Regioselective modifications of galactose-containing polysaccharides in aqueous media

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"Protect me from knowing what I don't need to know. Protect me from even knowing that there are things to know that I don't know.
Protect me from knowing that I decided not to know about the things that I decided not to know about.
Amen"

Douglas Adams, The Hitchhiker's Guide to the Galaxy

Abstract

Biorefining is defined as sustainable conversion of biomass into marketable products and energy. Forests cover almost one third of earth's land area, and account for approximately 40% of the total annual biomass production. In forest biorefining, the wood components are, in addition to the traditional paper and board products, converted into chemicals and biofuels. The major components in wood are cellulose, hemicelluloses, and lignin. The main hemicellulose in softwoods, which are of interest especially for the Nordic forest industry, is *O*-acetyl galactoglucomannan (GGM). GGM can be isolated in industrial scale from the waste waters of the mechanical pulping process, but is not yet today industrially utilized. In order to attain desired properties of GGM for specific end-uses, chemical and enzymatic modifications can be performed.

Regioselective modifications of GGM, and other galactose-containing polysaccharides were done by oxidations, and by combining oxidations with subsequent derivatizations of the formed carbonyl or carboxyl groups. Two different pathways were investigated: activation of the C-6 positions in different sugar units by TEMPO-mediated oxidation, and activation of C-6 position in only galactose-units by oxidation catalyzed by the enzyme galactose oxidase. The activated sites were further selectively derivatized; TEMPO-oxidized GGM by a carbodiimide-mediated reaction forming amides, and GO-oxidized GGM by indium-mediated allylation introducing double or triple bonds to the molecule. In order to better understand the reaction, and to develop a MALDI-TOF-MS method for characterization of regioselectively allylated GGM, α-D-galactopyranoside and raffinose were used as model compounds. All reactions were done in aqueous media.

To investigate the applicability of the modified polysaccharides for, e.g., cellulose surface functionalization, their sorption onto pulp fibres was studied. Carboxylation affects the sorption tendency significantly; a higher degree of oxidation leads to lower sorption. By controlling the degree of oxidation of the polysaccharides and the ionic strength of the sorption media, high degrees of sorption of carboxylated polysaccharides onto cellulose could, however, be obtained.

Anionic polysaccharides were used as templates during laccase-catalyzed polymerization of aniline, offering a green, chemo-enzymatic route for synthesis of conducting polyaniline (PANI) composite materials. Different polysaccharide templates, such as, native GGM, TEMPO-oxidized GGM, naturally anionic κ-carrageenan, and nanofibrillated cellulose produced by TEMPO-oxidation, were assessed. The conductivity of the synthesized polysaccharide/PANI biocomposites varies depending on the polysaccharide template; κ-CGN, the anionic polysaccharide with the lowest pK_a value, produces the polysaccharide/PANI biocomposites with the highest conductivity.

The presented derivatization, sorption, and polymerization procedures open new application windows for polysaccharides, such as spruce GGM. The modified polysaccharides and the conducting biocomposites produced provide potential applications in biosensors, electronic devices, and tissue engineering.

Keywords

Spruce galactoglucomannans; galactose-containing polysaccharides; regioselective modification; TEMPO-mediated oxidation; carbodiimide-mediated amidation; indium-mediated allylation; adsorption; conducting biocomposites

Sammanfattning

Bioraffinering definieras som hållbar förädling av biomassa till kommersiella produkter och energi. Nästan en tredjedel av jordens landareal är täckt av skog och skogen står för cirka 40 % av den årliga biomassaproduktionen. Vid bioraffinering av skog förvandlas trädens komponenter till biokemikalier och biobränsle, utöver de traditionella produkterna papper och kartong. Huvudkomponenterna i ved är cellulosa, hemicellulosor och lignin. I barrträd, som används i hög grad av pappersindustrin i norden, är den största gruppen av hemicellulosor *O*-acetyl galaktoglukomannaner (GGM). GGM kan isoleras bl.a. i industriell skala från processvatten från den mekaniska massaframställningen, men används inte ännu i kommersiellt syfte. Med hjälp av enzymatiska och kemiska reaktioner kan polysackaridernas egenskaper modifieras och nya potentiella användningsområden upptäckas.

Regioselektiva modifikationer av GGM, och andra galaktosinnehållande polysackarider, gjordes genom att kombinera oxidationsreaktioner med efterföljande derivatisering av de bildade karbonyl- eller karboxylgrupperna. Två olika rutter undersöktes: aktivering av C-6 positionen i olika sockerenheter genom TEMPO-förmedlad oxidering, och aktivering av C-6 positionen i endast galaktosenheterna genom oxidering katalyserat av enzymet galaktosoxidas (GO). De aktiverade positionerna derivatiserades sedan selektivt. TEMPO-oxiderat GGM modifierades vidare med en karbodiimidförmedlad reaktion där karboxylsyragrupperna tillsammans med aminer bildade amider. GO-oxiderat GGM modifierades med en indiumförmedlad allyleringsreaktion, under vilken antingen dubbel- eller trippelbindningar infördes till polysackariden. För att bättre förstå reaktionsförloppen och för att kunna utveckla en MALDI-TOF-MS analysmetod för karakterisering av regioselektivt allylerat GGM, användes α-D-galaktopyranosid och raffinos som modellsubstanser. Samtliga reaktioner utfördes i vattenmiljö.

Ett tänkbart användningsområde för modifierade polysackarider är funktionalisering av cellulosaytor genom adsorption. För att se hur oxidering påverkar affiniteten till cellulosa, undersöktes de modifierade polysackaridernas adsorption till fibrer. Karboxylering av polysackariderna påverkar sorptionstendensen märkbart; en högre oxidationsgrad leder till

sämre adsorption. Genom att utföra kontrollerad oxidation eller genom att justera den joniska styrkan av sorptionslösningen, kan en hög grad av adsorption av även karboxylerade polysackarider uppnås.

En grön, kemoenzymatisk metod för syntetisering av ledande polyanilin (PANI) utvecklades genom att använda lackasenzym som katalysator och anjoniska polysackarider som schabloner. Olika potentiella schabloner undersöktes, t.ex. omodifierad GGM, TEMPO-oxiderad GGM, naturligt anjoniskt κ-karragenan (κ-CGN) och nanofibrillerad cellulosa framställd med hjälp av TEMPO-oxidering. Ledningsförmågan hos de syntetiserade polysackarid/PANI-biokompositerna varierade beroende på den använda polysackaridschablonen; κ-CGN med det lägsta pK_a värdet, producerade polysackarid/PANI-biokompositer med bästa ledningsförmågan.

De presenterade derivatiserings-, adsorberings- samt polymeriseringsmetoderna öppnar upp nya användningsområden för polysackarider såsom GGM. Potentiella applikationer för de producerade polysackariderna och ledande biokompositerna finns inom elektronik, biosensorer, samt vävnadskonstruktion.

List of original publications

This thesis is a summary of the following original papers, referred to in the text with their corresponding Roman numerals. Some additional data are also presented.

I. Leppänen, A.-S., Xu, C., Eklund, P., Lucenius, J., Österberg, M., and Willför, S. (2013)**Targeted functionalization of spruce *O*-acetyl galactoglucomannans – TEMPO-oxidation and carbodiimide-mediated amidation.** *J. Appl. Polym. Sci.* Online, DOI: 10.1002/app.39528.

II. Leppänen, A.-S., Niittymäki, O., Parikka, K., Tenkanen, M., Eklund, P., Sjöholm, R., and Willför, S. (2010) **Metal-mediated allylation of enzymatically oxidized methyl α-D-galactopyranoside.** *Carbohydr. Res.* 345:2610-2615.

III. Leppänen, A.-S., Xu, C., Parikka, K., Eklund, P., Sjöholm, R., Brumer, H., Tenkanen, M., and Willför, S. (2012) **Targeted allylation and propargylation of galactose-containing polysaccharides in water.** *Carbohydr. Polym.* In press, DOI: 10.1016/j.carbpol.2012.11.053

IV. Parikka, K., Leppänen, A.-S., Xu, C. Pitkänen, L., Eronen, P., Österberg, M., Brumer, H., Willför, S., and Tenkanen, M. (2012) **Functional and anionic cellulose-interacting polymers by selective chemo-enzymatic carboxylation of galactose-containing polysaccharide.** *Biomacromolecules* 13:2418-1428.

V. Leppänen, A.-S., Xu, X., Liu, J., Wang, X., Pesonen, M., and Willför, S. (2013) **Anionic polysaccharides as templates for the synthesis of conducting polyaniline and as structural matrix for conducting biocomposites**. *Macromol. Rapid Comm.* 34:1056-1061.

Contribution of the author

The author of the thesis is the main author in four publications (I-III, V), and the second author in one (IV). In papers I-III, and V the author was responsible for the experimental design and did the experimental work together with co-authors, with the exception of the QCM-measurements (paper I), the oxidations (papers II-IV) and the preparation of NFC (paper V). In paper IV, the author was responsible for the bulk sorption tests. Some analytical work (NMR, SEM, TGA, electrochemical characterizations) was done with the help of specialists.

Related publications

Parikka, K., Leppänen, A.-S., Pitkänen, L., Reunanen, M., Willför, S., and Tenkanen, M. (2010) Oxidation of polysaccharides by galactose oxidase. *J. Agr. Food. Chem.* 58:262-271.

Xu, C., Leppänen, A.-S., Eklund, P., Holmlund, P., Sjöholm, R., Sundberg, K., and Willför, S. (2010) Acetylation and characterization of spruce (*Picea abies*) galactoglucomannans. *Carbohydr. Res.* 345:810-816.

Silver, S., Leppänen, A.-S., Sjöholm, R., Penninkangas, A., and Leino, R. (2005) Towards benign synthesis of indenes from indanones: zinc-mediated allylation of ketones in aqueous media as a source of substituted indenyl ligand precursors. *Eur. J. Org. Chem.* 6:1058-1081.

Leppänen, A.-S., Xu, C., and Willför, S. (2013) Anionic polysaccharides as templates for laccase catalyzed polymerization of aniline. The 17th International Symposium on Wood, Fibre and Pulping Chemistry, June 12-14, Vancouver, Canada, Proceedings. (Poster presentation)

Leppänen, A.-S., Parikka, K., Eklund, P., Sjöholm, R., Tenkanen, M., and Willför, S. (2011) Targeted derivatization of spruce galactoglucomannans in water. 3rd Nordic Wood Biorefinery Conference, March 22-24, Stockholm, Sweden, Proceedings pp. 257-258. (Poster presentation)

Leppänen, A.-S., Parikka, K., Eklund, P., Sjöholm, R., Tenkanen, M., and Willför, S. (2010) Targeted functionalization of spruce galactoglucomannans. 11th European Workshop on Lignocellulosic and Pulp, August 16-19, Hamburg, Germany, Proceedings pp. 93-96. (Oral presentation)

Leppänen, A.-S., Parikka, K., Tenkanen, M., Eklund, P., Sjöholm, R., and Willför, S. (2009) Chemical modification of oxidized *O*-acetyl-galactoglucomannan. Italic 5 - Science and Technology of Biomasses, 1-4 September, Varenna, Italy, Proceedings pp. 197-200. (Poster presentation)

List of important abbreviations

BKP	Bleached kraft pulp
CGN	Carrageenan
DO	Degree of oxidation
$DO_{\text{-CHO}}$	Degree of oxidation; the amount of aldehydes relative to the amount of Gal in the sample
$DO_{\text{-COOH}}$	Degree of oxidation; the amount of uronic acids relative to the total amount of sugars in the sample
EDC	*N*-ethyl-*N*'-(3-(dimethylamino)propyl)carbodiimide
FTIR	Fourier transform infrared spectroscopy
Gal	Galactose
$Gal_{\text{-CHO}}$	Galactose oxidized by GO containing an aldehyde at C-6
Gal*p*	Galactopyranosyl
GalA	Galacturonic acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GGM	*O*-acetyl galactoglucomannan
$GGM_{\text{-CHO}}$	GGM oxidized by GO
$GGM_{\text{-COOH}}$	GGM oxidized by galactose oxidase combined with I_2/KI
GGM_{PolyU}	GGM oxidized by TEMPO
Glc	Glucose
Glc*p*	Glucopyranosyl
GlcA	Glucuronic acid
GM	Guar galactomannan
$GM_{\text{-CHO}}$	GM oxidized by GO
GM_{PolyU}	GM oxidized by TEMPO
GO	Galactose oxidase
HMBC	Heteronuclear multiple bond correlation
ICP	Intrinsically conducting polymer
LBG	Locust bean gum
MALLS	Multi-angle laser-light-scattering
MALDI	Matrix-assisted laser desorption/ionization
Man	Mannose
Man*p*	Mannopyranosyl
ManA	Mannuronic acid
NFC	Nanofibrillated cellulose

NHS	*N*-hydroxysuccidimide
NMR	Nuclear magnetic resonance
PANI	Polyaniline
$Raf_{\text{-CHO}}$	Raffinose oxidized by GO
SEM	Scanning electron microscopy
TEMPO	2,2,6,6-Tetramethylpiperidine 1-oxyl
TGA	Thermogravimetric analysis
TMP	Thermomechanical pulp
TOF-MS	Time-of-flight mass spectrometry
UV-Vis	Ultraviolet-Visible spectroscopy
XG	Xyloglucan
$XG_{\text{-CHO}}$	XG oxidized by GO
$XG_{\text{-COOH}}$	XG oxidized by TEMPO
Xyl	Xylose
Xyl*p*	Xylopyranosyl

Contents

1. Introduction

The increasing awareness of the environmental impact of using non-renewable fossil resources for energy and chemicals has during recent years attracted a lot of attention to finding alternative sources of raw-materials. A key to sustainability is the utilization of renewable raw-materials. The renewing time of oil, gas and coal is estimated to be a in the order of 280 million years (Amidon and Liu, 2009). Ligno-cellulosic biomass, the most abundant organic source on earth, has a renewing time of only 5-80 years (Amidon and Liu, 2009; Liu et al. 2012). Forests cover ~32% of the earth's land area, and account for ~43% of the total annual biomass production.

Biorefining is defined as sustainable conversion of biomass into marketable products and energy (International Energy Agency, Bioenergy Task 42 Biorefinery). In forest biorefining, wood components are, in addition to the traditional construction materials, and paper and board products, converted into chemicals and biofuels. The main components of wood are cellulose, hemicelluloses, and lignin (Sjöström 1981). The type and amounts of the components vary somewhat between different tree species; approx. 40% of the wood dry weight consists of cellulose, 25-30% of lignin, and 20-30% of hemicelluloses. The rest consists of other polysaccharides (3-8%, for example, pectins and starch), extractives (<5%), proteins (0.5%), and inorganics (<1%). A major part of the hemicelluloses can be isolated from wood by extraction. Potential areas of application of the extracted hemicellulose are, e.g., as hydrocolloids, as water vapor, fat or oxygen barriers in packaging, and as sources of monosaccharides for biofuel production (Willför 2008; Amidon and Liu 2009; Liu et al. 2012; Mikkonen and Tenkanen 2012). Hemicelluloses having naturally a high affinity to cellulose can also, through adsorption to fibres, be used for cellulose surface modification and improvement of pulp properties (Hannuksela and Holmbom 2004a and b; Schwikal et al. 2011; Lindqvist et al. 2013).

Because of their non-toxicity, biocompatibility, good availability, and tunable physicochemical properties, such as water-solubility and viscosity, polysaccharides are today widely in use in the food, cosmetic, and pharmaceutical industries (Cui et al 2006; Rinaudo 2006; Liu et al. 2007; Yoon et al. 2008; Meghwal and Goswami 2012; Milani and Maleki 2012;

Jiangling and Shaoying 2013). Galactomannans, the groups of polysaccharides mainly discussed in this thesis, are used, for example, as dietary fibres (Elleuch et al. 2011), emulsifiers, stabilizers (Mudgil et al. 2011), and in packaging as eatable films (Cerqueira et al. 2011). In order to attain desired properties of the polysaccharides for specific end-uses, chemical and enzymatic modifications can be performed. One potential application area is, as mentioned, the use of polysaccharides for cellulose surface functionalization. The introduction of substituents to polysaccharides decreases their affinity to cellulose, but by performing controlled and selective derivatizations, some affinity can still be maintained. A huge amount of different polysaccharide modification reactions are available, of which only a minor part are regioselective. By developing new routes for selective derivatization and functionalization of polysaccharide in aqueous media, their properties can be tuned even more accurately in environmentally more benign conditions, thus further broadening the area of application.

2. Objective of the work

An efficient utilization of wood components would be economically beneficial for the Finnish forest industries. An example of a component that can be isolated from the waste waters from the pulping process, but still today lacks industrial applications, is the softwood hemicellulose *O*-acetyl galactoglucomannan (GGM). By performing chemical modifications the properties of GGM can be changed, enabling the discovery of novel application areas.

The objective of the work was to develop new routes allowing regioselective addition of substituents to water-soluble GGM, preferably such that can be done in aqueous environment. This required development of both the modification chemistry and some key analytical tools to verify the desired modifications. In order to better understand the novel polysaccharide reactions and the analysis thereof, methyl α-D-galactopyranoside and raffinose were employed as model compounds.

Further, the modified GGMs were tested in sorption onto cellulose fibres and as templates in production of conducting polysaccharide/polyaniline biocomposites by laccase-mediated polymerization.

3. Literature review

3.1 Spruce *O*-acetyl galactoglucomannan

Hemicelluloses are heteropolysaccharides built up by pentoses, hexoses, uronic acids, and deoxy hexoses (Fengel and Wegener 1984). Unlike cellulose that is a linear homopolysaccharide, hemicelluloses are often branched. Softwoods, that are of interest especially to the Nordic forest industry, contain 10-20% *O*-acetyl galactoglucomannan (GGM) making it the main hemicellulose in softwoods (Sjöström 1981; Fengel and Wegener 1984; Willför et al. 2005). GGM has a backbone consisting of (1→4)-linked *β*-D-mannopyranosyl (Man*p*) and *β*-D-glucopyranosyl (Glc*p*) units, with single (1→6)-linked *α*-D-galactopyranosyl (Gal*p*) units as side group (Capek et al. 2000; Willför et al. 2003a; Hannuksela and Hervé du Penhoat 2004) (Figure 3.1). Some of the Man units are naturally acetylated at C-2 or C-3, the degree of acetylation being approx. 0.3. Part of GGM is water-soluble and can be isolated in an industrial scale from the waste waters of mechanical pulping (Willför et al. 2003b). In addition to process waters, GGM can also be extracted from thermomechanical pulp (TMP) (Willför at al. 2003a) or even directly from wood by, e.g., pressurized hot water extraction (PHWE) (Song et al. 2008). The chemical composition of GGM varies somewhat depending on the raw-material and method of isolation used. The sugar ratio mannose:glucose:galactose (Man:Glc:Gal) of the water-soluble part of GGM is approximately 2.3-3.9:1:0.5-1.1, and the average molar mass is 21-40 kDa (Willför et al. 2003a; Xu 2008).

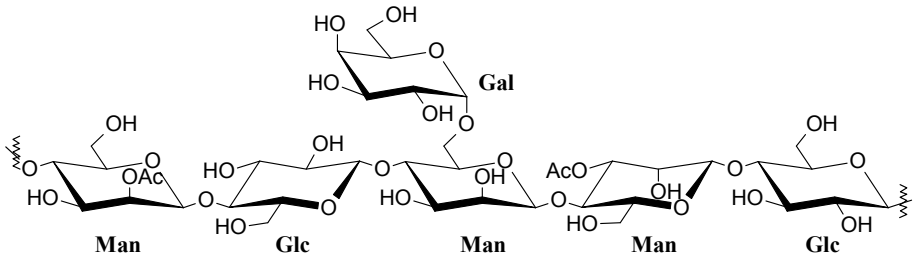


Figure 3.1. Structural features of water-soluble spruce GGM.

3.2 Galactose-containing polysaccharides from other sources

Galactomannans are polysaccharides with linear backbones built up of (1→4)-bonded *β*-D-Man*p* units with single (1→6)-linked *α*-D-Gal*p* as side groups (Nishinari et al. 2007). Galactomannans can be extracted from the endosperms of seeds from leguminous plants, and the Man-Gal ratio varies from 1:1 to 4:1, depending on the source of galactomannan. Examples of industrially utilized galactomannans are guar gum (guar galactomannan, GM), and locust bean gum (LBG), their chemical structures and Man:Gal ratios being presented in Figure 3.2. GM can be extracted from the tubers of guar beans, and LBG is extracted from the seeds of the carob tree. Both GM and LBG have several industrial applications, for example, in food and cosmetic industries as thickeners, stabilizers, and as dietary fibres.

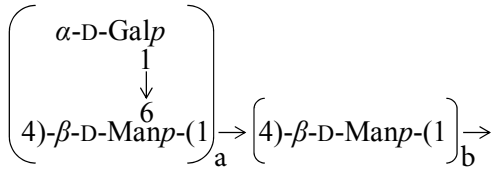


Figure 3.2. The structure of galactomannans. GM a=b; LBG a=1 and b=3.

Xyloglucan (XG) is the main hemicellulose in the primary cell wall of many vascular plants. XG isolated from the seeds of the tamarind tree is used, e.g., as thickener in food additives, as a gelling agent, and as a stabilizer (Nishinari et al. 2000). The backbone of XG consists of (1→4)-bonded *β*-D-Glc*p* units. The glucose (Glc) units are partially substituted with (1→6)-bonded *α*-D-xylopyranosyl (Xyl*p)* units. Some of the Xyl*p* units are further substituted with (1→2)-linked *β*-D-Gal*p* residues (York et al. 1993). The repeating units of XG, i.e. XXXG, XLXG, XXLG, and XLLG based on their substitution patterns, are presented in Figure 3.3. The ratio of these varies depending on the source of XG; tamarind XG contains ~13% XXXG, 39% XLXG and XXLG, and 48% XLLG (Nishinari et al. 2007). XG has a high affinity to cellulose (Vincken et al. 1995), and chemo-enzymatically modified XG has thus been used to introduce functionality to cellulose surfaces (Xu et al. 2012).

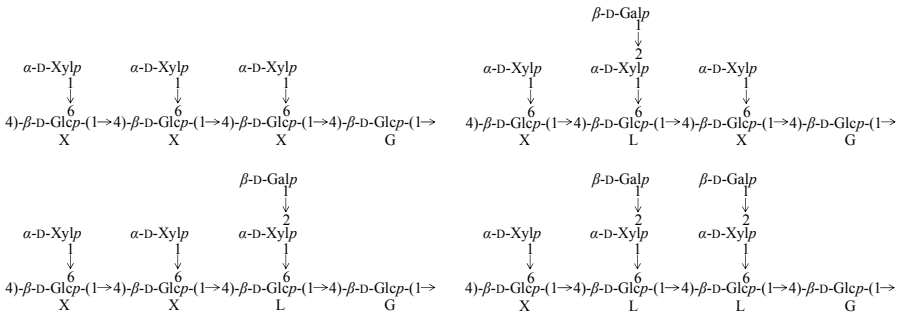


Figure 3.3.The chemical structures of the repeating units of XG.

Carrageenans (CGN) are naturally-occurring anionic polysaccharides obtained from red algae. CGN can be divided into two groups, the gel-forming κ-CGNs containing anhydro-units, and λ-CGNs with neither anhydro-units nor gelling properties (Rinaudo 2007). κ-CGNs include κ-CGN and ι-CGN. They consist of *β*-D-Gal*p* and *α*-D-3,6-anhydro-Gal*p* (3,6-anhydro-Gal*p*) units. CGNs are $(AB)_n$ copolymers with [*α*(1-3)-Gal*p*-*β*(1-4)-3,6-anhydro-Gal*p*-] as repeating unit (Figure 3.4). κ-CGN has one sulfate group at the A unit, whereas in ι-CGN both A and B units are sulfated. Since CGNs form gels they are, like many other polysaccharides, used as thickener and stabilizer in food products.

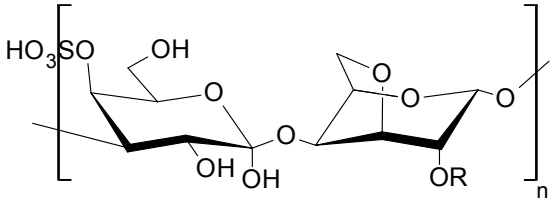


Figure 3.4. Structural features of carrageenan. R = H in κ-CGN; R=-SO_3H in ι-CGN.

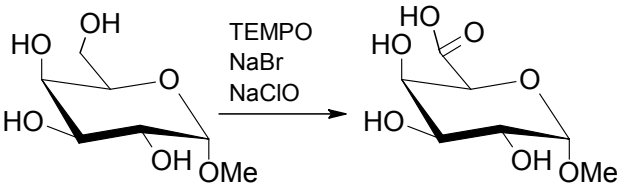
3.3 Selective modifications of polysaccharides

The physicochemical properties, such as solubility, hydrophobicity, and viscosity of polysaccharides can be tailored by chemical and enzymatic modifications. (1→4)-bonded hexose units in polysaccharides offer three potential reaction sites; the hydroxyls at C-2, C-3, and C-6. There are slight differences between the reactivity of these hydroxyls depending on the reaction conditions used. Even if the primary hydroxyl at C-6, being least sterically hindered, could be expected to be the most reactive, C-2 shows higher reactivity, for example, towards etherification (Lee et al. 1999). Typical polysaccharide modification reactions are esterifications, and etherifications of the hydroxyl groups, during which substituents are introduced randomly along the polysaccharide chains (Tomecko and Adams 1923; Mullen and Pascu 1942; Whistler and Wolfrom 1963; Whistler 1963; Ebringerová et al. 2005, El Seoud and Heinze 2005, Hartman et al. 2006; Cunha and Gandini 2010a and b; Xu et al. 2011; Kisonen et al. 2012; Kang et al 2013; Lindqvist et al. 2013).

Enzymes offer versatile tools for targeted and regioselective polysaccharide reactions (Tenkanen, 2004; Cheng and Gu 2012). Hydrolytic enzymes, i.e. endoglycanases and exoglycosidases, can be used to decrease the molar mass (M_w) and for removal of side-groups, while oxidases offer methods for selective introduction of carbonyl groups. Lipases, on the other hand, graft substituents to polysaccharides, and can thus also be applied for modification, but the reactions are often non-specific.

In addition to enzymes, regioselective modification of polysaccharides is achieved chemically by application of protective groups (Kobayashi and Sumitomo 1981, Felix 2001, Koschella et al. 2006) or by first activating certain positions for further reactions, e.g., by oxidations (Yalpani and Hall 1982; Ma et al. 2011; de Souza et al. 2011). Since the reactivity of carbonyls and carboxyls differs from the reactivity of hydroxyls, the oxidized groups can subsequently be selectively derivatized.

3.3.1 TEMPO-mediated oxidation of polysaccharides

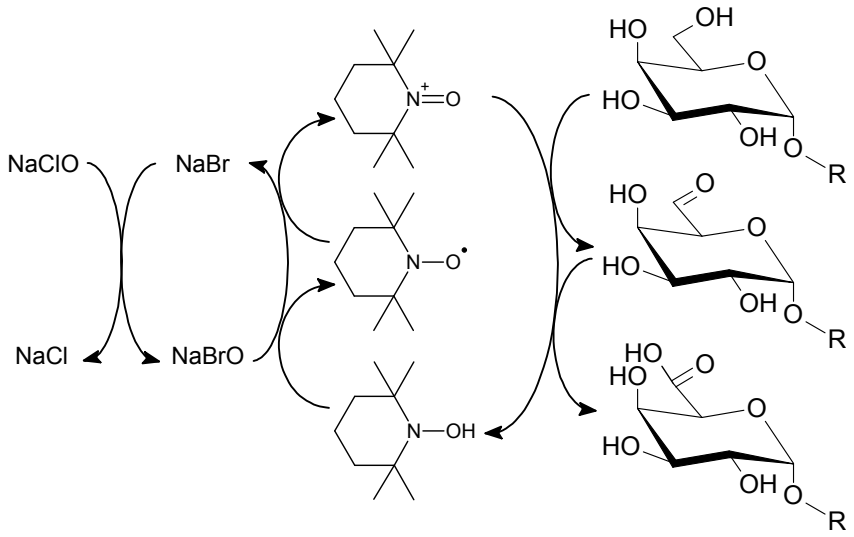


Scheme 3.1. TEMPO-mediated oxidation of methyl pyranosides.

The nitroxyl radical 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) catalyzes selective oxidation of primary alcohols to carbonyl compounds (Anelli et al. 1989). It is a highly selective method, and since the nitroxyl reagent is stable against humidity and air, the oxidations can be performed in aqueous systems. The selective oxidation of monosaccharide derivatives was first presented by Davis and Flitsch (1993) (Scheme 3.1). De Nooy et al. (1995) showed that the TEMPO-mediated system is also selective for primary alcohols in polysaccharides. TEMPO-mediated oxidation has been utilized for the oxidation of several different polysaccharides, such as cellulose, starch, GM, pullulan, and chitosan (Isogai and Kato 1998; Bragd et al. 2000; Bragd et al. 2004; Bordenave et al. 2008; Coseri et al. 2013).

A commonly applied TEMPO-mediated oxidation procedure involves using sodium bromide (NaBr) and sodium hypochlorite (NaClO) as regenerating oxidants: the *in situ* formed hypobromite regenerates TEMPO to its active form (Scheme 3.2) (Saito and Isogai 2006). Since one drawback of the TEMPO process is the environmental impact of using halogen-containing co-oxidants, methods involving other co-oxidants have been investigated. TEMPO-mediated oxidation of cellulose and starch has also been done using oxidative enzymes, e.g. laccase, as the primary oxidant (Viikari et al. 1999a and b; Mathew and Adlercreutz 2009; Aracri et al. 2011). The enzymatic method yielded a mixture of aldehydes and carboxylic acids, but the conversion obtained was low; less than 5% of the sugar units were oxidized. The degrees of oxidation of galactomannans oxidized by the laccase-TEMPO system also remained low; less than 7% of the sugar units were converted into aldehydes, and ~2% to carboxylic acids (Lavazza et al. 2011). Although the degree of oxidation (DO) remained low, the reaction yielded a thick gel. Laccase-TEMPO treatment has been used to increase the wet strength of sisal

pulp (Aracri et al. 2011). The increased viscosity or strength is assigned to hemiacetal bonding caused by the formed aldehydes.



Scheme 3.2. TEMPO-mediated oxidation of carbohydrates using NaBr and NaClO as primary oxidants.

Another approach to diminish the amount of halide-reagents is performing the oxidation in bromide-free conditions. Brochette-Lemoine et al. (1999) showed that the primary alcohols of carbohydrates can be oxidized without the use of NaBr, although the rate of oxidation was low. The active species of TEMPO can also be regenerated by direct oxidation by hypochlorite (Bragd et al. 2000). Since the rate of the bromide-free reaction is lower than that of the normal bromide-containing one, this procedure can be applied when performing controlled oxidations of polysaccharides, especially when aiming at low degrees of oxidation. Another benefit of the bromide-free method is a more economical oxidation procedure when using fewer chemicals. Ma et al. (2011) studied controlled TEMPO-mediated oxidation of polysaccharides. By adjusting the amount of reactants, different degrees of oxidation were obtained. However, the amounts of reactants needed for different polysaccharides could not be extrapolated from the results of a certain polysaccharide. Thus, optimization of the reaction parameters needs to be done separately for each polysaccharide.

To further decrease the environmental impact and cost of using TEMPO-mediated oxidation, Mao et al. (2010) studied recycling of the TEMPO reagents. During the oxidation TEMPO and NaBr function as catalysts, and NaClO as the oxidant. Therefore only NaClO is spent, and TEMPO and NaBr are still present in the solution at the end of the reaction. Thus, the spent liquor can be reused by addition of NaClO.

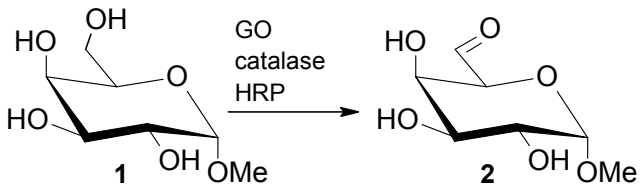
TEMPO-mediated oxidation of cellulose

Besides water-soluble polysaccharides, also cellulose can be oxidized by the TEMPO-mediated procedure (Isogai and Kato 1998). TEMPO-pretreatment of cellulose offers a cost effective method for preparation of nanofibrillated cellulose (NFC) (Saito et al. 2006). NFC is formed when cellulose fibres are disintegrated into individual nanofibres. NFC has high mechanical properties and potential applications are biocomposites, gas-barrier films, and flame-resistant materials (Isogai et al. 2011).

Fibrillation of cellulose is obtained through mechanical disintegration, or by chemical pretreatment followed by mechanical disintegration. The chemical pretreatment can be, for example, hydrolysis with 64% H_2SO_4 that introduces anionic groups on the surface of the fibrils (Siqueira et al. 2010; Isogai et al. 2011), the product is often referred to as nanocrystalline cellulose. Similarly, during TEMPO-mediated oxidation of native cellulose, anionic carboxylic acids are formed on the surface of the microfibrils softening up the hemicellulose-rich area between the fibrils (Saito et al. 2006). Individual nanofibers can then be obtained by mechanical disintegration. The repulsive effect between the microfibrils enhances the disintegration, and less energy is consumed than during disintegration by only mechanical treatment.

Another advantage of TEMPO-pretreatment is that the disintegration can be done after purification. Washing of the oxidized cellulose fibres can thus easily be done by normal suction filtration, and for industrial purposes it is easier to transport the NFC when water has been removed (Isogai et al. 2011). In addition, by TEMPO pretreatment nanofibers are produced in higher yields and with more uniform width, than by the H_2SO_4 pretreatment.

3.3.2 Oxidation of galactose-containing polysaccharides by galactose oxidase



Scheme 3.3. GO-catalyzed oxidation of methyl α-D-galactopyranoside.

The use of enzymes for polysaccharide modification is interesting since reactions are done in aqueous environment, and often at milder conditions than corresponding chemical reactions. Enzymes are non-toxic, biodegradable, and are usually highly chemo-, and regioselective, thus offering tools for controlled modifications. The enzyme galactose oxidase (GO, EC 1.1.3.9) catalyzes the oxidation of primary alcohols producing aldehydes and hydrogen peroxide (Whittaker 2003). The reaction is highly regioselective for the hydroxyl group at C-6 of Gal (Scheme 3.3).

GO exists in three different oxidation states; the active (oxidized), inactive, and fully reduced forms (Whittaker 2002). The first step in the catalytic oxidation cycle is reduction of the active form followed by reactivation by O_2. At the same time hydrogen peroxide is formed. During this cycle only approximately 5% of the enzyme is in the active form (Saysell et al. 1997). The oxidation of the inactive form of GO to the active form is improved by the use of peroxidases (Hartmans et al. 2004). Hydrogen peroxide inactivates GO and thus peroxide-degrading catalase is added to enhance the efficiency of the reaction (Hamilton et al. 1978).

During GO-catalyzed oxidation, in addition to the aldehyde (**2,** Scheme 3.3), also a dimeric compound, the α-β-unsaturated aldehyde, and the corresponding uronic acid derivative are formed (Ohrui et al. 1986; Matsumura et al. 1988; Basu et al. 2000). The formation of the by-products can be minimized by using optimized reaction conditions; methyl α-D-galactopyranoside (**1**, Scheme 3.3) and raffinose have been oxidized by GO producing the corresponding aldehydes in high yields (approx. 90%) in the

presence of catalase and horseradish peroxidase (HRP) (Parikka and Tenkanen 2009). In addition to working with monomers and oligomers, aldehydes have successfully also been introduced by GO oxidation to the galactose-containing polysaccharides LBG, GGM, GM, XG, larch arabinogalactan (AG), and corn fiber arabinoxylan (AX) (Yalpani and Hall 1982; Hartmans et al. 2004; Parikka et al. 2010, Xu et al. 2012; Parikka et al. 2012). Using the reaction conditions optimized for **1** and raffinose, the degree of oxidation ($DO_{\text{-CHO}}$, calculated as the amount of aldehyde groups relative to the total amount of galactose in the polysaccharides) of the oxidized polysaccharides $GGM_{\text{-CHO}}$, $GM_{\text{-CHO}}$, $AG_{\text{-CHO}}$, $AX_{\text{-CHO}}$, and $XG_{\text{-CHO}}$ varied between ~10-85% and are presented in Table 3.1.

Table 3.1 Degrees of oxidation of galactose-containing polysaccharides oxidized by GO (Parikka et al. 2010).

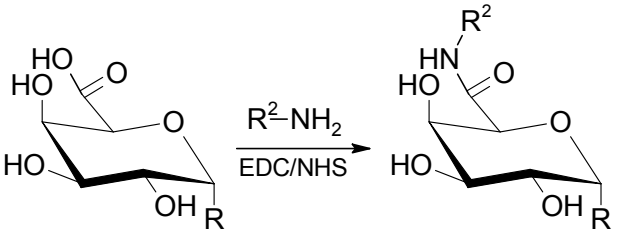
Polysaccharide	**$DO_{\text{-CHO}}$[a]**	**DO_{tot}[b]**
GGM	65	6
GM	40	16
AG	10	4
AX	20	2
XG	85	14

[a] The amount of oxidized Gal relative to the total amount of Gal in the sample

[b] The amount of oxidized Gal relative to the total amount of sugars in the sample

The aldehyde groups can form hemiacetal cross-linking, thus increasing the viscosity of the polysaccharide solutions (Parikka et al. 2010). Thermally stable gels have been formed through GO-oxidation of high molar mass polysaccharides (Parikka et al. 2012). The addition of $GM_{\text{-CHO}}$ to pulp has been shown to somewhat increase the strength of paper (Hartmans et al. 2004).

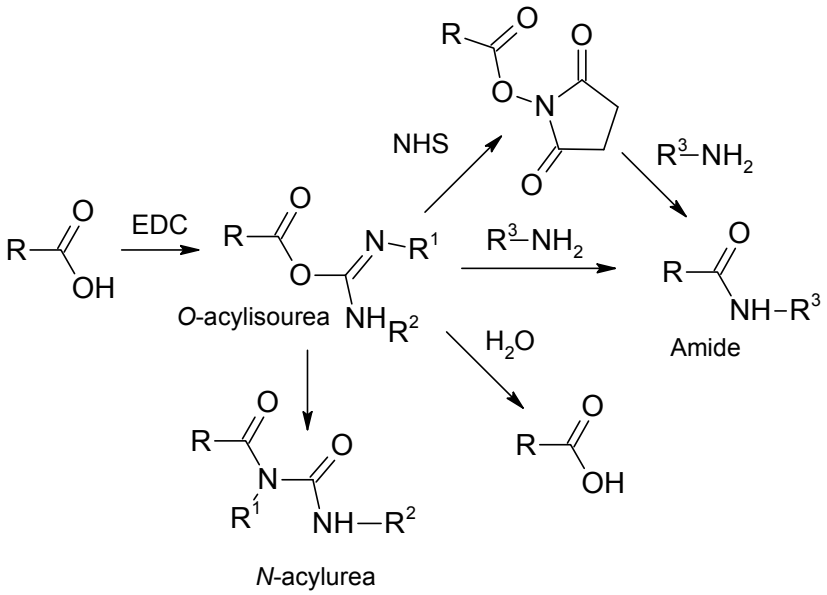
3.3.3 Carbodiimide-mediated amidation of polysaccharides



Scheme 3.4. Carbodiimide-mediated amidation of uronic acids.

Carbodiimides are frequently used reagents for activation of carboxylic acids towards esterification or amidation reactions (Scheme 3.4). The first synthetic applications were in peptide and nucleotide chemistry (Williams and Ibrahim 1981), and carbodiimides are still of the most used coupling reagents within peptide synthesis (Al-Warhi et al. 2012). In carbohydrate chemistry, carbodiimides first found application in the analysis of uronic acids. Acidic monosaccharides and polysaccharides can be labeled using water-soluble *N*-ethyl-*N*'-(3-(dimethylamino)propyl)carbodiimide (EDC) for fluorescent assay (Kobayashi and Ichishima 1990; 1992; 1994).

The simplified mechanism of carbodiimide-mediated amidation is presented in Scheme 3.5. The carbodiimide EDC reacts with carboxylic acids producing *O*-acylisourea, which then further reacts with the amine to form an amide (Montalbetti and Falque 2005). The intermediate *O*-acylisourea can also rapidly hydrolyze or undergo O→N rearrangement resulting in the formation of unreactive *N*-acylurea, decreasing the yield of the amidation reaction (Nakajima and Ikada 1995). By the addition of *N*-hydroxysuccidimide (NHS) the formation of *N*-acylurea can be significantly decreased (Grabarek and Gergely 1990; Bulpitt and Aeschilmann 1999, Araki et al. 2002). NHS forms an active ester which is more stable than *O*-acylisourea, and does not undergo O→N rearrangement, thus a higher amidation yield can be reached.



Scheme 3.5. Simplified mechanism of EDC/NHS-mediated amidation of carboxylic acids.

Benefits of the carbodiimide-mediated procedure compared to other amidation reactions are that no organic solvents are needed, and water-soluble polysaccharides can be amidated at mild conditions in aqueous solutions. Amidation of neutral polysaccharides is done by first introducing carboxylic acids to the polymer by, e.g., TEMPO-mediated oxidation. New biopolymers and biologically active materials have been developed by EDC/NHS-mediated amidation of e.g. chitin, chitosan, TEMPO-oxidized cellulose, and carboxymethyl cellulose (CMC) (Muzzarelli et al. 2001; Araki et al. 2002; Kast et al. 2003; Dominguez et al. 2005; Valdivia et al. 2005; Grabovac and Bernkop-Schnürch 2007; Follain et al 2008; Lasseuguette 2008; Barbucci 2010; Orelma et al. 2012).

3.3.4 Allylation and alkynylation of polysaccharides

One approach for chemical modification of polysaccharides is the introduction of highly reactive carbon-carbon double or triple bonds. These can be subsequently reacted enabling anchoring of desired functionalities to the polysaccharide, e.g. stimuli-responsive moieties, hydrophobic tails, bioactive, and UV-sensitive compounds. These may find applications in

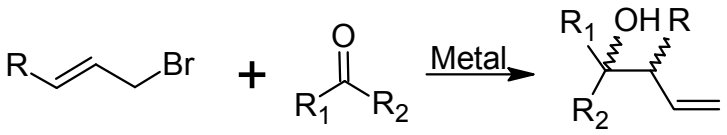
biomedical, food, intelligent packaging, and bio-detection applications (Maharjan et al. 2008; Cheng et al. 2010).

Allylation of carbohydrates was first reported by Tomecko and Adams (1923). In order to introduce reactive groups to polysaccharides, the authors formed allyl ethers of, e.g., dextrin, starch, and cellulose by reacting the polysaccharides with allyl bromide in alkaline conditions. Allyl ethers of polysaccharides have also been prepared using allyl glycidyl ether (Ameye et al. 2002; Duanmu et al. 2007; Huijbrechts et al. 2007) and in different solvent systems, such as *N,N*-dimethylacetamide and lithium chloride (LiCl/DMAc) (Lin & Huang 1992) or dimethyl sulfoxide/tetrabutylammonium fluoride trihydrate (Heinze et al. 2008).

Alkynylation of a variety of polysaccharides, such as, cellulose, GM, dextran, and starch, have been achieved through esterification, etherification, reductive amination of the reductive end, and amidation (Fenn et al. 2009, Tizotti et al. 2010, Elchinger et al. 2011, Mangiante et al. 2012). In addition, galactose units in XG and GGM have selectively been propargylated by combining GO-catalyzed oxidation of the galactose units with reductive amination of the formed aldehydes (Xu et al. 2012).

The double and triple bonds in polysaccharides have been further modified; allylated starch has been used to form epoxides (Huijbrechts et al. 2010) and for co-polymerization with acrylic monomers (Bhuniya et al. 2003), while the triple bonds of alkynylated polysaccharides have been further derivatized using "click-chemistry" (Tankam et al. 2007; Tizotti et al. 2010, Elchinger et al. 2011; Xu et al. 2012).

Indium-mediated allylation of carbohydrates



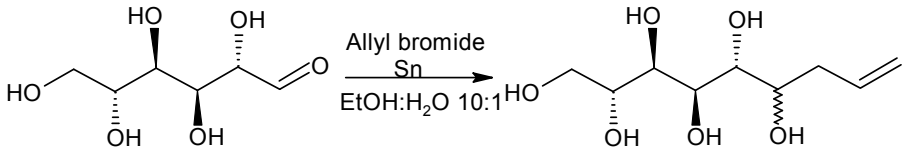
Scheme 3.6. Metal-mediated allylation of carbonyls.

Aldehydes and ketones can be modified by a Barbier-Grignard type allylation reaction in aqueous media, where the carbonyl group reacts with allyl halides in the presence of a metal mediator to form a homoallyl alcohol (Scheme 3.6) (Pétrier & Luche 1985; Li 1996). The metal-mediated allylation reaction was originally developed to form new carbon-carbon bonds in non-carbohydrate ketones and aldehydes. Schmid and Whiteside (1991) modified the procedure to unprotected carbohydrates. Using the tin-mediated reaction with allyl bromide, aldoses could be extended by three carbons. Later, Kim et al. (1993) reported the allylation of unprotected carbohydrates mediated by indium, tin, and zinc. In carbohydrate chemistry, the reaction has been used in the synthesis of bioactive compounds and functional lactones (Chan & Li 1992; Gordon & Whiteside 1993; Gao et al. 1994; Gao et al. 1996; Balla et al. 2007; Saloranta et al. 2008).

The rate of the allylation reaction is dependent both on the type of solvent system and on the mediating metal. Even if it is not the only factor affecting the reactivity of a metal (Andrews et al. 2001), the first ionization potential, i.e., the amount of energy required to remove an electron, gives a good indication of the reactivity. A lower first ionization potential increases the reactivity. Indium has a relatively low first ionization potential compared to zinc and tin, and the reactions proceed without the need for external proton sources, such as NH_4Cl. The use of saturated NH_4Cl solution decreases the solubility of carbohydrates, thus also decreasing the reactivity (Kim et al. 1993). Other advantages of indium are a relatively low toxicity and good tolerance towards moisture. Indium-mediated allylations of carbohydrates proceed at room temperature, and do not require reflux, which is the case for tin-mediated allylations. Indium-mediated reactions have also been reported to be more diastereoselective and form fewer byproducts than the tin-mediated ones (Kim et al. 1993).

Metal-mediated allylation reactions of non-carbohydrates have been done using water as the only solvent (Isaac and Chan 1995a), with organic co-solvents (e.g., ethanol, THF and DMF) (Li 1996; Prenner and Schmid 1996; Cheng and Loh 2005), in ionic liquids (Gordon and Ritchie 2002; Law et al. 2002) and even at solvent-free conditions (Andrews et al. 2001). Unprotected carbohydrates have been allylated in aqueous ethanol (Scheme 3.7), or by using, for instance, dioxane, THF, methanol, or 1-propanol as organic co-

solvent (Schmid and Whitesides 1991; Kim et al. 1993; Palmelund and Madsen 2005; Saloranta et al 2008).



Scheme 3.7. Tin-mediated allylation of D-mannose (Saloranta et al. 2008).

3.4 Sorption of mannans to cellulose surfaces

Research concerning the sorption of galactomannans onto cellulose fibres was originally concentrated on their application as refiner additives in the paper making process. By the addition of mannans, namely LBG and GM, the refining time could be shortened, and paper with better properties was obtained (Shriver et al. 1950). Sorption of polysaccharides is believed to occur on the surface of and also inside the fibre leading to higher flexibility and increased swelling. As a consequence, more effective fibrillation, and less cutting of the fibres was obtained (Laffend and Swenson 1968a). Later the research focus shifted to the dissolved hemicellulose present in the paper mill process waters. Resorption of the hemicelluloses onto fibres would lead to higher yield and better strength of the pulp. Nowadays, the interest around sorption is also within the utilization of modified hemicellulose, as well as other polysaccharides, as tools for cellulose surface modification. Soluble polysaccharides are often easier to modify than cellulose itself, and by sorption of derivatized polysaccharides functionalized cellulose fibres are obtained. In order to use modified polysaccharides for cellulose functionalization, a good understanding of how modifications of the polysaccharides affect their affinity to cellulose, is needed.

The sorption of hemicelluloses to cellulose fibres is often described by a four step model (Russo and Thode 1960). The first step is diffusion of hemicellulose molecules to the fibre surface. The rate of this step is dependent on the collision frequency, which is affected by the concentration of hemicelluloses in the solution, and the amount of available cellulose on the fibre surface (Gruenhut 1953). Also the size of the hemicellulose affects the rate; smaller molecules diffuse faster from solution to fibre surfaces (Laffend

and Swenson 1968b), although later in the process the larger molecules will replace the small ones on the surface. The following step is the attachment of the hemicellulose chains to the fibre surface. Hydrogen bonds may be one driving force since both cellulose and hemicellulose are capable of forming such bonds (Gruenhut 1953; Hansson 1970). The similar conformation of the mannan backbone and cellulose facilitates close contact between the molecules, thus also the formation of hydrogen bonds. The solubility of the hemicellulose is important, attachment take place if the polysaccharide-solvent attraction is lower than the polysaccharide-cellulose surface attraction (Laffend and Swenson 1968b). The third step of the sorption process is that the adsorbed polysaccharides diffuse to some extent into the fibre, forming monolayers on the internal parts accessible to hemicelluloses (Hansson 1970). The last step is detachment of polysaccharides from the fibres, that is, a reverse of the adsorption process. The rate of this step is believed to be quite limited.

Spruce GGM has naturally a high affinity to cellulose (Hannuksela et al. 2002 and 2003). The sorption is affected by the type of sorbent fibres; thermomechanical pulp (TMP) contains very little, if any at all, clean cellulose surface, and no sorption occurs, whereas nearly quantitative sorption is obtained for chemical pulps having more clean cellulose surfaces (Hannuksela et al. 2003). The sorption of galactomannans can be improved by removal of side-groups and substituents. Deacetylation decreases the solubility of polysaccharides resulting in high degree of sorption, even on TMP (Laffend and Swenson 1968a; Hannuksela and Holmbom 2004a). Less branching leads to higher sorption, thus removal of Gal side-groups influences the sorption significantly (Hannuksela et al. 2003). The influence of the side-groups was also shown by comparing the sorption of LBG and GM. LBG, containing less Gal side-groups than GM, sorbed much more effectively to cellulose (Gruenhut 1953).

The amount of polysaccharide that can attach to a cellulose surface is affected by the conformation of the polysaccharide in solution; side-groups and large substituents hinder the polysaccharide from being coiled, and less polysaccharide can fit on the surface. Polysaccharides containing anionic groups, e.g., hemicelluloses isolated from peroxide bleach thermomechanical pulp, are stretched out in water solutions, sterically hindering other polysaccharides chains to reach the surface (Tammelin et al. 2009).

Electrostatic interactions between charged substituents, such as carboxymethyl groups, hinder the polysaccharides from forming multi-layers, decreasing the sorption even further (Xu et al. 2011). By addition of salt to the sorption media, sorption of anionic polysaccharides onto cellulose can be obtained (Tammelin et al. 2009). Charges are neutralized, affecting the conformation of the polysaccharide. In addition the electrostatic repulsions between the slightly anionic cellulose surface and the polysaccharide decreases, enabling double-layer sorption.

3.5 Novel potential application areas for anionic polysaccharides

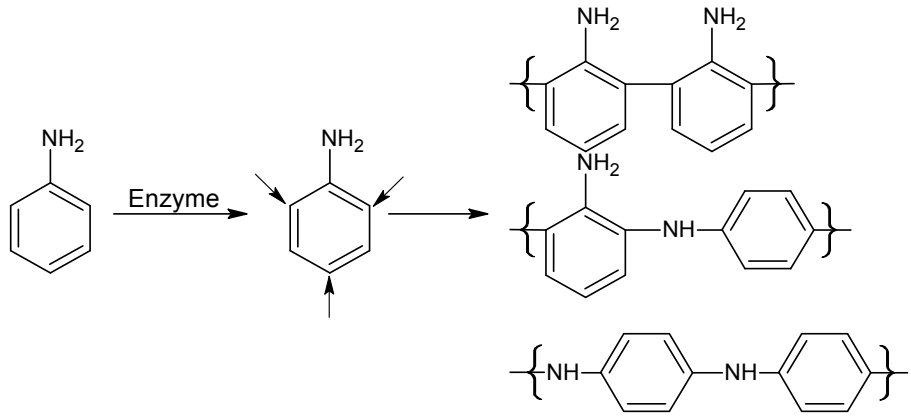
As previously mentioned, polysaccharides are already extensively utilized, and new application areas are enabled as novel plausible methods for polysaccharide modifications are developed.
Cellulose, a biodegradable and non-toxic polymer, offers a biocompatible platform for biomedical uses. By introducing carboxylic acid groups, either by sorption of carboxylated polysaccharides to cellulosic surfaces (Orelma et al. 2012) or by performing TEMPO-mediated oxidation directly on cellulose (Orelma et al. 2011), and by subsequent treatment with carbodiimides (EDC and NHS), bioactive molecules can be covalently bound to the surface. The products show potential as immunoassay sensors, and functionalization of cellulose thus offers an interesting field of applications for TEMPO-oxidized polysaccharides.

Another potential application field is the utilization of anionic polysaccharides as soft templates during enzymatic polymerization of aniline. Polyaniline (PANI) is one of the most studied intrinsically conducting polymers (ICPs). ICPs have numerous potential applications in electrical, chemical, biomedical, and sensing materials (MacDiarmid 2001; Bhadra et al. 2009; Ravichandran et al. 2010). Among the advantages of PANI compared to other ICPs, its chemical stability, tunable conductivity, and the ease to synthesize are highlighted. Still, limited water-solubility and poor mechanical properties are restricting the use in industrial scale. The mechanical strength of PANI can be improved by, for instance, formation of composites of polymers with desired mechanical properties and PANI, and the solubility has been increased by doping or by the use of templates or derivatives of

PANI. As a result, the PANI composites have demonstrated potential applications in biosensors, electronic devices, and tissue engineering (Gao et al. 2007; Huang et al. 2008; Bendrea et al. 2011; Pan et al. 2012).

PANI is generally synthesized chemically or electrochemically (Bhadra et al 2009). As a greener alternative to these routes, the use of enzymes as catalysts for polymerization has been suggested (Samuelson et al. 1998). Peroxidases and laccases from different sources are known to catalyze the polymerization of aniline (Samuleson et al. 1998; Karamyshev et al. 2003; Cruz-Silva et al. 2011). Laccase (benzediol: oxygen oxidoreductase; EC 1.10.3.2) is a multicopper oxidase that displays various functions, among others polymerization activity (Dwivedi et al. 2011). An advantage compared to peroxidases is that laccases use atmospheric oxygen as oxidizing agent, and the use of enzyme-deactivating hydrogen peroxide is avoided. Also, laccases show greater stability than peroxidases at the lower pH values needed for PANI synthesis.

At the pH used for the enzymatically-catalyzed reactions, the polymerization of aniline can occur both in ortho and para positions (Scheme 3.8). A lower degree of organization leads to lower conductivity. To avoid branching caused by the unselectivity of the reaction, template-assisted polymerization was introduced (Samuelson et al. 1998); Water-soluble conducting PANI was enzymatically synthesized using horseradish peroxidase in the presence of the anionic template sulfonated polystyrene (SPS). The template facilitates the alignment of the monomers so that the desired head-to-tail coupling is preferred. Besides this, the template also provides counter ions for doping of the synthesized PANI and helps to maintain the water-solubility of the product (Liu et al. 1999a and b; Cholli et al. 2000). Examples of anionic templates used are deoxyribonucleic acid (DNA), camphorsulfonic acid, poly(vinylsulfonic) acid, and polyvinylphosphonic acid (Liu et al. 1999b; Nagarajan et al. 2001; Rumbau et al. 2007; Vasil'eva et al. 2007; Gu et al. 2009; Streltsov et al. 2009; Shumakovich et al. 2010).



Scheme 3.8 Polymerization of aniline can occur both in ortho or para positions (indicated by arrows) leading to branched, and non-conductive, structures (Samuelson et al. 1998).

Suitable templates are typically strong polyelectrolytes with pK_a values that are lower than the pK_a of aniline (~4.6) (Liu et al. 1999b). The pK_a values of the polyuronates of Man and Gal are reminiscent of those of the corresponding monomers; 3.38, and 3.52, respectively (Cesàro et al. 1990). It can therefore be assumed, that the pK_a values of the polyuronic acids of GGM (GGM_{PolyU}), GM (GM_{PolyU}), and NFC, prepared by TEMPO-oxidation are lower than 4.6. The pK_a of the sulfate group in the naturally ionic κ-CGN is ~2. Thus, both TEMPO-oxidized polysaccharides and κ-CGN could function as templates during enzymatic polymerization.

The formation of electrically conducting composites from cellulose and PANI receives a growing interest, since cellulosic materials present one of the most promising renewable raw materials for large-scale production, due to their natural abundance and unique properties resource. Cellulose/PANI composites have been prepared by interfacial chemical polymerization (Lee at el. 2012), by chemical polymerization on bacterial cellulose (Hu et al. 2011; Marins et al. 2011; Müller et al. 2012; Shi et al. 2012), cellulose (Mo et al. 2009; Rußler et al. 2011), or carboxymethyl cellulose (Yin et al. 1997), and by mechanical mixing of the prepared polyaniline and cellulose or cellulose-derived substrates using different techniques (Pron et al. 1997; Lukasiewicz et al. 2007; John et al. 2010; Shariki et al. 2011).

4. Experimental

4.1 Materials

GGM was prepared from Norway spruce thermomechanical pulp (TMP) by a large laboratory-scale method modified from the method reported by Willför et al. (2003b). In short, a suspension of TMP in hot tap water was stirred for 3 h and the pulp was removed. The extract water was purified from colloidal wood resin, and aromatic residues using a cationic coagulant (Raifix 120, Raisio Chemicals Oy, Finland) and XAD-7 resin (Amberlite, Rohm and Haas, UK). The water was concentrated by ultra-filtration or by rotary evaporation before GGM was isolated by precipitation in ethanol and air-dried (I, III-V).

XG of reduced molar mass was prepared from tamarind seed xyloglucan (Innovasynth Technologies Ltd., India) by digestion with the xyloglucan-specific endo-glucanase (EGase, EC 3.2.1.151, specific activity 70680 U/25 g) from *Chrysosporium lucknowense* (xgl1). EGase was purchased from Dyadic NL, Wageningen, NL (II, IV).

Raffinose, methyl α-D-galactopyranoside, GM, and κ-CGN (commercial grade, type I) were purchased from Sigma-Aldrich, and were used without further purification (III, IV, V).

Fully bleached birch kraft pulp (BKP, in dry lap form) was obtained from a Finnish pulp mill. The pulp was suspended in distilled water, and was homogenized by a household mixer. The pulp suspension, with a fibre concentration of 13%, was stored at -18 °C (I, IV).

Nanofibrillated cellulose (NFC) was produced by TEMPO-mediated oxidation of BKP (birch) using a procedure optimized from the method published by Saito and Isogai (2004) (V).

All chemicals were of commercial grade. All, except aniline, were used without further purification.

4.2 Analytical methods

The carbohydrate content (I-V)

The carbohydrate contents of polysaccharides were determined by gas chromatography (GC) after methanolysis and silylation (Sundberg et al. 1996; Willför et al. 2009). GC analysis was done on a PerkinElmer AutoSystemXL instrument (Norwalk, USA) equipped with an HP-1 column.

GC-MS was done on an HP 6890-5973 GC-MSD instrument equipped with an HP-1 column.

The degree of oxidation $DO_{\text{-COOH}}$, *i.e.* the amount of uronic acid units relative to the total amount of sugar units, of TEMPO-oxidized polysaccharides was determined by GC after methanolysis and silylation (I, IV, V).

The degree of oxidation $DO_{\text{-CHO}}$, *i.e.* the amount of galactose units containing an aldehyde group ($Gal_{\text{-CHO}}$) relative to the total amount of Gal in, of GO-oxidized polysaccharides, was determined by deuterium labeling of the aldehydes by reduction with $NaBD_4$. After acid methanolysis and silylation, the DO could be calculated from the GC-mass spectra (MS) by comparing the abundance of the peaks at m/z 361 and m/z 362 (Parikka et al., 2010). The DO is defined as:

$$DO_{\text{-CHO}}\ \mathbf{(\%)} = ([\textit{Gal-CHO}] / ([\textit{Gal-CHO}] + [\textit{Gal}]))*100 \qquad (1)$$

where [Gal-CHO] is the amount of oxidized galactose units, and [Gal] is the amount of unoxidized galactose units (I, III, IV).

The degree of acetylation, DA (I)

Acetyl groups released from GGM were determined in the form of acetic acid by high performance liquid chromatography (HPLC) with a refractive index (RI) detector (Shimadzu Corporation, Japan. The GGM was firstly deacetylated by alkali treatment. The deacetylated GGM solution was titrated to pH 2.7 – 2.9 and then sampled for HPLC analysis. A Synergi 4u hydro-RP 80A column (Phenomenex, California, USA) equipped with a guard column was used.

Structural characterizations

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AV 600 instrument. Plain D_2O or a mixture of DMSO-d_6 and D_2O (1:2) was used as solvent (I-IV).

Weight average molar mass, M_w, and number average molar mass, M_n, were determined by Size-Exclusion Chromatography (SEC) in on-line combination with a Multi-Angle Laser-Light-Scattering (MALLS) instrument (miniDAWN, Wyatt Technology, Santa Barbara, USA) and with a refractive index (RI) detector (Shimadzu Corporation, Japan). A two-column system, 2×UltrahydrogelTM linear column (Waters, Milford, USA), in series was used (I, III).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) was performed with a SAI LT3 Plus MALDI-TOF mass spectrometer (SAI Ltd., Manchester, UK) equipped with a nitrogen laser of 337 nm and operated in positive ion mode (Xu et al. 2012). MALDI-TOF-MS samples were prepared by mixing the sample with matrix solution (2,5-dihydroxybenzoic acid in acetone) on the MALDI-TOF-plate. Raffinose was analyzed directly without any pretreatment, and the polysaccharides were enzymatically digested prior to the analysis (III).

Fourier Transform Infrared (FTIR) spectra were recorded on an FTIR spectrophotometer (Bruker ALPHA series) using a KBr disc. In addition, film samples were analyzed using an ALPHA platinum Attenuated Total Reflectance (ATR) single reflection diamond ATR module. The samples were directly placed on the ATR plate for measurement (I, V).

Ultraviolet-Visible (UV-Vis) spectra were recorded on Perkin-Elmer Lambda 40 UV/VIS spectrometer in the aqueous solutions (V).

Scanning Electron Microscopy (SEM) images were taken with a LEO Gemini 1530 with a Thermo Scientific UltraDry Silicon Drift Detector. The nonconductive samples were coated with carbon prior to the measurements. (V)

Thermogravimetric Analysis (TGA) of the NFC/PANI biocomposites was performed by Differential Scanning Calorimetry-Thermal Gravimetric

Analysis (DSC-TGA) (Q600, Ta Instruments). For the calculation of the weight content of PANI in the NFC/PANI composites, the differential weight loss of the NFC and NFC/PANI composites was calculated within the temperature range from 275 °C to 285 °C where both NFC and PANI were decomposed (V).

Electrochemical characterizations

The conductivity of the samples was characterized by 4-point probe measurements. A constant bias current was applied over the sample and the corresponding voltage was measured (Keithley 2400 SourceMeter®) (V).

4.3 Experimental procedures

TEMPO-mediated oxidation of GGM (I, IV, V)

GGM (200 mg) was dissolved in distilled water (40 mL). The oxidations were performed either at room temperature, or at 2-4 °C. TEMPO (2 mg), and NaClO (10-15% solution, pH adjusted to 10, 4 ml), and in some cases NaBr (300 mg), were added. The pH of the solution was kept at 10 by adding 0.5 M NaOH. The reaction was stopped by neutralization with 1 M HCl. The product was purified by dialysis against distilled water for three days (membrane cut-off 12000-14000 $gmol^{-1}$). The purified products were freeze-dried.

TEMPO-mediated oxidation of enzymatically hydrolyzed GM and XG (IV)

Hydrolyzed GM was obtained by treatment of GM with endo-1,4-β-mannanse, and hydrolyzed XG was obtained by treatment of XG with specific endo-1,4-β-xyloglucanase. The oxidations were performed at 2-4 °C. by addition of NaBr (150 mg), TEMPO (1 mg), and NaClO (10-15% solution, pH adjusted to 10, 2 mL) to a aqueous solution of the polysaccharide (100 mg). The pH was adjusted to 10. After 2.5 h the solution was neutralized by HCl (1M), and the product was precipitated in ethanol, redissolved in water, and freeze-dried.

Galactose oxidase (GO) catalyzed oxidation of methyl-Gal*p*, raffinose, and galactose-containing polysaccharides (II, III)

The oxidations were performed using GO in combination with horseradish peroxidase, and catalase, according to the methods previously reported

(Parikka and Tenkanen 2009; Parikka 2010; Xu et al. 2012). The oxidized polysaccharides were not isolated; the subsequent chemical modifications were performed directly in the solutions in which the oxidations were done.

Amidation of GGM_{PolyU} (I)

Amidation of GGM_{PolyU} was performed using a method reported earlier by Araki et al. (2001). Shortly, GGM_{PolyU} (150 mg, corresponding to approx. 0.8 mmol of uronic acids) was dissolved in distilled water (10 mL). NHS (140 mg, 1.2 mmol) was added, and the polysaccharide solution was stirred until the NHS was completely dissolved. The amine was added (1.5 equiv. relative to the amount of uronic acid groups), and the pH was adjusted to 7.5. EDC (220 mg, 1.2 mmol) was dissolved in distilled water (2 mL) and was added dropwise to the reaction solution. The pH was kept at ~7.5 by addition of NaOH (0.5 M), and the reaction was stirred at room temperature over night. Afterwards, the pH was lowered to 2, and the solution was dialyzed for two days (membrane cut-off 12000-14000 $gmol^{-1}$). The product was finally freeze-dried.

Indium-mediated allylation of GO-oxidized methyl-Gal*p* (2) (II)

Indium powder (2 equiv, related to the amount of 2 in the solution) was added to an aqueous solution of 2. If THF was used as co-solvent, the allylic halide (3 equiv, related to the amount of 2 in the solution) was dissolved in a small amount of the solvent, and was then added. Otherwise the allyl halide was added directly to the reaction solution. The suspension was stirred for 22-26 h at room temperature. The suspension was then neutralized with NaOH, filtered through a bed of kieselguhr, the solvent was removed by rotary evaporation, and the product was dried in vacuum. The homoallyl alcohols were purified by column chromatography (SiO_2). The yield was determined gravimetrically, and the diastereoselectivity was determined by ^1H NMR spectroscopy.

In the case of the crotylation and cinnamylation reactions, the stereoisomers were separated in pairs by column chromatography after acetylation.

Indium-mediated allylation and propargylation of raffinose and galactose-containing polysaccharides (III)

The amounts of reactants were related to the approximate molar amount of oxidized galactose units in the solution. Plain water was used as solvent in most reactions. Allylation reactions of oxidized raffinose were also done using methanol (MeOH) or tetrahydrofuran (THF) as co-solvents (co-solvent-H_2O 1:4 v/v). Oxidized GGM was also allylated in HCl (0.1M), or using THF as co-solvent (THF-H_2O 1:4 v/v).

The typical amount of sample was ca 10 mg. To the aqueous solution containing the oxidized polysaccharide (1-10 mg/mL), indium powder (5-10 equiv) and the allylic or propargylic halide (5-10 equiv) were added, and the solution was stirred at room temperature or at 55 °C for 24-48h. The allylated polysaccharide was isolated by precipitation in ethanol (ethanol-water 10:1 v/v), washed twice with ethanol-water (10:1 v/v), and dried by freeze-drying or under N_2.

Sorption of modified GGMs onto pulp fibres (I, IV)

Polysaccharide solution (~40 mg/g fibre) in distilled water was added to a suspension of BKP, giving a final fibre concentration of 1%. The suspension was stirred overnight, after which the fibres were removed by centrifugation. The amount of sorbed polysaccharides was calculated by subtracting the amount of polysaccharides left in the supernatant from the amount added. The effect of the ionic strength of the sorption medium was investigated by performing the sorption in water, and in 0.01 M, and 0.1 M NaCl. The samples were analyzed in duplicate, or triplicate.

Laccase-mediated polymerization of aniline (V)

Aniline was purified by extraction with HCl (1 M) from in diethyl ether (DEE) prior to use. Laccase from *Trametes versicolor* (EC 1.10.3.2, 13.6 U/mg) was obtained from Sigma-Aldrich. A stock solution of 5 mg/mL in distilled water was prepared, and it was stored in a freezer.

Polymerization of aniline was done in aqueous solution without any buffer. The dry content of the NFC suspension was 0.1%, while GGM, GGM_{PolyU}, and κ-CGN solutions were dissolved in distilled water at 10 mg/mL, and GM_{PolyU} at 5 mg/mL. First, the pH value of the polysaccharide solution/ NFC suspension containing the template was adjusted with phosphoric acid to 3-4. Aniline was added, the amount was related to the approximate molar amount

of anionic groups in the solution (the ratio of anionic group to aniline varied from 2:1 to 1:20). The pH was then readjusted to 3.7, and the solution/suspension was stirred at room temperature for 15 min before laccase was added. The solution was then stirred at room temperature over night. The dark-green polyaniline-polysaccharide was purified by dialysis against 1 mM HCl (pH 3) for three days (membrane cut-off 12000-14000 g/mol).

Patterning κ-CGN/PANI hydrogel by spray coating and casting (V)

For spray coating, an office copy paper was covered by a patterned mask using ELISA microarray as a template. The κ-CGN/PANI dispersion was sprayed using an air brush.

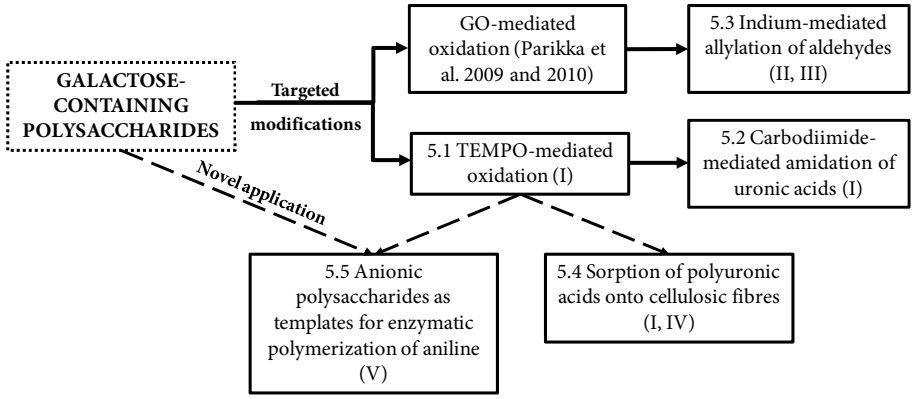
The κ-CGN/PANI and GGM_{PolyU}/PANI dispersion were cast onto Whatman no. 1 filter paper for conductivity and SEM characterizations. A weight was applied on top of the papers to smoothen any surface roughness and the samples were first air-dried, and then dried in a vacuum desiccator.

Preparation of NFC and NFC/PANI films (V)

The NFC/PANI suspensions were filtrated trough a Whatman Nuclepore Track-Etch polycarbonate membrane. A weight was applied on top of the films obtained and they were dried in vacuum desiccator.

5. Results and discussion

Scheme 5.1 illustrates the polysaccharide modifications paths discussed in the following sections. Two different routes were applied; activation of the C-6 positions in different sugar units by TEMPO-mediated oxidation, and activation of C-6 position only in Gal units by GO-mediated oxidation. The activated sites were then further selectively derivatized.



Scheme 5.1. The pathways used for targeted modification of polysaccharides. In addition to the modifications, also potential applications of anionic polysaccharides were investigated (dash line arrow).

5.1 TEMPO-mediated oxidation of GGM (I)

By TEMPO-mediated oxidation of polysaccharides, unsubstituted hydroxyls at the C-6 positions are converted into aldehydes or carboxylic acids. It is an efficient method, and complete oxidation can be obtained within minutes upon the addition of the reagents. The reaction rate and yield are dependent on the substrates and reaction parameters used. By adjusting the amount of reagents, or by using different co-oxidants, the rate and product (carbonyl or carboxyl) can be controlled (Bragd et al. 2004).

GGM was oxidized to its polyuronic acid derivatives (GGM_{PolyU}) by TEMPO-mediated oxidation (I). The highest DO, i.e. the amount of uronic acids compared to the total amount of sugars in the sample, obtained was approx. 80%. Some of the Man units are substituted with Gal side-groups, and cannot

therefore be oxidized. Part of the C-6 Man units, and also some in the Glc and Gal units remain unoxidized; the maximum DO of both Man and Glc was ~80%, whereas Gal only reached a DO of ~60%. No free C-6 signals could be seen in the ^{13}C NMR spectrum of GGM_{PolyU}, indicating that the remaining unoxidized sugar units were substituted at the C-6 position (Figure 5.1). Some of the C-6 substituted Glc units origin from glucan residues, and some Gal are likely to come from arabinogalactans (Willför et al. 2002). Also, it has been suggested, that GGM from Norway spruce may contain unidentified structural units resembling of hexoses with substituents on 6-position (Willför et al. 2003a)

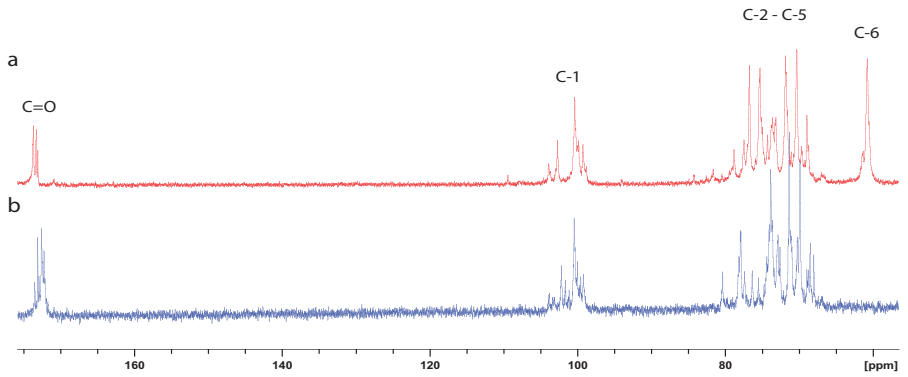


Figure 5.1. ^{13}C NMR spectra of GGM (a) and TEMPO-oxidized GGM (GGM_{PolyU}) (b). The carbonyl signals in (a) are from the acetyl groups in GGM.

TEMPO-mediated oxidation of polysaccharides is commonly performed at 2-4°C with NaClO and NaBr as co-oxidants. At these conditions, the oxidation of GGM proceeded fast, and high DO was reached within minutes; after 15 min the DO was already 65% (Figure 5.2). Performing the reaction at room temperature increased the rate, and the maximum DO of ~70% was achieved within 15 min. A notably slower conversion and thus a more controlled oxidation, was obtained at bromide-free conditions. At room temperature, a DO of only 40% was achieved and at 2-4°C only 16% was reached within the first 15 min. The lower reaction rate at the bromide-free conditions could be compensated by a longer reaction time, and an equally high DO was obtained within 120 min. The highest degrees of oxidation were reached when the oxidations were performed at 2-4°C.

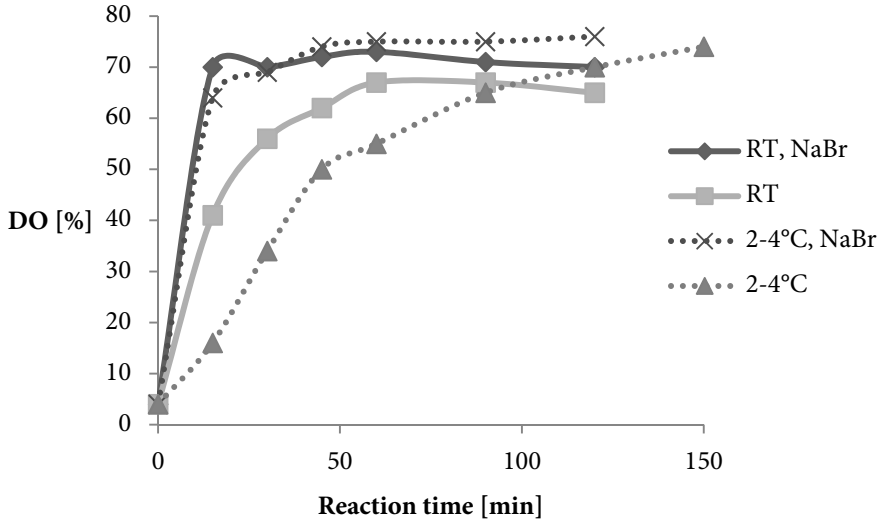


Figure 5.2. TEMPO-mediated oxidations done with or without NaBr and either at room temperature (RT) or at 2-4°C. All reactions were done in the presence of TEMPO and NaClO (I).

Differences in the oxidation rates of the different sugar species were observed. As can be seen in Figure 5.3, the rate of conversion of Man and Glc units to their corresponding uronic acids was similar, but the oxidation of Gal proceeded noticeably slower and the DO obtained was much lower. Although the lower conversion is partly explained by the existence of C-6 substituted non-GGM sugars in the solution, it has also been shown, that during the oxidation mediated by $TEMPO^+ BF_4^-$, the oxidation rate and conversion of Gal to the corresponding uronic acid was considerably lower than those of Man and Glc (Brenton et al. 2007). The authors speculated that the reason for this is the β-configuration of the C-4 in galactose that might sterically hinder the formation of the oxidation intermediates.

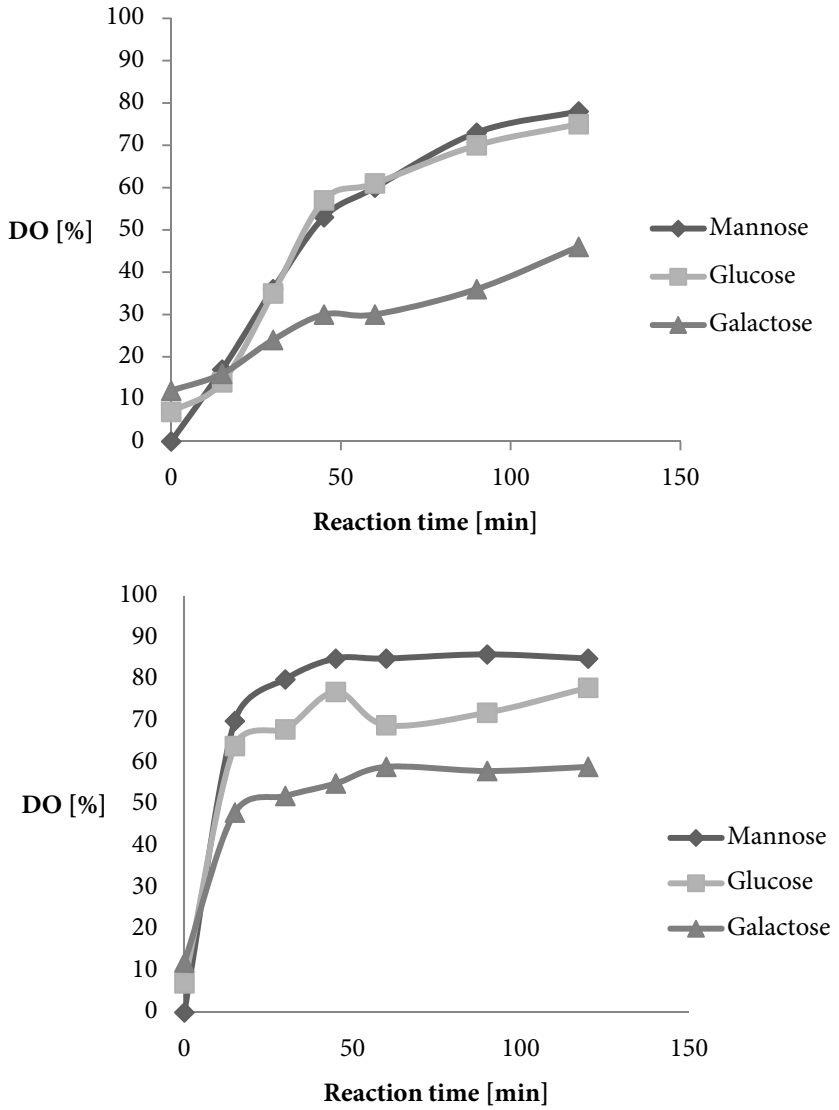


Figure 5.3. Oxidation rate of the different sugar units in GGM. Both reactions were performed at 2-4°C in the presence of TEMPO and NaClO. (a) was performed with and (b) without NaBr (I).

The aldehyde form is an intermediate in the TEMPO-mediated oxidation of alcohols to carboxylic acids. During TEMPO-mediated oxidation of GGM a few percent of the sugar units are in aldehyde form. At the 60 min, when maximum DO was reached when performing the oxidation without NaBr at

room temperature, approx. 10% of the remaining Gal units were in the aldehyde form. Since aldehydes form inter- and intramolecular hemiacetal bonds (Parikka and Tenkanen 2009) the aldehydes were reduced by $NaBH_4$ prior to SEC analysis to ensure that no aggregation through hemiacetal bonds would occur. Even if GGM contains (1→6)-bonded Gal side-groups that are accessible for cleavage during TEMPO-mediated oxidation, and even if some of the oxidations were performed at room temperature, no significant degradation could be observed during the oxidations (Figure 5.4) (I). Analysis of samples taken when the maximum DO of each method was reached, shows that GGM oxidized at room temperature in the presence of NaBr degraded the most. When oxidation was carried out at 2-4°C the M_w even slightly increased, which was caused by the formation of the carbonyl groups.

Because of the high pH, the acetyl groups of GGM are partly cleaved during TEMPO-mediated oxidation. When GGM was oxidized at 2-4 °C in the presence of NaBr, 20% of the acetyl groups were removed already after 5 min, and after 2.5 h the degree of deacetylation was 40% (IV). At room temperature, in the presence of NaBr, 20% of the acetyl groups were cleaved during the first 5 min (I). At 15 min, when the reaction had reached its maximum DO, approx. 30 % of the acetyl groups had been removed. Since the sodium-free reactions require longer time to be completed, also more deacetylation occur; by the time the maximum DO was reached at room temperature 50%, and at 2-4 °C up to 70% of the acetyl groups were removed.

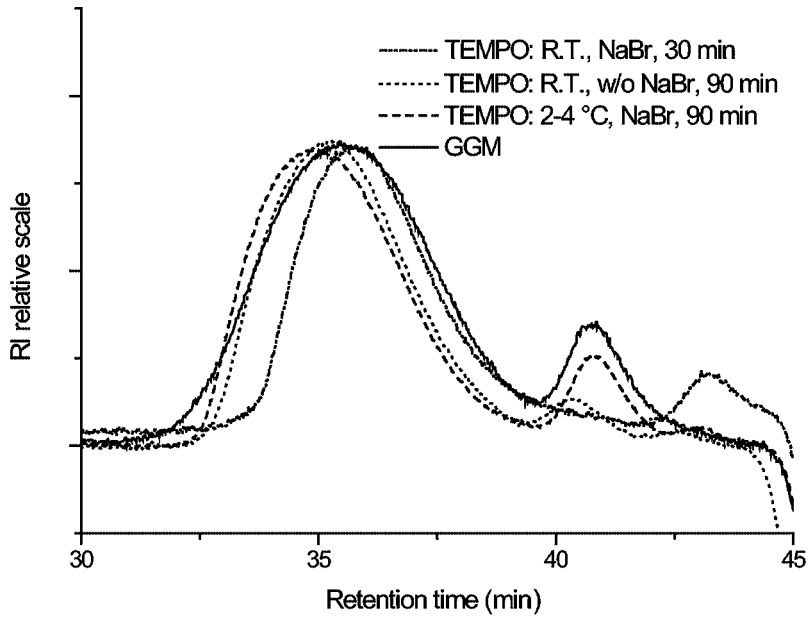


Figure 5.4. SEC-MALLS of TEMPO-oxidized GGMs (I).

Conclusions

TEMPO-mediated oxidation is an efficient way for selective introduction of carboxylic acids to spruce GGM. By performing the oxidation without NaBr, a more controllable reaction is obtained; polyuronic acids with degrees of oxidation reaching from 6% to 75% were formed (I). Although some of the acetyl groups are split of during the oxidation, the product remained readily water-soluble, thus offering a platform for further modifications in aqueous media.

5.2 Carbodiimide-mediated amidation GGM_{PolyU} (I)

GGM_{PolyU}, produced by TEMPO-mediated oxidation of GGM, was further functionalized by carbodiimide-mediated amidation, using *N*-ethyl-*N*'-(3-(dimethylamino)propyl)carbodiimide (EDC) and *N*-hydroxysuccidimide (NHS) as coupling reagents (I). Two structurally varying water-soluble amines were chosen as model compounds for the amidations; the amino acid arginine, and the aliphatic 1,6-diaminehexane. Arginine has been shown to

improve bioactive properties of chitosan, e.g. the anticoagulant activity (Liu et al. 2004). Derivatization with a diamine could lead to cross-linking, which is known to improve the strength properties of GGM films (Mikkonen et al. 2013).

In the FTIR spectrum of GGM_{PolyU}, besides the characteristic peaks of GGM, the formation of uronic acids resulted in the appearance of strong C=O stretching bands at 1603 cm^{-1} in its carboxylate form and at 1735 cm^{-1} in its acid form (Figure 5.5) (Liu et al. 2004) (I). When GGM_{PolyU} was reacted with 1,6-diaminohexane in the presence of the EDC and NHS, the band of C=O stretching at 1603 cm^{-1} shifted to 1637 cm^{-1} indicating the successful formation of amide bonds between the carboxylic acid groups of GGM_{PolyU} and 1,6-diaminohexane (GGM_{PolyU}-1,6-DAH). In addition, the appearance of absorption at 2865 and 2935 cm^{-1} is ascribed to symmetric and asymmetric H vibration of CH_2 groups in the alkyl chain of 1,6-diaminohexane.

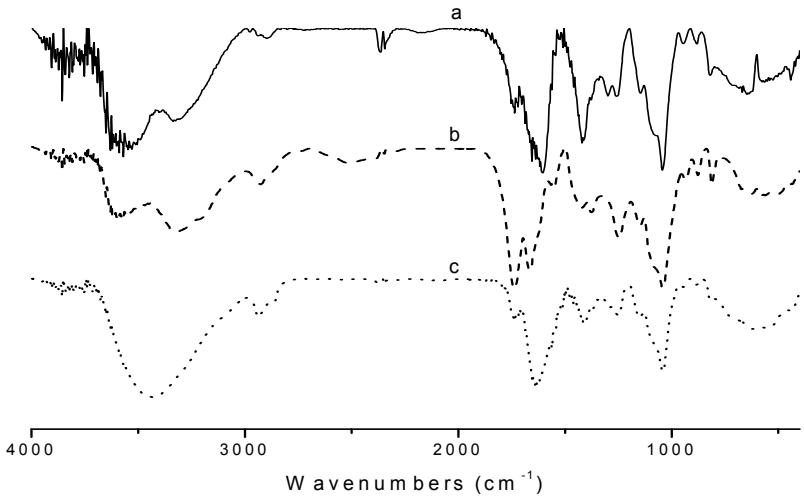


Figure 5.5. FTIR of TEMPO-oxidized GGM (GGM_{PolyU}) (a) and its amidation products of arginine (b) and 1,6-diaminohexane (c) (I).

NMR analysis also verified the formation of GGM_{PolyU}-1,6-DAH (I). The peaks between 27-40 ppm in the ^{13}C NMR spectrum are characteristic for the CH_2 groups of the amidated 1,6-diaminohexane (Figure 5.6) (Barbucci et al. 2006). The two-bond ^{1}H-^{13}C correlations in HMBC show a coupling between the carbonyl signals at 172-175 ppm and the amide CH_2 (C7) at 3.2-3.3 ppm,

indicating the formation of amide bonds (Figure 5.7). The lack of correlations between the carbonyl signals and the signals at 2.87 ppm (N-CH_2, C12) show that not all amines have cross-linked, and there are free amino groups left.

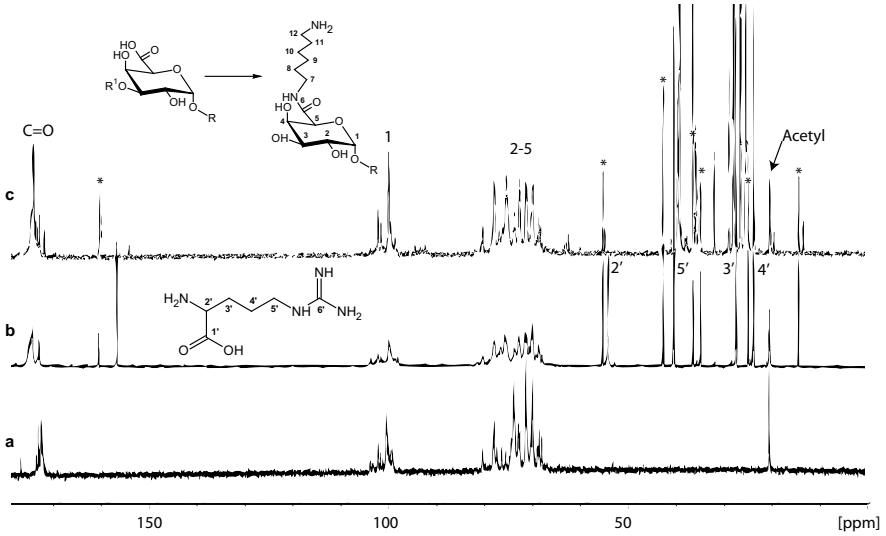


*Figure 5.6. ^{13}C NMR spectra of TEMPO-oxidized GGM (GGM_{PolyU}) (a) and its amidation products of arginine (b) and 1,6-diaminohexane (c). * Peaks assigned to the N-acylurea by-product (I).*

During EDC-mediated amidation, reactive *O*-acylisourea is formed when the EDC carbocation is attacked by the carboxylate, and this *O*-acylisourea can either react with primary amines to form amides or form *N*-acylurea through an intramolecular acyl transfer (Scheme 3.5, Section 3.3) (Nakajima and Ikada 1995). In the FTIR spectrum, the unreactive by-product *N*-acylurea has amide bands at ~1550 cm^{-1} and ~1650 cm^{-1}, and an imide band at ~1735 cm^{-1} (Gabarek and Gregerly 1990). These are undistinguishable from the bands of the desired amide products, but in the ^1H NMR spectrum the triplet at 1.1 ppm and the multiplet at 3.0-3.2 ppm can be assigned to *N*-acylurea (Kuo et al. 1991) (I). Thus, even if reaction conditions previously optimized for amidation of cellulose microcrystals were utilized (Araki et al. 2001), the formation of *N*-acylurea was not completely inhibited (I).

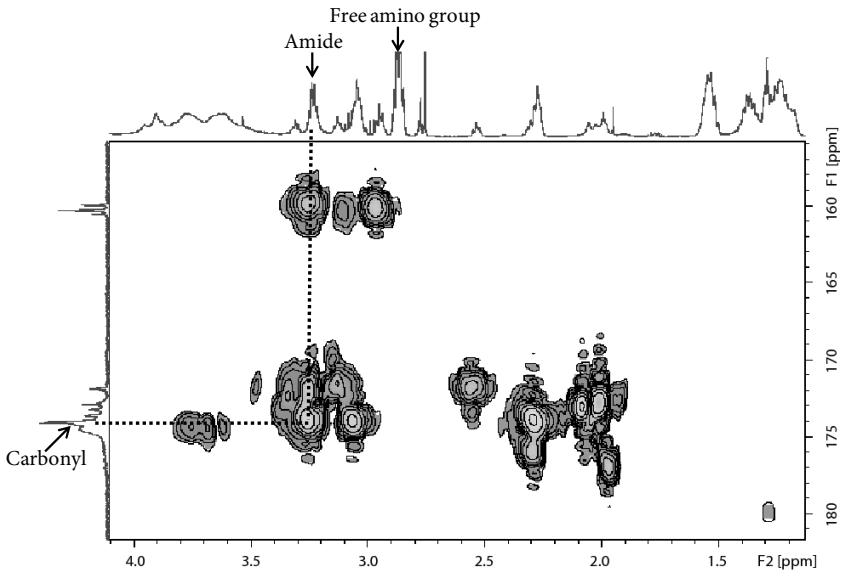


Figure 5.7 Part of the HMBC NMR spectra of GGM_{PolyU}-1,6-DAH. The correlation between the amide and the carbonyl signals is marked with a dashed line (I).

After amidation with arginine, the typical amide I (C=O stretching) at 1665 cm^{-1} and amide II at 1550 cm^{-1} (combination of N-H deformation and C-N stretching) were observed, and did reveal the successful addition of arginine to GGM_{PolyU} (Figure 5.5) (I). The dramatic increase in absorption at 1735 cm^{-1} is ascribed to introduction of carboxylate groups present in arginine, as well as the formation of *N*-acylurea. In the ^{13}C NMR spectrum, the peaks at 23.8 ppm (C4'), 27.5 ppm (C3'), 40.5 ppm (C5'), 54.2 ppm (C2'), 156.7 ppm (C6') can be assigned to arginine, whereas the peaks at 25 ppm, 36.4 ppm, 35.7 ppm, 55.3 ppm are from the CH group, and 14.4 ppm and 42.7 ppm the $-CH_3$ groups of *N*-acylurea (Figure 5.6) (I). The *N*-acylurea peaks can also be found in the ^{13}C NMR of 1,6-diaminohexane amidated GGM.

Conclusions

Carbodiimide-mediated amidation is a potential method for introducing functionality to GGM_{PolyU}. All steps are done in water. In order to minimize the formation of *N*-acylurea, thus maximizing the conversion of the reaction, a careful control of the reaction parameters needs to be done for the each different reagent used.

5.3 Indium mediated allylation of oxidized carbohydrates (II, III)

Aldehydes and ketones react with allyl halides in the presence of a mediating metal forming homoallyl alcohols (Scheme 3.6, Section 3.3). By combining enzymatic oxidation by galactose oxidase (GO) and indium mediated allylation, methyl α-D-Gal*p* (**1**, Figure 5.8) (II), raffinose, a trisaccharide containing an α-(1→6)-linked Gal group, and the galactose-containing polysaccharides GGM, GM, and XG were selectively derivatized (III). In addition to the allyl halides allyl bromide, crotyl chloride, and cinnamyl chloride, also propargyl bromide was applied (Figure 5.9).

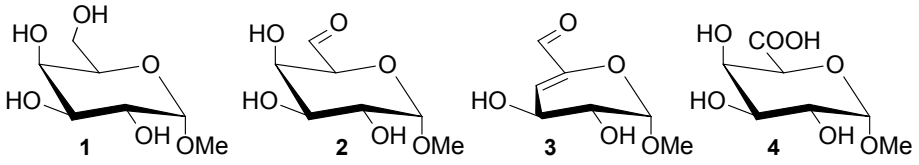


Figure 5.8. Methyl α-D-galactopyranoside (1) and the main (2) and the secondary products (3, 4) of the enzymatic oxidation of methyl-galactopyranoside. 2 = methyl α-D-galacto-hexodialdo-1,5-pyranoside, 3 = methyl (6-S)-3-deoxy-D-threo-hex-2-enodialdo-2,6-pyranoside, 4 = methyl α-D-galactopyranosiduronic acid (II) (Parikka et al. 2009).

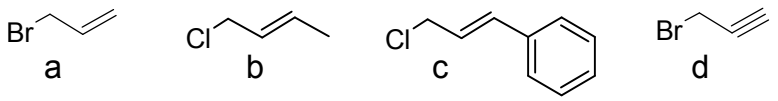


Figure 5.9. a) Allyl bromide; b) crotyl chloride; c) cinnamyl chloride; d) propargyl bromide

The formation of the desired homoallylic alcohols in the polysaccharides was verified by MALDI-MS analysis (III). Since polysaccharides require partial hydrolysis to oligomers prior to analysis, pre-screenings were done using GO-oxidized **1** (Figure 5.8) and raffinose as model substances (II, III). Because of the small size of the model substances, e.g., TLC and MALDI-MS analysis could be done directly on the reaction solutions, making the optimization of reaction parameters easier and faster.

5.3.1 Metal mediated allylation of oxidized methyl-Gal*p* and raffinose (II, III)

In order to activate the Gal groups to allylation reactions, GO-catalyzed oxidation was performed. The main product of the oxidation **1** (Figure 5.5) was *α*-D-galacto-hexodialdo-1,5-pyranoside (**2**, Figure 5.8) (Parikka and Tenkanen 2009). Methyl (6-*S*)-3-deoxy-D-*threo*-hex-2-enodialdo-6,2-pyranoside (**3**, Figure 5.8) and methyl *α*-D-galactopyranosiduronic acid (**4**, Figure 5.8) are secondary products that might be formed during oxidation or the following treatment of the aldehyde. By controlling the oxidation parameters **2** can be obtained in excellent yield, but during storage some dehydration occurs, and **3** can always be found in the aqueous solutions of oxidized **1**. The oxidation of raffinose was complete, that is, all primary hydroxyls in Gal units were oxidized to the corresponding aldehyde (Raf_{-CHO}).

Pre-screenings of the reaction between **2** and allyl bromide using zinc, tin, and indium showed that indium was the most efficient mediating metal (II). No reaction was observed when using tin or zinc at room temperature. Tin-mediated allylation of carbohydrates can be improved by heating the reaction solution (Kim et al. 1993). Unfortunately, heating of the aqueous solution of **2** lead to formation of secondary products, e.g., formation of **3** through dehydration of **2.** According to the TLC analysis that was used to follow the reaction, **3** also reacted in the allylation reaction, but this reaction was not investigated further. The uronic acid **4** did not react under these conditions (II).

The reactions were performed using water as the only solvent or with THF or methanol as organic co-solvent. According to TCL the rate of reaction between **2** and allyl bromide performed in plain water was somewhat higher than the one performed in THF-H_2O (1:1) (II). The conversion was also higher in plain water. A quantitative conversion of **2** to the corresponding allyl alcohol was obtained in water, whereas in THF-H_2O a 94% conversion was obtained. Addition of hydrochloric acid (HCl) increased the rate of the reaction significantly. When performing the indium-mediated allylation in 0.1 M HCl, no aldehyde could be observed by TLC analysis after 2 h. According to NMR analysis, the amount of **3** in the reaction mixture had slightly increased. The decrease in pH seems to increase the dehydration of **2**, thus decreasing the final yield of the reaction to some extent.

The reaction between $Raf_{\text{-CHO}}$ and allyl bromide proceeded well in plain water at room temperature (III). According to MALDI-MS analysis of the reaction solution, the reaction was complete after 24 h, and since only the expected product peaks of allylated raffinose (m/z = 567, Na^+ adduct; m/z = 583, K^+ adduct) were detected (Figure 5.10), the reaction was assumed to, similarly to the monomer **2**, yield in quantitative conversion to the product.

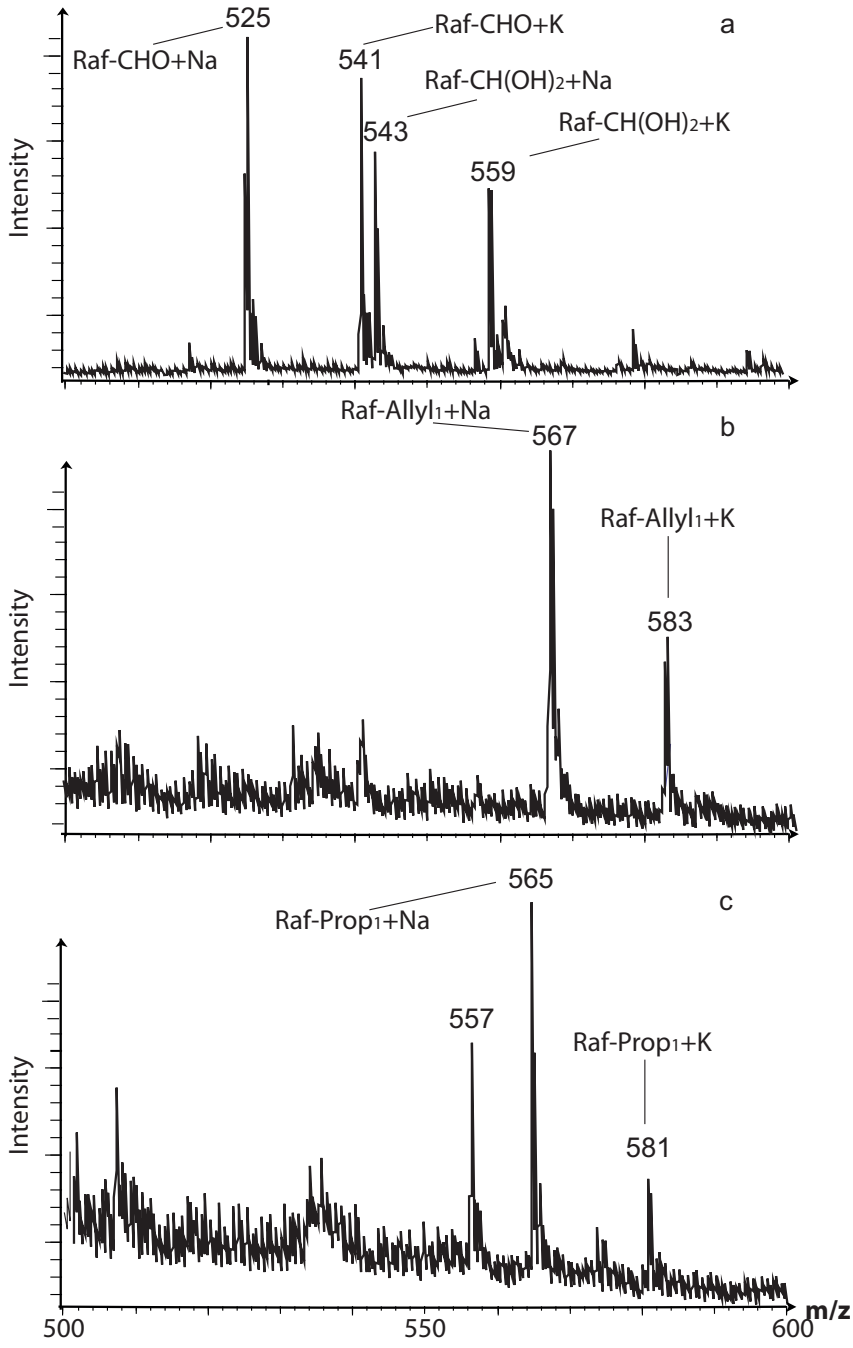


*Figure 5.10. MALDI-MS spectra of modified raffinose: (a) GO oxidized; (b) Allylated (Allyl); and (c) Propargylated (Prop) in Na^+ or K^+ adduct form (III). Raf-CHO = Raffinose containing an aldehyde group; * Hydrate of –CHO.*

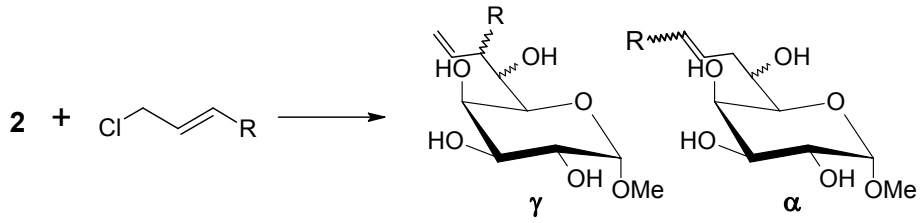
Even if crotyl chloride reacts with **2** to produce the corresponding homoallyl alcohol in moderate yield, no reaction could be observed with $Raf_{\text{-CHO}}$ (III). To enhance the solubility of crotyl chloride in the reaction media, the use of methanol as a co-solvent was attempted. Methanol did not improve the reaction and neither was it improved by performing the reaction at 55°C.

The conversion of the reaction of cinnamyl chloride and **2** in THF-H_2O was significantly higher than in plain water; 94% and 60%, respectively (II). $Raf_{\text{-CHO}}$ and cinnamyl chloride did also react in plain water at room temperature; however, unreacted starting material could still be detected even after 48 h. The conversion to product was not improved when raising the temperature to 55°C.

Propargyl bromide and $Raf_{\text{-CHO}}$ did not react at room temperature, but at 55 °C the reaction proceeded smoothly and almost no starting material was detected after 24 h (III). Even if the reaction proceeded well in just plain water, the rate could be increased slightly by using a co-solvent (MeOH or THF). MALDI-MS and ESI-MS analysis showed, in addition to only trace amounts of the starting material and its hydrate form, only the expected product peak (m/z = 565, Na+ adduct of propargylated raffinose) (Figure 5.10). Thus, it was concluded that, as in the case of allyl bromide, no unwanted side-reactions occurred during the reaction between $Raf_{\text{-CHO}}$ and propargyl bromide.

Regioselectivity

During the indium-mediated allylation reaction γ-substituted allyl halides can form either the α- or γ-adducts (Scheme 5.2). Besides the steric size of the γ-substituent of the allylic halide (Isaac and Chang 1995a), also the amount of water influences the regioselectivity significantly. By performing the reaction in 6 equiv of water relative to the amount of the aldehyde, the regioselectivity can be changed from γ-selective to almost completely α-selective (Cheng and Loh 2005). A higher amount of water in the reaction solution seems to inhibit the formation of the α-adducts, which is the case when **2** and crotyl chloride or cinnamyl chloride react in water. Both reactions are completely regioselective, and only the γ-adducts are obtained (II).



Scheme 5.2. Indium mediated allylation of GO oxidized methyl α-D-Galp (2). Only γ form was found after performing the reaction in water at room temperature. R = Me or Ph (II).

Propargylation of carbonyls theoretically produce a mixture of the homopropargyl and allenyl alcohols. The regioselectivity of propargylated $Raf_{\text{-CHO}}$ was not unambiguously assigned. However, indium mediated propargylation of non-carbohydrate aldehydes in water has been reported to produce mainly the propargylic product (Isaac & Chan 1995b), and zinc mediated propargylation of carbohydrate aldehydes produce the homopropargylic alcohol as major products, and only very little of the allenylic alcohols can be detected (Pakulski and Zamojski 1997; Paulsen and Madsen 2002).

5.3.2 Indium mediated allylation and propargylation of galactose-containing polysaccharides (III)

In GGM, approx. 10%, and in GM approx. 40%, of the sugar units are α-D-Gal*p* units attached to the Man*p* units of the backbone through (1→6) linkages (Willför et al. 2003a; Nishinari et al. 2007). In XG, approx. 16% of the sugar units are Gal*p* residues linked through (1→2) bonds to the Xyl*p* side-chains, attached to the glucose backbone (York et al. 1993; Nishinari et al. 2007). Reactive aldehyde groups ($Gal_{\text{-CHO}}$) were successfully formed at the C-6 of these terminal Gal*p* units by GO-mediated oxidation, producing the aldehyde-containing polysaccharides $GGM_{\text{-CHO}}$, $GM_{\text{-CHO}}$, and $XG_{\text{-CHO}}$ (Parikka et al. 2010). The degree of oxidation ($DO_{\text{-CHO}}$), determined by GC-MS after reduction of the oxidized galactose units with $NaBD_4$, was for $GGM_{\text{-CHO}}$ 60% (corresponding to approx. 6% of total sugars), and for $GM_{\text{-CHO}}$ 80% (approx. 32% of total sugars). XG was oxidized completely, that is, all terminal Gal units in $XG_{\text{-CHO}}$ were in the corresponding aldehyde form.

The allylation reactions were verified by MALDI-MS (III). Even if the analysis was not quantitative, the spectra still gave indications of the reaction efficiency; the existence of desired oligomers proved the reaction, the lack of starting material indicated a complete reaction (high efficiency), and the existence of oligomers originating from the starting material indicated an incomplete reaction (moderate or poor efficiency) (Table 5.1).

Table 5.1 Summary of indium mediated allylations and propargylations of GO-oxidized polysaccharides.

Polysaccharide	**Halide**[a]	**Reaction efficiency**[b]
$GGM_{\text{-CHO}}$	Allyl	+++
$GGM_{\text{-CHO}}$	Prop	+
$GGM_{\text{-CHO}}$	Cinn	+
$GGM_{\text{-CHO}}$	Crot	0
$GM_{\text{-CHO}}$	Allyl	+
$GM_{\text{-CHO}}$	Cinn	0
$XG_{\text{-CHO}}$	Allyl	+++
$XG_{\text{-CHO}}$	Prop	0
$XG_{\text{-CHO}}$	Cinn	+

[a] Allyl = Allyl bromide; Prop = Propargyl bromide; Cinn = Cinnamyl chloride; Crot = Crotyl chloride.

[b] +++ = high; + = moderate; 0 = poor

In addition to MALDI-MS, GC-MS was also used for monitoring the reactions. Even if some of the changes could be assigned to side reactions, such as dehydration occurring simultaneously to the allylation reactions, the decrease in DO gave an indication of the formation of allylated, or propargylated, products. The conversions of the allylation reactions, *i.e.* the decrease in the amount of $Gal\text{-}_{CHO}$ units, were calculated from the decrease in DO and are presented in Table 5.2 (III). In the case of XG, the amount of galactose detected after reduction by $NaBD_4$ was assumed to originate from unreacted Gal-CHO units, and the conversions were calculated directly from those values.

Table 5.2 Conversion of indium-mediated allylation reactions of GO-oxidized GGM and GM using different allyl halides. All reactions were carried out in water at 55 °C.

Polysaccharide	Halide[a]	DO(1)	DO(2)	Conversion [%][b]
GGM_{-CHO}	Allyl	60	0	100 (6)
GGM_{-CHO}	Prop	60	27	75 (5)
GGM_{-CHO}	Cinn	60	45	45 (3)
$GM_{-CHO(high\ DO)}$	Allyl	84	70	56 (19)
$GM_{-CHO(low\ DO)}$	Allyl	40	27	45 (6)

[a] Allyl = Allyl bromide; Prop = Propargyl bromide; Cinn = Cinnamyl chloride.

[b] % of Gal_{-CHO} that have reacted (% of total sugars).
DO(1) = DO of the starting material; DO(2) = DO of the product.

Allylation and propargylation of GGM_{-CHO} (III)

Allylation of oxidized GGM (GGM_{-CHO}) at room temperature in plain water was not successful. However, when increasing the temperature to 55°C, the reaction with allyl bromide was complete after 24 h. Already after 3 h, the DO had dropped to 20% (i.e. ~80% of the aldehydes had reacted), and after 24 h only trace amounts of aldehydes were left in GGM (Table 5.1). Allylation was verified by NMR by comparing ^{1}H NMR spectra obtained from the starting material and the product. The aldehyde proton signal at approx. 9.5 ppm had disappeared, and signals at approx. 2.3 ppm, 5.1 ppm and 5.8 ppm, corresponding to H-7, H-9, and H-8, respectively of allylated galactose, had appeared (Figure 5.11). The formation of homoallyl alcohols was also verified by MALDI-MS analysis after enzymatic digestion of the modified polysaccharides (Figure 5.12). The introduction of an allyl group could be observed as an increase in mass by 40 molar mass units (m/z = 725.5, 771.6, 813.6, 891.7, 933.7, 1017.8, 1095.7, 1137.8), cinnamyl as 116 mass units (m/z = 805.7, 847.5, 889.5, 1009.6, 1051.7, 1171.7), and propargyl as 38 mass units (m/z = 727.3, 769.4, 811.5, 931.6, 973.6, 1093.6, 1135.6). In the case of GGM, the mass difference of 40 mass units could theoretically also indicate the occurrence of oligomers containing both oxidized galactose units and an acetyl group. But since the DO was 0% after allylation of oxidized GGM, we

could safely assume that such oligomers were not present and the mass difference of 40 mass units could thus be assigned to the introduced allyl group.

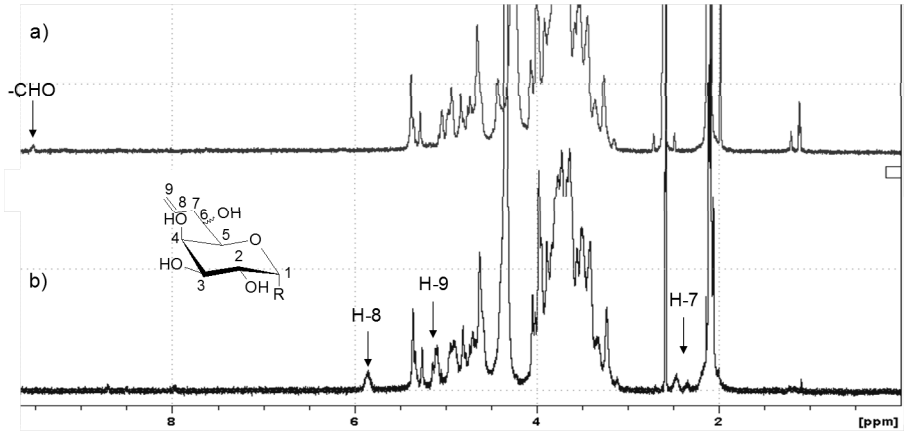


Figure 5.11. ^{1}H NMR spectra (in DMSO-d_6 / D_2O) of a) oxidized GGM and b) allylated GGM. R= GGM backbone (III).

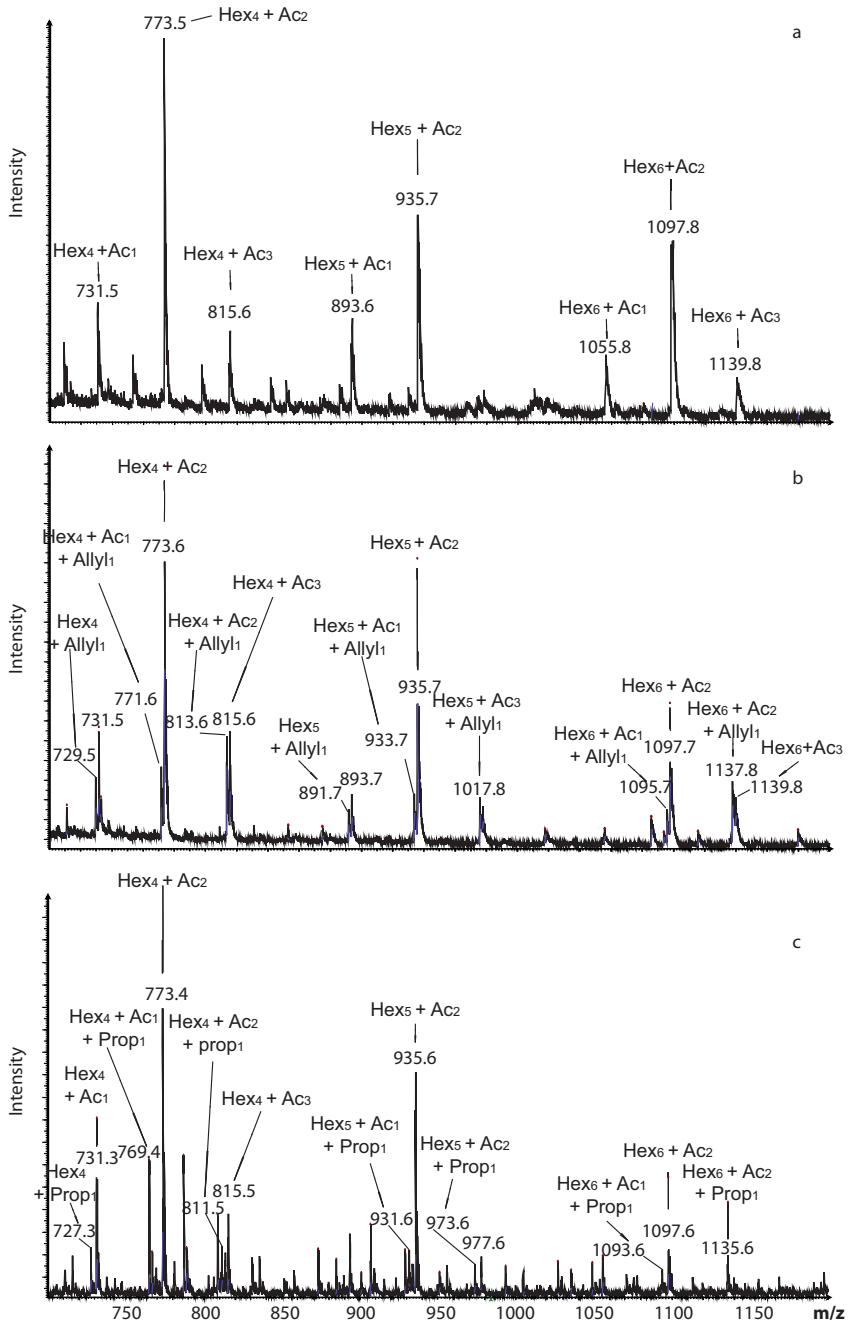


Figure 5.12. MALDI-MS spectra of modified GGMs. GGM oligosaccharides produced by mannanase hydrolysis of (a) native GGM, (b) allylated GGM, (c) propargylated GGM in Na^+ adduct form (III). Hex = Anhydrohexose; Ac = Acetyl.

Performing the reaction in 0.1 M HCl did also improve the rate of allylation of oxidized GGM; some reaction could be observed already at room temperature (III). After 24 h the DO had dropped to 49%, corresponding to a conversion of 38% of the available aldehyde groups. The decrease in pH causes some dehydration of the aldehyde. Although part of the decrease of DO in the reaction of oxidized GGM and allyl bromide in 0.1 M HCl can be assigned to the dehydration of aldehydes, the existence of the desired allylated galactose units could be verified by MALDI-MS and GC-MS analysis (III). Besides, according to the pre-screenings done for allylation of the oxidized Gal monomer **2** (Scheme 5.8), the dehydrated form **3** also reacts (III). Although that reaction was not investigated in detail, the reactivity of **3** indicates that dehydration does not necessarily reduce the amount of allylic moieties introduced to the polysaccharides.

Both cinnamyl chloride and propargyl bromide reacted with oxidized GGM at 55 °C (Figures 5.12 and 5.13) (III). The reaction with propargyl bromide was slightly more effective than the one with cinnamyl chloride. In the case of propargylation, 75% of the aldehydes had reacted, and for cinnamylation about 56% had reacted after 24 h (Table 5.2).

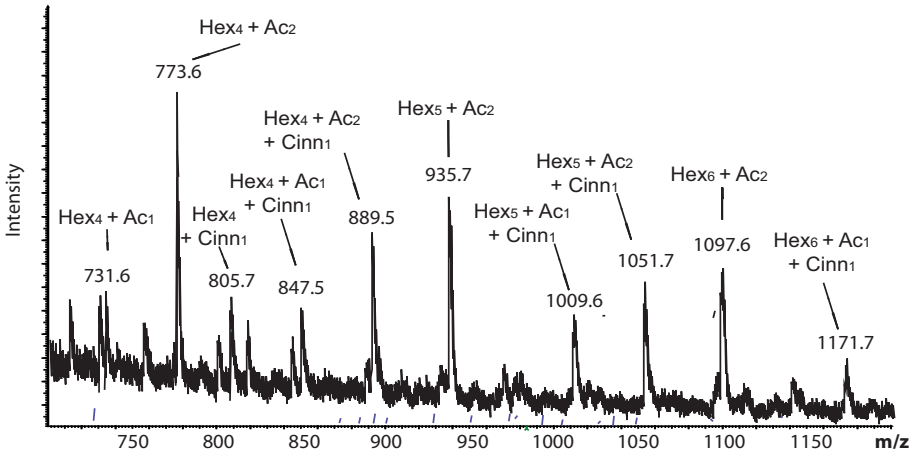


Figure 5.13 MALDI-MS spectra of GGM oligosaccharides produced by mannanase hydrolysis cinnamylated GGM in Na^+ adduct form (III). Hex = Anhydrohexose; Ac = Acetyl.

Allylation and propargylation of GM and XG (III)

To extend the usability of the indium mediated reaction also to other galactose-containing polysaccharides, GM_{-CHO} and XG_{-CHO} were allylated as well (III). All reactions were done in plain water and required 55°C. XG_{-CHO} reacted readily with allyl bromide, similarly to GGM_{-CHO} (Table 5.1). Following enzymatic digestion, mono-allylated derivatives of the $Glc_4Xyl_3Gal_1$ XGOs (m/z = 1287.6, $[M+Na^+]$; m/z = 1303.7, $[M+K^+]$) and only di-allylated derivatives of the $Glc_4Xyl_3Gal_2$ XGO (m/z = 1489.8, $[M+Na^+]$; m/z = 1505.8, $[M+K^+]$) were observed in the MALDI analysis. Oligomers containing the aldehyde group, $Glc_4Xyl_3Gal_1$ XGOs (m/z = 1245.8, $[M+Na^+]$) and $Glc_4Xyl_3Gal_2$ XGO (m/z = 1405.9, $[M+Na^+]$), disappeared, while, the non-galactose Glc4Xyl3 XGO (m/z = 1085.7, $[M+Na^+]$) remained as a reference. The reaction was complete within 24 h and almost no starting material was left in the solution (Figure 5.14). The ratio between Gal and Xyl in oxidized and allylated XG was determined by GC after reduction of the aldehydes. A decrease in this ratio during reactions was assumed to occur because of allylation of the Gal units. The Gal:Xyl ratio of the starting material XG_{-CHO} was 0.44. After allylation with allyl bromide, the ratio decreased to 0.06.

GM_{-CHO} formed a thick gel (Parikka et al. 2010), so the allylation reactions had to be performed in much more dilute solutions than allylation of the other polysaccharides (III). The lower concentration of aldehydes in the reaction solution might affect the outcome of the allylation; the reactivity of the aldehydes seemed to be remarkably lower than that of GGM_{-CHO} or XG_{-CHO}. The reaction was carried out on oxidized GMs with degrees of oxidation of 84% ($GM_{-CHO(high\ DO)}$) and 40% ($GM_{-CHO(low\ DO)}$). In both cases, the results were similar, and around 50% of the aldehydes reacted. Still, because of the high galactose content in GM, $GM_{-CHO(high\ DO)}$ produced the polysaccharide containing the largest amount of allyl groups.

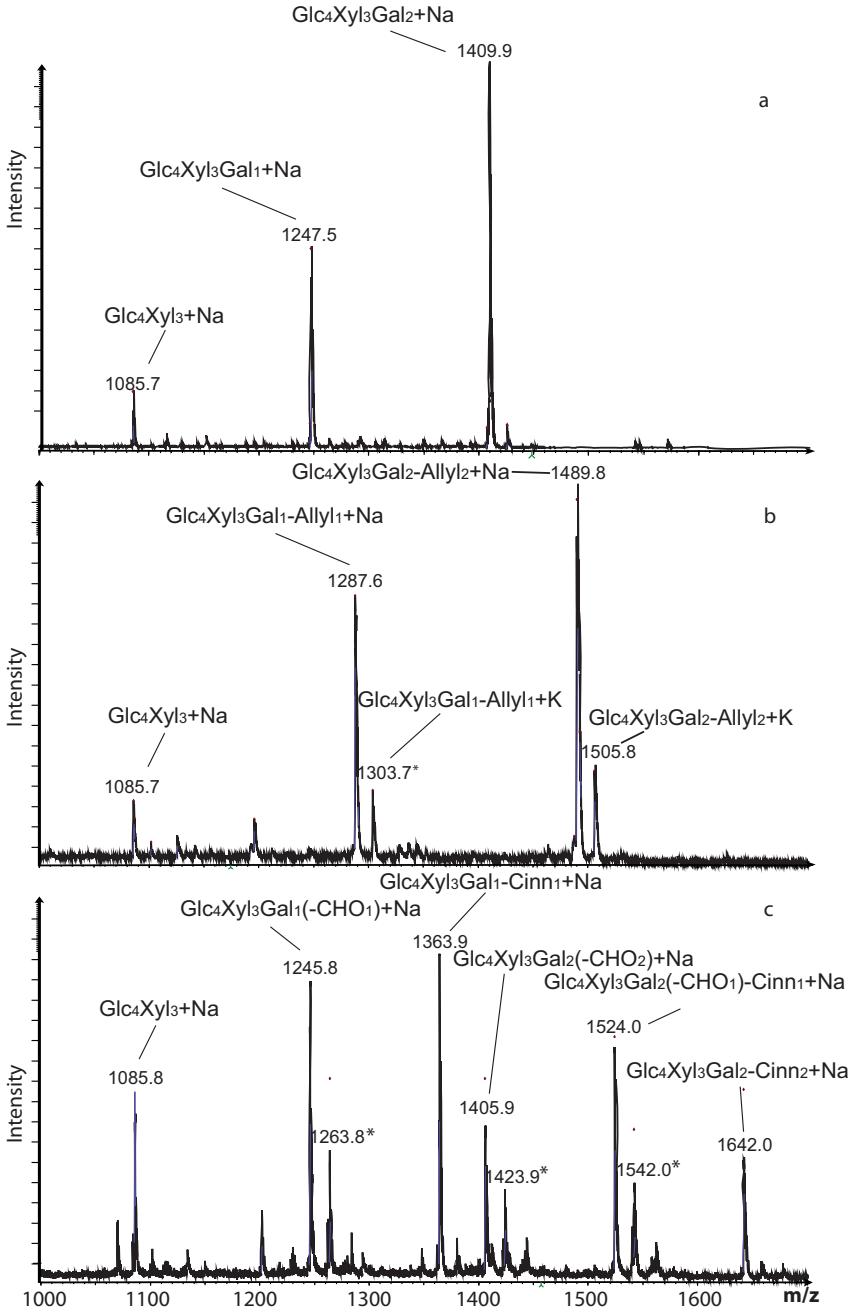


Figure 5.14. MALDI-MS spectra of XG derivatives. XG oligosaccharides produced by EGase hydrolysis of (a) native XG, (b) allylated XG, and (c) cinnamylated XG in Na^+ or K^+ adduct form (III). Glc = Glucose; Xyl = Xylose; Gal = Galactose.

$XG_{\text{-CHO}}$ reacted with cinnamyl chloride, but no reaction could be observed between $XG_{\text{-CHO}}$ and propargyl bromide (III) (Table 5.1). The reaction between $XG_{\text{-CHO}}$ and cinnamyl chloride led to a Gal:Xyl ratio of 0.24, corresponding to decrease of approx 45% of the amount of Gal units in the starting material. The reaction was verified by NMR analysis (Figure 5.15). Quantification by comparing the anomeric signals to the aromatic ones in ^{13}C NMR gave a DS of 0.03. Following enzymatic digestion, mono-cinnamylated derivatives of the $Glc_4Xyl_3Gal_1$ XGOs (m/z = 1363.9, $[M+Na^+]$), and both the mono- (m/z = 1524.0, $[M+Na^+]$) and di-cinnamylated derivatives of the $Glc_4Xyl_3Gal_2$ XGO (m/z = 1642.0, [M+Na]) were observed (Figure 5.14).

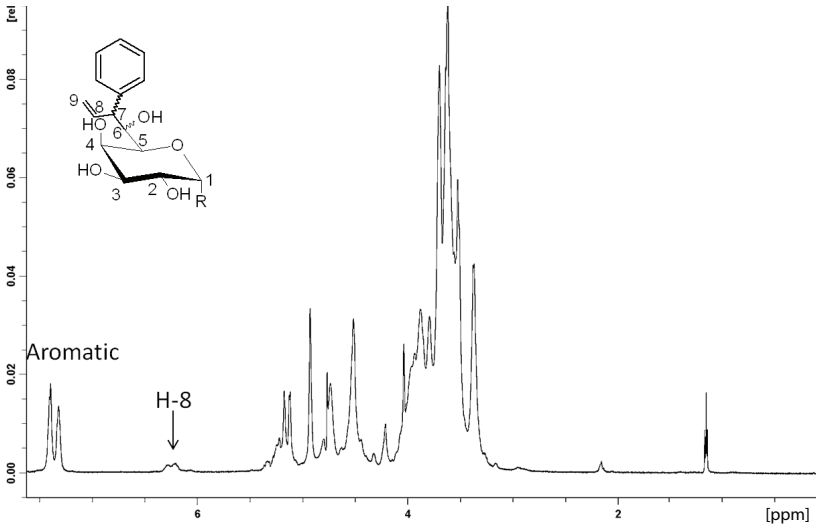


Figure 5.15 ^1H NMR spectrum of allylated XG.R = polysaccharide backbone.

The amount of galactose side groups affects the reactivity of the polysaccharide. GM, containing the largest amount of galactose, exhibited the lowest reactivity (III). The aldehyde and hydroxyl groups form hemiacetal, and in the case of oxidized GM this leads to the formation of a thick gel. The allyl halides, barely soluble in water, are prevented from penetrating into the polysaccharide network and are sterically hindered from reaching the free aldehydes. Only allyl bromide reacted with oxidized GM, whereas the other, even less soluble allyl halides showed no reactivity at all.

5.3.3 Conclusions

Enzymatically oxidized methyl α-D-galactopyranoside, raffinose, and the galactose-containing polysaccharides spruce galactoglucomannan, guar galactomannan, and tamarind xyloglucan can be chemically modified by an indium-mediated allylation reaction. The same reaction procedure can also be applied for spruce galactoglucomannan when using propargyl bromide as halide. All reactions were carried out by using water as the only solvent, thus the carbohydrates are functionalized in a one-pot reaction.

5.4 Sorption of modified polysaccharides to cellulose (I, IV)

5.4.1 Sorption of modified GGM onto chemical pulp

It is known that unmodified GGM has a high affinity towards cellulose, and that it sorbs well onto chemical pulps (Hannuksela et al. 2002 and 2003). Modifications, such as introduction of derivatives or altering the amount of side groups, will however affect the sorption tendency.

Unoxidized GGM in pure water were sorbed to bleached kraft pulp (BKP) in a high degree, the sorption being 65-80%, depending on the source of and the method of isolation of GGM. The highest sorption was obtained for GGM isolated by a large-scale laboratory method from TMP, whereas the sorption of GGM isolated from TMP process waters only reached a sorption of ~65%. The GGMs differ somewhat in chemical composition (sugar composition, degree of acetylation), M_w, and purity (Xu 2008). The sorption is influenced by the degree of acetylation, and the amount of impurities, such as lignin residues, both inhibiting the formation of hydrogen bonding between the GGM chain and the fibre surface. Carboxylic acids also affect the sorption; already a slight increase of the amount anionic groups changed the affinity; when GGM_{PolyU} with DO 6% was used, only approx. 30% sorption in electrolyte free conditions was obtained. For fully oxidized GGM_{PolyU} (DO 80%) the sorption was noticeably lower (Figure 5.16) (I, IV).

The effect of the ionic strength of the sorption media was investigated by performing experiments in NaCl (0.01 M and 0.1 M) solutions (I). As expected, the affinity of GGM_{PolyU} to fibres was enhanced with increasing ionic strength (first two bars in Figure 5.16). The sorption of low DO

GGM_{PolyU} (DO = 6%) was approx. 40% in 0.01 M NaCl (data not shown), and 55% in 0.1 M, compared to ~30% in water. The sorption remained equally high even when the DO increased from 6% to 10% (data not shown). The effect of the ionic strength was more noticeable when sorbing high DO GGM_{PolyU} (DO = 80%) to BKP; the sorption increased from 15% in water to approx. 50% in NaCl (0.1 M). The higher electrolyte concentration decreases the range of double-layer repulsion between the anionic polysaccharides and slightly anionic cellulose surface thus facilitating closer contact and van der Waals attraction. But more importantly, at high ionic strength anionic polysaccharides become more coiled, and more polysaccharide can fit to the surface (Tammelin et al. 2009).

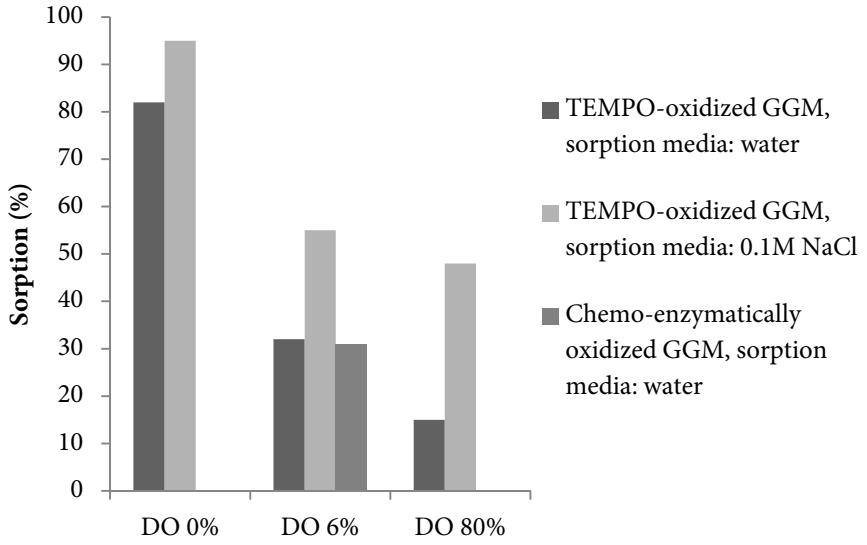


Figure 5.16. Sorption of GGMs with different degrees of oxidation onto chemical pulp. The sorption media was water or NaCl (0.1M). The oxidations were performed chemically using TEMPO or chemo-enzymatically using GO combined with I_2/KI. STDEV < 2% for all samples. (I, IV)

Charged groups located on the backbone of oxidized GGM could be assumed to interfere more with the interaction between the backbone of the polysaccharide and the cellulose chain than charged groups located only on the Gal side-group, thus decreasing the sorption. To investigate the role of the location of charge, the sorption of polysaccharides oxidized by two different methods were compared; TEMPO-mediated oxidation, where carboxylic

acids were formed on any primary alcohol, and GO-oxidation followed by I_2/KI oxidation of the aldehydes, where carboxylic acids were introduced only on the galactose side-groups (chemo-enzymatic oxidation) (I, IV). The maximum DO of GGM_{-COOH}, obtained by selective chemo-enzymatic carboxylation of the Gal groups, is 6% and the results were compared to the sorption of TEMPO-oxidized GGM_{PolyU} with a DO of 6%. For these low DO GGMs, the location of the charge did not seem affect the sorption (Figure 5.16, first and last bars of DO 6%). The amount of carboxylic acid groups was probably so low, that even if some of them are located on the back-bone, enough of carboxylic acid free segments still remained in the chain so that attachment could occur.

5.4.2 Sorption of galactose-containing polysaccharides onto chemical pulp (IV)

The bulk sorption of low-viscosity samples of GM, and XG was also investigated. Enzymatically hydrolyzed samples, GM50 (M_w ~50 kDa), and XG17 (M_w ~17 kDa), were prepared (IV). XG is known to have naturally a high affinity toward cellulose surfaces (Vincken et al. 1995). Indeed, almost quantitative sorption of hydrolyzed XG was obtained even in plain water. Similarly to GGM, the introduction of anionic groups disturbed the interaction with fibres, decreasing the sorption with increasing DO (Figure 5.17). However, because of the high amount of Xyl, which is not oxidized by TEMPO, in XG, only a slightly higher DO is obtained by TEMPO-mediated oxidation than by the chemo-enzymatic procedure. The sorption of XG is thus less affected than the sorption of GGM by the TEMPO-treatment.

The selective chemo-enzymatic oxidation affected the sorption of GM more than the sorption of GGM. GM contains more Gal side-groups, thus even if only Gal groups are oxidized, a DO high enough to inhibit attachment, and no sorption was obtained (Figure 5.17) (IV).

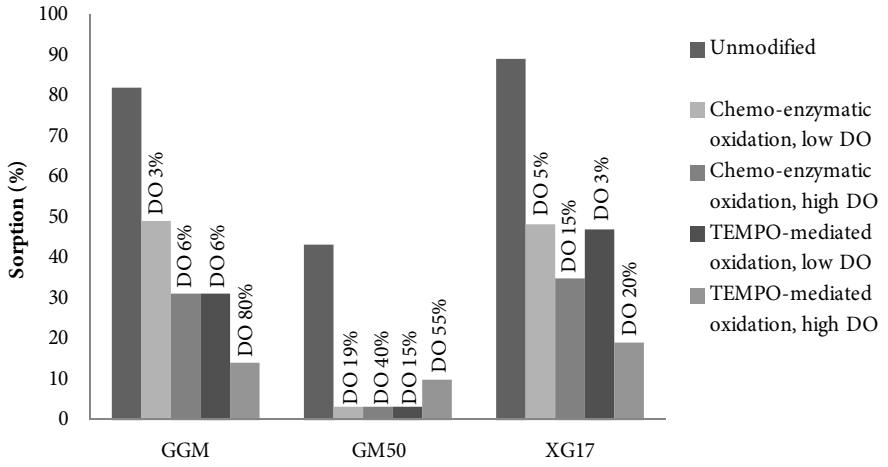


Figure 5.17. Sorption of GGM, and enzymatically hydrolyzed GM (GM50), and XG (XG17) onto chemical pulp. The polysaccharides were oxidized chemically using TEMPO or chemo-enzymatically using GO combined with I_2/KI (chemo-enzymatic). STDEV < 5% for all samples. (I, IV)

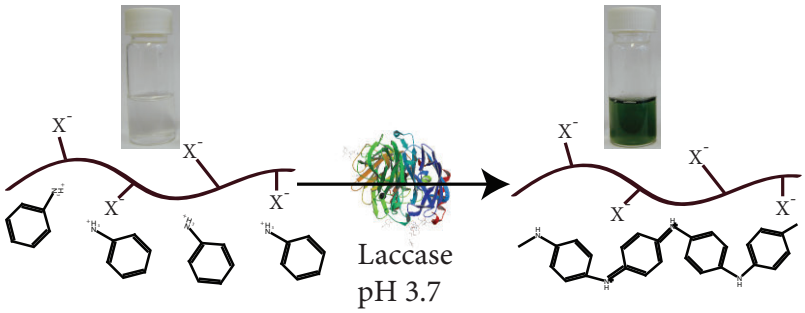
When the DO of GGM_{PolyU} reached 25%, and the DO of GM_{PolyU} 15%, no sorption in pure water could be observed (I, IV). Interestingly, some affinity to cellulose seemed to reoccur when the maximum DO of these polysaccharides was reached (80% and 55%, respectively) (IV). In both cases a sorption of approx. 10 % was measured (Figure 5.17). A possible explanation could be the removal of some of the (1→6)-bonded Gal side-chains caused by the TEMPO-treatment. A decrease in the amount of Gal side-groups increases sorption (Hannuksela et al. 2002). The sorption of GGM_{PolyU} could also be affected by the degree of acetylation. As was previously mentioned, TEMPO-treatment causes deacetylation of GGM_{PolyU}, and the degree of deacetylation increases with increasing treatment time. It is known that removal of acetyl groups lowers the water solubility (Xu et al. 2009), and a lower solubility would enhance sorption. Furthermore, when the molecule is deacetylated more hydroxyl-groups become available for hydrogen bonding.

5.4.3 Conclusions

The introduction of charges in the hemicelluloses affects their affinity to cellulose significantly. A higher DO leads to a lower sorption. By using ionic sorption media, charges on the polyuronic chain can be neutralized, changing the conformation of the polysaccharide and enabling more attachment to the cellulose surface.

5.5 Novel application area for anionic polysaccharides: templates for enzymatic polymerization of aniline (V)

The possibility to use anionic polysaccharides as templates during laccase-catalyzed polymerization of aniline was investigated (Scheme 5.3) (V). Firstly, polyuronic acids of GGM, and GM were formed by TEMPO-mediated oxidation (I). TEMPO-mediated oxidation was also applied to cellulose fibres, producing nanofibrillated cellulose (NFC), simultaneously introducing carboxylic acids to the fibre surfaces. In addition, the template properties of unoxidized GGM, and naturally anionic κ-CGN were also assessed.



Scheme 5.3. Template assisted enzymatic polymerization of aniline (V). X^- = anionic group.

The reaction parameters were adjusted according to the templates. The amount of aniline used in the reactions was related to the amount of anionic groups in the polysaccharide templates, and the ratio of anionic group to aniline was varied from 2:1 to 1:20. The DO of fully oxidized GGM_{PolyU} was 80%, corresponding to a carboxylic acid content of approx.4.5 mmol/g (I, IV). The amount of charged groups in GM_{PolyU} was approx 3.5 mmol/g, in NFC 0.8 mmol/g, and in κ-CGN 2.6 mmol/g.

The polymerization needs to be performed at a pH that is low enough for aniline to be protonated, but high enough for the polyelectrolyte to still be dissociated (Liu et al. 1999b). Moreover, the pH of the reaction should also be high enough not to deactivate the enzyme. The pK_a values of the GGM_{PolyU}, GM_{PolyU}, and NFC are lower than 3.7, and the pK_a of κ-CGN is ~2. Thus the reaction pH was adjusted to 3.7. Most importantly, the polyelectrolytes used are still dissociated to a large extent at this pH, but it is also the optimal pH for aniline polymerization using the laccase from *Trametes hirsuta* (Karamyshev et al. 2003). The laccase from *Trametes versicolor* (*T. versicolor*), has a high redox potential and catalytic activity high catalytic activity in the pH range 2.5-4 (Reinhammar 1972), and was therefore chosen for these experiments.

When polymerizations were done using GGM, GGM_{PolyU}, or GM_{PolyU} as template, green color, indicating the formation of the conducting emeraldine salt of PANI, was observed within 90 min upon addition of the enzyme (V). Polymerizations using NFC or κ-CGN proceeded much slower. During the first 5 hours of the κ-CGN reaction, no color change was visible, nor could anything be seen on UV-Vis. Still, when stirred over night all reactions, except the ones with GGM or GM_{PolyU}, yielded dark green products (Scheme 5.3). Although the GGM and GM_{PolyU} reactions in the beginning showed light green color, a brown precipitation was formed with time.
The concentration of anionic groups and reagents, and the viscosity of the reaction solution seemed to be the causes for differences in the polymerization rates. In the NFC suspension the concentration of anionic groups, and thus also aniline, was remarkably lower than that of the GGM_{PolyU} solution. The κ-CGN solution has higher viscosity than the GGM_{PolyU} solution, thus oxygen, that is the oxidizing agent used by laccase, was not as readily dissolved, and the polymerization rate decreased.

5.5.1 Chemical characterization of PANI

The formation of the conducting form of polyaniline (PANI) was seen in the UV-Vis spectra (Figure 5.18A). When polymerization was performed using κ-GCN, NFC, or GGM_{PolyU} as template, a broad absorption band between 700-800 nm and a shoulder at 400-420 nm slowly appeared (V). These absorption bands can be assigned to the polaron of conducting PANI (Shumakovich et al. 2010). As expected, unmodified GGM, containing very few charged groups, was a poor template. Absorption between 600-700 nm

with a maximum at approx. 650 nm, and a shoulder at 550 nm could be seen. The absorption between 400-600 nm is associated with the formation of branched, low-molar-mass PANI (Liu et al. 1999b). Also, the brown precipitation has previously been assigned as non-conducting branched PANI (Samuelson et al. 1998). The formation of branched PANI was also observed when using GM_{PolyU} as template. Even if it contains fairly high amount of carboxylic acids, the highly branched structure of GM_{PolyU} seems to hinder the formation of linear PANI.

When the pH of the water solution of κ-CGN/PANI complexes was increased from 3 to 10, the color gradually changed from green to blue (Figure 5.18B, insert), indicating the switch from emeraldine salt to base. In UV-Vis the polaron band around 750 nm started to disappear and another, originating from the quinoid of the emeraldine base, appeared around 550 nm (Figure 5.18B). The polysaccharide/PANI complexes could be reactivated by lowering the pH value to acidic medium.

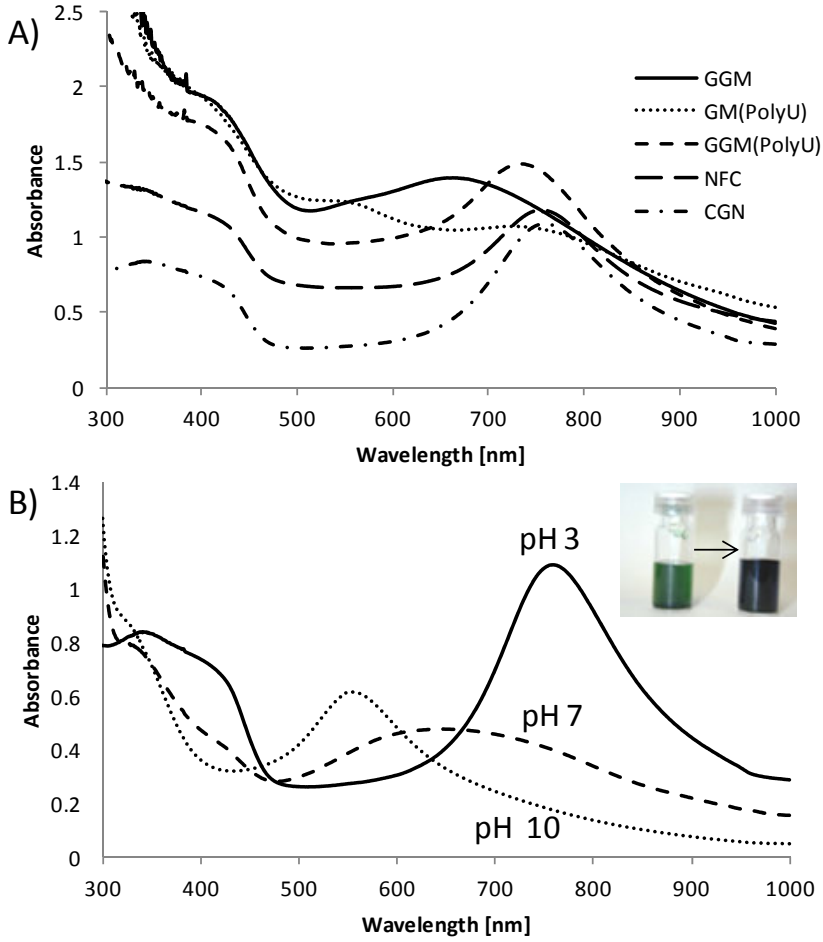


Figure 5.18. UV-Vis of PANI (A) polymerized using different polysaccharide templates and (B) PANI/κ-CGN at different pH values. Insert in (B): water solutions of polysaccharide/PANI at pH 3 (left) and 10 (right) (V).

In the FTIR spectra, the carbonyl stretching of the –COOH groups of NFC gave a band at 1729 cm^{-1} (Figure 5.19) (V). After polymerization, this band disappeared. The shoulder at 1482 cm^{-1} and the band at 1562 cm^{-1} are due to benzenoid ring and quinoid ring deformations. The successful head-to-tail coupling needed for the linear polymeric chain of PANI, was proven by the band at 880 cm^{-1} and the shoulder at 799 cm^{-1} (Tang et al. 1988; Liu et al 1999a).

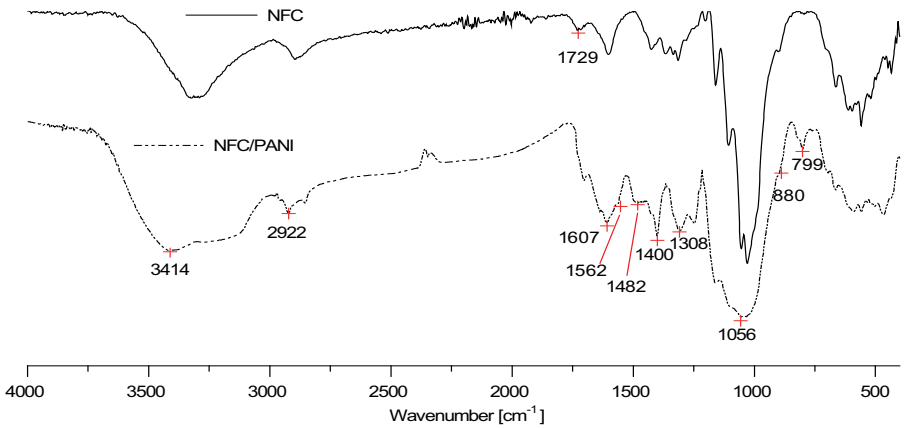


Figure 5.19. FTIR spectra of NFC and NFC/PANI biocomposites (V).

The amount of PANI in the NFC/PANI composites was calculated using thermogravimetric analyses (TGA) (V). Based on the differences in weight loss at 200-320°C between the NFC/PANI composites and the pure NFC film the amount of PANI was determined. TGA showed that the NFC/PANI composites had lower thermal stability than pure NFC below 320°C. The PANI content was approx. 4-5%. Even if aniline was used in the polymerization reactions readily in excess (up to 20 times the amount of anionic groups in NFC) the content in the end product increased only slightly, indicating that part of the polymerization occurred in solution and some of the formed PANI segments were not assembled by electrostatic forces onto the surface of NFC. These segments were washed away during the dialysis.

SEM micrographs of filter paper (Figure 5.20A) presented a typical structure of cellulose constituted by a 3-D network of nano- to micro-fibrils (V). After coating with κ-CGN/PANI, a layer of clews was formed on the top and covered the network of fibrils (Figure 5.20B). Similar types of structures have been observed in studies on polyaniline coated bacterial cellulose and polyaniline as such (Chao et al. 2005; Marins et al. 2011). SEM micrographs of NFC and NFC/PANI films showed different surface morphologies. NFC fibers were tightly associated together (Figure 5.20C). In NFC/PANI biocomposites, the NFC formed a matrix of nanofibers which was coated by

polyaniline during synthesis. The fibers were clearly better distinguished from each other (Figure 5.20D), indicating that PANI had been formed on the surface of the fibers.

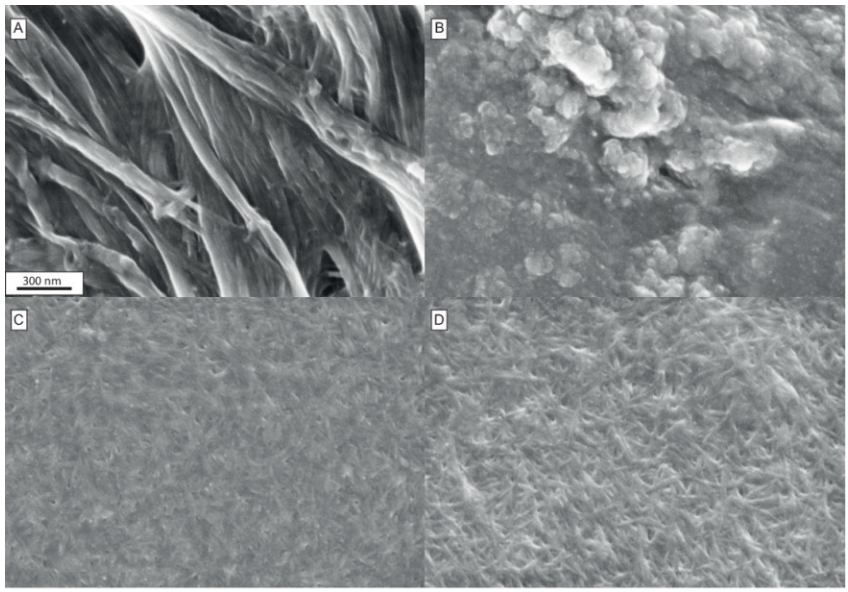


Figure 5.20. SEM images of (a) filter paper; (b) κ-CGN/PANI coated on filter paper; (c) NFC film; (d) NFC/PANI film (V).

5.5.2 Conductivity of PANI

The conductivity of the polysaccharide/PANI composites was measured by a four-point probing method. NFC/PANI biocomposites were measured as separate bulky films; GGM_{PolyU}/PANI and κ-CGN/PANI were deposited on filter paper for support (V). The highest conductivity of the polysaccharide/PANI compounds investigated was 5 mS/cm and was measured for the κ-CGN/PANI prepared by using a sulfate-aniline ratio of 1:2 (Table 5.3). When the ratio was 1:1, the conductivity dropped clearly; the amount of aniline was not sufficient to cover the κ-CGN chains uniformly. The highest values obtained for GGM_{PolyU}/PANI was measured for the composite prepared using aniline and anionic groups in 1:1 ratio, $1.4x10^{-7}$ S/cm.

Since NFC contains a lower molar amount of anionic groups/g than κ-CGN or GGM_{PolyU}, it required a higher molar ratio of aniline to reach its maximum

conductivity. Dosage of a –COOH-aniline ratio of 1:1 was clearly not enough. The highest conductivity of NFC/PANI biocomposites, $9x10^{-7}$ S/cm, was obtained at a ratio of 1:10. According to the TGA analysis, this corresponded to a PANI content of approx. 4.3% (V). When the ratio used was increased to 1:20, the PANI content increased somewhat (to approx. 4.8%) but the slight decrease in conductivity indicated that the amount and strength of the anionic groups were not high enough to facilitate the appropriate monomer alignment, and more nonconductive and branched PANI was formed. Although lower than the conductivity of κ-CGN/PANI, NFC/PANI biocomposites yielded conductivities higher than pure NFC ($3x10^{-8}$ S/cm), and are suitable for, e.g., electrical stimulation in vivo (Guo et al. 2011).

Table 5.3 Conductivities for PANI synthesized using polysaccharides templates and different anionic group — aniline ratios.

Template	Anionic group : Aniline[a]	Conductivity [S/cm]
BKP	- [b]	-
NFC	0	$3x10^{-8}$
NFC	1:10	$9x10^{-7}$
GGM_{PolyU}	1:1	$1x10^{-7}$
GGM_{PolyU}	2:1	$7x10^{-8}$
κ-CGN	2:1	$5x10^{-3}$
κ-CGN	1:1	$6x10^{-7}$

[a] Molar ratio

[b] The amount added aniline was 0.8 g/g pulp

The importance of the charged groups on the template properties of polysaccharides was also verified when the enzymatic polymerization was done directly on unmodified bleached kraft pulp (BKP). The amount of anionic groups in BKP is less than 70 μmol/g (Fardim et al. 2002). During the polymerization, the color of BKP/PANI became brownish and no conductivity could be detected on final product.

Of the two linear and high molar mass templates investigated (NFC and κ-CGN), κ-CGN, containing the anionic groups with the lowest pK_a value, showed superior template properties. The charge density was also remarkably higher in κ-CGN than in NFC; in κ-CGN every second sugar unit contain a sulfate group, whereas in NFC only approx. 1/8 glucose units were carboxylated. Thus, increasing the amount of carboxylic acids or introducing groups with lower pK_a values, could enhance the template properties of NFC.

5.5.4 Potential processing means

The good water solubility of the polyaniline-based biocomposites prepared using polysaccharides as templates offers a variety of facile processing means for further application. κ-CGN/PANI was micropatterned on office copy paper by mask-spray coating using normal ELISA microarray plates as template (Figure 5.21a) (V). In addition, an even layer of κ-CGN/PANI was also obtained by deposition on filter paper. As NFC has high mechanical properties and has been widely used in biocomposites, NFC/PANI was directly processed to film (Figure 5.21b). NFC itself formed a transparent film with high strength. GGM_{PolyU}/PANI and κ-CGN/PANI also formed films by casting, although the mechanical properties of these films were not as good as that of NFC/PANI films.

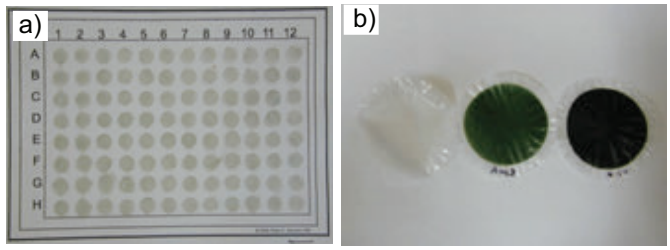


Figure 5.21. a). A dot array of κ-CGN/PANI mask-spray coated on office copy paper with dot diameter about 7 mm; b) Films of NFC; NFC/PANI (-COOH-aniline 1:2); NFC/PANI (-COOH-aniline 1:10) (from left to right) (V).

5.5.5 Conclusions

Polysaccharides, abundant and naturally occurring materials, can function as templates for enzymatic polymerization of conducting polyaniline. The conductivity of the enzymatically synthesized polysaccharide/PANI biocomposites varies depending on the polysaccharide templates. The polysaccharide/PANI biocomposites have illustrated various possibilities of facile processing means. The resulted conducting biocomposites provide a broad range of potential applications in biosensors, and tissue engineering. This approach also opens a new area of application of polysaccharides.

6. Summary

In order to broaden the scope of application for hemicelluloses in general and especially for *O*-acetyl galactoglucomannans (GGM) from wood, new modification methods are needed.

Regioselective modifications of GGM and other galactose-containing polysaccharides can be done by combining regioselective oxidations with subsequent reactions of the formed carbonyl or carboxyl groups. In this thesis two different pathways were investigated: activation of the C-6 positions in different sugar units by TEMPO-mediated oxidation, and activation of the C-6 position in only Gal-units by GO-mediated oxidation. The activated sites were then further selectively derivatized. The reactions were done in aqueous environment, even with water as the only solvent, thus avoiding time consuming drying and re-dissolution steps.

TEMPO-mediated oxidation of GGM is an efficient method for converting C-6 hydroxyl groups to carboxyl groups; fully oxidized GGM (DO ~80%) can be obtained within minutes upon addition of the reagents. By performing the oxidations in NaBr-free conditions, the reaction is more easily controlled, and polyuronic acids with varying degrees of oxidation (6%-75%) can be obtained. The products remain readily water-soluble, thus enabling further modifications in aqueous environment. The carboxyl groups of oxidized GGM can be further functionalized by a carbodiimide-mediated amidation reaction using EDC and NHS as coupling reagents. In order to minimize the formation of the unwanted side-product *N*-acylurea, thus maximizing the conversion of the reaction, a careful control of the reaction parameters needs to be done for the each different reagent used.

Aldehydes formed through GO-mediated oxidation can be chemically modified by an indium-mediated allylation reaction. A new carbon-carbon bond is formed, and since the reaction can be done using both allyl and propargyl halides, it is possible to introduce double or triple bonds selectively to the Gal-units of GGM. The allylation reaction is also applicable on other Gal-containing polysaccharides, i.e., guar galactomannan, and tamarind xyloglucan. Because of the heterogeneity of the GGM material used, characterization of the allylation product by NMR was challenging. Instead

MALDI-TOF-MS proved to be useful tool; after enzymatic digestion of the modified polysaccharide, it can be used for proving the formations of the allylation products of GGM.

A potential application field for modified polysaccharides is their use for cellulose surface functionalization through sorption. The introduction of substituents to GGM decreases the sorption, but by doing controlled modifications, some affinity to cellulose can still be maintained. The introduction of carboxylic acids to polysaccharides affects their affinity to cellulose significantly. High DO leads to almost no sorption in pure water, but by careful control of the reaction, degrees of oxidation suitable for sorption can be produced. By using ionic sorption media also high DO polysaccharides are sorbed onto cellulose.

Anionic polysaccharides can be used as templates during laccase-catalyzed polymerization of aniline, offering a green route for synthesis of conducting polyaniline (PANI). Different polysaccharide templates, such as, GGM, TEMPO-oxidized GGM, naturally anionic κ-carrageenan, and nanofibrillated cellulose produced by TEMPO-mediated oxidation, were assessed. The conductivity of the synthesized polysaccharide/PANI biocomposites varies depending on the polysaccharide templates; κ-CGN, containing the anionic groups with the lowest pK_a value, produced the polysaccharide/PANI biocomposites with the highest conductivity (5 mS/cm).

The presented derivatization, sorption, and polymerization procedures open new application platforms for non-cellulosic wood polysaccharides, such as spruce GGM. The modified polysaccharides and the conducting biocomposites produced provide potential applications in a variety of products applications such as biosensors, electronic devices, and specifically engineered tissue.

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Affi

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