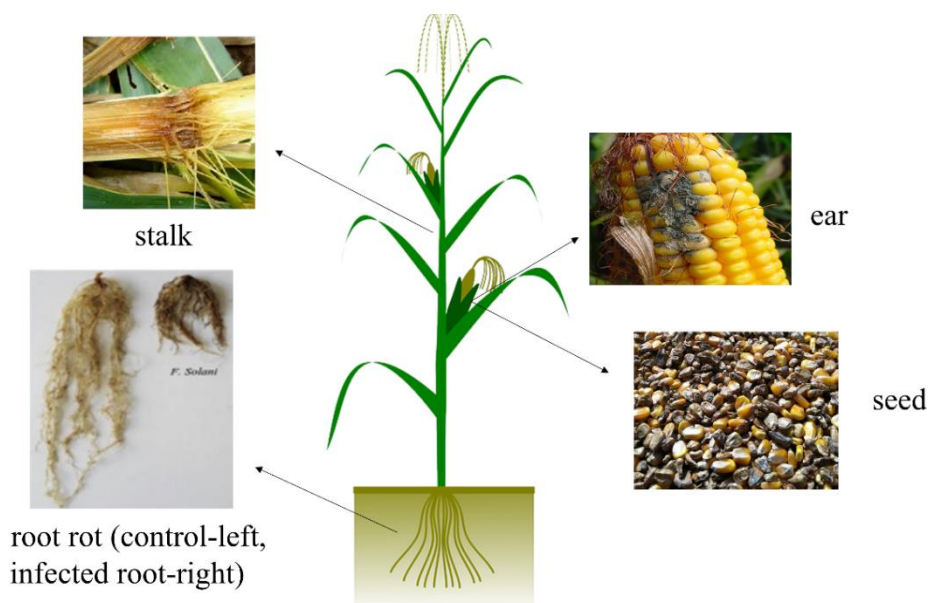


Figure 5.13: The effect of maize contamination with the *Fusarium* fungus.

Chitin, chitosan and their derivatives have previously been applied in the agriculture sector in many different roles which can mainly be attributed to their antiviral, antibacterial and antifungal properties.³³ These polymers can be used to control or reduce the spread of plant disease, boost the plants innate defence mechanisms and to promote plant growth. In agriculture, these polymers are typically used to combat bacterium, fungi, insects, oomycetes and viruses. The polymer can be administered through a variety of methods, such as a seed coatings, foliar treatment agents and soil amendments.³³ There are various mechanisms through which chitosan acts to reduce plant diseases. The currently understood mechanisms include:

- direct activity against plant pathogens,
- acting as a physical barrier around pathogen penetration sites,
- chelation of nutrients and minerals to prevent pathogen access,
- activation of defence related compounds, proteins and events,
- and an increase in the antioxidant content such as flavones, etc.³³

The review by Sharp looked at the effect of chitin and its derivatives in agriculture, specifically the modification of plant-microbial interactions and crop yield. This review noted a number of publications on the activity of chitosan against the fungus *Fusarium* in different plants such as date palms, tomatoes, pigeon peas and cucumbers.³⁴ A study by Al-Hetar *et al.* demonstrated the activity of chitosan against *Fusarium oxysporum f. sp. cubense*, a common cause of banana wilt.³⁵ The study reported an increased inhibition of 76% at a concentration of 8 mg/mL on potato dextrose agar, where inhibition was found to be directly proportional to the concentration of the polymer. When tested in potato dextrose broth complete inhibition was observed.³⁵ Another study looked at the effect of chitosan seed treatment as a resistance elicitor for *Fusarium graminearum* (*F. graminearum*) in wheat. Treated and untreated seeds were exposed to the fungus and the activity of enzymes related to plant defence were studied. It was noted that the seeds treated with chitosan displayed lower levels of disease, thus making chitosan an alternative to the use of conventional antifungals.³⁶

Owing to the literature available on the antifungal activity of chitosan and its application in crop protection, the activity of 6-deoxy-6-amino chitosan was of interest. The antifungal activity of diamino chitosan derivatives has previously been reported on various strains of fungi. Yang *et al.* reported on the activity of 6-deoxy-6-amino chitosan against the fungus *A. niger* where the modified derivative displayed inhibition at 0.1% (w/v) compared to the inactive chitosan.² Luan *et al.* later looked at the antifungal activity of 6-deoxy-6-amino chitosan against four plant pathogens, *F. oxysporum f. sp. cucumerium*, *F. oxysporum f. niveum*, *Phomopsis asparagi* and *Gibberella zeae*. Results indicated that the modified polymer displayed a higher degree of inhibitory activity compared to the parent polymer, where the activity had improved more than 20% at 0.5 mg/mL against the pathogens tested.³⁷

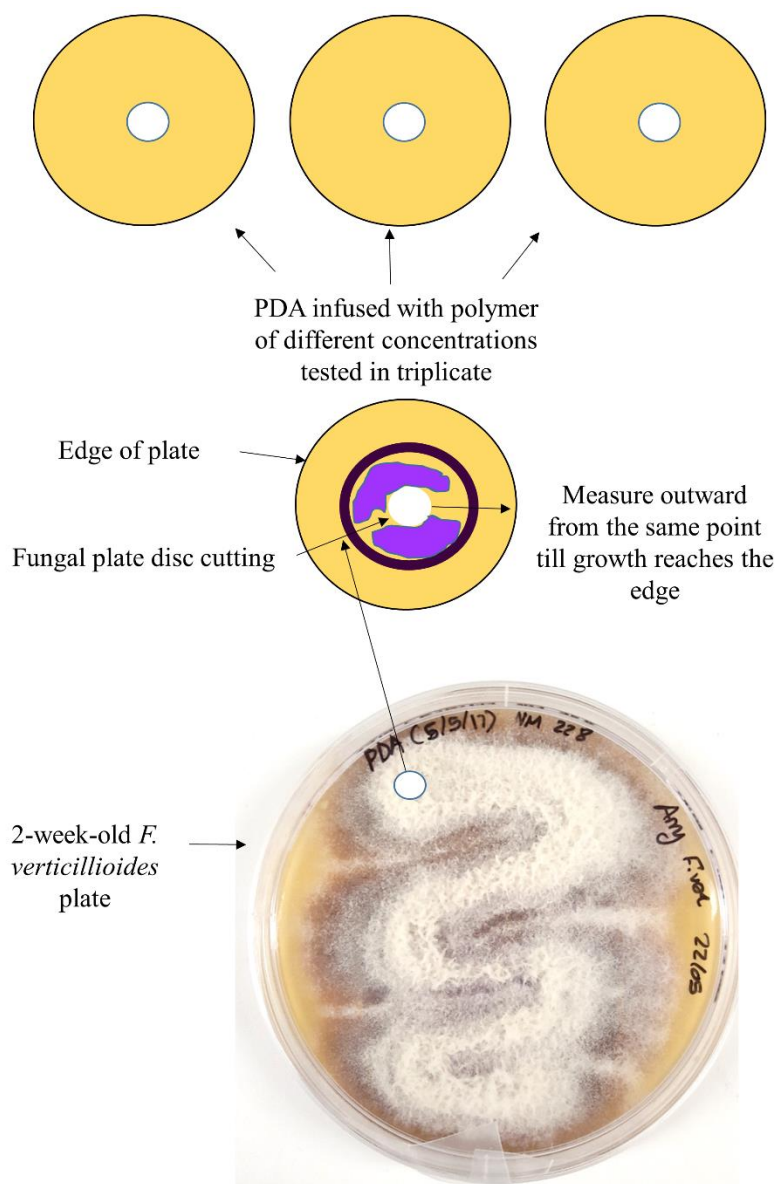
Antifungal assessment of the activity of chitosan and 6-deoxy-6-amino chitosan against *Fusarium verticillioides* (*F. verticillioides*)

The fungicidal activity of chitosan (**1.2**) and 6-deoxy-6-amino chitosan (**1.15**) was determined by a radial hyphal growth bioassay as reported by Al-Hetar *et al.*³⁵ Due to the insolubility of chitosan in water, a 5% chitosan solution was made up in 1% acetic acid and diluted to the appropriate concentrations (Table 5.1). 6-Deoxy-6-amino chitosan (**1.15**) was dissolved in water and diluted as needed. These concentrations were achieved by diluting the polymer solutions with potato dextrose agar (PDA). The polymer plates were compared to controls consisting of PDA only, a 0.1% acetic acid control and a water control.

Table 5.1: Polymer concentrations (w/v) tested for antifungal activity.

Chitosan (%)	6-Deoxy-6-amino chitosan (%)
0.5	1
0.1	0.5
0.05	0.1
0.01	0.05
	0.01

A sample of *F. verticillioides* was taken from a 14 day old colony and seeded onto the plates which were incubated at 30 °C. All samples were tested in triplicate. Figure 5.14 gives an illustration of the preparation of the plates for testing.

**Figure 5.14:** The preparation of the plates for antifungal assessment.

The plates were monitored visually and the radial colony growth was measured daily using ImageJ software³⁸, until the leading edge of the fastest growing colony had reached the edge of the plate. In all experiments, the media control cultures of *F. verticillioides* reached the edge of the plate after four days of incubation at 30 °C. Measurements were only taken from day two as no growth was observed on day one for any of the plates, including the controls.

The percentage of inhibition was calculated based on percentage inhibition of radial growth (PIRG%) as follows:

$$PIRG\% = \frac{R1 - R2}{R1} \times 100\%$$

where, R1 = radial growth of *F. verticillioides* on the control plate, and R2 = radial growth of *F. verticillioides* on the treated plate.

The *F. verticillioides* fungus was characterised by the formation of white mycelia spreading toward the edge of the plate. The micrograph in Figure 5.15 shows the growth of *F. verticillioides* on PDA media. This fungus possesses species-specific monophialides (projections from the vesicles of fungi) with spherical, sticking spores at the ends ("false heads") which can be seen in the figure.



Figure 5.15: The *F. verticillioides* fungus with the ("false heads "indicated by the arrow).

After four days the media controls, were covered with fungus growing outward. The base of the fungus appeared brown due to the media used and the characteristic white mycelia was

observed. Upon treatment with the polymers this growth pattern was altered. The graph in Figure 5.16 shows the average radial growth of the fungus for the different concentrations of chitosan tested (0.01, 0.05, 0.1 and 0.5%) as well as the controls.

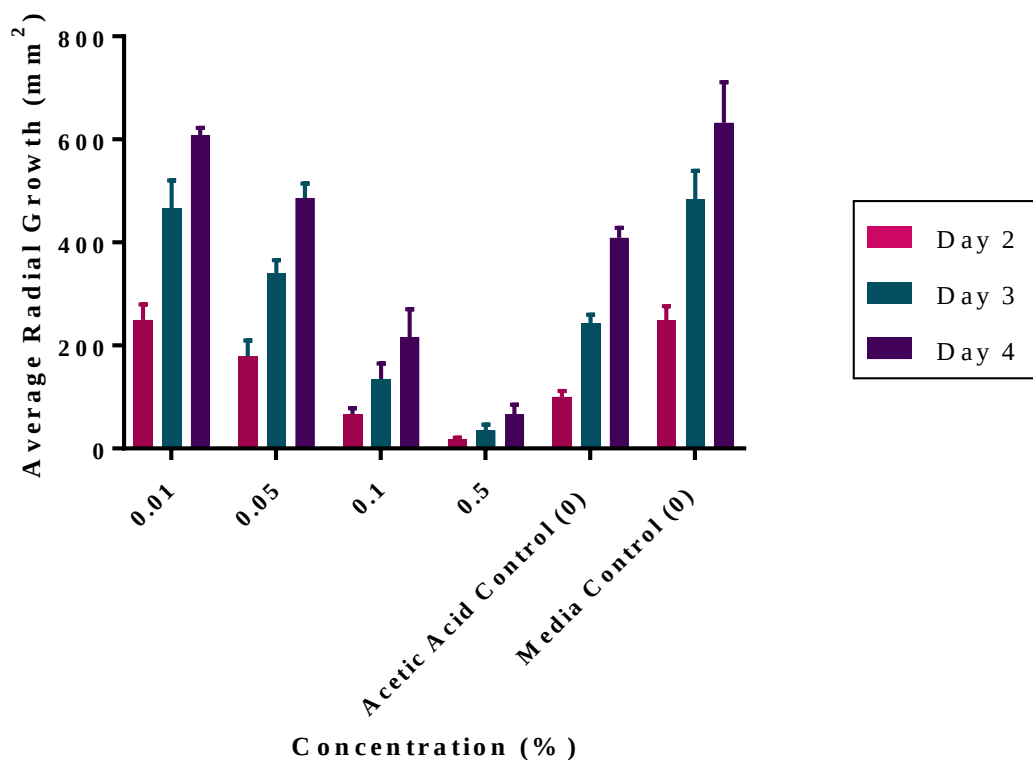


Figure 5.16: Effect of chitosan at various concentrations on mycelial growth after four days of incubation. An average of three biological replicates was used and a Two-way ANOVA analysis was performed on the data using GraphPad Software.³⁹

The percentage inhibition of radial growth (PIRG%) for chitosan is given in Table 5.2.

Table 5.2: Antifungal activity of chitosan against *F. verticillioides*.

Concentration				
Day 2	0.01	0.05	0.1	0.5
PIRG%	-0.69	28.15	73.00	92.95
Day 3	0.01	0.05	0.1	0.5
PIRG%	3.64	29.52	72.03	92.71
Day 4	0.01	0.05	0.1	0.5
PIRG%	3.78	23.00	65.84	89.40

At a concentration of 0.01% minimal activity on inhibition of fungal growth was observed after four days of incubation. When the concentration of the polymer was increased to 0.05% there were signs of inhibition starting on day two of incubation. The white mycelia did not reach the

edge of the plate in all three replicates, indicating a degree of inhibition to a certain extent. This inhibitory activity was clearly evident at concentrations of 0.1% and 0.5% across all three days. At a concentration of 0.1% the radius of the fungal growth was half that of the control and the percentage inhibition had increased to approximately 65% on day four. At a concentration of 0.5% almost no growth was observed beyond the original *Fusarium* plug, where the percentage inhibition was approximately 89%. In addition, the fungus appeared to be growing vertically instead of spreading outward which is not typical behaviour.

However, inhibitory activity was also seen for the acetic acid media control (Figure 5.17). Two way ANOVA analysis of the data (Table A4 in the Appendix), showed that the acetic acid control does affect the growth of the fungus significantly compared to the media control from day 2 onwards. It is important to note that the pH of the acetic acid control was adjusted to 5.6 using 2N NaOH prior to any testing to discount for any effect of the acetic acid as suggested by Al-Hetar *et al.*³⁵ The inhibitory activity of acetic acid at various concentrations was subsequently tested in order to verify this result. Concentrations of 0.1, 0.5 and 1% acetic acid all showed inhibition of the fungus to some degree. This result was further supported by the study reported by Yildirim-Aksoy *et al.* which stated that although acetic acid is commonly used for the dissolution of chitosan, it has intrinsic antibacterial properties which is a drawback as its use as a solvent in the assessment of antimicrobial activity.⁴⁰ This inhibitory effect of the acetic acid control was noteworthy and there have been previous reports of the antifungal activity of acetic acid alone.⁴¹ Therefore, the observations for the different chitosan concentrations cannot be used as an accurate measurement of the inhibitory activity of the polymer. In addition, the majority of studies did not account for the effect of the acetic acid. This indicated that the previously reported results were questionable due to the action of the acid in addition to the polymer.

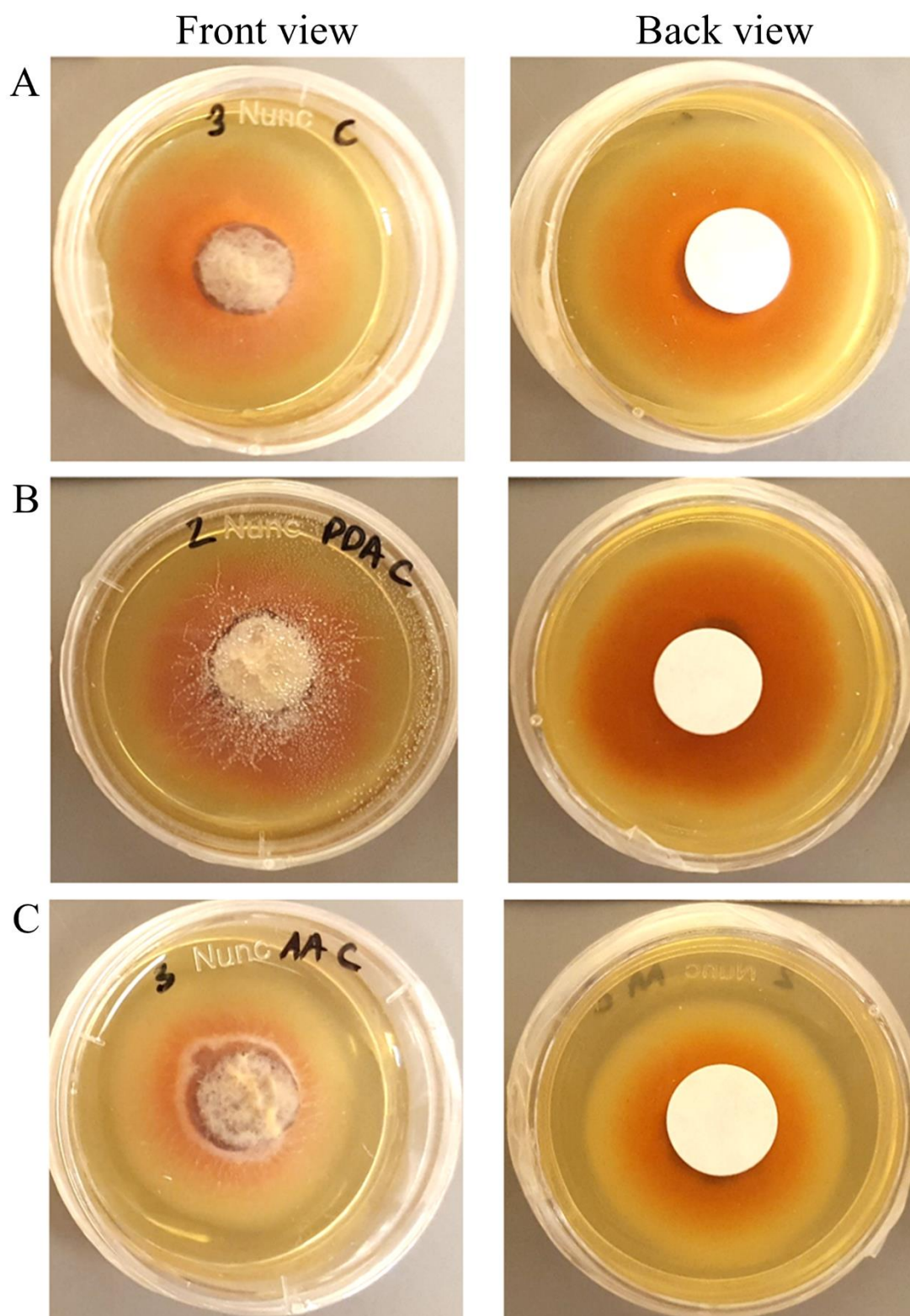


Figure 5.17: The control plates used in the assessment of the activity of *F. verticillioides*, (A) water control, (B) PDA control and (C) acetic acid control.

The plates in Figure 5.18 illustrate the effect of chitosan of different concentrations on the fungi after four days of incubation.

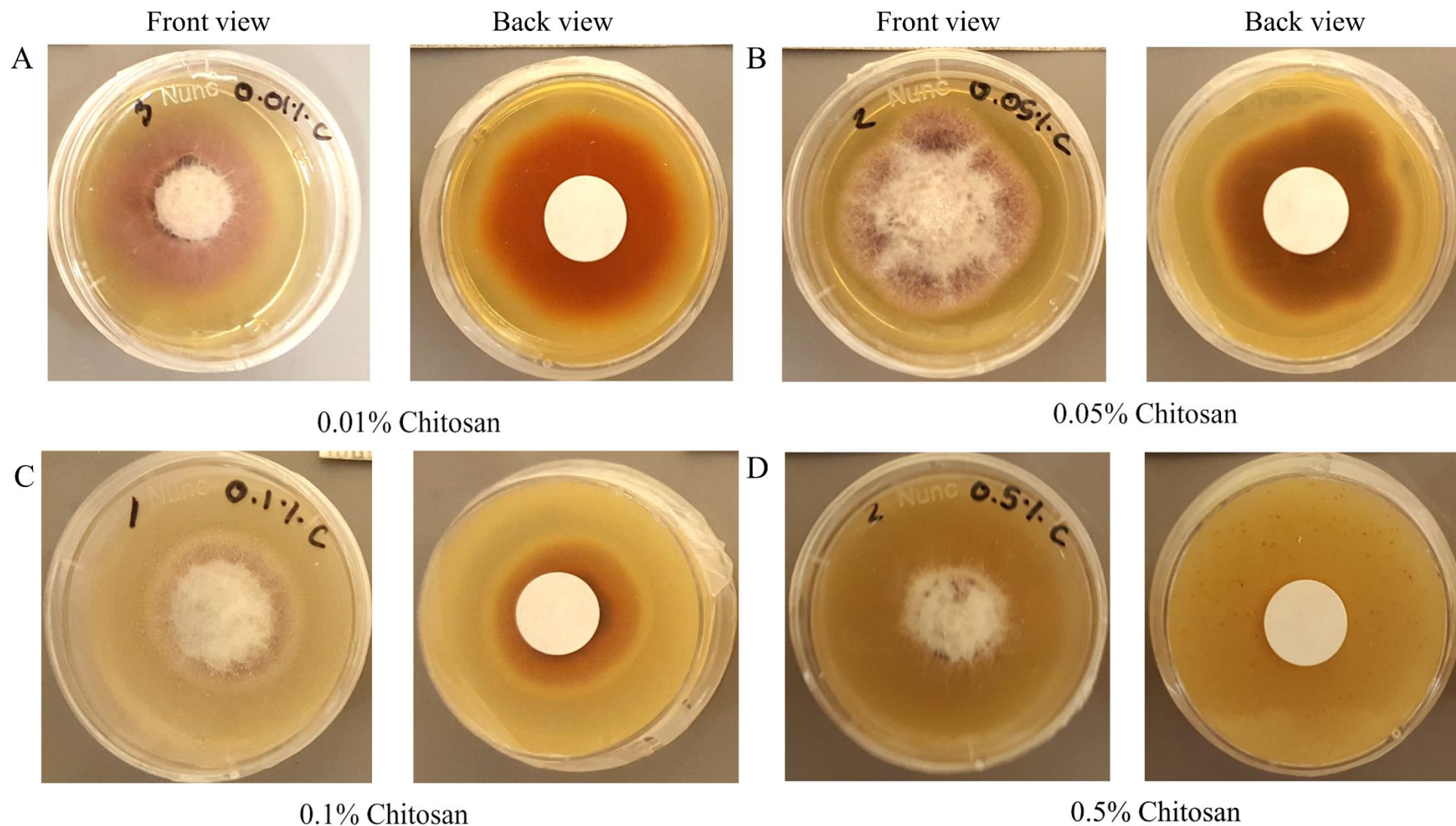


Figure 5.18: The antifungal activity of different concentrations of chitosan dissolved in 1% acetic acid, (A) 0.01% chitosan, (B) 0.05% chitosan, (C) 0.1% chitosan and (D) 0.5% chitosan against *F. verticillioides* at day four of incubation.

6-Deoxy-6-amino chitosan (**1.15**) was dissolved in water and diluted as needed to give final concentrations of 0.01, 0.05, 0.1, 0.5 and 1% respectively. Thereafter, the polymer was mixed with PDA and the plates were allowed to set. The inhibitory activity of the different concentrations of 6-deoxy-6-amino chitosan were monitored over the course of the time period, and the fungal inhibition calculated and illustrated in the graph in Figure 5.19.

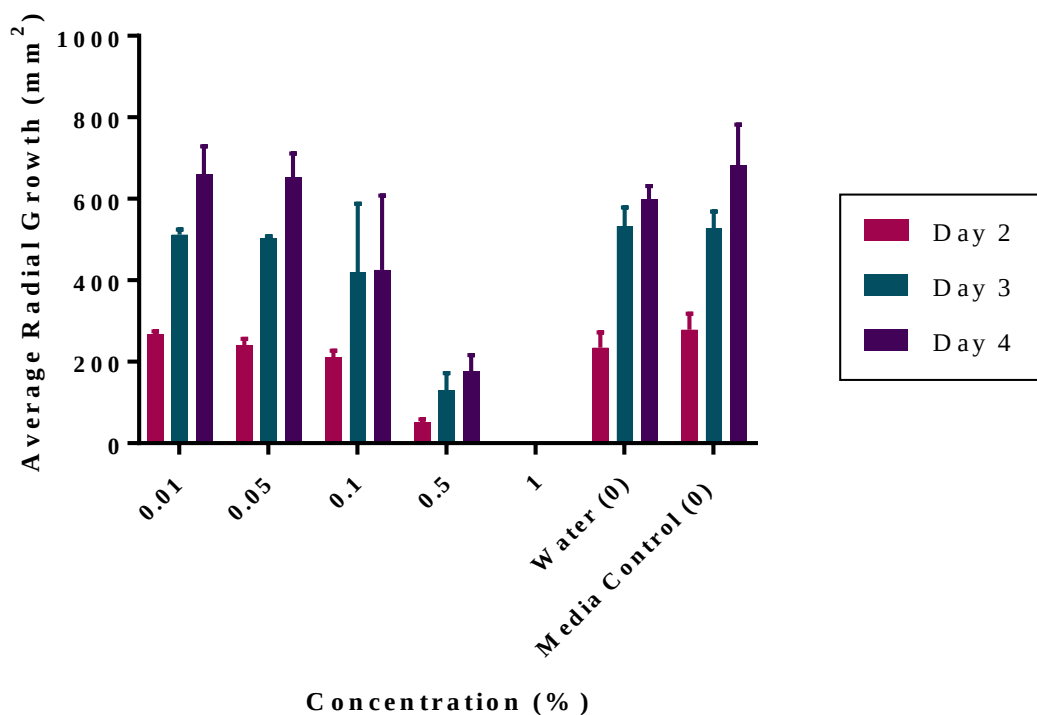


Figure 5.19: Effect of 6-deoxy-6-amino chitosan (**1.15**) at various concentrations on mycelial growth after four days of incubation. An average of three biological replicates was used and a Two-way ANOVA analysis was performed on the data using GraphPad Software.³⁹

The percentage inhibition of radial growth (PIRG%) is given in Table 5.3 for 6-deoxy-6-amino chitosan across the different days for each concentration.

Table 5.3: Antifungal activity of 6-deoxy-6-amino chitosan against *F. verticillioides*.

Concentration					
Day 2	0.01	0.05	0.1	0.5	1
PIRG%	3.68	13.44	24.52	81.73	100
Day 3	0.01	0.05	0.1	0.5	1
PIRG%	2.89	4.79	20.41	75.23	100
Day 4	0.01	0.05	0.1	0.5	1
PIRG%	3.39	4.50	37.78	74.14	100

From the graph, it can be seen that there was no noticeable inhibitory activity for 0.01 and 0.05%, potentially due to the low concentration of the polymer. After day four of incubation, only 3.39% and 4.50% percentage inhibitions were observed for 0.01 and 0.05%, respectively. Chitosan followed a similar trend; however, since the acetic acid was shown to have an effect on fungal growth this result was inconclusive. Inhibition was observed from 0.1%; however, the standard deviations for day 3 and 4 were greater compared to the other concentrations, therefore this result was not statistically significant. The percentage inhibition for a concentration of 0.1% reached 37.78% at day four. At a concentration of 0.5% and above, significant inhibition was observed. A concentration of 0.5% showed a marked inhibition, further supporting the inhibitory activity (74.14% at day four) observed. At 1%, complete inhibition was observed compared to the standard controls. Two way ANOVA analysis of the data confirmed the significance of the inhibitory activity of the 0.5% and 1% solutions from day two onwards (data given in the appendix in Table A5). Additionally, the analysis indicated that the 0.1% solution displayed significant inhibition on day 4.

It has been reported that the antifungal activity of chitosan was directly related to the positive charge of the polymer. As a result of the additional amine present on 6-deoxy-6-amino chitosan the positive charge was expected to be higher which in turn affected the antifungal activity observed. This result was in agreement with Luan *et al.* who demonstrated a greater fungal activity for 6-deoxy-6-amino chitosan against selected fungi compared to chitosan.³⁷ This study used 0.35% acetic acid for the dissolution of the polymer prior to diluting the sample to the appropriate concentration. It is not clear whether the presence of acetic acid in the solutions was taken into account, regardless of the low concentrations present.³⁷

Controls consisting of water and PDA are shown in Figure 5.20 for comparative purposes.

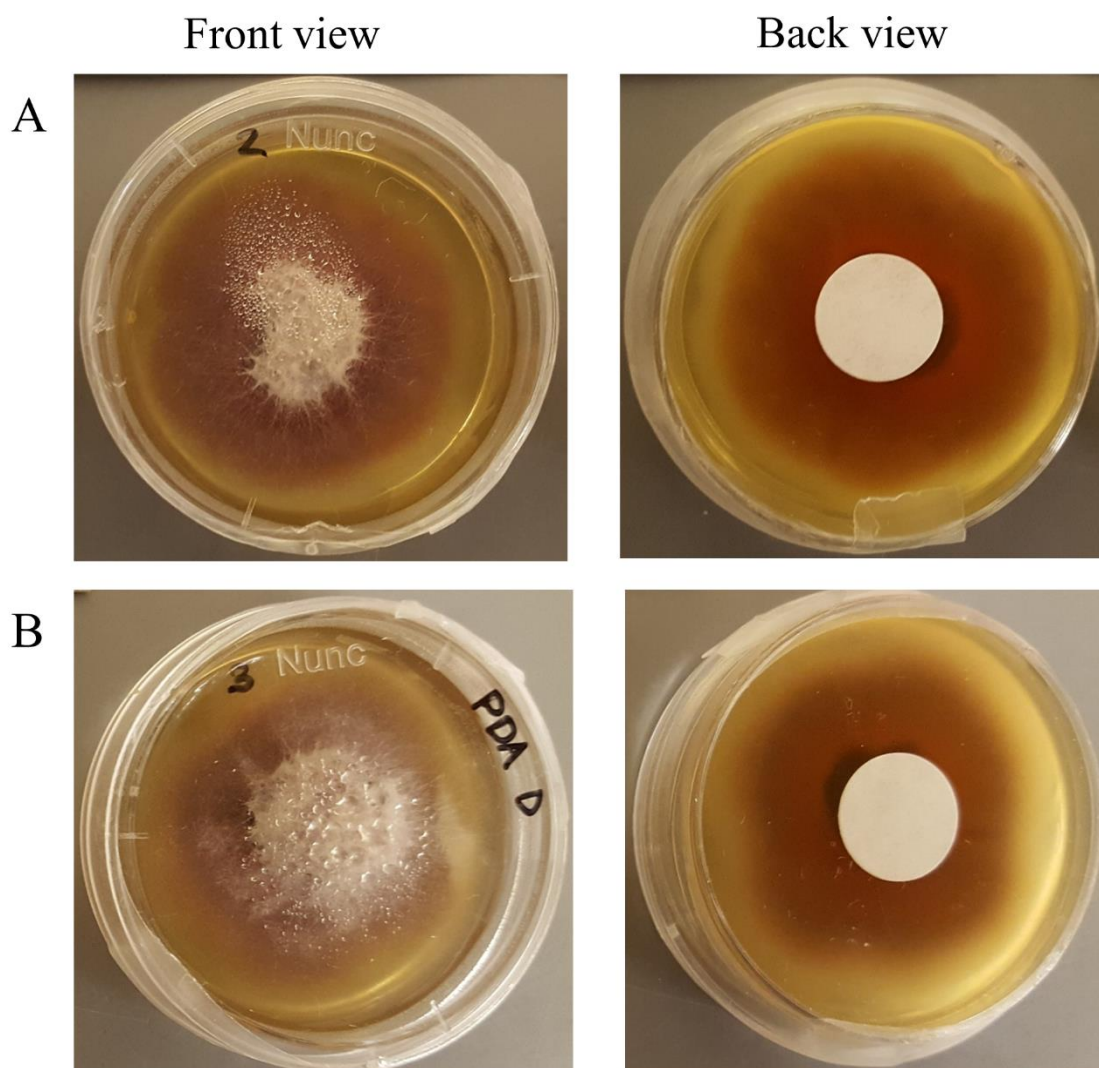


Figure 5.20: The control plates used in the assessment of the activity of *F. verticillioides*, (A) water control and (B) PDA control.

The plates containing different concentrations of 6-deoxy-6-amino chitosan and their effect on fungi after four days of incubation are shown in Figure 5.21 and 5.22.

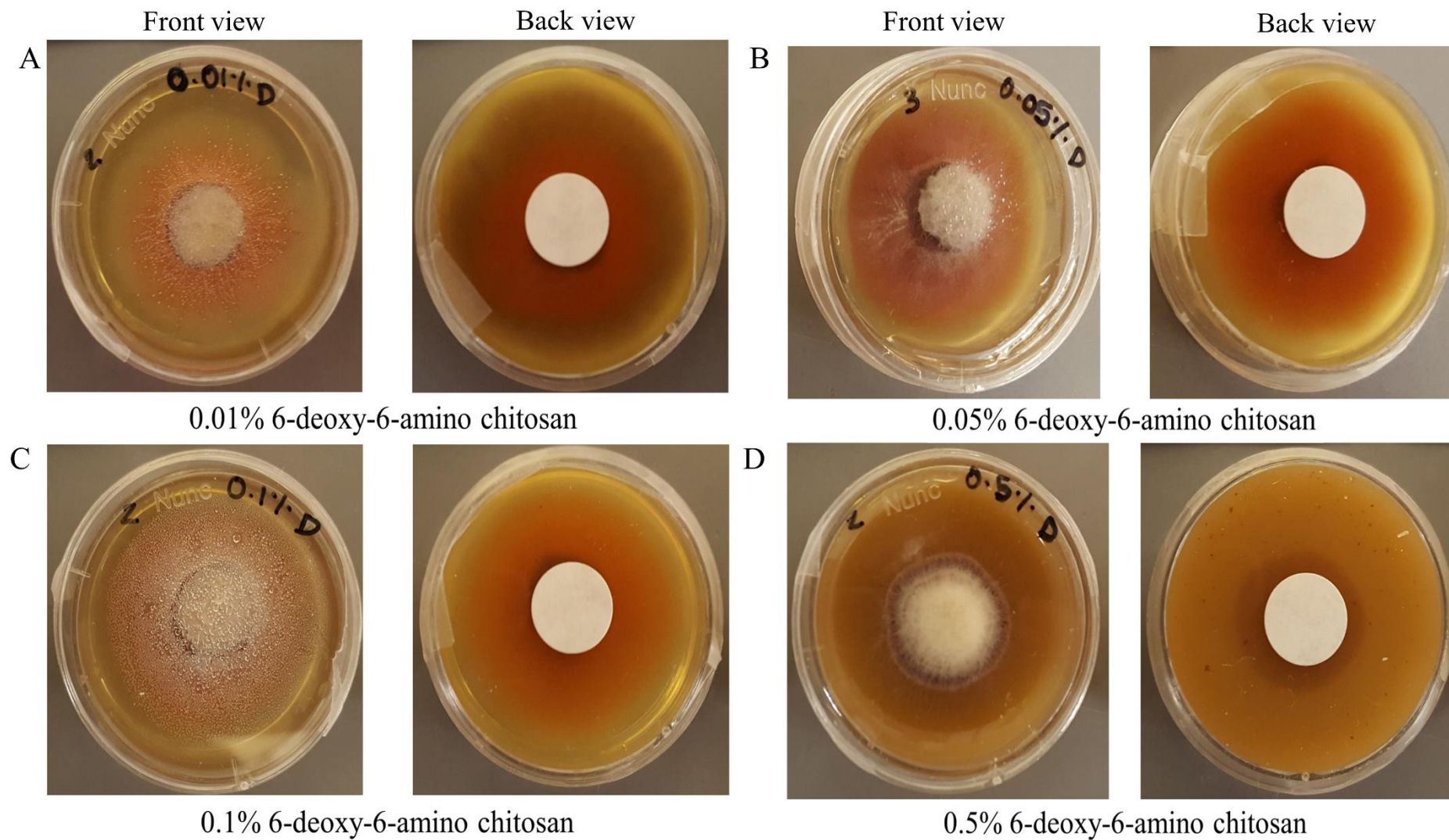


Figure 5.21: The antifungal activity of different concentrations of 6-deoxy-6-amino chitosan dissolved in H₂O (A) 0.01% 6-deoxy-6-amino chitosan, (B) 0.05% 6-deoxy-6-amino chitosan, (C) 0.1% 6-deoxy-6-amino chitosan and (D) 0.5% 6-deoxy-6-amino chitosan against *F. verticillioides* at day four of incubation.

The plate treated with a 1% concentration of the polymer is shown separately to highlight the effect of the polymer on the fungus.

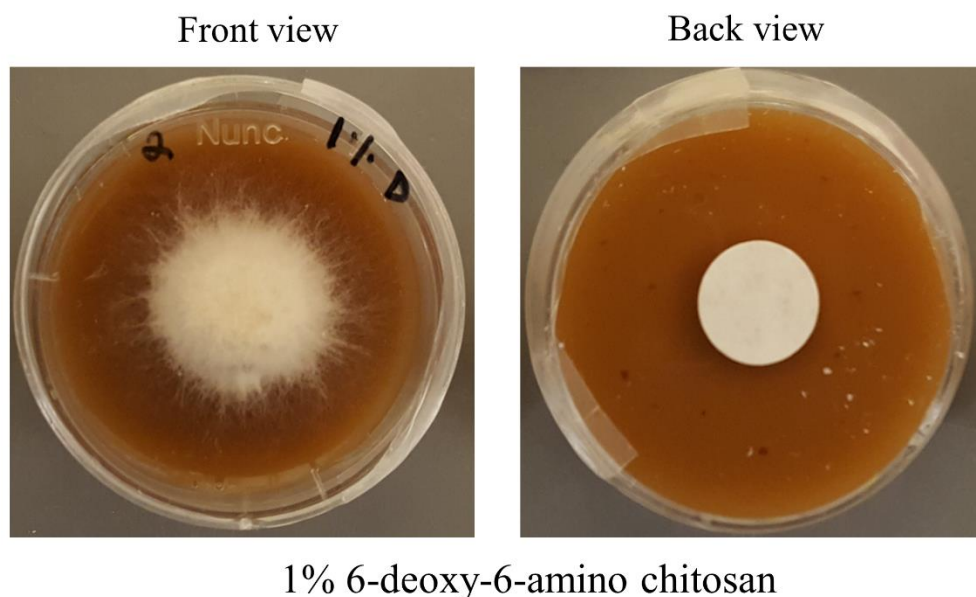


Figure 5.22: The antifungal activity of 1% of 6-deoxy-6-amino chitosan dissolved in H₂O against *F. verticillioides* at day four of incubation.

From Figure 5.22 it can be seen that the fungal mycelia have grown upwards instead of outward as is the case with the control plates. This can potentially be attributed to the fungus attempting to avoid the polymer by growing vertically and thereby exposing only a small part of its structure to the polymer infused agar. To confirm this hypothesis, a plate treated with 0.5% of 6-deoxy-6-amino chitosan was viewed under a microscope. The micrographs in Figure 5.23 shows the section of the plate on the edge of the original fungal plug.

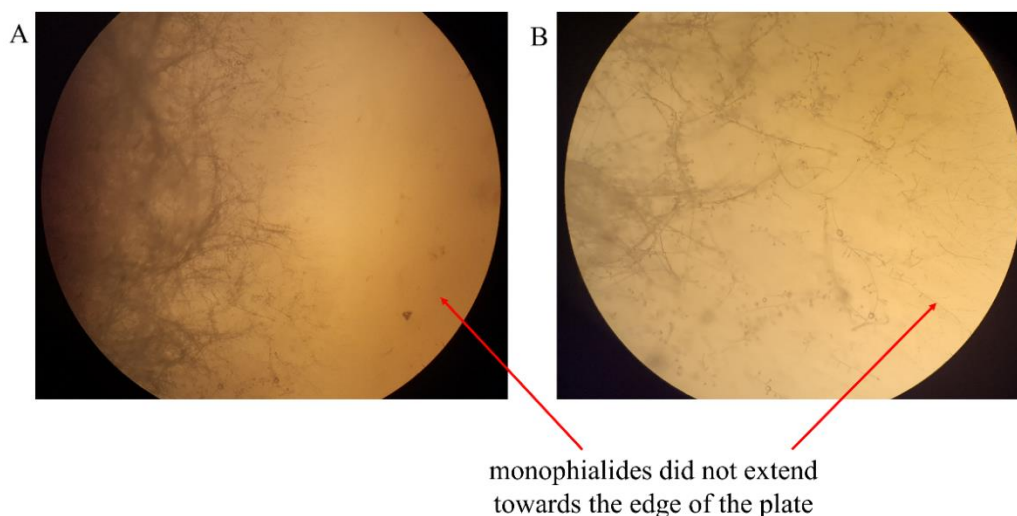


Figure 5.23: Light micrograph of a fungal plate treated with 0.5% 6-deoxy-6-amino chitosan, (A) magnification 40X and (B) magnification 100X.

It was noted that the monophialides (projections from the vesicles of fungi) did not extend towards the edge of the plate, where very little fungal hyphae were present as shown by the micrograph in Figure 5.23 B. This lack of growth outwards confirmed the inhibitory activity of the polymer.

The inhibitory activity displayed by 6-deoxy-6-amino chitosan is promising in the development of antifungals from a bio-derived source. It is advantageous that 6-deoxy-6-amino chitosan is water soluble and allows for evaluation at a high concentration in the absence of an acid, without being highly viscous, which is key for the future application of this polymer.

The current data can be expanded on by testing the effect of the polymer as a preventative or a curative in the treatment of maize plants. The next step in this study would be to apply the polymer to the maize plants and assess the effect on plant growth and plant defence responses when the plant is exposed to fungi such as *F. verticillioides*. Other avenues of research include the use of 6-deoxy-6-amino chitosan in the synthesis of copper nanoparticles, where the copper-polymer complex can be investigated for its effect on the defence responses and plant growth in maize plants following a similar study recently reported by Choudhary *et al.* for chitosan.³¹ Additionally, the difference in the effect of oligomers versus the polymers on plant growth and fungus inhibition would be an area of interest. The application of the polymer and COS on maize plants is discussed below.

Oligomers of chitin and chitosan (COS) possess properties which differ from the parent polymers. Upon hydrolysis the solubility of the polymer fragments are enhanced which affects its inherent properties. Studies have shown that oligomers possess enhanced antitumor, antifungal, antibacterial and anti-inflammatory properties. The review by Verlee *et al.* reported on the recent developments in the antibacterial and antifungal activity of chitosan and its derivatives, where the difference in the activity between the polymer and oligosaccharides was highlighted.⁴² Muanprasat and colleagues provided an in depth analysis of the biological activities and the potential therapeutic applications stemming from this.⁴³ This review noted that the antifungal activity of COS has been reported; however, it is generally lower than chitosan.⁴³

Production of oligosaccharides through hydrolysis

Oligomers or chitooligomers are derived from chitosan which demonstrate interesting properties correlated by its molecular weight. Well defined oligomers are generally produced by chemical (HCl, HNO₂, HF, H₂O₂), enzymatic (chitinase, chitosanase, lysozyme) and

physical (hydrothermal, microwave, ultrasonication, γ -ray) methods. The end product of the exhaustive chemical/acid hydrolysis are the monomers, acetic acid and various other small molecules including oligomers of differing chain lengths (Figure 5.24).⁴⁴

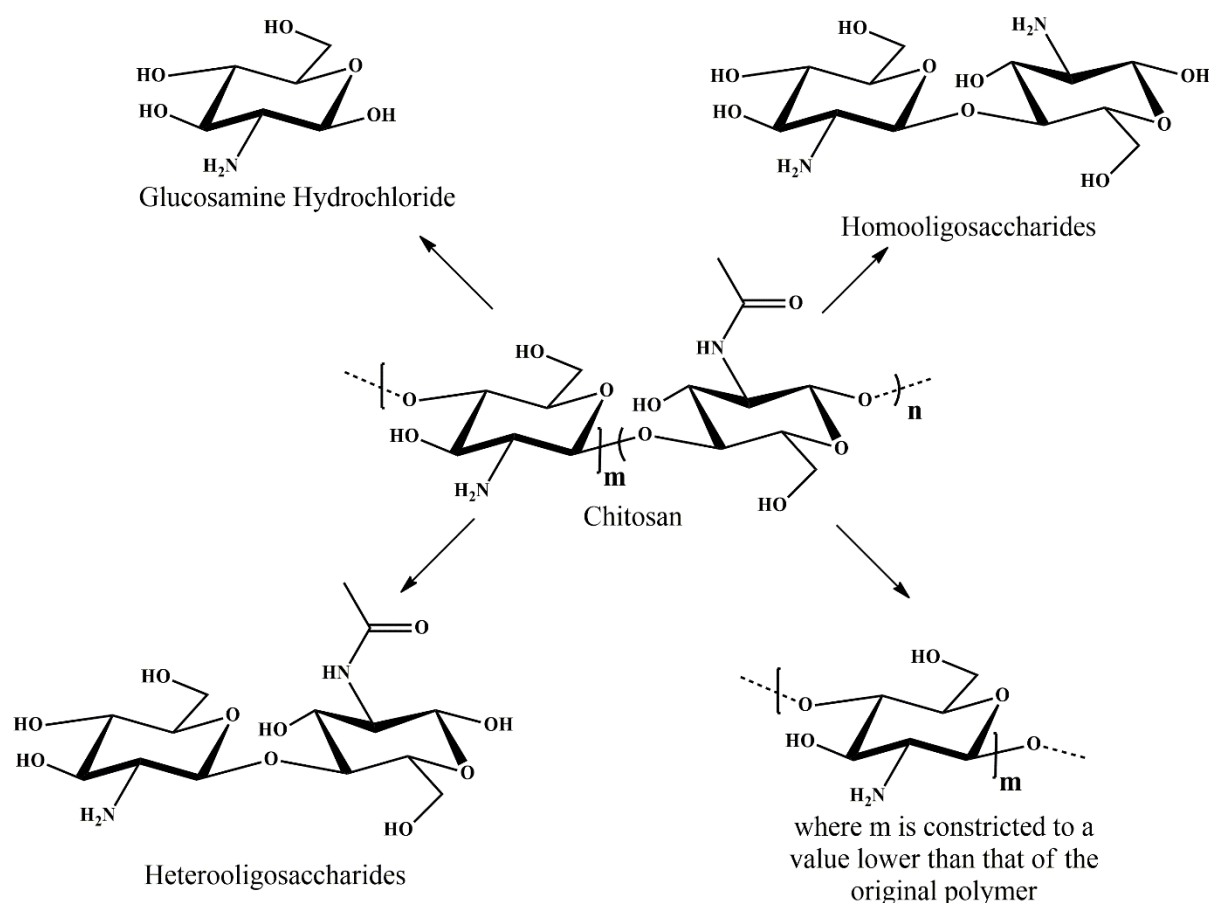


Figure 5.24: The products obtained from the hydrolysis of chitosan.

There are two steps which occur during the acid hydrolysis of chitosan, cleavage of the glycosidic linkages (polymer backbone) and N -acetyl linkages (side chains).⁴⁴ Kasaai *et al.* studied the fragmentation of chitosan by acids and proposed the mechanism of hydrolysis where the overall fragmentation of the polymer was initiated by a proton attaching itself to the glycosidic linkage which cleaved the larger molecules into shorter chains.⁴⁵

Acid hydrolysis was used in order to produce oligomers based on chitosan and its derivative, 6-deoxy-6-amino chitosan (**1.15**). Concentrated hydrochloric acid (32%) was used in the depolymerisation of the polymers as this method was the simplest and avoided extra steps in isolation of the products. The polymers were stirred in concentrated HCl at 70 °C for 2.5 hours and thereafter cooled. The oligomers were obtained by precipitation with cold acetone, and the resulting solid dissolved in water and the pH adjusted to 7 before freeze drying.⁴⁶ The samples

were subsequently characterised to determine if the basic structure of the polymer remained intact after hydrolysis. The first indication of the decrease in the molecular weight was the increase in the solubility of the samples obtained. Oligosaccharides of chitosan (5.2) and 6-deoxy 6-amino chitosan (5.3) dissolved immediately in water.

Analysis of the products obtained by ^1H NMR spectroscopy indicated that depolymerisation had occurred to some extent as evidenced by the sharpening of the peaks observed. The spectra were similar to that of the polymers themselves; however, there was a noticeable decrease in the peak related to the acetyl group of residual chitin residues due to acid treatment. In the case of chitosan, two peaks are observed for H-1 at 5.52 and 4.94 ppm where these are attributed to the anomeric proton of the reducing and non-reducing β -glucosamine units.⁴⁷ The ^1H NMR spectrum for the hydrolysed 6-deoxy-6-amino chitosan is shown as a representative (Figure 5.25). In addition, the peaks attributed to H-2 – H-6 appear sharper overall and indicated the preservation of the polymer backbone in the production of oligosaccharides. Peaks attributed to the formation of a Schiff base formed from the aldehyde at the anomeric proton and the amine were not observed.

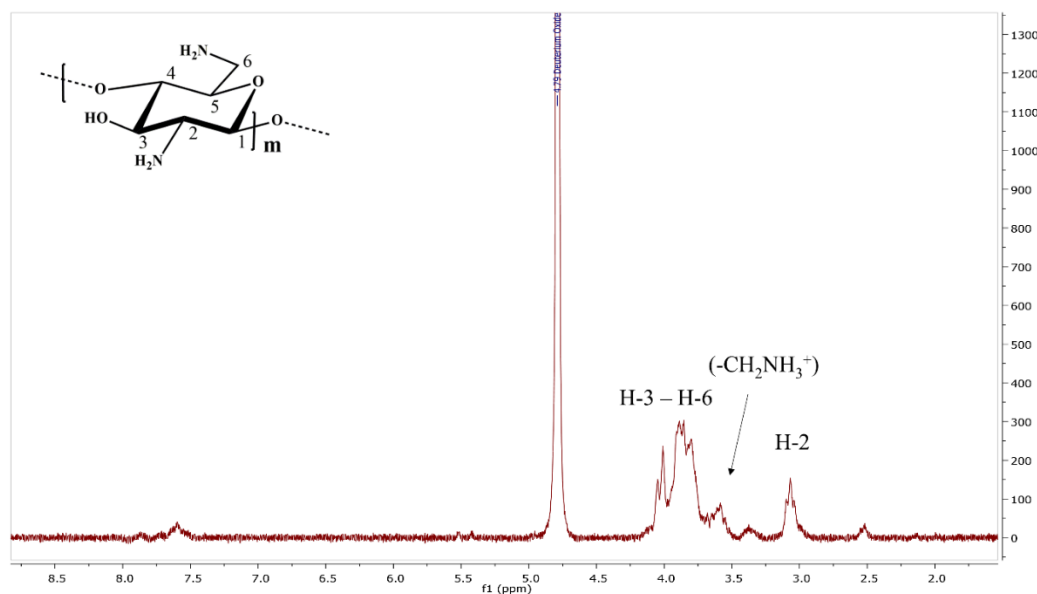


Figure 5.25: ^1H NMR spectrum of 6-deoxy-6-amino chitosan (5.3) oligomers after acid hydrolysis in D_2O .

The oligomers were also analysed using high resolution mass spectrometry to determine the size of the fragments obtained *via* hydrolysis (Figure 5.26 A-B).

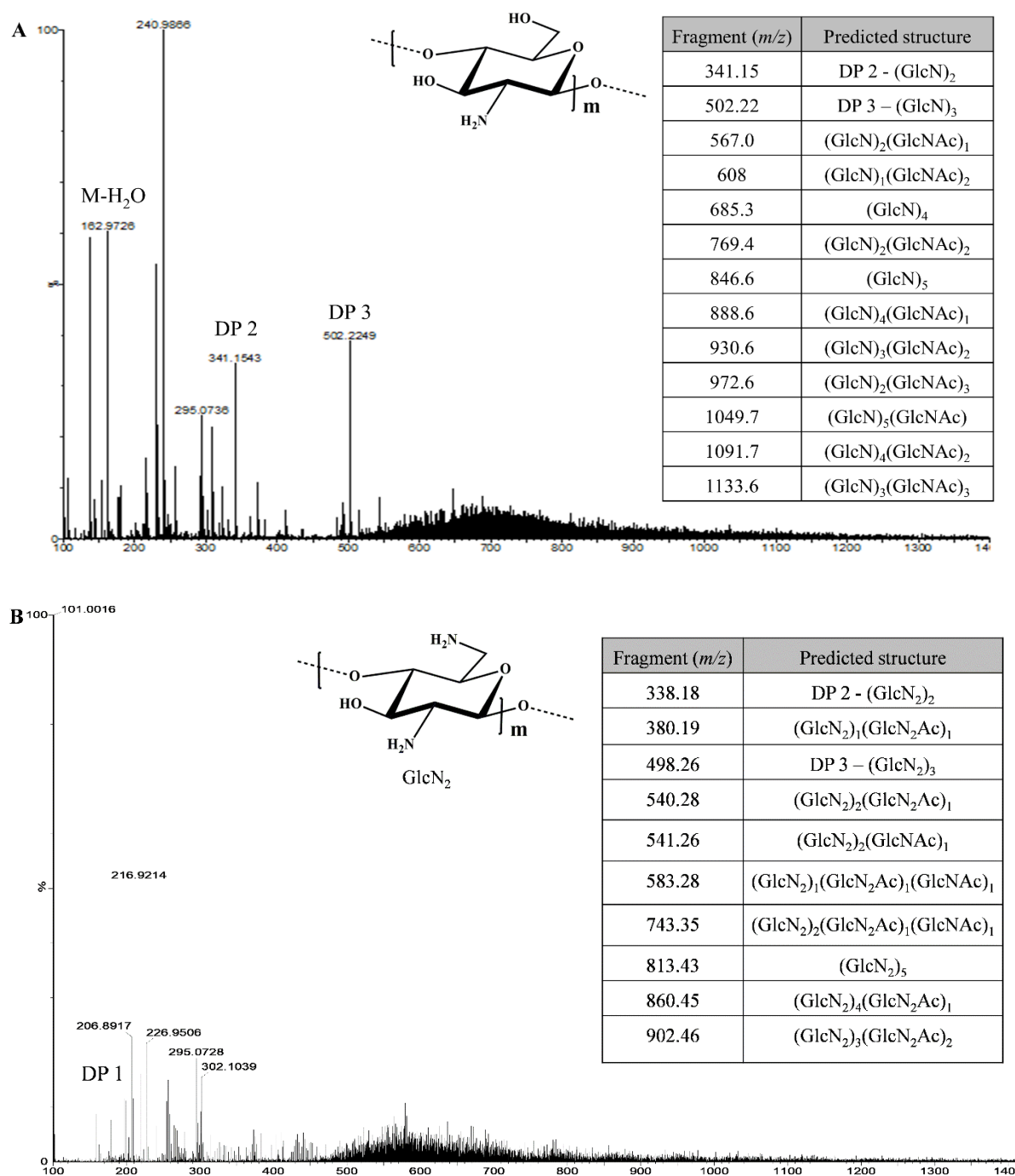


Figure 5.26: HRMS spectra of hydrolysed (A) chitosan and (B) 6-deoxy-6-amino chitosan.

For chitosan a degree of polymerisation (DP) of 1 refers to one glucosamine unit where a DP of 2 refers to a dimer or (GlcN)₂. For 6-deoxy-6-amino chitosan (5.3) a DP of 1 refers to one diamine monomer. From the data, it can be seen that the oligomers obtained contain a mix of fragments with the DP ranging from 1 to roughly 9. However, there was a range of oligomers with masses between m/z 500 – 1000 which were challenging to assign as the peaks overlapped. These can potentially be assigned to oligomers of a higher DP.⁴⁸ The centre of this range is

roughly m/z 680 for chitosan and m/z 580 for 6-deoxy-6-amino chitosan which could possibly correspond to a tetrasaccharide for chitosan and a $(\text{GlcN}_2)_1(\text{GlcN}_2\text{Ac})_1(\text{GlcNAc})_1$ fragment for 6-deoxy-6-amino chitosan. A similar result was obtained in the study by Ibrahim *et al.* where monomers with a DP of 3 to 6 were found in this region.⁴⁹ The study by Embaby *et al.* identified the fragments present in this region using MALDI-TOF-MS which were obtained by the hydrolysis of chitosan by an *Aspergillus sp.*⁵⁰ The fragments together with their predicted structures from this particular study are given in the Figure 5.26.⁵⁰ The fragments identified by Embaby *et al.* can potentially be used to identify the fragments obtained in the current study in the region m/z 500 – 1000.⁵⁰

There were also peaks corresponding to the loss of one or two water molecules due to the method of analysis used. The mass spectra for the oligomers obtained from 6-deoxy-6-amino chitosan appear to have a smaller average size compared to that of chitosan. The majority of oligomers lie in the region of m/z 600 – 900 for chitosan and m/z 500 – 700 for 6-deoxy-6-amino chitosan. Enzymes can be used to produce tailored oligomers; however, the goal of the current application was to investigate the difference in the effect of oligomers or more specifically monomers of 6-deoxy-6-amino chitosan on the antifungal activity of the polymer.

In a preliminary study the effects of the application of 6-deoxy-6-amino chitosan and the oligomers thereof on maize plants exposed to *F. verticillioides* was investigated. The effect of polymer and oligomer application on the plants was determined by the study of the changes in four key phytohormones related to a defence response namely, jasmonic acid, salicylic acid, abscisic acid and indole-3-acetic acid.⁵¹ Initial results indicated that there was an induction of a plant defence response when used as either a curative or preventative treatment in a time trial analysis for both the polymer and oligomers. This result was compared to the control which consisted of maize treated with *F. verticillioides*. While the control did induce a plant defence response, the response of the plants when treated with the polymers and oligomers was higher. Therefore, it can be inferred that the polymer and oligomers acted to prime the plants when used as both a curative and preventative. This result was in agreement with the review by Sharp which noted that there are physiological responses from plants treated with chitosan.³⁴

5.2.4 The applicability of 6-deoxy-6-amino chitosan as an edible food coating

There have been numerous reports on the use of chitosan and its derivatives as edible antifungal coatings in the food and beverage industry. The use of edible coatings is a popular method to preserve produce where the coating can act as a barrier to moisture and oxygen during

processing, handling and storage. In addition, the growth of microorganisms can be retarded. Chitosan films and coatings have also been reported to prevent the spoilage of fresh fruit and vegetables without compromising aesthetics and nutritional quality which typically declines during storage. These nutritional qualities include natural antioxidants, ascorbic acid, anthocyanins and polyphenols.⁵² The review by Yuan *et al.* covered the extensive application of chitosan films and coatings in the presence of essential oils, as well as their antioxidant and antimicrobial activity together with their application in food systems.⁵³ Satoh *et al.* demonstrated that 6-deoxy-6-amino chitosan displayed similar cytotoxicity to chitosan.⁵⁴ A later study of low molecular weight 6-deoxy-6-amino chitosan synthesised by Kim *et al.* showed that this polymer did not show any cytotoxicity on the Normal Human Dermal Fibroblast (NHDF) 3T3 proliferation under 100 µg/mL.⁵⁵ Therefore, since chitosan and 6-deoxy-6-amino chitosan have been shown to be non-cytotoxic the use of these polymers as edible coatings is possible.

Strawberries, a much loved fruit, are perishable as they are easily damaged, quick to suffer from desiccation, decay and physiological disorders upon storage. Thus, the treatment of strawberries is a popular subject for research. The major challenge in the distribution of strawberries is the time from harvest to supermarket shelf. Furthermore, the treatment of strawberries with any agent to minimise damage and delay the onset of fungal growth should not affect the overall aesthetics and taste of the fruit. Strawberries are particularly challenging to work with as their skin is particularly thin and moisture is easily lost through respiration and evaporation of moisture through the skin to air.⁵⁶

Studies have shown that *Botrytis cinerea* and *Rhizopus stolonifera* are the two fungi most commonly known to affect strawberries during storage. Chitosan has previously been studied as a post-harvest treatment for strawberries.⁵⁷ Vu *et al.* studied the antifungal properties of palmitoylated chitosan in conjunction with limonene and peppermint as bioactive edible coatings, where the chitosan-limonene combination was found to have superior activity.⁵⁷ Han and co-workers studied the sensory evaluation of strawberries coated with chitosan, since the acid used in the dissolution can contribute to an astringent taste which is undesirable.⁵⁶ Wang *et al.* reported on the effect of a chitosan based coating on the antioxidants, antioxidant enzyme system, and post-harvest fruit quality of strawberries.⁵⁸ This study found that chitosan treated fruit maintained a high antioxidant and ascorbic acid content and the microbial defence mechanism of the fruit was enhanced.⁵⁸

Since 6-deoxy-6-amino chitosan has demonstrated improved antifungal activity compared to chitosan, its use as an edible antifungal food coating in the treatment of strawberries was investigated.³⁷ The full experimental details are given in Chapter Seven section 7.8. In this preliminary study, strawberries (*Fragaria ananassa*) from a local supplier were treated with chitosan (1% w/v in 1% acetic acid) and 6-deoxy-6-amino chitosan (1% w/v in H₂O). Two methods of application were used, namely spraying and dipping. Strawberries were randomly distributed into three groups. Two groups were assigned to the 2 treatments (spraying and dipping) while the third group provided as the untreated control (Table 5.4). The treated strawberries were thereafter stored at room temperature.

Table 5.4: The sample groups and treatment protocols used.

Application method	Untreated (control-water)*	Untreated (control - 1% acetic acid)*	Treated (chitosan)*	Treated (6-deoxy-6-amino chitosan)*
Spraying	1	1	3	3
Dipping	1	1	3	3

* refers to the number of strawberries in that particular test group

The chitosan solution was quite viscous compared to the 6-deoxy-6-amino chitosan solution, making application through spraying for chitosan difficult. An added advantage of the use of the water soluble 6-deoxy-6-amino chitosan is that it allows for the application of a high concentration without affecting the viscosity of the solution. All strawberries were selected to be a semi-uniform set and were acquired from commercial sources. After the polymer solutions had been applied to the strawberries, the groups were stored at room temperature and the fungal growth was assessed daily *via* visual inspection. Figure 5.27 shows a representative of the control and treated strawberries on day one.

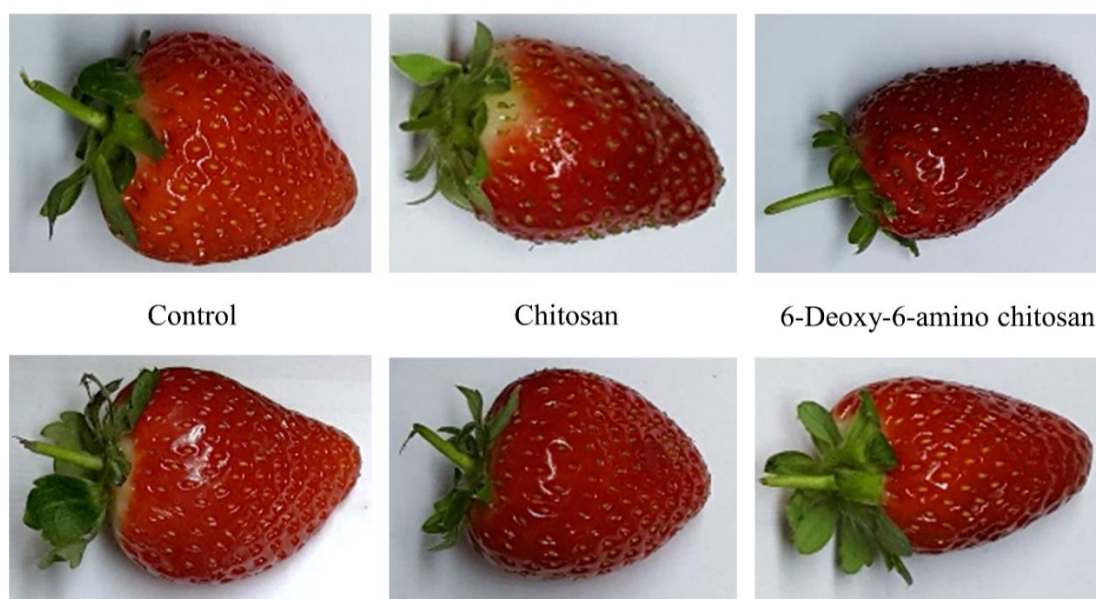


Figure 5.27: Treated strawberries on day one, dipped (top), sprayed (bottom).

The samples were monitored daily and no change was observed during days one to three. On day four, a softening of the fruit was observed for all samples, where the polymer treated samples appeared to have a thin film surrounding the fruit. The control exhibited signs of decay where the trend was the same for both sprayed and dipped samples. On day five there was a visible darkening of the samples and the first signs of decay were noted on polymer treated samples, where the control was almost completely covered in fungus (Figure 5.28).

As the days progressed the polymer coated samples appeared to shrink, possibly due to moisture loss and the development of fungus was observed. At the end of day 5 it was noted that both chitosan and 6-deoxy-6-amino chitosan delayed the onset of fungus compared to the control. The strawberries treated with 6-deoxy-6-amino chitosan appeared firmer and more aesthetically pleasing compared to those treated with chitosan.

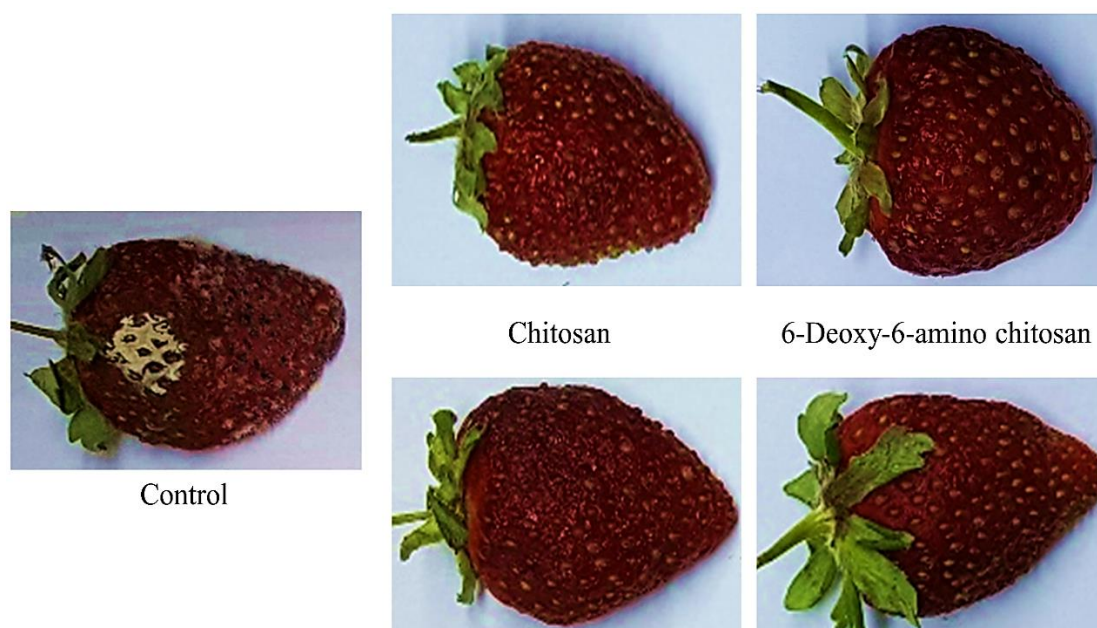


Figure 5.28: Polymer treated samples, dipped (top), sprayed (bottom) versus the control on day five.

It was noted that the fungus developed faster on the sprayed samples compared to the dipped sample set. This can potentially be attributed to the formation of a film over the whole of the strawberry when the sample is dipped compared to when the polymer is sprayed on. However, the exposure of strawberries to excess moisture *via* dipping was not ideal, since strawberries readily absorb moisture which contributes to the development of mottled or blotchy fruit. This also makes the fruit more sensitive to mechanical damage. Additionally, fungal growth is accelerated in the presence of moisture which shortens the shelf life of these berries.⁵⁹ Therefore, an application method which relies on dipping or soaking of the strawberries in the polymer solution may not be practical. An alternative formulation could be the application of the polymer solution as a fine mist by spraying the fruit. The extension of the shelf life was also dependent on when the strawberries were treated, either directly after harvesting or once they have reached the supermarket shelf. The berries used in this study were treated after purchase. Spraying immediately after harvest would provide a true reflection of the effect of polymer treatment. None the less, it is clear that polymer treatment delayed the onset of mould.

Comparing the results obtained here to that in literature was challenging, due to the difference in the source of the chitosan used in addition to the differences in the species of strawberry tested. However, at this junction it cannot be conclusively stated that 6-deoxy-6-amino chitosan displayed a higher degree of inhibition compared to chitosan, as a bigger sample size would be required to confirm this. Since the current study was preliminary this application should be

expanded upon in future. In order to analyse the effect of coating samples with 6-deoxy-6-amino chitosan, various analyses can be performed. These include: HPLC analysis of berry anthocyanins and phenolic compounds, total anthocyanin and total phenolic content, assays of antioxidant activities, assays of antioxidant enzymes, determination of ascorbate and glutathione, determination of β -1,3-glucanase activity and protein determination.⁵⁸ In addition, the sensory evaluation of the polymer coated samples should be conducted since 6-deoxy-6-amino chitosan was dissolved in water for application, it should not suffer from the astringent taste sometimes associated with the use of chitosan dissolved in acetic acid.⁵⁶

5.3 The application of 6-deoxy-6-amino chitosan as a solid support for catalysis

5.3.1 Background to the application

A common theme of today's research is the valorisation of carbohydrate waste; however, there is very little reported on the valorisation of protein based waste due to the complexity of protein processing. In terms of the biorefinery concept, protein waste can be processed into useful products or energy. Since the main components of proteins is amino acids, this is typically the focus of recovery efforts where the amino acids can be converted to useful platform molecules.⁶⁰ Examples of amino acids being converted to useful materials includes the conversion of phenylalanine to styrene *via* deamination and decarboxylation reactions. Additionally, lysine can be used in the production of caprolactam which is a valuable precursor to Nylon 6.⁶¹

A common application of modified chitosan polymers is their use as solid supports in catalytic reactions. 6-Deoxy-6-amino chitosan in particular, has been functionalised with iminopyridyl and iminophosphine moieties and subsequently reacted with rhodium and palladium. The metal immobilised polymer supports were used as catalysts for Suzuki-Miyaura and Heck cross-coupling reactions. The study found that the catalysts showed high activity for both reactions in organic and aqueous media and could be reused up to five times without significant loss of activity.⁶²

The focus of the current study was on the immobilisation of pyridoxal 5'-phosphate (PLP) (5.4, Figure 5.29 A). PLP is the active form of vitamin B6 which acts as a co-factor for many enzymes. The active site around the PLP co-factor is given in Figure 5.29 B.⁶³ PLP dependent enzymes are involved in a variety of biological applications including, amino acid metabolism, in the synthesis of amino sugars and in the metabolism of homocysteine. Additionally, deficiencies of PLP can be used as a biomarker for certain syndromes and diseases.⁶⁴

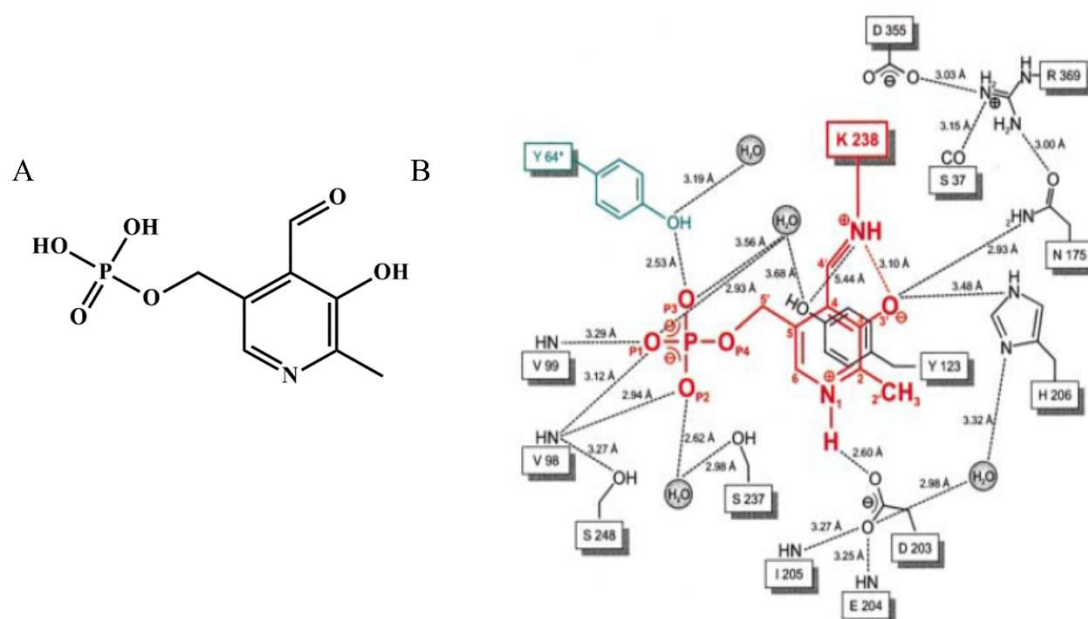


Figure 5.29: (A) Chemical structure of pyridoxal 5'-phosphate (5.4, PLP) and (B) the active site around the PLP co-factor (red), residues contributing from the neighbouring subunits (green) together with hydrogen bonds and interatomic distances (Å).⁶³

The review by Percudani *et al.* referred to PLP as ‘*the most versatile organic cofactor in biology*’ to try and highlight its importance.⁶⁵ PLP is typically found bound to proteins *via* a Schiff base referred to as the internal aldimine. An external aldimine is formed when PLP binds with the incoming substrate and the enzymatic reactions relevant to that substrate are carried out.⁶⁴ The PLP moiety is typically anchored *via* strong ionic bonding interactions (Figure 5.29 B, Y64, V98, V99 and S248). PLP acts as an electrophilic catalyst by stabilising the various carbanionic reaction intermediates formed, where it has been reported that more than 140 distinct enzyme activities are PLP dependent.⁶⁵ The chemical transformations which are carried out include, decarboxylation, transamination, racemisation, α - β and β - γ eliminations as well as retro-aldol cleavages.⁶⁶

While natural enzymes are typically large macromolecules, the majority of enzyme models and mimics are small molecules. The review by Marchetti *et al.* discussed the field of biomimetic chemistry with an emphasis on the importance of biomimetic catalysis defined as chemical catalysis that mimics certain key features of enzymatic systems.⁶⁷ Mimics of vitamin B6-dependent enzymes in transamination is a well-studied topic, where researchers look to nature for inspiration in the design of enzyme like molecules. Transamination takes place when a α -carbon bond of an amino acid is hydrolysed to produce an α -keto acid. Thereafter, pyridoxamine phosphate reacts with a second α -keto acid substrate forming an intermediate ketamine which then releases the corresponding amino acid. A number of artificial enzymes

has been reported by covalently linking pyridoxamine with β -cyclodextrin at primary or secondary edges for use in half-transamination reactions. Polyethyleneimine (PEI) is another synthetic polymer which has been modified for use as an enzyme mimic. Using modified PEI the non-enzymatic biomimic of the full transamination was realised. Zhang *et al.* used a PLP catalysed non-covalent cyclodextrin-keto acid inclusion complex in the catalysis of an enantioselective full transamination in the pure aqueous phase to produce various amino acids.⁶⁸

The immobilisation of PLP onto various stationary phases is an area of great interest especially in the area of affinity chromatography for protein purification. In the majority of studies, PLP is covalently linked to the support *via* the formation of a Schiff base. Ikeda *et al.* reported on the immobilisation of Sepharose-bound derivatives of PLP, pyridoxine 5'-phosphate and a pyridoxal 5'-phosphate-tryptophan complex (Figure 5.30).⁶⁹ These stationary phases were used for the affinity chromatography of apotryptophanase from *E. coli*, where the enzyme was successfully separated and eluted from the column.⁶⁹ More recently, Wang *et al.* used PLP as an immobilised metal affinity chromatography ligand through grafting of silica, oxidised carbon nanotubes and silica coated magnetic iron nanoparticles together with metals such as iron (Fe^{3+}), gallium (Ga^{3+}) and titanium (Ti^{4+}).⁷⁰ These immobilised metal affinity ligands were used in the enrichment of phosphopeptides before MS analysis.⁷⁰

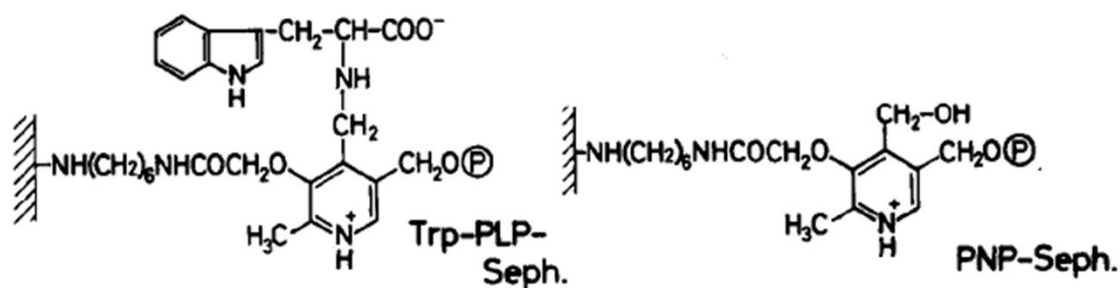


Figure 5.30: Sepharose immobilised PLP.⁶⁹

Renewed interest in the immobilisation of PLP was seen with the report of PLP bound to a lysine modified polyamidoamine (PAMAM) dendrimer. In addition, it was recently reported that PAMAM dendrimers are flexible with regards to their conformation and density depending on their environment, making these molecules similar to enzymes.⁶⁴ Filipowicz *et al.* synthesised bioconjugates of PAMAM third generation (G3) dendrimers with trans-retinal, pyridoxal, and pyridoxal phosphate, where the ability of these conjugates to permeate the skin was studied (Figure 5.31).⁷¹ In a later study, the *in vitro* cytotoxicity of a PAMAM G3–

pyridoxal–biotin bioconjugate was investigated. The results indicated that the conjugate displayed a lower cytotoxicity compared to the dendrimer itself.⁷² The latest research studied the effect of PAMAM G3–pyridoxal–biotin bioconjugates on the cell morphology, motility and ATP levels in normal and cancer cells in the hopes of finding a safe vitamin carrier.⁷³

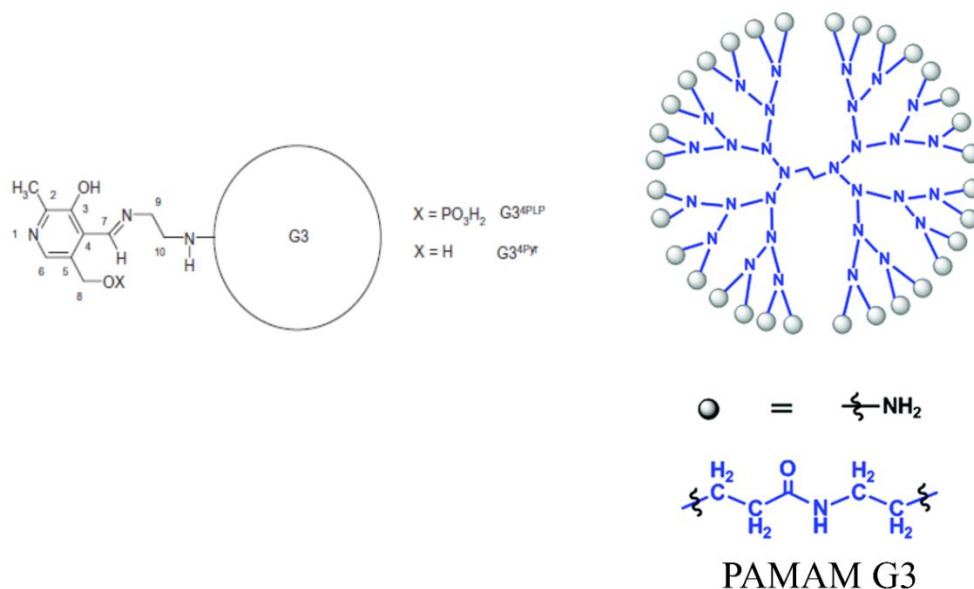


Figure 5.31: PAMAM G3 dendrimer conjugates of PLP.⁷¹

Chitosan has previously been modified by PLP through the treatment of chitosan cryogels with a PLP solution. Chitosan was first crosslinked with glutaraldehyde and the cryogel was subsequently formed. These gels were modified directly with PLP after deprotonation of the free amino groups. The cryogels were used in a study to determine whether the adsorption of ions such as copper and uranyl was improved.⁷⁴ The structure of the crosslinked chitosan cryogels functionalised with PLP is shown in Figure 5.32.

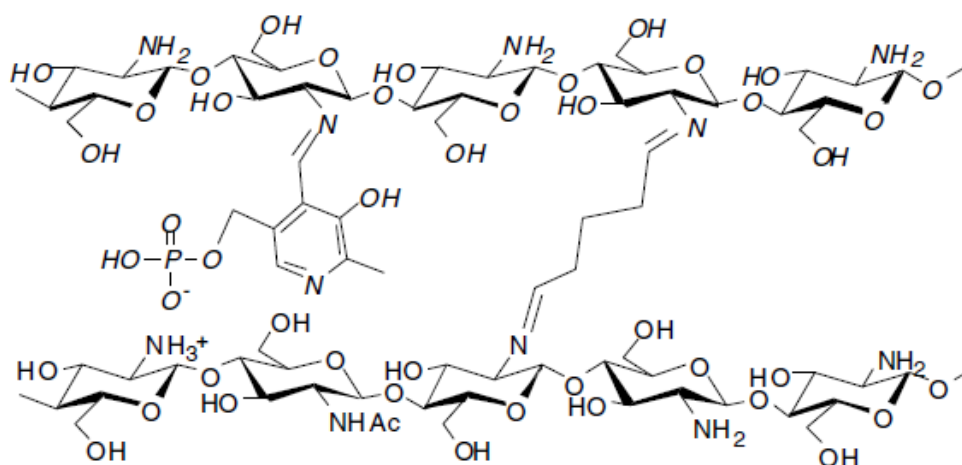


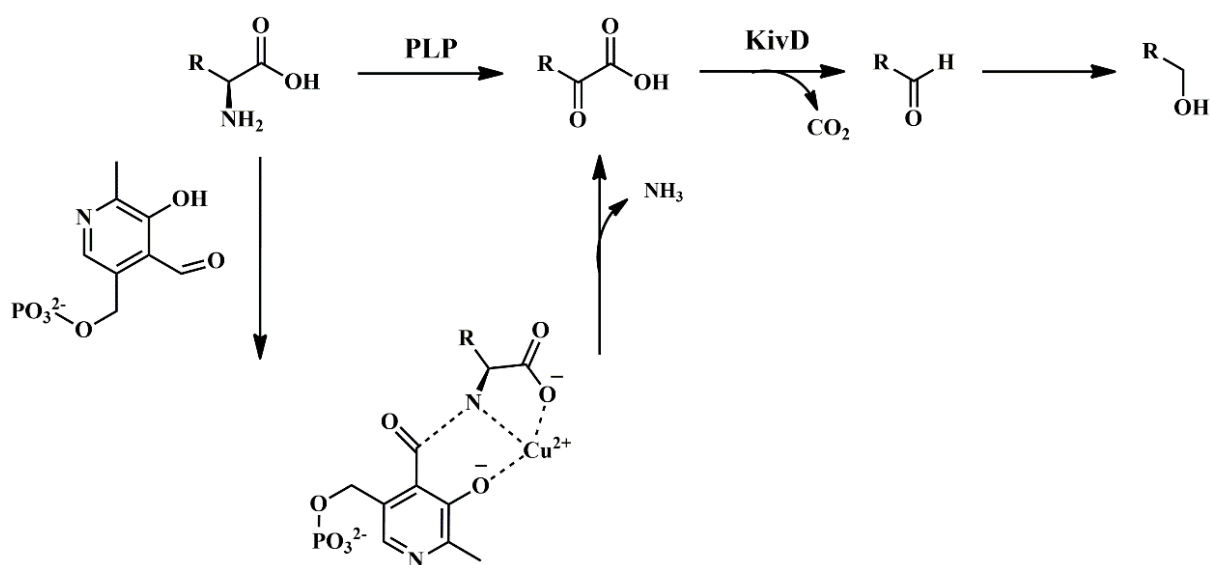
Figure 5.32: The structure of the reaction product between crosslinked chitosan and pyridoxal phosphate.⁷⁴

Prior PLP conjugates were used mostly for affinity chromatography and delivery of PLP (vitamin B6) and not much research has been published on the use of conjugates for enzyme mimicry since the position of attachment differs. Therefore, it is of interest whether PLP conjugates would behave as enzyme mimics in specific reactions.

PLP dependent deamination

Of the many PLP dependent reactions available, deamination is particularly important. In diverse biological systems, one of the most important forms of nitrogen transfer, is the conversion of an amino acid to an α -keto acid *via* transamination. PLP has been shown to convert amino acids to α -keto acids. These α -keto acids are important intermediates in biological processes including the production of amino acids and are industrially important for use in medicines, food-feed additives and in agricultural products. One of their main functions is to regulate protein turnover in metabolism which makes them indispensable for human and animals. The concentration of these acids in blood and urine can be used in the identification and treatment of several diseases, such as the diagnosis and treatment of phenylketonuria which is linked to a metabolic defect in amino acid metabolism.^{65, 66}

The study by Choi demonstrated the formation of alcohols using a PLP-copper dependent oxidative deamination of model amino acids and sequential enzymatic reduction by 2-keto acid decarboxylase (KivD) and alcohol dehydrogenase (ADH).⁷⁵ Scheme 5.1 illustrates the proposed pathway for this conversion, supported by experimental results which showed that upon formation of the 2-keto acids the reducing steps occurred simultaneously.⁷⁵



Scheme 5.1: The conversion of amino acids to alcohols as proposed by Choi.⁷⁵

The study by Liu *et al.* investigated polyethyleneimine functionalised with pyridoxamine to convert a keto acid to form the corresponding amino acid and PLP.⁷⁶ This polymer functioned as an enzyme mimic which could be recycled up to 2.5 times.⁷⁶ Shi and co-workers reported on the use of modified chiral pyridoxals in the asymmetric transamination of α -keto acids to α -amino acids. This work served as the first reported chiral-pyridoxal-catalysed asymmetric biomimetic transamination of α -keto acids involving two half-transaminations.⁷⁷

Due to the abundance of literature on PLP dependent processes and the development of enzyme mimics, it is a point of interest whether 6-deoxy-6-amino chitosan functionalised with PLP could behave as an enzyme mimic and be applied in a specific reaction such as deamination to produce α -keto acids. Additionally the influence of a covalent or ionic bond between the polymer and PLP on the outcome of deamination reactions is of interest.

5.3.2 Schiff base linked PLP to chitosan and 6-deoxy-6-amino chitosan

Chitosan and 6-deoxy-6-amino chitosan form covalent links with PLP through the formation of a Schiff base.

Chitosan – PLP – Cu (5.5)

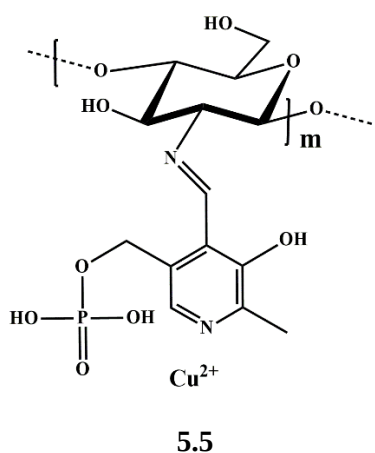
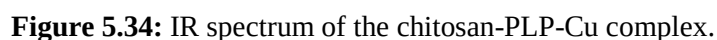


Figure 5.33: The structure of the chitosan-PLP complex (5.5).

The Schiff base between the polymer and PLP (5.5, Figure 5.33) was formed by stirring PLP in the presence of chitosan and removing excess PLP through dialysis. In the case of chitosan, PLP and copper sulphate were added to the solution of chitosan in 1% acetic acid, followed by sodium tripolyphosphate to precipitate the polymer. The solid was isolated by centrifugation, washed with methanol, dried and obtained with a moderate yield of 47%.

The polymer obtained was subsequently characterised; however, a ^1H NMR spectrum was not obtained due to the paramagnetic nature of the copper complex. In the IR spectrum of the



The diagram illustrates the chemical structure of a polymeric complex. The repeating unit is shown within brackets with a subscript 'm'. The polymer backbone consists of a 1,2,3,4-tetrahydro-1H-benzotriazin-4-ylidene moiety linked via its 1-position to a 2,6-dimethyl-4-hydroxy-5-(hydroxymethyl)pyrimidin-3-ylidene moiety. The pyrimidine ring is substituted with two methyl groups at the 2 and 6 positions and a hydroxyl group at the 4 position. The 5-position of the pyrimidine ring is linked to a hydroxymethyl group, which is further linked to a phosphonate group (O=P(OH)2). The phosphonate group is coordinated to a copper ion (Cu²⁺). The pyrimidine ring is also linked to a hydroxyl group, which is further linked to a phosphonate group (O=P(OH)2). The phosphonate group is coordinated to a copper ion (Cu²⁺). The pyrimidine ring is also linked to a hydroxyl group, which is further linked to a phosphonate group (O=P(OH)2). The phosphonate group is coordinated to a copper ion (Cu²⁺).

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The conjugation of 6-deoxy-6-amino chitosan with PLP is of interest, due to the presence of the additional amine at C-6 the loading of PLP was expected to be double compared to that of chitosan. The Schiff base between 6-deoxy-6-amino chitosan and PLP (**5.6**, Figure 5.35) was formed through stirring the polymer in a saturated solution of PLP. The PLP-polymer Schiff base approximates the internal aldimine found in the enzyme. Due to the conjugation of PLP to 6-deoxy-6-amino chitosan, the polymer was rendered insoluble in neutral H₂O. The ¹H-NMR spectrum of this polymer is given in Figure 5.36.

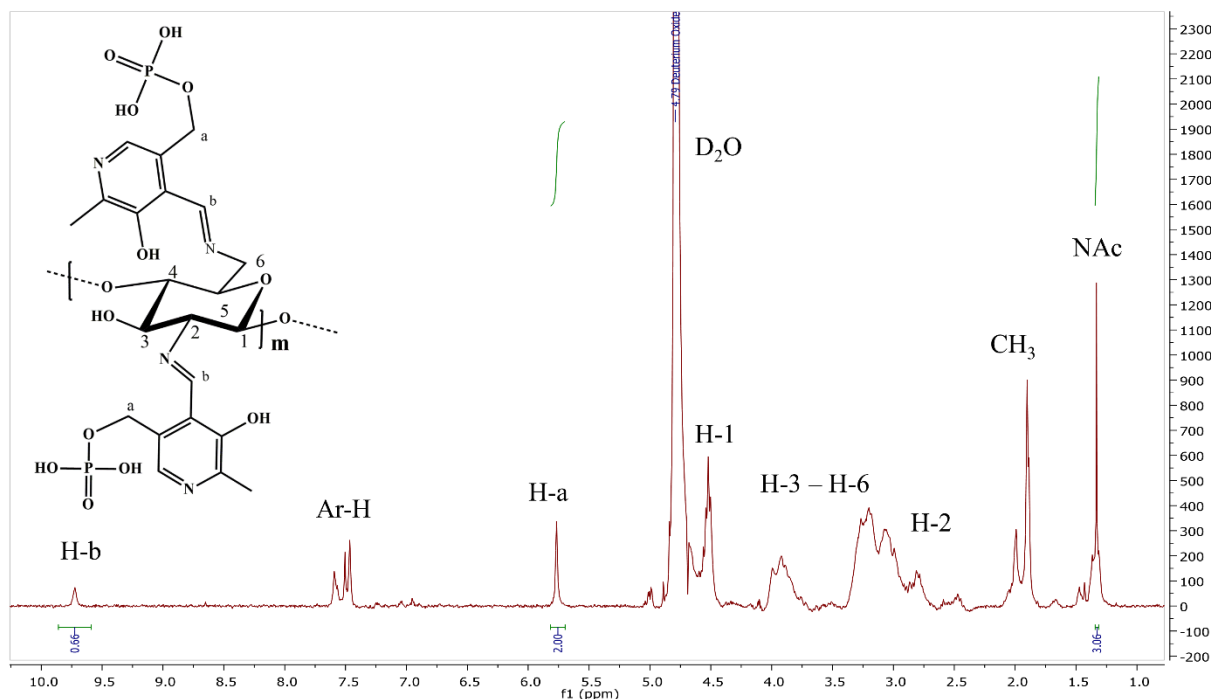


Figure 5.36: ¹H-NMR of 6-deoxy-6-amino chitosan-PLP complex in 2% DCl/D₂O.

Stability of the Schiff base is questionable at a low pH; however, the relative integrals in the ¹H-NMR show that H-b attributed to the imine is still present at an acidic pH. Peaks assigned to the aldehyde are noticeably absent potentially due to the neutralisation of the acid by the positive charge on the polymer. In the ¹H-NMR the peak at 9.72 ppm was attributed to the imine formed between PLP and the polymer while the H-a proton resonated at 5.74 ppm. The signals present between 2.52 and 4.0 ppm were assigned to the polymer backbone. The signal between 1.86 and 2.05 ppm was assigned to the methyl group of PLP. The peak assigned to the aldehyde of PLP was absent at 10.6 ppm further confirming the formation of an imine between PLP and the polymer and the absence of hydrolysis in the presence of DCl.

The ³¹P NMR spectrum of the polymer (Figure 5.37) displayed two peaks at -0.57 and -1.35 ppm compared to free PLP which displayed two peaks at -0.61 and -1.39 ppm at the same pH.

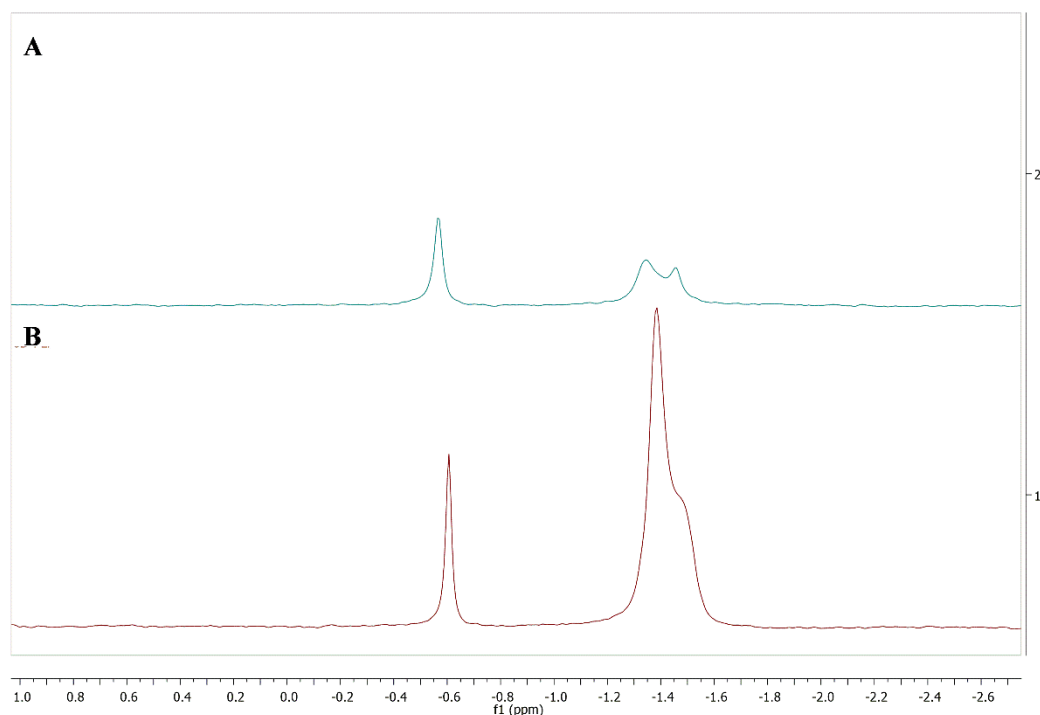
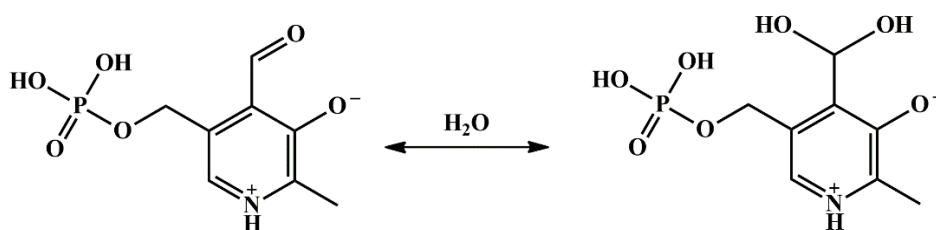


Figure 5.37: The ^{31}P NMR of the 6-deoxy-6-amino chitosan-PLP complex (**A**) versus free PLP (**B**) in 2% DCl/D $_2$ O.

The shifts are quite similar where PLP exists in two forms in an acidic aqueous solution (Scheme 5.2). Additionally, the peak at -1.35 ppm is more resolved compared to free PLP where one broad peak with a shoulder is observed. This indicated that a complex was formed with a different environment to the free PLP. This may be due to the substitution of PLP at either or both C-2 and C-6. In addition, the IR spectrum confirmed the presence of the characteristic imine at 1532 cm^{-1} supporting substitution of the polymer.

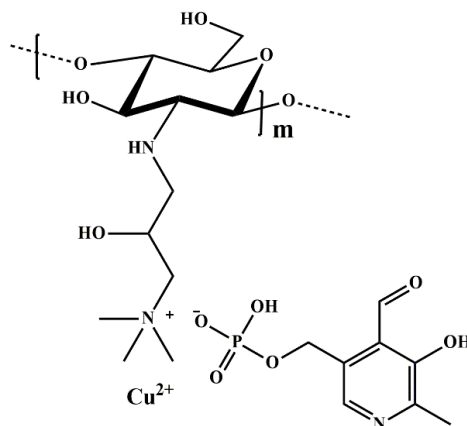


Scheme 5.2: The different forms of PLP in an acidic environment.

5.3.3 The formation of an ionic PLP complex

Ionic complex of PLP and 3-Trimethylammonium-2-hydroxypropyl-N-chitosan (5.7)

The polymer chosen to form the ionic complex with PLP was 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (5.7, Figure 5.38).

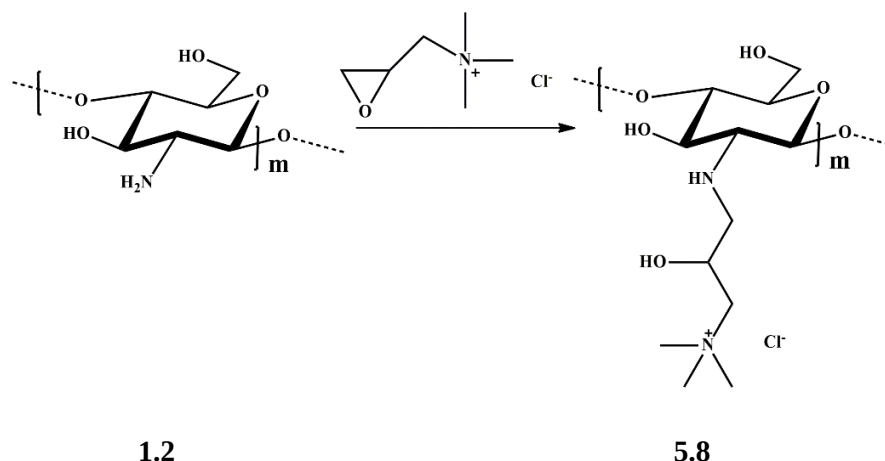


5.7

Figure 5.38: The structure of the 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride-PLP complex.

In the enzyme structures, PLP is complexed ionically using the phosphate group as an anchor (Figure 5.29 B). Here, a quaternary trimethyl ammonium chitosan derivative was proposed as an anchor for PLP. Binding to 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride is considered to be through an ionic interaction with PLP, whereby the positive charge on the amine and the negatively charged phosphate group interact.

This quaternised derivative of chitosan has received significant attention due to its improved properties such as solubility, antimicrobial activity and cytotoxicity.⁷⁸ 3-Trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (5.8) was synthesised by reacting chitosan with glycidyl trimethylammonium chloride (Scheme 5.3). The polymer was recovered with a yield of 75% and subsequently characterised. The ¹H NMR spectrum showed the introduction of a peak at 3.60 ppm attributed to the methyl groups attached to the quaternary nitrogen as well as the other groups related to the alkyl chain. The NMR spectra agreed with that reported in the literature.⁷⁹



Scheme 5.3: The synthesis of 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride. Reagents and conditions: glycidyl trimethylammonium chloride, H₂O, 60 °C, 24 hr, 75%.

IR analysis of the sample supported the introduction of the functional group on the polymer backbone as evidenced by the absorbance at 1478 cm⁻¹ which corresponded to the C-H bending of the trimethyl ammonium group (Figure 5.39).

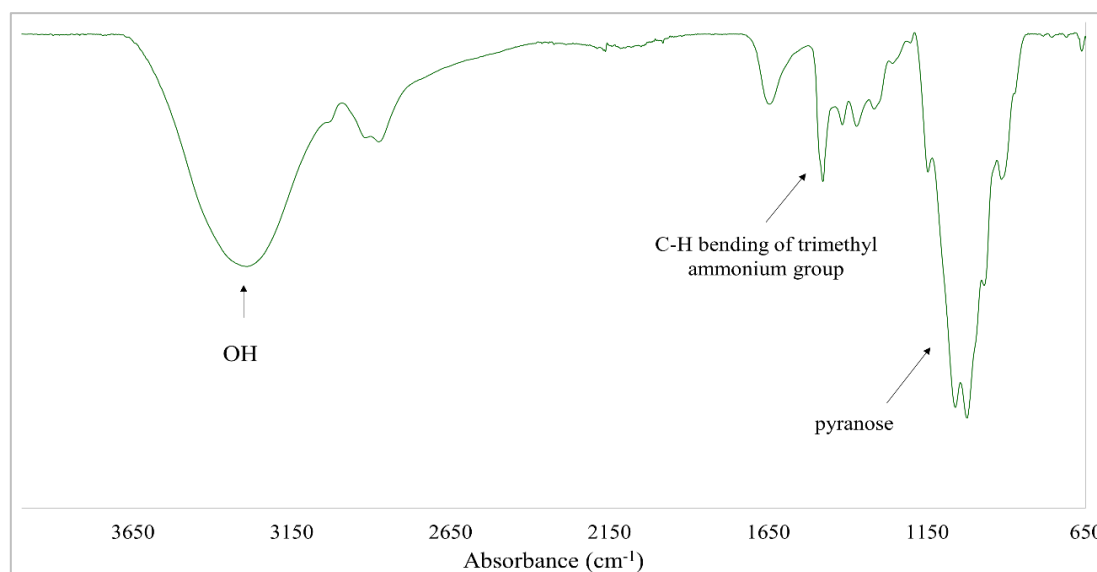
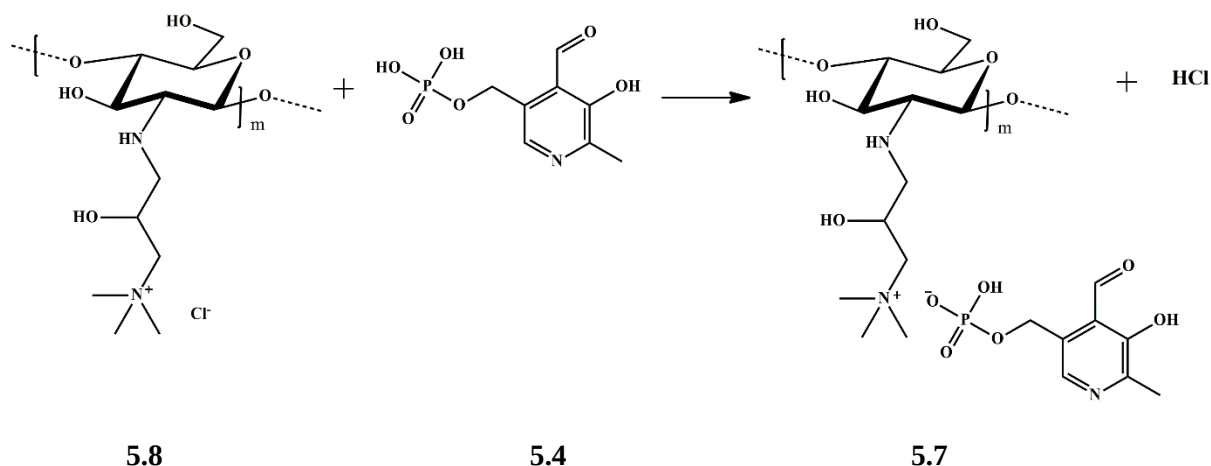


Figure 5.39: IR spectrum of 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (**5.8**).

The polymer was allowed to stir with excess PLP overnight at 25 °C and thereafter dialysed against water. The reaction of PLP with 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride produced a complex due to the interaction of the quaternary nitrogen and the negative phosphate group, with an exchange of the Cl⁻ and the PO₄⁻ groups (Scheme 5.4).



Scheme 5.4: Formation of the complex between 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan and PLP.

After complexation the solubility of the polymer decreased and thus a ¹H NMR spectrum was obtained by lowering the pH of the solution (Figure 5.40).

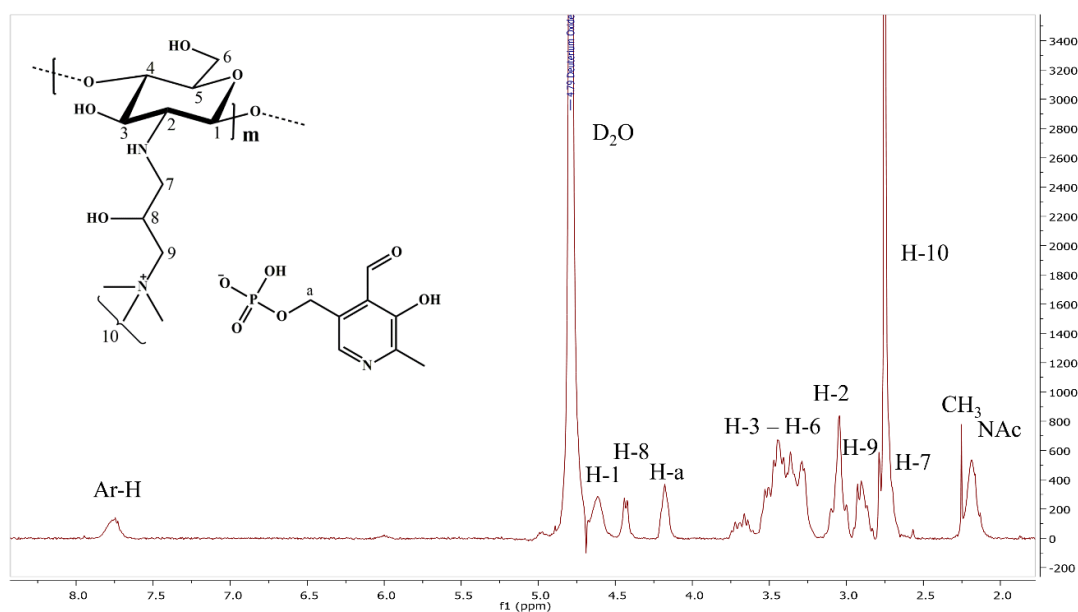


Figure 5.40: ¹H-NMR spectrum of 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan-PLP complex (5.7) in 2% DCl/D₂O.

In the ¹H NMR peaks assigned to the polymer and PLP are all accounted for, with an upfield shift seen for the peaks assigned to H-1 and H-3 – H-6 of the polymer. The peaks attributed to PLP shifted slightly upfield compared to free PLP, where the peak at 4.18 ppm was assigned to the CH₂ of PLP while the signal between 7.62 and 7.92 ppm corresponded to the aromatic protons of PLP. The proton assigned to the aldehyde in free PLP was absent in the spectrum supporting the complexation. Furthermore, hemiacetal formation is restricted in pyridoxal phosphate, therefore, no evidence was observed for this in the ¹H NMR spectrum.

The ^{31}P NMR spectrum of the polymer (Figure 5.41) displayed two peaks at -0.54 and -1.40 ppm compared to free PLP which displayed two peaks at -0.61 and -1.39 ppm at the same pH. The shifts are quite similar where PLP exists in two forms in an acidic aqueous solution (Scheme 5.2). Additionally, the peak at -1.40 ppm was a single well defined peak compared to free PLP where one broad peak with a shoulder is observed. This indicated that a complex was formed with a different environment to the free PLP. This may be due to the ionic complexation of PLP to the quaternised chitosan.

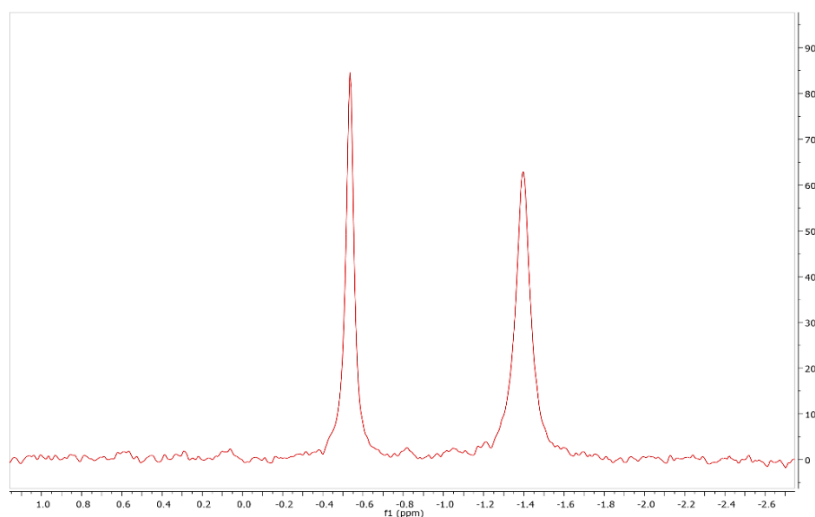


Figure 5.41: The ^{31}P NMR of 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan-PLP complex in 2% DCl/D₂O.

The IR spectrum further confirmed the presence of both PLP and the polymer with absorptions bands due to PLP assigned to C=C – CH₃, P=O, P-OH and P-O-C visible together with the peak attributed to the C-H bending of trimethyl ammonium group.

Deamination reactions

5.3.4 Non-enzymatic PLP – mediated conversion of amino acids to α -keto acids

One of the more widely used commercial conversions of amino acids to α -keto acids is that of phenylalanine to phenylpyruvic acid. This reaction is particularly useful as phenylpyruvic acid (PPA) is widely used in the pharmaceutical, food, and chemical industries. PPA serves as a useful intermediate in the synthesis of D-phenylalanine which in turn is used in the production of chiral pharmaceuticals and food additives. Phenyllactic acid which has found use as an antimicrobial, food preservative and flavour enhancer, is obtained from PPA. Aspartame the widely used sweetener is also derived from PPA.⁸⁰ As a result of the widespread use of PPA, its synthesis method is of importance. Commercial PPA is typically produced *via* chemical

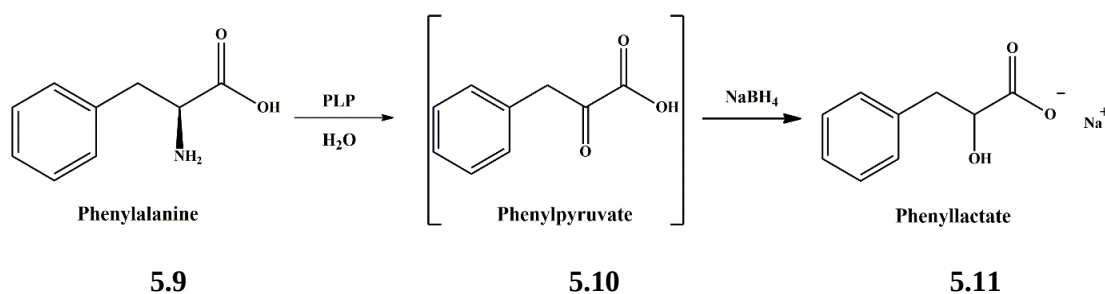
synthesis using several methods, where these methods cause environmental pollution, produce various by-products, and have relatively low yields.⁸⁰

There have been efforts to produce PPA using alternative routes such as enzymatic transformations. Phenylalanine was used in the production of benzaldehyde *via* phenylpyruvic acid. This study used a cell extract from *Lactobacillus plantarum* which converted the phenylalanine to PPA with the use of an aminotransferase. PPA was subsequently found to be converted to benzaldehyde by chemical oxidation.⁸¹ The study by Hou *et al.* expressed a membrane bound gene, L-amino acid deaminase (L-AAD) from *Proteus mirabilis* in *E. coli* which was subsequently used to produce PPA.⁸⁰ A later study by the same authors used the engineered *E. coli* in a two-step production of PPA *via* the growing and resting of cells and a two-stage temperature control strategy. The method was optimised to perform the reaction in a 3 L bioreactor where a mass conversion rate of 94% was achieved.⁸²

Due to the increasing commercial interest in the production of PPA, a milder more efficient method is sought after. In the current study, the ionic or covalent immobilisation of PLP on chitosan and derivatives thereof was investigated. The conversion of phenylalanine to PPA was subsequently studied using these modified polymers.

Standard conversion, polymer free - PLP – Cu – Phenylalanine

The conversion of phenylalanine to the sodium salt of phenyllactate was carried out in phosphate buffered saline in the presence of copper. The conversion is shown in Scheme 5.5.



Scheme 5.5: The conversion of phenylalanine to phenylpyruvate.

L-phenylalanine is generally used in preference to D-phenylalanine as it is the naturally occurring form of the amino acid. In the design of this study, it must be remembered that the natural versus synthetic production of phenylpyruvate should be cost effective. Synthetic production of PPA is currently very expensive due to the methods used. One synthetic method involves the reaction of benzaldehyde and glycine; however, toxic by-products of this reaction have been reported.⁸⁰ Another method, reacts benzyl chloride and carbonic oxide under high

pressure in the presence of cobalt octacarbonyl which serves as the catalyst. Once again toxic by-products are obtained in addition to low yields making these synthetic pathways unfavourable.⁸⁰

In the current study, the reaction of L-phenylalanine with PLP in the presence of copper, was allowed to proceed for 24 hours at 37 °C. Thereafter, the intermediate phenylpyruvic acid was subsequently reduced with NaBH₄ due to the instability of PPA. The reaction was washed with saturated sodium chloride and extracted with ethyl acetate. After drying over anhydrous magnesium sulfate and reducing the solvent *in vacuo*, phenylpyruvate was recovered in 11% yield as an off-white solid. If the remainder is only starting material the method can be optimised to obtain a higher conversion since the current process used is a batch process; however, if a continuous flow process is used any unreacted starting material may be recycled.

The ¹H NMR spectrum for the material obtained is shown in Figure 5.42. The multiplet resonating between 7.37 and 7.45 ppm was assigned to the protons of the aromatic ring. The signal at 4.58 ppm was assigned to H-2 while the two multiplets at 3.06 and 3.24 ppm were attributed to the protons of H-3.

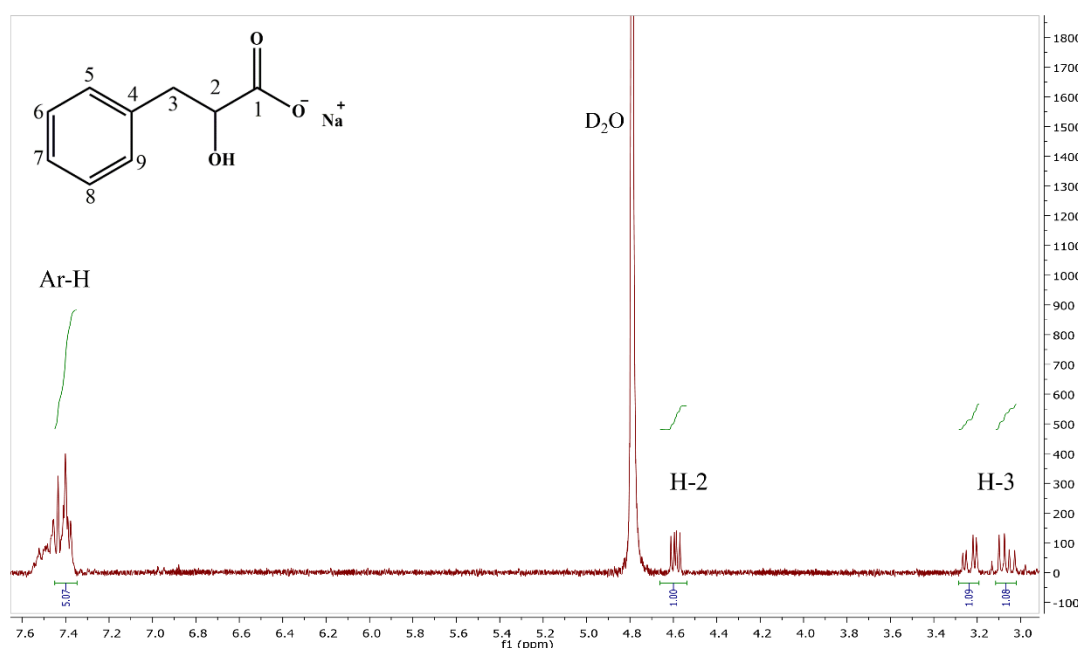


Figure 5.42: ¹H NMR spectrum of the sodium salt of phenyllactate (5.11) in D₂O.

Stereoselectivity (in the presence of a stereogenic centre) and more specifically enantioselectivity also comes into play in the transamination of α -keto acids, where the metal used generally fixes the geometry so that the proton transfer occurs on one face.⁸³ The study

by Koszelewski *et al.* investigated the synthesis of α -chiral primary amines through transamination, where the stereoselectivity of the ω -transaminases used was a key part of the study.⁸⁴ Generally a hydride reduction of an enantiotopic carbonyl in the absence of any molecules that induces asymmetry, leads to the formation of a racemic mixture of enantiomers which are not resolved by spectroscopy. It is therefore not possible to determine if one enantiomer was produced in preference to the other from the ^1H NMR obtained.

Polymer bound PLP conversion

Covalently linked PLP polymers, chitosan and 6-deoxy-6-amino chitosan

Phenylalanine was converted to phenylpyruvate using the polymer bound PLP in the presence of copper. The procedure followed the same method for that of the polymer free conversion; however, once the reaction was complete the sample was centrifuged and the polymer pellet removed before the reduction of phenylpyruvic acid.

Chitosan – PLP – Cu – Phenylalanine

The polymer bound PLP (0.351 mmol) and L-phenylalanine (0.353 mmol) were reacted and the intermediate reduced once the polymer had been removed. In the case of chitosan the polymer already contained copper therefore, additional copper was not added. For the chitosan bound PLP conversion, the yield of phenyllactate was 21% versus the 11% of the polymer free reaction. The material recovered was analysed and the ^1H NMR spectra was comparable to that obtained for the polymer free conversion. The multiplet resonating between 7.37 and 7.48 ppm was assigned to the protons of the aromatic ring. The signal at 4.64 ppm was assigned to H-2 while the two multiplets at 3.10 and 3.26 ppm were attributed to the protons of H-3.

6-Deoxy-6-amino chitosan – PLP – Cu – Phenylalanine

The polymer bound PLP (0.349 mmol), copper (0.332 mmol) and L-phenylalanine (0.326 mmol) were reacted and the intermediate reduced once the polymer had been removed. For the 6-deoxy-6-amino chitosan bound PLP conversion, the yield of phenyllactate was 24% versus the 11% of the polymer free reaction. The material recovered was analysed and the ^1H NMR spectra was comparable to that obtained for the polymer free conversion. The multiplet resonating between 7.37 and 7.42 ppm was assigned to the protons of the aromatic ring. The signal at 4.60 ppm was assigned to H-2 while the two multiplets at 3.06 and 3.23 ppm were attributed to the protons of H-3.

*Ionic linked PLP - 3-trimethylammonium-2-hydroxypropyl-N-chitosan chloride**3-Trimethylammonium-2-hydroxypropyl-N-chitosan chloride – PLP – Cu – Phenylalanine*

The ionic complex (0.350 mmol), copper (0.355 mmol) and L-phenylalanine (0.354 mmol) were reacted and the intermediate reduced once the polymer had been removed. For the PLP ionic complex, the yield of phenyllactate was 29% versus the 11% of the polymer free reaction. The material recovered was analysed and the ^1H NMR spectra was comparable to that obtained for the polymer free conversion. The multiplet resonating between 7.40 and 7.58 ppm was assigned to the protons of the aromatic ring. The signal at 4.61 ppm was assigned to H-2 while the two multiplets at 3.09 and 3.26 ppm were attributed to the protons of H-3.

Recyclability of 6-deoxy-6-amino chitosan-PLP in the conversion of phenylalanine

Moreover, the recyclability of the 6-deoxy-6-amino chitosan-PLP complex in the conversion of L-phenylalanine was studied. The reaction was carried out using a similar procedure, where the polymer pellet isolated after centrifugation was used directly in a subsequent reaction. The pellet was used for three cycles, where on the third cycle conversion was not obtained. This decrease in activity after the second cycle can possibly be attributed to the loss of PLP after each cycle, where after the second cycle minimal PLP remained complexed leading to incomplete conversion. In order to verify this theory, IR analysis of the polymer-PLP-Cu complex was performed after the second and third cycle and compared to the starting polymer. The IR spectra obtained is shown in Figure 5.43.

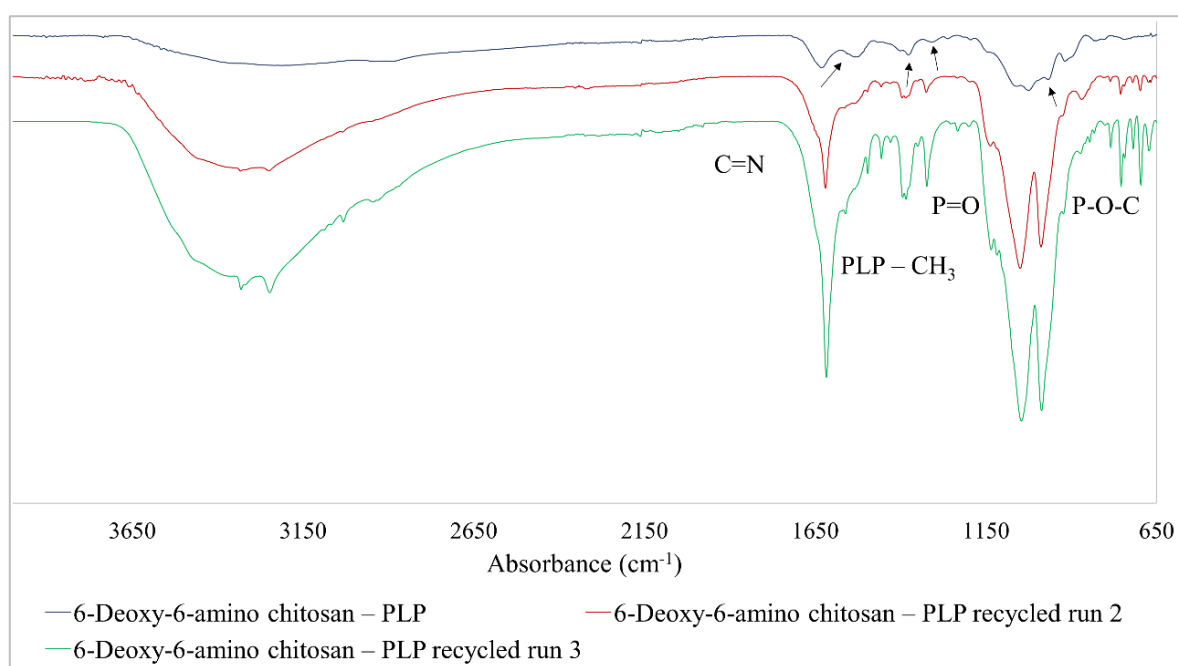


Figure 5.43: IR spectra of recycled 6-deoxy-6-amino chitosan-PLP complex.

In the starting polymer the peaks attributed to 6-deoxy-6-amino chitosan and PLP can be seen. After the second cycle of using the polymer-PLP complex in the conversion of L-phenylalanine it can be noted that the characteristic imine at 1532 cm^{-1} assigned to the Schiff base between the polymer and PLP noticeably weakens. In addition, there is also a shift and increase in intensity in the peaks related to $\text{C}=\text{C}-\text{CH}_3$, $\text{P}=\text{O}$ and $\text{P}-\text{O}-\text{C}$ from 1375 , 1304 and 967 cm^{-1} to 1385 , 1323 and 988 cm^{-1} respectively. After the polymer had been recycled three times, the imine band was indistinguishable from the amine band assigned to the polymer and the intensities of the peaks related to $\text{PLP}-\text{CH}_3$, $\text{P}=\text{O}$ and $\text{P}-\text{O}-\text{C}$ increased. A possible explanation would be that the PLP leaches off the polymer after every cycle due to the cleavage of the imine bond binding PLP to the polymer.

An additional advantage of this polymer supported conversion of phenylalanine, is the heterogeneous nature of the reaction. In industry, heterogeneous catalysts are preferred over homogeneous catalysts. Heterogeneous catalysts are easily separated from the product, typically more robust and easier to re-use. Drawbacks associated with heterogeneous catalysts include a lower selectivity. Homogeneous catalysts generally display superior selectivity and specificity, although separating the product from the catalyst can be challenging.⁸⁵

The difference in activity between the covalent versus ionically bound polymer were found to be not significant. Optimisation of reaction parameters may improve the yield and the turn over frequency of the polymers. Preliminary results demonstrated that the polymers can be bound to PLP either through a Schiff base reaction or through an ionic link to chitosan, 6-deoxy-6-amino chitosan and 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride. In addition, the conversion of an amino acid to an α -keto acid was demonstrated together with the reusability of a 6-deoxy-6-amino chitosan-PLP complex. This simple conversion of phenylalanine offers a useful alternative to the conventional synthesis of PPA. The advantage of the method reported here, is the use of a biorenewable polymer as the solid support for PLP and the minimised costs and issues related to the use of toxic agents since the biopolymers used are biodegradable. Additionally, the 6-deoxy-6-amino chitosan-PLP complex could be reused twice in the conversion of L-phenylalanine. This activity can potentially be improved through covalent immobilisation of PLP and optimisation of the reaction conditions.

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Chapter Six

Conclusion and Future Outlook

The aim of this work was to optimise the synthesis of 6-deoxy-6-amino chitosan, which is synthesised from the polymers chitin and chitosan. The source of the chitin and chitosan used in the current work is shellfish which was converted and marketed by an international supplier. However, there is scope for the application of chitin and chitosan from a local source where a stable and consistent supply of these polymers will encourage their use in various sectors. There is sufficient tonnage (> 1000 MT/annum) available locally to make the development of a sustainable production facility feasible. Additionally, a chitosan biorefinery is a possibility where Figure 6.1 shows the various applications which can be explored.

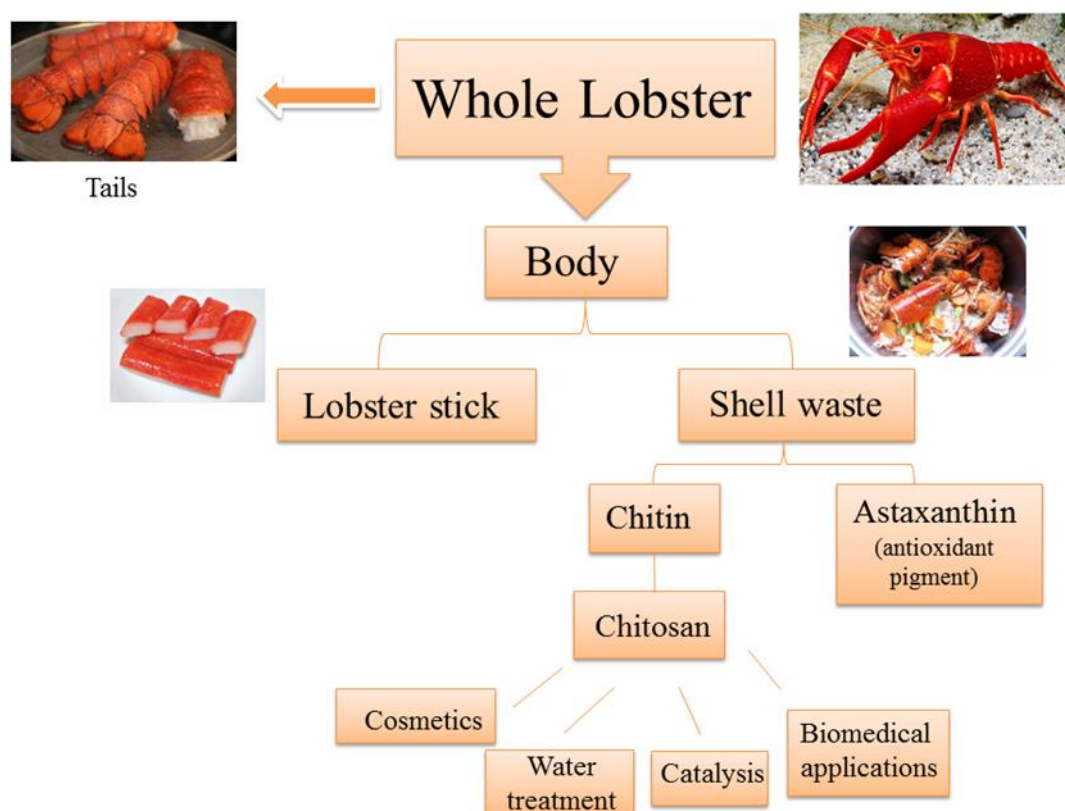
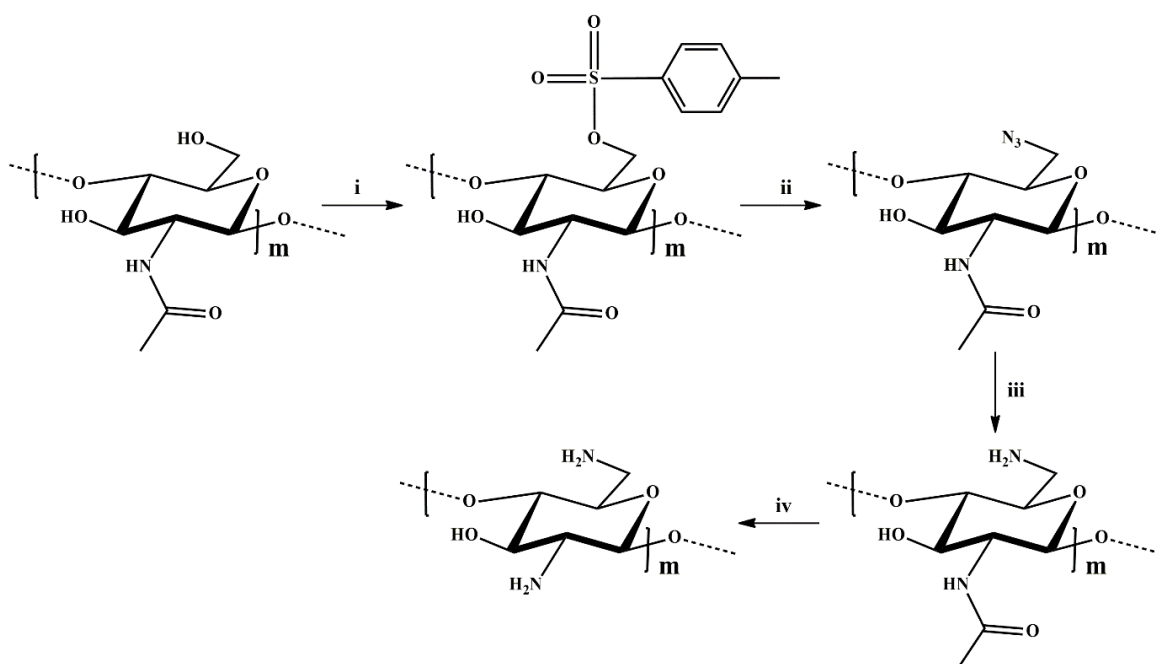


Figure 6.1: The possibilities available from the cultivation of chitin rich crustaceans.

6-Deoxy-6-amino chitosan is an interesting derivative which has been receiving significant attention due to its interesting properties such as its use as an antimicrobial agent. The large scale synthesis of this polymer has been challenging as reported methods utilise hazardous reagents and solvents. Owing to the lack of an acceptable method for the large scale synthesis of 6-deoxy-6-amino chitosan an alternative method was sought. Initially, the synthesis was

carried out according to literature methods (which have their own disadvantages) for comparison of the end product and scalability of the sequence.

Consequently, an alternative pathway starting from chitin was considered using the same route to introduce the amine *via* reduction of an azide group. The synthesis of 6-deoxy-6-amino chitosan was achieved through the tosylation of chitin in a mixture of DMAc/LiCl. The polymer was subsequently reacted with sodium azide and then reduced to the amine using triphenylphosphine. 6-Deoxy-6-amino chitin was thereafter deacetylated using aqueous NaOH to yield 6-deoxy-6-amino chitosan. The synthetic pathway starting from chitin is shown in Scheme 6.1.

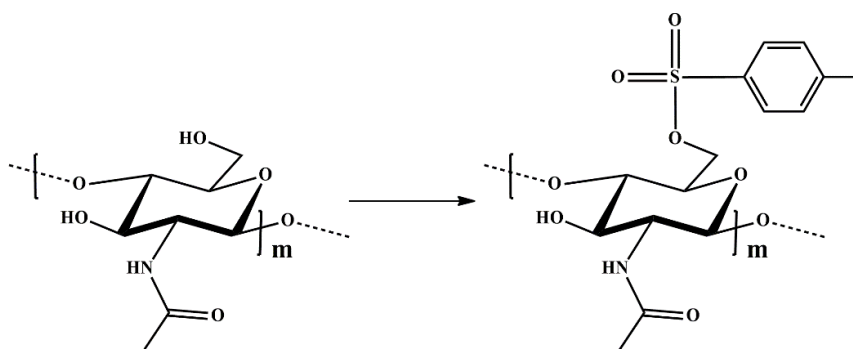


Scheme 6.1: Synthesis of 6-deoxy-6-amino chitosan starting with chitin. Reagents and yields: (i) *p*-Toluenesulfonyl chloride, Et₃N, DMAc/LiCl, 99%; (ii) NaN₃, DMAc, 86%; (iii) PPh₃, DMAc, H₂O, 95%; (iv) NaOH (45% w/v), 61%.

One of the most challenging aspects related to the use of chitin and chitosan, is the lack of environmentally friendly solvents which can be used in the modification of these polymers. A secondary goal of the study was to assess alternative solvents for the functionalisation of chitin and chitosan. The solubility of chitin and chitosan in various solvents was assessed through the use of Hansen solubility parameters. Potential solvents were identified such as BMIMAc, Cyrene™, DMAc, DMF, DMSO, glycerol, propylene carbonate, NMP and sulfolane. Further studies using computational methods such as DFT calculations, MD simulations, QTAIM analysis, NBO analysis and RDFs calculations were performed and the solubility of a model

compound was calculated in BMIMAc, Cyrene™, DMF, DMSO and NMP. The results showed that BMIMAc had the strongest interactions of these solvents.

A further solubility study of chitin/LiCl in 23 solvents from different classes was carried out. BMIMAc, Cyrene™, DMAc, sulfolane, and Tamisolve® gave promising dispersibility and were thereafter applied in a model reaction. The model reaction chosen was the tosylation of chitin (Scheme 6.2).



Scheme 6.2: Tosylation of chitin.

Results showed that only the reactions using the DMAc/LiCl combination achieved tosylation suggesting the heterogeneous nature of the reaction could be attributed for this result. Therefore, the tosylation of a homogenous mixture of chitin in NaOH/urea was attempted, where the material recovered once again did not indicate successful tosylation. Owing to this, the tosylation of chitin and *N*-phthaloyl chitosan in mixtures of the ILs BMIMCl and BMIMAc in the presence of pyridine was attempted. These reactions did not yield the expected tosylated product and attempts were made to substitute the co-salt in the DMAc tosylation of chitin. NaCl, BMIMCl and BMIMAc were tested and the reactions were unsuccessful, suggesting again that the possible heterogeneous nature of the reactions are the issue. This further confirms the hypothesis that a homogenous reaction medium is required for tosylation to occur. Therefore, the utilisation of DMAc was unavoidable and instead other means of introducing the amine at C-6 was investigated.

In the pursuit of an alternative synthesis of 6-deoxy-6-amino chitosan, the modification of glucosamine was considered in order to determine optimised reaction conditions and in order to monitor the progress of the reactions. Glucosamine was functionalised with an additional amine at C-6 using potassium phthalimide as the amine source. The reaction conditions optimised on the monomer model reaction was applied to both chitin and *N*-phthaloyl chitosan in the synthesis of 6-deoxy-6-amino chitosan. This novel pathway successfully produced a

water soluble polymer while avoiding the generation of large quantities of waste and by-products. The overall yields compared favourably to the previously reported methods and the amount of residual phthaloyl groups was lower compared to that reported in the literature. Additionally, the model study using a diphtaloyl protected monomer demonstrated the difficulty in deprotection. However, it was quite clear that phthaloyl deprotection is successful on the polymer. Preliminary molecular modelling studies shed some light on this phenomenon also explaining the relatively small amount of residual phthaloyl present after the deprotection step.

As an extension of this study, an additional amine was introduced at C-6 through functionalisation with an alkylamino group. Tosyl chitin and *N*-phthaloyl chitosan were reacted with ethylenediamine which served as both the reagent and solvent as well as the deprotecting agent in the case of the chitosan by removing the phthaloyl group. Thereafter, the chitin derivative was deacetylated to produce 6-deoxy-6-(2-aminoethyl) amino chitosan. This pathway is shorter and produces less hazardous waste compared to the methods used in literature. Therefore, this method serves as a promising route to the introduction of an amino alkylamino group which can be synthesised on a large scale. The applications of this polymer have not been fully explored and further studies would enable the full use of this appealing polymer.

The more soluble 6-deoxy-6-amino chitosan was subsequently tested in a broad range of applications to determine its use as a bio-degradable alternative to synthetic materials typically utilised. The applications were split into two sections, namely, testing of the antimicrobial activity of the polymer in various forms and the use of the polymer as a solid support in catalysis.

The formation of nanofibres based on 6-deoxy-6-amino chitosan was studied and it was found that 6-deoxy-6-amino chitosan did not form nanofibres by itself and had to be co-spun with polyethylene oxide (PEO). Chitosan was also co-spun with PEO for comparative purposes. Beading was observed in the formation of the nanofibres; however, spinning conditions were optimised and the beading was minimised. The nanofibres produced were in the range of 54.4 nm – 89.6 nm for chitosan and 77.5 nm – 131.0 nm for 6-deoxy-6-amino chitosan. These nanofibres were further crosslinked with glutaraldehyde to prevent dissolution. The loading of these nanofibres with silver nanoparticles was conducted using the polymer as the capping and reducing agent. Furthermore, 6-deoxy-6-amino chitosan did not require heat for the formation

of nanoparticles making the synthesis simple. It was noted that 6-deoxy-6-amino chitosan acted as a better dispersing agent compared to chitosan producing uniform nanoparticles. The size of the nanoparticles for both chitosan and 6-deoxy-6-amino chitosan were below 20 nm. These silver loaded nanofibres were subsequently applied in antimicrobial assessments where the activity against *E. coli* was assessed. The nanofibres themselves did not inhibit the growth of *E. coli* potentially due to the small amount of polymer on the sample tested. However, when loaded with silver nanoparticles, clear inhibition zones were seen for both the chitosan and 6-deoxy-6-amino chitosan loaded fibres.

A further potential application of 6-deoxy-6-amino chitosan is its use as an antifungal agent in crop protection and as an edible food coating. In this application, chitosan and 6-deoxy-6-amino chitosan were tested against the fungus *Fusarium verticillioides*. Both polymers displayed inhibitory activity against the fungus; however, acetic acid which was used for the dissolution of chitosan also showed inhibitory activity. Therefore, the comparison of the activity of these polymers was challenging. Although, 6-deoxy-6-amino chitosan displayed inhibitory activity when dissolved in water alone and prevented the fungus from spreading further compared to the control. The difference in the activity of the polymer versus oligomers produced through acid hydrolysis was also studied. It was found that both the polymer and oligomers acted as a curative when applied to infected maize plants.

Chitosan and 6-deoxy-6-amino chitosan were further assessed as edible coatings for strawberries. The treated fruit displayed a slower onset of fungal growth compared to the control, where the polymers appeared to form a film over the fruit. In terms of the two application methods used, spraying versus dipping, the samples which were dipped appeared to decay slower compared to those which were sprayed. A better formulation, application method and a bigger sample set would be needed to conclusively state that 6-deoxy-6-amino chitosan was effective as an antifungal edible coating making these preliminary results encouraging for future development.

A separate application involved the immobilisation of pyridoxal 5'-phosphate (PLP) onto chitosan, 6-deoxy-6-amino chitosan and a quaternary polymer 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride as solid supports for catalysis. The chitosan and 6-deoxy-6-amino chitosan bound PLP polymers were formed through Schiff base formation whereas the 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride bound PLP was formed through an electrostatic interaction. These PLP bound polymers were applied in the conversion

of amino acids to α -keto acids, specifically the conversion of L-phenylalanine to phenylpyruvic acid. The polymer bound PLP was successfully used in the conversion of L-phenylalanine to phenylpyruvic acid where the yields compared favourable to the reaction of free PLP. Additionally, the recyclability of the 6-deoxy-6-amino chitosan bound PLP was tested and the polymer could be used twice before a significant decrease in activity was observed. Analysis of the polymer bound PLP indicated that the PLP had leached and there was insufficient quantities to catalyse any further reactions.

Finally, this thesis reports the successful synthesis of an aqueous soluble 6-deoxy-6-amino chitosan, where the method used was comparatively greener and more environmentally friendly compared to previous reports. This makes the polymer more accessible to various diverse applications in the agricultural and biomedical sectors.

Future Outlook

In terms of the synthesis of 6-deoxy-6-amino chitosan, with the development of alternative, environmentally cost effective solvents, potential replacements for the hazardous solvents used in the current study could be found. This in turn could potentially lead to a reduction in the amount of waste generated. Recycling of certain solvents should also be considered in order to minimise waste production. Additionally, the substitution of the hydroxyl at C-6 with a reactive group needs to be expanded where alternative bio-derived reagents to *p*-toluenesulfonyl chloride can be utilised including *p*-cymenesulfonyl chloride.

With regards to the applications reported here, the electrospinning conditions can be further optimised to decrease the size of the nanofibres formed. Spinning parameters may be further modified in order to lower the amount of poly(ethylene) oxide required for successful production of nanofibres. Furthermore, the study of the silver nanoparticle loaded fibres should be expanded to include other bacterial species and the use of 6-deoxy-6-amino chitosan in the production of nanoparticles using other metals to consider as heterogeneous catalysts.

The antifungal application of 6-deoxy-6-amino chitosan can be expanded to include a more in depth study on the effect of the polymer and oligomers on the defence mechanisms of the plant. The edible food coating study can also be expanded to include a bigger sample set and other fruits in order to definitively state that 6-deoxy-6-amino chitosan displays as good or better

delay of the onset of decay compared to chitosan. Here, solubility is the key attribute where an increase in activity would serve as an added bonus.

The use of 6-deoxy-6-amino chitosan as a solid support for PLP immobilisation can be extended to include polymers linked *via* click chemistry to PLP (Figure 6.2) and the application of this polymer in the conversion of amino acids to α -keto acids can be evaluated.

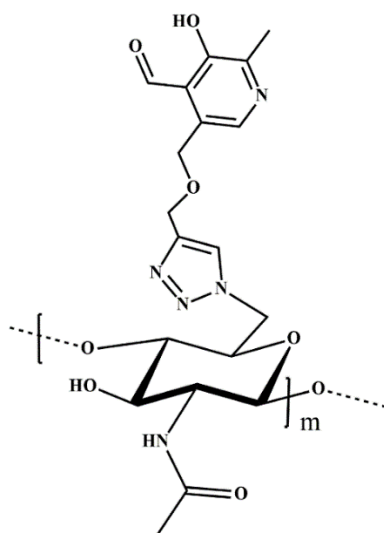


Figure 6.2: PLP linked to chitin *via* a click reaction.

The covalent link would exclude the possibility of PLP leaching under reaction conditions. The conversion of other useful amino acids to α -keto acids using bound PLP can potentially be exploited. These α -keto acids are useful intermediates in the synthesis of clinically important molecules, therefore their synthesis using a greener process compared to the current synthetic strategies is of importance.

This thesis described selected applications of this versatile polymer that can be further explored in order to bring the full potential of 6-deoxy-6-amino chitosan to the fore.

Chapter Seven

Experimental Procedures

7.1 General Remarks

All reagents and solvents used were purchased from commercial suppliers (Sigma-Aldrich, Fluka, Merck, Kimix) and used as received. Distilled/Milli-Q H₂O was used in all reactions, unless otherwise stated.

Low molecular weight (LMW) chitosan extracted from crabs which was purchased from AK Scientific was used in all reactions. All calculations were based on one unit of chitosan with a DDA > 90 %. Chitin extracted from shrimp shells was purchased from Sigma Aldrich and used directly. Polymers were purified by ultra-filtration using a dialysis bag where indicated, with a cut off molecular weight of 14 kDa.

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Unity XR400 MHz (¹H at 400 MHz, ¹³C at 101 MHz), Varian Unity XR300 MHz (¹H at 300 MHz, ¹³C at 75 MHz) or a Bruker Ultrashield 400 Plus spectrometer (¹H at 400 MHz, ¹³C at 101 MHz, ³¹P at 162 MHz). Solid-state ¹³C NMR was recorded on an Agilent VNMRS Wide Bore 500 MHz spectrometer with a ¹³C frequency of 125 MHz. ¹H and ¹³C NMR chemical shifts were recorded with reference to tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) in this thesis are reported in ppm, and *J* values in Hz. The numbering scheme given for each compound is for assignment purposes only and not necessarily consistent with IUPAC naming convention.

All reactions were performed under a nitrogen atmosphere; unless stated otherwise. Reactions were monitored by thin layer chromatography (TLC) using aluminium backed Merck silica-gel 60 F₂₅₄ plates and were visualised by ultraviolet light or staining with acidic ethanolic solutions of anisaldehyde (2.5% solution of anisaldehyde in a mixture of sulfuric acid and ethanol (1:10 v/v)) or ninhydrin (3% glacial acetic acid in ethanol solution) and heated.

A ninhydrin assay was used to indicate the presence of an amine group by reacting selected samples in a mixture of water and methanol with ninhydrin at 70 °C for 30 minutes and thereafter visually assessing the sample. The sample was thereafter compared to the blank and a colour change was recorded as a positive which was in the majority of cases the development of a purple colour.¹

Infrared absorptions (IR) were measured on a Perkin-Elmer Spectrum One FT-IR Spectrometer using Attenuated Total Reflectance (ATR).

Microanalyses for C, H, N and S were carried out on a Thermo Flash 1112 Series CHN Analyser and the EA Euro 3000.

High resolution mass spectrometry (HRMS) was performed on a Waters Synapt G2 with direct injection into a stream of acetonitrile. Source: Electrospray positive/negative, Cone Voltage 15 V.

Melting points were determined on a Reichert-Jung Thermovar hot-stage microscope and are uncorrected.

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.

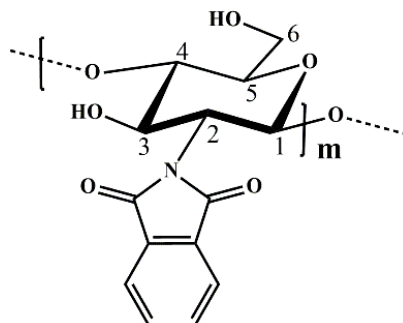
Powder X-ray diffraction spectra were recorded on a Bruker AXS D8 Advance diffractometer. The XRD data was collected using a $\text{CoK}\alpha$ radiation source of wavelength 1.79 Å, the diffractometer operated in the $\theta/2\theta$ mode primarily in the 30 – 100 ° (2θ) range with a step-size of 0.03°. All powdered samples were analysed directly.

Hot stage microscopy (HSM) was performed using a Linkam TP92 manual temperature control unit connected to a Linkam THMS600 hot stage which was mounted on a Nikon SMZ-10 stereoscopic microscope. For thermogravimetric analysis (TGA), the experiments were performed on a TA Instrument TA-Q500 with Universal Analysis 2000 software and a dry nitrogen flow rate of 60 cm³.min⁻¹. Differential scanning calorimetry (DSC) data was collected on a TA Instrument TA-Q200 Differential Scanning Calorimeter with Universal Analysis 2000 software and a dry nitrogen gas flowrate of 60 cm³.min⁻¹. HSM was used to corroborate and confirm the TGA and DSC data obtained.

Scanning electron microscopy (SEM) was performed on a Nova NanoSEM 230. Transmission electron microscopy (TEM) measurements were performed on a LEO EM 912 operating at 120 kV to visualise nanoparticles produced.

7.2 Chitin and Chitosan Polymer synthesis

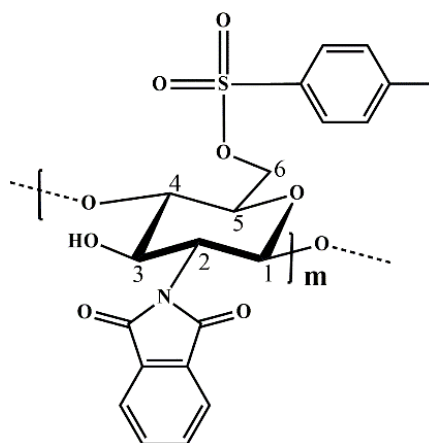
N-Phthaloyl chitosan (**1.18**)²



1.18

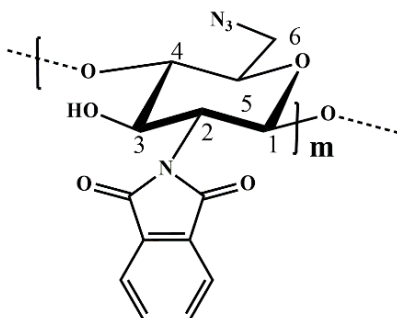
Chitosan (1.18 g, 7.37 mmol) was refluxed with phthalic anhydride (2.75 g, 18.6 mmol) in a 10% AcOH/H₂O solution (50 mL) at 120 °C for 24 hr. The mixture was allowed to cool to room temperature, and the precipitate collected by centrifugation. The solid was washed with water (50 mL), ethanol (50 mL) and thereafter allowed to stir in acetone (50 mL) to dissolve unreacted phthalic anhydride. *N*-phthaloyl chitosan (**1.18**) was recovered as a brown solid (1.71 g, 81%).

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.04 – 7.49 (4H, m, Ar-H), 5.31 – 4.05 (6H, m, H-1, H-3 – H-6), 3.32 (1H, s, H-2), 2.10 (s, acetylated units); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 170.7 (C=O), 134.9 (Ar-q), 131.9 (Ar-C), 130.8 (Ar-C), 98.7 (C-1), 76.1 (C-3), 74.3 (C-4), 69.6 (C-2), 69.1 (C-5), 59.6 (C-6), 55.4 (OAc); IR: ν (cm⁻¹) 3409 (broad strong band, OH), 2926 (sharp band, CH aliphatic), 1775 and 1704 (phthalimido), 1632, 1520, 1320 (strong bands, NH), 1092 (broad band, pyranose), 715 (phthaloyl aromatic); Elemental Analysis (%): C, 52.14; H, 4.99; N, 4.99; Ninhydrin test: negative vs blank.

6-Deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (1.19)²**1.19**

N-Phthaloyl chitosan (**1.18**) (7.89 g, 27.1 mmol) was suspended in anhydrous pyridine (190 mL) and cooled to 0 °C, followed by the addition of *p*-toluenesulfonyl chloride (20.4 g, 107 mmol). The mixture was allowed to warm to RT and stirred for 48 hr. The brown viscous solution was added to ice water (560 mL) and the resulting precipitate was filtered and washed with ethanol (700 mL) and diethyl ether (700 mL). Drying under reduced pressure to a constant mass gave 6-deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) as a brown solid (12.0 g, 99%).

IR: ν (cm⁻¹) 3448 (broad band, OH), 2948 (sharp band, CH aliphatic), 1774, 1716 (sharp band, imide C=O), 1174 (sharp band, S=O), 1069 (broad band, pyranose), 815 (sharp band, C-O-S), 718 (sharp band, phthaloyl-aromatic); Elemental Analysis (%): C, 43.92; H, 4.22; N, 4.43; S, 5.07.

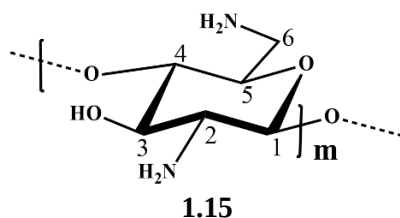
6-Deoxy-6-azido *N*-phthaloyl chitosan (1.20)²**1.20**

6-Deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) (9.12 g, 20.5 mmol) was suspended in NMP (700 mL) followed by the addition of NaN₃ (16.9 g, 260 mmol). The resulting mixture was allowed to stir at 80 °C for 4 hr. Thereafter, the reaction mixture was allowed to cool and

the upper layer of NMP decanted. EtOH (200 mL) was poured into the remaining mixture which was left overnight to allow a precipitate to settle. The supernatant was removed and 40% ethanol-water (v/v) (200 mL) was added and the resulting mixture was centrifuged. The precipitate was washed with acetone, collected and dried under reduced pressure. The resulting brown solid was further dried under reduced pressure to a constant mass (6.39 g, 99%).

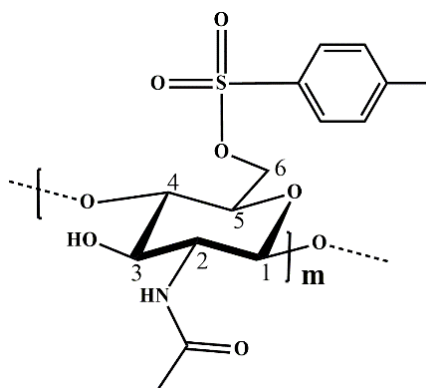
IR: ν (cm^{-1}) 3435 (broad band, OH), 2939 (sharp band, CH aliphatic), 2103 (sharp band, azido), 1772, 1718 (sharp bands, imide C=O), 1660 (sharp band, C=O stretch of secondary amide), 1174 (weak band, residual S=O), 1107 (broad band, pyranose), 718 (phthaloyl-aromatic); Elemental Analysis (%): C, 50.20; H, 4.23; N, 12.78.

6-Deoxy-6-amino chitosan (**1.15**) Method reported by Jardine *et al.*²



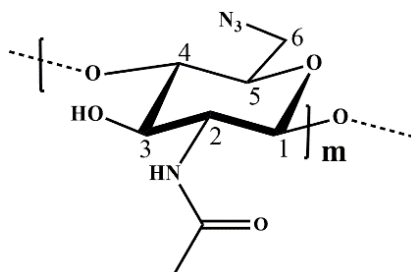
Triphenylphosphine (13.2 g, 50.3 mmol) was added to a solution of 6-deoxy 6-azido *N*-phthaloyl chitosan (**1.20**) (7.93 g, 25.1 mmol) in NMP (80 mL), and the reaction was allowed to stir at room temperature for 15 hr. Hydrazine monohydrate (8.00 mL, 257 mmol) and water (56 mL) were added and the reaction mixture was allowed to stir at 100 °C for an additional 4 hr. The excess water in the reaction was decanted and the remaining mixture added to ethanol (160 mL). The precipitate was collected by centrifugation, washed with ethanol and dried by suction filtration. The resulting precipitate was suspended in water and dialysed against water using a membrane with a cut-off weight of 14 kDa. The product was obtained and lyophilised to yield (**1.15**) as a brown solid (4.02 g, 100%).

¹H NMR (400 MHz, 2% DCl/D₂O) δ 5.36 (1H, s, H-1), 4.51 – 4.03 (5H, m, H-3 – H-6), 3.83 – 3.93 (2H, m, H-6, $-\underline{\text{C}}\text{H}_2\text{NH}_3^+$), 3.67 (1H, s, H-2); IR: ν (cm^{-1}) 3379 (broad strong band, OH, NH), 2922 (sharp band, CH aliphatic), 1653, 1591 (sharp, strong bands, NH₂), 1060 (broad band, pyranose); Elemental Analysis (%): C, 41.50; H, 6.70; N, 13.57; Ninhydrin test: positive vs blank.

6-Deoxy-6-*p*-toluenesulfonyl chitin (2.2)³**2.2**

Chitin (5.97 g, 29.4 mmol) and LiCl (26.1 g, 616 mmol) were dried separately at 50 °C overnight and at 130 °C for 4 hr. The solids were allowed to cool to 25 °C, LiCl was allowed to stir in DMAc (260 mL) until all the solid had dissolved. Chitin was subsequently added and the mixture was allowed to stir at room temperature until a viscous gel formed (12 hr). The reaction mixture was allowed to cool to 0 °C followed by the addition of Et₃N (72.0 mL, 520 mmol) and a solution of *p*-toluenesulfonyl chloride (51.1 g, 268 mmol) in DMAc (120 mL) which was added over 90 min (13.5 mL in 10 minute intervals). The reaction was allowed to stir at 5 °C for a further 48 hr. Water (1.50 L) was added and the precipitate washed with ethanol (700 mL) and the resulting product was lyophilised to give 6-deoxy-6-*p*-toluenesulfonyl chitin (**2.2**) as a light brown solid (10.4 g, 99%).

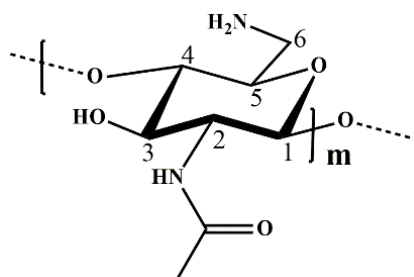
IR: ν (cm⁻¹) 3396 (broad band, OH), 2936 (sharp band, C-H aliphatic), 1655 (sharp band, C=O stretch of secondary amide), 1160 (sharp band, S=O), 1063 (broad band, pyranose), 812 (sharp band, C-O-S); Elemental analysis (%): C, 44.70; H, 6.85; N, 5.30; S, 5.75; Ninhydrin test: negative vs blank.

6-Deoxy-6-azido chitin (2.3)²**2.3**

To a suspension of 6-deoxy-6-*p*-toluenesulfonyl chitin (**2.2**) (0.520 g, 1.46 mmol) in DMAc (20 mL) was added NaN₃ (1.24 g, 19.1 mmol) and the mixture allowed to stir at 80 °C for 4 hr. After cooling to 25 °C, ethanol (20 mL) was added to the solution to form a precipitate which was left overnight to allow settling. The supernatant was removed and to the residue was added 40% ethanol-water (v/v) (11 mL). The resulting solution was centrifuged, the supernatant discarded and the precipitate washed with acetone, collected and dried under reduced pressure. The resulting light brown solid dried under reduced pressure to a constant mass (0.290 g, 86%).

IR: ν (cm⁻¹) 3277 (broad band, OH), 2873 (sharp band, CH aliphatic), 2099 (sharp band, azido), 1649 (sharp band, C=O stretch of secondary amide), 1022 (broad band, pyranose); Elemental Analysis (%): C, 41.88; H, 6.67; N, 16.87; Ninhydrin test: negative vs blank.

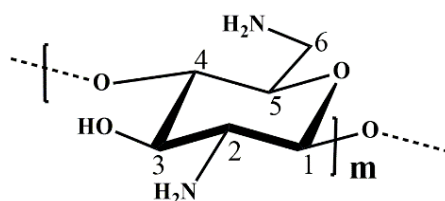
6-Deoxy 6-amino chitin (**1.12**) via azide substitution²



1.12

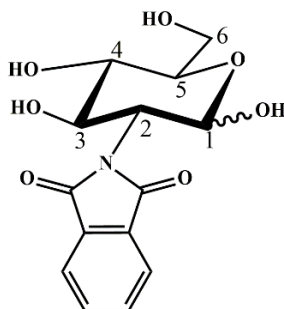
Triphenylphosphine (0.324 g, 1.23 mmol) was added to a mixture of 6-deoxy 6-azido chitin (**2.3**) (0.231 g, 1.01 mmol) in DMAc (10 mL), at room temperature and allowed to stir for 15 hr. Subsequently water (2 mL) was added and the mixture was allowed to stir overnight. Excess solvent in the reaction mixture was decanted and ethanol (10 mL) was added. The precipitate was collected by centrifugation, washed with ethanol and filtered. The product was obtained and lyophilised to yield a light brown solid (0.195 g, 95%).

IR: ν (cm⁻¹) 3273 (broad strong band, OH, NH), 2869 (sharp band, CH aliphatic), 1645 (sharp band, C=O stretch of secondary amide), 1550 (sharp, strong band, NH₂), 1022 (broad band, pyranose); Elemental Analysis (%): C, 47.39; H, 6.69; N, 8.14; Ninhydrin test: positive vs blank.

6-Deoxy-6-amino chitosan (1.15) obtained using chitin as the starting material⁴**1.15**

NaOH (45%, 40 mL) was added to 6-deoxy 6-amino chitin (**1.12**) (0.195 g, 0.964 mmol) and the mixture refluxed at 100 °C for 5 hr. Thereafter, the mixture was allowed to cool to 25 °C and the resulting solid collected under reduced pressure. The precipitate was washed with water until the washings were neutral, and lyophilised to yield 6-deoxy-6-amino chitosan (**1.15**) as a brown solid (0.0936 g, 61%).

IR: ν (cm⁻¹) 3282 (broad strong band, OH, NH), 1647, 1572 (sharp strong band, NH₂), 1022 (broad band, pyranose); Elemental Analysis (%): C, 44.24; H, 6.89; N, 9.40; Ninhydrin test: positive vs blank.

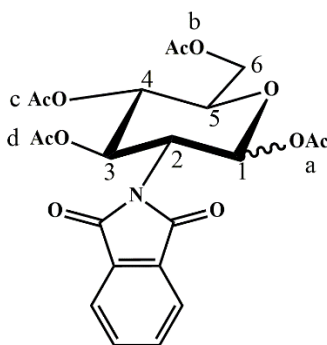
Introduction of an C-6 amine using glucosamine as a model for the polymer C-6 substitution reaction**2-Deoxy-2-phthalimido- α,β -D-glucopyranoside (4.2)⁵****4.2**

NaHCO₃ (4.28 g, 50.9 mmol) was added slowly to a solution of D-glucosamine hydrochloride (5.41 g, 25.1 mmol) and phthalic anhydride (3.76 g, 25.4 mmol) in water (12.5 mL). The solution was allowed to stir for 18 hr at 25 °C. The pH of the solution was thereafter adjusted to pH 2 using 5% HCl, resulting in a white precipitate that was collected and washed with water to produce 2-deoxy-2-phthalimido- α,β -D-glucopyranoside (**4.2**) as a white solid (3.52 g, 45%).

¹H NMR (400 MHz, DMSO-*d*₆) δ 7.96 (1H, d, *J* = 8.1 Hz, Ar-H), 7.77 – 7.72 (1H, m, Ar-H), 7.58 – 7.53 (2H, m, Ar-H), 6.34 (1H, br s, H-1), 5.08 (1H, br s, OH), 4.91 (1H, br s, OH), 4.46

(2H, br s, 2 x OH), 3.82 – 3.48 (5H, m, H-3 – H-6), 3.25 – 3.15 (1H, m, H-2); ^{13}C NMR (101 MHz, DMSO- d_6) δ 169.0 (C=O), 168.7 (C=O), 131.5 (Ar-q), 131.4 (Ar-q), 129.7 (Ar-C), 129.4 (Ar-C), 129.2 (Ar-C), 128.5 (Ar-C), 91.1 (C-1), 72.6 (C-3), 71.4 (C-4), 71.0 (C-2), 61.7 (C-5), 55.5 (C-6); IR: ν (cm^{-1}) 3253 (broad band, OH), 2877 (sharp peak, CH aliphatic), 1708 (sharp peak, imide C=O), 1615 (sharp peak, NH bending), 1557 (sharp band, C=O stretch of secondary amide) 1108 (sharp band, pyranose), 1037 (C-N vibration), 706 (sharp peak, phthaloyl-aromatic); Mp: 191–193 °C (Lit. Mp.197 – 198 °C)⁵.

2-Deoxy-2-phthalimido- α,β -D-glucopyranoside tetraacetate (**4.3**)⁵



4.3

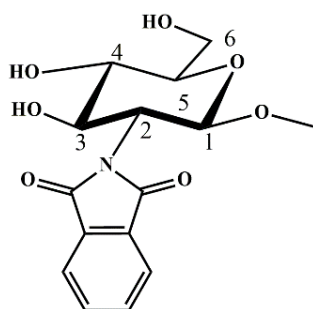
Acetic anhydride (14.3 mL, 150 mmol) was added to a solution of 2-deoxy-2-phthalimido- α,β -D-glucopyranoside (**4.2**) (5.03 g, 16.3 mmol) in pyridine (21 mL) and allowed to stir for 18 hr at 25 °C. The resulting solution was added to ice to allow precipitation of the product. The solid collected was allowed to stir in diethyl ether (30 mL) for 1 hr at 25 °C. Thereafter, the solvent was removed followed by drying *in vacuo*. The residue was allowed to stir in ethanol (20 mL) and the solid was filtered and dried to yield 2-deoxy-2-phthalimido- α,β -D-glucopyranoside tetraacetate (**4.3**) (6.66 g, 86%). A white solid was obtained as mixture of $\alpha:\beta$ anomers.

$\alpha:\beta$ Anomers: 1:0.4; ^1H NMR (400 MHz, CDCl_3) α – isomer: δ 7.87 – 7.77 (4H, m, Ar-H), 6.55 (1H, m, H-3), 6.27 (1H, d, J = 3.3 Hz, H-1), 5.15 (1H, t, J = 9.6, H-4), 4.71 (1H, dd, J = 11.6, 3.4 Hz, H-2), 4.34 (1H, m, H-6), 4.14 (2H, m, H-5, H-6'), 2.13 (3H, s, OAc-Hd), 2.10 (3H, s, OAc-Hc), 2.08 (3H, s, OAc-Hb), 1.89 (3H, s, OAc-Ha);

β – Anomer: δ 7.87 – 7.77 (4H, m, Ar-H), 6.50 (1H, d, J = 8.9 Hz, H-3), 5.87 (1H, dd, J = 10.6, 9.2 Hz, H-1), 5.20 (1H, t, J = 9.3, H-4), 4.46 (1H, dd, J = 10.6, 8.9 Hz, H-2), 4.35 (1H, dd, J = 12.1, 3.9, H-6), 4.29 (1H, m, H-6'), 4.19 – 4.14 (1H, m, H-5), 2.13 (3H, s, OAc-Hd), 2.10 (3H, s, OAc-Hc), 2.08 (3H, s, OAc-Hb), 1.89 (3H, s, OAc-Ha); ^{13}C NMR (101 MHz, CDCl_3) δ 170.7 (C=O), 169.8 (C=O), 169.5 (C=O), 169.3 (C=O), 168.6 (C=O), 167.4 (C=O),

137.8 (Ar-q), 134.5 (Ar-q), 131.6 (Ar-C), 131.2 (Ar-C), 124.4 (Ar-C), 123.7 (Ar-C), 90.6 (C-1), 70.2 (C-5), 69.5 (C-4), 67.1 (C-6), 61.6 (C-3), 52.9 (C-2), 20.9 (C-OAc), 20.7 (C-OAc), 20.6 (C-OAc), 20.3 (C-OAc); IR: ν (cm⁻¹) 1748 (C=O), 1712 (sharp peak, imide C=O), 1469 (NH bending frequencies), 1365, 1213 (C-N bending), 1031 (C-N vibration), 723 (sharp peak, phthaloyl-aromatic); Mp: 100-101 °C (Lit. Mp. 105 – 110 °C)⁵.

Methyl 2-deoxy-2-phthalimido- β -D-glucopyranoside (**4.5**)⁵



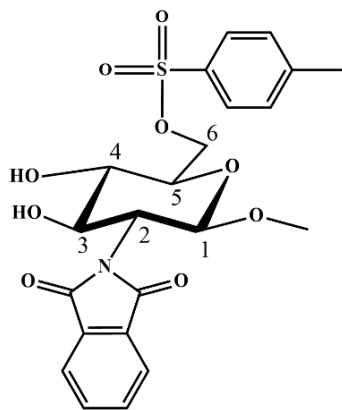
4.5

2-Deoxy-2-phthalimido- α,β -D-glucopyranoside tetraacetate (**4.3**) (3.06 g, 6.40 mmol) was added to a solution of HBr in acetic acid (33%, 12 mL) followed by the addition of acetic anhydride (1 mL) and the mixture was allowed to stir for 90 minutes at 25 °C. The reaction mixture was extracted with DCM (120 mL) and the organic layer washed with ice-water (120 mL x 3), saturated NaHCO₃ (120 mL x 2) and brine (60 mL). The organic layer was isolated and dried over anhydrous MgSO₄, followed by evaporating to give the bromo substituted sugar (**4.4**) as a brown residue. The residue was subsequently dissolved in MeOH and left to stand overnight. The solution was neutralised with a Dowex® 1X2 HCO₃⁻ ion exchange resin, and the filtrate removed *in vacuo* and washed with chloroform. The solvent was removed *in vacuo* and methyl 2-deoxy-2-phthalimido- β -D-glucopyranoside (**4.5**) was isolated as an off-white solid (1.48 g, 71%).

¹H NMR (400 MHz, CD₃OD-*d*₄) δ 7.99 – 7.85 (4H, m, Ar-H), 5.16 (1H, d, *J* = 8.5 Hz, H-1), 4.30 (1H, dd, *J* = 10.7, 8.4 Hz, H-3), 4.07 – 3.98 (2H, m, H-2, H-6), 3.82 (1H, dd, *J* = 11.9, 5.2 Hz, H-6), 3.52 – 3.46 (5H, m, OMe, H-4, H-5); ¹³C NMR (101 MHz, CD₃OD-*d*₄) δ 168.4 (C=O), 134.3 (Ar-q), 134.1 (Ar-q), 131.7 (Ar-C), 122.8 (Ar-C), 99.3 (C-1), 76.9 (C-5), 71.3 (C-3, C-4), 61.4 (C-6), 57.1 (C-2), 55.6 (OMe); IR: ν (cm⁻¹) 3313 (broad band, OH), 2940 (sharp peak, CH aliphatic), 1704 (sharp peak, imide C=O), 1563 (sharp band, C=O stretch of secondary amide), 1385 (C-H bending, C-CH₃ deformation), 1066 (sharp band, pyranose),

1018 (C-N vibration), 711 (sharp peak, phthaloyl-aromatic); Mp: 190-191 °C (Lit. Mp: 190 °C)⁵.

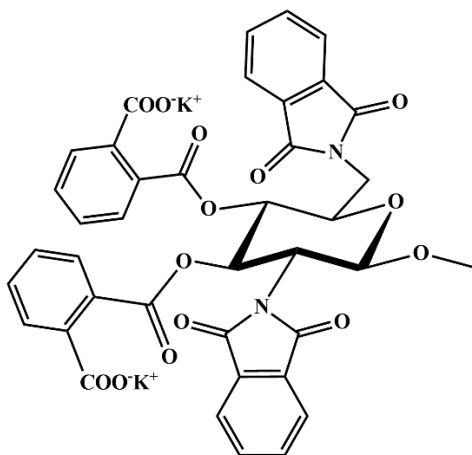
Methyl 2-deoxy-2-phthalimido-6-O-*p*-toluenesulfonyl-β-D-glucopyranoside (4.6)⁶



4.6

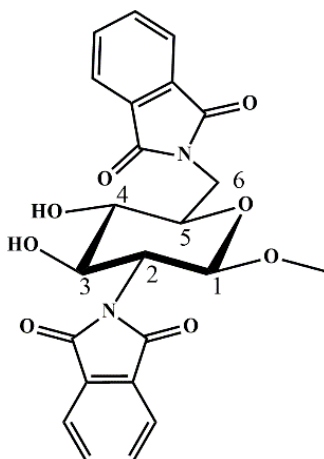
p-Toluenesulfonyl chloride (4.30 g, 22.5 mmol) was added to a solution of methyl 2-deoxy-2-phthalimido-β-D-glucopyranoside (**4.5**) (2.62 g, 8.1 mmol) in anhydrous pyridine (165 mL) at - 60 °C. The reaction mixture was allowed to stir at - 60 °C for 6 hr, after which time the mixture was poured into conc. HCl-ice (1:1) and extracted with CHCl₃ (100 mL x 3). The organic layer was successively washed with conc. HCl-ice (1:2), ice-water followed by saturated NaHCO₃. The organic layer was separated and dried over anhydrous MgSO₄ followed by removal of the solvent *in vacuo* to give an off-white solid (3.09 g, 80%).

¹H NMR (400 MHz, CDCl₃) δ 7.84 – 7.77 (4H, m, Ar-H), 7.74 – 7.69 (2H, d, *J* = 8.0 Hz Ar-H), 7.36 (2H, d, *J* = 8.0 Hz, Ar-H), 5.11 (1H, d, *J* = 8.4 Hz, H-1), 4.44 – 4.36 (2H, m, H-3, H-6), 4.29 (1H, dd, *J* = 10.6, 8.3 Hz, H-6), 4.05 (1H, dd, *J* = 10.9, 8.4, H-2), 3.67 – 3.59 (1H, m, H-5), 3.60 – 3.52 (1H, m, H-4), 3.38 (3H, s, OMe), 2.47 (3H, s, CH₃); ¹³C NMR (101 MHz, CDCl₃) δ 168.3 (2 x C=O), 145.1 (Ar-q), 134.1 (Ar-C), 132.8 (Ar-q), 131.7 (2 x Ar-q), 129.9 (2 x Ar-C), 128.0 (2 x Ar-C), 123.5 (2 x Ar-C), 99.2 (C-1), 73.5 (C-5), 71.6 (C-3), 71.4 (C-4), 68.6 (C-6), 56.7 (C-2), 56.2 (OMe), 21.6 (CH₃); IR: ν (cm⁻¹) 3472 (broad band, OH), 2912 (sharp band, CH aliphatic), 1774, 1698 (sharp band, imide C=O), 1173 (sharp band, S=O), 1082 (sharp band, pyranose), 812 (sharp band, C-O-S), 719 (sharp band, phthaloyl-aromatic); Mp: 152-155 °C (Lit. Mp (anomeric mixture): 97 – 100 °C).⁶

Methyl 3,4-diphthalate-2,6-diphthalimido- β -D-glucopyranoside (4.12)⁷**4.12**

Potassium phthalimide (0.480 g, 2.60 mmol) was added to a solution of methyl 2-deoxy-2-phthalimido-6-O-*p*-toluenesulfonyl- β -D-glucopyranoside (**4.6**) (0.750 g, 1.56 mmol) in DMAc (21 mL) and heated at 110 °C until TLC analysis indicated the complete consumption of the starting material. After 4.5 hr, water (20 mL) was added, and the solution extracted with diethyl ether (30 mL x 3). The combined organic layers were washed with water and dried over anhydrous MgSO₄. The solvent was removed *in vacuo* to produce an off-white solid, which was subsequently recrystallised from methanol to give methyl 3,4-diphthalate-2,6-diphthalimido- β -D-glucopyranoside (**4.12**) as a white solid (0.570 g, 49%).

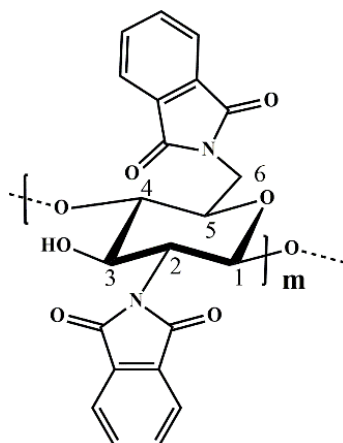
¹H NMR (400 MHz, CDCl₃) δ 7.70 – 7.93 (16H, m, Ar-H), 5.11 (1H, d, *J* = 8.4 Hz, H-1), 4.42 (1H, dd, *J* = 10.8, 8.8 Hz, H-3), 4.26 (1H, dd, *J* = 14.6, 3.6 Hz, H-6), 4.09 – 4.04 (2H, m, H-2, H-6), 3.72 (1H, dt, *J* = 9.8, 3.3 Hz, H-4), 3.41 (3H, s, OMe), 3.32 – 3.38 (1H, m, H-5); ¹³C NMR (101 MHz, CDCl₃) δ 169.1 (C=O), 168.2 (C=O), 167.8 (C=O), 134.3 (Ar-q), 134.0 (Ar-q), 132.6 (Ar-C), 131.8 (Ar-C), 123.7 (Ar-C), 123.4 (Ar-C), 99.0 (C-1), 73.8 (C-3), 72.8 (C-2), 70.3 (C-5), 56.3 (C-2), 56.1 (OMe), 37.8 (C-6); IR: ν (cm⁻¹) 3380 (broad band, OH), 2940 (sharp band, CH aliphatic), 1774, 1704 (sharp band, imide C=O), 1389 (sharp band, C-N), 1068 (sharp band, pyranose), 719 (sharp band, phthaloyl-aromatic); ESI-MS Calculated: 475.1100 [M – 2(C₈H₅O₃ + Na)]⁺ for C₃₉H₂₆K₂N₂O₁₄, Observed: 475.1114; Mp: 120 – 124 °C.

Methyl 2,6-dideoxy-2,6-diphthalimido- β -D-glucopyranoside (4.13)⁶**4.13**

Methyl 3,4-diphthalate-2,6-diphthalimido- β -D-glucopyranoside (**4.12**) (0.109 g, 0.240 mmol) and hydrazine hydrate (1 mL) was allowed to reflux in absolute ethanol (4 mL) at 95 °C. The reaction was allowed to proceed until TLC analysis indicated complete consumption of the starting material. The mixture was allowed to cool and ethanol (10 mL) was added. The solution was filtered to remove the precipitated hydrazide salt and the solvent reduced by co-evaporating with toluene to yield an off-white solid (0.0630 g, 82%).

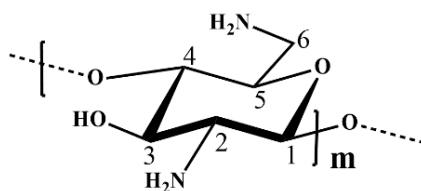
¹H NMR (400 MHz, D₂O) δ 8.25 – 7.89 (8H, m, Ar-H), 4.42 (1H, d, J = 8.3 Hz, H-1), 3.73 – 3.63 (1H, m, H-3), 3.61 (3H, s, CH₃), 3.54 (1H, dd, J = 11.0, 2.4 Hz, H-6) 3.45 – 3.50 (1H, m, H5), 3.36 – 3.43 (1H, m, H-4), 3.19 (1H, dd, J = 13.4, 9.1 Hz, H-6), 2.72 – 2.78 (1H, m, H-2); ¹³C NMR (101 MHz, D₂O) δ 169.8 (C=O), 132.7 (Ar-C), 128.9 (Ar-q), 125.7 (Ar-C), 103.4 (C-1), 74.7 (C-5), 72.1 (C-3), 71.6 (C-4), 57.4 (CH₃), 56.2 (C-2), 40.7 (C-6); IR: ν (cm⁻¹) 3170 (broad band, OH, NH), 2908 (sharp band, CH aliphatic), 1629, 1567 (sharp, strong bands, NH₂), 1371 (sharp band, C-N), 1082 (sharp band, pyranose), 706 (sharp peak, phthaloyl-aromatic); ESI-MS: m/z 444.0615 ([M – CH₄O + Na]⁺); Mp: 225 °C (decomp.).

For the computational modelling and calculations, Materials Studio 2016 version 16.1.0.21 was used. The modelling involved the use of the geometry optimisation function using ultra fine quality and the Dreiding forcefield.

6-Deoxy-2,6-bis(phthalimido) chitosan (4.17)⁷**4.17**

6-Deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) (0.188 g, 0.420 mmol) and potassium phthalimide (0.0595 g, 0.300 mmol) were suspended in DMAc (4 mL) and refluxed at 120 °C for 4.5 hr. The mixture was allowed to cool to 25 °C, water (5 mL) was then added and the resulting precipitate filtered, washed with diethyl ether (5 mL) and water (5 mL) respectively. The residue was dried under reduced pressure to obtain the polymer (0.152 g, 86%).

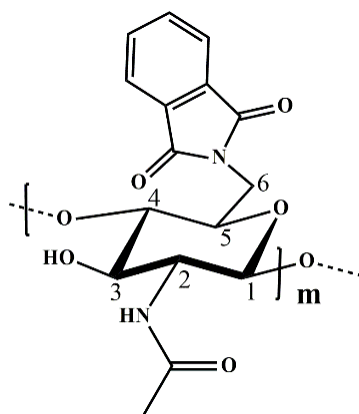
IR: ν (cm⁻¹) 3412 (broad band, OH), 2892 (sharp band, CH aliphatic), 1774, 1708 (sharp band, imide C=O), 1383 (sharp band, C-N), 1062 (broad band, pyranose), 719 (sharp band, phthaloyl-aromatic); Elemental Analysis (%): C, 50.88; H, 5.26; N, 6.02; Ninhydrin test: negative vs blank.

6-Deoxy-6-amino chitosan (1.15) via bisphthalimido chitosan**1.15**

6-Deoxy-2,6-bis(phthalimido) chitosan (**4.17**) (0.0984 g, 0.230 mmol) was allowed to stir in NMP (3 mL) followed by treatment with hydrazine monohydrate (3.00 mL, 62 mmol). The mixture was allowed to stir at 100 °C for 4 hr and was thereafter allowed to cool to 25 °C. EtOH (6 mL) was added and the resulting precipitate was collected by filtration. The polymer was obtained as a brown solid (0.0366 g, 98%).

^1H NMR (400 MHz, 2% $\text{DCl}/\text{D}_2\text{O}$) δ 7.76 – 8.09 (5H, m, residual *N*-phthaloyl), 4.35 (1H, s, H-1), 3.44 – 3.84 (5H, m, H-3 – H-6), 3.05 (1H, s, H-2), 2.46 (3H, s, acetylated units); IR: ν (cm^{-1}) 3273 (broad strong band, OH, NH), 2904 (sharp band, CH aliphatic), 1641, 1587 (sharp, strong bands, NH_2), 1034 (broad band, pyranose); Elemental Analysis (%): C, 40.71; H, 7.25; N, 12.02, Ninhydrin test: positive vs blank.

6-Deoxy-6-phthalimido chitin (4.18)⁷

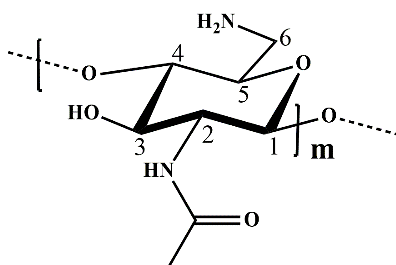


4.18

6-Deoxy-6-*p*-toluenesulfonyl chitin (2.2) (2.28 g, 6.37 mmol) and potassium phthalimide (1.06 g, 5.73 mmol) was suspended in DMAc (44 mL) and heated at 120 °C for 4.5 hr. The mixture was allowed to cool to 25 °C and water (60 mL) was added. The resulting precipitate was filtered and washed with diethyl ether (50 mL), water (50 mL) followed by drying under reduced pressure to obtain the polymer as a light brown solid (1.67 g, 79%).

Solid-state ^{13}C CPMAS NMR (5 kHz) δ 210.6 ($\text{NHC}=\text{O}$), 170.6 ($\text{C}=\text{O}$), 163.9 ($\text{C}=\text{O}$), 131.0 (Ar-q), 124.0 (Ar-C), 98.5 (C-1), 73.0 (C-3 – C-5), 57.0 (C-2), 38.7 (C-6), 21.1 (CH_3); IR: ν (cm^{-1}) 3365 (broad band, OH), 1774, 1702 (sharp band, imide $\text{C}=\text{O}$), 1387 (sharp band, C-N), 1062 (broad band, pyranose), 721 (sharp band, phthaloyl-aromatic); Elemental Analysis (%): C, 49.79; H, 5.52; N, 7.19; Ninhydrin test: negative vs the blank.

6-Deoxy-6-amino chitin (1.12) via 6-deoxy-6-phthalimido chitin ²

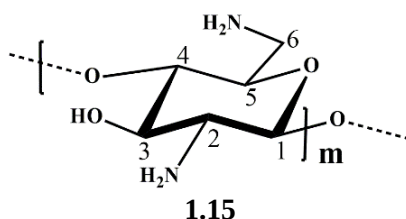


1.12

6-Deoxy-6-phthalimido chitin (**4.18**) (5.13 g, 15.5 mmol) and hydrazine monohydrate (18 mL, 372 mmol) were refluxed in absolute ethanol (120 mL) at 95 °C for 4 hr. The mixture was allowed to cool to 25 °C and EtOH (300 mL) was added. The resulting precipitate was collected by filtration. The polymer was obtained as a dark brown solid (3.10 g, 99%).

IR: ν (cm⁻¹) 3305 (broad strong band, OH, NH), 2876 (sharp band, CH aliphatic), 1647, 1585 (sharp, strong bands, NH₂ and N-acetyl), 1062 (broad band, pyranose); Elemental Analysis (%): C, 46.69; H, 6.69; N, 8.22; Ninhydrin test: positive vs the blank.

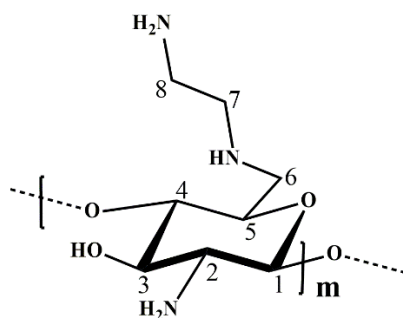
6-Deoxy-6-amino chitosan (**1.15**) via 6-deoxy-6-phthalimido chitin ⁴



NaOH (45% w/v, 40 mL) was added to 6-deoxy-6-amino chitin (**1.12**) (1.76 g, 8.69 mmol) and refluxed at 100 °C for 1 hr. Thereafter, the mixture was allowed to cool to 25 °C and the resulting solid collected under filtration. The pH of the solution was adjusted to 7 using 1 M HCl and subsequently dialysed against water for 24 hr. The contents were lyophilised to yield 6-deoxy-6-amino chitosan (**1.15**) as a brown solid (1.38 g, 99%).

¹H NMR (400 MHz, D₂O) δ 7.80 – 7.55 (5H, m, residual *N*-phthaloyl), 4.44 (1H, s, H-1), 4.18 – 3.26 (5H, m, H-3 – H-6), 2.93 (1H, s, H-2), 2.17 (3H, s, acetylated units); ¹³C NMR (101 MHz, D₂O) δ 173.5 (residual C=O), 102.1 (C-1), 78.3 (C-4), 75.1 (C-5), 72.6 (C-3), 60.1 (C-2), 22.2 (NHCH₃); IR: ν (cm⁻¹) 3345 (broad strong band, OH, NH), 2924 (sharp band, CH aliphatic), 1633, 1532 (sharp, strong bands, NH₂), 1024 (broad band, pyranose); Elemental Analysis (%): C, 40.55; H, 7.25; N, 12.52; Ninhydrin test: positive vs the blank.

6-Deoxy-6-(2-aminoethyl) amino chitosan (**4.19**)

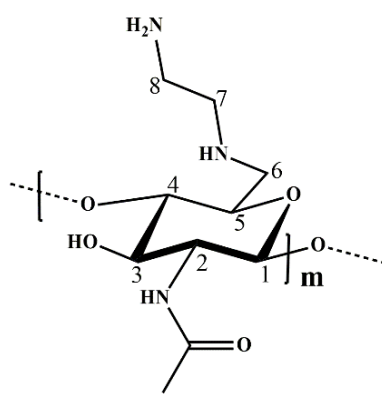


4.19

6-Deoxy-6-*p*-toluenesulfonyl *N*-phthaloyl chitosan (**1.19**) (1.16 g, 2.59 mmol) was allowed to reflux in ethylenediamine (15.0 mL, 0.300 mmol). After 6 hr the solution was allowed to cool to 25 °C and isopropanol (160 mL) added to allow for precipitation. The product was collected and further washed with ethanol (30 mL x 4). Following filtration and drying, (**4.19**) was obtained as a brown solid (0.490 g, 93%).

IR: ν (cm⁻¹) 3313 (broad strong band, OH, NH), 2869 (sharp band, CH aliphatic), 1659, 1579 (sharp strong band, NH₂), 1028 (broad band, pyranose); Elemental analysis (%): C, 44.85; H, 8.16; N, 20.29; Ninhydrin test: positive vs blank.

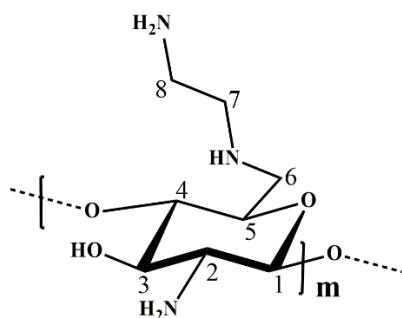
6-Deoxy-6-(2-aminoethyl) amino chitin (**1.13**)



1.13

6-Deoxy-6-*p*-toluenesulfonyl chitin (**2.2**) (0.710 g, 2.00 mmol) was refluxed in ethylenediamine (15.0 mL, 0.230 mmol). After 6 hr the solution was allowed to cool to 25 °C and isopropanol (100 mL) added to allow for precipitation. The product was collected and further washed with ethanol (20 mL x 4). Following filtration and drying under reduced pressure, 6-deoxy-6-(2-aminoethyl) amino chitin (**1.13**) was obtained as a brown solid (0.401 g, 83%).

IR: ν (cm⁻¹) 3261 (broad strong band, OH, NH), 1661 (sharp strong band, NH₂), 1555 (sharp band, C=O stretch of secondary amide), 1065 (broad band, pyranose); Elemental analysis (%): C, 42.44; H, 8.03; N, 11.85; Ninhydrin test: positive vs blank.

6-Deoxy-6-(2-aminoethyl) amino chitosan (4.19) using chitin as the starting material⁴**4.19**

A mixture of 6-deoxy-6-(2-aminoethyl) amino chitin (**1.13**) (2.41 g, 9.82 mmol) and NaOH (45% w/v, 100 mL) was allowed to reflux at 100 °C for 5 hr. The mixture was then allowed to cool to 25 °C and the resulting solid collected under filtration. The pH was adjusted to pH 7 using 1 M HCl. The resulting solid was collected under filtration while washing with H₂O and lyophilised to yield 6-deoxy-6-(2-aminoethyl) amino chitosan (**4.19**) as a brown solid (1.58 g, 79%).

¹H NMR (400 MHz, 2% DCl/D₂O) δ 4.93 (1H, s, H-1), 4.61 – 4.12 (5H, m, H-3 – H-6), 3.37 – 3.73 (1H, m, H-2), 3.33 – 2.79 (4H, m, H-7, H-8), 1.72 (3H, s, acetylated units); ¹³C NMR (101 MHz, 2 % DCl/D₂O) δ 72.7 (C-5), 64.5 (C-3), 52.0 (C-2), 48.6 (C- 6), 42.6 (C-7), 39.7 (C-8); IR: ν (cm⁻¹) 3281 (broad band, OH, NH), 2888 (sharp band, CH aliphatic), 1645, 1582 (sharp band, NH₂), 1030 (broad band, pyranose); Elemental analysis (%): C, 45.77; H, 8.81; N, 13.54; Ninhydrin test: positive vs blank.

7.3 Computational modelling for the dissolution of chitosan in alternative solvents

7.3.1 Quantum Mechanics (DFT, QTAIM and NBO)

1,4-Dimethoxyglucosamine was used in the DFT calculations due to the large size of the chitosan chains and the complexity involved. All molecular structures were fully optimised using the M06-2X functional with the def2-SVP basis set (i.e. M06-2X/def2-SVP). Vibrational frequencies were calculated at the same level of theory, to confirm that the optimised structures correspond to a true local minimum, i.e. all structures lacked imaginary vibrational frequencies. Natural bond orbitals (NBO) analysis was performed at the M06-2X/def2-SVP level of theory, to obtain the charges of the atoms in each molecular structure. Due to the anomeric effect, during optimisation the methoxy group at position C-1 of 1,4-dimethoxyglucosamine was in the axial orientation and consequently the hydrogen in an equatorial orientation, resulting in an α -conformer.⁸ To prevent this, the dihedral angle N2–C2–C1–O1 was frozen at - 60.00 ° for all DFT calculations performed.

The interaction energies (E_{int}), including the basis set superposition error (BSSE) correction using the counterpoise method, was calculated using a larger def2-TZVP basis set. When calculating the interaction energy between BMIMAc and 1,4-dimethoxyglucosamine, the cation and anion of the ionic liquid were taken as one fragment, even though 1,4-dimethoxyglucosamine interacts predominantly with the anion, as reported in previous studies.^{9, 10} This avoided a three-body problem, where the interaction between fragment 1 and fragment 2, fragment 1 and fragment 3 and fragment 2 and fragment 3 has to be computed. There is no exact solution to a three-body problem therefore the anion and cation were grouped such that only two fragments (and the interactions between them) are dealt with.

A quantum theory of atoms in molecules (QTAIM) analysis was performed, at the M06-2X/def2-TZVP level of theory. QTAIM calculations confirm the existence of hydrogen bonds by identifying bond critical points and bond paths. This method also provides the electron density associated with each hydrogen bond interaction. In order to quantify the hydrogen bond strength, scans with 20 steps of size 0.1 Å in the hydrogen-donor distance were performed on the optimised geometries of each of the following pairs:

- acetamide – methanol
- acetate – methanol
- acetate – methylamine
- acetone – methanol

- acetone – methylamine
- dimethylether – methanol
- dimethylether – methylamine
- DMSO – methanol
- ethanol – methylamine
- methanamide – methanol
- trimethylamine – methylamine

These specific pairs were chosen as they were considered to be the smallest fragments that could be used to model the hydrogen bonds, such that the values obtained would minimise the role of additional components (e.g. overall dispersion, electrostatics and induction), all of which increase with system size. Interaction energies and electron density at the bond critical point (BCP $\rho(r)$) were extracted at each scan point for each pair and a calibration plot (plot of interaction energy against BCP $\rho(r)$) plotted for each pair. A third degree polynomial was fit to the data points of each calibration plot and the BCP $\rho(r)$ obtained from QTAIM analysis for each hydrogen bond formed between 1,4-dimethoxyglucosamine. Each solvent molecule was then substituted into the equation of the appropriate calibration curve to determine the corresponding interaction energy of that specific hydrogen bond. Table 7.1 gives the hydrogen bond which were modelled. The QTAIM analysis was done using the AIMAll software.¹¹

Table 7.1: The hydrogen bonds studied and their corresponding calibration pair.

Studied pair	Hydrogen bond	Calibration pair
1,4-dimethoxyglucosamine – Ionic liquid	O1 _{AC} ...H3–O3 O2 _{AC} ...H2–N2	acetate – methanol acetate – methylamine
1,4-dimethoxyglucosamine – Cyrene [®] (orientation I) ^a	O1 _{Cyr} ...H3–O3 O3 _{Cyr} ...H21–N19	dimethylether – methanol acetone – methylamine
1,4-dimethoxyglucosamine – Cyrene [®] (orientation II)	O3 _{Cyr} ...H3–O3 O1 _{Cyr} ...H2–N2	acetone – methanol dimethylether – methylamine
1,4-dimethoxyglucosamine – Cyrene [®] (orientation III)	O3 _{Cyr} ...H3–O3 O1 _{Cyr} ...H2–N2 N2...H1 _{Cyr} –C1 _{Cyr}	acetone – methanol dimethylether – methylamine ethanol + methylamine
1,4-dimethoxyglucosamine – DMF	O _{DMF} ...H3–O3	Methanamide (formaldehyde) – methanol
1,4-dimethoxyglucosamine – DMSO	O _{DMSO} ...H3–O3	DMSO – methanol
1,4-dimethoxyglucosamine – NMP	O _{NMP} ...H3–O3 N2...H1 _{NMP} –C1 _{NMP}	Ethanamide (acetamide) – methanol trimethylamine – methylamine

^aThe 1,4-dimethoxyglucosamine + cyrene[®] pair was examined in three orientations.

Natural bond orbitals (NBO) analysis was performed at the M06-2X/def2-SVP level of theory and from this data the charges of the individual atoms in a molecule and the second order perturbation stabilisation energies were obtained. The NBO 3.1¹² routines included with Gaussian 09 was used. All the remaining quantum mechanical computations were carried out with the Gaussian 09 package.¹³

7.3.2 Molecular dynamics (MD)

DL_POLY version 2.20¹⁴ was used to perform molecular dynamics simulations. Simulations were performed in periodic cubic boxes consisting of one 1,4'-dimethoxychitobiose, centred in the cubic boxes containing (a) 150 BMIMAc ion pairs (150 1-buty-3-methylimidazolium cations and 150 acetate anions) (b) 400 CyreneTM (c) 600 DMF, (d) 500 DMSO, and (e) 400 NMP solvent molecules. The number of molecules/ions were chosen so as to give a simulation box that was approximately 40×40×40 Å³ in size, in each case. The final box sizes of the equilibrated cubic solvent boxes are reported in Table 7.2.

Table 7.2: Composition and dimensions of the different solvent boxes.

Solvent box	Solvent	Length of sides of box (Å)
a	150 BMIMAc	36.12
b	400 Cyrene [®]	40.98
c	600 DMF	43.12
d	500 DMSO	39.08
e	400 NMP	40.43

CL&P¹⁵ force field parameters available in literature were used for BMIMAc, and parameters for DMF, DMSO, NMP, Cyrene[®] and 1,4'-dimethoxyglucosamine were generated from OPLS-2005.¹⁶

A pre-equilibration run, consisting of 5 simulations of 100 ps at high temperature and pressure, was performed. This was done to overcome high energy barriers in the initial, randomly generated starting configurations of slightly lower densities than the target values. This was accomplished using the PACKMOL software¹⁷, and to accelerate the approach to equilibrium. These simulations were started at a temperature of 700 K and a pressure of 10.0 kbar which was gradually decreased to 300 K and 1 bar. This was followed by an equilibration run of 1.5 ns in the NPT (isothermal-isobaric) ensemble with the temperature set to 300 K and the pressure to 1 bar. A 1 ns production run in the NVT (canonical) ensemble was then performed at a temperature of 300 K, with the box size taken from the average over the final 1 ns of the NPT simulation. Configurations were saved every 1 ps.

For all simulations performed, the cut-off distance for the van der Waals interactions was set to 12 Å and the Ewald summation method, with an accuracy of 10⁻⁶ was used for the electrostatic interactions. A time step of 1 fs was used to integrate the equations of motion. All covalently linked hydrogen bonds were kept fixed using the SHAKE algorithm.¹⁸

The VMD package¹⁹ was utilised for visualisation of the solvent boxes.

7.3.3 General procedure used for the dissolution tests based on the computational analysis

Chitosan (0.0440 g, 0.274 mmol) was allowed to stir in either [BMIMAc] (4.00 mL, 22.0 mmol), Cyrene[®] (1.25 mL, 7.8 mmol), DMF (1.62 mL, 22.0 mmol), DMSO (1.58 mL, 22.0 mmol), and NMP (2.12 mL, 22.0 mmol). DMAc/LiCl (3.04 mL, 22.0 mmol) was used as a control for comparative solubility purposes. The mixtures were allowed to stir at 60 °C for 2

hr and thereafter visually assessed. The samples were centrifuged at 3500 rpm for 5 min and the supernatant withdrawn and weighed.

7.4. Potential replacement of DMAc as the solvent for the tosylation of chitin

General method

The desired solvent (2.5 mL) and chitin (0.050 g, 0.247 mmol) were combined with LiCl (0.130 g, 3.07 mmol) for 24 hr with shaking. Thereafter solubility was assessed visually. The solvents used in this study are given in Table 7.3. These include solvents from the following classes: polar aprotics, hydrocarbons, medium polarity solvents and polar protic solvents.

Table 7.3: Selected solvents used for dissolution studies of chitin.

Polar aprotics		Hydrocarbons	Medium polarity
Acetone	DMSO	Cyclohexane	DCM
Acetonitrile	γ -Valerolactone	<i>p</i> -Cymene	1,3-Dioxolane
Cyclopentanone	NMP	Toluene	Ethyl acetate
Cyrene TM	Propylene carbonate		THF
Dimethyl carbamate	Sulfolane		
DMAc	Tamisolve [®]		
DMF			

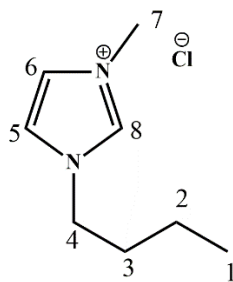
From the solvents tested, four (CyreneTM, DMAc, Sulfolane and Tamisolve[®]) were used in the reaction of chitin with tosyl chloride. The procedure used was that for the tosylation of chitin as reported by Zou *et al.* with some modifications.³

Chitin (0.250 g, 1.23 mmol) and LiCl (0.650 g, 15.3 mmol) were added to the desired solvent (13 mL) and the mixture was allowed to stir at room temperature for 12 hr. The reaction mixture was allowed to cool to 0 °C, followed by the addition of Et₃N (3.60 mL, 26.0 mmol) and a solution of *p*-toluenesulfonyl chloride (2.50 g, 13.1 mmol) in the selected solvent (5 mL). The reaction was allowed to stir at 0 °C for a further 24 hr. The mixture was then allowed to precipitate by addition of acetone (50 mL). The resulting residue was washed with methanol (75 mL), water (50 mL) and acetone (50 mL). The isolated solids were lyophilised and characterised by IR spectroscopy.

Potential replacement of LiCl as the co-salt for the tosylation of chitin

In the reaction of tosyl chloride and chitin, LiCl was substituted with NaCl, 1-butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium acetate respectively. The same procedure was followed as for the synthesis of 6-deoxy-6-*p*-toluenesulfonyl chitin (**2.2**), where chitin in DMAc together with the salt was treated with tosyl chloride in the presence of triethylamine.

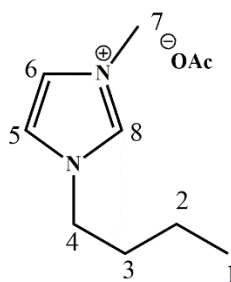
1-Butyl-3-methylimidazolium chloride (BMIMCl) (**3.7**)^{20, 21}



3.7

1-Chlorobutane (90.0 mL, 0.860 mol) and 1-methylimidazole (117 mL, 1.47 mol) were heated at 100 °C for 7 days. The progress of the reaction was monitored using a colorimetric method based on the determination of 1-methylimidazole with copper(II) chloride in ethanol. A yellow coloured solution indicates the completion of the reaction.²¹ Upon completion of the reaction, the top layer containing unreacted starting material was decanted followed by the addition of ethyl acetate (90 mL). The bottom layer was decanted when the 2 solutions separated. This step was repeated twice to ensure that any unreacted starting material was removed. The mixture was reduced *in vacuo* to remove any residual ethyl acetate and dried under vacuum to produce BMIMCl (**3.7**) as an off-white hygroscopic solid (129 g, 86%).

¹H NMR (400 MHz, CDCl₃) δ 10.6 (1H, s, H-8), 7.65 (1H, t, *J* = 1.8 Hz, H-5), 7.48 (1H, t, *J* = 1.8 Hz, H-6), 4.25 (2H, t, *J* = 7.4 Hz, H-4), 4.04 (3H, s, H-7), 1.88 – 1.76 (2H, m, H-3), 1.36 – 1.22 (2H, m, H-2), 0.86 (3H, t, *J* = 7.4 Hz, H-1); ¹³C NMR (101 MHz, CDCl₃) δ 136.1 (C-8), 123.1 (C-5), 121.4 (C-6), 48.6 (C-4), 35.6 (C-7), 31.2 (C-3), 18.4 (C-2), 12.5 (C-1); IR: ν (cm⁻¹) 3092, 2969 (sharp strong band, CH aliphatic), 1560, 1466 (strong bands, C=N), 1337 (sharp band, -C-CH₃), 1163 (sharp strong band, C-N), 898, 790 (sharp strong band, C-H); Mp: 67 – 70 °C (Lit. mp: 65 °C)²².

1-Butyl-3-methylimidazolium acetate (BMIMAc) (3.3)²³**3.3**

1-Butyl-3-methylimidazolium chloride (**3.7**) (1.01 g, 5.80 mmol) and potassium acetate (0.635 g, 6.50 mmol) were allowed to stir in ethanol (3.14 mL) at 40 °C for 6 hr. The solution was filtered and the solvent removed *in vacuo*. The resulting liquid was stored at - 10 °C for 24 hr to further allow precipitation of the potassium acetate and sodium chloride. The precipitate was filtered off and washed with acetone. The solvent was removed *in vacuo* and 1-butyl-3-methylimidazolium acetate (**3.3**) was isolated as a pale yellow oil (0.920 g, 80%).

¹H NMR(400 MHz, CDCl₃) δ 10.2 (1H, s, H-8), 7.42 (1H, t, *J* = 1.6 Hz, H-5), 7.30 (1H, t, *J* = 1.7 Hz, H-6), 4.15 (2H, t, *J* = 7.3 Hz, H-4), 3.90 (3H, s, H-7), 1.75 (3H, s, OAc), 1.73 – 1.66 (2H, m, H-3), 1.30 – 1.15 (2H, m, H-2), 0.81 (3H, t, *J* = 7.4 Hz, H-1); ¹³C NMR (101 MHz, CDCl₃) δ 177.4 (C=O), 138.4 (C-8), 123.5 (C-5), 121.7 (C-6), 49.3 (C-4), 36.1 (C-7), 32.0 (C-3), 25.0 (CH₃CO), 19.3 (C-2), 13.3 (C-1); IR: ν (cm⁻¹) 3146 (broad band, C=C), 2956 (sharp strong band, CH aliphatic), 1559, 1466 (strong bands, C=N), 1391 (sharp band, -C-CH₃), 1167 (sharp strong band, C-N), 879, 750 (sharp strong band, C-H).

7.5. Production of oligosaccharides through acid hydrolysis

General hydrolysis procedure²⁴

Chitosan and 6-deoxy-6-amino chitosan (**1.15**) were added to conc. HCl (32%) separately and heated to 70 °C for 2.5 hr. Thereafter the reaction mixture was allowed to cool to 25 °C and washed with cold acetone (10 mL x 3). The reaction mixture was centrifuged and the pellet washed 3 times with cold acetone (10 mL x 3). The solids obtained, were then dissolved in water (5 mL) and the pH of the solution adjusted with 5 M NaOH to pH 7. Thereafter the samples were lyophilised to give the acid hydrolysis products as solids.

Chitosan hydrolysis (5.2)

Chitosan (0.235 g, 1.46 mmol) and conc. HCl (32%, 4 mL) gave the acid hydrolysis product as a brown solid (0.237 g, 90%).

¹H NMR (400 MHz, D₂O) δ 5.54 – 5.50 (1H, m, H-1'), 5.05 – 4.94 (1H, m, H-1), 4.10 – 3.71 (5H, m, H-3 – H-6), 3.32 – 3.16 (1H, m, H-2); IR: ν (cm⁻¹) 3325 (broad strong band, OH, NH), 2884 (sharp band, CH aliphatic), 1623, 1522 (sharp, strong bands, NH₂), 1064 (broad band, pyranose); Ninhydrin test: positive vs blank.

6-Deoxy-6-amino chitosan hydrolysis (5.3)

6-Deoxy-6-amino chitosan (0.114 g, 0.650 mmol) and conc. HCl (32%, 2 mL) gave 6-deoxy-6-amino chitosan hydrolysis product as a light brown solid (0.103 g, 93%).

¹H NMR (400 MHz, D₂O) δ 4.05 – 3.51 (5H, m, H-3 – H-6), 3.13 – 2.97 (1H, m, H-2); IR: ν (cm⁻¹) 3301 (broad strong band, OH, NH), 2908 (sharp band, CH aliphatic), 1627, 1514 (sharp, strong bands, NH₂), 1060 (broad band, pyranose); Ninhydrin test: positive vs blank.

7.6. Electrospinning

Preparation of polymer solutions

A poly(ethylene oxide) (5.1, PEO) (MW 600,000) solution was prepared by dissolving PEO (0.100 g, 1.67×10^{-4} mmol) in acetic acid (0.5 M, 3.5 mL) with vigorous stirring. Separately a chitosan/6-deoxy-6-amino chitosan solution was prepared by dissolving the polymer (1.8 g, 11.2 mmol) in acetic acid (0.5 M, 3.5 mL). Once all solids had dissolved, the polymers were combined in a 1:1:1 weight ratio, chitosan/diamino:PEO:0.5 M AA, and stirred for 2 hr.

Preparation of fibre mats

The above solution was employed as a spinning solgel for electrospinning using a digital electrospinning apparatus EC-DIG (IME Technologies, Eindhoven, Netherlands). The electrospinning was performed at room temperature, and a relative humidity of 50%. The solution was placed into a plastic syringe (3 mL) and forced through a blunt needle with a diameter of 0.4 mm at a flowrate of $165 \mu\text{L} \cdot \text{hr}^{-1}$. The applied voltage was fixed at 15 kV and the tip-to-collector distance was fixed at 20 cm. The nanofibrous mats were collected on the surface of aluminium foil fixed on the grounded copper current collector and dried at room temperature for 24 hr. Fibres were also spun onto cellulose filter paper (25 mm, $0.25 \mu\text{m}$ pore size).

IR: ν (cm^{-1}) 3325 (broad strong band, OH, NH), 2924 (sharp band, CH aliphatic), 1651, 1560 (sharp, strong bands, NH_2), 1465 (sharp band, asymmetric and symmetric CH_2 deformation), 1342 (sharp band, CH_2 wagging), 1278, 1239 (sharp bands, asymm. and symm. CH_2), 1098 (broad band, pyranose), 963 (sharp band, asymmetric CH_2 rocking, CH_2 and C-C stretch), 840 (sharp band, rocking CH_2 , C-O-C deformation).

Cross-linking of the electrospun fibre mats²⁵

Nanofibre mats were placed in an empty vapour chamber at room temperature in a well ventilated hood. The nanofibres were not removed from the foil or filter paper. A glutaraldehyde solution (4 mL, 25%) was placed in the base of the chamber and evenly dispersed. A vacuum was applied and the fibres were crosslinked by vapour deposition for 2 hr.

IR: ν (cm^{-1}) 3361 (broad strong band, OH, NH), 2873 (sharp band, CH aliphatic), 1639, (sharp, strong band, NH_2), 1587 (sharp band, C=N), 1462 (sharp band, asymmetric and symmetric CH_2 deformation), 1340 (sharp band, CH_2 wagging), 1278, 1242 (sharp bands, asymmetric and

symmetric CH₂), 1102 (broad band, pyranose), 963 (sharp band, C-C stretch), 836 (sharp band, rocking CH₂, C-O-C deformation).

Silver loading of the electrospun fibre mats

The synthesis of silver nanoparticles was carried according to the procedure reported by *Wei et al.*²⁶ Silver nitrate was reduced using chitosan or 6-deoxy-6-amino chitosan as the reducing agent. In a typical preparation of these nanoparticles, a silver nitrate solution (52 mM) was stirred together with the polymer until a homogenous solution was obtained. Thereafter, the solution was incubated at 95 °C overnight in the case of chitosan and at room temperature for 6-deoxy-6-amino chitosan. The presence of silver nanoparticles was confirmed by UV analysis of the solution and TEM visualisation of the prepared particles. These nanoparticles were applied to the prepared NFs and thereafter allowed to dry.

Evaluation of the nanofibres and the silver loaded nanofibres as antimicrobial agents

The antibacterial activity of the silver-embedded nanofibres was examined by inhibition zone method. Polymer:PEO nanofibres without Ag-NPs were tested as a control. The Gram-negative bacteria *E. coli* was used as model strain. Sterilised nutrient agar medium was used for bacteria growth. To evaluate the antibacterial activity, the *E. coli* bacteria culture with concentration of 10⁵ colony forming unit per ml (CFU/ml) was spread onto the agar plate. The materials both chitosan and 6-deoxy-6-amino chitosan nanofibres with and without silver were applied as 25mm² sections on the inoculated Petri dishes in duplicate. The clear zone of inhibition was assessed after incubation at 37 °C for 24 hr.

7.7. 6-Deoxy-6-amino chitosan as an antifungal agent in crop protection

The fungus, *Fusarium verticillioides* (*F. verticillioides*) was supplied by the Molecular and Cell Biology Department at the University of Cape Town. *F. verticillioides* MRC826 strain (Gelderblom et al. 1988), obtained from Pannar Seed (Pty) Ltd., South Africa was streaked from 30% glycerol stocks (stored at -80 °C) onto potato dextrose agar plates (9.75 g potato dextrose agar, 250 mL deionised water) and placed in a 30 °C incubator for 14 days. At the end point, the fungus reached a purple stage of growth (sexual stage).

Effects of chitosan and 6-deoxy-6-amino chitosan on mycelial radial growth

Fungicidal activity was determined by a radial hyphal growth bioassay as reported by *Al-Hetar et al.*²⁷ Initially a 5% chitosan solution was made up in 1% acetic acid and heated to 40 – 60 °C, with stirring for 24 hr. Separately, **1.15** was dissolved in water with stirring and sonication to give a 10% solution. A 3.9% solution of potato dextrose agar (PDA) was made by dissolving PDA (9.75 g) in water (250 mL). The chitosan, 6-deoxy-6-amino chitosan and PDA solutions were autoclaved separately at 120 °C for 15 min. The final polymer concentrations tested, are given in Table 7.4.

Table 7.4: Polymer concentrations (w/v) tested for antifungal activity.

Chitosan (%)	6-deoxy-6-amino chitosan (%)
0.5	1
0.1	0.5
0.05	0.1
0.1	0.05
	0.01

These final concentrations were achieved by diluting the stock polymer solutions with the PDA solution and pouring into sterile 35 mm petri dishes. Control plates consisted of PDA only, a 0.1% acetic acid control and a water control. A 10 mm diameter plug from the advancing margins of a 14 day old colony of *F. verticillioides* on PDA was seeded centrally onto the prepared plates. Testing was done in triplicate for all concentrations evaluated. All plates were incubated at 30 °C in completely randomised design (CRD). The radial colony growth was measured daily using ImageJ software²⁸, until the leading edge of the fastest growing colony had reached the edge of the plate. The percentage of inhibition was calculated based on percentage inhibition of radial growth (PIRG%) as follows:

$$PIRG\% = \frac{R1 - R2}{R1} \times 100\%$$

where, R1 = radial growth of *F. verticillioides* on the control plate, and R2 = radial growth of *F. verticillioides* on the treated plate.

7.8. Edible food coating testing protocol²⁹

Strawberries (*Fragaria ananassa*) were purchased from commercial sources. The strawberries were selected for uniform size and colour and free from physical damage. Strawberries were randomly distributed into 3 groups.

Two groups were assigned to the 2 treatments (spraying and dipping) while the third group provided as the untreated control (Table 7.5). The solutions tested included:

- chitosan solution (1% w/v) in 1% acetic acid
- 6-deoxy-6-amino chitosan (1% w/v) in H₂O

Method

The dipping treatment consisted of immersing fruits for 1 minute at room temperature in the chitosan solution and thereafter the strawberries were allowed to air dry. In the spraying treatment the strawberries were sprayed with 2 mL of the polymer solution. The strawberries were stored at room temperature. Testing was done in triplicate and the strawberries monitored visually for the appearance of fungal growth.

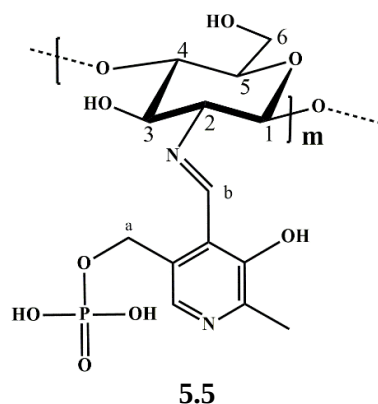
Table 7.5: The samples groups and treatment protocols used in the edible food coating experiments.

Application method	Storage conditions	Untreated (control-water)*	Untreated (control- 1% acetic acid)*	Treated (chitosan)*	Treated (6-deoxy-6-amino chitosan)*
Spraying	RT	1	1	3	3
Dipping	RT	1	1	3	3

* refers to the number of strawberries in that particular test group

7.9 Pyridoxal phosphate immobilised polymers and their application as polymer supports in the conversion of amino acids to α -keto acids

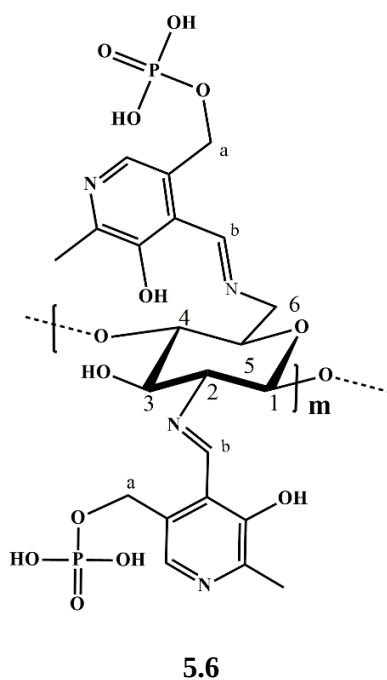
Chitosan – PLP – Cu (5.5)³⁰



CuSO₄ (0.323 g, 2.02 mmol) and PLP (0.504 g, 1.90 mmol) were added to a solution of chitosan (1.02 g, 6.33 mmol) in acetic acid (1%, 100 mL). The pH was adjusted to between pH 6 and pH 7 using 1% aq. sodium triphosphate. The mixture was allowed to stir for 18 hr at 25 °C. Thereafter the mixture was centrifuged and the supernatant discarded. The pellet was washed with MeOH (80 mL) and filtered to produce a green solid (1.66 g, 47%).

IR: ν (cm⁻¹) 3234 (strong broad band, OH), 2916 (sharp band, CH aliphatic), 1629 (sharp band, C=O stretch of secondary amide), 1544 (sharp band, C=N), 1377 (sharp band, PLP – CH₃), 1308 (P=O), 1058 (broad band, pyranose), 969 (sharp band, P-O-C).

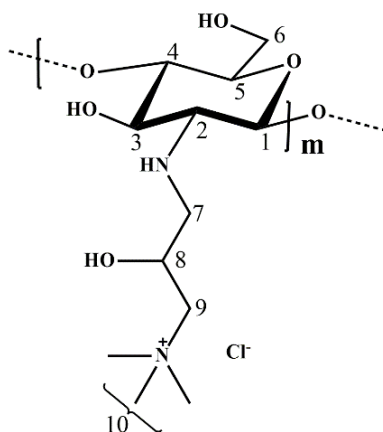
6-Deoxy-6-amino chitosan – PLP Schiff base (5.6)³¹



6-Deoxy-6-amino chitosan (0.70 g, 4.39 mmol) was allowed to stir in an ammonium acetate buffer (0.07 mM, 21 mL, pH 9) followed by the portion-wise addition of PLP (0.363 g, 1.38 mmol). The reaction mixture was allowed to stir for 18 hr at 40 °C and thereafter dialysed against water, changing the dialysis buffer regularly until no visible release of unbound PLP (yellow colour) was observed. The solution was centrifuged and the pellet dried to produce a brown solid (0.740 g, 29%).

^1H NMR (400 MHz, 2% DCl/D₂O) δ 9.72 (s, H-b), 7.65 – 7.39 (m, Ar-H), 5.74 (2H, s, H-a), 4.61 – 4.50 (1H, m, H-1), 4.03 – 2.92 (5H, m, H-3 – H-6), 2.77 (1H, s, H-2), 2.05 – 1.86 (3H, m, CH₃), 1.32 (s, acetylated units); ^{31}P NMR (81 MHz, 2% DCl/D₂O) δ - 0.57, - 1.35; IR: ν (cm⁻¹) 3222 (strong broad band, OH), 2912 (sharp band, CH aliphatic), 1631 (sharp band, C=O stretch of secondary amide), 1532 (sharp band, C=N), 1375 (sharp band, PLP – CH₃), 1304 (P=O), 1060 (broad band, pyranose), 1026 (broad band, P-OH), 967 (sharp band, P-O-C).

3-Trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (Q188) (5.8)³²

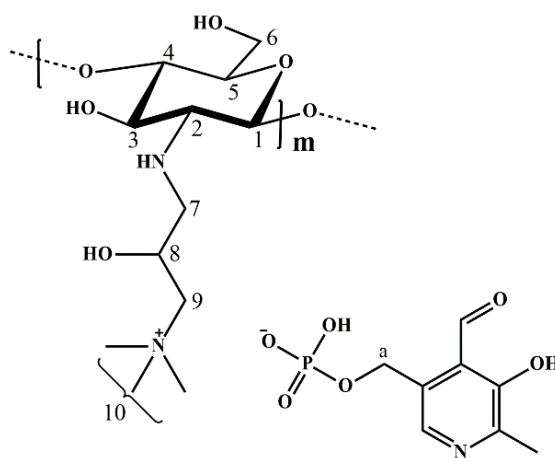


5.8

Chitosan (5.08 g, 31.7 mmol) was suspended in H₂O (100 mL), followed by the addition of glycidyl trimethylammonium chloride (11.4 g, 75.2 mmol) in 3 portions over a period of 8 hr. The reaction mixture was allowed to stir at 60 °C for 24 hr. Once cooled, the solution was adjusted to pH 5 using conc. 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 polymer was precipitated in 4:1 acetone:EtOH (250 mL) and the resulting precipitate filtered, washed with EtOH and dried under vacuum to afford (5.8) as an off white solid (7.38 g, 75%).

^1H NMR (400 MHz, D_2O) δ 5.20 (1H, s, H-1), 4.71 – 4.61 (1H, m, H-8), 4.37 – 3.92 (5H, m, H-3 – H-6), 3.75 (1H, s, H-2), 3.60 (9H, s, H-10), 3.39 (2H, m, H-9), 2.91 (2H, s, H-7), 2.00 (s, acetylated units); ^{13}C NMR (101 MHz, D_2O) δ 75.1 (C-5), 73.5 (C-4), 69.6 (C-3), 65.8 (C-9), 61.1 (C-8), 60.8 (C-6), 54.8 (C-2), 52.3 (C-10); IR: ν (cm^{-1}) 3305 (strong broad band, OH), 2928 (sharp band, CH aliphatic), 1651 (sharp band, C=O stretch of secondary amide), 1478 (sharp band, C-H bending of trimethyl ammonium group), 1054 (broad band, pyranose); Elemental Analysis (%): C, 37.90; H, 7.07; N, 6.52.

3-Trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride – PLP electrostatic complex (5.7)



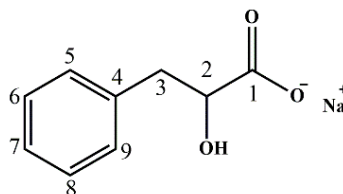
5.7

3-Trimethylammonium-2-hydroxypropyl-*N*-chitosan chloride (1.10 g, 3.56 mmol) was allowed to stir in an ammonium acetate buffer (0.07 mM, 33 mL, pH 9) followed by the portion-wise addition of PLP (0.503 g, 1.90 mmol). The reaction mixture was allowed to stir for 18 hr at 25 °C and the solid isolated and dialysed against water, changing the dialysis buffer regularly until no visible release of unbound PLP (yellow colour) was observed. The solution was centrifuged and the pellet dried to produce an orange solid (5.7) (1.62 g, 50%).

^1H NMR (400 MHz, 2% $\text{DCl}/\text{D}_2\text{O}$) δ 7.92 – 7.62 (1H, m, Ar-H), 4.58 (1H, s, H-1), 4.48 – 4.38 (1H, m, H-8), 4.18 (2H, s, H-a), 3.74 – 3.20 (5H, m, H-3 – H-6), 3.05 (1H, s, H-2), 2.81 – 2.96 (2H, m, H-9), 2.78 (1H, m, H-7), 2.73 (9H, s, H-10), 2.25 (3H, s, CH_3), 2.19 (s, acetylated units); ^{31}P NMR (81 MHz, 2% $\text{DCl}/\text{D}_2\text{O}$) δ - 0.54, - 1.40; IR: ν (cm^{-1}) 3266 (strong broad band, OH), 2928 (sharp band, CH aliphatic), 1629 (sharp band, C=O stretch of secondary amide), 1476 (sharp band, C-H bending of trimethyl ammonium group), 1401 (sharp band, PLP – CH_3), 1294 (P=O), 1064 (broad band, pyranose), 1026 (broad band, P-OH), 967 (sharp band, P-O-C).

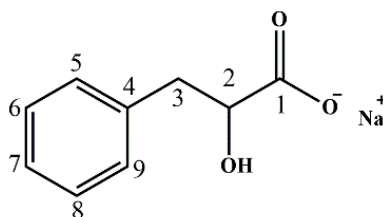
Non-enzymatic PLP – mediated conversion of amino acids to α -keto acids^{33, 34}

L-Phenylalanine was converted to phenylpyruvate (**5.10**) in phosphate buffered saline (PBS) (0.1 M, pH 7) consisting of NaCl (80 g), KCl (2 g), Na₂HPO₄·7H₂O (21.7 g) and KH₂PO₄ (2.5 g), diluted to 1 L and the pH adjusted using 1 M HCl. PLP-mediated conversion of L-phenylalanine was performed to measure the standard conversion with free PLP versus the polymer bound PLP.

Standard conversion, polymer free**5.11***PLP – Cu – Phenylalanine*

To phosphate buffer (35 mL), was added PLP (10 mM, 0.103 g, 0.408 mmol), CuSO₄ (0.0445 g, 0.279 mmol) and L-phenylalanine (0.0382 g, 0.229 mmol). The reaction mixture was allowed to stir at 37 °C for 24 hr and thereafter allowed to cool to 25 °C. Thereafter, NaBH₄ (0.0112 g, 0.283 mmol) was added *in situ* at 50 °C and the solution stirred for 10 min. The solution was placed in ice and the pH adjusted to below 2 using 2 N HCl, followed by saturation with solid NaCl. The solution was extracted with EtOAc (50 mL x 3) and the combined organic layers were dried over anhydrous Na₂SO₄. The solvent was removed *in vacuo* to yield an off-white solid (0.00482 g, 11%).

¹H NMR (400 MHz, D₂O) δ 7.37 – 7.45 (5H, m, Ar-H), 4.58 (1H, dd, J = 7.5, 4.6 Hz, H-2), 3.24 (1H, dd, J = 14.0, 4.6 Hz, H-3), 3.06 (1H, dd, J = 14.0, 7.5 Hz, H-3); ¹³C NMR (101 MHz, D₂O) δ 177.2 (C-1), 136.9 (C-4), 129.5 (C-6, C-8), 128.7 (C-5, C-9), 127.1 (C-7), 71.3 (C-2), 39.6 (C-3).

Polymer bound conversion**5.11**

Chitosan – PLP – Cu – Phenylalanine

To phosphate buffer (35 mL), was added chitosan – PLP – Cu (0.199 g, 0.351 mmol), and L-phenylalanine (0.0583 g, 0.353 mmol). The reaction mixture was allowed to stir at 37 °C for 24 hr and thereafter allowed to cool to 25 °C. The mixture was centrifuged at 3 500 rpm for 5 min and the polymer pellet removed. Thereafter, NaBH₄ (0.0160 g, 0.423 mmol) was added at 50 °C and the solution stirred for 10 min. The solution was placed on ice and the pH adjusted to below 2 using 2 N HCl, followed by saturation with solid NaCl. The aqueous solution was extracted with EtOAc (50 mL x 3) and the combined organic layers were dried over anhydrous Na₂SO₄. The solvent was removed *in vacuo* to yield an off-white solid (0.0138 g, 21%).

¹H NMR (400 MHz, D₂O) δ 7.48 – 7.37 (5H, m, Ar-H), 4.64 (1H, dd, *J* = 7.6, 4.8 Hz, H-2), 3.26 (1H, dd, *J* = 14.1, 4.8 Hz, H-3), 3.10 (1H, dd, *J* = 14.1, 7.6 Hz, H-3').

6-Deoxy-6-amino chitosan – PLP – Cu – Phenylalanine

To phosphate buffer (35 mL), was added 6-deoxy-6-amino chitosan – PLP (0.2047 g, 0.349 mmol), CuSO₄ (0.0530 g, 0.332 mmol) and L-phenylalanine (0.0538 g, 0.326 mmol). The reaction mixture was allowed to stir at 37 °C for 24 hr and thereafter allowed to cool to 25 °C. The mixture was centrifuged at 3 500 rpm for 5 min and the polymer pellet separated. Thereafter, NaBH₄ (0.0211 g, 0.558 mmol) was added at 50 °C and the solution stirred for 10 min. The solution was placed on ice and the pH adjusted to below 2 using 2 N HCl, followed by saturation with solid NaCl. The aqueous solution was extracted with EtOAc (50 mL x 3) and the combined organic layers were dried over anhydrous Na₂SO₄. The solvent was reduced *in vacuo* to yield an off-white solid (0.0145 g, 24%).

¹H NMR (400 MHz, D₂O) δ 7.42 – 7.37 (5H, m, Ar-H), 4.60 (1H, dd, *J* = 7.6, 4.8 Hz, H-2), 3.23 (1H, dd, *J* = 14.1, 4.8 Hz, H-3), 3.06 (1H, dd, *J* = 14.1, 7.6 Hz, H-3').

3-Trimethylammonium-2-hydroxypropyl-N-chitosan chloride – PLP – Cu – Phenylalanine

To phosphate buffer (35 mL), was added 3-trimethylammonium-2-hydroxypropyl-N-chitosan chloride – PLP (0.193 g, 0.350 mmol), CuSO₄ (0.0567 g, 0.355 mmol) and L-phenylalanine (0.0585 g, 0.354 mmol). The reaction mixture was allowed to stir at 37 °C for 24 hr and thereafter allowed to cool to 25 °C. The mixture was centrifuged at 3 500 rpm for 5 min and the polymer pellet separated. Thereafter, NaBH₄ (0.0184 g, 0.486 mmol) was added at 50 °C and the solution stirred for 10 min. The solution was placed on ice and the pH adjusted to below 2 using 2 N HCl, followed by saturating the solution with solid NaCl. The aqueous solution was extracted with EtOAc (50 mL x 3) and the combined organic layers were dried over

anhydrous Na₂SO₄. The solvent was reduced *in vacuo* to yield an off-white solid (0.0186 g, 29%).

¹H NMR (400 MHz, D₂O) δ 7.58 – 7.40 (5H, m, Ar-H), 4.61 (1H, dd, *J* = 7.6, 4.7 Hz, H-2), 3.26 (1H, dd, *J* = 14.1, 4.7 Hz, H-3), 3.09 (1H, dd, *J* = 14.1, 7.6 Hz, H-3').

6-Deoxy-6-amino chitosan – PLP recyclability in the non-enzymatic PLP – mediated conversion of amino acids to α-keto acids

The conversion of L-phenylalanine to phenylpyruvate using 6-deoxy-6-amino chitosan bound PLP was reported above. The recyclability of this polymer was evaluated. The reaction was carried out using the procedure outlined above. The polymer pellet obtained was allowed to dry without any further purification. The solid obtained was subsequently used in a second and third conversion of L-phenylalanine to phenylpyruvate.

7.10. References

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Appendix

- Table A1: Computed interaction energies of the different 1,4-dimethoxyglucosamine (1,4-dg)-solvent pairs.
- Table A2: Donor-acceptor NBO interactions and perturbative estimates.
- Table A3: Average interaction energies obtained from molecular dynamics simulations.
- Table A4: Two way ANOVA analysis of antifungal activity of chitosan.
- Table A5: Two way ANOVA analysis of antifungal activity of 6-deoxy-6-amino chitosan.

Table A1: Computed interaction energies of the different 1,4-dimethoxyglucosamine (1,4-dg)-solvent pairs.

Pair	Total E_{int} (kJ/mol)	H-bond description	H...A (Å)	A...H-D (°)	BCP $\rho(r)$ (e/a_0^3)	QTAIM E_{hb} (kJ/mol)
1,4-dg + BMIMAc	- 111.88	O1 _{AC} ...H3 – O3	1.56	170.45	6.62×10^{-2}	- 97.67
		O2 _{AC} ...H2 – N2	1.97	169.45	2.35×10^{-2}	- 58.41
1,4-dg + Cyrene™ (orientation I)	- 29.08	O1 _{Cyr} ...H3 – O3	1.92	162.17	2.44×10^{-2}	- 24.28
		O3 _{Cyr} ...H21 – N19	2.27	156.01	1.24×10^{-2}	- 18.73
1,4-dg + Cyrene™ (orientation II)	- 38.41	O3 _{Cyr} ...H3 – O3	1.93	164.50	2.49×10^{-2}	- 28.16
		O1 _{Cyr} ...H2 – N2	2.54	115.23	9.44×10^{-3}	- 15.68
1,4-dg + Cyrene™ (orientation III)	- 48.07	O3 _{Cyr} ...H3 – O3	2.00	162.89	1.91×10^{-2}	- 27.59
		O1 _{Cyr} ...H2 – N2	2.64	111.35	—	—
		N2...H1 _{Cyr} – C1 _{Cyr}	2.46	128.27	1.17×10^{-2}	13.90
1,4-dg + DMF	- 49.29	O _{DMF} ...H3 – O3	1.86	154.03	2.77×10^{-2}	- 29.92
1,4-dg + DMSO	- 59.04	O _{DMSO} ...H3 – O3	1.73	163.89	3.84×10^{-2}	- 45.86
1,4-dg + NMP	- 48.53	O _{NMP} ...H3 – O3	1.77	167.32	3.06×10^{-2}	- 33.83
		N2...H1 _{NMP} – C1 _{NMP}	2.35	179.78	1.38×10^{-2}	- 24.76

* H...A – hydrogen bond acceptor, H-D - hydrogen bond donor, BCP - bond critical point

Table A2: Donor-acceptor NBO interactions and perturbative estimates.

Pair	Donor (i)	Acceptor (j)	E(2) kJ/mol
1,4-dimethoxyglucosamine + BMIMAc	LP1 (O1 _{AC})	$\sigma^*(\text{O3} - \text{H3})$	39.79
	LP2 (O1 _{AC})	$\sigma^*(\text{O3} - \text{H3})$	249.53
	LP3 (O1 _{AC})	$\sigma^*(\text{O3} - \text{H3})$	6.28
	LP1 (O2 _{AC})	$\sigma^*(\text{N2} - \text{H2})$	18.24
	LP2 (O2 _{AC})	$\sigma^*(\text{N2} - \text{H2})$	27.32
	LP1 (O3)	$\sigma^*(\text{C1}_{\text{IL}} - \text{H1}_{\text{IL}})$	9.08
	LP2 (O3)	$\sigma^*(\text{C1}_{\text{IL}} - \text{H1}_{\text{IL}})$	31.34
1,4-dimethoxyglucosamine + Cyrene TM (orientation I)	LP1 (O1 _{Cyr})	$\sigma^*(\text{O3} - \text{H3})$	37.40
	LP2 (O1 _{Cyr})	$\sigma^*(\text{O3} - \text{H3})$	4.52
	LP1 (O3 _{Cyr})	$\sigma^*(\text{N2} - \text{H2})$	5.94
	LP2 (O3 _{Cyr})	$\sigma^*(\text{N2} - \text{H2})$	10.92
1,4-dimethoxyglucosamine + Cyrene TM (orientation II)	LP1 (O3 _{Cyr})	$\sigma^*(\text{O3} - \text{H3})$	32.26
	LP2 (O3 _{Cyr})	$\sigma^*(\text{O3} - \text{H3})$	17.28
	LP1 (O1 _{Cyr})	$\sigma^*(\text{N2} - \text{H2})$	1.92
	LP2 (O1 _{Cyr})	$\sigma^*(\text{N2} - \text{H2})$	1.46
1,4-dimethoxyglucosamine + Cyrene TM (orientation III)	LP1 (O3 _{Cyr})	$\sigma^*(\text{O3} - \text{H3})$	11.8
	LP2 (O3 _{Cyr})	$\sigma^*(\text{O3} - \text{H3})$	5.27
	LP1 (O1 _{Cyr})	$\sigma^*(\text{N2} - \text{H2})$	1.84
	LP1(N2)	$\sigma^*(\text{C1}_{\text{Cyr}} - \text{H1}_{\text{Cyr}})$	7.03
1,4-dimethoxyglucosamine + DMF	LP1 (O _{DMF})	$\sigma^*(\text{O3} - \text{H3})$	29.79
	LP2 (O _{DMF})	$\sigma^*(\text{O3} - \text{H3})$	0.50
1,4-dimethoxyglucosamine + DMSO	LP1 (O _{DMSO})	$\sigma^*(\text{O3} - \text{H3})$	46.74
	LP2 (O _{DMSO})	$\sigma^*(\text{O3} - \text{H3})$	32.89
	LP3 (O _{DMSO})	$\sigma^*(\text{O3} - \text{H3})$	25.73
1,4-dimethoxyglucosamine + NMP	LP1 (O _{NMP})	$\sigma^*(\text{O3} - \text{H3})$	51.46
	LP1 (N2)	$\sigma^*(\text{H1}_{\text{NMP}} - \text{C1}_{\text{NMP}})$	20.42

Table A3: Average interaction energies obtained from molecular dynamics simulations.

Pair	Coulomb energy (kJ/mol)	vdW ^a energy (kJ/mol)	E _{total} (kJ/mol)	N ^b	E _{total} /N (kJ/mol)
a	-490.97 ± 24.43	-18.56 ± 14.70	-509.53 ± 28.51	150	-3.40 ± 0.190
b	-117.53 ± 23.12	-218.37 ± 12.46	-335.90 ± 26.26	400	-0.84 ± 0.066
c	-121.86 ± 24.12	-210.14 ± 12.83	-332.00 ± 27.32	600	-0.55 ± 0.046
d	-155.53±29.83	-226.94 ± 13.16	-382.47 ± 32.59	500	-0.76 ± 0.065
e	-121.76 ± 22.20	-226.50 ± 13.14	-348.26 ± 25.80	400	-0.87 ± 0.065

^avan der Waals, ^bNumber of solvent molecules

Table A4: Two way ANOVA analysis of the antifungal activity of chitosan.

Within each row	compare columns (simple effects within rows)								
Number of families	3								
Number of comparisons per family	5								
Alpha	0.05								
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value				
Day 2									
Media Control (0) vs. 0.01	-1.703	-76.65 to 73.25	No	ns	0.9999				
Media Control (0) vs. 0.05	69.85	-5.097 to 144.8	No	ns	0.0748				
Media Control (0) vs. 0.1	181.2	106.2 to 256.1	Yes	****	0.0001				
Media Control (0) vs. 0.5	230.7	155.7 to 305.6	Yes	****	0.0001				
Media Control (0) vs. Acetic Acid Control (0)	147.5	72.56 to 222.5	Yes	****	0.0001				
Day 3									
Media Control (0) vs. 0.01	17.56	-57.39 to 92.51	No	ns	0.9578				
Media Control (0) vs. 0.05	142.6	67.63 to 217.5	Yes	****	0.0001				
Media Control (0) vs. 0.1	347.9	272.9 to 422.8	Yes	****	0.0001				
Media Control (0) vs. 0.5	447.7	372.8 to 522.7	Yes	****	0.0001				
Media Control (0) vs. Acetic Acid Control (0)	240.7	165.7 to 315.6	Yes	****	0.0001				
Day 4									
Media Control (0) vs. 0.01	23.88	-51.07 to 98.83	No	ns	0.8692				
Media Control (0) vs. 0.05	145.4	70.43 to 220.3	Yes	****	0.0001				
Media Control (0) vs. 0.1	416.2	341.2 to 491.1	Yes	****	0.0001				
Media Control (0) vs. 0.5	565	490.1 to 640	Yes	****	0.0001				
Media Control (0) vs. Acetic Acid Control (0)	223.1	148.1 to 298	Yes	****	0.0001				
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF	
Day 2									
Media Control (0) vs. 0.01	248.2	249.9	-1.703	28.48	3	3	0.05981	36	
Media Control (0) vs. 0.05	248.2	178.3	69.85	28.48	3	3	2.453	36	
Media Control (0) vs. 0.1	248.2	67	181.2	28.48	3	3	6.361	36	
Media Control (0) vs. 0.5	248.2	17.5	230.7	28.48	3	3	8.099	36	
Media Control (0) vs. Acetic Acid Control (0)	248.2	100.7	147.5	28.48	3	3	5.18	36	
Day 3									
Media Control (0) vs. 0.01	483	465.4	17.56	28.48	3	3	0.6165	36	
Media Control (0) vs. 0.05	483	340.4	142.6	28.48	3	3	5.007	36	
Media Control (0) vs. 0.1	483	135.1	347.9	28.48	3	3	12.22	36	
Media Control (0) vs. 0.5	483	35.21	447.7	28.48	3	3	15.72	36	
Media Control (0) vs. Acetic Acid Control (0)	483	242.3	240.7	28.48	3	3	8.451	36	
Day 4									
Media Control (0) vs. 0.01	632	608.2	23.88	28.48	3	3	0.8387	36	
Media Control (0) vs. 0.05	632	486.7	145.4	28.48	3	3	5.105	36	
Media Control (0) vs. 0.1	632	215.9	416.2	28.48	3	3	14.61	36	
Media Control (0) vs. 0.5	632	67.02	565	28.48	3	3	19.84	36	
Media Control (0) vs. Acetic Acid Control (0)	632	409	223.1	28.48	3	3	7.833	36	

Table A5: Two way ANOVA analysis of the antifungal activity of 6-deoxy-6-amino chitosan.

Within each row	compare columns (simple effects within rows)								
Number of families	3								
Number of comparisons per family	6								
Alpha	0.05								
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value				
Day 2									
Media Control (0) vs. 0.01	10.26	-134.1 to 154.6	No	ns	0.9997				
Media Control (0) vs. 0.05	37.44	-106.9 to 181.8	No	ns	0.9576				
Media Control (0) vs. 0.1	68.28	-76.08 to 212.6	No	ns	0.6408				
Media Control (0) vs. 0.5	227.6	83.23 to 371.9	Yes	***	0.0007				
Media Control (0) vs. 1	278.5	134.1 to 422.8	Yes	****	0.0001				
Media Control (0) vs. Water (0)	43.73	-100.6 to 188.1	No	ns	0.9177				
Day 3									
Media Control (0) vs. 0.01	15.22	-129.1 to 159.6	No	ns	0.9996				
Media Control (0) vs. 0.05	25.23	-119.1 to 169.6	No	ns	0.9938				
Media Control (0) vs. 0.1	107.6	-36.79 to 251.9	No	ns	0.2123				
Media Control (0) vs. 0.5	396.4	252.1 to 540.8	Yes	****	0.0001				
Media Control (0) vs. 1	527	382.6 to 671.3	Yes	****	0.0001				
Media Control (0) vs. Water (0)	-5.801	-150.2 to 138.6	No	ns	0.9999				
Day 4									
Media Control (0) vs. 0.01	23.19	-121.2 to 167.6	No	ns	0.9955				
Media Control (0) vs. 0.05	30.76	-113.6 to 175.1	No	ns	0.983				
Media Control (0) vs. 0.1	258.1	113.8 to 402.5	Yes	***	0.0001				
Media Control (0) vs. 0.5	506.6	362.2 to 650.9	Yes	****	0.0001				
Media Control (0) vs. 1	683.3	538.9 to 827.6	Yes	****	0.0001				
Media Control (0) vs. Water (0)	84.31	-60.05 to 228.7	No	ns	0.436				
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF	
Day 2									
Media Control (0) vs. 0.01	278.5	268.2	10.26	53.97	3	3	0.1901	42	
Media Control (0) vs. 0.05	278.5	241	37.44	53.97	3	3	0.6937	42	
Media Control (0) vs. 0.1	278.5	210.2	68.28	53.97	3	3	1.265	42	
Media Control (0) vs. 0.5	278.5	50.88	227.6	53.97	3	3	4.217	42	
Media Control (0) vs. 1	278.5	0	278.5	53.97	3	3	5.16	42	
Media Control (0) vs. Water (0)	278.5	234.7	43.73	53.97	3	3	0.8103	42	
Day 3									
Media Control (0) vs. 0.01	527	511.8	15.22	53.97	3	3	0.2819	42	
Media Control (0) vs. 0.05	527	501.8	25.23	53.97	3	3	0.4674	42	
Media Control (0) vs. 0.1	527	419.4	107.6	53.97	3	3	1.993	42	
Media Control (0) vs. 0.5	527	130.6	396.4	53.97	3	3	7.345	42	
Media Control (0) vs. 1	527	0	527	53.97	3	3	9.764	42	
Media Control (0) vs. Water (0)	527	532.8	-5.801	53.97	3	3	0.1075	42	
Day 4									
Media Control (0) vs. 0.01	683.3	660.1	23.19	53.97	3	3	0.4298	42	
Media Control (0) vs. 0.05	683.3	652.5	30.76	53.97	3	3	0.57	42	
Media Control (0) vs. 0.1	683.3	425.2	258.1	53.97	3	3	4.783	42	
Media Control (0) vs. 0.5	683.3	176.7	506.6	53.97	3	3	9.386	42	
Media Control (0) vs. 1	683.3	0	683.3	53.97	3	3	12.66	42	
Media Control (0) vs. Water (0)	683.3	599	84.31	53.97	3	3	1.562	42	