Master's thesis

NTNU
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Chitin Oligomers and Chitin-based Block Polysaccharides: Terminal Conjugation of Dextran to Water Insoluble Chitin Oligomers

Master's thesis in Biotechnology (MBIOT5)
Supervisor: Bjørn E. Christensen
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Trine Muren

Abstract

Recent research has had a shift in focus from exploitation of synthetic polymers to exploitation of biopolymers. Creating block polysaccharides is a relatively new field of research which has great potential due to the versatility and abundance of polysaccharides. The work in this thesis focused on chitin and the challenges regarding the solubility due to its interesting properties, such as its immunogenic activity for development of novel therapies. Additional work included optimization of already established protocols.

Fully N-acetylated water-soluble and water insoluble chitin oligosaccharides of the type A_nM were produced by degradation of chitosan with an excess of nitrous acid. Dextran oligosaccharide conjugates (dextran-PDHA) were obtained by hydrochloric acid hydrolysis of high molecular weight dextran and conjugated to PDHA by reductive amination. The mixtures were fractioned by size exclusion chromatography (SEC) and characterized by proton nuclear magnetic resonance (^{1}H-NMR) and SEC with multi-angle laser light scattering (SEC-MALLS).

The main focus of this thesis was directed towards the water insoluble A_nM oligosaccharides, as research is limited due to the solubility challenges. First, simultaneous nitrous acid degradation of chitosan and conjugation to PDHA was attempted. The assumption was that by conjugating to PDHA, a terminal charge would be introduced, possibly preventing the longer oligosaccharides from the initial precipitation. The reaction was not successful as it generated chitosan with no evidence of conjugation.

Alternative solvents were tested, whereas N,N-dimethylacetamide (DMAc) with a few percent of LiCl proved as a viable solvent system. A preliminary study showed successful conjugation of water-soluble A_nM to A_nM-PDHA in 8wt% LiCl/DMAc. Furthermore, water insoluble A_nM was successfully conjugated to dextran-PDHA. Dextran was chosen as the second block due to the hypothesis that conjugation to a compound of high solubility would keep the finished conjugate water-soluble, which indeed it did. ^{1}H-NMR and SEC-MALLS analyses confirmed successful conjugation.

^{1}H-NMR using deuterated DMAc was attempted to allow for studies of the reaction kinetics of the conjugation in LiCl/DMAc. Comprehensive studies gave inconclusive results, which meant that the method of choice had to be purification of the reaction mixture and redissolve it in D_2O for ^{1}H-NMR analyses.

Reaction kinetics analyses performed aside from ^{1}H-NMR showed that the conjugation in DMAc happens at a fast rate, with equilibrium yields of roughly 60% and 40% for water-soluble and water insoluble A_nM (respectively) obtained within 15 minutes. Additionally, using an excess of chitin resulted in an equilibrium yield of 84%.

Additionally, the kinetic parameters for the reductive amination of water-soluble A_nM to dextran-PDHA in standard conditions were established. When using chitin as the second block, the reaction was one order of magnitude faster than with dextran as the second block. However, the reduction was time consuming in both cases.

As a side project, the kinetics of the periodate oxidation of chitin was attempted elucidated by optical rotation measurements. Periodate oxidation selectively targets the vicinal diol of the non-reducing end, creating a dialdehyde, possibly changing the rotation of the molecule. Nonetheless, no change in the optical rotation was detected, hence this method is not appropriate to determine the degree of periodate oxidation of chitin.

The development of a protocol for conjugation of water insoluble chitin to dextran to produce a water-soluble conjugate, serves as a basis for further research on the water insoluble fraction and related bioactivity.

Sammendrag

Nyere forskning har hatt et skifte i fokus fra utnyttelse av syntetiske polymerer til utnyttelse av biopolymerer. Produksjon av blokk polysakkarider er et relativt nytt felt med høyt potensial grunnet allsidigheten og mangfoldet av polysakkarider. Arbeidet til denne masteroppgaven fokuserte på kitin og utfordringene rundt løseligheten til dette polysakkaridet grunnet interessen i dets egenskaper, som dets immunogene aktivitet for utvikling av nye medisinske behandlinger. Annet arbeid inkluderte optimalisering av allerede etablerte protokoller.

Fullt N-acetylerte vannløselige og vann uløselige kitin oligosakkarider av typen A_nM ble produsert ved degradering av kitosan med et overskudd av salpetersyrling. Dextran oligosakkarid konjugater (dextran-PDHA) ble produsert ved syrehydrolyse av høymolekylær dextran, og konjugert til PDHA ved reduktiv aminering. Reaksjonsblandingene ble fraksjonert med størrelseseksklusjons-kromatografi (SEC) og karakterisert med proton kjernemagnetisk resonans spektroskopi (^{1}H-NMR) og SEC med multivinkel laser lysspredning (SEC-MALLS).

Hovedfokuset til denne oppgaven var rettet mot de vann-uløselige A_nM oligosakkaridene, da det er begrenset med forskning på disse grunnet utfordringene rundt løselighet. Først ble degradering av kitosan kombinert med konjugering til PDHA. Antakelsen var at ved konjugering til PDHA ville en terminal ladning bli introdusert, som muligens kunne hindre de lengre oligosakkaridene fra den initielle utfellingen. Reaksjonen var ikke vellykket da sluttproduktet var kitosan uten bevis på konjugering.

Alternative løsemidler ble testet, hvor N,N-dimethylacetamide (DMAc) med noen prosent LiCl viste seg å være et passende system. Preliminære studier viste suksessfull konjugering av vannløselig A_nM til A_nM-PDHA i 8wt% LiCl/DMAc. Videre ble vann-uløselig A_nM vellykket konjugert til dextran-PDHA. Dextran ble valgt som den andre blokken basert på hypotesen om at konjugering til et stoff med høy løselighet ville øke løseligheten til det ferdige konjugatet, som viste seg å stemme. ^{1}H-NMR og SEC-MALLS analyser bekreftet konjugeringen.

^{1}H-NMR med deuterert DMAc som løsemiddel ble testet for å kunne utføre studier av reaksjonskinetikken til konjugeringen i LiCl/DMAc. Omfattende forsøk ga ingen tydelige resultat. Metoden for resten av arbeidet for denne masteroppgaven ble derfor å rense prøven og løse den i D_2O for ^{1}H-NMR analyser.

Reaksjonskinetikk utført uten real time NMR viste at konjugeringen i DMAc skjer raskt, med likevekts utbytte på henholdsvis 60% og 40% for vannløselig og vann-uløselig A_nM, oppnådd innen 15 minutter. I tillegg kan likevekts utbyttet økes til 84% ved å bruke et overskudd av kitin.

De kinetiske parameterne for reduktiv aminering av vannløselig A_nM til dextran-PDHA ved standardbetingelser ble bestemt. Reaksjonshastighetskonstantene viste at ved bruk av kitin som den andre blokken så var reaksjonen en størrelsesorden raskere enn ved bruk av dextran som den andre blokken. Derimot var reduksjonen tidkrevende i begge tilfeller.

Som et sideprosjekt ble det forsøkt å bestemme reaksjonskinetikken av perjodatoksidering av kitin basert på målinger av optisk rotasjon. Perjodatoksidering angriper selektivt de vicinale diolene på den ikke-reduserende enden av kitin og produserer et dialdehyd. Dette ble tenkt å endre rotasjonen av molekylet. Ingen endring i optisk rotasjon ble målt, det ble dermed konkludert at måling av optisk rotasjon er ikke en passende metode for å bestemme graden av perjodatoksidering av kitin.

Utvikling av en protokoll for konjugering av vann-uløselig kitin til dextran som gir et vannløselig konjugat, kan være grunnlaget for videre forskning på vann-uløselig kitin og relatert bioaktivitet.

Abbreviations

$(A_nM)_{wis}$	Water insoluble fraction after nitrous acid degradation of chitosan to yield chitin oligomers (DP>9)
^{1}H-NMR	Proton nuclear magnetic resonance
AcOH	Acetic acid
AmAc	Ammonium acetate
A_nM	Fully N-acetylated chitin with 2,5-andhydro-D-mannose at the reducing end
A-unit	N-acetyl-D-glucoseamine, GlcNAc
DMAc	N,N-dimethylacetamide
DMSO	Dimethylsulfoxide
D_nM	Fully N-deacetylated chitosan with 2,5-anhydro-D-mannose at the reducing end
DP	Degree of polymerization
DP_n	Number average degree of polymerization
D-unit	D-glucoseamine, GlcN
EDTA	2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid
F_A	Fraction of N-acetylated units
GlcN	D-glucoseamine
GlcNAc	N-acetyl-D-glucoseamine
HCl	Hydrogen chloride
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
HMF	5-hydroxymethylfurfural
HMW	High molecular weight
HONO	Nitrous acid
HPLC	High pressure liquid chroamtography
LiCl	Lithium chloride
LMW	Lowe molecular weight
MAMP	Microbe-associated molecular pattern
M_n	Number average molecular weight
MQ-water	MilliQ-water, ultrapure water
M	2,4-anhydro-D-mannose
M’	The alternative form of 2,4-anhydro-D-mannose formed upon degradation

M_w	Weight average molecular weight
MWCO	Molecular weight cut-off
NaAc	Sodium acetate
$NaBH_3CN$	Sodium cyanoborohydride
NaCl	Sodium Chloride
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NRE	non-reducing end
PB	2-methylpyridine borane complex, a-picoline borane, pic-BH_3
PDHA	O,O'-1,3-propanediylbishydroxylamine dihydrochloride
PEO	Polyethylene oxide
pK_A	Acid dissociation constant
PRR	Pattern recognition receptors
rcf	relative centrifugal force
RI	Refractive index
RT	Room temperature
SEC	Size exclusion chromatography
SEC-MALLS	Size exclusion chromatography with multi-angle laser light scattering
TEA	Triethylamine
TLR2	Toll-like receptor 2
TSP	2,2,3,3,-d(4)-3(trimethylsilyl)propionic acid

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1 Introduction

1.1 General Background and Motivation

Polymer science has been an important area within the field of research for several decades. In general, polymers are readily subjected to alteration and modification to obtain desired properties for exploitation within fields such as pharmaceutics, biomedicine, agriculture and biotechnology. Recently, with the motion towards green chemistry and environmentally friendly research, increasing interest has been drawn to the move from synthetic polymers towards natural biopolymers.

One class of biopolymers that are inexpensive, abundant and renewable, are the polysaccharides. Polysaccharides inhabit desired properties such as biocompatibility and biodegradability which are of significance considering sustainable chemistry (1). Recently, block polysaccharides have been sought as the rise of a new and important class of biomaterials. By linear conjugation of two polysaccharides, a plethora of properties can be tailored – with the intrinsic properties still intact (1). The magnitude of combinations that can be performed gives a solid foundation to produce biomaterials with different properties which could be of great importance for industrial and medicinal uses.

One of such naturally abundant polysaccharides is chitin, which is in fact estimated to be the second most abundant biopolymer in nature. Chitin is a polysaccharide found in the exoskeleton of crustaceans and insects (amongst other sources), which is already harvested as by-products of fishery. Chitin elicits biodegradability and non-toxicity, leaving it as an interesting polysaccharide for a range of industrial applications (2).

Moreover, chitin has been shown to elicit immunogenic activity in relevance to fungal infection and airway inflammation during asthma (3). However, this seems to be a size dependent system in which only chitin with chains of minimum six subunits trigger a downstream response. Research in this area is limited due to chitin becomes gradually less water-soluble above a degree of polymerization (DP) of 5 and is essentially water insoluble at DP > 9 (2). It is thus of great interest to find alternative ways to overcome the difficulty in obtaining oligomeric chitin above DP 9. To overcome this challenge would possibly be a step in the direction to the development of novel therapies addressing chitin-mediated inflammatory disease conditions.

1.2 Aims

Aim 1: Preparation and characterization of chitin oligosaccharides and dextran conjugates

Initial work will include preparation of chitin and dextran oligosaccharides and conjugates from already established protocols for further use later in the work for this thesis. Chitin oligosaccharides will be prepared by nitrous acid degradation of chitosan with a high fraction of N-acetylated units (F_A=0.48) to obtain fully N-acetylated oligosaccharides of the type A_nM. Dextran conjugates will be obtained by acid hydrolysis of high molecular weight dextran with subsequent conjugation to PDHA by reductive amination.

Aim 2: Determine the kinetic parameters of the conjugation of chitin oligosaccharides to dextran-PDHA

The kinetic parameters for the conjugation of dextran oligosaccharides to chitin conjugates of the type A_nM-PDHA by reductive amination have previously been described (4). Due to the difference in the nature of the reducing end of the two sugars, it was proposed that conjugation of chitin oligosaccharides to dextran-PDHA would be more effective. Thus, the kinetic parameters using chitin as the second block will be assessed to allow for comparison of the already established kinetic parameters with dextran as the second block.

Aim 3: Assess change in optical rotation as a method to determine the reaction kinetics of the periodate oxidation of the non-reducing end of chitin oligosaccharides

Periodate oxidation is a novel approach to create a platform to exploit the non-reducing end (NRE) of chitin oligosaccharides. Periodate selectively oxidize the NRE by attacking the vicinal diols to produce a dialdehyde which can further be used as a target by reductive amination to produce more complex block polysaccharides. As of now, the kinetics behind the periodate oxidation are unelucidated, with incomplete oxidation with 4 equivalents periodate to oligomer for 24 h (5). As the oxidation opens the NRE residue and creates a dialdehyde, it is speculated that the conformational change will be measurable with a polarimeter. Hence, optical rotation measurements throughout the reaction will allow to determine the degree of oxidation at different time points to increase the understanding of the reaction kinetics.

Aim 4: Solubility of chitin: Assess alternative solvent systems and develop protocol for conjugation of water insoluble chitin to dextran-PDHA for increased solubility

Fully N-acetylated chitin oligosaccharides becomes gradually less water-soluble with increasing degree of polymerization (DP) and is essentially water insoluble at DP > 9. The longer oligosaccharides are of increasing interest due to the bioactive properties. Nonetheless, there is limited research on fully N-acetylated chitin due to its water insolubility. The main focus of this thesis will be to explore alternative pathways to solubilize the water insoluble fraction. First, a reaction combining degradation of chitosan to obtain chitin oligosaccharides with simultaneous conjugation to PDHA will be attempted. The background for this reaction is that conjugation to PDHA will provide a terminal charge and possibly keep the longer oligosaccharides from precipitation. Secondly, alternative solvent systems will be assessed. If an appropriate solvent system gets established, the focus will be to prepare and optimize a protocol for terminal conjugation to dextran. The basis is that conjugation to a highly soluble component will increase the overall solubility of the conjugate allowing for subsequent analysis and further research on the water insoluble chitin oligosaccharides.

2 Theory

2.1 Block Polymers

A polymer is a macromolecule consisting of repeating units called monomers in different degrees and compositions. A biopolymer is one of which originates from a natural source, ranging from microorganisms to trees, insects and algae. For several decades, polymers, often synthetic, have been extensively researched as they are of great importance for industrial applications. Polymers in general are readily subjected to modification to alter the properties such as making graft polymers and/or altering the degree and composition of the polymerization. Less described, and perhaps a bit underestimated, is the plethora of properties that can be obtained by terminal conjugation of two polymers creating a linear diblock (1).

Recent research have shown that by conjugating two polymers one can obtain widely different properties, such as self-assembled, stable aggregates with a range of possibilities compared to the abilities of the polymer alone (1). Self-assembly is a process in which the molecules spontaneously organize themselves into stable aggregates and structures by noncovalent interactions such as electrostatic forces, van der Waals, hydrophobic interactions and hydrogen bonds. These self-assembled structures are dependent on several intrinsic factors including polymer block composition and concentration, as well as external factors including solvent, additional ions and pH (Figure 2-1) (6).

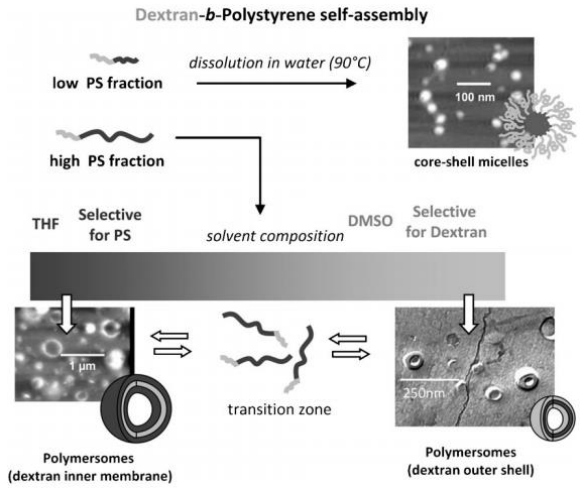

Figure 2-1: Example of the different properties of a block polymer (here dextran-b-polystyrene) depending on intrinsic and external factors (1).

2.1.1 Block Biopolymers Consisting of Polysaccharides

Block polysaccharides are the rise of a new class of biomaterials with advantageous properties compared to synthetic block polymers (1,2). They are inexpensive, abundant and renewable making them interesting compounds for explotation within the field of sustainable chemistry, such as for plastics and detergents (7). Furthermore, several biological phenomena depend on oligo- and polysaccharides. Glycoconjugates, polysaccharides conjugated to other chemical species (protein, lipids etc.), are highly involved in cellular processes including fertilization, cancer metastasis, pathogen infection and cellular recognition (1). Additionally, polysaccharides have important functions in energy storage and the extracellular matrix (1). The bioactivity constructs a platform where natural biomaterials can be exploited for biomedical, pharmaceutical and biotechnological applications such as gene therapy, drug delivery, vaccination and diagnostics (Figure 2-2) (1).This provides the motivation for conducting further research on this class of natural biomaterials.

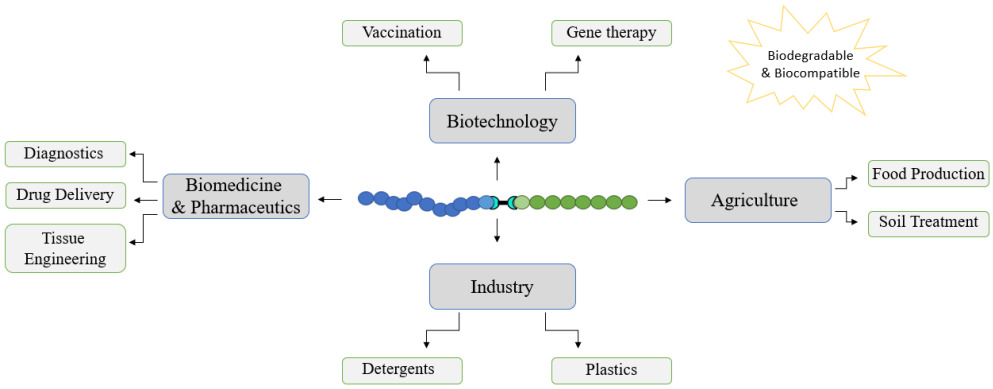

Figure 2-2: Potential applications of polysaccharide block biopolymers.

2.1.2 Block Polysaccharides Syntheses

There are mainly three methods to prepare polysaccharide block biopolymers. The first method is the extension of the polysaccharide block by radical polymerization (7). Second is the enzymatic extension of the block (7). And third, which is the basis of this thesis, is the end-to-end coupling of two blocks. A bifunctional linker molecule is often used for addition of a second block through the reducing ends to create antiparallel linear diblocks of the type A-*b*-A or A-*b*-B (where A and B are different polymers, and *b* represents the linker molecule) (1). A reaction extensively used to achieve this is the use of an amine as a linker with a subsequent reduction to form stable products, this is called reductive amination and is explained in depth later in this thesis. Illustration of the products of different syntheses are shown in Figure 2-3.

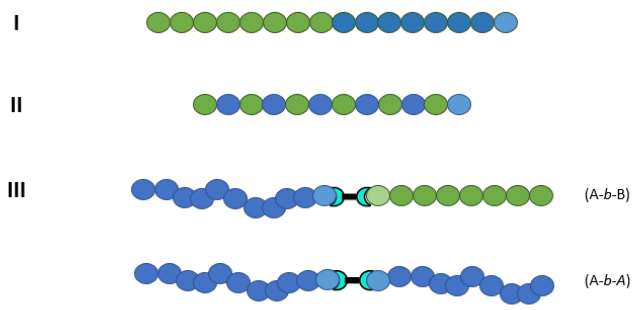

Figure 2-3: Illustration of the product from different syntheses of block polymers. I and II being polymerization techniques while III is end-to-end coupling.

2.2 Chitin

Chitin is claimed to be the second most abundant biopolymer found naturally (cellulose being the most abundant) (2). The polysaccharide is of increasing interest due to its physiochemical and biological activity. It is accessible and renewable, making the exploitation of chitin highly relevant as we move towards increased focus on green chemistry and environmentally friendly research. Chitin possesses advantageous properties including bioactivity, biodegradability and non-toxicity, leaving it as an interesting polysaccharide for a range of industrial applications (2). The intrinsic physiochemical and biological properties of chitin might be useful within fields including (but not limited to) antimicrobial agents, food preservatives, medicines and biomedical materials (8).

2.2.1 Physiochemical Properties of Chitin

The β-(1-4)-linked N-acetyl-D-glucosamine (GlcNAc, denoted A) homopolymer (Figure 2-4) is found in the exoskeleton of crustaceans and insects, as well as in the cell wall of fungi, yeast and green algae (9,10). Due to the structural chemistry including N-acetyl-groups but no charges, chitin becomes gradually less soluble above a degree of polymerization (DP) of 5 and is essentially water insoluble at DP > 9. Chitin is a relatively stiff polymer, with a persistence length of 125 Å (12.5 nm) (11). It is generally insoluble in both organic and aqueous solvents. Nonetheless it can be dissolved in alkali solutions under certain conditions with a lower critical solution temperature of ~30 ^{o}C (12), as well as in some concentrated acids at higher DP (10,13). However, the latter is at the expense of depolymerization and some degree of deacetylation. Additionally, chitin has been dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and dimethylacetamide containing a few percent LiCl (LiCl/DMAc) (2,14).

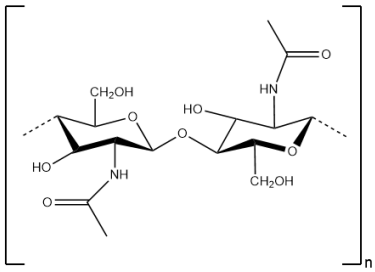

Figure 2-4: Chemical structure of chitin consisting of repeating units of β-(1-4)-linked N-acetyl-D-glucosamine (GlcNAc).

The crystalline nature of chitin can be explained from its ability to form secondary, tertiary, and quaternary architectural structures, as many other polysaccharides. Polymerization of the monosaccharides can produce α, β, and γ sheets contributing to the crystallinity (Figure 2-5) (13). The polymer chains arranged in an antiparallel fashion, the α-sheet, is the most abundant form found in nature. Less common is the β-sheet in which all chains are parallel and the γ-sheet which is a mix of the α- and β-conformation. These allomorphs are stable consisting of a strong network based on inter- and intrachain hydrogen bonds between the acetyl groups of the same and/or adjacent chains (-NH…O=C and -OH…O=C). Additionally, in the α-sheet interchain hydrogen bonds are formed between the hydroxymethyl groups at C6 making it more rigid than the two other structures (13).

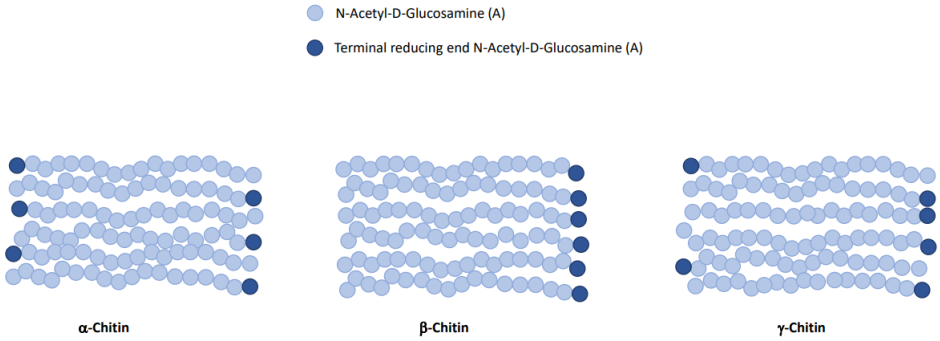

Figure 2-5: Schematic representation of different chitin conformations (α-, β- and γ-sheet).

The interest in expanding the knowledge and continue the research on this biopolymer roots back to the wide abundance, exploitation of the by-products of fishery, and the fact that as a naturally occurring biopolymer it is nontoxic and biodegradable (15). Further research regarding the biological and physiochemical properties of chitin as well as overcoming the challenge of the solubility is highly relevant for progression in assessing potential biomedical and biotechnological applications.

2.2.2 Size-Dependent Immunogenic Activity of Chitin

Chitin has been shown to elicit immunogenic activity in relevance to fungal infection and airway inflammation during asthma (3). Chitin constitutes a microbe-associated molecular pattern (MAMP) which is typically sensed by binding to pattern recognition receptors (PRRs). Chitin appears to directly bind to the PRR toll-like receptor 2 (TLR2), a system which is present in humans and other mammals such as mice, as well as plants, thus it is believed to be highly conserved between kingdoms. This is the primary fungal chitin sensor in the innate immune system of human and murine immune cells. TLR2 senses microbe pathogens and triggers a downstream immune response by production of pro-inflammatory cytokines and chemokines. Interestingly, research indicates that this is a size dependent system, in which the size of the oligomer will either promote or restrict immune activation (3). To the best of our knowledge, minimum six-subunit-long chitin chains are the smallest immunologically active motifs for triggering downstream activation. Furthermore, research suggest that shorter chains (< 6 GlcNAc) have the potential to reduce the immune activation triggered by longer chains. This indicates that two or more receptors need to bridge to initiate signaling, which can be inhibited by shorter-non-stimulatory chains occupying the TLR2 binding pocket (3).

The interaction between chitin and TLR2 is a potential target for novel therapies addressing chitin-mediated inflammatory disease conditions. Due to the size dependent system, it is of great interest to overcome the difficulties in obtaining oligomeric chitin above DP 6. This encourages further research on alternative solvent systems for chitin and attempt to increase the solubility in aqueous solutions by for example conjugation to produce block polysaccharides.

2.2.3 N,N-Dimethylacetamide Containing LiCl as Solvent for Chitin

N,N-Dimethylacetamide containing lithium chloride (LiCl/DMAc) is a common solvent for cellulosic materials (16). Indeed it dissolves chitin as well (14). Chitin can somehow be considered as a derivate of cellulose having one acetamide group substituting a hydroxyl group. The two biopolymers have some similar properties contributing to the challenge of dissolution, namely inter- and intramolecular hydrogen bonding (as described above).

A possible mechanism of dissolution of cellulose in LiCl/DMAc has been proposed by Zhang *et al.* (17), shown in Figure 2-6. The LiCl dissociate and the free ions themselves play an important part in the dissolution. Li^+ ions associate with DMAc molecules and form a complex of one Li^+ surrounded by several DMAc molecules. DMAc in turn associates with the Cl^- ions which forms hydrogen bonds to the OH groups of the cellulose chain. This

spreads the molecule physically and prevents the cellulose chains to form intermolecular hydrogen bonds – thus it becomes dissolved. The mechanism enlightened here describes why cellulose readily dissolves in LiCl/DMAc compared to a system only containing DMAc. As the solubility of cellulose and chitin is both mainly attributed to the intra- and intermolecular hydrogen bonds, it is highly probable that this mechanism applies to chitin as well.

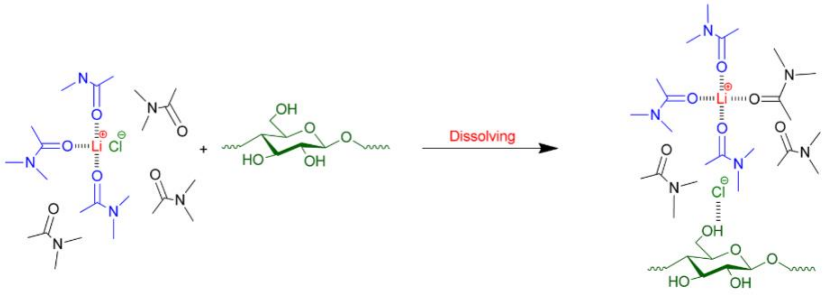

Figure 2-6: Proposed mechanism of dissolution of cellulose in N,N-dimethylacetamide containing lithium chloride (17).

2.3 Dextran

Many polysaccharides and other biopolymers are synthesized naturally in bacteria and other microorganisms. One of such is dextran, a polysaccharide synthesized from sucrose by bacteria such as *Leuconostoc*, *Streptococcus* and *Lactobacillus* species (18). Dextran (Figure 2-7) is made up of a α-(1-6)-linked D-glucose backbone, and might contain some short branches of one to three residues of the same structure coupled to O3 of the backbone (19). The α-(1-6)-linkage contributes to the random coil structure and the high solubility of dextran as it is virtually free rotation around three single bonds (19). This flexibility, manifested in a persistence length of 4 Å (0.4 nm), leads to low intrinsic viscosity and low radius of gyration (R_G) even at high molecular weights (19,20). Additionally, properties such as it is non-ionic and flexible makes the polymer of commercial importance within biotechnological applications including (but not limited to) plasma expanding, standards for other polymers, or stationary phases for gel filtration (19).

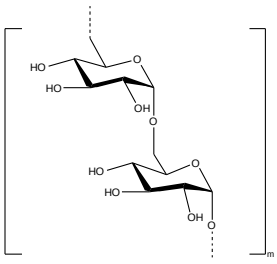

Figure 2-7: The backbone of dextran consisting of repeating units of α-(1-6)-linked D-glucose.

2.4 Nitrous Acid Degradation of Chitosan to Obtain Chitin- and Chitosan Oligosaccharides

Biopolymers, including polysaccharides, exist naturally in different lengths and can be chemically, physically or biologically depolymerized to smaller fragments. As length and molecular weight affect the physiochemical properties of the biopolymer, cleavage into smaller fragments is often desired. With the motive to produce block polysaccharides, a special type of degradation has been shown to be advantageous for making chitin- and chitosan oligosaccharides as it produces a reactive reducing end residue, namely nitrous acid degradation (21).

Nitrous acid (HONO) degradation is a rapid, homogenous reaction with the advantages of being selective and easily controlled (22). The nitrous acid deaminate the D-residues producing a chain cleavage whereas the reactive 2,5-anhydro-D-mannose (denoted M) is left at the reducing end of the new oligosaccharide (Figure 2-8) (21,23). As a result, the degree of scission (α) depends on the molar amount of HONO in respect to the molar amount of D-units and is directly related to the degree of deacetylation (1-F_A) (21).

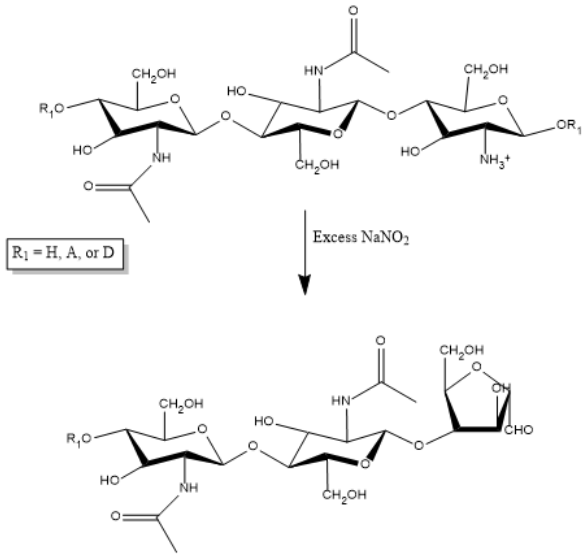

Figure 2-8: Nitrous acid degradation of chitosan to obtain chitin or chitosan oligosaccharides with a reactive 2,5-anhydro-D-mannose (M) unit at the reducing end.

When a chitosan with high F_A (e.g. $F_A > 0.4$) is degraded by an excess of HONO, all the D-units will be deaminated and converted to M-units (21,23). This will yield fully N-acetylated water-soluble short chitin oligosaccharides with a M-unit at the reducing end (A_nM) as well as

an water insoluble fraction of high molecular weight A_nM (21,23). However, if chitosan oligosaccharides are of interest, chitosan with a low F_A can be treated with substoichiometric amounts of HONO to D-units (21,23). This will produce chitosan hetero-oligosaccharides consisting of both A- and D-residues with a M-unit at the reducing end (A_nD_mM) or homo-oligosaccharides of the type D_nM depending on F_A (21,23). The relationship between F_A and molar amount of HONO is illustrated in Figure 2-9. The M-unit at the reducing end is often preferred due to the lack of mutarotation in solution and the aldehyde group is readily available for further reactions such as reductive amination compared to that of the reducing end of native polysaccharides as it does not form intramolecular hemiacetals (21).

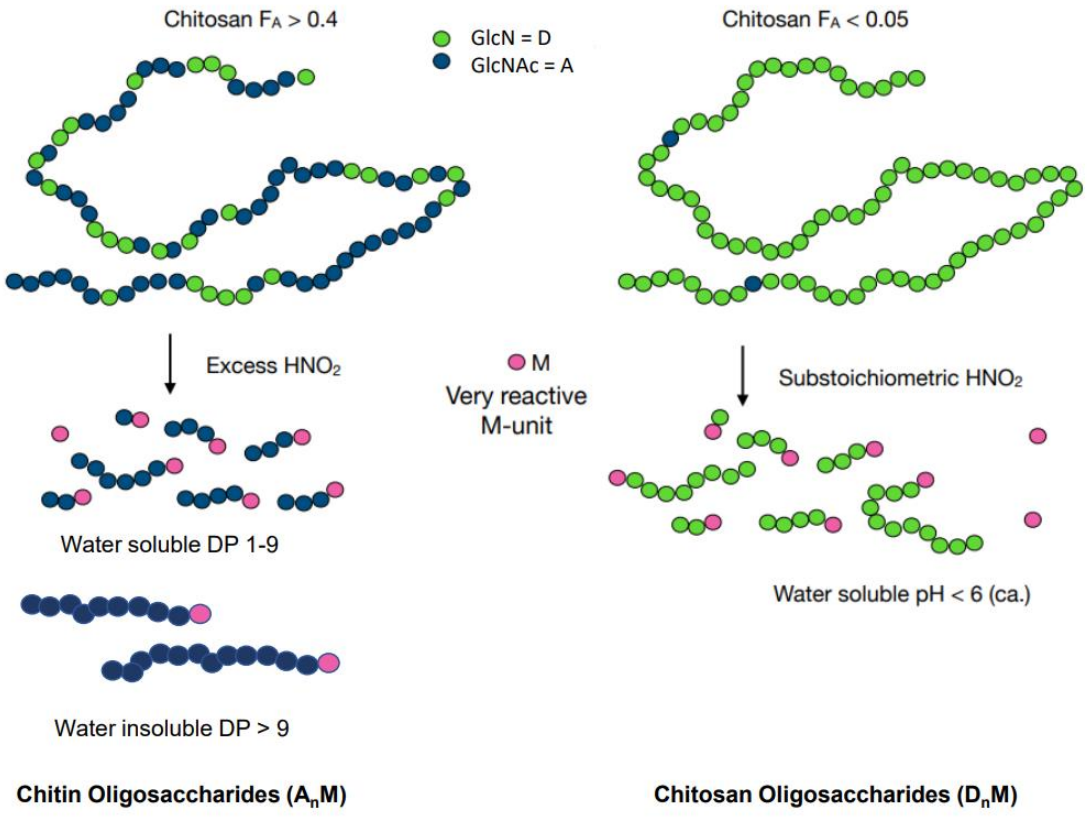

Figure 2-9: Illustration of how the degree of acetylation (F_A) and the molar amount of HONO affects which product is obtained by nitrous acid degradation of chitosan.

2.5 Reductive Amination Through Oxime Click Chemistry

Reductive amination is a classical method employed in organic chemistry for the reversible conversion of carbonyl groups into amines followed by the irreversible reduction to secondary amines (24). Depending on the type of amine used, the amination will produce a imine, oxime or hydrazone, also known as Schiff bases (25,26).

One group of much used amines for reductions are the oxyamines, whereas the reaction is popularly called oxime-click. The reactions of polysaccharides with oxyamines have been applied as a coupling method for e.g. modifying gold nanoparticles, in biomaterial scaffolds and introduce fluorescent tags (27,28). Oxime click is a reversible process where the primary oxyamine attacks the carbonyl group of the open-ring polysaccharide to produce a hemiaminal (27). Subsequent elimination of water produces acyclic oximes in both (E)- and (Z)-configuration, as well as cyclic N-glycosides (27). The general reaction mechanism is shown in Figure 2-10 and an example considering polysaccharides is shown in Figure 2-11.

The emergence of this technique has provided a faster and more stable reaction with a higher yield of oximes due to higher nucleophilicity of the oxyamine compared to the amines used in classical reductive amination processes (26,27). Additionally, in many cases there is no need for the reduction step to form a secondary amine as the oxymes have a sufficient hydrolytic stability due to lower electrophilicity on C1 (29).

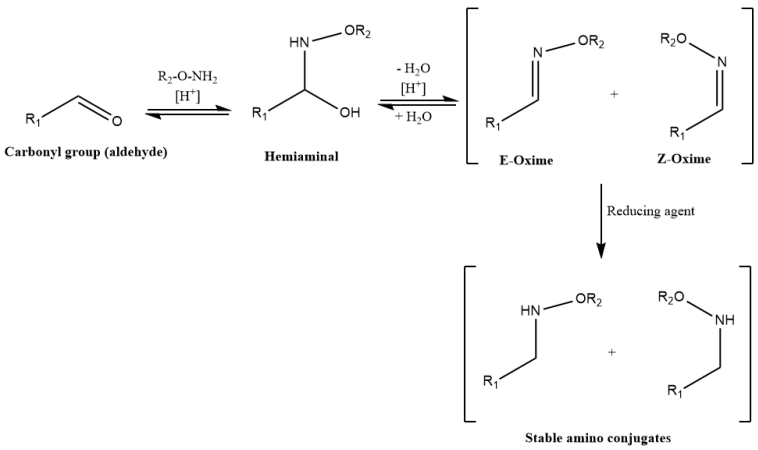

Figure 2-10: General mechanism of reductive amination by primary oxyamines (R_2-O-NH_2).

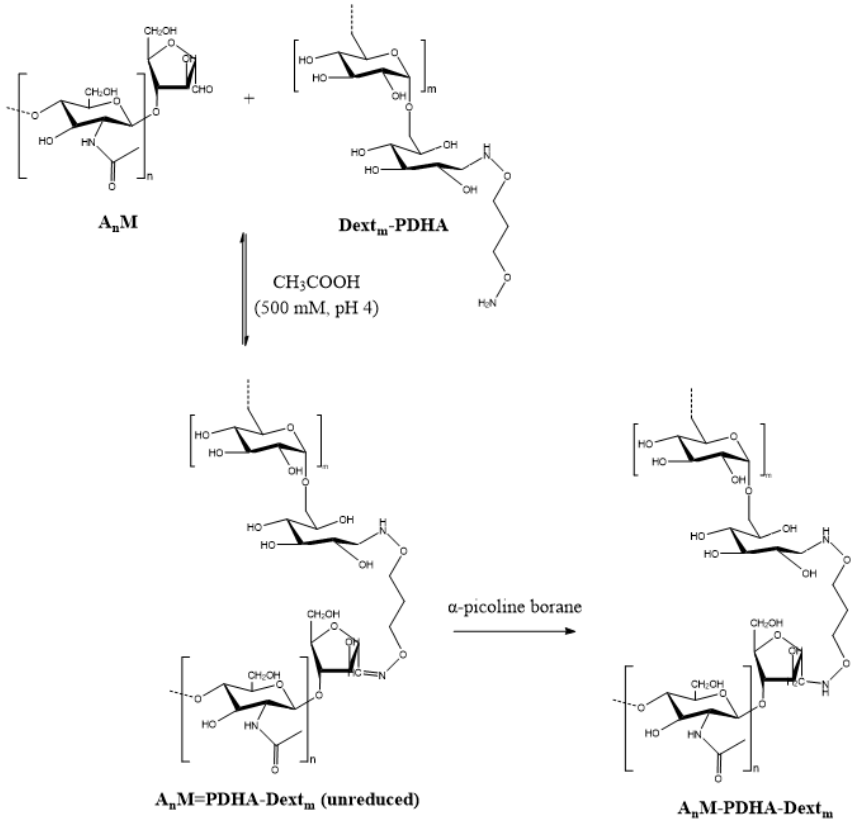

Figure 2-11: Demonstration of reductive amination here using chitin oligosaccharides (A_nM) and dextran activated by the oxyamine O,O'- propanediyl-bishydroxyl-amine (PDHA) ($dext_m$-PDHA) but generally true for polysaccharides (here only Z-oxime shown). PDHA can be coupled to both polysaccharides in no necessary order. n and m denotes the degree of polymerization for A-units and dextran-units, respectively.

Nonetheless, reduction is often employed to ensure a stable product and to increase the yields. For most compounds, reductive amination is employed as a one-pot reaction, where the amination and subsequent reduction happens at the same time in the same container. Hence, the selection of the reducing agent is extremely important as it must reduce the imine/oxime/hydrazone without affecting the carbonyl group substantially. However, the nitrous acid degradation of chitin and chitosan gives an exposed aldehyde sensitive to the reduction using sodium cyanoborohydride or α-picoline borane which normally does not affect polysaccharides with a native reducing end. The protocol has thus been changed to a two-pot reaction performing amination first with a subsequent reduction step when complete amination has been achieved (4).

Sodium cyanoborohydride ($NaBH_2CN$) has been widely used as a reducing agent as it is soluble in a range of solvents, poses the desirable selectivity and it is stable in acidic medium (25). However, $NaBH_2CN$ has its drawbacks as it produces toxic by-products during

hydrolysis, affecting humans and the environment and is thus limited to only small quantities (25). Recently, α-picoline borane (PB, Figure 2-12) has emerged as a substitute for $NaBH_2CN$. PB has properties that complies with the selectivity, stability and solubility demands and the spontaneous decomposition is approximately 20 times slower than of $NaBH_2CN$ (25). Indeed it has low solubility in aqueous solutions, however the reaction rates have been shown to increase with either stirring in room temperature or simply increasing the temperature of the reaction to 40°C (4). This implies the reaction takes place on the surface of the particles. Moreover, it achieves the same results as $NaBH_2CN$ but with lower toxicity, being more sustainable from an environmental perspective (25).

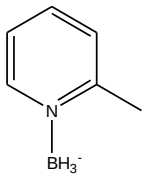

Figure 2-12: Chemical structure of α-picoline borane (PB).

2.5.1 Chemical Linker O,O'-1,3-propanediyl-bishydroxyl-amine

The growing interest in the application potentials of glycoconjugates has led to the development of a range of paths involved in activation of the reducing end of polysaccharides for further reactions to obtain e.g. block polysaccharides (30). The use of oxyamines has become increasingly attractive due to their high nucleophilicity in different pH and commercial availability as well as being more efficient than many other amines (27,31).

The conjugation by reductive amination of the symmetrical dioxyamine O,O'-1,3-propanediyl-bishydroxyl-amine (PDHA) to oligosaccharides has recently been described as a powerful tool in the pathway of producing advanced oligosaccharide materials (26). This method has an advantage due to the fact that there is no need for a catalyst or to process it under extreme conditions (7).

PDHA consists of a C3 backbone with an oxyamine group ($-O-NH_2$) at each end (Figure 2-13). Due to the bifunctional symmetrical structure, PDHA can act as a chemical crosslinker between two molecules containing carbonyl groups (26). Conjugation of PDHA to the reducing end of polysaccharides produces an oxime in both (E) and (Z) configuration, as well as a fraction of cyclic β-N-pyranosides (26,27).

Mo *et al.* (4) have investigated the effect of the molar ratio of substituent relative to PDHA. Studies done in pH 3-5 with 2 equivalents of PDHA to substitute showed that the yield

increased with the pH, but unfortunately so did the reaction time. Thus, it has been concluded that pH 4 gives the best compromise between yield and a reasonable reaction time. However, when increasing the molar ratio of PDHA from 2 equivalents to 10 equivalents two benefits occur; (I) faster reaction time, and (II) lower production of disubstituted species. As this is a way of chemically link two molecules containing carbonyl groups, monosubstituted PDHA is usually preferable as one end of the chemical linker will still be free for further coupling to a molecule of interest. Nonetheless, the authors found that some amount of disubstituted species will occur in any case of the investigated protocols. However, this might not be a problem as they are easily removed by chromatography and have also been shown to not remarkably affect further reactions.

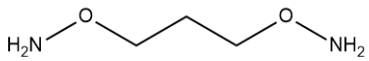

Figure 2-13: Chemical structure of the bifunctional linker O,O'-1,3-propanediyl-bishydroxyl-amine (PDHA).

2.6 Periodate Oxidation of Carbohydrates

Periodate oxidation is a widely used method for structure elucidation and chemical modifications in carbohydrate chemistry. It is a reaction performed in relatively mild conditions, such as aqueous solutions (32). This is advantageous as many polysaccharides are water-soluble as well as it is suitable for sensitive polysaccharides.

The periodate ion (IO_4^-) attacks vicinal diols and other closely related structures with high selectivity (32,33). A prerequisite needed for the oxidation to happen is that at least one of the vicinal hydroxyl groups is in equatorial position. The oxidation leads to a break in the C-C-bond, leaving the moiety with a dialdehyde (Figure 2-14).

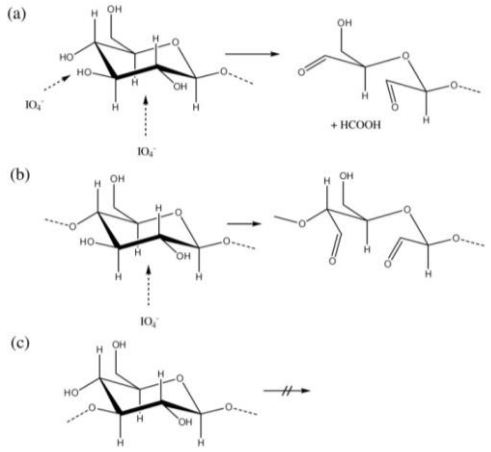

Figure 2-14: General mechanism of periodate (IO_4^-) oxidation of polysaccharides, here with β-D-glucose as an example (34).

2.6.1 Periodate Oxidation of the Non-Reducing End of Chitin Oligosaccharides

Interest has been drawn to periodate oxidation of fully N-acetylated chitin oligosaccharides prepared by nitrous acid degradation (A_nM). These chitin oligosaccharides are structured with vicinal diols only at the non-reducing end (NRE), providing an excellent basis for functionalization of the NRE without affecting the remaining structure. The oxidation provides two aldehyde groups at C3 and C4 at the NRE (Figure 2-15), making reactive hot spots for further conjugation by for example reductive amination to produce block polysaccharides. Interestingly, it has been found that the dialdehyde formed at the NRE was highly reactive when subjected to reductive amination, it is even suggested it was more reactive than the aldehyde of the M-residue at the reducing end (I. V. Mo *et al*., submitted to Carbohydrate Polymers, 2021).

A new method to selectively equip the susceptible NRE with reactive aldehydes opens the possibility of creating precursors for a range of new glycoconjugates, including more advanced block polysaccharides such as triblocks of the type ABC or ABA (where A, B and C are different type of polysaccharides).

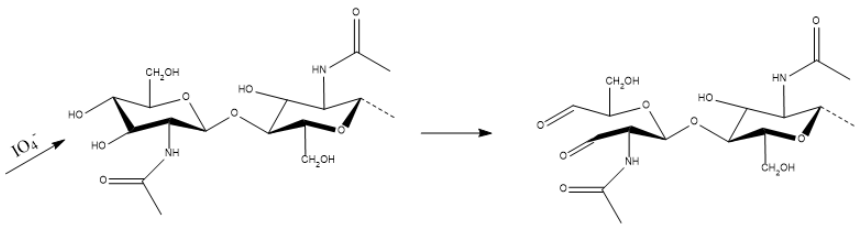

Figure 2-15: Periodate (IO_4^-) oxidation at the non-reducing end of chitin oligosaccharides.

2.7 Analytical methods

2.7.1 Size Exclusion Chromatography

Several separation methods are of importance for fractionation and quantification of the contents of a substance in organic chemistry. This includes electrophoresis, equilibrium centrifugation, mass spectrometry and chromatography (19). Chromatography is an analytical method extensively used for the separation of organic molecules (19). Separation and fractionation of the substance is based on different chemical properties, depending on the substance of interest (35). The method is particularly useful when it comes to biopolymers as they are often disperse.

One such chromatography method is called size-exclusion chromatography (SEC), also known as gel permeation chromatography (GPC) or gel filtration chromatography (GFC). SEC is a high pressure liquid chromatography (HPLC) separation technique based on that the effective hydrodynamic volume of the constituents of a sample will determine the time used to passively diffuse through a column (19,35). This is different than most other chromatography techniques, such as partition, ion exchange and adsorption, in which separation is based on interaction with the stationary phase (36).

Briefly, the system consists of a buffer, HPLC pump, minimum one column and a detector (Figure 2-16) (19). The column is often made up of polyacrylamide or another cross-linked neutral polymer and makes up the stationary phase (19). The choice of the material of the column decides the pore size in which the mobile phase (the buffer) including the sample of interest needs to diffuse through (35). The separation quality of the sample content depends on the length of the column, the material of the stationary phase and the flow time of the mobile phase through the system (36). Moreover, coupling of several columns in series have been shown to improve the separation process (36). Several detectors can be used whereas the most common ones are UV, fluorescence or refractive index detector (19). Once the constituents of the mobile phase have diffused through the column and been detected, the data is plotted against the retention time in a chromatogram.

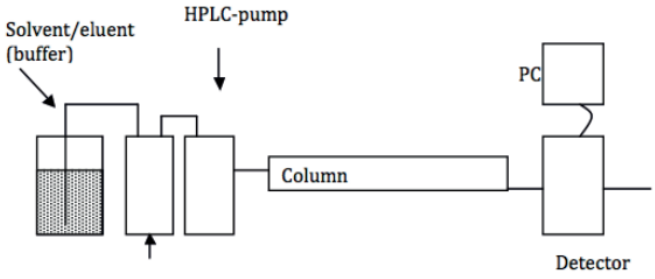

Figure 2-16: Standard set-up for a size-exclusion chromatography system (37). Several columns can be connected for better separation and resolution, as well as a fraction collector can be installed.

The principle behind the sample separation is based on the stationary phase. The sample of interest is dissolved in the mobile phase and injected into the system. The sample will diffuse through the column and molecules with larger hydrodynamic volume than the pore size of the stationary phase will be excluded from the pores and elute faster through the column(s) due to little or no retention (19). Conversely, molecules with smaller hydrodynamic volume will only be partially excluded from the pores. When the molecules diffuse into the pores, a local equilibrium establishes between the stationary phase and the mobile phase and the particles will be retained until they diffuse out (19). It is important to note that size in this context refers to the hydrodynamic volume, i.e. how the molecule acts in solution depending on chemical properties such as branching, charge etc., as it does not necessary correlate to the molecular weight. Altogether, the largest molecules will elute first and subsequent molecules elute according to decreasing hydrodynamic volume (35). The principle of the separation mechanism is illustrated in Figure 2-17.

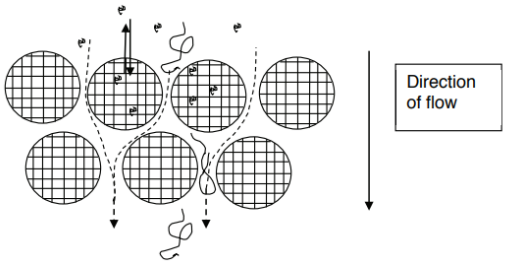

Figure 2-17: Demonstration of the principle behind size exclusion chromatography of a disperse mixture of molecules (37).

When using SEC or other column chromatography techniques, there are two different ways of doing it – analytical and preparative. In an analytical run, a small sample is injected to achieve an overview of the constituents of the sample. This technique is often applied to elucidate unknown components of a sample by comparing to a curve of known analytes (35). In a

preparative run, the intention is to separate the sample. This is done by injecting a greater sample content and connect the system to a fraction collector.

2.7.1.1 Size Exclusion Chromatography with Multi-Angle Laser Light Scattering

Size Exclusion chromatography with multi-angle laser light scattering (SEC-MALLS) is a way to determine the weight distributions and averages of a sample by recording how the contents scatter light (38). It has its basis on the size exclusion chromatography principle as described above but has two on-line detectors instead; a concentration sensitive detector (ultra violet (UV) or refractive index (RI) detector) and a light scattering detector able to monitor up to 18 angles (~30-150°) at the same time (38). Additionally, other detectors can also be connected, e.g. a viscosity detector. The software records raw data from the detectors at regular intervals which are stored and processed separately, the output is shown in Figure 2-18. By scattering light on a polymer chain an intensity distribution is produced from which the spatial extension of the molecule can be deduced (39). Altogether, this technique makes it possible to study the molecular weight dependence of radius of gyration (R_G) and it will provide the information about the shape of the polymer in solution (38).

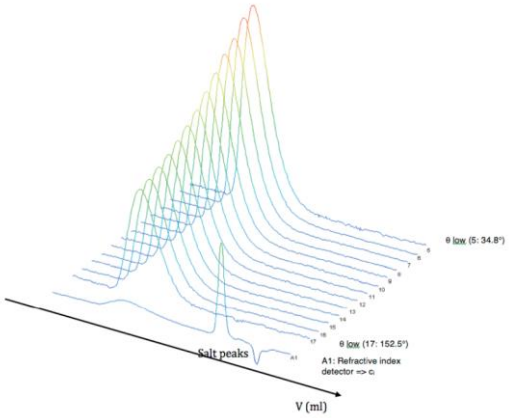

Figure 2-18: Output from a SEC-MALLS experiment (37).

2.7.2 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is one of the most widely used tools to elucidate, characterize and/or verify the structure and composition of organic molecules or components in a mixture (40). Recently, NMR has become increasingly important in polymer chemistry as it is an indispensable tool to elucidate macromolecules compositions, sequences, polymerization, chain conformations, as well as kinetics and mechanisms of polymerization reactions. The NMR spectrometer is based upon four components; (I) a helium cooled

superconductive cryomagnet, (II) a radiofrequency emitter, (III) a receiver, and (IV) a computer (Figure 2-19).

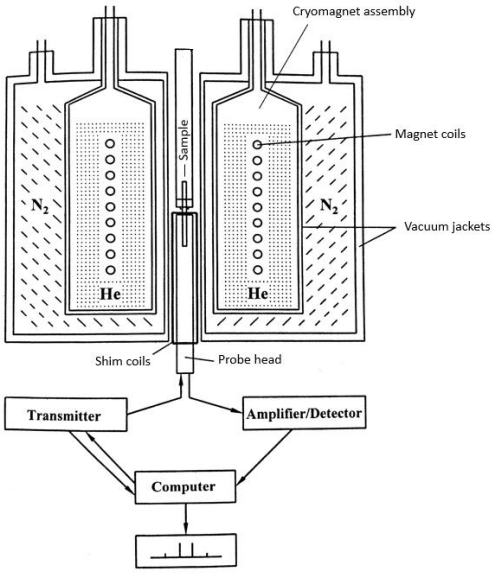

Figure 2-19: Schematic diagram of a typical NMR-instrument (adapted from Friebolin (40)).

NMR is a form of absorption spectrometry where the absorbing species are atomic nuclei (41). Resulting from the absorption is a spectrum showing a plot of the frequencies of the absorption peaks versus peak intensities (41).

The standard method of recording NMR spectra is the pulsed-Fourier transform method (41). The sample gets placed in a probe in the magnetic field and the nuclei will precess around the stationary magnetic field in a somewhat random fashion. The frequency of this precession is called the Larmor frequency. The nuclei will resonate when irradiated by a radiofrequency pulse with the right frequency. This frequency is known as the resonance frequency or chemical shift. When irradiated, the exited spins will precess around the external magnetic field, creating a current that can be detected, producing a free induction decay (FID). The information from FID, a function of time, gets converted to a spectrum with a frequency domain, using Fourier transformation (41).

2.7.2.1 Proton Nuclear Magnetic Resonance Spectroscopy

A widely used method of NMR is one-dimensional mode of observation. This mode produces spectra with just one frequency axis and the second being the intensity of the signal (40). One of these types which is important for polysaccharide characterization is Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy. ^{1}H-NMR spectroscopy gives information of the chemical environment surrounding the different hydrogens in the molecule (41). Electrons

surrounding the nuclei can alter the magnetic field around a certain proton, thus different protons will have different chemical shift values due to different magnetic shielding (40,41). In the resulting spectrum the coupling between adjacent protons are observable and the intensities of the signals are roughly correlated to the ratios of the respective protons in the molecule (40). It is important to note that oligosaccharides are mobile and have more or less rotation around the glycosidic linkage. In practice this means that the observed values of the couplings are averaged over several different conformations (40).

On this basis, ^{1}H-NMR is helpful in determining which monomers are present in the oligosaccharide or polysaccharide, how the monomers are linked together and what is the conformation of the chain (40). To decide how many monomers and which monomers are present in the oligosaccharide it is valuable to look at the anomeric protons (H1). These are usually separate from each other as well as the other ring protons and have a chemical shift of ~4.5-5.5 ppm (40). The intensity of this peak relative to the intensity of a peak from an internal proton roughly correlates to how many monosaccharides the oligo- or polysaccharide is composed of.

2.7.2.1.1 ^{1}H-NMR Spectrum of A_nM Oligosaccharides

^{1}H-NMR spectra of fully N-acetylated chitin oligosaccharides have previously been annotated by Boyd *et al.* (42). The resonances of the M-residue obtained by nitrous acid degradation of chitosan have been annotated by Tømmeraas *et al.* (21). The free aldehyde group of the M residue did not have the resonance expected (9-10 ppm). Interestingly, the aldehyde group revealed itself as a gem-diol. This is a result of water being added creating a hydrated aldehyde group, with a following resonance of ~5 ppm. The chemical shifts of the different protons in the A and M units of A_nM are given in Table 1, the structure with the corresponding protons is shown in Figure 2-20 and annotated spectra are shown in Figure 2-21.

Table 1: ^{1}H-NMR chemical shifts (ppm) for the protons in the A- and M-residues of fully N-acetylated chitin oligosaccharides assigned by Boyd *et al.* (42) and Tømmeraas *et al.* (21) , respectively.

Unit	H1	H2	H3	H4	H5	H6a	H6b
A	4.59	3.74-3.77	3.59-3.72	3.46-3.63	3.98	3.43	3.49
M	5.01	3.76	4.35	4.13	3.98	3.43	3.49

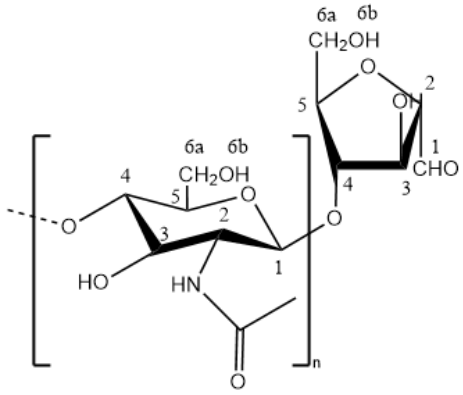

Figure 2-20: Corresponding protons from Table 1 marked on the structure of chitin oligosaccharides of the type A_nM obtained by nitrous acid degradation.

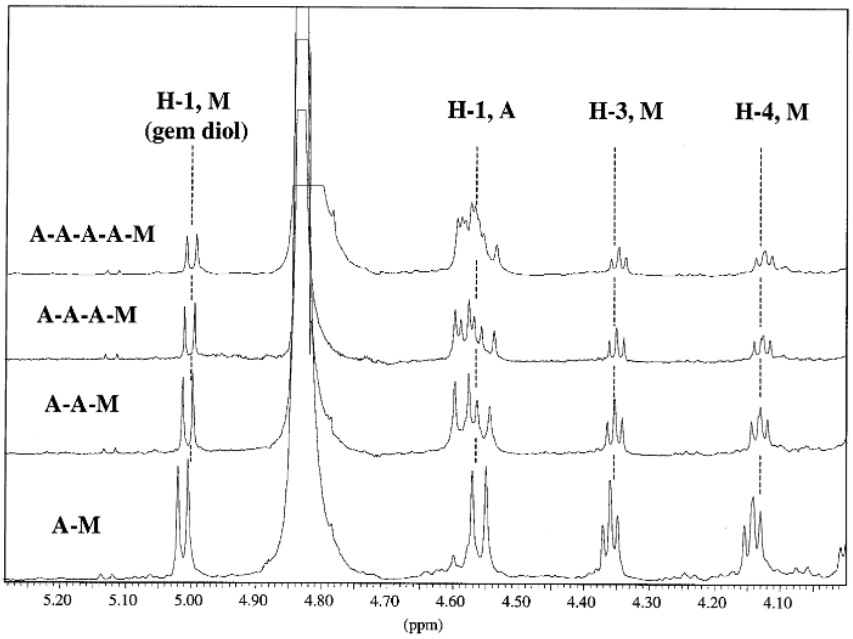

Figure 2-21: ^{1}H-NMR (400.13 MHz, 298 K, pH 5.0) spectra of fully N-acetylated chitin oligosaccharides of the type A_nM obtained from nitrous acid degradation of chitosan (21).

2.7.2.1.2 ^{1}H-NMR Spectra of A_nM Oligosaccharides Conjugated to PDHA

Moussa *et al*. (30) and Mo *et al*. (4) have previously annotated ^{1}H-NMR spectra of A_nM conjugated to PDHA and the product of the following reduction as shown in Figure 2-22 and Figure 2-23, respectively. Comparing the spectrum of A_nM conjugated to PDHA with the spectrum of A_nM alone several deviations appear. At first, the reducing end gem-diol signal is remarkably reduced. The signals at higher ppm (7-7.5 ppm) show the occurrence of CH_2=N protons as a mixture of Z- and E-oximes along with a minor signal from an alternative form of the M-unit (4,30). In the spectrum solely consisting of A_nM there were no signals in this range. Furthermore, when the conjugated oximes are reduced the H1,M of E- and Z- oximes

disappear and H1 of M appears as a signal at 3.0-3.2 ppm.

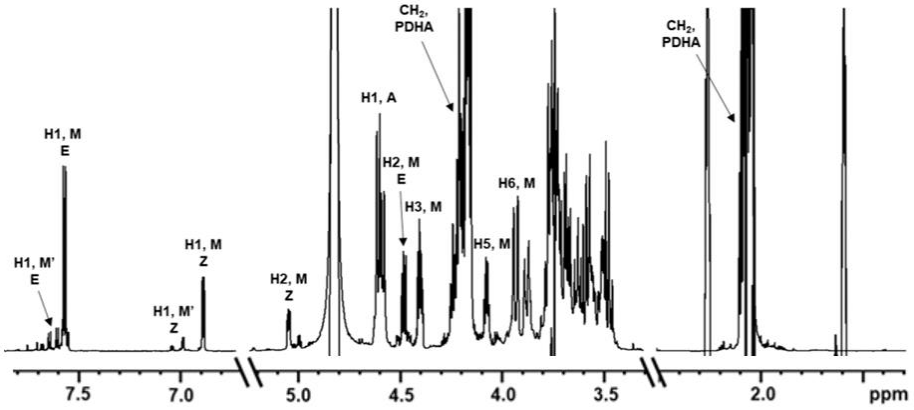

Figure 2-22: [1]H-NMR (600MHz, 300K) spectrum of chitin oligosaccharides of the type A_nM conjugated to PDHA (A_nM-PDHA) (4).

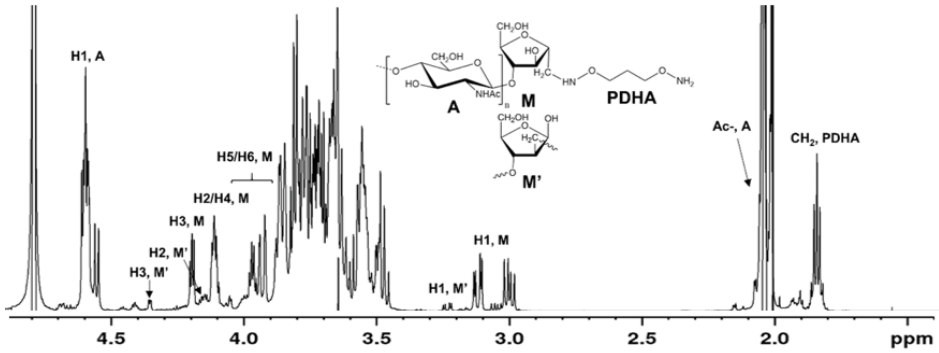

Figure 2-23: [1]H-NMR (600MHz, 300K) spectrum of reduced A_nM-PDHA (4).

2.7.2.1.3 [1]H-NMR Spectrum of Dextran

[1]H-NMR spectra of dextran have previously been annotated by Seymour, Knapp and Bishop (1979). The internal H-residues (H2-H6) are found in the 3-4 ppm area while the resonances necessary to decide degree of polymerization and branching are found in the 4-6 ppm area. The proton attached to C1 (H1) of the internal glucose residues corresponds to the doublet at 4.9 ppm. Along with the anomeric protons which are present in both α and β configuration, at ~5.2 and ~4.6 ppm (respectively), the degree of polymerization can be determined. Additionally, the resonance at ~5.3 ppm corresponds to the H1 at branching points, from which the degree of branching can be determined. A [1]H-NMR spectrum of dextran with annotated peaks are shown in Figure 2-24 along with the structure of the polysaccharide.

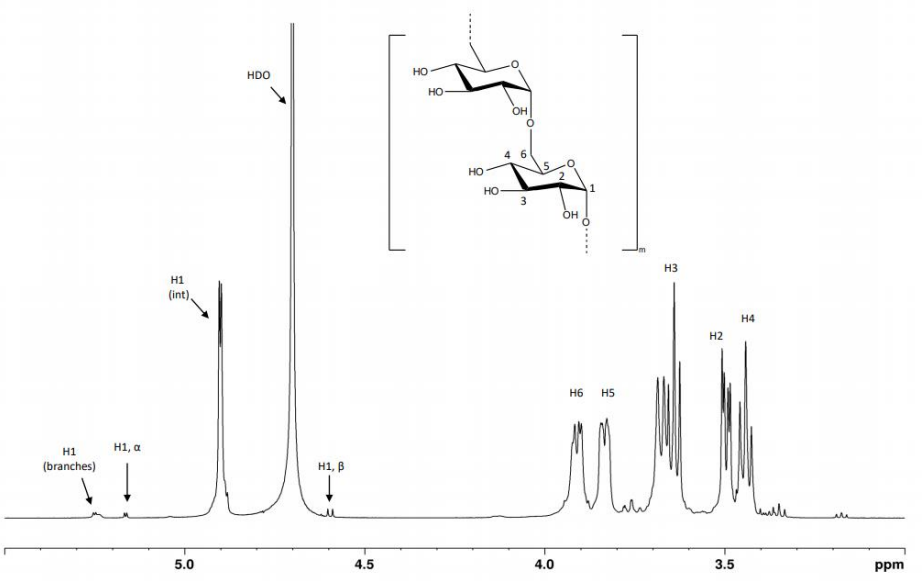

Figure 2-24: ^{1}H-NMR (600 MHz, 298K, D_2O) of dextran including numbered structure and annotated peaks.

3 Materials and Methods

3.1 Materials

Chitosan (F_A=0.48, M_w=722 kDa, M_n=345 kDa) was obtained from Advanced Biopolymers (Norway). Dextran T-2000 was purchased from Pharmacia Fine Chemicals. O,O'-1,3-propanediylbishydroxylamine dihydrochloride (PDHA), 2-methylpyridine borane complex (α-picoline borane, PB) were purchased from Sigma Aldrich. All other chemicals were obtained from commercial sources.

3.2 Size Exclusion Chromatography

Analytical or preparative SEC were conducted by dissolving 8-10 mg or 200 mg of sample (respectively) in 4 mL ammonium acetate either 0.1 M (pH=6.9) or 0.15M (pH=4.5) and filtered through 0.45 μm before injection. The mobile phases varied due to adaption to anionic and cationic polymers, however as chitin is neutral both systems could be used based on availability.

The SEC system was composed of three HiLoad 26/60 columns, 26 mm x 60 cm, packed with Superdex 30 connected in series. The flow rate of the system was set to 0.8 mL/min and the analyses were carried out at ambient temperature. The oligomers were detected by a refractive index (RI) detector (SHODEX R1-101). The data acquisition was performed by Windaq software and the resulting chromatogram shows RI (mV) as a function of elution time (min). For preparative runs the oligomers were collected by a fraction collector (either LKB 2111 Multirac KS1 or FRAC 100) and freeze dried five times to remove the ammonium acetate.

3.3 Size Exclusion Chromatography with Multi Angle Laser Light Scattering

The sample (2-5 mg/mL) was dissolved in MQ to 2x concentration and put on shaking for 24 h. Before analysis the sample was diluted to 1x concentration with mobile phase and filtered through 0.45 μm. The run time was set to 80-100 min depending on the sample, with two injections of 100 μL to assure reproducibility. Pullulan (DP_n=137), MQ water and polyethylene oxide (PEO) were used as standards.

The systems were composed of a precolumn and two serial connected columns (TSKgel 4000 PWXL and TSKgel 2500 PWXL or TSKgel 4000 PWXL and TSKgel 3000 PWXL). The mobile phases were either 0.15 M $NaNO_3$ with 10 mM EDTA (pH=6.0) or 0.2 M ammonium acetate (pH=4.5) depending on if the samples were anionic or cationic (respectively). The

flow rate was set to 0.5 mL/min and the analyses were carried out in ambient temperature. The oligomers were detected by two on-line detectors; multiangle light scattering photometer Dawn DSP at λ_0 = 633 nm (Wyatt, USA) and refractive index SHODEX RI-501. Some samples were additionally analyzed by a Viscotek TDA 301 viscosity detector.

The software ASTRA 7.3.2 (Wyatt, USA) was used for recording and processing of the data. Dn/dc and A2 was set to 0.148 ml/g and $2x10^{-4}$ for pure dextran samples, these values were also assumed for dextran-A_nM conjugates.

3.3.1 SEC-MALLS in 0.9% LiCl/DMAc

This part was outsourced to the laboratory of Prof. Antje Potthast, Universität für Bodenkultur, Vienna.

The samples were dissolved in 0.9% LiCl/DMAc (5 mg/mL) except from $(A_nM)_{wis}$ which was dissolved in 8% LiCl/DMAc (50 mg/mL). The samples were filtrated and 100 µl was injected. The system was composed of one pre-column and four serial coupled columns (PL gel mixedA ALS (now Agilent), 20µm, 7.5x300 mm). The flow rate was 1.00 mL/min. The samples were detected by a multiangle light scattering photometer Dawn DSP at λ_0 = 488 nm (Wyatt, USA) and a refractive index (RI) detector Shodex RI 71. Dn/dc and A2 was set to 0.136 ml/g and 0, respectively.

3.4 Nuclear Magnetic Resonance Spectroscopy

The sample of interest (~5 mg) was dissolved in 500-600 µL D_2O (99.9%) or deuterated sodium acetate (500 mM, pH=3.8, pD=4.2) containing 2,2,3,3-d(4)-3(trimethylsilyl)propionic acid (TSP, 2 mM) and transferred to a 5 mm NMR-tube. The spectra were recorded in a Bruker Ascend 14.1T 600 MHz spectrometer with Avance III HD electronics and a 5 mm Z-gradient CP-TCI cryogenic probe (Bruker BioSpin AG, Fällanden, Switzerland) at 298K or 355K and processed and analyzed by the software TopSpin 4.0.9.

3.5 Preparation and Characterization of Chitin Oligosaccharides and Conjugates

3.5.1 Nitrous Acid Degradation of Chitosan to Yield A_nM Oligosaccharides

Chitosan (F_A = 0.48, M_w=722 kDa, M_n=345 kDa, free amine form) was dissolved in acetic acid (2.5 vol %, 20 mg/mL) by stirring overnight. The solution was bubbled with nitrogen gas (N_2) for approximately 15 minutes to remove dissolved oxygen. A solution of sodium nitrite ($NaNO_2$) (20 mg/mL, 1.3 equivalents of $NaNO_2$ to D-units) was bubbled with N_2 and added in

3 intervals of 45 minutes. The reaction was proceeded in the dark at 4°C overnight while stirring. The solution was centrifuged (10 min, 7142 rcf) 3 times and the pellet was washed with acetic acid (2.5 vol%) in between. The supernatant containing the soluble low molecular weight A_nM oligosaccharides was filtered (5 and 0.45 µm) before it was freeze dried. The sample was fractionated by SEC and characterized by ^{1}H-NMR as described in section 3.2 and 3.4 respectively.

3.5.2 Processing of the Water Insoluble Fraction of A_nM Oligosaccharides

The water insoluble fraction of A_nM ($DP_n > 9$, hereafter called $(A_nM)_{wis}$) from the experiment described in section 3.5.1 was centrifuged (7142 rcf, 10 min) and washed with MQ water two times to remove residual acetate before freeze drying.

3.5.3 Conjugation of A_nM to PDHA and Subsequent Reduction

A_nM (20.1 mM) and 10 equivalents of PDHA (201 mM) was dissolved in sodium acetate (500 mM, pH=4). The reaction was left in RT for a minimum of 6 h on a shaking device. 20 molar equivalents of α-picoline borane (PB) were added and left to react for 48 h in RT on a shaking device. The mixture was dialyzed against NaCl (50 mM) (molecular weight cut off (MCWO)=100-500 kDa) until the PB particles were dissolved. The mixture was freeze dried and purified by SEC and characterized by ^{1}H-NMR as described in section 3.2 and 3.4.

3.5.3.1 Optimization of Reduction Protocol

Increasing the temperature is widely known to increase the reaction kinetics of certain reactions. Thus, it was attempted to increase the temperature of the reduction step of the reductive amination to 40°C to allow for faster reduction. The protocol above was followed, only it was done at 40°C in deuterated sodium acetate buffer to allow for ^{1}H-NMR measurements during the time course of the reaction.

3.6 Preparation and Characterization of Dextran Conjugates

3.6.1 Acid Hydrolysis to Obtain Dextran Oligosaccharides

Dextran T-2000 (50 mg/mL, M_w = 2 000 000 g/mol) was dissolved in MQ water by stirring for 24 h. Equivalent volume of HCl (0.1 M) was added to the dextran solution to a final concentration of 25 mg/mL dextran and 0.05 M HCl in a preheated container (~ 95°C). The reaction was left at 95°C for 90 minutes. To terminate the reaction the solution was instantly cooled down to RT followed by pH-neutralization using NaOH for stabilization. Excessive fluid was removed using a rotavapor before dialysis (MCWO=3.5 kDa) against MQ water to

remove any salt in the sample to a conductivity of < 3 µS/cm. The hydrolysate was freeze dried.

3.6.2 Conjugation of Dextran Oligosaccharides to PDHA

The acid hydrolyzed dextran (20.1 mM) was dissolved in sodium acetate (500 mM, pH=4.0). 10 equivalents of PDHA were added and allowed to react for 24 h in RT. 20 equivalents of α-picoline borane were added and the reaction was continued for 69 h at 40°C. Excessive α-picoline borane was removed by dialysis against NaCl (50 mM, MCWO=3.5 kDa), followed by dialysis against MQ water to remove excessive salt to a conductivity < 3 µS/cm. The sample was freeze dried before it was characterized by ^{1}H-NMR (section 3.4), fractionated by SEC (section 3.2), and the fractionated samples were characterized by SEC-MALLS (section 3.3).

3.7 Reaction Kinetics of the Reductive Amination of Chitin to Dextran-PDHA

3.7.1 Reductive Amination of A_5M to $Dext_{15}$-PDHA

Purified A_5M (3.5 mM) was dissolved in deuterated sodium acetate buffer (500 mM, pH=4) containing TSP as internal standard. Equimolar amounts of $dext_{15}$-PDHA were added and the reaction was left at RT. The reaction was studied by regularly obtaining ^{1}H-NMR measurements until stabilized. 3 equivalents of α-picoline borane were added to the reaction mixture and shaken. The reaction was left at RT with regularly obtaining ^{1}H-NMR measurements until the reduction was completed.

RT-NMR was used to study the course of the conjugation by monitoring the appearance of E- and Z-oxime resonances (6.8 ppm – 7.5 ppm), and to study the reduction by monitoring the disappearance of the same resonances as well as the increased intensity of the secondary amine resonances (2.9 ppm – 3.6 ppm). For the reduction, resonances from PB shielded the E-oxime signal. However, as all the resonances corresponding to PB have the same intensities, the integral of the E-oxime resonance was obtained by subtraction. All spectra were calibrated by TSP and all resonance intensities were related to H1,Glc (int) as this was assumed constant throughout the reaction.

3.7.2 Optimization of the Reduction Protocol of A_nM-PDHA-Dextran Diblocks

Purified A_5M (20.1 mM) was dissolved in deuterated sodium acetate buffer (500 mM, pH=4) containing TSP as internal standard. Equimolar amounts of $dext_{15}$-PDHA were added and the

reaction was left at RT overnight. 20 equivalents of α-picoline borane were added to the reaction mixture and shaken. The reaction was left at 40°C with regularly obtaining ^{1}H-NMR measurements until the reduction was completed.

RT-NMR was used to study the course of the reduction by the same analysis method as described above.

3.8 Periodate Oxidation of Chitin Oligosaccharides

3.8.1 Calibration of Appropriate Concentration

A_2M was dissolved in MQ water to final concentrations of 1 mg/mL, 5 mg/mL and 10 mg/mL. The solution was bubbled with N_2 (g) for 10 min to remove dissolved oxygen. Optical rotation was measured at 589 nm using Anton Paar MCP5100 polarimeter.

3.8.2 Optical Rotation Measurements of Periodate Oxidation of A_2M Oligosaccharides

A_2M was dissolved in MQ water (10 mg/mL) and bubbled with N_2 (g) for 10 min to remove dissolved oxygen. A freshly prepared solution of sodium metaperiodate ($NaIO_4^-$) was bubbled with N_2 (g) and added to give a final molar ratio of 1:2, 1:1, and 2:1 ($NaIO_4^-$: A_2M). The reaction was left in the dark at 4°C. Optical rotation was measured at 589 nm every three hours using Anton Paar MCP5100 polarimeter. After 40 h ethylene glycol (10 molar equivalents to $NaIO_4^-$) was added and left for 30 min to terminate the reaction. The reaction mixture was dialyzed (MCWO=100-500 Da) against water to a conductivity of 0 µS/cm and freeze-dried.

This experiment was also conducted using glucose (10 mg/mL) with 1 molequivalent of sodium metaperiodate, serving as a control.

The specific rotation was calculated according to

$$\alpha = \frac{[\alpha]_\lambda^T \times 100}{c \times l} \qquad \text{(I)}$$

where α is the optical rotation measured by the instrument, $[\alpha]_\lambda^T$ is the specific rotation at temperature T and wavelength λ, c is concentration of the sample in g/100 mL and l is the length of the sample cell in dm.

3.9 Simultaneous Nitrous Acid Degradation and Conjugation to PDHA

3.9.1 Nitrous Acid Degradation of Chitosan with Simultaneous Conjugation to PDHA

Chitosan (F_A=0.48, 40 mM) was dissolved in acetic acid (2.5 vol%) by stirring overnight in RT. The solution was bubbled with N_2 for 15 min to remove dissolved oxygen. 10 equivalents of PDHA were added followed by addition of a solution of sodium nitrite ($NaNO_2$) (40 mM) in three intervals of 45 min. The reaction was run for 70 h in the dark at 4°C while stirring. The pH was adjusted to pH=6 (NaOH) to terminate the reaction. The solution was centrifuged (15 min, 4226 rcf) 3 times and the pellet was washed with sodium acetate (500 mM, pH=6 to avoid oxime hydrolysis) in between. The supernatant was dialyzed (MCWO=100-500 kDa) against MQ water to a conductivity of < 2 µS/cm. The sample was freeze dried and characterized by SEC and ^{1}H-NMR. The insoluble fraction was processed as described in section 3.5.2.

3.10 Dissolution of the Water Insoluble Fraction of Chitin Oligosaccharides

3.10.1 Exploratory Study of Dissolution of the Water Insoluble Chitin Oligosaccharides

$(A_nM)_{wis}$ (water insoluble fraction of A_nM oligosaccharides) (5 mg/mL) produced in section 3.5.2 was dissolved in dimethylsulfoxide (DMSO), dimethylacetamide (DMAc), DMAc containing 8% LiCl, 5 M sodium hydroxide (NaOH), 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and H_2O (which served as negative control). The samples were shaken for several days with regular visual observation. Additionally, the sample dissolved in DMAc was heated to 100°C after 48 h, whilst the samples dissolved in 8 wt% LiCl-DMAc and DMSO were heated to 60°C.

3.10.2 Concentration Dependence of A_nM Dissolved in 8 wt% LiCl/DMAc

$(A_nM)_{wis}$ (5 mg/mL) was dissolved in different ratios of water and 8wt% LiCl/DMAc by heating to 60°C and investigated by microplate assay. The percentage of water in the solvents examined was 1%, 2.5%, 5%, 10%. Additional samples containing A_nM dissolved in 100% LiCl/DMAc and 100% H_2O served as control samples. The microplate assay was performed by Tecan Plate Reader at 600nm and the experimental set up is shown in Table 2.

Table 2: The content of each well assayed by microplate. Column 1 contained $(A_nM)_{wis}$ (5 mg/mL) in each well with different concentrations of solvent while column 2 contained pure solvent. All wells had a volume of 100 µL.

	1	2
A	100% LiCl/DMAc	Pure 100% LiCl/DMAc control
B	1% H_2O, 99% LiCl/DMAc	
C	2.5% H_2O, 97.5% LiCl/DMAc	
D	5% H_2O, 95% LiCl/DMAc	
E	10% H_2O, 90% LiCl/DMAc	
F	100% H_2O	Pure 100% H_2O as control

3.11 Preparation of Water-Soluble Chitin-based Block Polysaccharides in LiCl/DMAc

3.11.1 Conjugation of A_4M to A_4M-PDHA in LiCl/DMAc

A_4M (20.1 mM) was dissolved in 8 wt% LiCl/DMAc containing 1% H_2O and briefly heated to 60°C for complete dissolution. Equimolar amounts of fully reduced A_4M-PDHA were added and the reaction was left in 40°C for 48 h. The reaction mixture was diluted 2x with MQ water before it was dialyzed (MCWO=100-500 Da) against MQ water to a conductivity of 0 µS/cm. The water used for dialysis was kept above pH=6 to avoid possible oxime hydrolysis. The solution was freeze dried.

3.12 Preparation of Water Insoluble Chitin-based Block Polysaccharides in LiCl/DMAc

3.12.1 Conjugation of Equimolar Amounts of $(A_nM)_{wis}$ to Dextran-PDHA in LiCl/DMAc

$Dext_m$-PDHA (40.1 mM, m=DP_n=23 or 37) was dissolved in 8wt % LiCl/DMAc containing 1% water. The solution was heated briefly to 90°C for complete dissolution. $(A_nM)_{wis}$ (40.1 mM, assumed DP_n=12) was dissolved in the same solvent and briefly heated to 90°C for complete dissolution. The components were mixed to a final concentration of 20.1 mM, and the reaction was performed at 40°C for 44 h. The mixture was diluted 2x with MQ water and dialyzed (MCWO=100-500 Da) against MQ water (pH > 6) to a conductivity of 0 µS/cm. The mixture was centrifuged (7142 rcf ,10 min) and washed two times to remove excess $(A_nM)_{wis}$ before freeze drying.

3.12.1.1 Triethylamine as Catalyst in the Preparation of Diblocks in LiCl/DMAc

With inspiration from Röhrling *et al*. (28) the experiment described in section 3.12.1 was conducted again with triethylamine (TEA) as a base catalyst. The experiment was performed as described above, but with addition of equimolar amounts of TEA to PDHA as well as the reaction time was shortened to 6 h and 24 h (in two separate experiments).

3.12.2 Conjugation of an Excess of $(A_nM)_{wis}$ to $Dext_m$-PDHA in LiCl/DMAc

$Dext_{37}$-PDHA (40.1 mM) was dissolved in 8wt % LiCl/DMAc containing 1% water. The solution was heated briefly to 90°C for complete dissolution. 3 equivalents of $(A_nM)_{wis}$ (120.6 mM, assumed DP_n=12) was dissolved in the same solvent and briefly heated to 90°C for complete dissolution. The components were mixed to a final concentration of 20.1 mM and 60.3 mM for $dext_m$-PDHA and $(A_nM)_{wis}$, respectively, and equimolar amounts of TEA to PDHA was added. The reaction was performed at 40°C for 6 h. The mixture was diluted 2x with MQ water and dialyzed (MCWO=100-500 Da) against MQ (pH > 6) to a conductivity of 0 µS/cm. The mixture was centrifuged (7142 rcf, 10 min) and washed two times with MQ water to remove excess $(A_nM)_{wis}$ before freeze drying.

3.13 Real Time NMR with Deuterated 8wt% LiCl/DMAc as Solvent

Reduction and conjugation of A_2M (separate reactions) were performed by dissolving A_2M (17.6 mM) in 8wt% LiCl/DMAc (deuterated) followed by addition of either 3 equivalents of α-Picoline Borane or 2 equivalents of PDHA, respectively. Reduction of $(A_nM)_{wis}$=PDHA-$Dext_{23}$ ("=" means unreduced) was performed by dissolving the diblock (2.6 mM) in 8wt% LiCl/DMAc (deuterated) with 1% D_2O (99.9%) followed by addition of 3 equivalents of PB. A small amount of TSP was added to all tubes for calibration. All three reactions were performed in RT with regularly obtained ^{1}H-NMR (298K an 355K, 600 MHz) spectra.

The concentrations used were based on the mass of sample needed to obtain adequate ^{1}H-NMR spectra, thus it deviates from standard concentration of 20.1 mM.

3.14 Reaction Kinetics of Chitin-based Block Polysaccharides in LiCl/DMAc

3.14.1 Water-soluble A_nM-based Block Polysaccharides

A_2M-PDHA (40.2 mM) was dissolved in 8wt % LiCl/DMAc containing 1% water. The solution was heated briefly to 60°C for complete dissolution. Equimolar amounts of A_2M (40.2 mM) were dissolved in the same solvent and briefly heated to 60°C for complete

dissolution. The components were mixed to a final concentration of 20.1 mM and 1 molequivalent of triethylamine (to linker) was added. The reaction was conducted at 40°C for 23 h, whilst aliquots of the mixture were obtained and terminated after 1 h, 3 h, 6 h, 10 h and 23 h. Each sample was diluted 2x with MQ water and dialyzed (MCWO=100-500 Da) against MQ (pH > 6) to a conductivity of 0 µS/cm before freeze drying and ^{1}H-NMR characterization.

The experiment was reproduced only varying the time for which aliquots of the mixture were obtained to 0.25 h, 0.5 h, 0.75 h, 1 h, 1.5 h and 3 h.

^{1}H-NMR was used for determination of the conjugation yield in the different samples. The resonance corresponding to H1,A was used as internal standard to identify the relative oxime yield in each sample.

3.14.2 Water Insoluble A_nM-based Block Polysaccharides

$(A_nM)_{wis}$ (40.2 mM, assumed DP_n=12) was dissolved in 8wt % LiCl/DMAc containing 1% water. The solution was heated briefly to 90°C for complete dissolution. Equimolar amounts of $dext_{37}$-PDHA (40.2 mM, M_n=6000 g/mol) were dissolved in the same solvent and briefly heated to 90°C for complete dissolution. The components were mixed to a final concentration of 20.1 mM and 1 equivalent of triethylamine (to linker) was added. The reaction was conducted at 40°C for 6 h, whilst aliquots of the mixture were obtained and terminated after 0.25 h, 0.5 h, 0.75 h, 1 h, 3 h and 6 h. Each sample was diluted 2x with MQ water and dialyzed (MCWO=100-500 Da) against MQ (pH > 6) to a conductivity of 0 µS/cm and freeze dried. The freeze dried sample was redissolved in a small amount of water and centrifuged (7142 rcf, 10min) and washed 2 times to remove any precipitate. The supernatant was freeze dried and characterized by ^{1}H-NMR.

^{1}H-NMR was used for determination of the conjugation yield in the different samples. The resonance corresponding to H1,Glc (int) of dextran was used as internal standard to identify the relative oxime yield in each sample.

4 Results and Discussion

4.1 Characterization of Chitin Oligosaccharides and Chitin Conjugates

Chitosan (F_A=0.48, M_w=723 kDa, M_n=345 kDa, free amine form) was degraded by an excess of nitrous acid (relative to D-units) to obtain chitin oligosaccharides with a reactive M-unit at the reducing end (A_nM oligosaccharides). Chitin conjugates were produced by conjugating A_nM to PDHA with subsequent reduction by α-picoline borane. The water-soluble A_nM oligosaccharides and the A_nM-PDHA conjugates were separated by SEC and characterized by ^{1}H-NMR.

4.1.1 Characterization of Chitin Oligosaccharides Obtained by Nitrous Acid Degradation

Fully N-acetylated chitin oligosaccharides with 2,5-anhydro-D-mannose at the reducing end (A_nM) was obtained by nitrous acid degradation of chitosan (F_A=0.48) as described in section 3.5.1. The degradation yields A_nM oligomers with various degrees of polymerization due to the random distribution of A- and D-units in the parent chitosan (44). Oligomers with DP < 5 are readily soluble in aqueous solutions, whereas 5 < DP < 9 becomes gradually less soluble and DP > 9 are essentially water insoluble.

The sample with the mixture of the different soluble A_nM oligosaccharides was characterized by ^{1}H-NMR spectroscopy as described in section 3.4 (Figure 4-1). Lack of signals characteristic for the D-units in chitosan, such as H2,D at ~3 ppm (21), confirmed that the degradation was complete yielding solely fully N-acetylated chitin oligomers.

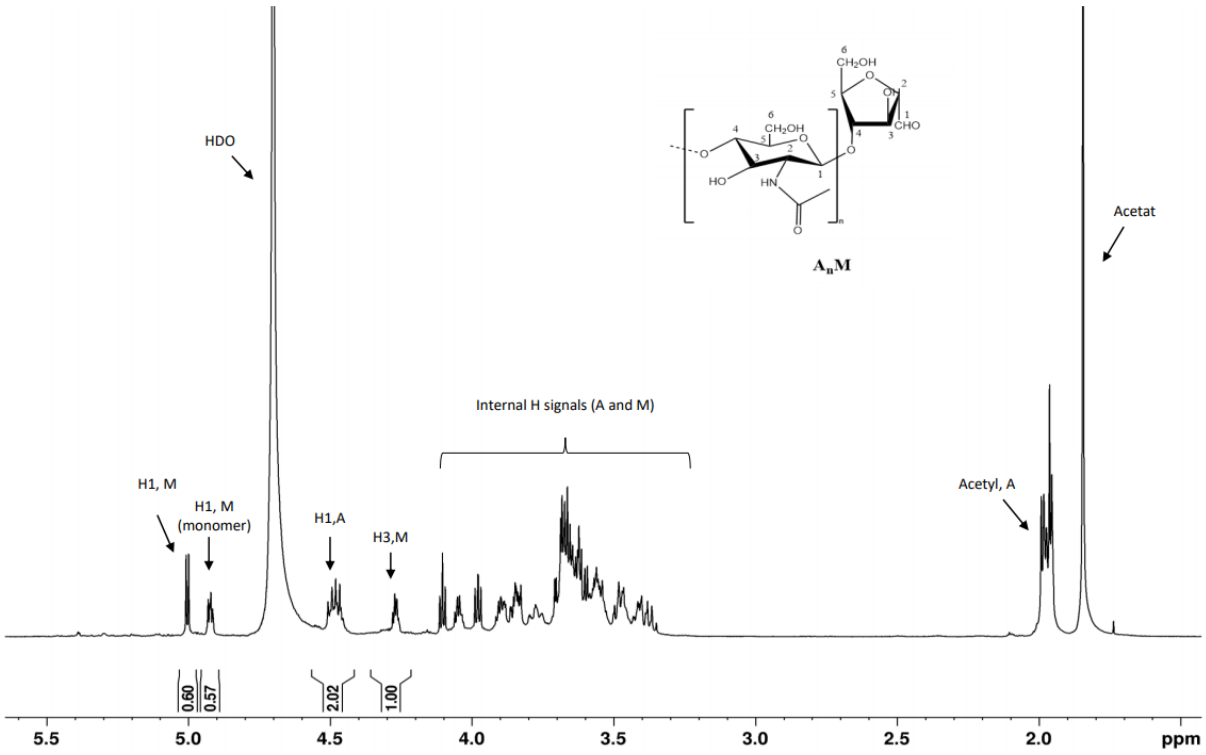

Figure 4-1: ^{1}H-NMR (600MHz, 298K, D_2O) of unfractionated fully N-acetylated A_nM oligosaccharides obtained by nitrous acid degradation of chitosan (F_A=0.48). M and A represents the respective monomers in the oligosaccharides.

The mixture of oligosaccharides with different degree of polymerization was fractionated by preparative SEC as described in section 3.2 using three series coupled HiLoad 26/60 columns packed with Superdex 30 and 0.1 M ammonium acetate (pH=6.9) as mobile phase. The resulting chromatogram is shown in Figure 4-2 along with how the fractions were collected (F1-F7). The chromatogram is in accordance with the findings by Mo *et al.* (4).

^{1}H-NMR of fraction 3 (marked in Figure 4-2) was used to determine and calibrate the DP within the respective fractions (Appendix A). The ratio between the H1,A resonance and the H3,M resonance was 5:1, meaning there are five A residues for each M residue - A_5M (DP=6).

Evident from the chromatogram, complete baseline separation was not achieved. As a result of this, there was a slight possibility of not obtaining completely monodisperse samples. As

an example, the trimer fraction (F6) might be (to a small degree) contaminated by the adjacent eluted samples; tetramer (F5) and dimer (F7).

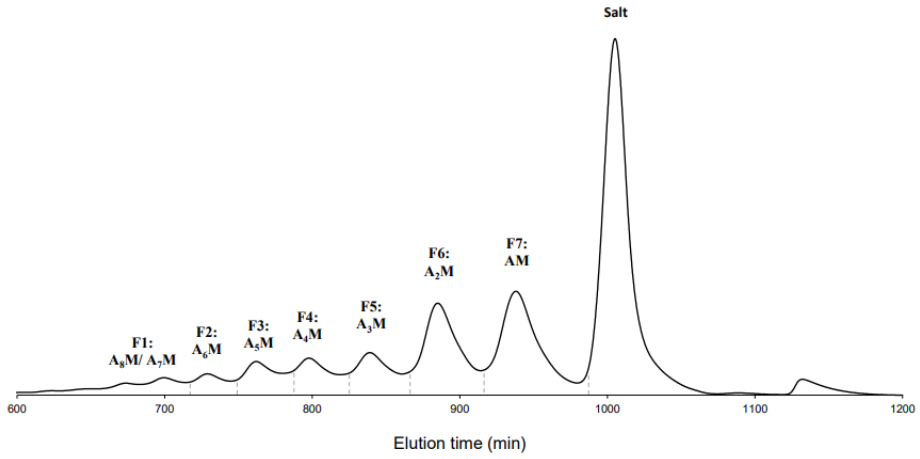

Figure 4-2: Size exclusion chromatogram (0.1 M AmAc, pH=6.9) of fully N-acetylated chitin oligosaccharides with 2,5-anhydro-D-mannose at the reducing end (A_nM) obtained by nitrous acid degradation of chitosan (Fa=0.48). Including how the fractions were collected as well as degree of polymerization determined by ^{1}H-NMR analysis.

During the experiment an interesting observation was made. After the centrifugation the supernatant was transferred into three centrifuge tubes whereas two of them had a brown-yellow color after being freeze dried (Figure 4-3). This is due to the free M-monomers undergoing a reaction similar to Maillard browning. As the starting chitosan had a F_A of 0.48 and was treated with an excess of nitrous acid to D-units, it is obvious that the transformation of D-units to M-units will give rise to a certain number of free M-monomers. All D-units which does not directly bind to an A-unit will be cleaved and transformed to M-monomers. The M-monomer undergoes acid catalyzed dehydration producing the compound 5-hydroxymethylfurfural (HMF) which gives a distinct brown-yellow color (21,45).

After the first centrifuge run there was an excess of supernatant which was filtered and distributed into two tubes. This supernatant probably contained more of the readily soluble shorter oligosaccharides, as well as the free M-units which explains the color development. The last tube contained supernatant from the three rounds of washing and is believed to contain more of the longer oligosaccharides which are more challenging to get in solution and thus it did not develop a color. The sample was purified by size exclusion chromatography, where HMF was removed, and the color disappeared along with it.

Figure 4-3: Color development during processing of nitrous acid degraded A_nM due to formation of 5-hydroxymethylfurfural.

4.1.2 Conjugation of A_nM to PDHA with Optimization of the Reduction Protocol

Conjugation of A_nM to PDHA was conducted according to the protocol described in section 3.5.3. The standard protocol of the subsequent reduction of chitin-conjugates is to react for 48 h in RT. Here, increasing the temperature to 40°C was attempted to increase the reaction rate (protocol described in section 3.5.3.1).

^{1}H-NMR spectra were obtained for the A_5M oligomer in D_2O before reaction initiation, after 6 h of conjugation to PDHA, and after 18 h of reduction at 40°C (Figure 4-4). The relative yield of the conjugation was calculated from the appearance of the E- and Z-oxime resonances (7.6 and 7.0 ppm, respectively) relative to H1,A to be 97% after 6 h, in agreement with previous results (4). Reducing agent was added and allowed to react for 18 h at 40°C. ^{1}H-NMR spectroscopy was conducted and lack of signals from E and Z-oxime and the presence of secondary amine resonances showed that the compound was fully reduced.

Minor resonances appeared slightly upfield of the main resonances for the H1,M signals both in gem-diol form and as oxime conjugations. These resonances are attributed to an alternative M form (M') created during nitrous acid degradation, which has the aldehyde located at C2 (5,46). Nonetheless, it seems to react with PDHA in the same manner as the normal M, thus it poses no problem.

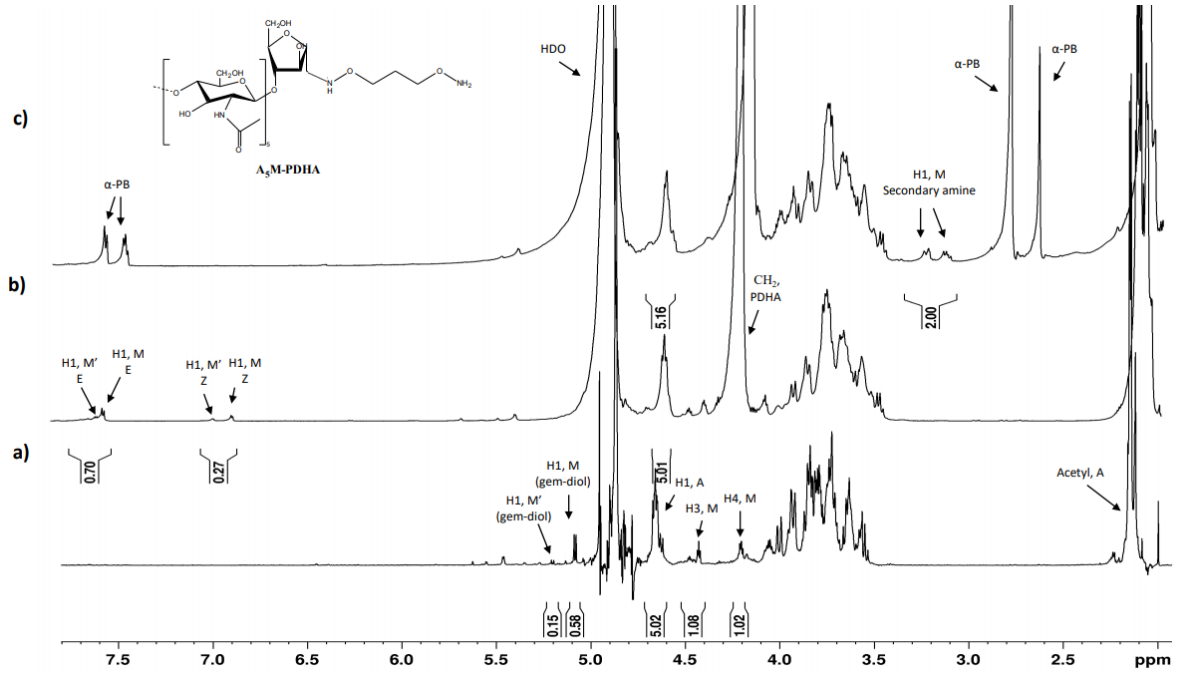

Figure 4-4: ^{1}H-NMR (298 K, 600 MHz) spectra of a) A_5M in D_2O, b) A_5M=PDHA ("=":unreduced) in deuterated NaAc buffer (500 mM, pH=4) after 6 h conjugation in RT c) A_5M-PDHA in deuterated NaAc buffer after 18 h of reduction at 40°C. TSP was used as internal standard for chemical shift reference (0 ppm) in b) and c). Including structure of the finished conjugate. M' represents the alternative form of the M-residue.

Based on these and previous results, complete reduction of A_nM-PDHA conjugates can be obtained by addition of 20 equivalents of α-picoline borane and a reaction time of either 48 h in RT or 18 h at 40°C. Both protocols were used throughout the work of this master thesis depending on time- and instrument-availability.

4.1.3 Fractionation of A_nM-PDHA Conjugates

After conjugation and reduction to obtain A_5M-PDHA conjugates, the sample was fractionated by SEC to remove any diblock formation and unconjugated oligosaccharides (section 3.2). The chromatogram is shown in Figure 4-5, whereas the conjugate is eluting slightly earlier than pure A_5M due to the attachment of PDHA, at 720-750 min. A small amount of disubstituted species were formed (A_5M-PDHA-MA_5) as seen at approximately 600-650 min, as well as some traces of unconjugated A_5M at 750-800 min. Minor traces of oligomers with lower DP were also detected (> 800 min), accounting for the slight polydispersity in the sample due to incomplete baseline separation during the original fractionation of the acid degraded oligosaccharide mixture (section 4.1.1). The sample had

previously been stated to be fully conjugated and reduced by the ^{1}H-NMR spectra obtained during the reactions (section 4.1.2, Figure 4-4).

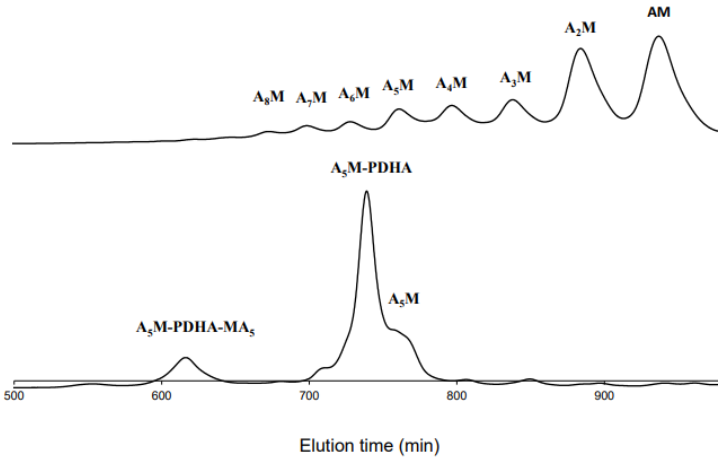

Figure 4-5: Size exclusion chromatogram (0.1M AmAc, pH=6.9) of the reaction mixture of A_5M-PDHA Fractionation of the mixture of fully N-acetylated A_nM oligomers included for comparison.

The activation and purification of A_nM-PDHA conjugates described in this section was a part of the sample preparation conducted in the beginning and throughout when needed for later use in the work in this master thesis.

4.2 Characterization of Dextran Conjugates

This experiment was done in collaboration with other master students. Dextran T-2000 (M_w = 2 000 000 g/mol) was degraded by acid hydrolysis to obtain dextran oligosaccharides. The dextran oligosaccharides were coupled to PDHA by reductive amination before the mixture was separated using SEC and characterized by ^{1}H-NMR and SEC-MALLS.

4.2.1 Characterization of Dextran-PDHA Obtained by Acid Hydrolysis with Subsequent Reductive Amination

After degradation of high molecular weight dextran and reductive amination to prepare dext$_m$-PDHA conjugates (m=DP_n) (described in section 3.6), ^{1}H-NMR spectrum was obtained of the unfractionated sample (Figure 4-6). The aim of this analysis was to determine if the sample had been fully conjugated and reduced, as well as determine the DP_n. There were no traces of E/Z-oxime resonances around 6.8 ppm and 7.4 ppm, assuring the sample had been fully reduced. This was also supported by the resonances occurring at 2.8-3.1 ppm belonging to the CH_2 of the secondary amines created by reduction, and no reducing end signals. The DP_n was

calculated to be 41 based on the relationship between H1 in the internal glucose residues and the resonances from the secondary amines.

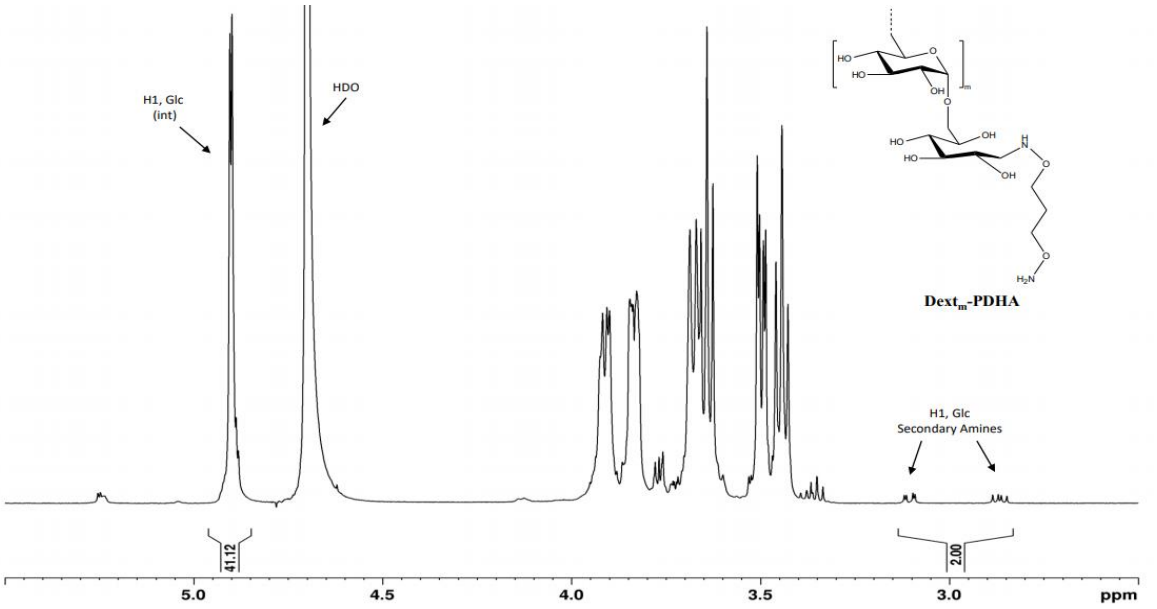

Figure 4-6: ^{1}H-NMR (600 MHz, 298K) spectrum of the unfractionated reduced dextran-PDHA conjugates (DP_n=41) including the structure of the conjugate.

4.2.2 Fractionation and Characterization of Dextran-PDHA Conjugates

Preparative SEC (section 3.2) by three series coupled Hiload 26/60 columns packed with Superdex30 with 0.1 M ammonium acetate (pH=6.9) of the dextran-PDHA mixture (DP_n=41) was employed to obtain fractions with more narrow distributions. The resulting chromatogram is shown in Figure 4-7, along with how the fractions were collected.

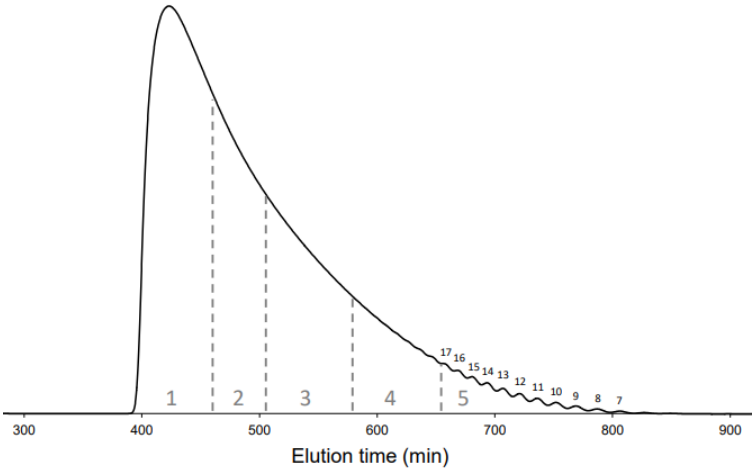

Figure 4-7: Analytical size exclusion chromatogram (0.1 M AmAc, pH=6.9) of dextran-PDHA (DP_n = 41). Including areas which were pooled together as one fraction to narrow the dispersion.

The degree of polymerization of the smaller oligomers are included, obtained from isolation of a narrow peak to obtain a monodisperse sample. The peak eluting at 732-747 min was collected for ^{1}H-NMR analysis (Appendix A). The DP of this sample was determined to be 11 by the relationship between the resonance of H1 of the internal glucose residue relative to the secondary amine resonances.

The five fractions were analyzed by SEC-MALLS to determine molecular weight, polydispersity and DP_n. It was evident from the chromatogram (Appendix A) that fraction 5 (containing the smallest conjugates) had been contaminated with fraction 1 (containing the longest conjugates), thus this fraction was separated with SEC again to remove any contamination.

The results are summarized in Table 3 and confirmed successful fractionation. As expected, there is a decrease in molecular weight from fraction 1-5 and satisfactory low polydispersity within the respective fractions.

Table 3: Physical properties of the fractions of dextran-PDHA collected as shown in Figure 4-7 and analyzed by SEC-MALLS (0.2 M AmAc, pH=4.5, TSKgel 4000 and 3000).

Fraction	M_n (kDa)	M_w (kDa)	Polydispersity (M_w/M_n)	DP_n (M_n/M_0)
1	16	17	1.1	96
2	8.4	8.5	1.0	52
3	5.8	6.0	1.0	37
4	3.8	3.9	1.0	23
5	2.6	2.6	1.0	15

4.3 Reaction Kinetics of the Reductive Amination of Chitin Oligosaccharides to Dextran-PDHA

The reaction kinetics of the reductive amination of water-soluble chitin oligosaccharides (A_nM) to dextran-PDHA was studied by real time NMR (RT-NMR). The reaction kinetics of the reductive amination of A_nM-PDHA to $dext_m$ have previously been characterized by Mo *et al.*(4). The authors proposed that initial PDHA-activation of dextran and subsequent conjugation to A_nM would be more time efficient. This due to the reactivity of the reducing end M-unit which does not participate in the formation of hemiacetals as dextran does.

4.3.1 Kinetic Modelling of the Reductive Amination of Oligosaccharides

The model applied in this section is developed by Mo *et al.*(4) and a detailed description of the model development is given in supplementary information S3 of this article.

The first step of reductive amination is a reversible amination (conjugation) to produce E- and Z-oximes (in the case of M at the reducing end, native reducing ends will additionally produce N-pyranosides). This is followed by an irreversible reduction to form stable secondary amines. A general reaction scheme for the reductive amination of oligosaccharides is given in Figure 4-8. Briefly, assuming first order kinetics with respect to the concentration of each reactant, along with input of experimental data, a model can be achieved by simulating the concentration at successive time increments throughout the reaction. The model was fitted to the data by minimizing the sum of squares of the rate constants.

The objective of the model is to obtain concentration-independent rate constants for a system which serves as a powerful tool to simulate reactions at different concentrations, and as such, to predict optimized reaction conditions.

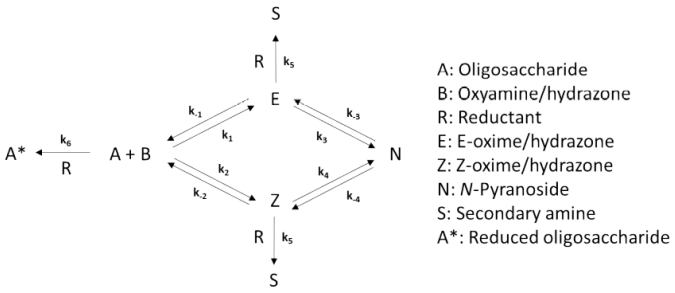

Figure 4-8: General reaction scheme for reductive amination of oligosaccharides with native reducing ends (4). Rate constants are assigned for each reaction step. For chitin and chitosan with a M-unit at the reducing end, no N-pyranosides are formed in which the model is simplified to not contain this step.

4.3.2 Conjugation of A_5M to $Dext_{15}$-PDHA

Conjugation of purified A_5M to fully reduced $dext_{15}$-PDHA was performed under equimolar conditions as described in section 3.7.1. The data collected from integrating the ^{1}H-NMR spectra obtained during the reaction as well as a fitted model to obtain the kinetic parameters of the reaction is presented in Figure 4-9 (^{1}H-NMR spectra are given in Appendix B). The kinetic parameters estimated from the fitted model are summarized in Table 4 along with previous completed comparable experiments. The equilibrium yield was established within 2.5 h, at which the combined yield was 63%.

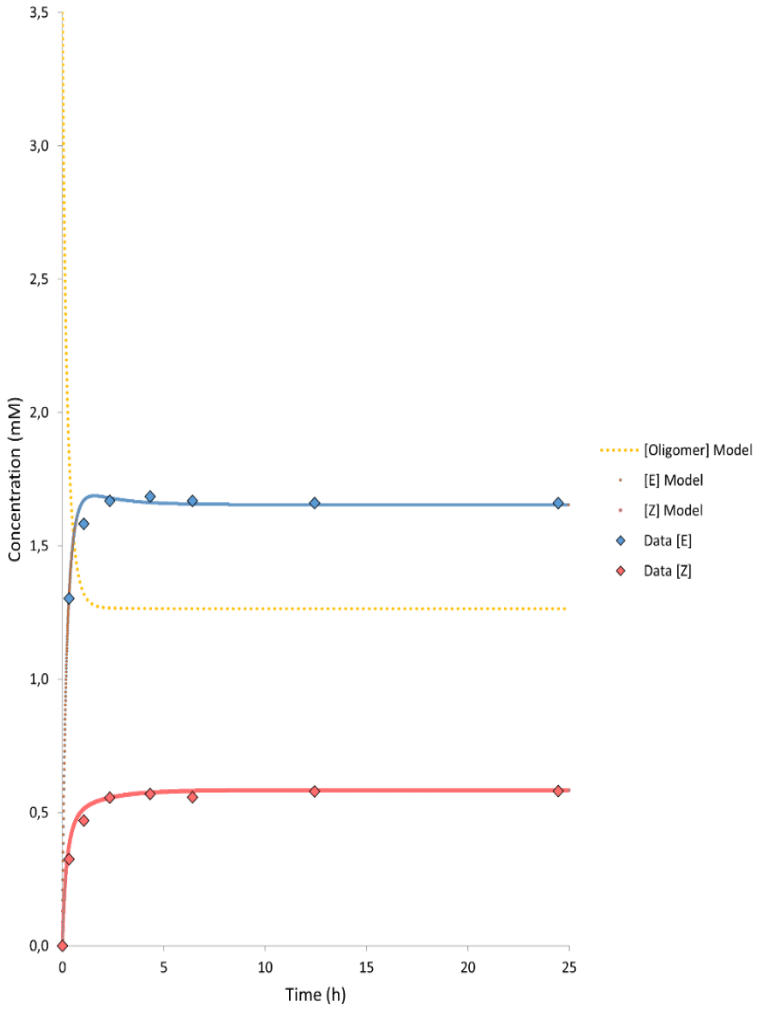

Figure 4-9: Reaction kinetics of the conjugation of A_5M (3.5 mM) to equimolar amounts of $dext_{15}$-PDHA in RT (500 mM NaAc, pH=4).

Table 4: Kinetic parameters obtained from the modelling of the conjugation of A to B. All initial experiments were performed at RT in NaAc buffer (500 mM, pH=4). (E=E-oxime, Z=Z-oxime, P=N-pyranosides).

A	B	c_A (mM)	Ratio	$A+B \leftrightarrow E$		$A+B \leftrightarrow Z$		$A+B \leftrightarrow E+Z+P$		$t_{0.5}$ (h)	$t_{0.9}$ (h)	Equilibrium yield (%)
			A:B	k_1 (h^{-1})	k_{-1} (h^{-1})	k_2 (h^{-1})	k_{-2} (h^{-1})	K_T(h^{-1})	K_{-T} (h^{-1})			
A_5M	$Dext_{15}$-PDHA	3.5	1:1	7.30×10^{-1}	7.06×10^{-1}	2.05×10^{-1}	5.61×10^{-1}	8.86×10^{-1}	6.58×10^{-1}	0.15	0.60	63
[a]A_5M	$Dext_{15}$-PDHA	20.1	1:1	-	-	-	-	-	-	0.03	0.18	
[b]$Dext_6$	A_5M-PDHA	20.1	1:1	1.1×10^{-2}	5.0×10^{-2}	1.5×10^{-3}	1.0×10^{-1}	1.34×10^{-2}	4.72×10^{-2}	2.07	8.36	66
[b]A_2M	PDHA	20.1	1:2	2.4×10^{-2}	2.2×10^{-1}	1.0×10^{-2}	2.2×10^{-1}	-	-	0.24	0.87	91

[a] Simulated from the rate constants obtained at 3.5 mM

[b] Data obtained from Mo *et al*. (4)

The conjugation of A_nM to PDHA-activated dextran was significantly faster than the conjugation of dextran to PDHA-activated A_nM. Evident from Table 4, the rate constants are

generally order(s) of magnitude higher for the former reaction than of the latter. By simplifying the kinetic model, the rate constant k_T describes the rate at which the total of E- and Z-oxime and N-pyranosides are produced, i.e. the rate at which the combined yield is formed. The value of which indicates that the conjugation of A_5M to PDHA-activated dextran is 10 times faster than of the conjugation the other way around. Despite the reduced concentration (3.5 mM vs 20.1 mM), the time taken to reach 90% of equilibrium yield ($t_{0.9}$) was significantly shorter, highlighting the high reactivity obtained by this route.

Furthermore, reaction outcomes from different starting concentrations can be simulated assuming a proportional rate-concentration relationship and concentration independent rate constants. Simulations of $t_{0.5}$ and $t_{0.9}$ at 20.1 mM, conducted with the estimated rate constants from the 3.5 mM reaction, revealed an impressive 70-fold decrease in $t_{0.5}$ and a 45-fold decrease in $t_{0.9}$. Thus, compared with the findings of Mo *et al.* (4) the conjugation of A_nM to PDHA-activated dextran is evidently faster, even at much lower concentrations.

The increased reaction rate of the conjugation of A_nM to $dext_m$-PDHA compared to $dext_m$ to A_nM-PDHA can be explained by the structural chemistry of the reducing end of the two oligosaccharides. The reducing end of dextran is participating in an aldehyde/hemiacetal equilibrium in which the conjugation is only happening when the aldehyde is available. A_nM however, has a pending aldehyde that does not take place in a hemiacetal equilibrium, consequently it is readily available for conjugation. Thus, initial activation of dextran can be performed with a high excess of PDHA (e.g. 10x) to shift the equilibrium which will increase the reaction rate of the first step. Subsequent attachment of A_nM as the second block exploits the high reactivity of the M-residue as the second step is often performed in lower ratios (1:1-3). Indeed, there is also an advantage in that the second block has been shown to attach faster compared to the initial PDHA-activation.

Comparing this reaction to the parameters obtained by conjugating A_2M to free PDHA (with 2xPDHA), there is an increase in the reaction rate as well. There is a ten-fold increase in the rate constants and a significant decrease in $t_{0.5}$ and $t_{0.9}$ (evident when simulating 1:2 molar equivalents). This supports the previous findings that attachment of the second block seems to be more time efficient on an overall basis regarding several polysaccharides, as the free end of the amine linker seems to have increased reactivity after the first attachment (5).

Altogether, this experiment (first mentioned by Mo *et al.* (4)) shows another approach to produce A_nM-PDHA-dextran diblocks than previously described. By initial PDHA-activation

of dextran, an excess can be used to surpass the time-consuming factor of the aldehyde which participates in a hemiacetal equilibrium. With subsequent attachment of A_nM, the high reactivity of the pending aldehyde of the M-residue is exploited. The results demonstrates that the second attachment in this protocol is significantly faster, even at much lower concentrations.

4.3.3 Reduction of A_5M=PDHA-$Dext_{15}$

After equilibrium was established at 63% conjugation (that is 2.2 mM reducible diblock), 3 equivalents of α-picoline borane (PB) were added to assess the reduction rate of A_5M=PDHA-$dext_{15}$ ("=" represents unreduced conjugate). The reduction was terminated at 300h (RT, pH=4) with a yield of 88%.

Due to the time-consuming reduction, an optimized protocol with 20.1 mM oligosaccharides, 20 equivalents PB at 40°C was performed as described in section 3.7.2. A conjugation yield of 63% (that is 12.7 mM reducible diblock) was obtained before reduction was initiated. The identical equilibrium conjugation yields show that the results from the conjugation with A_nM as the second block are reproducible. The reaction was complete after approximately 72 h with 100% reduced conjugates.

A model of the reaction kinetics was fitted to the experimental data (Figure 4-10) for both conditions, assuming the reduction is irreversible and that the rate is proportional to the concentration of the reactants. The ^{1}H-NMR spectra are given in Appendix A.

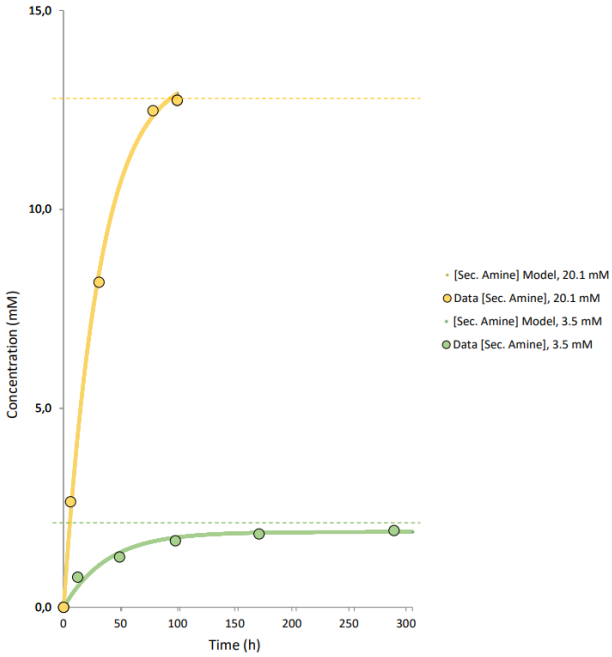

Figure 4-10: Experimental data and a fitted models of the formation of secondary amine (mM) during reduction of A_5M=PDHA-$Dext_{15}$ inn NaAc (500 mM, pH=4) with different conditions: (I) 20.1 mM starting material, 20 x PB, 40°C (yellow) and (II) 3.5 mM starting material, 3 x PB, RT (green). The dotted lines represent maximum reduction yield of the respective reactions.

The reduction yield was calculated from the decrease in E- and Z-oxime resonances relative to the resonance from H1,Glc of dextran as the latter is assumed constant throughout the reaction in both cases. In the first reduction (3.5 mM) the reduction yield was also calculated from the increased signal intensity from the secondary amine resonances. However, it is to be noted that it was not possible to follow the change of intensity of the secondary amine resonances of the optimized reduction due to minor impurities overlapping the signals. The concentration of the secondary amine formation was thus determined by the conversion change of E- and Z-oxime, assuming the intensity decrease of the oxime resonances corresponds to the intensity increase in the secondary amine resonances.

The reduction was initially fast, but the rate of conversion slowed down over time. This can be explained with initial addition of PB there is a bigger excess which yields faster reduction and over time as PB gets oxidized the reaction will deaccelerate. Mo *et al*. (4) studied the relative reducing power of PB in deuterated NaAc (500 mM, pH=4) over time, and at 100 h the relative reducing power has decreased from 100% to ~80% while at 300 h it has decreased to almost 50%. For time consuming reductions this effect could be circumvented by incorporating multiple additions of reducing agent throughout the reaction.

The advantage of using dextran as the second block is that once reduction is initiated, conjugation can proceed as the background reduction of unreacted dextran is low. The equilibrium conjugation yields for the conjugation reactions were 63% and 66%, whereas Mo *et al.* (4) found that after adding reducing agent the conjugation persisted resulting in a final diblock yield of 92%. On the contrary, the unreacted A_nM gets promptly reduced upon addition of PB due to the pending aldehyde that does not participate in a hemiacetal equilibrium – resulting in no further conjugation during reduction. Thus, an important advantage of using dextran as the second block is that higher yields of the diblock are obtained.

In either case, an excess of one of the oligosaccharides (either the activated one or the second block) would result in higher yields. When using this strategy, it is important to consider which oligosaccharide is used in excess. For instance, a surplus of inactivated A_nM would render the unconjugated oligosaccharides inactive after the reduction. To surpass this, an excess of the PDHA-activated oligosaccharide would be preferable as the linker will protect the oligosaccharide and it can thus be recycled at a later point (4). Alternatively, an excess of the oligosaccharide with native reducing end with slow background reduction could be used.

Altogether, the conjugation of A_nM to initially activated dextran is substantially faster than the conjugation of dextran to initially activated A_nM. Although the reductions are time-consuming in both cases, increasing the temperature and surplus of reducing agent, completely reduced diblocks are obtained after ~72 h. With the initial activation taken into account, approximately 4 days for dext-PDHA and 2 days for A_nM-PDHA, the protocols for the total reductive aminations are 6-7 days for both approaches. Not considering purification steps in between. Nonetheless, with using initially activated A_nM and dextran as the second block, higher yields are obtained due to the slow background reduction of unconjugated dextran (92% vs 63%). Hence, the choice of protocol should be based on available starting material, taken the difference of final diblock yield into account.

4.4 Periodate Oxidation of Chitin Oligosaccharides

Periodate oxidation is a novel approach for activation of the non-reducing end (NRE) of fully N-acetylated chitin oligomers. The oxidation attacks vicinal diols, which is only present at the NRE of chitin oligomers. This creates a dialdehyde which is available for further reactions, such as conjugation to oxyamines for preparation of block polysaccharides.

Previous experiments (I.V. Mo, submitted to Carbohydrate Polymers, 2021) have shown that obtaining fully oxidized conjugates is challenging, even with the use of up to 4 equivalents of sodium metaperiodate to A_nM oligosaccharides.

4.4.1 Reaction Kinetics of Periodate Oxidation of A_nM by Optical Rotation Measurements

The reaction kinetics of the periodate oxidation of the NRE of chitin oligosaccharides was assessed following the protocol described in section 3.8.

The results from the measurements are presented in Figure 4-11 including oxidation of glucose using equimolar amounts of periodate as a positive control. As mentioned, previous experiments (I.V. Mo, submitted to Carbohydrate Polymers) have not successfully obtained completely oxidized oligosaccharides, although the majority of the oligosaccharides seems to be oxidized within 24 h. Nonetheless, there was no evident rotational change of A_2M during the time span of the reaction (40 h).

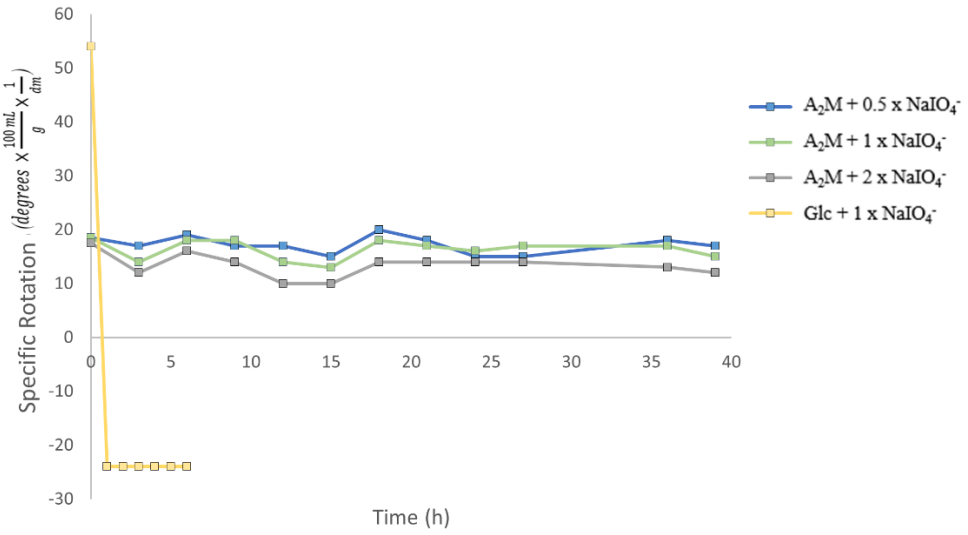

Figure 4-11: Calculated specific rotation from optical rotation measurements (Anton Paar MCP5100 polarimeter) as a function of time for periodate oxidation of A_2M oligosaccharides using different amounts of sodium metaperiodate ($NaIO_4^-$). Including oxidation of glucose with 1 equivalent $NaIO_4^-$ for comparison.

In preparation for the experiment, several concentrations of A_nM were measured to calibrate the appropriate amount needed for the measurements (section 3.8.1). This excludes concentration as a source of error as it was sufficient to receive signals. Some deviance occurred in the measurements from hour to hour, however this is attributed to oscillation in the instrument as the signal was reset to an empty cell between each measurement.

In contrast, glucose was rapidly oxidized with equimolar amounts of periodate yielding measurable results. At time zero the specific rotation of pure glucose in water was measured to be 54˚, whilst after 1 h it had changed to -24˚ due to the oxidation. The sample was measured every hour the next 6 h resulting in -24˚ in each measurement, hence it was concluded that the reaction was completed in less than 1 h.

As no significant change in rotation was measured after 40 h, it was concluded that this is not an appropriate method for determining the reaction kinetics of periodate oxidation of A_nM oligosaccharides. It is not clear why this approach did not work as it requires more detailed experimental procedures. However, a speculation is that the positive and negative rotational changes from the oxidation are cancelling the signals evenly.

4.5 Simultaneous Nitrous Acid Degradation and Conjugation to PDHA

When preparing chitin oligosaccharides (A_nM) there is a limitation in which only the short oligosaccharides are water-soluble and the longer (and more interesting) oligosaccharides are water insoluble. Here, increasing the solubility was attempted by introducing a positive charge by the terminal amino group of PDHA. Thus, this experiment was conducted on the basis that a new protocol for material preparation including degradation and conjugation in one step has the potential to increase the solubility of the longer A_nM oligosaccharides to possibly avoid precipitation as insoluble material.

4.5.1 Characterization of the Product from Simultaneous Nitrous Acid Degradation and Conjugation

Simultaneous degradation and conjugation to yield fully N-acetylated chitin oligosaccharides with 2,5-anhydro-D-mannose at the reducing end conjugated to PDHA was attempted (A_nM-PDHA, described in section 3.9). The sample was characterized by ^{1}H-NMR spectroscopy (section 3.4) and is shown in Figure 4-12. It is important to note that a pH-adjustment to pH=3.5 (using deuterated acetic acid) was necessary to completely dissolve the sample in D_2O.

The spectrum revealed unexpectedly broad signals and confirmed that no conjugation was obtained by the lack of signals from the E- and Z-oximes at 6.8 and 7.5 ppm. The signal at 3 ppm is characteristic of H2 of the D-unit in chitosan which indicates that the degradation has not been complete. The presence of D-units would greatly be impacted by pH, which explains why the sample was not soluble at neutral pH but dissolved readily with the pH adjustment. An increase to pH=9 was conducted for further examination, which resulted in precipitation of the sample. As fully N-acetylated chitin is a neutral biopolymer it would not be affected by pH, further supporting that the sample contains a certain number of D-units.

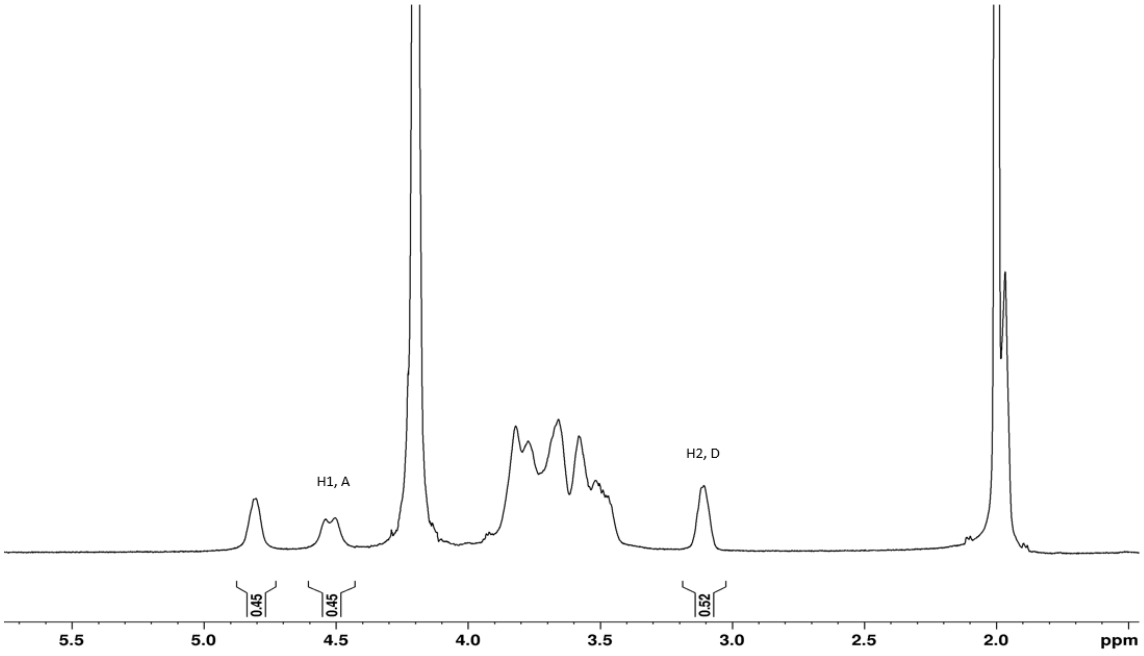

Figure 4-12: ^{1}H-NMR (600MHz, 355K pH=3.5, D_2O) spectrum of the sample obtained by simultaneous nitrous acid degradation and conjugation to PDHA (described in section 3.9).

Upon closer examination, the integral ratio of the H1,A and H2,D suggested that the sample had been minimally degraded. The parent chitosan had a F_A=0.48, whereas the integral ratios here suggest 52% D-residues and 45% A-residues. Additionally, no reducing end resonances were observed. Initially, the resonance at 4.8 ppm was assigned to H1,M(gem-diol), however as the M-residues appears by converting the D-residues, this was discarded due to the intensities of the signals does not support this. The H2,D resonance would have been drastically decreased with the appearance of a H1,M signal of this intensity. The lack of reducing end resonances is attributed to if the parent chitosan with high molecular weight (M_n=722 kDa) was not particularly degraded, the reducing end resonances are minimal compared to the internal proton resonances, thus not observable in the ^{1}H-NMR spectrum.

An analytical SEC run was conducted to assess the size of the chains in the sample (Figure 4-13). By comparison to the same starting material with the same degradation protocol (given that conjugation was not successful) as shown in Figure 4-2 (section 4.1.1), the sample eluted considerably faster. This corresponds to a sample containing biopolymers with higher hydrodynamic volume, indicating longer chain lengths and higher molecular weight. This underlines that the sample was not fully degraded into A_nM oligosaccharides both by the indication of size, as well as the solubility of the sample as fully N-acetylated chitin oligosaccharides of this size would not be soluble in the mobile phase used for this SEC system.

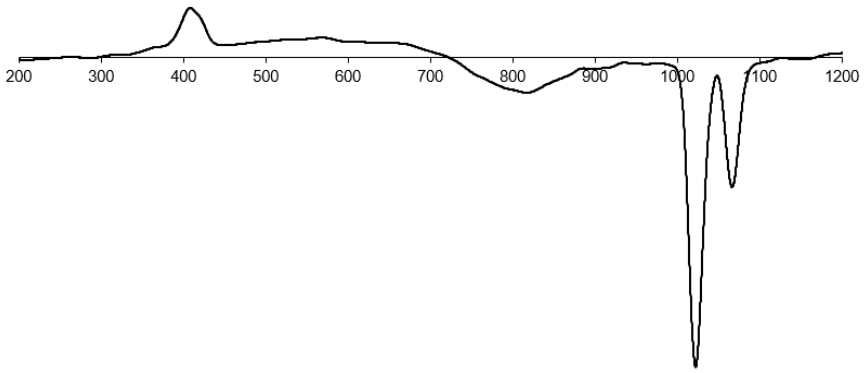

Figure 4-13: Analytical size exclusion chromatogram (0.15 M AmAc, pH=4.5) of the sample obtained by simultaneous degradation of chitosan (F_A=0.48) and conjugation to PDHA as described in section 3.9.

In the literature it was found that nitrous acid has the potential to oxidize the oximes to the parent aldehyde or ketone (47). With the results presented here, it is probable that some nitrous acid was used to reverse the conjugation by oxidation of the oximes. As a result, there was not enough nitrous acid present to turn all the D-residues into M-residues – yielding chitosan. Additionally, oximes are sensitive to acidic hydrolysis (7) making it possible that the potential conjugation was reversed during the time span of the reaction or when lowering the pH for complete dissolution in preparation for ^{1}H-NMR analysis.

4.6 Solvents for Water Insoluble Chitin Oligosaccharides

The nitrous acid degradation of chitosan (F_A=0.48) yields fully N-acetylated chitin oligosaccharides of various degree of polymerization. The oligosaccharides with DP < 9 are water-soluble while oligosaccharides with DP > 9 are water insoluble. The latter is hereafter called $(A_nM)_{wis}$. $(A_nM)_{wis}$ is insoluble in most common aqueous and organic solvents. This makes it challenging to acquire knowledge about the properties of longer

oligosaccharides/polysaccharides alone and/or in conjugation with other molecules. The potential applications and possibilities arising if the water insoluble fraction could be dissolved provided the motivation for this experiment. The water insoluble fraction prepared in section 3.5.2 was dissolved in different solvents and studied.

4.6.1 Dissolution of Water Insoluble A_nM Oligosaccharides

$(A_nM)_{wis}$ was dissolved in different solvents to a concentration of 5 mg/mL and observed visually over a time period of several days (section 3.10.1). The oligosaccharides remained insoluble in H_2O, DMSO and DMAc but dissolved in HFIP, NaOH (5M) and 8 wt% LiCl/DMAc under certain circumstances. A summary of the observations is included in Table 5.

Table 5: Observations and details of dissolution of the water insoluble fraction of A_nM oligosaccharides in different solvents. Every sample was made to a final concentration of 5 mg/mL A_nM.

Solvent	25 °C	60 °C	100 °C	Additional comments
H_2O	Insoluble	-	-	
DMSO	Insoluble	Insoluble	-	
DMAc	Insoluble	Insoluble	Insoluble	
HFIP	Soluble	-	-	Soluble after 24h on shaking
5 M NaOH	Soluble	-	-	Developed brown color
8 wt% LiCl/DMAc	Insoluble	Soluble	Soluble	Stayed soluble down to 4 °C after initial heating to 60 °C

The sample dissolved in 5 M NaOH immediately developed a brown color, indicating occurrence of a reaction. Strong bases may be used to de-N-acetylate chitin to yield chitosan (5), in which this solvent is unsuitable if fully N-acetylated chitin is to remain intact. A possible reason for the color development is that a reaction might occur with the de-N-acetylated subunits that leads to formation of HMF (5).

As expected, chitin dissolved in 8 wt% LiCl/DMAc upon brief heating to 60°C (14). It stayed soluble and stable afterwards in temperatures down to 4°C (lower temperatures have yet to be described). This was a promising result, as LiCl/DMAc is widely used as solvent in the treatment of cellulosic materials (16). It was thus of interest to further examine the solubility of chitin oligomers in LiCl/DMAc and this will be the preferred solvent for $(A_nM)_{wis}$ hereon in the work in this master thesis.

4.6.2 Concentration Dependence of $(A_nM)_{wis}$ Dissolved in 8 wt% LiCl/DMAc

As the $(A_nM)_{wis}$ oligomers were shown to be dissolved in 8 wt% LiCl/DMAc, further studies were conducted to identify the concentration dependence of the dissolution. The aim was to assess the percentage of water which can be added before the oligomers precipitate. The microplate assay experimental set up is shown in Table 2 (section 3.10.2) and the results from the assay is shown in Figure 4-14.

Obviously, A_nM dissolved better in the concentration range assayed (0-10% water in LiCl/DMAc) than in 100% water which served as a negative control. However, some turbidity in the sample containing 10% water was observed, demonstrating that it is not completely soluble in this composition. It was found that A_nM had satisfactory dissolution in 8 wt% LiCl/DMAc containing up to 5% water.

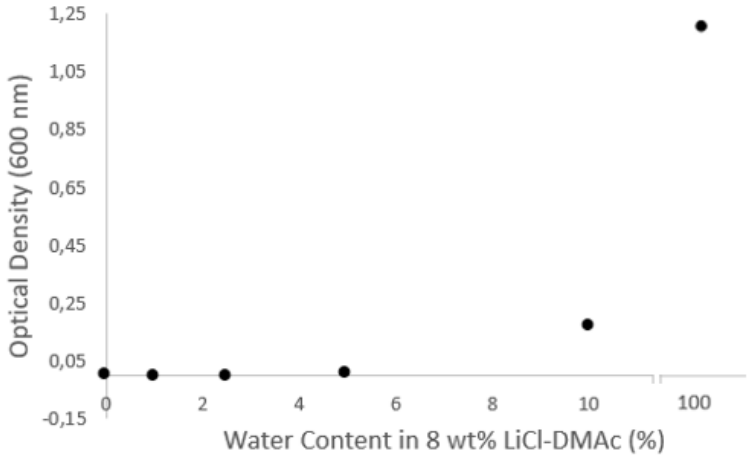

Figure 4-14: Optical density measured at 600nm (Tecan Plate Reader) of chitin oligosaccharides in 8 wt% LiCl/DMAc with different percentages of water.

4.6.3 Dissolution of Dextran in 8 wt% LiCl/DMAc

It is proposed that conjugation of $(A_nM)_{wis}$ to dextran might lead to the diblock being soluble in water, as dextran is a readily soluble biopolymer. Thus, it was of interest to assess the solubility of dextran in 8 wt% LiCl/DMAc to obtain homogenous conjugation in this solvent.

Acid degraded dextran (DP_n =42, 5 mg/mL) was dissolved in 8 wt% LiCl/DMAc containing 1% water. As the solution did not dissolve in RT, it was heated to 60°C. After 16 h, the dextran was still not dissolved. The temperature was increased to 80°C resulting in complete dissolution after a couple of minutes.

4.7 Preparation of Water-soluble Chitin-based Block Polysaccharides in LiCl/DMAc

Röhrling *et al.* (28) have previously conducted a reaction in LiCl/DMAc in which conjugation of an oxyamine bearing a fluorophore was used to determine the intra-chain carbonyl content of oxidized cellulose. Based on these results and that the water insoluble chitin oligosaccharides ($(A_nM)_{wis}$) were shown to be soluble in DMAc with some LiCl added, it was of interest to attempt a conjugation reaction in this solvent. As Röhrling *et al.* (28) stated that a water content up to 2.5% did not interfere with the conversion rate, 8wt% LiCl/DMAc with 1% water (V/V) was chosen as the standard solvent system for the subsequent reactions completed in this master thesis.

A preliminary experiment of conjugation of water-soluble A_nM oligosaccharides (DP < 9) to fully reduced A_nM-PDHA was attempted. This due to practical reasons as the water-solubility allowed for the subsequent ^{1}H-NMR analysis whether successfully conjugated or not.

4.7.1 Preparation of A_4M=PDHA-M_4A Block Polysaccharides in LiCl/DMAc

Conjugation of A_4M to A_4M-PDHA in 8 wt% LiCl/DMAc was attempted by following the protocol described in section 3.11.

Prior to reaction initiation ^{1}H-NMR analysis was conducted of A_4M-PDHA to assure the conjugate was fully reduced before conjugation to A_4M to produce the diblock A_4M=PDHA-M_4A (where "=" indicates unreduced E/Z-oxime). This would certify that any signals resulting from conjugation without reduction, that is E/Z-oximes, would only arise if A_4M was successfully conjugated to A_4M-PDHA. The spectrum is presented in Figure 4-15 and showed close to fully reduced conjugate with negligible minor traces of E-oximes.

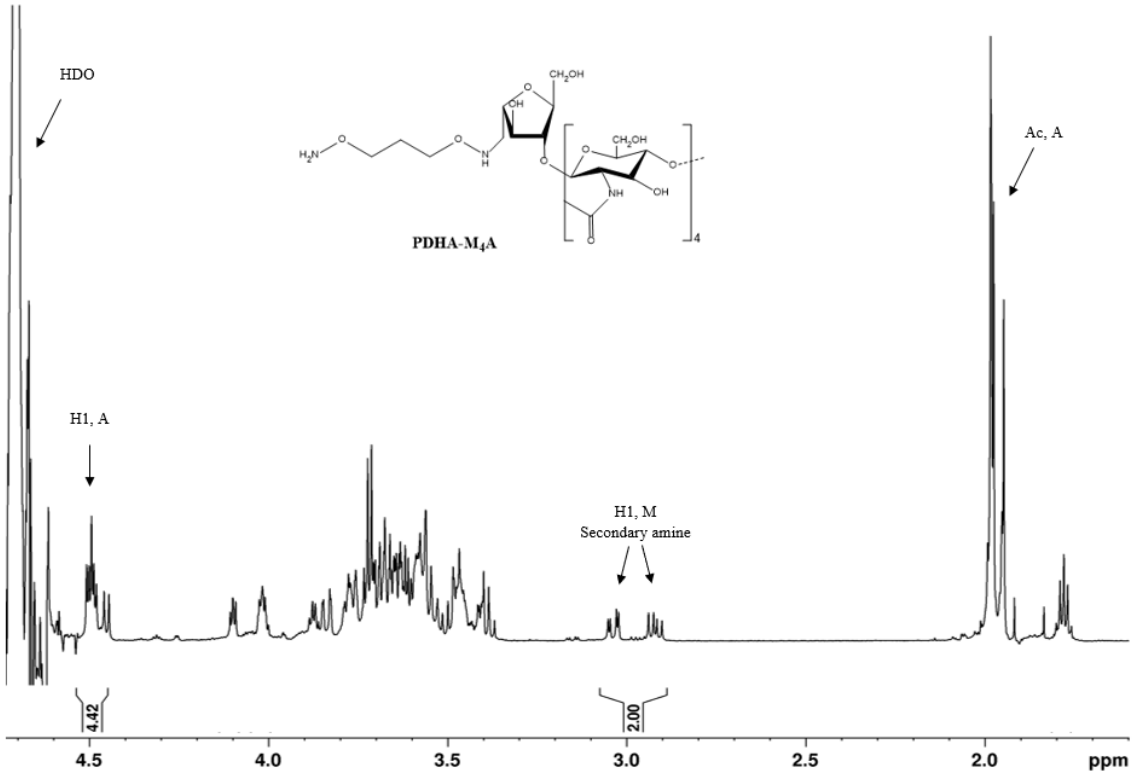

Figure 4-15: ^{1}H-NMR (600 MHz, 298K, D_2O) spectrum of reduced A_4M-PDHA.

The ^{1}H-NMR analysis performed of the purified reaction mixture after the conjugation in LiCl/DMAc showed successful conjugation (Figure 4-16). Key resonances that substantiate successful conjugation is found in the presence of E- and Z-oxime signals at 7.4 and 6.8 ppm (respectively), as well as the increased intensity of the H1,A resonance.

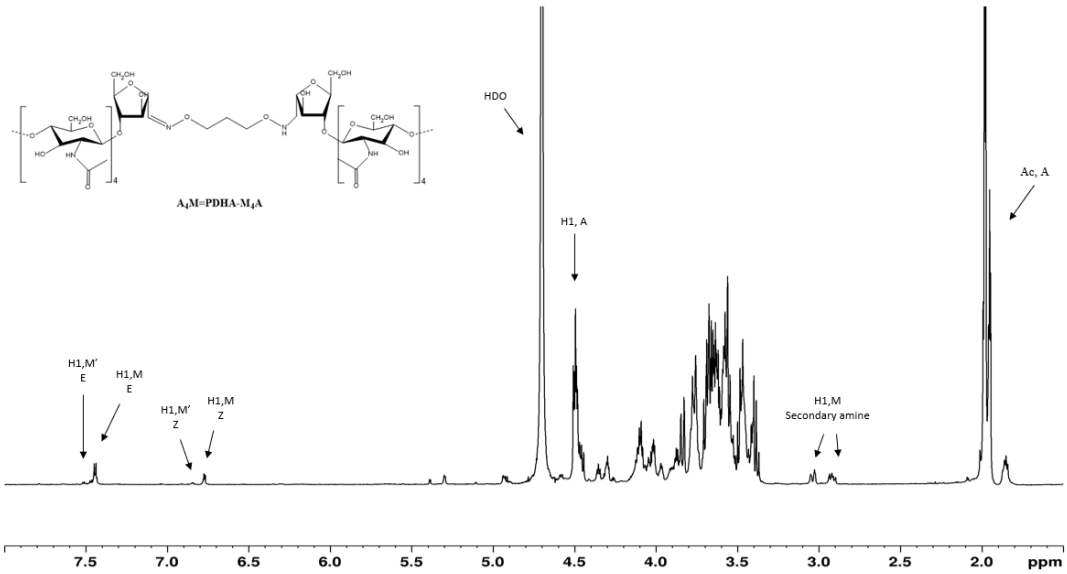

Figure 4-16: ^{1}H-NMR (600 MHz, 298K, D_2O) spectrum of A_4M=PDHA-MA_4 after reaction in LiCl/DMAc. The "=" indicates unreduced E/Z-oximes. M' represents the alternative M-unit formed upon degradation.

The results show that LiCl/DMAc is a viable solvent for oxime click reactions for chitin oligosaccharides of the type A_nM to the free end of PDHA. On this basis, it is reasonable to assume this solvent system is applicable to oxime conjugation of A_nM to other PDHA-activated oligo- or polysaccharides that are soluble in LiCl/DMAc, such as dextran.

4.8 Preparation of Water Insoluble Chitin-based Block Polysaccharides in LiCl/DMAc

Due to successful conjugation of water-soluble A_nM (DP < 9) to A_nM-PDHA in 8 wt% LiCl/DMAc, it was of interest to attempt conjugation of water insoluble A_nM (referred to as $(A_nM)_{wis}$, DP > 9). Earlier studies have shown that conjugation of chitosan to a compound of high solubility increases the overall solubility. Both linear reducing end conjugation of polyethylene glycol (PEG) to chitosan (48), and lateral conjugation of dextran to the amino groups of chitosan (49), have been shown to increase the solubility compared to chitosan alone. Thus, dextran was chosen as the second block, due to the idea that conjugation of $(A_nM)_{wis}$ to a conjugate of high solubility would increase the overall solubility of the diblock in aqueous solutions, allowing for subsequent ^{1}H-NMR analyses.

4.8.1 Conjugation of $(A_nM)_{wis}$ to $Dext_m$-PDHA

$(A_nM)_{wis}$ was conjugated to $dext_m$-PDHA (m=DP_n) following the protocol described in section 3.12.

The first experiment conducted was conjugation of equimolar amounts of $(A_nM)_{wis}$ (where *n* is assumed to be 11) to $dext_{23}$-PDHA. The ^{1}H-NMR spectrum of $dext_{23}$-PDHA obtained prior to conjugation to $(A_nM)_{wis}$ is shown in Figure 4-17 b. The spectrum revealed that no resonances were present in the region 6.8 and 7.5 ppm confirming there were no residual oximes present, hence the sample was fully reduced.

The ^{1}H-NMR spectrum of the purified product of the preparation of $(A_nM)_{wis}$=PDHA-$dext_{23}$ in LiCl/DMAc is presented in Figure 4-17 c. Key resonances demonstrating conjugation are the E- and Z-oxime signals at 7.4 and 6.8 ppm, respectively. The presence of $(A_nM)_{wis}$ is manifested by the H1,A resonance at 4.5 ppm. The equilibrium yield was found to be 38% from the integrals of the oxime resonances relative to the integral of the secondary amine, in agreement with the resonance from unreacted H1,M (gem-diol) which was found to be 62% (Figure 4-17 d and e).

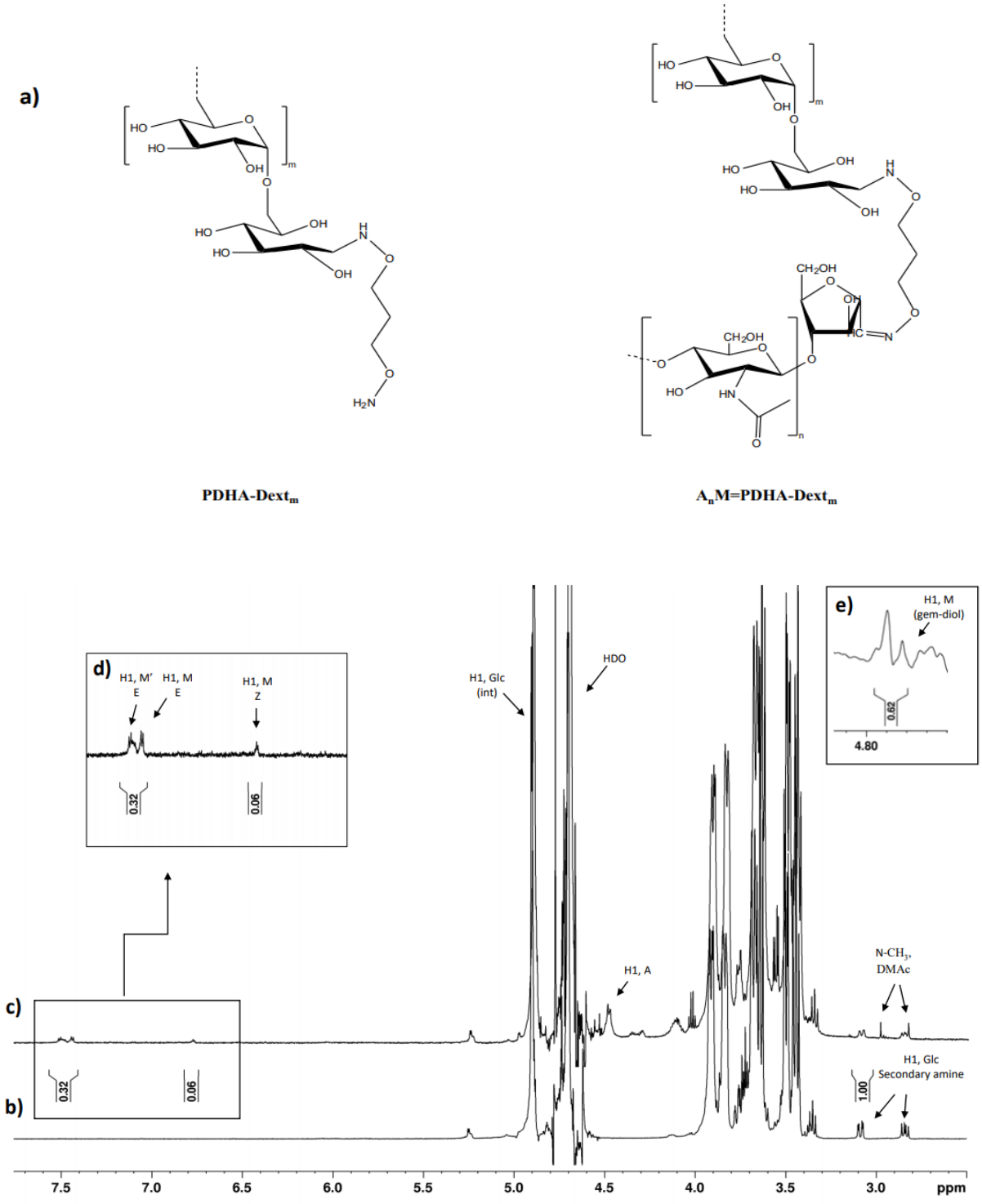

Figure 4-17: a) General structure of the of the compounds. b) and c) ^{1}H-NMR (600MHz, 298K, D_2O) spectra of reduced dext$_{23}$-PDHA and $(A_nM)_{wis}$ =PDHA-dext$_{23}$ (where n is assumed to be 11) after reaction in LiCl/DMAc, respectively. The "=" indicates unreduced E/Z-oximes. c) Expanded view of the region that confirmed successful conjugation by E- and Z-oxime resonances. e) Expanded view of the region with the resonance of H1,M (gem-diol) from unreacted A_nM.

Two new singlets arose at 2.8-3.0 ppm, one of which overlapped one of the secondary amine protons of the reduced $dext_{23}$-PDHA. This is traces of DMAc in the solution as the two methyl groups next to the nitrogen corresponds to these resonances when dissolved in D_2O (50). In addition, a signal appearing just above 2 ppm was assigned to the methyl group next to the carbonyl of DMAc. However, integration and calculation of the yield was still achievable as one of the secondary amine signals was without interruption.

$(A_nM)_{wis}$ has previously been shown to dissolve by brief heating to 60°C in 8 wt% LiCl/DMAc (section 4.6). However, in this experiment a temperature of 80-90°C was necessary for complete dissolution. This is attributed to the increased concentration used for the conjugation protocol (20.1 mM corresponds to roughly 50 mg/mL, given DP_n=12) compared to the dissolution experiment (5 mg/mL).

It is important to mention that the challenge of the solubility of the water insoluble chitin fraction leads to limited access to analyze the composition. Thus, an assumption has been made that the DP_n is 12 (n=11). With the assumption follows some uncertainty regarding the reactions performed with molar equivalents of reactants as it might be under- or overestimated.

Röhrling *et al.* (28) has conjugated oxidized cellulose to the hydroxylamine Carbazole-9-carboxylic Acid (CCOA) in LiCl/DMAc. On the contrary to aqueous solutions, where this reaction is acid-catalyzed, they found that in LiCl/DMAc it is base-catalyzed. With triethylamine (TEA) as base catalyst, the reaction time was reduced to 6 h. It was thus of interest to assess whether the reaction time in this case also could be reduced using this catalyst.

^{1}H-NMR of the product obtained by the 6 h conjugation of $dext_{23}$-PDHA to $(A_nM)_{wis}$ with TEA as catalyst is shown in Figure 4-18 (protocol described in section 3.12.1.1). Presence of E- and Z-oxime signals revealed successful conjugation with an equilibrium yield of 48%. This reaction was also repeated with the same conditions but a longer reaction time of 24 h to maximize the equilibrium yield. Nonetheless, no significant change in yield was achieved, showing that a reaction time of 6 h is sufficient.

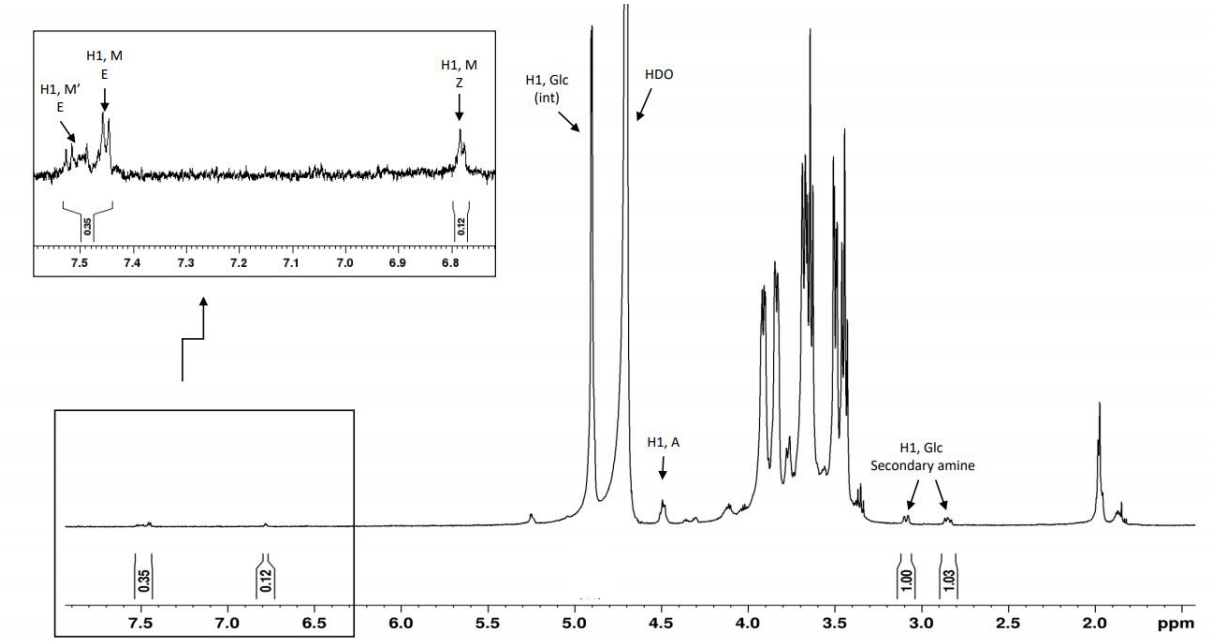

Figure 4-18: ^{1}H-NMR (298K, 600 MHz, D_2O) spectrum of the product of the conjugation of reduced $Dext_{23}$-PDHA to $(A_nM)_{wis}$ (n assumed to be 11) in 8wt% LiCl/DMAc with triethylamine as base catalyst.

A summary of the abovementioned experiments and further experiments varying parameters such as reaction time, molar ratios, and catalyzed by TEA or not have been conducted and are presented in Table 6.

Table 6: Results from conjugation of A to B in 8wt% LiCl/DMAc containing 1% water including concentration, molar ratio, catalyzed or not, temperature, reaction time and yield.

A	B	c_A(mM)	A:B	TEA	T(°C)	t(h)	Yield (%)
A_4M	A_4M-PDHA	20.1	1:1	No	40	48	-
*A_2M	PDHA	20.1	1:2	No	RT	420	58
$(A_nM)_{wis}$	$Dext_{23}$-PDHA	20.1	1:1	No	40	48	38
$(A_nM)_{wis}$	$Dext_{23}$-PDHA	20.1	1:1	Yes	40	6	48
$(A_nM)_{wis}$	$Dext_{23}$-PDHA	20.1	1:1	Yes	40	183	48
$(A_nM)_{wis}$	$Dext_{37}$-PDHA	20.1	1:1	Yes	40	24	43
$(A_nM)_{wis}$	$Dext_{37}$-PDHA	60.3	3:1	Yes	40	6	84

* Experiment and result explained in section 3.13 and 4.9.2.

Results of the product obtained by the 6 h conjugation of $dext_{37}$-PDHA to a 3x molar excess of $(A_nM)_{wis}$ is presented in Table 6 (protocol described in section 3.12.2). Presence of E- and Z-oxime signals revealed that conjugation had happened with a yield of 84%, a substantial

increase compared to the reactions executed with molar equivalents (~40%). Showing that an excess of one of the reactants would maximize the yield.

$(A_nM)_{wis}$ was chosen as the reactant in excess as the non-conjugated surplus is readily removed by precipitation in water. Nonetheless, there are some challenges regarding recycling of the material. Due to the inability to characterize the precipitate, it can not be assured that it is composed of pure chitin oligosaccharides. Interestingly, the precipitate did not dissolve when resuspended in 8 wt% LiCl/DMAc. Moreover, there is a challenge in purifying the compound from unreacted dextran as well, leaving (what is here called) the purified sample composed of $(A_nM)_{wis}$=PDHA-$dext_m$ diblock as well as an unknown amount of unreacted $dext_m$-PDHA. Thorough purification was not reviewed any further in the work in this thesis due to time limitation.

Additionally, an experiment was performed for 183 h to assess whether exceedingly long reaction times could increase the equilibrium yield. This was inspired by that Röhrling *et al.* (28) had yields of up to 90% with reaction times of 500 h. Nonetheless, the equilibrium yield of the reaction was calculated to be 48%, showing no improvement from the reactions performed for 6 h, further stating that the reaction happens within 6 h.

The results show that LiCl/DMAc is a viable solvent system for oxime click reactions of water insoluble chitin oligosaccharides of the type A_nM to the free end of PDHA. This is presumably the first evidence presented of terminal conjugation of dextran to water insoluble chitin to produce a water-soluble conjugate.

4.9 Deuterated N,N-Dimethylacetamide with LiCl as Solvent for ^{1}H-NMR

Due to successful conjugation reactions in 8 wt% LiCl/DMAc, it was of interest to attempt these reactions in deuterated DMAc to be able to measure reaction kinetics through real time NMR (RT NMR).

4.9.1 ^{1}H-NMR with Deuterated 8wt% LiCl/DMAc as Solvent

^{1}H-NMR was obtained of A_2M and $(A_nM)_{wis}$ in 8 wt% LiCl/DMAc (Figure 4-19). The spectra had some similarities in the resonances where peaks were present, aside from $(A_nM)_{wis}$ that had broadening of the peaks which is expected for a compound with higher DP. By comparison to A_2M in D_2O there were some trends in peaks arising between 3 ppm and 5 ppm, however they could not be characterized without further experiments.

Some deviances were present in the area above 5 ppm as the samples in LiCl/DMAc had several resonances in this area which were non-existent with D_2O as solvent. It was hypothesized that the resonance appearing at 9.5-10 ppm belongs so the proton of the aldehyde group at the reducing end M-unit, due to the fact that it would not appear as a gem-diol at ~5 ppm given that DMAc is not an aqueous solvent. Furthermore, it was explored whether a rise in temperature would affect the presentation of the resonances (Figure 4-20). At 355K the solvent peak at ~4.5 ppm had a downfield shift to ~4.0 ppm, revealing additional resonances in the 4.5 ppm area.

2D-NMR spectra of A_2M were acquired (COSY, HSQC, HMBC) in the attempt of spectrum annotation (Appendix C). The 2D spectra gave inconclusive results and mediated no further progress in assigning the peaks.

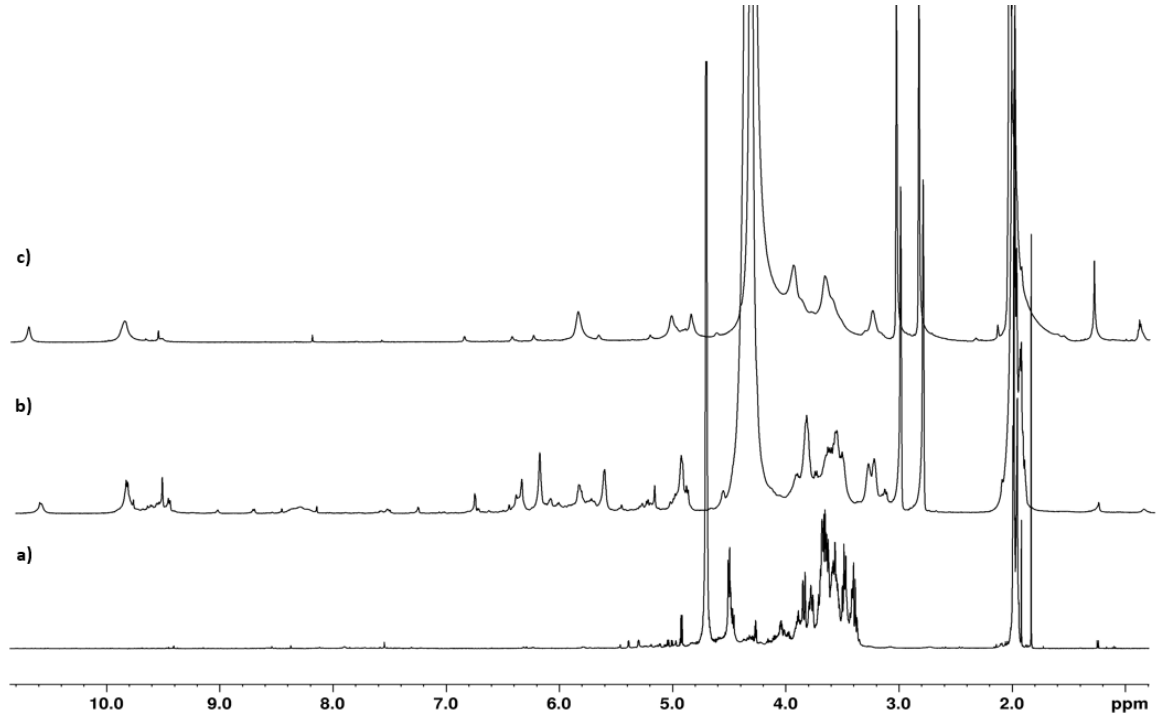

Figure 4-19: ^{1}H-NMR (298K, 600 MHz) of a) A_2M in D_2O b) A_2M in 8 wt% LiCl/DMAc c) $(A_nM)_{wis}$ in 8 wt% LiCl/DMAc (b and c calibrated by TSP).

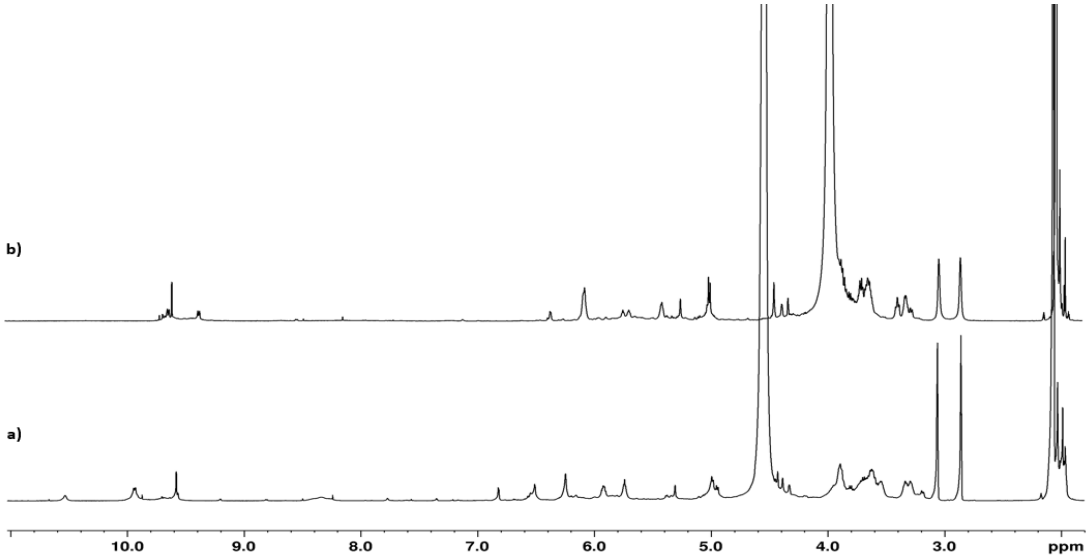

Figure 4-20: ^{1}H-NMR (600 MHz) of A_2M in 8 wt% LiCl/DMAc with 1% H_2O at a) 298K and b) 355K.

^{1}H-NMR in 8wt% LiCl/DMAc was also obtained of the compound previously conjugated in LiCl/DMAc (section 4.8); $(A_nM)_{wis}$=PDHA-dext$_{23}$ (Figure 4-21). As expected from the previous spectra, it was not comparable to the spectrum of the same compound obtained in D_2O. Additionally, the resonance at 9.5-10 ppm was also present in this spectrum, suggesting it does not belong to the proton of the aldehyde at the M-unit as it would disappear once conjugated. Nonetheless, as the reaction yielded 48% conjugation, the resonance may be from the unconjugated A_nM oligosaccharides as the compound had not been purified.

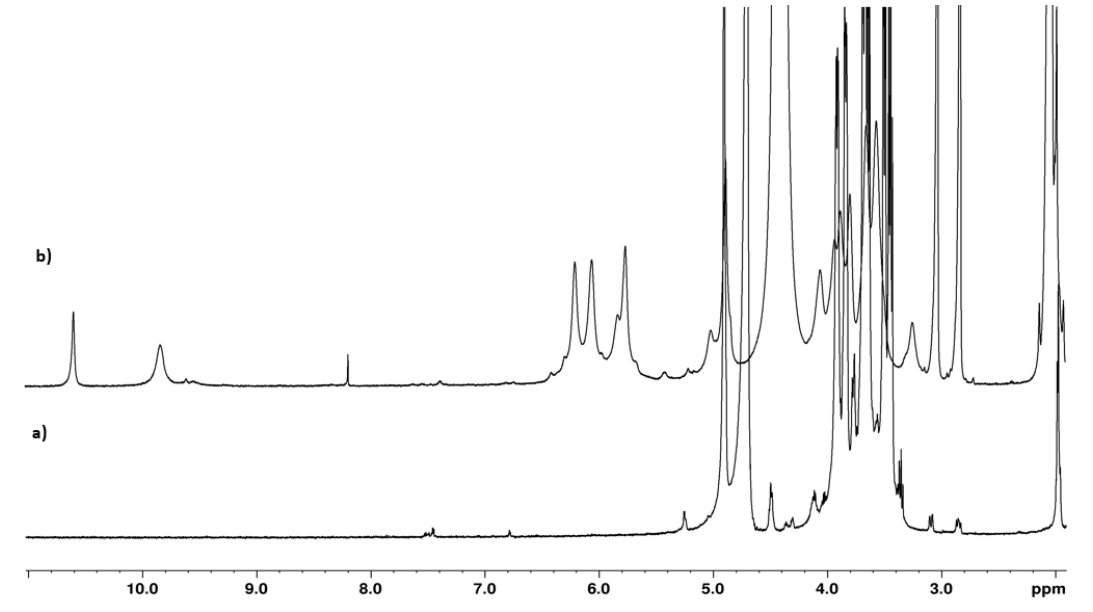

Figure 4-21: ^{1}H-NMR (298K, 600 MHz) of $(A_nM)_{wis}$=PDHA-dext$_{23}$ in a) D_2O and b) 8 wt% LiCl/DMAc (calibrated by TSP).

4.9.2 Reactions in 8wt% LiCl/DMAc studied by Real Time NMR

As the spectra obtained in deuterated DMAc (with LiCl) were not comparable to previous spectra obtained in D_2O and the 2D spectra obtained for spectrum annotation gave limited information, another approach for peak characterization was attempted. Different reactions with known outcomes were initiated and followed by real time ^{1}H-NMR (RT ^{1}H-NMR). This approach would allow for peak annotation by observing the change of intensity of the peaks belonging to the protons affected by the reactions.

The reactions performed with aims and outcomes are summarized in Table 7. The reactions were conducted as described in section 3.13. The ^{1}H-NMR spectra obtained during the different reactions in deuterated DMAc, as well as the ^{1}H-NMR spectra of the purified compounds redissolved in D_2O are given in Appendix C.

Table 7: Reactions followed by real time ^{1}H-NMR in deuterated DMAc containing 8% LiCl for peak assignment of NMR spectra. All reactions were followed for 48 h. PB=α-picoline borane.

Reaction	Aim / Peak(s) to assign	Outcome
Reduction of A_2M by PB (3x)	H1,M (of CHO)	No change in resonances observed
Conjugation of A_2M to PDHA (2x)	H1,M (of CHO), E- and Z-oxime	No change in resonances observed
Reduction of $(A_nM)_{wis}$=PDHA-$Dext_{23}$ by PB (3x)	E- and Z-oxime, secondary amine	No change in resonances observed

Neither reduction of A_2M oligosaccharides nor reduction of $(A_nM)_{wis}$=PDHA-$dext_{23}$ over a period of 48 h resulted in change of peak intensities in the spectra acquired in deuterated DMAc. However, the peaks arising from PB itself seemed to have a slight downfield shift over time. This was found due to the intensity of the original peaks from PB decreased in order with the increase of similar peaks slightly downfield. This led to the speculation that PB is somehow decomposing over time in LiCl/DMAc, as it does in sodium acetate (4). However, further examination is needed in order to substantiate this speculation.

Furthermore, purification of the $(A_nM)_{wis}$=PDHA-$dext_{23}$ diblock and redissolving it in D_2O for ^{1}H-NMR analyses revealed that no reduction had taken place by the continuous presence of E- and Z-oxime with roughly the same intensities as before reduction initiation. This observation was substantiated by no change in the intensity of the secondary amine resonances as well. Suggesting that PB might not be a suitable reducing agent in this solvent system. Interestingly, PB has previously been stated as a viable reducing agent in DMSO (also an organic solvent), however these experiments were performed in the presence of acetic

acid (24). It could thus be interesting to assess if complete reductive amination could be achieved with addition of acetic acid.

However, lack of reduction in LiCl/DMAc will not be a problem for further experimental work as conjugation and reduction can be performed in a two-pot system, changing the solvent after conjugation to one appropriate for reduction. This can be done due to the initial findings that after conjugation of $(A_nM)_{wis}$ to dextran the conjugate is water-soluble.

Regarding the conjugation, it was surprising that no changes in peak intensities were observed over a period of 48 h in deuterated DMAc as this reaction had previously been successful aside from NMR. It was speculated that since the previous experiments were performed at 40°C, it might be a necessary temperature condition for the reaction to occur. Nonetheless, by purifying the sample and redissolving it in D_2O, the following spectrum revealed that the reaction had been successful with a conjugation yield of 58%, stating that high temperature is not necessary for the reaction to occur. However, the sample was left for 7 days before purification, thus this experiment can not be used to determine if higher temperatures increase the reaction rate.

Due to time limitation and inconclusive results, this part of the project concerning NMR with deuterated DMAc as solvent was not further pursued. The previous method of purifying the reaction mixture and redissolve it in D_2O for ^{1}H-NMR characterization has been successful and was the method of choice for the remainder of the experiments executed with DMAc as solvent.

4.10 Reaction Kinetics of Chitin-based Block Polysaccharides in 8wt% LiCl/DMAc

The reaction kinetics of the formation of chitin-based diblocks in 8wt% LiCl/DMAc was studied. First, to calibrate the experiments, reaction kinetics of the conjugation of water-soluble A_2M (DP < 9) to PDHA-activated A_2M was assessed. Thereafter, the protocol for the water-soluble A_2M with some changes for the time points of the samples was applied to the conjugation of $(A_nM)_{wis}$ (water insoluble A_nM, $DP_n > 9$) to dextran-PDHA.
The reaction kinetics for both reactions were analyzed by ^{1}H-NMR by integration of the peaks of interest to find the relative yield at different time points throughout the reaction.

4.10.1 Reaction Kinetics of the Conjugation of A_2M to A_2M-PDHA

The reaction kinetics of the conjugation of A_2M to A_2M-PDHA (fully reduced) in 8wt% LiCl/DMAc containing 1% H_2O was performed as described in section 3.14.1. Aliquots of the mixture were taken regularly, and the reaction was terminated at defined time points to allow for following the reaction through a time period of 23 h. Due to the fast reaction rate, the experiment was reproduced, and aliquots of the mixture were taken at short intervals within the first hour.

^{1}H-NMR was used to analyze the samples obtained at the different time points. The integrals of the E- and Z-oxime resonances (7.5 and 6.8 ppm, respectively) relative to the integral of H1, A (~4.5 ppm) were used to determine the relative yields of conjugates and are presented in Figure 4-22 as a function of time.

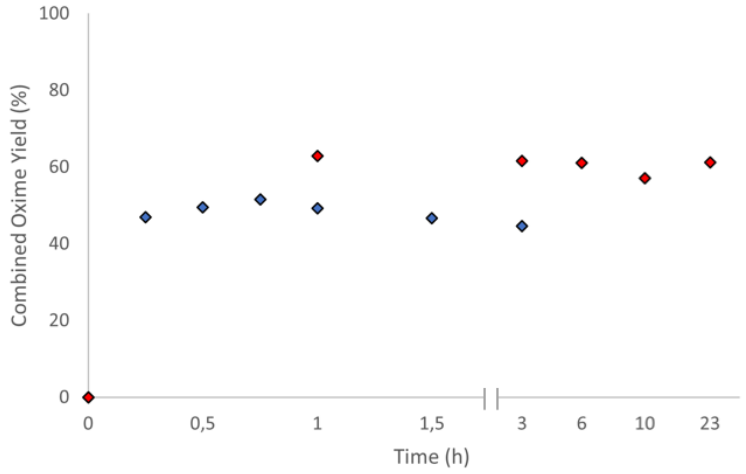

Figure 4-22: Combined E- and Z-oxime formation during conjugation of A_2M (20.1 mM) to equimolar amounts of A_2M-PDHA in 8 wt% LiCl/DMAc + 1% H_2O at 40°C at two separate occasions (red and blue). Note the adapted x-axis after 1.5 h.

The first attempt to assess the reaction rate revealed that equilibrium yield was reached within 1 h, calculated to be 61%. The minor deviance in yield observed at 10 h (57%) is attributed to the uncertainty in the ^{1}H-NMR measurements. A follow up experiment was performed in which samples were taken frequently within the first hour. Equilibrium was reached within 15 minutes at 50% showing that the reaction is indeed fast.

The deviance in the equilibrium yields for the two separate experiments is attributed to practical difficulties of the protocol at the wet lab. Although the equilibrium yields were not

reproducible, the similarities in the trends of the reaction rate where it is complete within 1 h indicates that the conjugates are formed at a high rate.

This preliminary study shows that conjugation to produce A_nM-diblocks (DPn < 9) in 8wt% LiCl/DMAc containing 1% H_2O with triethylamine as base catalyst happens at a high rate. The reaction is essentially complete within 15 minutes giving respectable equilibrium yields. It was thus of interest to assess whether the fast kinetics is also applicable to the conjugation of water insoluble A_nM to dextran.

4.10.2 Reaction Kinetics of the Conjugation of $(A_nM)_{wis}$ to Dextran-PDHA

The reaction kinetics of the conjugation of $(A_nM)_{wis}$ to $dext_{37}$-PDHA (fully reduced) in 8wt% LiCl/DMAc containing 1% H_2O was performed as described in section 3.14.2. Aliquots of the mixture were taken regularly and the reaction was terminated at defined time points to follow the reaction through a time period of 6 h. Previous experiments (section 4.7 and 4.8) have established that 6 h is sufficient to obtain maximum equilibrium yield. Reaction kinetics of water-soluble A_nM diblocks (results above) revealed that equilibrium yield was obtained within 15 minutes. Two separate experiments were performed to assess the reproducibility of the results.

^{1}H-NMR of $dext_{37}$-PDHA was obtained prior to the conjugation to assure that is was fully reduced, as well as to characterize the integral of the H1,Glc (int) resonance. The H1,Glc (int) resonance was assumed constant throughout the reaction and was thus used as internal standard for determination of conjugation yield.

^{1}H-NMR spectrum was acquired of each sample obtained during the reaction, and the spectra were calibrated by TSP. The sample obtained after 15 min in the first experiment had some deviances in the integral values of known peaks and was thus considered invalid and removed from the data collection. The relative yield of each sample was determined from the integral of the E- and Z-oxime resonances (7.4 and 6.8 ppm, respectively) relative to the previously established integral of H1,Glc(int). The results are presented in Figure 4-23.

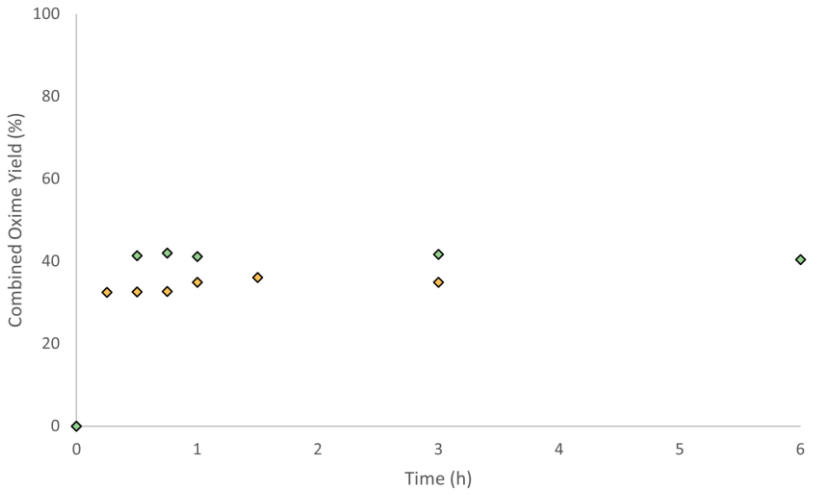

Figure 4-23: Combined E- and Z-oxime formation during conjugation of $(A_nM)_{wis}$ (20.1 mM) to equimolar amounts of $dext_{37}$-PDHA in 8 wt% LiCl/DMAc + 1% H_2O at 40°C. Two separate experiments were performed at different occasions (green and yellow).

The equilibrium yield was calculated to be 40% and 34% for the two separate experiments, obtained within 30 minutes and 15 minutes, respectively. From the results obtained in section 4.10.1 and in this section, the equilibrium yield in the first experiment (green) was in all likelihood reached within 15 minutes as well. Nonetheless, due to the difficulties in obtaining a valid ^{1}H-NMR spectrum of the sample this can not be quantitatively verified. As mentioned in the previous section, the deviance in the equilibrium yield is attributed to the practical difficulties at the wet lab.

Interestingly, this is somewhat comparable to the results obtained in section 4.3, whereas conjugation of water-soluble A_nM (A_5M) to dextran-PDHA in standard conditions (500 mM NaAc, pH=4) 90% of equilibrium yield is obtained at 10 minutes with the same concentration of reactants. The equilibrium yield was 63%, which is indeed comparable to the reactions with water-soluble A_nM in DMAc (~60%). Although a direct comparison can not be done due to different solvents as well as different fractions of A_nM oligosaccharides (water insoluble vs water-soluble), this can be of significance for the choice of protocol.

The results presented here indicates that the conjugation of the water insoluble fraction of fully N-acetylated chitin with M at the reducing end to dextran-PDHA (DP_n=37) in 8wt% LiCl/DMAc is essentially complete within 15 min with equimolar ratio at 40°C with TEA as base catalyst.

4.11 SEC-MALLS Analyses of Water Insoluble Chitin Conjugates

Successful conjugation of dextran to water insoluble chitin to produce a water-soluble chitin-dextran conjugate allowed for further examination of the physical properties of the conjugate. SEC-MALLS analyses of chitin-dextran diblocks were performed as described in section 3.3 (with 0.15 M NaNO3 with 10 mM EDTA, columns TSKgel 4000 and 2500) and compared to previously obtained results of pure dextran-PDHA (presented in section 4.2.2). Additionally, the samples were sent to the laboratory of Prof. Antje Potthast, Universität für Bodenkultur, Vienna, for SEC-MALLS analyses with 0.9% LiCl/DMAc as solvent (section 3.3.1). The resulting chromatograms are given in Figure 4-24.

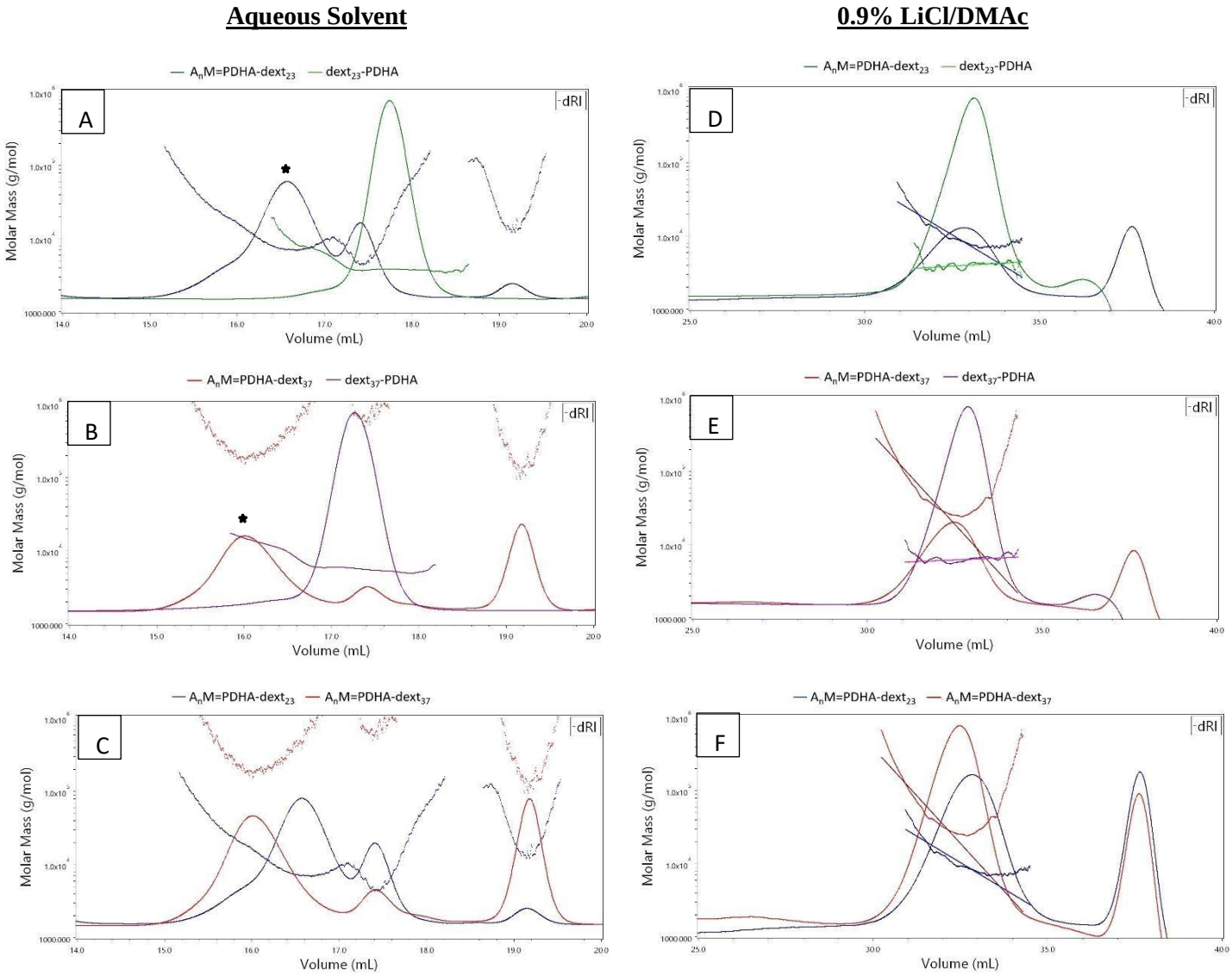

Figure 4-24: SEC-MALLS chromatograms of molar mass vs elution volume for $(A_nM)_{wis}$=PDHA-dext$_m$ (m=23 or 37) and dext$_m$-PDHA (m=23 or 37) in aqueous solvent and in organic solvent (0.9% LiCl/DMAc) (left and right column, respectively). Physical properties of the content of the samples are given in Table 8 whereas for the diblocks in A and B are given for the peaks marked with a star only.

SEC-MALLS analyses of $(A_nM)_{wis}$=PDHA-dext$_m$ (m=23 and 37) and of pure unconjugated dext$_m$-PDHA obtained in 0.9% LiCl/DMAc were performed as chitin readily dissolves in LiCl/DMAc. The chromatograms are presented in Figure 4-24 D-F. All peaks were symmetrical with no sign of aggregation. The pure dextran-PDHA samples also served as control samples, to assure that correct molecular weights were obtained. Evident from the chromatograms, the molecular weights have increased with the attachment of $(A_nM)_{wis}$. A slight shift in elution volume is also observed due to successful conjugation. However, the columns used have not been of appropriate size to separate the unconjugated dextran from the diblocks in the samples, resulting in one peak. Hence, the unconjugated dextran might influence the calculated molecular weights of the diblocks.

SEC-MALLS chromatograms of $(A_nM)_{wis}$=PDHA-dext$_m$ (m=23 and 37) compared to pure unconjugated dext$_m$-PDHA obtained in aqueous solvent are presented in Figure 4-24 A-C. Two different solvent were used (0.15 M $NaNO_3$ with 10 mM EDTA and 0.2 M AmAc) due to the availability of the systems. However, this is assumed to not affect the analyses due to the solvent change will not affect the behavior of dextran-PDHA. Successful conjugations are evident in the chromatograms, whereas one peak is present for the conjugates and a separate peak eluting later corresponds to residual unconjugated dext$_m$-PDHA. In Figure 4-24 C, the two diblocks are presented together, showing the similarities in the peaks. As expected, a slight shift is observed as $(A_nM)_{wis}$-PDHA-dext$_{37}$ eluted earlier than $(A_nM)_{wis}$-PDHA-dext$_{23}$ due to longer chain lengths.

An unexpected peak was eluting at 19-19.5 mL. First, it was assumed a contamination due to the low concentration in the $(A_nM)_{wis}$-PDHA-dext$_{23}$ sample (Figure 4-24 A), however it had a substantially higher concentration in the $(A_nM)_{wis}$-PDHA-dext$_{37}$ sample (Figure 4-24 B). The analyses showed that the content has a high molecular weight and high polydispersity (Mn=12 kDa, Mw=20 kDa), but it did not scatter any light. Nonetheless, it eluted later than the diblock, which means it has a lower hydrodynamic volume. This indicates aggregation of some of the components in the sample, however further examination is needed in order to verify the details of the content.

Furthermore, a low intensity peak eluted at ~10-14 mL (depending on diblock) (Figure 4-25). Interestingly, the content corresponding to this peak had a high light scattering signal, extremely high molecular weight (M_w=30 000 kDa and 20 000 kDa, for the dext$_{37}$- and dext$_{23}$-diblock, respectively) and low intrinsic viscosity (13 ml/g). These results led to the

speculation that it might be a soluble aggregation of chitin or chitin-dextran conjugates. The earlier elution time for this component in the sample of $(A_nM)_{wis}$-PDHA-dext$_{37}$ compared to $(A_nM)_{wis}$-PDHA-dext$_{23}$ indicated the latter. The interest in this aggregated component compared to the one described above is attributed to the high light scattering signal and the large hydrodynamic volume. Along with the physical properties, it is speculated that this might be an ordered structure. The lack of this signal in the samples obtained in organic solvent indicates that it is an aggregation as a result of the aqueous surroundings. Which means it is probable that it is attributed to the inherent ability of chitin to self-assemble by inter- and intrachain hydrogen bonding – leading to the suggestion of a self-assembled chitin core with a dextran surrounding. This is presumably the first evidence of a possibly ordered self-assembly of a conjugate of water insoluble chitin and dextran. However, this is only a speculation, any detailed information about the ordering or the content can not be deduced from these analyses. Forthcoming studies should thus include further examination of this component, by e.g. microscopy techniques.

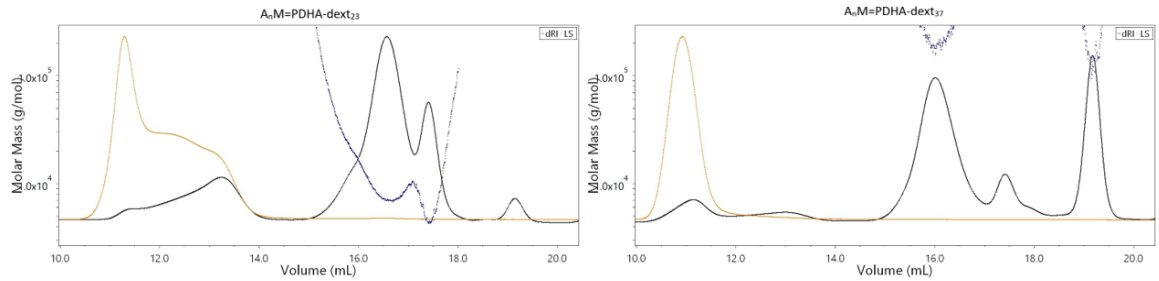

Figure 4-25: SEC-MALLS chromatograms of molar mass vs elution time, including light scattering (yellow) for $(A_nM)_{wis}$=PDHA-dext$_m$ (m=23, 37, left and right respectively).

The physical properties obtained from the analyses are presented in Table 8. For the pure dextran-PDHA samples and the $(A_nM)_{wis}$ =PDHA-dext$_{23}$ diblock the molecular weight obtained in the different solvents were roughly the same. Moreover, the weight of $(A_nM)_{wis}$=PDHA-dext$_{23}$ was roughly the sum of $(A_nM)_{wis}$ and dext$_{23}$-PDHA alone, as expected. However, accurate numbers were not obtained for $(A_nM)_{wis}$ =PDHA-dext$_{37}$. The molecular weights were much higher than anticipated and is probably a result of an inhomogeneous sample.

The polydispersity is higher in both diblocks compared to dextran-PDHA, both in organic and aqueous solvent. This is attributed to the differing chain lengths of $(A_nM)_{wis}$, as well as the lack of separation of unconjugated dextran-PDHA in the samples obtained in organic solvent. Additionally, the mass recoveries of the diblock samples in DMAc were low compared to the

pure dext-PDHA samples (32% and 39% vs 74% and 80%), as well as the mass recovery of $(A_nM)_{wis}$ was only 24%. The reason for this is unknown.

Table 8: Physical properties obtained from the SEC-MALLS analyses of water insoluble chitin conjugates and dextran-PDHA in organic and aqueous solvent. The physical properties of the diblocks in aqueous solvents are given only for the peaks marked with stars in Figure 4-24.

	Organic Solvent				Aqueous Solvent			
Sample	**M_n (kDa)**	**M_w (kDa)**	**Polydispersity (M_w/M_n)**	**Mass Recovery (%)**	**M_n (kDa)**	**M_w (kDa)**	**Polydispersity (M_w/M_n)**	**Mass Recovery (%)**
$(A_nM)_{wis}$=b-dext$_{23}$	7.8	10.3	1.3	32	8.1	11.4	1.4	77
Dext$_{23}$-PDHA	4.0	4.0	1.0	74	3.8	3.9	1.0	63
*$(A_nM)_{wis}$ =b-dext$_{37}$	15.5	37.2	2.4	39	280	492	1.8	69
Dext$_{37}$-PDHA	6.2	6.2	1.0	80	5.8	6.0	1.0	99
$(A_nM)_{wis}$	3.8	5.2	1.4	24	-	-	-	-

*Inaccurate information, possibly due to inhomogeneous sample

The opportunity to conduct SEC-MALLS with LiCl/DMAc as solvent allowed for analyses of the water insoluble chitin fraction (Figure 4-26). The sample did not dissolve in 0.9% LiCl/DMAc which was the solvent for the SEC-MALLS system, so it was dissolved in 8% LiCl/DMAc and injected. Based on the results, $(A_nM)_{wis}$ seems to have a higher number average degree of polymerization than first assumed, suggesting a DP_n of 19 (M_n/M_0). All reactions in this thesis using this fraction have been performed based on the assumption that it has a DP_n of 12. The effect of this estimate on the equimolar reactions performed is a 1.6x under- or overestimation depending on whether the calculations were performed based on a certain molar amount of dext$_m$-PDHA or A_nM, respectively. Nonetheless, there are some insecurities whereas A2 and dn/dc for chitin is unknown, in which the value here is set to the same as for cellulose (51) – 0 and 0.136 ml/g, respectively.

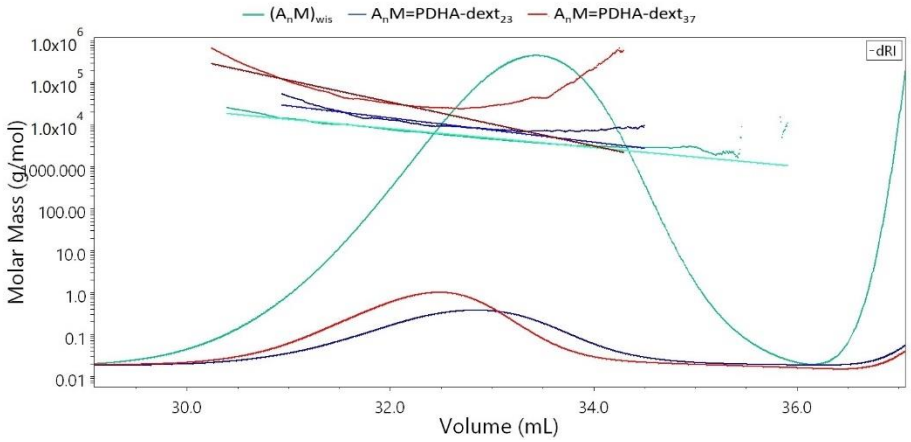

Figure 4-26: SEC-MALLS chromatograms of molar mass vs elution time of $(A_nM)_{wis}$, $(A_nM)_{wis}$=PDHA-dext$_{23}$ and $(A_nM)_{wis}$=PDHA-dext$_{37}$ in 0.9% LiCl/DMAc.

5 Concluding Remarks and Future Perspectives

The work for this master thesis was a collection of expanding the information related to already established protocols as well as exploratory studies for the development of new protocols for future importance.

The kinetic parameters for the conjugation and reduction of water-soluble chitin to dextran-PDHA were elucidated. Optical rotation was dismissed as a method to characterize the reaction kinetics of the periodate oxidation of the non-reducing end of chitin oligosaccharides. Furthermore, and the main focus of the thesis, an alternative solvent system for oxime click reactions of water insoluble chitin oligosaccharides (DP > 9) was established; N,N-dimethylacetamide (DMAc) containing a few percent LiCl. Oxime click reactions with PDHA as linker were successful at high rates. Reduction, however, was not successful using α-picoline borane as reducing agent. Nonetheless, this is not limiting as the amination and reduction can be performed in a two-pot system.

5.1 Reaction Kinetics of the Reductive Amination of A_5M to $Dext_{15}$-PDHA

The reaction rate constants for the conjugation of A_5M to $dext_{15}$-PDHA were elucidated and the subsequent reduction of the diblock was optimized. The conjugation of A_nM to initially activated dextran was significantly faster than the conjugation of dextran to initially activated A_nM. Attributed to the high reactivity of the pending aldehyde of the reducing end M-residue. Nonetheless, the reduction was time consuming in both cases. However, by increasing the temperature and surplus of reducing agent, completely reduced conjugates were obtained after 72 h. With using initially activated A_nM and dextran as the second block, higher yields are obtained due to the slow background reduction of unconjugated dextran (92% vs 63%). Hence, the choice of protocol should be based on available starting material, taken the difference of final diblock yield into account. Additionally, using a molar excess of one of the components would increase the yield in any case.

5.2 Optical Rotation to Measure Kinetics of the Periodate Oxidation of A_nM Oligosaccharides

Measuring optical rotation was attempted as a method to determine the reaction rate of the periodate oxidation of the non-reducing end of chitin oligosaccharides. Previous studies on periodate oxidation of chitin oligomers (I.V. Mo, submitted to Carbohydrate Polymers, 2021) have shown that within 24 h a substantial amount have been oxidized. Comprehensive studies stated that optical rotation is not an appropriate method to determine the degree of oxidation

at the non-reducing end of chitin oligosaccharides, as no change in rotation was detected over a time period of 40 h.

5.3 Simultaneous Nitrous Acid Degradation and Conjugation to PDHA

Simultaneous degradation of chitosan by nitrous acid and conjugation to PDHA was attempted. The aim of the experiment was to attach PDHA while the fully N-acetylated chitin oligomers were produced in an attempt to increase the solubility of the longer oligosaccharides (DP > 9) before precipitation. ^{1}H-NMR and SEC analyses revealed that the degradation was not complete, yielding chitosan with no evidence of conjugation. The conclusion was that some conjugation had been obtained, but the nitrous acid reversed the conjugation, following there was no longer an excess of nitrous acid to fully degrade the chitosan.

5.4 Dissolution of Water insoluble Chitin Oligosaccharides

An exploratory study using alternative solvents was conducted to dissolve the water insoluble fraction of chitin oligosaccharides ($(A_nM)_{wis}$). $(A_nM)_{wis}$ successfully dissolved in N,N-dimethylacetamide containing a few percent LiCl (LiCl/DMAc) upon brief heating. This solvent system is widely used for cellulosic materials and was chosen as the preferred solvent for further studies.

5.5 Deuterated N,N-Dimethylacetamide as Solvent for ^{1}H-NMR Analyses of Water insoluble Chitin Oligosaccharides and Conjugates

^{1}H-NMR using deuterated N,N-dimethylacetamide (DMAc) as solvent was attempted to allow for studies of the reaction kinetics of oxime click in LiCl/DMAc. Preliminary ^{1}H-NMR spectra of known compounds dissolved in deuterated DMAc with LiCl were incomparable with ^{1}H-NMR spectra obtained with D_2O as solvent. 2D-NMR gave inconclusive results and mediated no further progress in peak assignment. Another approach to peak assignment was attempted by performing different reactions with known outcomes and follow it with real time NMR (RT-NMR). However, no change in resonances were observed for any of the reactions, making it an unsuitable method for peak annotation. Due to inconclusive results and time limitation, ^{1}H-NMR with deuterated DMAc as solvent was not further pursued as it need thorough analysis to continue with this method.

5.6 Terminal Conjugation of Dextran to Water Insoluble Chitin in LiCl/DMAc

In this thesis, N,N-dimethylacetamide (DMAc) with a few percent LiCl is proposed as a viable solvent system to produce chitin-based block polysaccharides by oxime-click to the free end of a bifunctional linker (PDHA). Terminal conjugation of dextran to water insoluble chitin produced a water-soluble chitin-dextran conjugate.

Preliminary studies with water-soluble A_nM (DP < 9) showed successful conjugation with yields of up to 60% with molar equivalents. Conjugation of $(A_nM)_{wis}$ to dextran-PDHA was successful with a yield of roughly 40%. An important finding was that conjugation of $(A_nM)_{wis}$ to highly soluble dextran, yielded a water-soluble conjugate. Systematic studies revealed that the reactions reached equilibrium yield within 15 minutes. By using an 3x excess of one of the blocks a yield of 84% was obtained. Here, it was found beneficial with an excess of $(A_nM)_{wis}$ due to lack of methods to purify the reaction mixture of unconjugated dextran-PDHA. Unconjugated $(A_nM)_{wis}$ however could readily be removed by precipitation.

^{1}H-NMR and SEC-MALLS analyses verified successful conjugation. Additionally, SEC-MALLS analyses conducted in aqueous solvent revealed an interesting component of the sample which had a high molecular weight, high light scattering signal and low intrinsic viscosity. It is speculated that it might be an ordered structure with a chitin core and dextran surrounding, however, further examination is needed in order to verify the details.

During the previous studies of using deuterated DMAc for RT-NMR, it was found that α-picoline borane (PB) is not an appropriate reducing agent in LiCl/DMAc. Nonetheless, this will not hinder further progress as the reductive amination of A_nM type oligomers are generally performed in a two-pot system. The complete reductive amination can be performed by conjugation in LiCl/DMAc with subsequent purification, followed by reduction in an appropriate solvent (such as sodium acetate). The drawback of this two-pot system is however that it would be a time-consuming protocol, as the reaction mixture need thorough purification by dialysis after both the oxime click to remove DMAc, as well as the reduction to remove PB. Nonetheless, the initial findings that conjugation of the water insoluble chitin fraction to highly soluble dextran produces a chitin-dextran conjugate which is soluble in aqueous solutions renders this as a viable method.

5.7 Future Perspectives

Forthcoming studies should include more detailed analyses of the reaction rate of chitin-based block polysaccharides in LiCl/DMAc, including maximation of yield, different concentrations of reactants, varying temperatures, and different percentages of LiCl. Additionally, a thorough review of the purification process of the $(A_nM)_{wis}$-PDHA-dextran reaction mixture is necessary to secure completely purified diblocks. This thesis has proven successful oxime click reactions in LiCl/DMAc with PDHA as linker, making it possible to conjugate to other polysaccharides that are soluble in LiCl/DMAc, such as beta glucans and cellulose.

Furthermore, it is of interest to assess the properties of the newly produced $(A_nM)_{wis}$-PDHA-dextran block polysaccharides. Properties such as stability at different temperatures, solvents, pH, and self-assembly should be reviewed. It is of special interest to examine the content of the possible self-assembled structures in aqueous solutions (as discussed in section 4.11).

The presumably first successful conjugation of water-insoluble chitin to dextran which resulted in a water-soluble conjugate produced in this thesis, might be of importance for further biochemical analyses of the previously described chitin-TLR2 relationship. By terminal conjugation (compared to lateral) it is probable that the intrinsic properties of chitin remain intact. Forthcoming studies should include bioanalysis to assess the potential of the conjugate to trigger downstream immune responses through possible binding to TLR2. The results from this thesis could be of great importance for further studies of this relationship, potentially mediating the development of novel therapies addressing chitin-mediated inflammatory diseases such as fungal infection and airway inflammation during asthma.

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Appendix A: Characterization of Chitin Oligosaccharides and Dextran Conjugates

The ^{1}H-NMR spectrum of fraction 3 presented in the chromatogram from the separation of fully N-acetylated chitin oligomers (A_nM) from nitrous acid degradation of chitosan (section 4.1, Figure 4-2) is shown in Figure A-1. The degree of polymerization (DP) was calculated from the ratio between H1,A and H3,M to be 6 (A_5M), which was used to calibrate the DP within all the collected fractions.

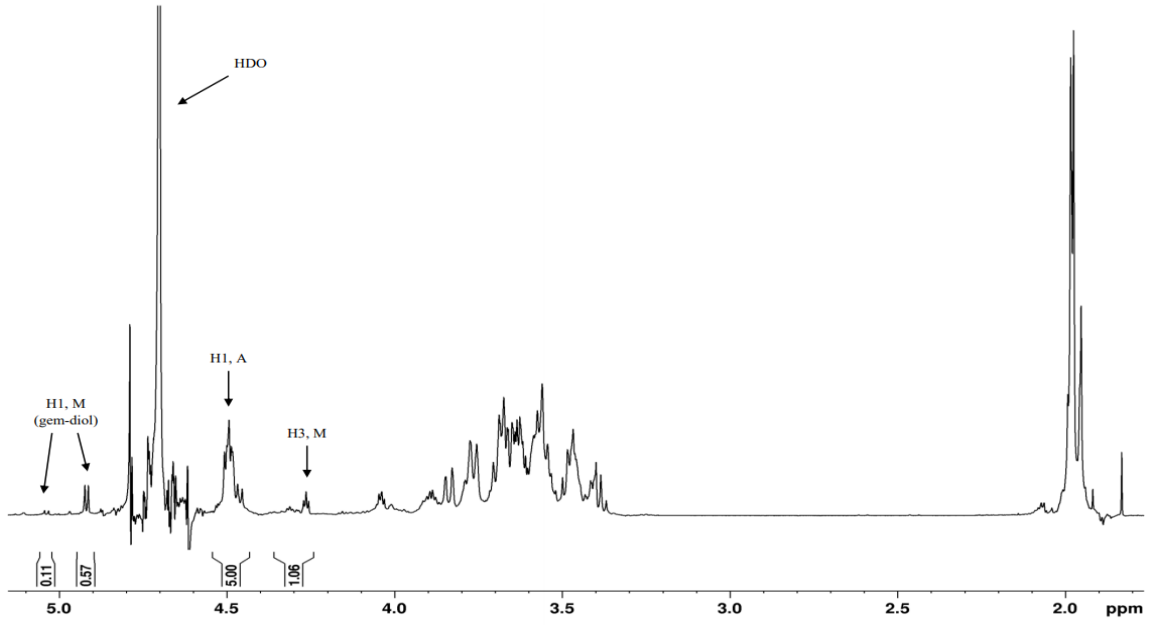

Figure A-1: ^{1}H-NMR (600 MHz, 298K, D_2O) of fraction 3 of the separated nitrous acid degraded A_nM (chromatogram shown in Figure 4-2, section 4.1.1) for determination of DP=6 (A_5M).

The ^{1}H-NMR spectrum of isolation and purification of a monodisperse fraction (elution time: 732-747 min) from the separation of dextran-PDHA conjugates (section 4.2) is given in Figure A-2. The DP of the sample was determined to be 11 by the relationship between the resonance of H1 of the internal glucose residue relative to the secondary amine resonances.

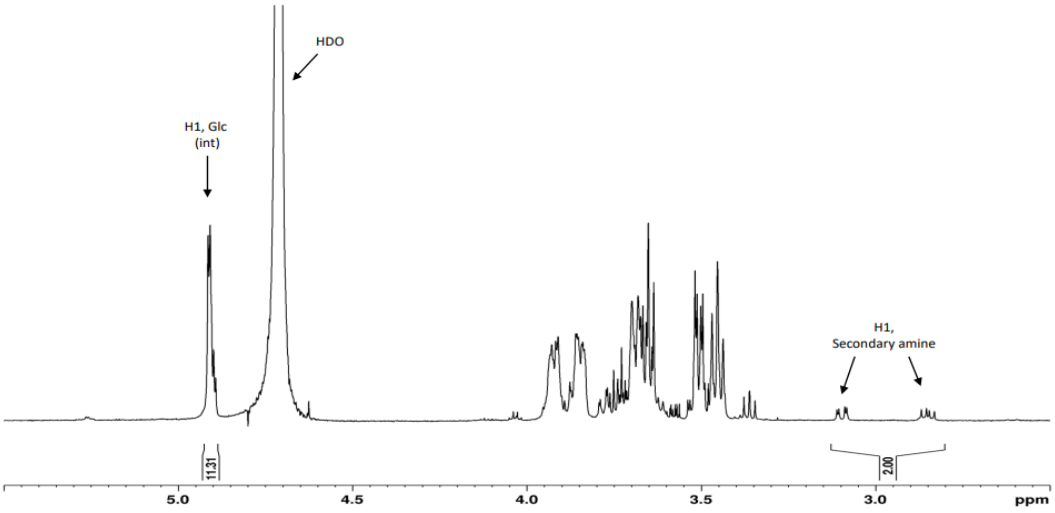

Figure A-2: ^{1}H-NMR (600 MHz, 298K, D_2O) of the collection and purification of a narrow peak (elution time: 732-747 min) of the smaller oligomers of the SEC fractionation of dextran-PDHA (section 4.2.2) for determination of DP=11.

Dextran-PDHA (DP_n=41) was separated by SEC and collected as five fractions to obtain samples with more narrow distributions (section 4.2.2). The five fractions were analyzed by SEC-MALLS to determine the molecular weight, polydispersity and number average degree of polymerization (DP_n). The resulting chromatogram is shown in Figure A-3. Fraction 5 was contaminated by fraction 1, so this fraction was separated by SEC again to remove the contamination.

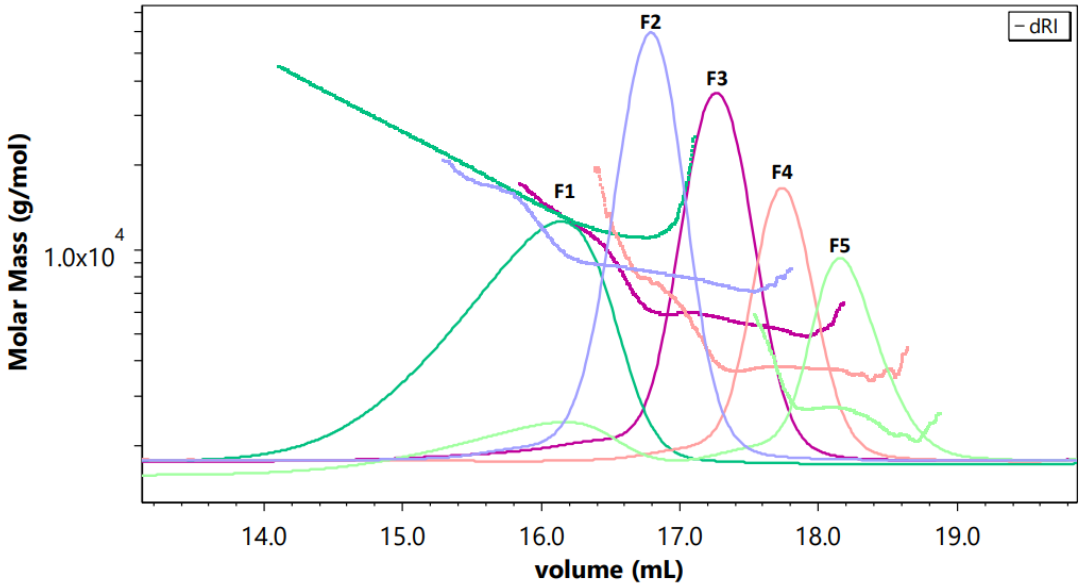

Figure A-3: SEC-MALLS (0.2 M AmAc, pH=4.5, A2=2x10^{-4}, dn/dc=0.148 ml/g) analyses of the molecular weight distribution of the collected dextran-PDHA fractions (F1-F5).

Appendix B: ^{1}H-NMR Spectra from the Reaction Kinetics of A_5M to $Dext_{15}$-PDHA

^{1}H-NMR spectra from the conjugation of A_5M to $dext_{15}$-PDHA (section 4.3) are given in Figure B-1. The ^{1}H-NMR spectra from the subsequent reduction of the resulting A_5M-PDHA-$dext_{15}$ diblock is given in Figure B-2. The relative yield of E- and Z-oxime at each time point was used to fit a model of the reaction kinetics of the conjugation reaction. Interestingly, the presentation of the secondary amine signals changes after conjugation to A_5M due to a change in the environment when attachment happens. The relative change in yield for E- and Z-oximes as well as the secondary amine resonances for each time point was used to fit a model of the reaction kinetics of the reduction.

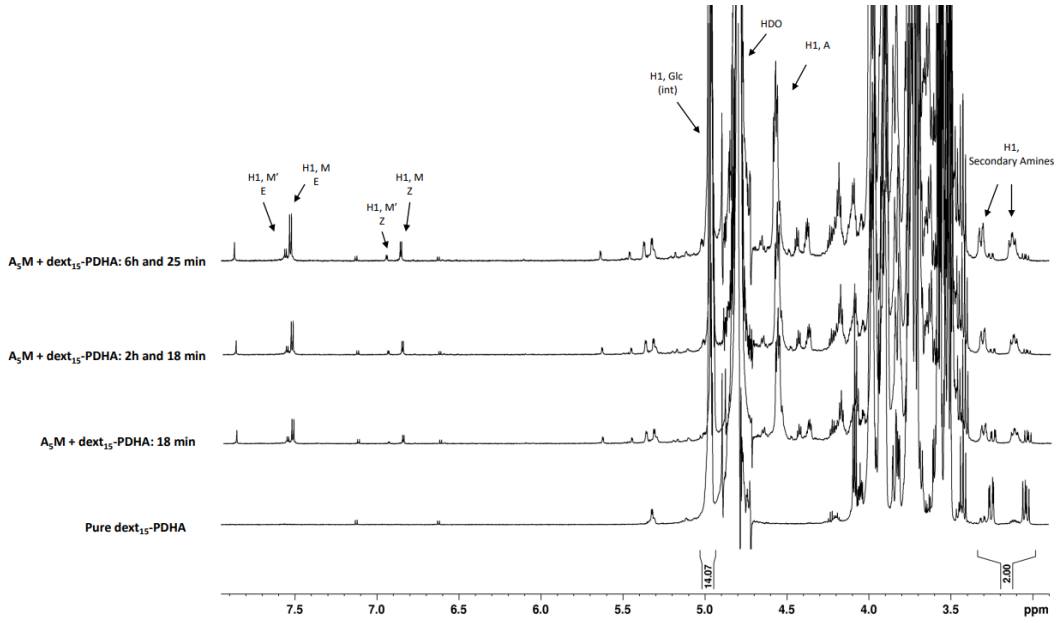

Figure B-1: ^{1}H-NMR (600 MHz, 298K, 500 mM NaAc) of pure $dext_{15}$-PDHA (3.5 mM) and at different time points after addition of equivalent amounts of A_5M.

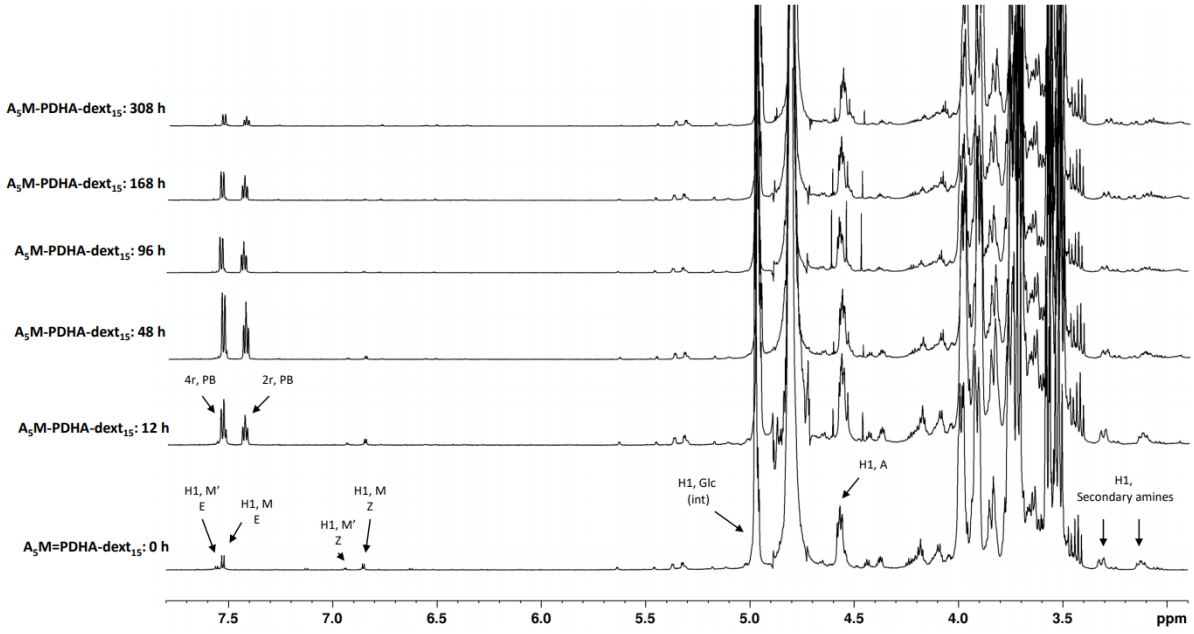

Figure B-2: ^{1}H-NMR (600 MHz, 298K, 500 mM NaAc) of unreduced A_5M=PDHA-$Dext_{15}$ (3.5 mM, "=":unreduced) and at different time points after addition of 3 equivalents α-picoline borane.

Appendix C: ^{1}H-NMR in Deuterated N,N-Dimethylacetamide

2D ^{1}H-NMR Spectra of A_2M in deuterated N,N-dimethylacetamide

The acquired 2D NMR (HMBC, HSQC, COSY) spectra of A_2M in deuterated DMAc containing 8% LiCl are given in Figure C-1, Figure C-2 and Figure C-3 (section 4.9).

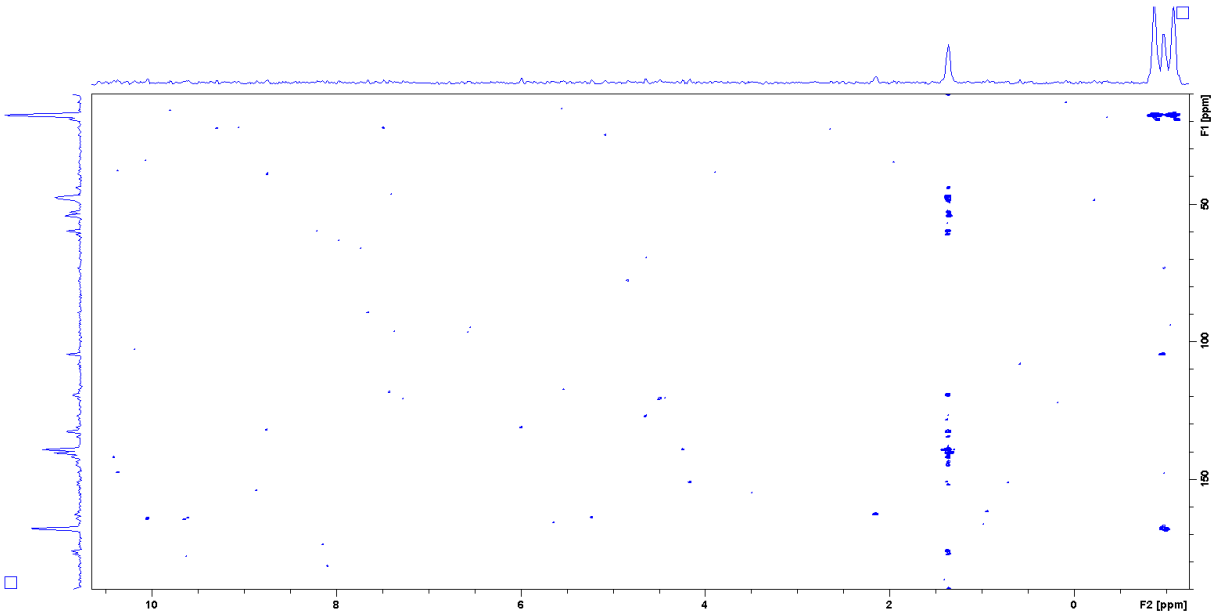

Figure C-1: ^{1}H-NMR HMBC (600 MHz, 355K) of A_2M in deuterated DMAc containing 8% LiCl.

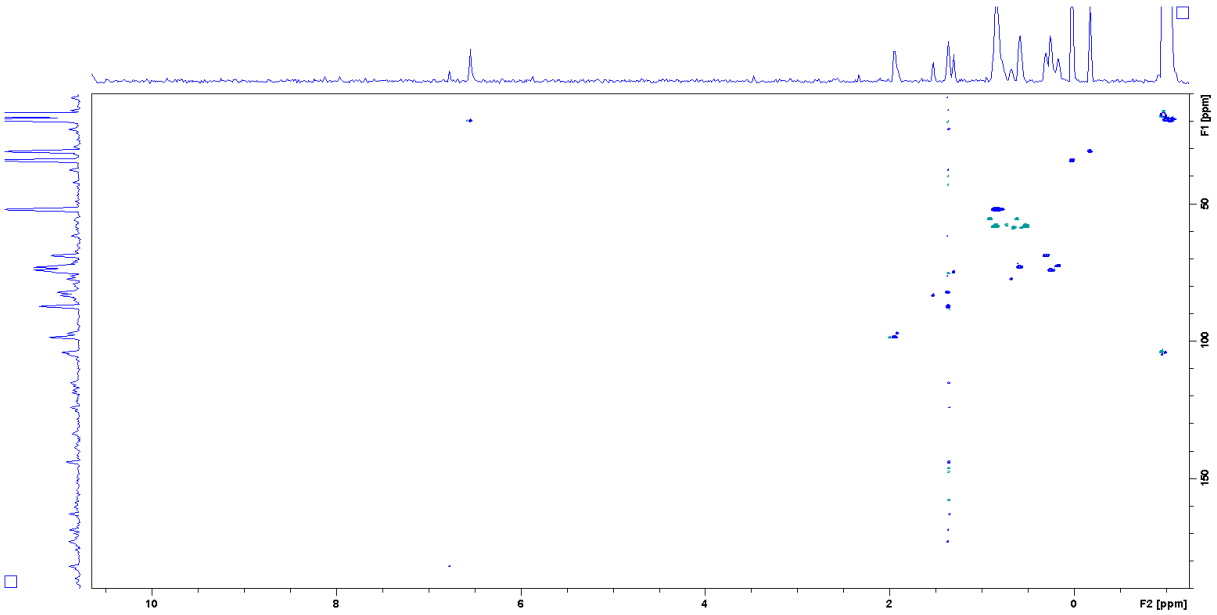

Figure C-2: ^{1}H-NMR HSQC (600 MHz, 355K) of A_2M in deuterated DMAc containing 8% LiCl.

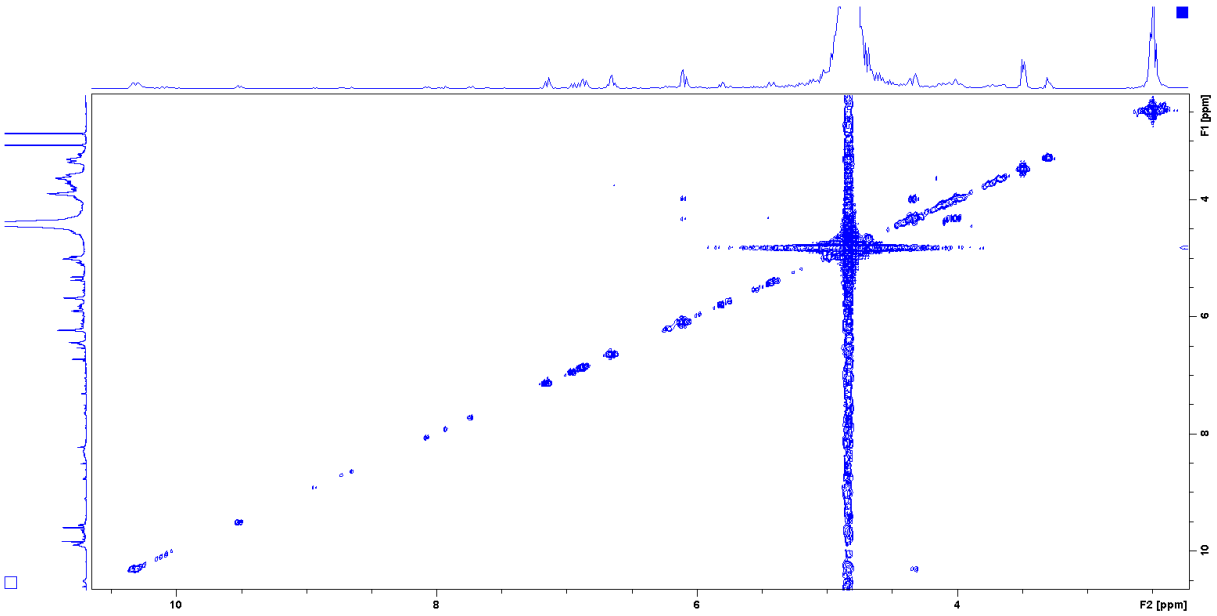

Figure C-3: ^{1}H-NMR COSY (600 MHz, 355K) of A_2M in deuterated DMAc containing 8% LiCl.

^{1}H-NMR spectra of reactions in deuterated N,N-dimethylacetamide

Different reactions were executed in deuterated N,N-dimethylacetamide with 8% LiCl with the aim of assigning peaks due to change of intensity in ^{1}H-NMR spectra (section 4.9.2). During the time span of the reactions all spectra were obtained at both 298K and 355K due to the downfield shift of the solvent peak at 355K to allow for observation of all resonances. However, only spectra obtained at 298K are given here because there were no substantial differences in the spectra recorded at the two different temperatures.

Reduction of A_2M by α-picoline borane is shown in Figure C-4. Conjugation of A_2M to PDHA is shown in Figure C-5, while the purified compound redissolved in D_2O is shown in Figure C-6. The reduction of $(A_nM)_{wis}$=PDHA-dext$_{15}$ is shown in Figure C-7, while the purified compound redissolved in D_2O is shown in Figure C-8.

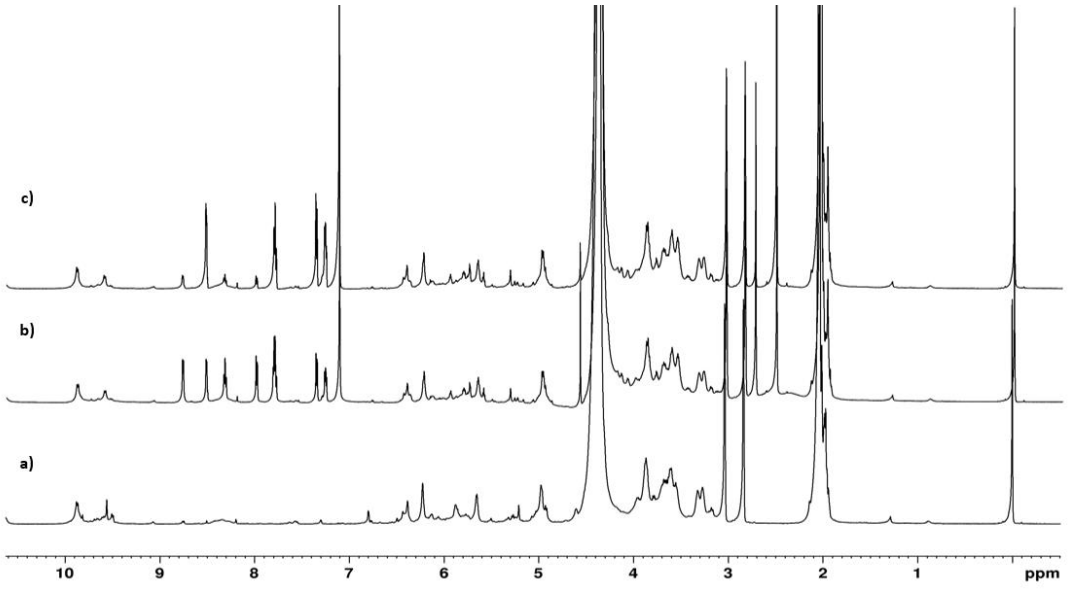

Figure C-4: ^{1}H-NMR (298K, 600 MHz) of a) A_2M in 8 wt% LiCl/DMAc b) A_2M with 3 molar equivalents of α-picoline borane at 0h and c) after 48h (calibrated by TSP).

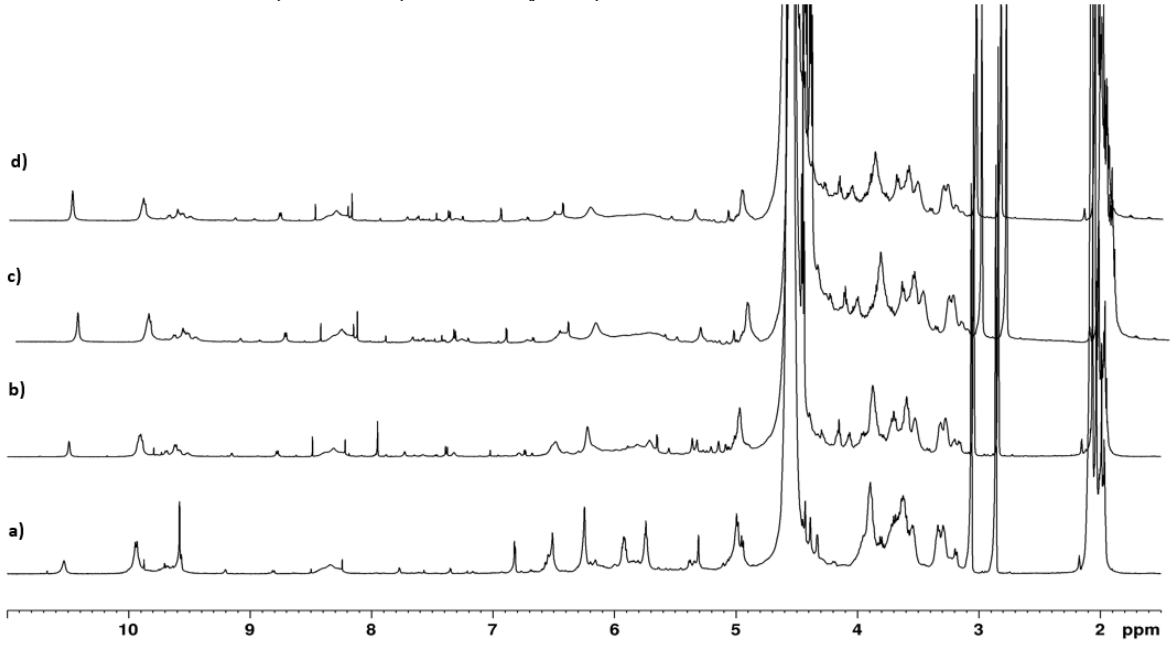

Figure C-5: ^{1}H-NMR (298K, 600 MHz) of a) pure A_2M in 8 wt% LiCl/DMAc + 1% H_2O and b), c) and d) are after addition of 2 equivalents PDHA at t=0h, t= 24h and t=48h, respectively. All spectra are calibrated by TSP.

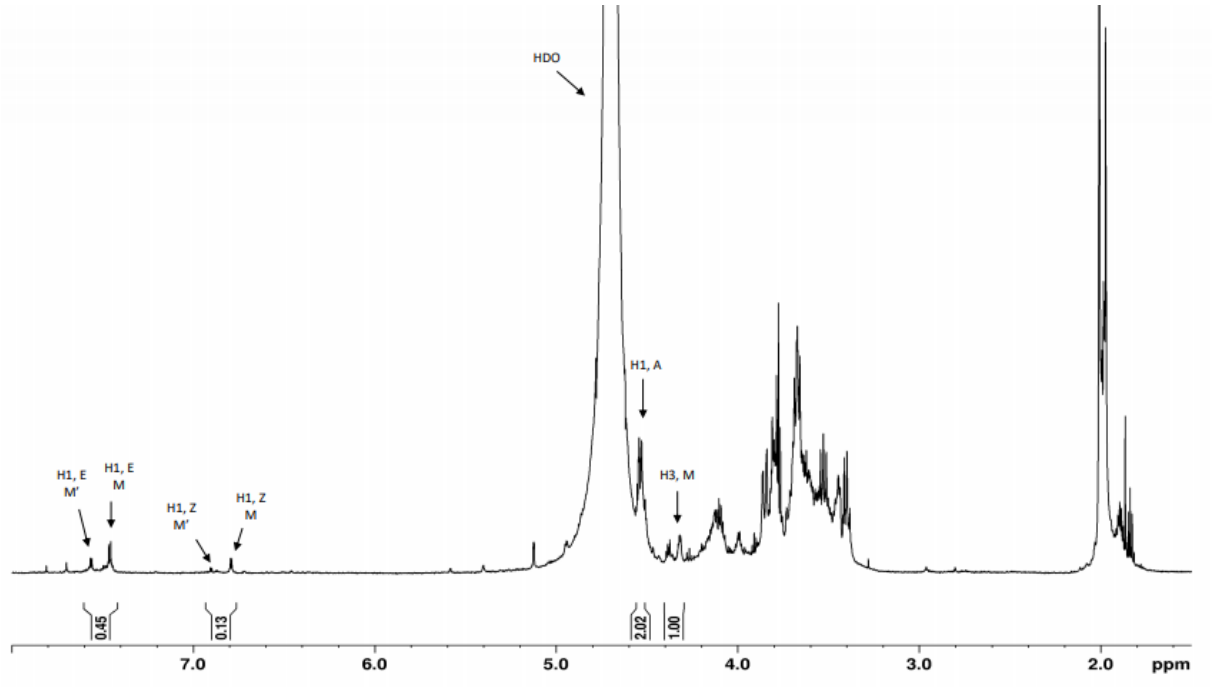

Figure C-6: ^{1}H-NMR (298K, 600 MHz) of purified A_2M=PDHA in D_2O (after conjugation in LiCl/DMAc).

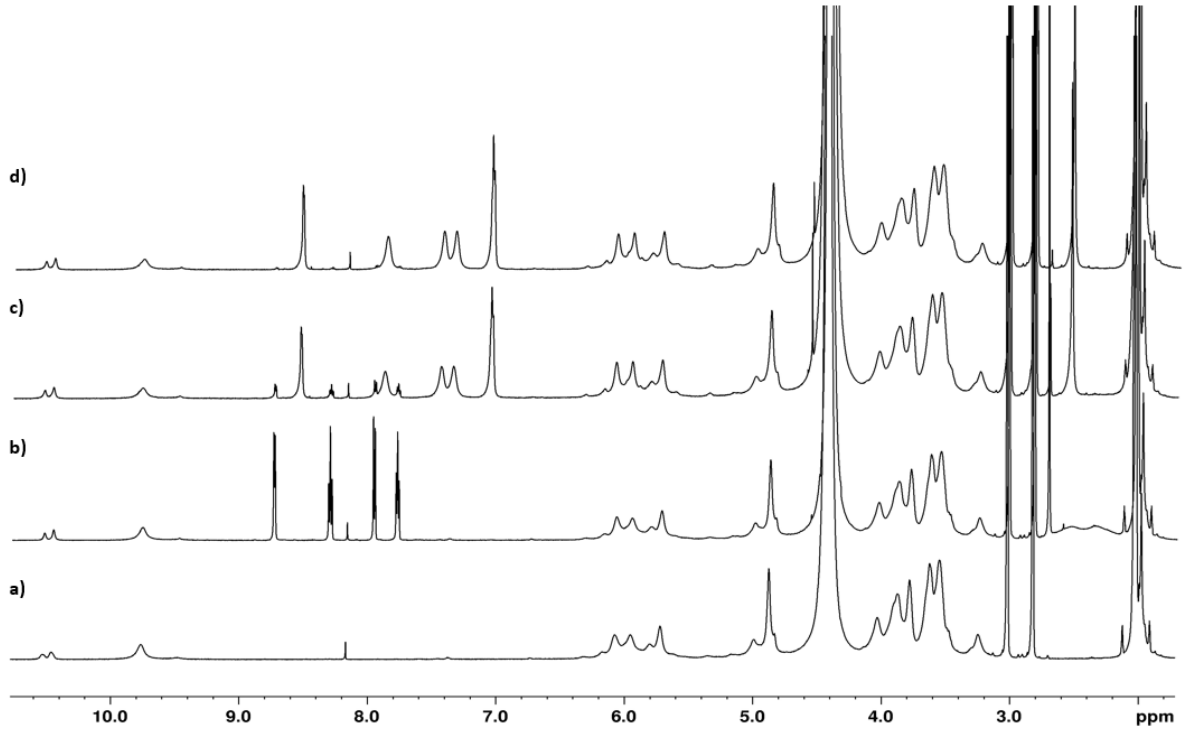

Figure C-7: ^{1}H-NMR (298K, 600 MHz) of a) pure $(A_nM)_{wis}$=PDHA-$Dext_{23}$ in 8 wt% LiCl/DMAc + 1% D_2O and b), c) and d) are after addition of 3 equivalents α-Picoline Borane at t=0h, t= 24h and t=48h, respectively. All spectra are calibrated by TSP.

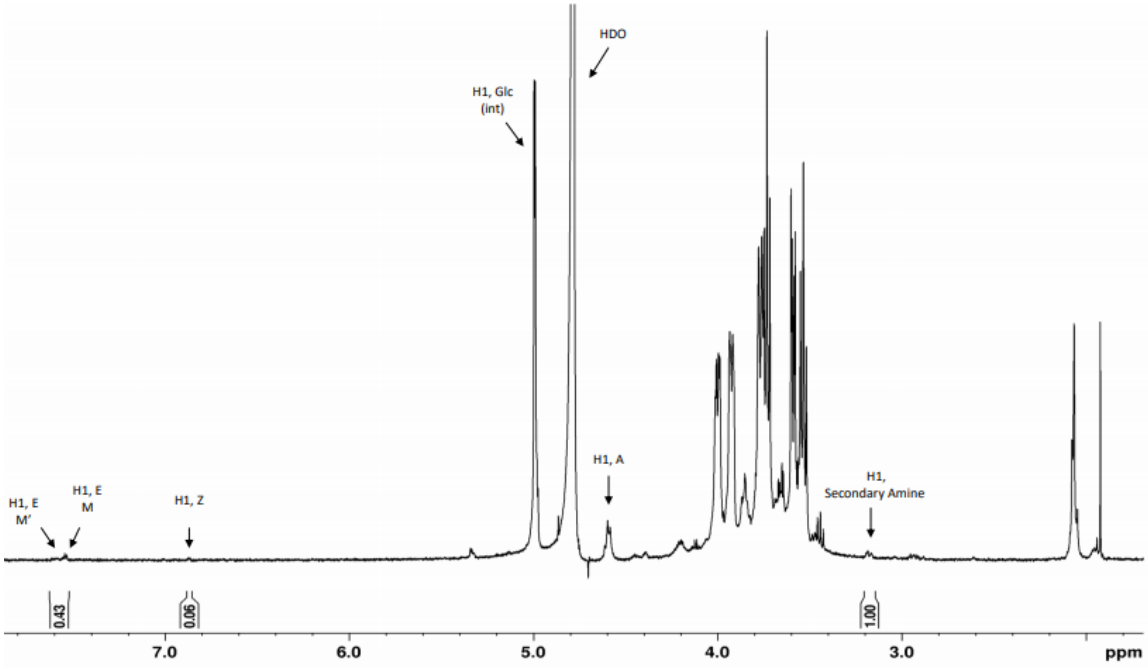

Figure C-8: ^{1}H-NMR (298K, 600 MHz) of purified $(A_nM)_{wis}$=PDHA-Dext$_{23}$ in D_2O (after attempting reduction by 3xPB in LiCl/DMAc).

Trine Muren

Chitin Oligomers and Chitin-based Block Polysaccharides

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