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SUMMARY

Polysaccharides are important biopolymer materials, which have vital functions in almost all living systems. They serve as energy storage, play active parts in cell signalling processes and/or offer mechanical structure to cell walls. Today, we also rely on polysaccharides in many commercial products, often to give them a specific texture.

A carbonyl group or more precisely an aldehyde (predominantly), is present in all polysaccharides masked as a hemiacetal at its reducing end. In some cases additional carbonyls may also be introduced. Such carbonyls (aldehydes or ketones) can be formed naturally by oxidation in the presence of oxygen and light, or be introduced as a result of chemical processing deliberately or un-deliberately. The chemical stability and structural properties are affected by the introduction of carbonyl groups. Several methods exist for their detection, but the majority of these have a low detection limit and/or provide only a measure of the average carbonyl content. Because of this, these methods offer limited information for polydisperse polysaccharide samples containing small amounts of carbonyl functionalities.

This thesis deals with the detection of carbonyl group profiles within polysaccharides, and the use of periodate oxidation to introduce carbonyl groups into different alginate materials, resulting in altered chemical and physical properties.

In the first part of the study, the carbonyl structure and content in the pectic polysaccharide sphagnan was re-investigated. Previous literature describes the presence of a novel monosaccharide named 5-keto-D-mannuronic acid (5-KMA) in sphagnan, and points it out as the main reason for the preservation of organic material in peat bogs. Through the work of this thesis and other recent publications it is shown that the carbonyl content in sphagnan is a tenfold lower than previously published, and that 5-KMA do not exist in sphagnan. The latter was demonstrated by reacting sphagnan with different amino compounds. A flaw in the original colorimetric assay for detecting 5-KMA was also revealed. The tanning ability of sphagnan was tested, but it was found that it did not meet the demands to be classified as a true ‘tanning agent’. Although the carbonyl content was found to be lower than first anticipated, it was higher than in other polysaccharides, possessing only the reducing end carbonyl group. During this work it became clear that a better general method for carbonyl profile detection in polysaccharides dissolved in water was needed. Such a method was

therefore developed. The method involves labelling of the polysaccharide with either tritium or fluorescent labels. The labelling technique should be chosen depending on the structure of the polysaccharide. After labelling, the polysaccharide is analysed by size exclusion chromatography (SEC) with multiangular laser light scattering (MALLS) coupled with tritium or fluorescence detection.

The chain stiffness and extensions of various in vitro epimerized alginates, and periodate oxidized alginates, were studied using SEC-MALLS with on-line viscosity detection. The chain conformation and extensions were evaluated using the exponent in the Mark-Houwink-Sakurada (MHS) relationship and the persistence length (*q*) from Bohdanecký analysis. The study revealed no significant difference in chain stiffness and extension between alginates with varying content of mannuronic acid (M) and guluronic acid (G), while alginates subjected to partial periodate oxidation and reduction showed a pronounced difference. For instance, a reduction of 35% in the MHS exponent *(a*) was observed between an unoxidized and a 44 mol% oxidized sample.

In the last part of the study, periodate oxidized and borohydride reduced mannuronan were studied by NMR and the main peaks in the spectrum assigned. Later, oxidized/reduced mannuronan was used as a substrate for the C-5 epimerases AlgE4 or AlgE6. The fraction of G-residues (F_G) decreased as the fraction of oxidized residues (P_0) increased. The main reason for this was that the stretches of M-residues, which the enzyme could attach, were shortened. Finally, mannuronan was oxidized/reduced (P_0 = 0.02-0.08) before or after epimerization and studied by NMR, SEC-MALLS with on-line viscometry and small-strain oscillatory measurements. The results from the latter method showed that the dynamic storage modulus (G') decreased rapidly as P_0 increased. By comparing the oxidized/reduced alginates with alginate from *Laminaria hyperborea* (leaf and stipe) and *Durvillea antarctica* with similar molecular weight (MW) and average G-block length ($N_{G>1}$), it was concluded that the decrease in G' was mainly due to an increased flexibility of the polymer and not simply a reduction in MW and $N_{G>1}$.

LIST OF APPENDIX PAPERS

I. Kristiansen, K. A., Ballance, S., Potthast, A. & Christensen, B. E. (2009). An evaluation of tritium and fluorescence labelling combined with multi detector SEC for the detection of carbonyl groups in polysaccharides. *Carbohydrate Polymers*, 76, 196-205.

II. Kristiansen, K. A., Schirmer, B. C., Aachmann, F. L., Draget, K. I., Skjåk-Bræk, G. & Christensen, B. E. Novel alginates prepared by independent control of chain stiffness and distribution of G-residues: Structure and gelling properties. Accepted for publication in: *Carbohydrate Polymers.*

III. Ballance, S., Kristiansen, K. A., Holt, J., & Christensen, B. E. (2008). Interactions of polysaccharides extracted by mild acid hydrolysis from the leaves of *Sphagnum papillosum* with phenylhydrazine, *o*-phenylenediamine and its oxidation products, or collagen. *Carbohydrate Polymers*, 71, 550-558.

IV. Vold, I. M. N., Kristiansen, K. A. & Christensen, B. E. (2006). A study of the chain stiffness and extension of alginates, in vitro epimerized alginates, and periodate-oxidized alginates using size-exclusion chromatography combined with light scattering and viscosity detectors. *Biomacromolecules*, 7, 2136-2146.

ABBREVIATIONS AND SYMBOLS

2-AA	-	2-aminobenzoic acid
2-AB	-	2-aminobenzamide
CCOA	-	Carbazole-9-carboxylic acid [2-(2-aminooxyethoxy)ethoxy)]amide (Carbazole carbonyl oxyamine)
CDO	-	Calculated degree of oxidation
COSY	-	Homonuclear correlation spectroscopy
DMAc/LiCl	-	*N*,*N*-dimethylacetamide/LiCl
DP	-	Degree of polymerisation
DPM	-	Disintegrations per minute
DP_n	-	Number average degree of polymerisation
F_G/F_M	-	Fraction guluronan/mannuronan residues
G’	-	Dynamic storage modulus
G’’	-	Dynamic loss modulus
GDL	-	D-glucono-δ-lactone
G	-	Guluronic acid
HSQC	-	Heteronuclear single quantum coherence
M	-	Mannuronic acid
MHS	-	Mark-Houwink-Sakurada (relationship)
MW	-	Molecular weight
M_n	-	Number average molecular weight
M_w	-	Weight average molecular weight
$N_{G>1}$	-	Average G-block length (larger than one guluronan residue)
NMR	-	Nuclear magnetic resonance
NOESY	-	Nuclear Overhauser effect spectroscopy
P_0	-	Fraction of oxidized units within the polymer
P_t	-	Theoretical fraction of oxidized units within the polymer
ROESY	-	Rotational frame nuclear Overhauser effect spectroscopy
RT	-	Room temperature
SMV	-	SEC combined with MALLS and viscosity detection

Notation used in ^{1}H-NMR spectra of alginate

The notation used in ^{1}H-NMR spectra of alginate follows the coding system introduced by Grasdalen (1983). Some examples are:

G-1: denotes the peak arising from the proton linked to the C1 carbon on a guluronic acid residue (G).

MG-5M: denotes the peak arising from the proton linked to the C5 carbon on a guluronic acid (G) residue having a neighbouring mannuronic acid residue (M) on each side in the chain.

In some cases the subtext *red* is used meaning the reducing end, or *nonred* meaning the non reducing end residue.

Special notation used:

M-1O_M	-	the peak arising from the proton linked to the C1 carbon on an M-residue having an oxidized/reduced M-unit as its neighbour in the chain.
O_M-1M	-	the peak arising from the proton linked to the C1 carbon on an oxidized/reduced M-unit having an M-residue as its neighbour in the chain.
G-5O_G	-	the peak arising from the proton linked to the C5 carbon on a G-residue having an oxidized/reduced G-unit as its neighbour in the chain.

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1. INTRODUCTION

This thesis deals with the part of biotechnology known as biopolymer chemistry. Biopolymers in nature, fronted by proteins and polysaccharides, are pinnacles of function guided design. The reasons for studying biopolymers are numerous, and in the years to come they are predicted to have a large impact on the biotechnology sector in the form of biosensors and new modified materials with enhanced features over those obtained directly from nature (Handel et al., 2001). In this thesis, biopolymers in the form of polysaccharides are studied with or without chemical modification.

1.1 Polysaccharides

Polysaccharides (glycans) are the most abundant biopolymers found on our planet serving as energy storage, structural scaffolds and protective shells. They also represent diverse epitopes for molecular recognition between cells (Handel et al., 2001). The building blocks of polysaccharides are monosaccharides, such as aldoses and ketoses and a wide variety of derivatives. Derivation includes oxidation, deoxygenation, introduction of other substituents, alkylation and acylation of hydroxy groups, and chain branching (www.iupac.org). The polysaccharides consist of ten to hundreds of monosaccharides joined together through glycosidic linkages and the overall structure may be linear, branched, or even cyclic. The biomedical importance of this class of molecules has directed significant attention to their synthesis and structural analysis (Handel et al., 2001).

1.2 Carbonyl groups in polysaccharides

A carbonyl group, or more specifically an aldehyde (predominantly), is part of every polysaccharide structure and known as the reducing end. The aldehyde is usually hydrated or masked as a hemiacetal. In some cases carbonyl groups can be introduced into the polysaccharide after its biosynthesis. This can be a result of a chemical reaction in nature or chemical/physical processing in the laboratory. In the latter instance, these functional groups may occur deliberately or un-deliberately.

In nature the introduction of carbonyl functionalities into polysaccharides is usually a result of a number of auto-oxidative processes. The oxidation is catalysed by light and free oxygen, and takes place via a free radical mechanism (Potthast et. al., 2006). Natural oxidation is of

great importance for cellulose materials and has been studied in great detail for this polysaccharide. In cellulose, hydroperoxyl radicals react under H-atom abstraction, which finally leads to the formation of carbonyl or carboxyl groups. Several metal ions can trigger oxidation, and chromophores can serve as activators along with an increase in temperature (Potthast et. al., 2006).

As mentioned, carbonyl groups can also be introduced by various chemical methods mostly through bleaching and oxidative processes. Hypochlorite bleaching is known to introduce carbonyl groups in polysaccharides when preformed in the pH range 2-8. Another example is acidic sulphite pulping of cellulose. Carbonyls may be introduced specifically by for instance periodate oxidation or 2,2,6,6-tetramethylpiperidine-1-oxy radial (TEMPO)-mediated oxidation. The latter is often simply referred to as TEMPO oxidation. In this procedure, a mixture of NaBr, NaClO and TEMPO (catalyst) is used (Potthast et al., 2006; Saito et al., 2006). Periodate oxidation splits α-glycol groups resulting in two aldehyde groups, while TEMPO oxidation introduces one carbonyl group at the C6 position in the monosaccharide unit (Potthast et al., 2006). Periodate oxidation is utilized to a great extent in this thesis and is more closely described in the section below.

A physical method known to introduce carbonyl groups is high energy radiation, for instance γ-irradiation or β-irradiation, but this is not a method that is often used since polysaccharides exposed to this type of radiation tend to depolymerise (Potthast et. al., 2006).

1.2.1 Aldehyde structures introduced by periodate oxidation

Periodate oxidation was discovered by Malaprade in 1928. The periodate ion can split α-glycols, which results in aldehyde groups (Guthrie, 1961). When splitting α-glycols it is generally considered that the cleavage reaction involves reversible formation of a cyclic complex or intermediate, via an acyclic ester, and that the intermediate decomposes via a cyclic transition state to the products (Perlin, 2006). A suggested reaction scheme describing the periodate oxidation of a mannuronic acid residue in the alginate chain is showed in Figure 1. The carbonyl groups formed may exist hydrated, as acyclic aldehydes, hemiacetals or hemialdals, or as combinations of these. The hemiacetal formation may be intermolecular as well as intramolecular (Gutherie, 1961; Painter & Larsen, 1970).

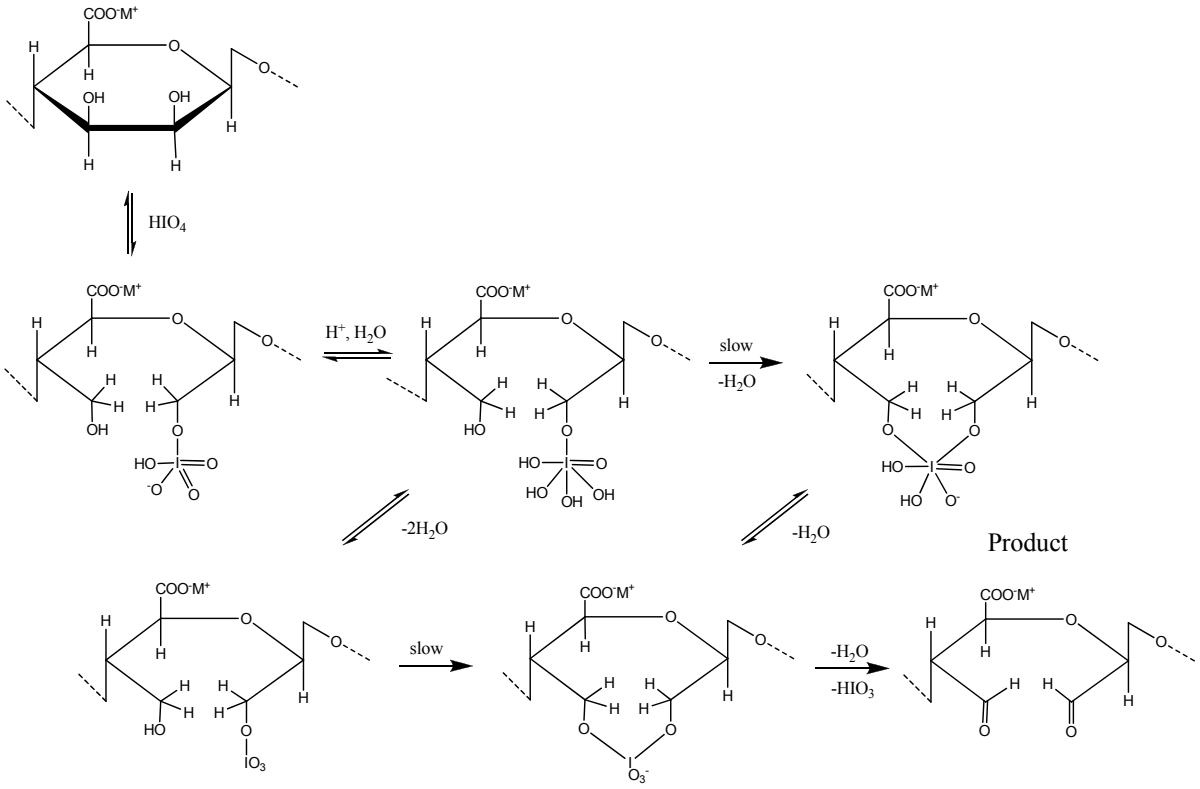

Figure 1: Suggested reaction scheme describing the periodate oxidation of a mannuronan residue within the alginate chain (modified from Buist et al. (1966) and Perlin (2006)).

Periodate oxidation has been a useful tool in glycochemistry for a long time. It has been used as a method for basic structural elucidation of polysaccharides, such as starch and xylan, and also to elucidate the structure of oligosaccharides in glycoproteins. For the latter application the Smith technique is often used, which involves immediate reduction by borohydride after oxidation followed by mild acid hydrolysis (Abdelakher, 1952; Sharon, 1975).

For the seaweed polysaccharide alginate, periodate oxidation results in the cleavage of the C2-C3 bond in the monosaccharide unit and a dialdehyde is formed (Figure 1). Although the periodate oxidation offers an interesting way of changing the chemical structure of alginate and making it more reactive, it leads to some depolymerisation. The degradation mechanism involved is presumably a free radical mediated mechanism and the degradation seems to be unavoidable even in the presence of a free radical scavenger (Balakrishnan et al., 2005; Painter & Larsen, 1970). Periodate oxidation is known to act randomly upon alginate (Painter & Larsen, 1970).

1.3 Polysaccharides studied in this work

Alginate, sphagnan, pullulan and dextran are exploited in this study. The two latter polysaccharides are used mostly as reference materials (standards) in the work regarding carbonyl detection.

1.3.1 Alginate

Alginate was first described by the British chemist E. E. C. Stanford in 1881, and is a linear polysaccharide consisting of β-D-mannuronic acid (M) and α-L-guluronic acid (G) (Figure 2), connected by 1→4 glycosidic linkages (Draget et al., 2002; Smidsrød & Draget, 1996). The pKa values of M and G measures to 3.38 and 3.65 respectively, in 0.1 M NaCl (Haug, 1964).

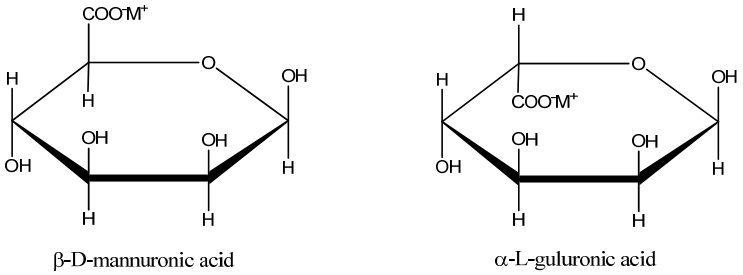

Figure 2: Alginate chemical structure. Haworth formulas of the two monomers of alginate: β-D-mannuronic acid (M) and α-L-guluronic acid (G).

The main source of alginate is from marine brown algae (Phaeophyta) (Painter, 1983a), but a polymeric material with a similar structure to alginate is also produced by *Azotobacter vinelandii* and several species of *Pseudomonas* (Gorin & Spencer, 1966; Govan et al., 1981; Linker & Jones, 1966). In particular the *Pseudomonas aeruginosa* is known as an alginate producing bacterium, and often colonises the mucus in the lungs of people with cystic fibrosis (Pedersen et al., 1990).

The biosynthesis of alginate is special in the sense that the epimerases converting M-residues to G-residues work *post* polymerisation. A similar phenomenon is only found in heparin/heparin sulphate and dermatan sulphate, where D-glucuronic acid is converted into L-iduronic acid (Lindahl et al., 1972; Malmstrøm et al., 1975; Valla et al., 2001). The epimerization in alginate is preformed by a series of enzymes, known as the AlgE enzymes. Seven of these enzymes have been recombinantly expressed in *E. coli*, and their action upon

poly-M results in different products as shown in Table 1. Pure poly-M is also obtainable, by 'knocking out' the epimerase genes.

Table 1: The main characteristics of the seven AlgE epimerases from *Azotobacter vinelandii*[a] (Draget et al., 2002).

Type	Molar mass (kDa)	Modular structure	Products
AlgE1	147.2	A1 R1 R2 R3 A2 R4	Bi-functional G-blocks + MG-blocks
AlgE2	103.1	A1 R1 R2 R3 R4	G-blocks (short)
AlgE3	191	A1 R1 R2 R3 A2 R4 R5 R6 R7	Bi-functional G-blocks + MG-blocks
AlgE4	57.7	A1 R1	MG-blocks
AlgE5	103.7	A1 R1 R2 R3 R4	G-blocks (medium)
AlgE6	90.2	A1 R1 R2 R3	G-blocks (long)
AlgE7	90.4	A1 R1 R2 R3	Lyase activity + G-blocks + MG-blocks

[a]A - 385 amino acids; R - 155 amino acids

The main task of alginate in seaweed is to bring strength and flexibility to the plant. This can be done foremost because of the alginates ability to form hydrogels with divalent cations. The content and distribution of G-residues are important in this process. The ion binding properties was reported by Smidsrød (1974) to be in the following order:

GG-blocks: $Ba^{2+} > Sr^{2+} > Ca^{2+} >> Mg^{2+}$

MM-blocks: $Ba^{2+} > Sr^{2+} \sim Ca^{2+} \sim Mg^{2+}$

MG-blocks: $Ba^{2+} \sim Sr^{2+} \sim Ca^{2+} \sim Mg^{2+}$

The complexation of alginate with divalent ions is described by the 'egg-box' model, in which each divalent ion interacts with two adjacent residues as well as with two G-residues in an opposing chain (Grant et al., 1973). Two different methods are used for making hydrogels of alginate by ionic cross-linking *in vitro*, depending on the way the divalent ions are introduced. In the first method, known as the diffusion method, the divalent ions diffuse into a reservoir of alginate. This method is preferred in the food industry, since it is rapid, but often results in inhomogenous gels (Onsøyen, 1996). In the second method, $CaCO_3$ (powder) is added to a alginate solution, where $c_{Alg} \geq c_{Alg}^*$ (c* - critical overlap concentration), followed by D-glucono-δ-lactone (GDL), which gradually makes the mixture acidic, thus slowly releasing Ca^{2+}-ions in order for the gel to set homogenously (Draget et al., 1990). Gels of alginate may also be made by gradual protonation of the carboxyl groups of M and G, resulting in an acid gel (Draget et al., 1994).

In industry, alginate has been exploited foremost because of its gelling, viscosifying and stabilizing properties (Onsøyen, 1996).

1.3.2 Sphagnan

The name sphagnan is used for the polysaccharides extracted from chlorite treated leaves of *Sphagnum papillosum* by mild acid hydrolysis. Their structure resembles rhamnogalacturonan Type I pectins, both with respect to sugar content and a high degree of branching. The weight average molecular weight (M_w) is in the range of 35-48 kDa, measured by size exclusion chromatography with multiangular laser light scattering (SEC-MALLS). Sphagnan contains mostly galacturonic acid (38 mol%), rhamnose (22 mol%), glucose (15 mol%), galactose (10 mol%) and xylose (9 mol%), but mannose and glucuronic acid is also present to a minor extent (Ballance et al., 2007).

Sphagnum mosses have a history of preserving organic material. Food is known to be well preserved in peat, a fact that has been exploited since the Viking age (Painter, 1995). A famous example of the preserving ability of *Sphagnum* is the Lindow Man, which was found in 1984 in Manchester, UK. He had been buried in the peat for approximately 2000 years and the remains were indeed very well preserved. Similar examples exist from other places in northern Europe (Painter, 1991). *Sphagnum* mosses have also had a number of other applications, such as surgical dressings during the First World War (Elliott, 1964).

The reason for the preserving properties of *Sphagnum* has been attributed to a combination of factors such as; low pH, lack of oxygen and an antimicrobial poly-phenolic component known as *sphagnum* acid (Painter, 1991a, b, 1995). However, some years ago it was claimed that sphagnan was responsible for the antimicrobial effect of *Sphagnum*, and that it contained a novel monosaccharide residue named 5-keto-D-mannuronic acid (5-KMA). The keto function in this residue was supposed to be the reason why sphagnan had a unique tanning ability. The keto group was expected to react with the amino groups in proteins and ultimately tan the material via Maillard browning (Ballance, 2004; Painter, 1983). Recently, the presence of 5-KMA has been refuted (Ballance, 2004a, b, 2007, c, 2008).

1.3.3 Pullulan and dextran

Pullulan and dextran are both polymers of glucose, but their linkage pattern differs significantly. Pullulan consists of α-(1→4) linked maltotriose units, which are further linked by (1→6) linkages. Dextran on the other hand is a α-D-(1→6) linked glucan with mainly α-(1→3) linkages to the backbone of the polymer (Smidsrød & Moe, 1995). Both polysaccharides are widely used as narrow molecular weight standards for calibration of size exclusion chromatography (SEC) as a method to measure molecular weight.

1.4 Main methods

Multi-detector size exclusion chromatography and NMR are the main methods used in almost every aspect of this thesis. Methods like small-strain oscillatory measurements, differential scanning calorimetry (DSC) and electrophoretic mobility combined with dynamic light scattering (DLS) are also applied, but less widely. A short presentation of the two main methods follows, focusing on their application for polysaccharides.

1.4.1 Multi-detector SEC

Size exclusion chromatography (SEC) separates molecules on the basis of their hydrodynamic volume. When injected onto the column, big molecules elute prior to smaller ones. This is because molecules with a small hydrodynamic volume penetrate the pores in the packing material to a larger extent than bigger molecules. SEC is an important tool in the analysis of polysaccharides, since these usually are polydisperse (Chi-San, 1995).

The combination of SEC with RI, UV, light scattering (LS) photometers, viscometry (VISC) or fluorescence detectors makes it possible to characterise the whole range of molecular weights in polysaccharides. By combining a concentration sensitive detector and a multiangular laser light detector (MALLS) with SEC, both the molar mass and the radius of gyration can be determined (the second virial coefficient (A_2) might also be obtained using only the RI and MALLS detector). The molar mass and radius of gyration (R_G) can be determined since the amount of light scattered is directly proportional to the polymer molar mass and concentration, while the angular variation of the scattered light is directly related to R_G. However, the light scattered by particles smaller than the incident light is the same for all angles and equal to the plane perpendicular to polarization. This is the reason why R_G cannot

be determined for molecules much smaller than the wavelength of the incident light (approx. 20 nm for I = 632 nm). The above information can be expressed through the Rayleight-Gans-Debye equation;

$$I_{scattered}(\theta) \propto R(\theta) = KcMWP(\theta)\left[1 - 2A_2MWcP(\theta)\right] \quad (1)$$

R(θ) is the excess Rayleigh ratio, meaning the ratio of the scattered and incident light intensity, corrected for the scattering volume size and the distance from the scattering volume. MW abbreviates molecular weight and c the concentration in g/mL. A_2 is the second virial coefficient, describing solvent-solute interactions. A positive A_2 value indicates a 'good solvent'. The form factor (P(θ)), also referred to as the scattering function, relates the angular variation in scattering intensity to the R_G of the particle. Finally, K is equal to $(4\pi_2 n_0^2/N_A\lambda_0^4)/(dn/dc)^2$, where n_0 is the solvent refractive index, N_A is Avogadro's number, λ_0 is the incident wavelength in vacuum and dn/dc the specific refractive index increment (Potschka & Dublin, 1996; Wyatt, 1993).

In addition to the light scattering detector, a viscosity (VISC) detector may be combined with SEC enabling more information about molecular shape. This is particularly useful when R_G is below 20 nm, since the viscosity data then becomes the only source of information regarding shape. The VISC detector measures the pressure difference between the inlet and outlet of the measuring cell, using the principle of the 'Wheatstone bridge'. From this, the specific viscosity (η_{sp}) of the solution can be calculated (Potschka & Dublin, 1996). The η_{sp} is equal to the intrinsic viscosity [η] at sufficiently low concentrations, thus [η] is defined as;

$$[\eta] \equiv \lim_{c \to 0} \frac{\eta_{sp}}{c} \quad (2)$$

In data analysis the chromatographic profile can be divided into a series of slices, which is assumed monodisperse. By measuring the viscosity of each slice, information regarding shape can be found from the Mark-Houwink-Sakurada relationship; $[n] = k \cdot M^a$. If a = 0, the molecule resembles a sphere, while a = 1.8 is consistent with a stiff rod. Values in between are intermediate states and when a = 0.5-0.8, the polymer is characterised as a random coil (Smidsrød & Moe, 1995).

Analysing unbranched polysaccharides with random coil behaviour, the SEC-MALLS-VISC (SMV) setup provides data that allows for predictions of the persistence length (q). The q is a

measure of the polysaccharide extensions. The Bohdanecký approach, which is a simplification of the wormlike chain model, is often used to find q (Bohdanecký, 1983). According to the model, $(M^2/[\eta])^{1/3}$ is a linear function of $M^{1/2}$.

$$\left(\frac{M^2}{[\eta]}\right)^{1/3} = A_\eta + B_\eta M^{1/2} \qquad (3)$$

$$A_\eta = A_0 M_L \Phi_{0,inf.}^{-1/3}$$

$$B_\eta = B_0 \Phi_{0,inf.}^{-1/3} \left(\frac{2q}{M_L}\right)^{-1/2}$$

$\Phi_{0,\,inf.}$ is the Flory parameter and equals $2.86 \cdot 10^{23}$. A_0 and B_0 are known functions of the reduced hydrodynamic diameter (d_r), and B_0 can in practice be replaced by a mean value (1.05). M_L is the molar mass per unit of contour length (Mendichi et al., 2003; Vold et al., 2006).

1.4.2 NMR and alginate structure analysis

NMR (nuclear magnetic resonance) gives information about molecular structure based on transition states between different states of nuclear spin. Atoms with uneven numbers possess spin. The molecules are exposed to an outer magnetic field and resonance occurs when the frequency of the source corresponds to the energy difference between the different states of spin. Some atoms are shielded by other atoms and this result in a chemical shift, which is characteristic for the different atoms in the molecule (Claridge, 1999). In this study, NMR has mainly been used to characterize alginate along with its oxidized, and 2-aminobenzamide (2-AB) substituted derivatives.

The main features of the high resolution ^1H and ^{13}C NMR spectrum of alginate were assigned in the early 1980's by Grasdalen et al. Since the chemical shift of protons/carbons within a monosaccharide in alginate is affected by its neighbouring monomers, sequence information is available through NMR. The fraction of the four diad frequencies (F_{GG}, F_{GM}, F_{MG}, F_{MM}) and the eight possible triad frequencies (F_{GGG}, F_{GGM}, F_{MGG}, F_{MGM}, F_{MMM} and F_{GMG}) can be determined. F_G can be obtained as the sum of F_{GG} and F_{MG}, while F_M is the sum of F_{MM} and F_{MG} (Grasdalen, 1983).

For sufficiently long chains contributions from the reducing end groups can be neglected and $F_{MG} = F_{GM}$. For such chains the average G-block length ($\overline{N}_{G>1}$) can be calculated by the expression (Grasdalen, 1983);

$$\overline{N}_{G>1} = \frac{F_G \quad F_{MGM}}{F_{GGM}}$$

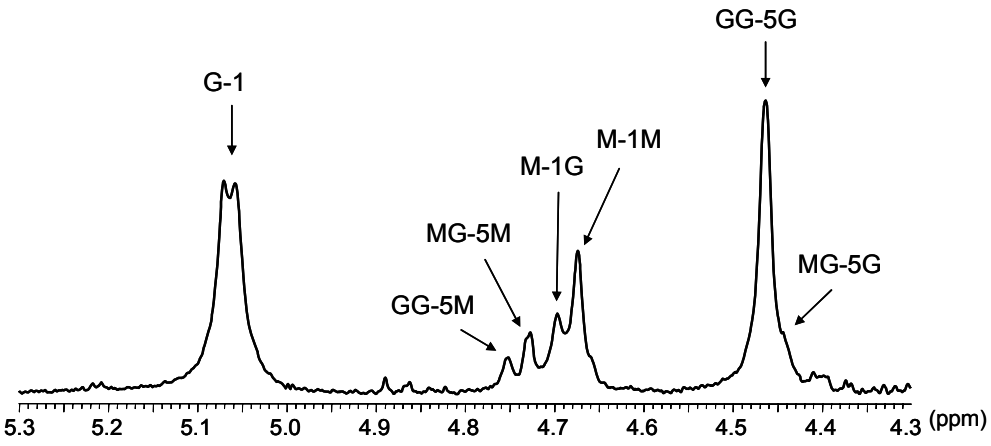

Figure 3: ^{1}H-NMR spectrum of a *Laminaria hyperborea* stipe alginate ($F_G > 0.7$)

Figure 3 shows a typical ^{1}H-NMR spectrum of alginate represented by an alginate from *Laminaria hyperborea*. As strategies for chemical modifications of alginate become more interesting, 2D NMR experiments like COSY, HSQC and NOESY offers great opportunities to provide evidence for the existence of different structures. Finally, it should be mentioned that a combination of specific alginate lyases together with high performance anion exchange chromatography (HPAEC) with pulsed amperiometric detection (PAD) and/or various mass spectrometry (MS) techniques also offers extremely important information in the studies of alginate structure (Ballance et al., 2005; Holtan et al., 2006a). Together with NMR this nearly completes the toolbox for alginate analysis.

1.5 Tools for carbonyl detection

The history of detecting carbonyl groups in oligosaccharides/polysaccharides starts with the colorimetric methods, such as the reducing end assay of Nelson-Somogyi (Somogyi, 1952). In this method Cu^{2+} (blue) is reduced to Cu^{+} (red) and thus serves as an indicator of a reducing end. Shortly after, other strategies evolved applying amino-substances (often hydrazines and hydroxylamines), which could react with the carbonyl function in question. The quantification was made possible by C/N-analysis or fluorescence spectroscopy (Maekawa & Koshijima,

1991; Ramsey et al., 2001). The reaction between an amino group and a carbonyl function is frequently used in many aspects of glycochemistry and the strategy known as reductive amination is often applied to obtain a stable linkage between the two, as shown in Figure 4. In reductive amination the double bond (Schiff base) formed between the carbonyl and the amine is reduced by the cyanoborohydride anion. If the amination and the reduction are performed in 'one pot' it is known as direct reductive amination, while the two step procedure refers to indirect reductive amination (Baxter & Reitz, 2002; Borch et al., 1970).

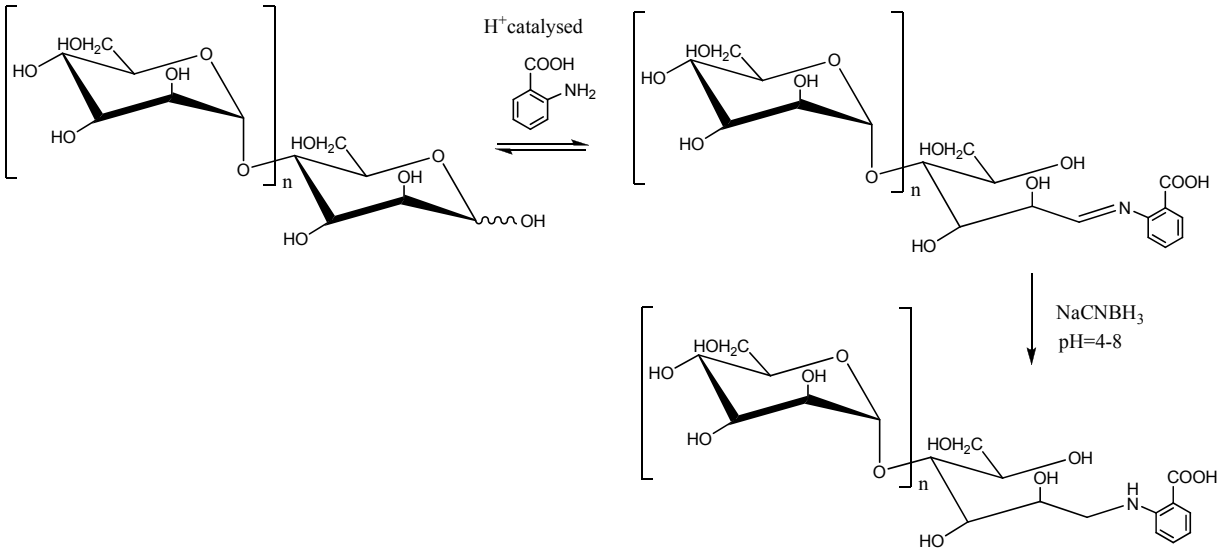

Figure 4: Indirect reductive amination of the reducing end of a glucan structure using the fluorescent label 2-aminobenzoic acid (2-AA).

The reduction of carbonyls by tritiated sodium borohydride (NaB^3H_4), thereby incorporating one tritium atom per carbonyl group, which could be detected by liquid scintillation counting (LSC), have also been exploited (Kongruang & Penner, 2004; Kongruang et al., 2004; Mclean et al., 1973). This strategy is however, hampered by the difficulty of removing excess tritium.

NMR and infrared spectroscopy (IR) are direct physical methods for carbonyl detection in polysaccharides. Viscometry has also been used to indirectly prove the existence of such structural features. For instance, Calvini et al. (2004; 2006) subjected periodate oxidized cellulose to alkaline degradation and measured the viscosity before and after degradation, thereby obtaining a measure of the fraction of oxidized residues (P_0).

The methods mentioned above struggle with several fundamental drawbacks. They all offer average estimates, which yields limited information for polydisperse polysaccharide samples.

Some are lacking the sensitivity needed (NMR, IR), while others offer only indirect evidence of carbonyls (Nelson-Somogyi method, viscosity measurements). In the assay of Nelson-Somogyi for instance, 'back oxidation' is a well known problem (Hodge & Hofreiter, 1962).

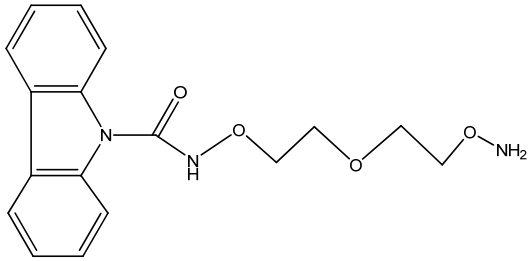

Figure 5: The fluorescent label carbonyl carbazole oxyamine (CCOA).

Well aware of the above challenges, Röhrling et al. (2002a; 2002b) searched for a method for carbonyl quantification in cellulose. No method existed for determining small amounts of carbonyls over a distribution of molecular weights, so a method was developed using a fluorescent marker, named carbonyl carboxyl oxyamine (CCOA) in combination with SEC-MALLS. Due to the poor solubility of cellulose in water the method needed to be compatible with the solvent *N*,*N*-dimethylacetamide (DMAc)/LiCl (Röhrling et al., 2002a). The marker (CCOA) (Figure 5) was synthesized, but at present it is not commercially available (Röhrling et al., 2001). CCOA reacts in the same way as 2-aminobenzoic acid (2-AA) shown in Figure 5, however the reduction step is omitted since the linkage between nitrogen and the *o*-substituted hydroxylamine (CCOA) is regarded as non-reversible under the experimental conditions. The CCOA also has a spacer region to make the fluorescence signal independent of the probe location on the polymer. Today, the CCOA method is probably the most accurate method for carbonyl detection in polysaccharides dissolved and analysed in DMAc/LiCl (Röhrling et al., 2002a; Potthast et al., 2003).

1.6 The importance of carbonyl groups in polysaccharides

The reasons for studying carbonyl groups in polysaccharides are numerous. In general, such functional groups can alter the stability and stiffness of polysaccharides, and serve as anchor points for cross-linking or connecting other molecules to the polymer backbone. When connecting molecules to a carbonyl group it is mainly their reaction with amines that are utilized (e.g. hydrazines or *o*–substituted hydroxylamines). The following are some examples describing how the reactivity of carbonyls in polysaccharides have been, or could be utilized.

The monosaccharides containing carbonyls often represent 'hot spots' for degradation along the polymer chain. Usually this is regarded as a drawback and the estimation of carbonyls are important to predict the stability of the material (e.g. in the study of deterioration of paper (Henniges et al., 2006)). However, in special cases controlled degradation is important, and deliberate introduction of carbonyls might be a strategy to obtain easily degradable polysaccharide materials (Bouhadir et al., 2001).

The attachment of hydrophobic molecules, such as long alkyl chains or cholesterol moieties can alter the surface of polysaccharides and make them amphiphilic. This is shown by Akiyoshi et al. (1993) for hydrophobized pullulan creating self-aggregating nanoparticles for drug delivery. Although the linking of cholesterol did not involve a carbonyl group, such groups have great potential as anchor points creating nanoparticles.

A study that did involve carbonyl groups for making nanoparticles was the study by Halkes et al. (2005). They used the reducing ends of oligosaccharides as an anchor point to make gold glyconanoparticles, utilizing reductive amination. In this work they proved this strategy to be a facile and elegant way to make such particles.

Finally, the carbonyl function can be used as an anchor point for fluorescent labelling. Tracing oligosaccharides and polysaccharides, when for instance introduced to living systems, is important. Although utilizing a different type of chemistry to connect fluorescent probes, the group of Bertozzi at University in California, Berkeley, showed the importance of this through several of their publications (Laughlin et al., 2007a, b, 2008). However, even if the carbonyl function might have a great potential for attaching such probes, the difficulty in this situation is of course on the level of biological recognition.

2. AIM OF THE THESIS

The aim of this thesis can be split into two sub-aims, which is closely inter-connected through the general topic ‘carbonyl functionalities in polysaccharides’.

The first aim was to establish a method for the detection of carbonyl group profiles in polysaccharides dissolved in an aqueous solvent. This work was initiated for two reasons. First, such a method could be useful in the characterisation of all water soluble polysaccharides. Secondly, such a method was needed in the further investigation of sphagnan, which was claimed to have special properties because of its alleged high content of carbonyl functionalities. As a part of this work, the reactivity of sphagnan with phenylhydrazine, *o*-phenylenediamine and collagen was re-investigated.

The second aim was to investigate whether the alginate C-5 epimerases AlgE4 and AlgE6 could act upon periodate oxidized and borohydride reduced mannuronan. It was also examined whether this material could be used to form alginate hydrogels with Ca^{2+}-ions. Depending on the outcome of these investigations new easily degradable alginate gels could potentially be formed. The degradability and gel strength might be ‘tuned’ using alginates with different degree of oxidation. As a background for this work the chain stiffness and extensions of alginates with different chemical composition or periodate oxidation was investigated.

3. DETECTION OF CARBONYL GROUP PROFILES

This section starts with a re-investigation of reactive carbonyl groups in sphagnan. During this work it became evident that a method for detecting carbonyl group profiles in water soluble polysaccharides was needed. Since no such method could be found directly from literature it became one of the main aims of this thesis to develop such a method. The solution became three different strategies, which could be used depending on the type/structure of polysaccharide analysed. All three strategies were later applied to sphagnan.

3.1 Carbonyl functionalities in sphagnan

A large number (~25 mol%) of carbonyl groups were claimed to be present in sphagnan in the form of a novel monosaccharide called 5-keto-D-mannuronic acid (KMA) (Painter, 1983b). However, recent studies have rejected the presence of 5-KMA (Ballance et al., 2004a, b, 2007). A high content of carbonyl functionalities in sphagnan, regardless if present in 5-KMA or not, could be of great biological and biotechnological interest. Therefore a re-investigation of the carbonyl functionalities in sphagnan was initiated. The importance of high carbonyl content would be reflected in a great potential in binding nitrogen as well as possibly making sphagnan a good tanning agent.

3.1.1 A re-investigation of reactive carbonyl groups in sphagnan

In previous literature the existence of 5-KMA, and thus carbonyl groups, was mainly proved by a colorimetric assay and the formation of a quinoxalinol when reacted with O-phenylenediamine (Painter, 1983b; Smidsrød & Painter, 1984). The tanning ability of sphagnan, thus presumably reflecting its high content of reactive carbonyl groups, was 'proved' by treating collagen with sphagnan followed by differential scanning calorimetry. According to Painter (1991), this showed an increase in the thermo stability of the treated collagen compared to untreated collagen.

The colorimetric assay for 5-KMA detection was repeated. In this assay sphagnan was mixed with excess phenylhydrazine, under conditions favouring imine formation, to label the keto function in 5-KMA. After this step sphagnan was hydrolysed in 12 M HCl for 24 h at RT, and the absorbance measured at 275 nm. It was found that control samples of both sphagnan and

other commercial pectins yielded absorbance at this wavelength, because of the formation of furans during the strong acid hydrolysis (Martinez et al., 2000) (Figure 6). If the absorbance peak at 275 nm for acid hydrolysed sphagnan (not treated with phenylhydrazine) was compared against a standard curve of phenylhydrazine, the assay showed a false result implying a content of 5-KMA in the order of approximately 25 mol%.

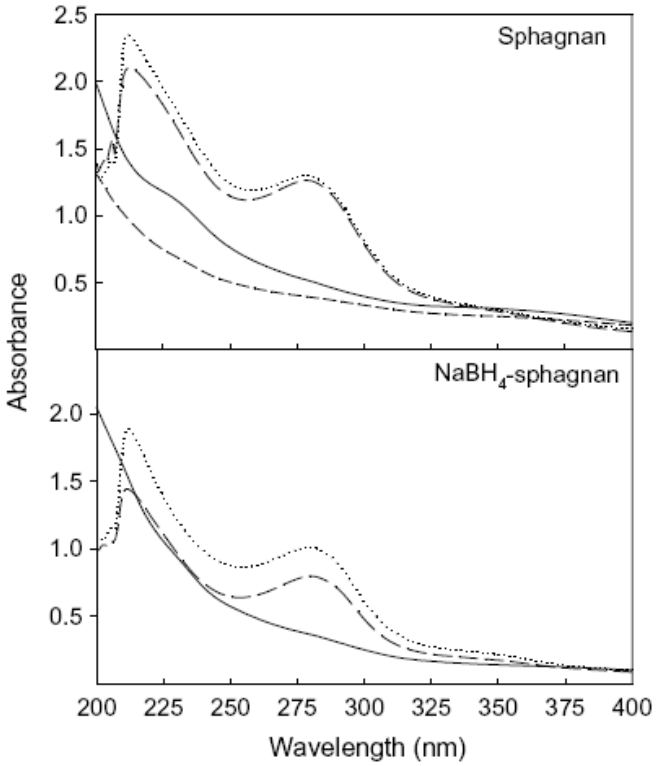

Figure 6: UV spectra (200–400 nm) of native sphagnan, sphagnan labelled with phenylhydrazine and sphagnan reduced by 20% (w/v) borohydride, from chlorite-treated leaves. Dotted lines are samples incubated with phenylhydrazine at 60 °C for 2 h, hydrolysed in concentrated HCl for 24 h, and diluted to 6 M HCl. Solid lines are samples treated with phenylhydrazine, but not with acid, and dissolved in water. Long dashed lines are native samples hydrolysed in concentrated HCl for 24 h, and diluted to 6 M HCl. Short dashed lines represent spectra of native samples dissolved in water.

According to Painter et al. (1991) a fluorescent quinoxalinol is formed when the free acid-form of sphagnan was mixed with O-phenylenediamine (OPD) (calculated pK_a 4.46). These experiments were repeated, but the fluorescent quinoxalinol was not observed. An attempt to prove the latter structure by NMR was inconclusive.

The interactions between sphagnan and gelatin were investigated using dynamic light scattering to determine size, while titrated in the pH range 6.0-2.0. Complex coacervates were detected in the pH range 2.0-4.8, which is typical for polyelectrolyte complexes formed by

electrostatic interactions of oppositely charged polymers, rather than a covalent reaction between a carbonyl group and an amino group. Since the interaction between sphagnan and gelatine was mainly electrostatic, it seemed unlikely that sphagnan could serve as a true tanning agent.

The tanning ability of sphagnan was investigated. A true tannage elevates hydrothermal stability with a concomitant rise in shrinkage temperature, whereas 'leathering', typical of oil, brains and smoke-treated collagen, just renders a material non-putrescible and changes its physical appearance (Covington, 1997). Measurements of hydrothermal stability, as denaturation temperature, of bovine hide powder collagen treated with sphagnan (from chlorite-treated leaves) at pH 4.0–4.5 for 24 h showed little difference compared to an untreated control (Table 2). Furthermore, the sphagnan treated collagen was readily degraded by collagenase. From these results it can be concluded that sphagnan do not meet the criteria of a true tanning agent.

Table 2: Denaturation temperatures (n = 3) of untreated hide powder collagen and hide powder collagen treated with sphagnan from chlorite-treated leaves (A), sphagnan from acetone/methanol-treated leaves (B) and two polyphenolic vegetable tannins. Corresponding denaturation temperatures after 4 wetting and drying cycles are given in brackets.

Hide powder treatment	Denaturation temp. (°C)
Untreated	44.0 (42.0)
Sphagnan A	46.0 (44.0)
Sphagnan B	54.0 (48.0)
Chestnut-extract	57.5 (57.5)
Mimosa-extract	63.5 (63.5)

Finally, the carbonyl content of sphagnan was estimated by reaction with hydroxylamine at pH 4.0 - 4.5, followed by carbon/nitrogen (C/N) analysis. The total nitrogen content was determined to 0.63% ± 0.085 (n = 4). Comparing this result with untreated sphagnan (N content 0.2 %), showed that less than 1 of 18 sugars contained a reactive carbonyl group or less than 5.6 carbonyl groups per 100 monomers were present. However, this was a rough estimate, since the C/N-analysis was on the absolute borderline of instrument detection.

From the studies made of sphagnan with phenylhydrazine, OPD, hydroxylamine, or collagen it can be concluded that the carbonyl content of sphagnan was overestimated in earlier studies

(Painter, 1983b; Smidsrød & Painter, 1994). Together with additional evidence (Ballance, 2004a, b, 2007) it also proves the non-existence of 5-KMA. However, no exact measure of the carbonyl content in sphagnan was found and there was yet no knowledge about the carbonyl profile. If a certain molecular weight fraction was enriched in carbonyl functionalities, the fraction could be isolated and potentially be of biotechnological interest. Since no method could be found in literature for precise measurement of carbonyl group profiles for polysaccharides dissolved in water, the development of such a method was initiated.

3.2 Method development: Carbonyl profile detection

Strategies for tritium or fluorescence labelling were utilized for carbonyl detection. The use of these approaches in combination with SEC-MALLS makes it possible to determine the carbonyl group profiles within polysaccharides dissolved in an aqueous solvent. Several challenges need to be addressed in the development of such a method.

The first thing to consider is the labelling conditions, which should not degrade the polymer in question. It is also important that the labelling reaction reaches complete conversion within reasonable time limits. Secondly, when using a fluorescent probe, several demands are required. The probe must:

1. be water soluble (at low concentrations)
2. cannot be too hydrophobic (otherwise the polymer becomes amphiphilic at high DS and insoluble aggregates are formed)
3. not interact significantly with the column material
4. not interfere with the MALLS detection, meaning that its emission wavelength must not overlap with the laser wavelength (632 nm)
5. possess an emission wavelength, which is not dependent on its location on the polymer

With this as a prerequisite, we tested several labelling strategies for carbonyl detection in combination with SEC-MALLS. Tritium labelling via NaB^3H_4 reduction and fluorescent labelling by carbonyl carbazole oxyamine (CCOA) and 2-aminobenzamide (2-AB) was found appropriate. However, before using the selected labelling strategies, the reaction kinetics and above criteria had to be evaluated.

3.2.1 Tritium labelling via NaB^3H_4 reduction

The reduction of carbonyl groups to alcohols within polysaccharides using a fixed ratio (expressed as the specific activity in the reaction mixture) of $NaB^3H_4/NaBH_4$ is a useful way to detect carbonyl groups. This introduces a certain fraction (depending on activity) of tritium atoms to the carbon atoms originally bearing the carbonyl groups.

The polysaccharide structure is minimally affected by the tritium labelling compared to the attachment of 'bulky' groups for UV or fluorescence detection. Borohydride reduction of carbonyl groups in water is known to be fast, but with ketones reduced slightly slower than aldehydes (McMurry, 1988).

It was important that the specific activity of the NaB^3H_4 were kept as low as possible because of the strict regulations for tritium release in Norway. Some consideration was therefore put into finding the optimum labelling conditions, with regard to sensitivity, stability of the borohydride and reaction kinetics. The use of 0.10 M NaB^3H_4 (spec. act. 2.5 mCi/mmol) in the reaction mixture was chosen since this gave sufficient sensitivity, and was previously shown to completely convert glucose to glucitol within 24 h at room temperature (Hansson et al., 1969). The pH was kept equal to 12.5 in order to prevent decomposition of borohydride (Mochalov et al., 1965).

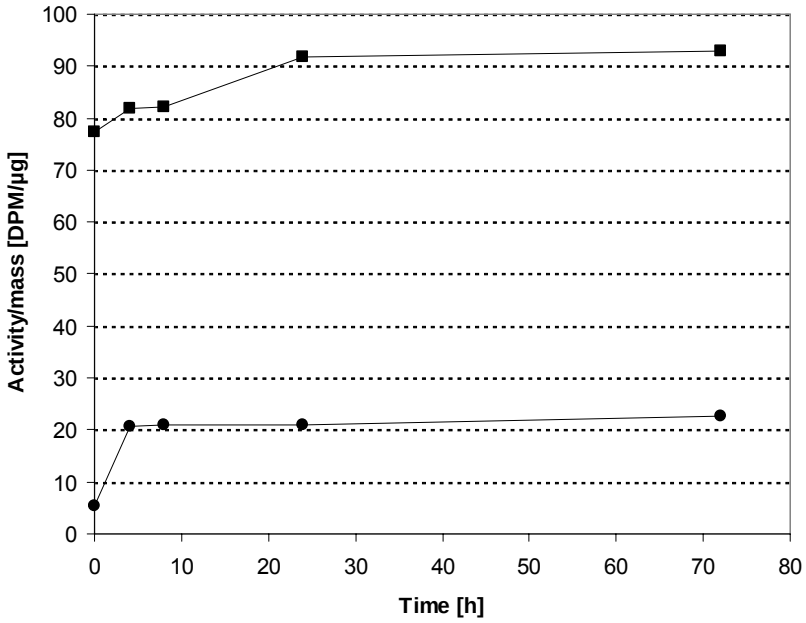

Figure 7: Reaction kinetics of pullulan (●) and sphagnan (■), when reduced by 0.3 M NaB^3H_4 (specific act. 2.5 mCi/mmol) at RT, pH 12.5. Every datapoint is a result of one SEC-MALLS injection with off-line tritium detection.

The reaction kinetics were investigated for the polysaccharides pullulan and sphagnan, showing that the reaction was complete after 24h (Figure 7). A pullulan (DP~10) and alginate oligomer (DP~16) were also studied by ^{1}H-NMR. The spectrum showed a disappearance of the reducing end signals, when treated by borohydride using the above mentioned conditions (unpublished data). No significant degradation during reduction was observed for pullulan, dextran, alginate or sphagnan (SEC-MALLS). However, periodate oxidized alginate and pullulan were degraded due to strong alkaline conditions (pH 12.5), and the approach was deemed not suitable for these samples.

3.2.2 Fluorescence labelling

Different strategies for fluorescence labelling were evaluated. This work was initiated, since not all carbonyl containing polysaccharides could withstand the alkaline conditions used during borohydride reduction. A complementary method was also needed to confirm the results of the reduction approach.

The number one choice was to adapt the CCOA method to an aqueous system, since this was already a validated carbonyl detection method for cellulose dissolved in DMAc/LiCl. However, this method did have two serious drawbacks when applied to polymers dissolved in water. First, the reaction time for complete conversion in zinc acetate buffer (pH 4, 40°C) was found by Röhrling et al. (2001) to be 168 h, which makes the labelling a tedious procedure. Secondly, the labelling of polysaccharides with a carbonyl content of 2-4 mol% formed insoluble aggregates in water caused by the high hydrophobicity of the CCOA label (Figure 5). Despite these inherent limitations the approach could be used to label polysaccharides with low carbonyl content.

The fluorescent label 2-aminobenzoic acid (2-AA) was considered as an alternative to CCOA, but failed the fifth criteria above, and the emission was slightly dependent on the location of the fluorescent probe. Instead 2-aminobenzamide (2-AB) was tested, which has been previously applied for fluorescent labelling of both glucan and pectin oligosaccharides (Bigge et al., 2002; Ishii et al., 2002). It was found that 2-AB fulfilled the criteria previously mentioned, and it could be connected to the carbonyl group via direct reductive amination. To reach complete conversion the reaction conditions were optimized. The first step in reductive amination, the imine formation, is acid catalysed and rate limiting, while reduction of imines

by $NaCNBH_3$ is known to be specific for pH values in the range 4-10 (Borch et al., 1970). Oxidized polysaccharide structures are sensitive to alkaline degradation (Bouhadir et al., 2001; Whistler et al., 1959) and temperatures above room temperature were therefore avoided in order to slow down such processes. The optimum labelling conditions was found to be pH 5.8 for 24 h at RT and the reaction kinetics was investigated for all polysaccharides involved (Figure 8). The reactions were found to follow pseudo first order kinetics (R^2 = 0.99 – 0.96).

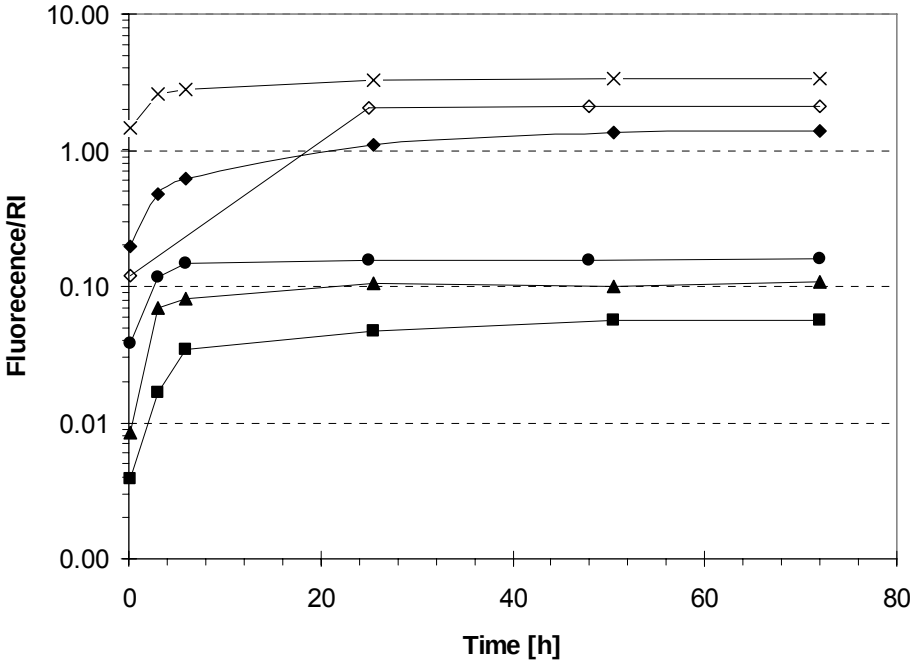

Figure 8: Reaction kinetics for 2-AB labelling of pullulan (■), alginate (▲), mannuronan (●), sphagnan (♦), mannuronan periodate oxidized (P_0=0.06) (◊) and periodate oxidized (P_0=0.08) alginate (40% guluronic acid) (x). The polysaccharides were labelled using 0.05 M 2-AB and 0.25 M $NaCNBH_3$ at 24 h, pH 5.8.

The degree of substitution (DS) of the reducing end was examined by ^{1}H-NMR of a mannuronan oligomer (Figure 9B). Using an oligomer with predetermined DP (= 20) the signal from the four protons observed from 2-AB was compared with the signal due to the C1 proton on mannuronic acid (M-1), this showed complete substitution of the reducing ends (DS=1) (Figure 9). In addition, the disappearance of the reducing end signals was additional evidence for complete conversion.

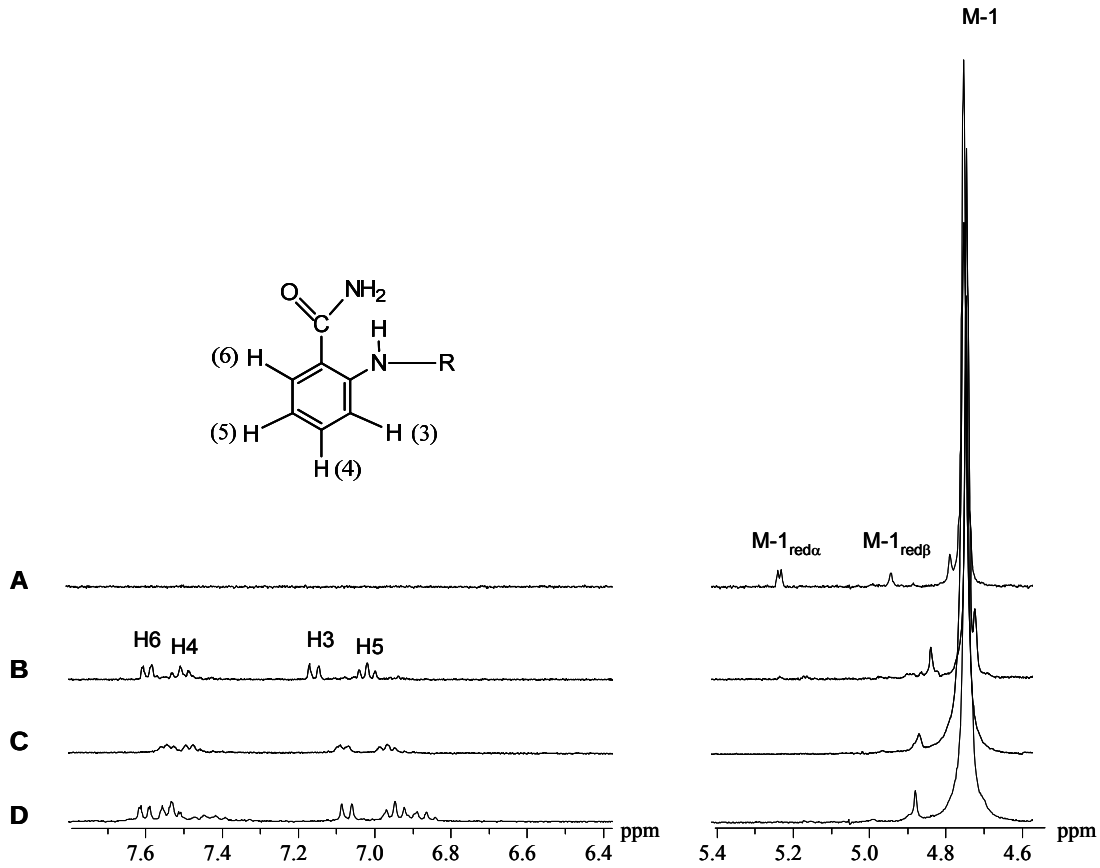

Figure 9: ^{1}H-NMR spectra of a mannuronan oligomer are shown before (A) and after (B) labelling with 2-aminobenzamide (2-AB) at the reducing end. Spectra C and D show a periodate oxidized mannuronan oligomer with P_0=0.04 and P_0=0.08 (borohydride reduced before oxidation) after labelling with 2-AB, respectively. All samples were labelled for 24 h. The peaks appearing in the 6.8-7.7 ppm region belong to the four protons on 2-AB as indicated. The chemical shifts of the protons on 2-AB were assigned according to Ishii et al. (2002) and modelling using ChemBioDraw Ultra version 11.0. Abbreviation; R = anchor point for carbonyl group.

A mannuronan oligomer (DP ~ 20) was reduced by borohydride and periodate oxidized (P_0 = 0.04 and 0.08), followed by 2-AB labelling and studied by ^{1}H-NMR (Figure 9C and 9D). The aim was to determine the degree of substitution (DS) of the dialdehydes. The DS was determined by comparing the average signal intensity from the four protons of 2-AB with the signal from the C-1 proton on internal mannuronic acid residues (M-1). A DS of 0.5 was found, indicating that only one of the two aldehydes formed upon periodate oxidation reacted with 2-AB. It is not clear whether this was due to the fact that 2-AB cannot react with an aldehyde group when another 2-AB molecule was located in its close vicinity, or if one of the aldehydes was simply not reactive. When substituted hydroxylamines were reacted with cellulose possessing low degrees of oxidation (~ P_0 = 0.01), both aldehydes seemed to be reactive (Potthast et al., 2007). On the other hand when periodate oxidized alginate was substituted with a polyether via direct reductive amination, only 0.12 mol polyether was

incorporated per mol of uronic acid as opposed to 0.40 mol, if complete conversion was to be obtained (Carré et al., 1991).

3.2.3 SEC-MALLS combined with tritium or fluorescence detection

After labelling the polysaccharide with tritium, CCOA or 2-AB it was analysed by SEC-MALLS with either off-line tritium or on-line fluorescence detection as shown in Figure 10.

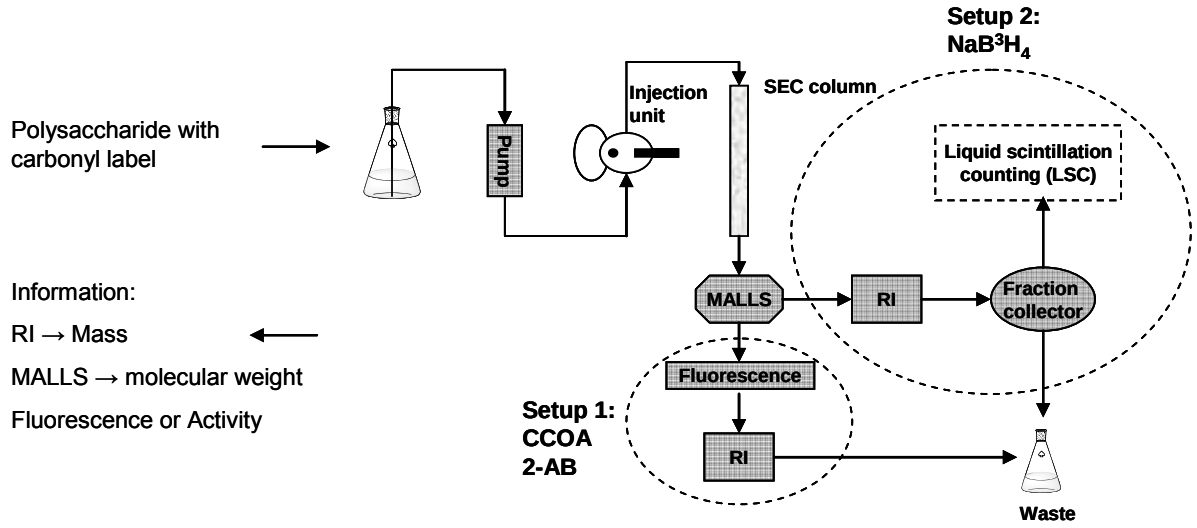

Figure 10: Setup for the analysis of carbonyl profiles in polysaccharides utilizing fluorescent or tritium labelling of the carbonyl group.

In this setup the molecular weight, mass and fluorescence/activity is obtained for a series of fractions or slices throughout the chromatographic profile. Analysing a polysaccharide containing only the reducing end carbonyl group, the signal (disintegrations per minute (DPM) or fluorescence) per mass should be inversely proportional to DP throughout the chromatographic profile. In order for the approach to be valid, the latter statement has to be independent on the type of polysaccharide.

The different labelling approaches combined with SEC-MALLS with fluorescence or tritium detection are from now on simply referred to as the tritium, CCOA or 2-AB approach.

3.3 The tritium approach

Using this approach the polysaccharide was labelled with tritium as described earlier and analysed with SEC-MALLS combined with off-line liquid scintillation counting (LSC). ^{3}H was measured, in the form of DPM, for fractions collected across the molecular weight profile. The number average degree of polymerisation (DP_n) was further calculated for each fraction along with the mass. Pullulan, dextran and alginate, presumably containing only the reducing end carbonyl, were analysed. Figure 11 shows that the calculated DPM/µg was inversely proportional to DP_n across the DP range and overlapping for the three polysaccharides. Thus, these (and possibly several others) can serve as standards for carbonyl quantification. It should be pointed out that it is particularly important that a standard prepared at the same time as the unknown samples are included, since the activity of the borohydride may change as a consequence of disintegration of the isotope.

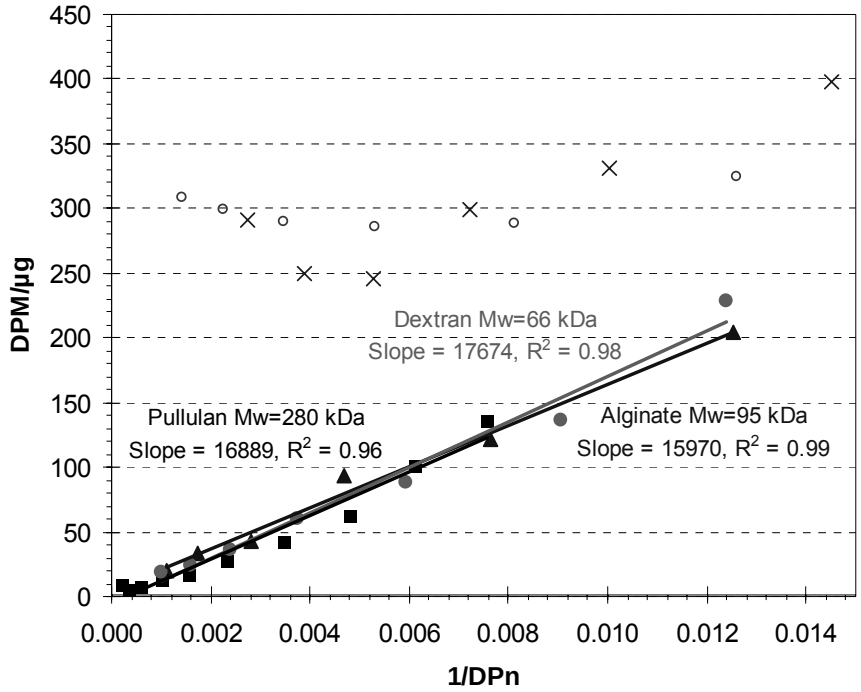

Figure 11: Detection of carbonyl profiles applying the tritium approach on pullulan (■), dextran (●), alginate (▲), sphagnan (x) and sphagnan extracted without the use of chlorite (○).

Sphagnan was analyzed along with the standards (Figure 11), and it can be observed that the carbonyl content increased with increasing DP. The average carbonyl content was 2.9 carbonyls per 100 monomers (quantified using a pullulan standard). Sphagnan extracted with or without the use of chlorite was analysed, since it was suspected that the chlorite treatment

might introduce additional carbonyl groups. However, no significant difference was observed between the two samples (Figure 11).

3.4 The CCOA approach

The polysaccharides with CCOA labelled carbonyl groups were analysed by SEC-MALLS with on-line fluorescence detection. The data were treated in the same way as in the tritium approach, with one exception. In this case the fraction/slices were assumed monodisperse and the DP was calculated instead of DP_n for the tritium approach. The assumption is valid, since the slice volume was sufficiently low.

The use of the CCOA approach was seriously hampered by the formation of insoluble aggregates during labelling of samples with high carbonyl content. However, both pullulan and dextran could be utilized as standards for quantification (Figure 12). Alginate was severely acid hydrolysed during labelling and could therefore not be analyzed.

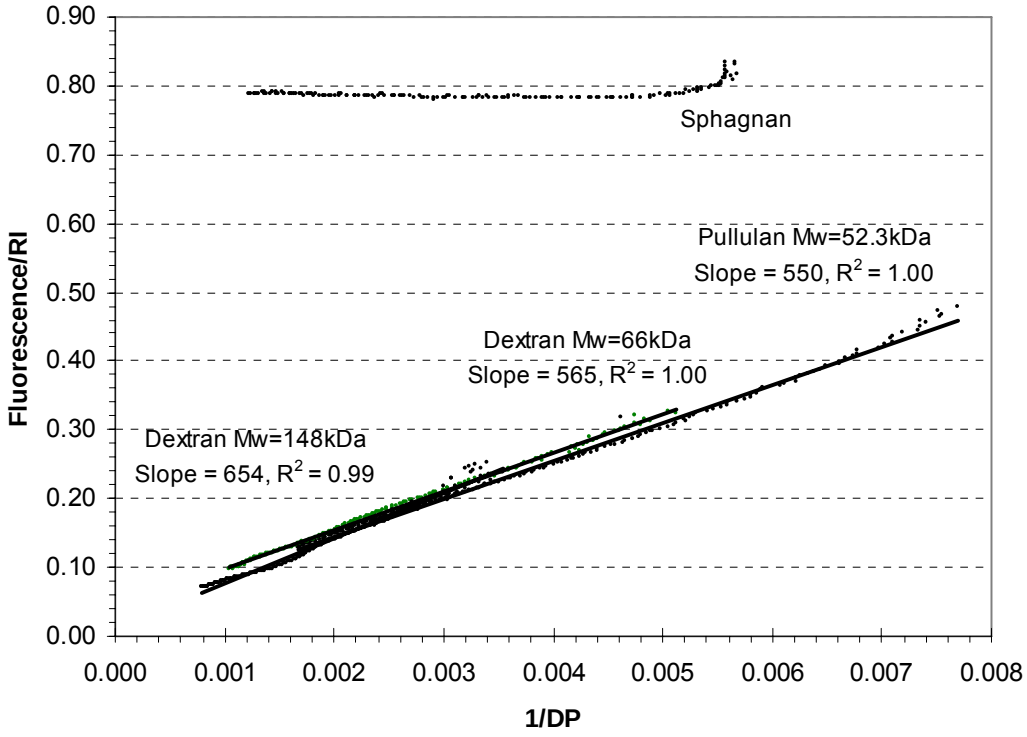

Figure 12: Detection of carbonyl profiles in pullulan, dextran, alginate and sphagnan using the CCOA approach.

It was also found that excess CCOA (not bound to the polysaccharide) was absorbed onto the column material. This phenomenon was attributed to a hydrophobic interaction between

CCOA and the column packing material (network of polyhydroxy methacrylate co-polymers). A potential solution to this problem is to add a modifier to the mobile phase. Several modifiers were tested. The best result was obtained using 0.05 M Na_2SO_4/0.01 M EDTA containing 20% (v/v) acetonitrile. However, even in the presence of the modifier, the unbound CCOA eluted several minutes after the salt peak. As a consequence of this, a delay was introduced between injections.

Sphagnan was analysed by the CCOA approach (Figure 12), but the formation of aggregates in the high molecular weight region affected the molar mass determination and an overall increase in M_n of 8% was observed. The average carbonyl content was found to be 1.4 carbonyls per 100 monomers (determined by comparison with pullulan standards), a factor of 2.1 lower than in the tritium approach.

3.5 The 2-AB approach

In the 2-AB approach the data collection and processing was identical to the CCOA approach (except for different excitation (340 nm) and emission (450 nm) wavelengths). The results were similar to the tritium approach for the reference samples (pullulan, dextran and alginate), and for sphagnan. It was found that the 2-AB approach had some positive features compared to the CCOA approach. First, the 2-AB label is much less hydrophobic compared to CCOA, which facilitates the detection in polysaccharides with high carbonyl content (> 2 mol%). Secondly, the reaction conditions are milder, which is important when analysing acid/alkaline labile structures. Finally, the labelling was complete after 24 h as opposed to 168 h for CCOA.

Due to the mild labelling conditions, the 2-AB approach allows detection of aldehyde groups in periodate oxidized alginate (< 10% oxidation). Assuming that the dialdehydes formed upon periodate oxidation of the polysaccharides examined are monosubstituted with 2-AB, the degree of oxidation (D_{ox}) can be described by the following expressions:

Fluorescence $\propto n_e + n_{ox}$

$$\text{Fluorescence} = A\left(n_e + n_{ox}\right) = A\left(\frac{n_0}{DP} + n_0 D_{ox}\right) = An_0\left(\frac{1}{DP} + D_{ox}\right) \qquad (1)$$

$RI = mass = M_0 n_0$

$$\frac{\text{Fluorescence}}{\text{RI}} = \frac{An_0}{M_0 n_0} \cdot \left(\frac{1}{DP} + D_{ox} \right) = \frac{A}{M_0} \cdot \left(\frac{1}{DP} + D_{ox} \right) \qquad (2)$$

A = constant, n_e = number of reducing end residues, n_{ox} = number of oxidized residues, n_0 = number of monomers, M_0 = monomer weight, D_{ox} = degree of oxidation (= n_{ox}/n_0).

The constant A, expressing the relationship between fluorescence intensity and number of carbonyl groups, could be obtained from the slope of the linear plot (A = slope · M_o), while the degree of oxidation could be estimated from the intercept (D_{ox} = Intercept/(A/M_0)).

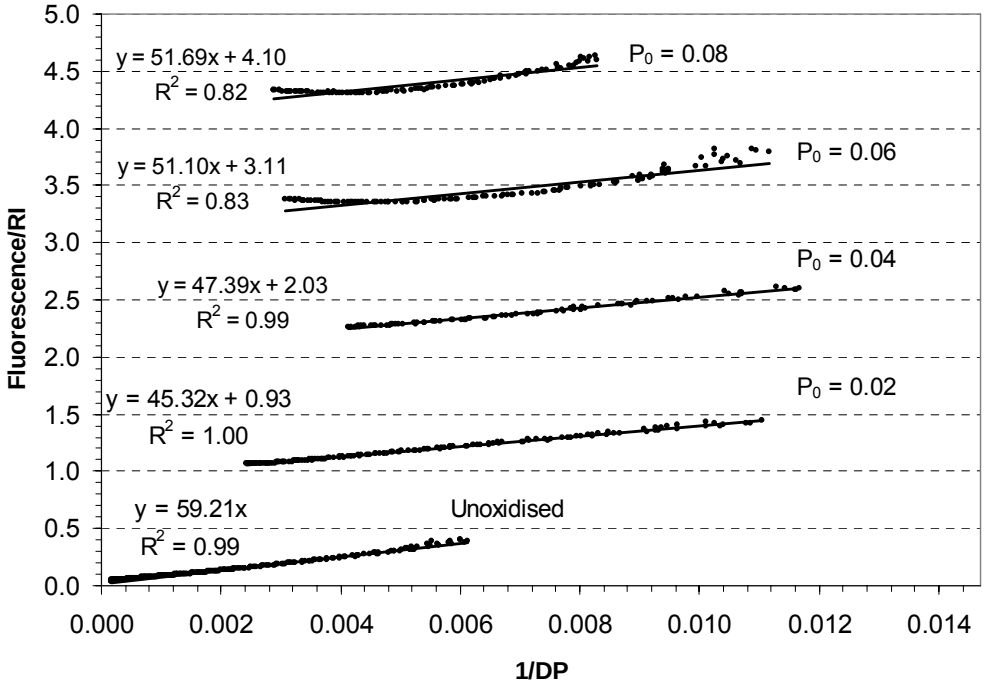

Figure 13: Carbonyl detection using the 2-aminobenzamide (2-AB) approach applied to alginate (F_G = 0.46) partially oxidized with P_0 equal to 0.02, 0.04, 0.06 and 0.08. Monomer weight was taken as 198 g/mol for both oxidized and unoxidized alginate residues.

Figure 13 shows the analysis of partially oxidized alginates (P_t = 0.02-0.08). The fraction of oxidized units was calculated using equation 2. A good correlation was found between the theoretical fraction of oxidized units (P_t) and the measured fraction of oxidized units (P_0) (R^2 = 0.998). Periodate oxidized samples thereby served as a positive control and confirmed that all periodate added was consumed (no over-oxidation).

An interaction of unbound 2-AB with the column material was observed, analogues to the one described for CCOA, however the interaction involving 2-AB was weaker than the one involving CCOA. This confirmed that the interaction was likely to be hydrophobic, since 2-

AB is less hydrophobic than CCOA. Using the mobile phase containing 20 % (v/v) acetonitrile, free 2-AB eluted immediately after the salt peak.

3.6 Results of the carbonyl distribution analysis of sphagnan

The carbonyls in sphagnan were quantified and it was found that both the tritium and the 2-AB approach gave identical results with respect to the average content and distribution of carbonyl groups (Figure 14).

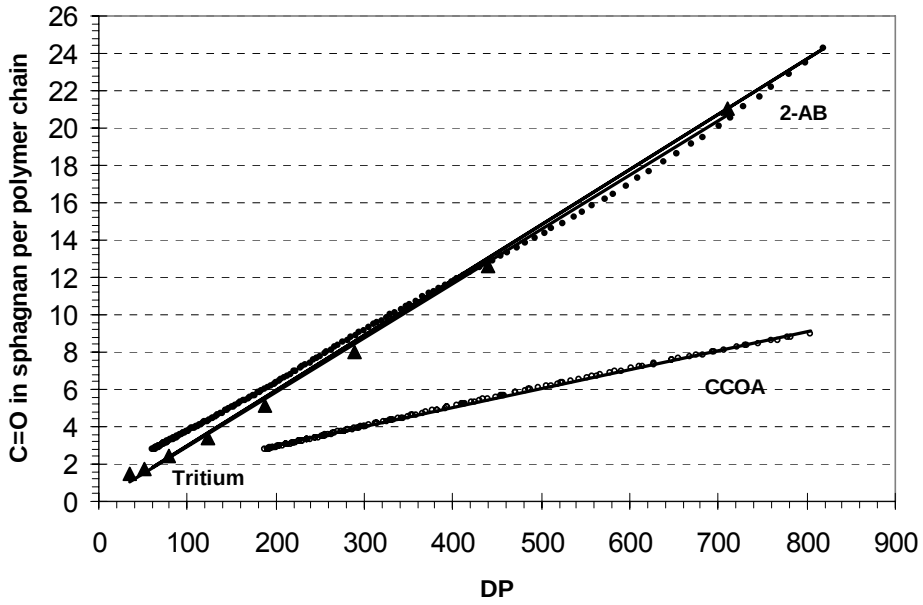

Figure 14: The carbonyl profile in sphagnan determined using the tritium (▲), 2-AB (●) and CCOA (○) approach. The total number of carbonyls per chain is found by dividing the signal (fl./µg or DPM/µg) obtained for sphagnan by the signal obtained for the pullulan standard. The data in the figure can be fitted to linear functions; slope 2AB and tritium approach = 0.03 (R^2 = 0.99), slope CCOA approach = 0.012 (R^2 = 0.96). The 2-AB approach cannot detect ketones.

The average carbonyl content of sphagnan, using the tritium and 2-AB strategies, was 2.9 carbonyls per 100 monomers. The CCOA approach on the other hand underestimated the carbonyl content throughout the DP range studied (Figure 14), and the average carbonyl content was underestimated by 52%. The reason for this could probably be found in a combination of different factors. Sphagnan aggregated upon labelling and thus M_w was overestimated by 25%. Aggregation might also affect the reaction rate between the label and the carbonyl functionalities in the polymer. If labels are closely located in space this could also lead to fluorescence quenching resulting in a lower fluorescence signal (Miller, 2005). All in all the CCOA approach does not seem to be the appropriate strategy of choice for this sample.

Sphagnan was claimed to contain the novel monosaccharide 5-KMA containing a α-keto group (Painter, 1983b). If 5-KMA were to be present in sphagnan a difference between the tritium approach and the 2-AB approach should have been detected, since the 2-AB does not react with keto groups presumably present in 5-KMA. However, no such difference was observed. This observation was taken as further proof of the non-existence of 5-KMA in sphagnan.

4. CHEMICAL AND PHYSICAL STUDIES OF NOVEL ALGINATE MATERIALS

The chapter describes periodate oxidation as a tool to create alginate materials with increased chain flexibility. Some of these materials can be used to make hydrogels with new properties compared to gels made of unoxidized alginate.

4.1 The impact of periodate oxidation on alginate chain stiffness and extension

The chain stiffness and extensions of alginates, in vitro epimerized alginates, and periodate oxidized alginates were analysed using SEC-MALLS-VISC (SMV) and static light scattering (LALLS) with off-line viscometry. The motivation for this analysis was to clarify the picture of alginate chain stiffness and extension, since the literature data describing this topic varies greatly (Berth, 1992; Mackie et al., 1980; Martinsen et al., 1991; Smidsrød et al., 1973; Smidsrød & Haug, 1968).

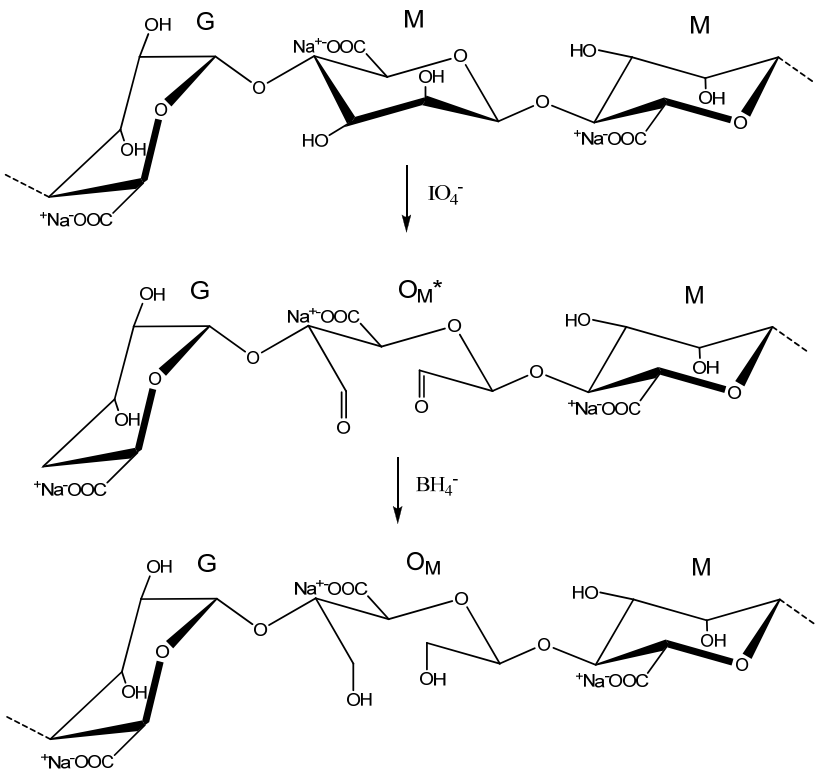

Figure 15: Chemical structure of an alginate fragment (..GMM..) before and after partial periodate oxidation (oxidation occurs randomly) and the corresponding structure obtained after aldehyde reduction.

A broad range of alginate samples were studied, including samples made by epimerization of mannuronan, using the C-5 epimerases in vitro. Periodate oxidized and borohydride reduced alginate (Figure 15) was also studied, giving valuable background information to the study of oxidized/reduced alginate hydrogels.

The chain stiffness was evaluated on the background of the Mark-Houwink-Sakurada (MHS) relationship and the persistence length (q) obtained from Bohdanecký analysis. The static light scattering with off-line viscometry gave the MHS relation: $[\eta] = 0.00504 \cdot M^{1.01}$, for alginate from *Laminaria hyperborea* leaf and stipe. Using SMV the MHS plot was similar, independent of the chemical composition of M and G. The plot however possessed a slight curvature for all alginates analysed. The main reasons for this curvature could most likely be ascribed to the influence of non-Newtonian behaviour (shear thinning), since the on-line viscometer operated at a rather high shear rate, but could also be a result of the change in hydrodynamic volume with M, previously observed for hyaluronic acid (Mendichi et al., 2003). Due to this curvature the MHS equation was split into three regions describing different molecular weight regions (Vold et al., 2007):

20 000 Da < M < 100 000 Da: $[\eta] = 0.0051 \cdot M^{1.00}$

100 000 Da < M < 300 000 Da: $[\eta] = 0.0349 \cdot M^{0.83}$

300 000 Da < M < 1 000 000 Da: $[\eta] = 0.0305 \cdot M^{0.66}$

Periodate oxidized alginate showed a decrease in intrinsic viscosity independent on molecular weight (MW). For instance a threefold decrease was observed for a sample oxidized 44 mol% and also the MHS exponent was reduced from 1.00 to 0.65. This indicates that the polymer has become more flexible, which was expected considering previous data (Lee et al., 2002; Smidsrød & Painter, 1973). The Bohdaneký analysis verifies these results and a *q* of 15 nm was calculated for all alginates, while *q* of periodate oxidized alginate was considerably lower (below 4 nm for a 44% oxidized sample). Figure 16 shows alginate $q_{(I=0.17)}$ as a function of P_0. It should however be noted that the absolute values can be somewhat questionable for the most oxidized samples, which approach the behaviour of random coils, possibly causing an overestimation of chain stiffness.

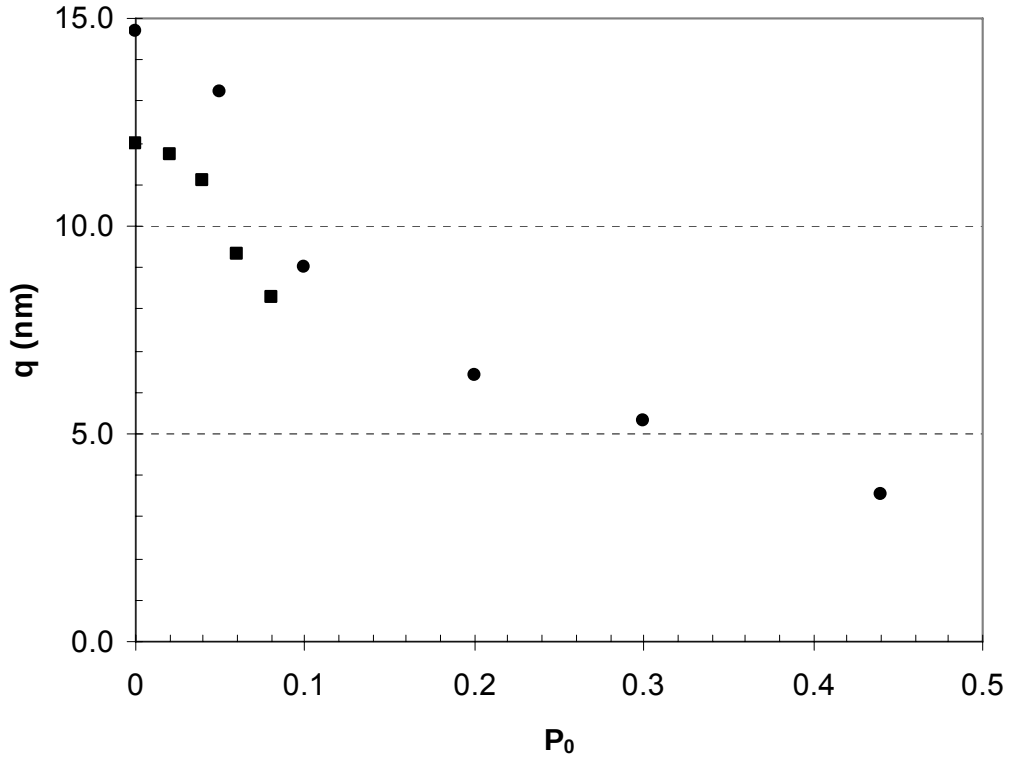

Figure 16: The dependence of the degree of periodate oxidation on the calculated persistence lengths (q) of alginate from *Laminaria hyperborea* leaf (F_G = 0.55) (•) and mannuronan epimerized by AlgE6 (F_G=0.90) (■). The ionic strength (I) was 0.17 M.

Since *q* is influenced by the electrostatic interactions between chains, it might differ when determined at different ionic strengths. One strategy to deal with this variability is to introduce an electrostatic term (q_{el}) into the equations for determining *q*. In this work however, a different approach was introduced by exploiting the empirical relationship between intrinsic viscosity and ionic strength, known as the Smidsrød B parameter. The relationship between the intrinsic viscosity and the ionic strength is:

$[\eta] = [\eta]_\infty + B[\eta]_{0.1}^{\upsilon}(I^{-0.5})$

In this way the viscosity, and thus also *q,* can be calculated at infinite ionic strength (q_∞). Such a value might be of interest when comparing *q* between charged and uncharged polymers. For the various alginates analyzed with different composition of M and G, q_∞ was found to be 12 nm.

A general observation in the analysis of alginates, commercial alginates in particular, was an upturn in the log M - V plot near the void volume. This could be due to incomplete separation of the largest molecules, the presence of aggregates, or both. In most cases it was due to incomplete separation, since no deviating geometries were indicated in the R_G - M curve.

Since the commercial software did not have an option fitting data to models that could account for both the upturn and at the same time extrapolate properly in the low molecular weight region, a 'split peak' approach was adapted after exporting the data to an Excel spreadsheet. Small amounts of particulate material found in some samples might explain the variance in previous data describing alginate chain stiffness.

In the light of the structural changes periodate oxidation has upon alginate, it was thought interesting to try to epimerize oxidized/reduced mannuronan using the C-5 epimerases. In this way alginates with higher chain flexibility could be tailored. Some of these materials could be interesting to study as building blocks for alginate hydrogels. First, however the chemical structure of periodate oxidized mannuronan was more closely investigated.

4.2 Chemical characterization of periodate oxidized mannuronan

Periodate oxidized mannuronan (with or without borohydride reduction) was analyzed by ^{1}H-NMR before attempting to add a second level of complexity by epimerizing this substrate. Since the acid lability of oxidized/reduced alginate is of interest, the spectrum after mild acid hydrolysis was also investigated.

The spectrum of a periodate oxidized mannuronan oligomer, DP_n~30, (Figure 17B) showed a series of peaks, particularly in the region 4.7 ppm - 5.2 ppm. These peaks were not present in mannuronan (Figure 17A). The reason for this complex peak pattern was probably the formation of inter and intra residue hemiacetals between reactive aldehydes and the alcohol groups (Painter & Larsen, 1970).

When the aldehydes were reduced by 20% (w/v) borohydride the spectrum was simplified and two new peaks appeared at 4.75 ppm and 4.85 ppm (Figure 17C). It was assumed that one of these peaks was due to the C1 proton in the oxidized unit (O_M-1M) and the other peak originated from the C1 proton in the neighbouring residue of an oxidized unit (M-1O_M). When the sample was exposed to mild acid hydrolysis (Smith degradation) the peak at 4.75 ppm disappeared from its position in the spectrum and a triplet at 5.05 ppm appeared. During acid hydrolysis the oxidized unit was split, causing its protons to shift or disappear from the spectrum as a new compound was formed. The disappearing peak was therefore assigned to O_M-1M, while the peak at 4.85 ppm was assigned to M-1O_M.

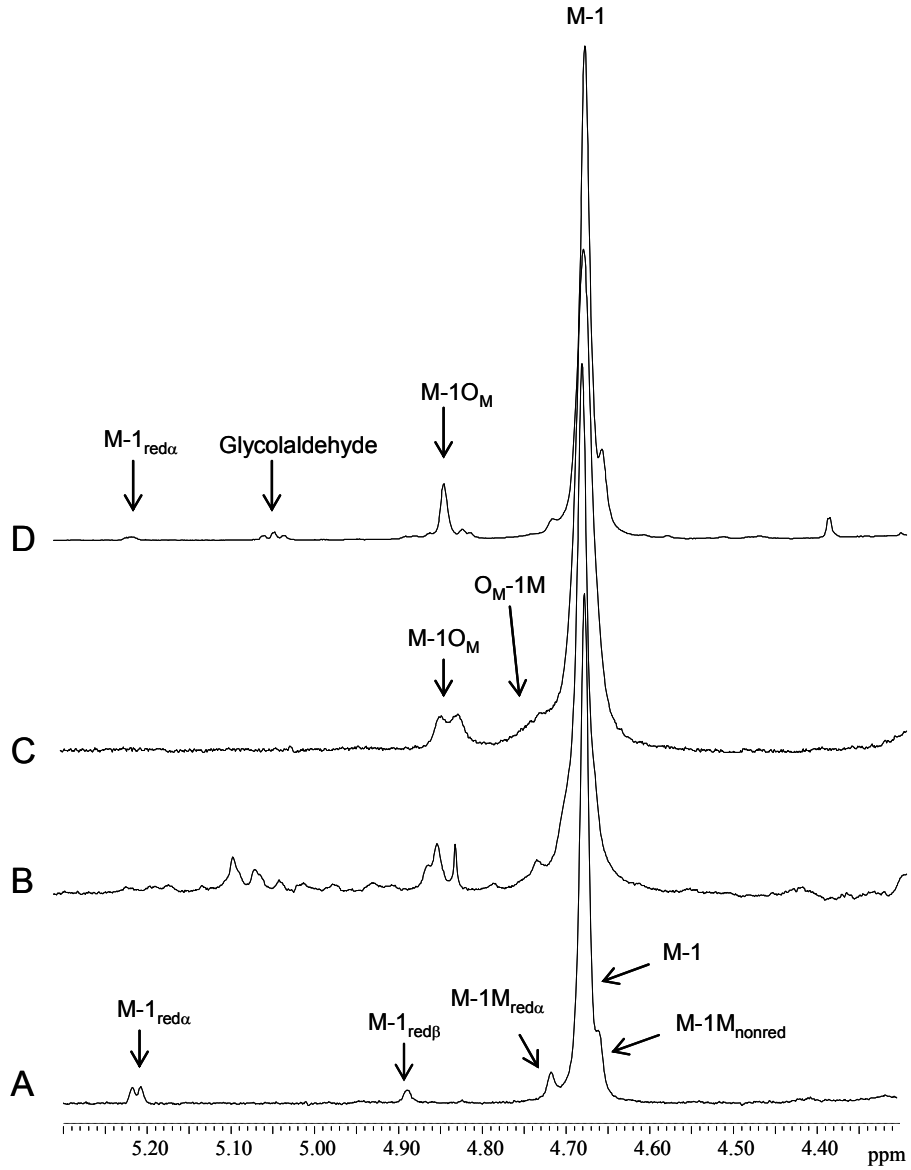

Figure 17: The ^{1}H-NMR spectra of mannuronan oligomers (DP_n~30); untreated (A), P_0 = 0.10 (B), P_0 = 0.10, borohydride reduced (C), P_0 = 0.10, borohydride reduced and acid degraded (D). Peaks were assigned according to Grasdalen (1983) except for the peaks M-1O and O_M-1M (see text).

The Smith degradation of mannuronan can tentatively be described as in Figure 18. The triplet found at 5.05 ppm in the spectrum was therefore attributed to either one of the protons on the remains of the oxidized unit still connected to the polymer or to glycolaldehyde, which was formed during degradation.

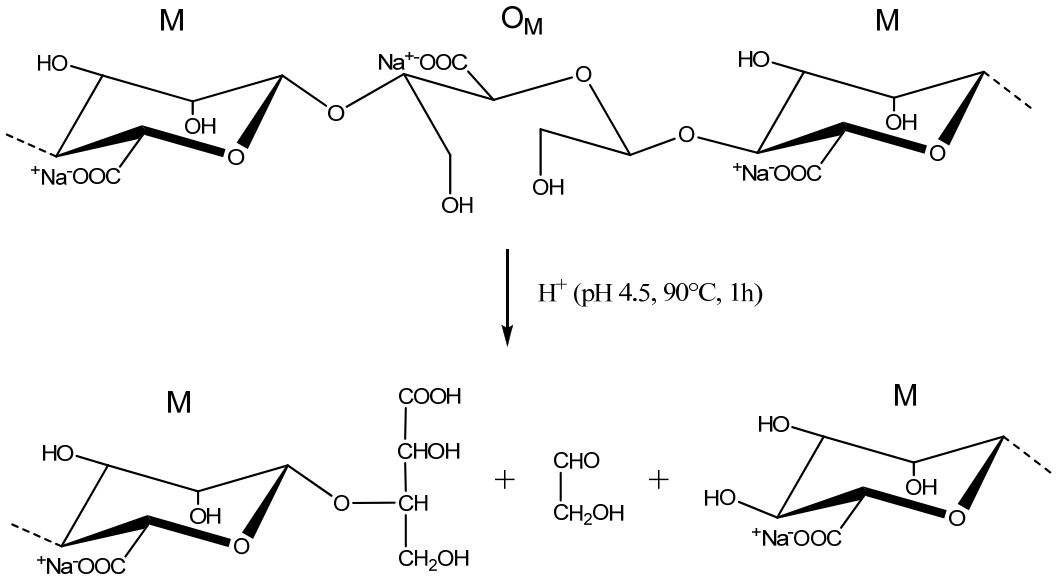

Figure 18: Mannuronan treated with sodium periodate, sodium borohydride and degraded by mild acid hydrolysis. Suggested reaction products based upon Fransson & Carlstedt (1974) and Sharon (1975). The glycolaldehyde shown exists in equilibrium with its hemiacetal form in water.

A further investigation of the Smith degradation of mannuronan was carried out in the NMR tube at pD 4.5. Signs of degradation could be observed already after 15-30 minutes. In addition to a weak signal from M-$1_{red\alpha}$, the triplet signal at 5.05 ppm was observed. It was formed almost instantly and was thereafter constant, while the reducing ends increased as the hydrolysis proceeded. It is previously reported that the oxidized and reduced units of methyl α-glucoside are hydrolysed at a rate 10^5 times faster than the unoxidized residues (Sharon, 1975). It was therefore assumed that the oxidized unit was split immediately and that the increasing reducing end signal was observed as a consequence of the slower hydrolysis of glycosidic bonds. A ^{1}H 2D COSY experiment was conducted and it was found that the protons seen as a triplet at 5.05 ppm did not couple with any of the protons on the polymer, but had a cross peak at 3.51 ppm, pointing towards the existence of glycolaldehyde. This was further verified by a ROESY experiment, where no cross peaks were found to the protons on the polymer suggesting that the signals at 5.05 ppm and 3.51 ppm originated from a single compound. Finally, to prove the existence of glycolaldehyde a ^{13}C HSQC was recorded. In this experiment the protons at 5.05 ppm coupled to a carbon atom with chemical shift of 90.54 ppm, which could be assigned directly to the hemiacetal group of glycolaldehyde. The doublet peak at 3.51 ppm coupled to a carbon atom with chemical shift of 65.29 ppm, which fits with the expected chemical shift for the alcohol group. The peak assignment was further in accordance with the listed spectrum (accession # bmse000258) deposited in the Biological

Magnetic Resonance Databank (http://www.bmrb.wisc.edu/) for glycolaldehyde in D_2O and with previous literature describing glycolaldehyde in its hydrated form (Amyes & Richard, 2007; Glushonok et al., 2000).

4.3 Periodate oxidized/reduced mannuronan as substrate for AlgE4 and AlgE6

Using the C-5 epimerases upon oxidized/reduced mannuronan would make it possible to design alginates with different patterns of M and G residues separated by flexible units (Figure 19). The AlgE6 enzyme converts M-residues into long G-blocks, while AlgE4 epimerize every second M-residue resulting in strictly alternating sequences of M and G.

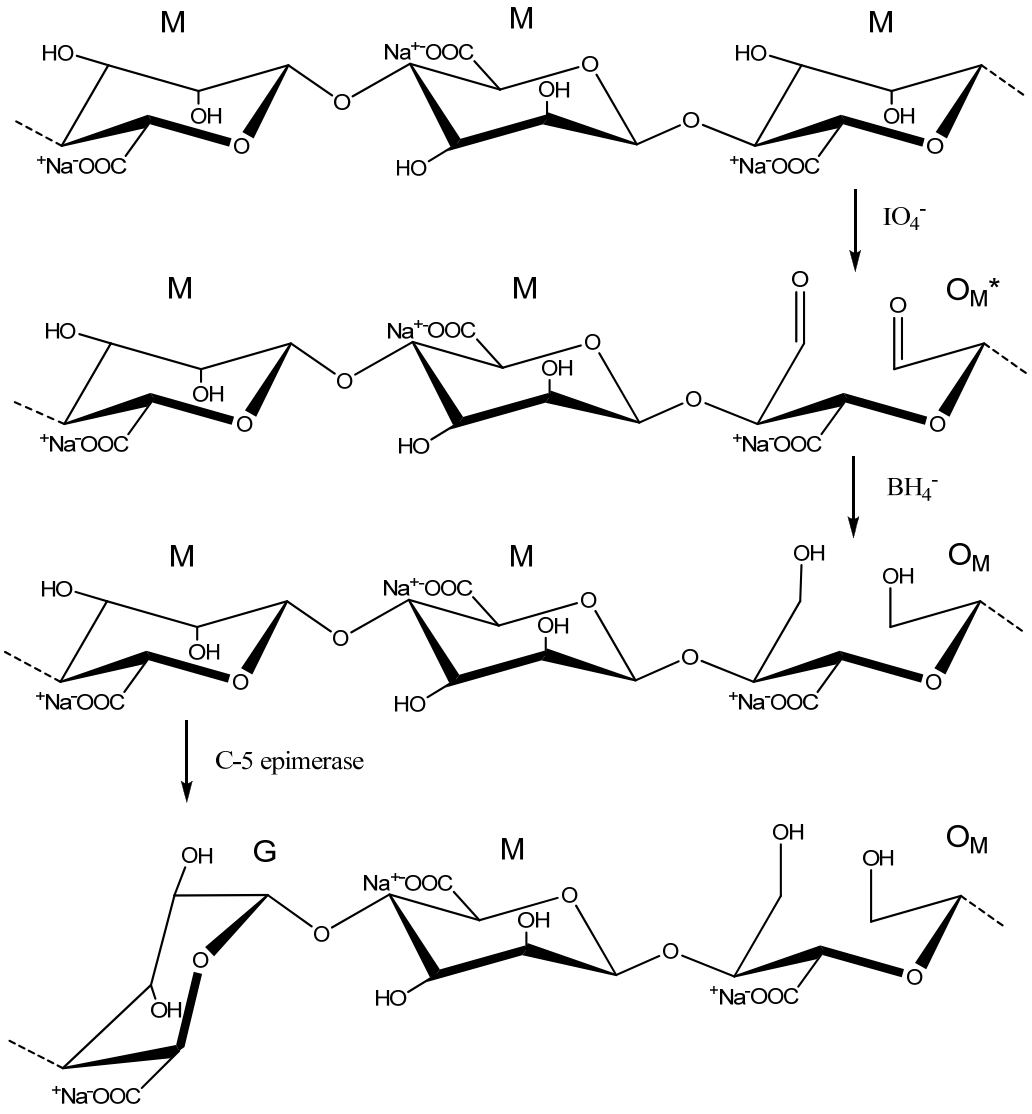

Figure 19: Structure of a mannuronan fragment (…MMM…) followed by periodate oxidation, borohydride reduction, and epimerization (…GMM…).

Epimerization was attempted using AlgE4 and AlgE6 and it was found that both enzymes indeed could epimerize the oxidized/reduced mannuronan substrate, for P_0 equal to 0.05 and 0.10 as shown in Table 3. The epimerization conditions introduced 46 % G in the case of AlgE4, and 90% G for AlgE6, when acting on pure mannuronan. It was assumed that the enzyme was not able to epimerize oxidized units, which seems to be supported by the results, since the fraction of G units (F_G) decreased as P_0 increased.

Table 3: The fraction of guluronic acid (F_G) and G-block length ($N_{G>1}$) are shown for periodate oxidized/borohydride reduced mannuronan epimerized with AlgE4 (i) and AlgE6 (ii).

Sample	P_0	F_G	$N_{G>1}$
i	0.00	0.46	0.00
i	0.05	0.26	0.00
i	0.10	0.14	0.00
i	0.20	0.00	0.00
ii	0.00	0.40	5.80
ii	0.05	0.34	4.80
ii	0.10	0.29	3.00
ii	0.20	n.d.	n.d.

n.d. - not detected

The action of AlgE4 and AlgE6 upon mannuronan oligomers has been investigated previously. It was found that AlgE4 needs a minimum of six consecutive M-residues to start epimerization, while eight residues are needed for AlgE6 (Campa et al., 2004; Holtan et al., 2006a). Assuming random oxidation, the distribution of blocks consisting of contiguous unoxidized M-residues can be calculated by directly applying the theory of random depolymerisation (or polymerisation) of linear polymers (Tanford, 1961). In this case the degree of oxidation (P_0) replaces the degree of chain scission (α) used in the original formula. We assume this approach is valid for $P_0 < 0.5$, which is the well-known oxidation limit of alginate (Larsen & Painter, 1969). The weight fraction of blocks with *n* contiguous M-residues ($W(n_M)$) for an indefinitely long chain then follows the expression:

$$W(n_M) = n_M P_0^{\,2} (1 - P_0)^{(n_M - 1)}$$

n_M - number of unoxidized mannuronic acid residues.

When P_0 is 0.10, $W(n_M \geq 8)$ is 0.81 indicating that the enzymes have several M-stretches to attach and if P_0 is increased to 0.20, $W(n_M \geq 8)$ is reduced to 0.50. It seems difficult to predict the F_G obtained by epimerizing an oxidized/reduced mannuronan substrate solely based upon this information, however it does explain why F_G is decreasing rapidly with increasing P_0. It was also not evident whether M units neighbouring an oxidized/reduced mannuronan unit could be epimerized, or if the increased flexibility of the polymer affected epimerization to any extent. These questions await further investigations.

4.4 Mannuronan oxidized before or after epimerization with AlgE6

Mannuronan was periodate oxidized/reduced before (Strategy I) or after (Strategy II) epimerization with AlgE6 (Figure 20). The aim was to make two different materials that could potentially form easily degradable and soft alginate gels. First, mannuronan was periodate oxidized to P_0 = 0.02-0.08 and epimerized by AlgE6 (Strategy I). Secondly, mannuronan was epimerized by AlgE6 to F_G = 0.90 and oxidized in the same range (Strategy II).

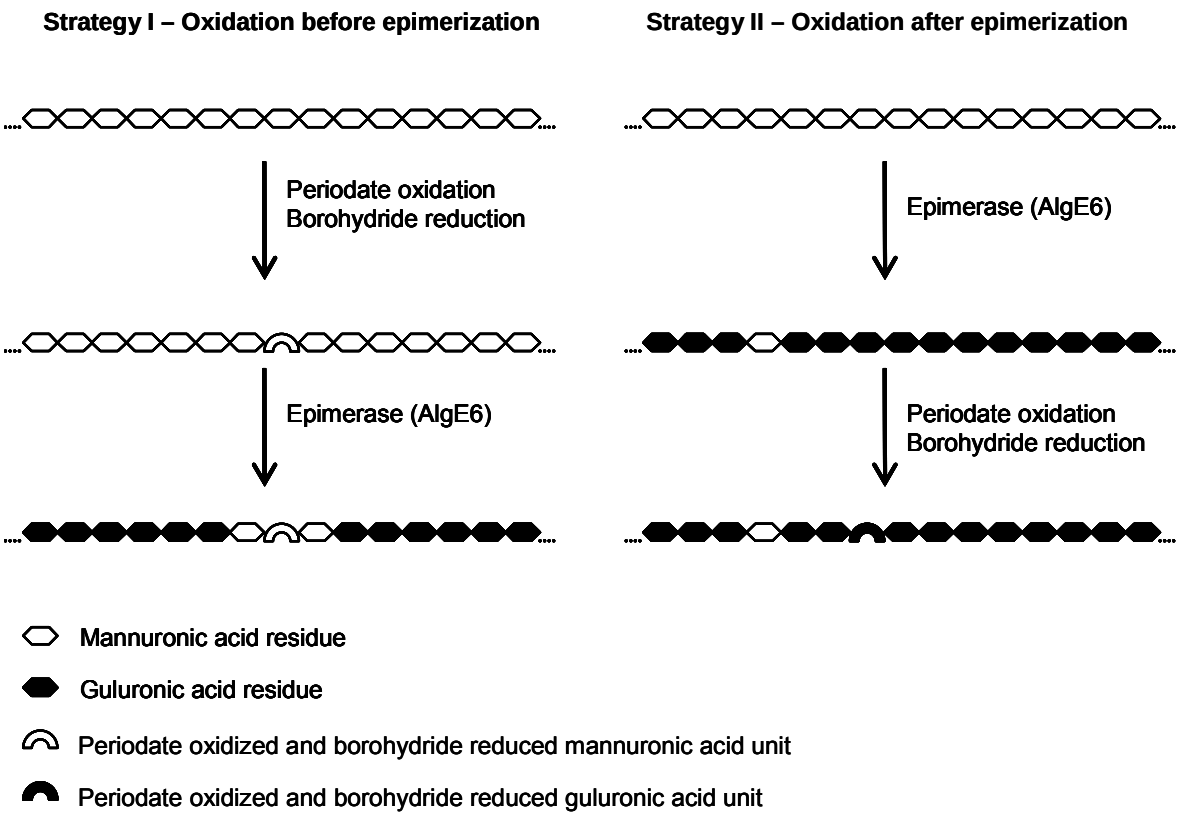

Figure 20: Schematic illustration of two strategies for making new alginate materials.

4.4.1 Mannuronan oxidized/reduced before epimerization (Strategy I)

The mannuronan oxidized/reduced before epimerization (Strategy I) was analysed by NMR. Figure 21 show the resulting ^{1}H-NMR spectrum of a sample with $P_0 = 0.08$. The peaks were assigned according to Grasdalen et al. (1983) and Holtan et al. (2006b). Similar to the spectrum of oxidized mannuronan the peak assigned as M-1O_M appear, while the peak O_M-1M does not appear, since the sample was exposed to mild acid hydrolysis to improve the quality of the NMR spectrum. The F_G could be calculated in the usual way (Grasdalen, 1983), but it should be mentioned that the peak originating from glycolaldehyde was present overlapping with the G-1 peak. Its area was, however < 2% of the G-1 signal in samples with $P_0 < 0.10$ and $F_G \geq 0.40$, and its presence was therefore ignored in the calculations. The calculated degree of oxidation (CDO) was obtained by dividing the area of the M-1O_M peak by the total signal originating from M and G units. Since the CDO fits well with the theoretical degree of oxidation it was concluded that all periodate was consumed in the P_0 range studied and that any over-oxidation could be neglected. A decrease in the fraction of G-residues and G-block length was observed as the degree of oxidation increased (Table 4). The main reason for this was that the enzyme had fewer sites for attack; a minimum of eight intact mannuronan residues is required for AlgE6 as stated earlier.

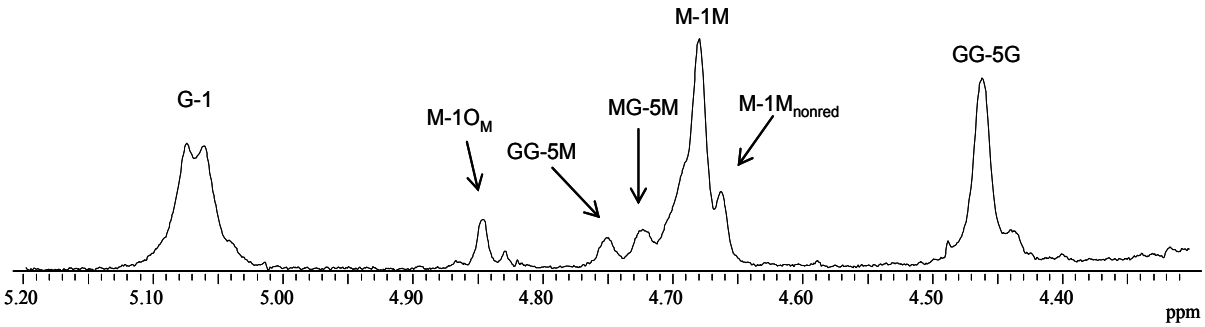

Figure 21: The ^{1}H-NMR spectrum of mannuronan with $P_0 = 0.08$ followed by epimerization with AlgE6 to $F_G = 0.43$. All samples are reduced with 20% (w/v) borohydride after oxidation and acid degraded (60 min pH 5.6 followed by 45 min pH 3.8, 90°C) before NMR analysis. Peaks were assigned according to Grasdalen (1983) except for the peak M-1O_M (see text).

4.4.2 Mannuronan oxidized/reduced after epimerization (Strategy II)

The mannuronan oxidized/reduced after epimerization (II) was analysed by NMR. Because of the high content of G-residues and the fact that the oxidation is assumed to be random (Painter & Larsen, 1970), mainly G-residues were oxidized. This was reflected in the spectrum as shown in Figure 22 of a sample with $P_0 = 0.08$. The peak M-1O_M was now

replaced by a peak at 4.61 ppm assigned to the proton on C-5 in the G-residue neighbouring an oxidized G-residue (G-5O_G). Since the probability of oxidizing an M-residue in the sample with $P_0 = 0.08$ was low (0.008%), assuming random oxidation (Painter & Larsen, 1970), the M-1O_M peak was not observed. It was therefore further assumed that all oxidized units were G-units. In analogy with the description of the above material (Strategy I), the peak of the H-1 proton of an oxidized G-unit (O_G-1G) was not observed, since it was hydrolysed before NMR analysis. The G-block length ($N_{G>1}$) can be calculated, but some assumptions must be made to the original expression given by Grasdalen (1983), since the oxidized G-units (G*) were now taken to terminate a G-block in the same way as an M residue. The following expression was suggested:

$$N^*_{G>1} = \frac{n_G - n_{MGM}}{n_{GGM} + n_{MGG^*} + n_{GGG^*}}$$

Due to low abundance and/or poor resolution of the signals some assumptions were made in order to simplify this expression.

1). n_{MGM} can be neglected since $n_{MGM} << n_G$. AlgE6 is a G-block forming enzyme and few single G-units are present.

2). All the oxidized guluronan residues (G*) are mainly within a G-block and the probability of the sequence MGG* is low, hence $n_{MGG^*} \sim 0$.

3). The signal from glycolaldehyde overlapping with the signal from H-1 on guluronic acid is ignored on the same grounds as described above.

The expression was then simplified to:

$$N^*_{G>1} = \frac{n_G}{n_{GGM} + n_{GGG^*}} = \frac{F_G}{F_{GGM} + F_{GGG^*}}$$

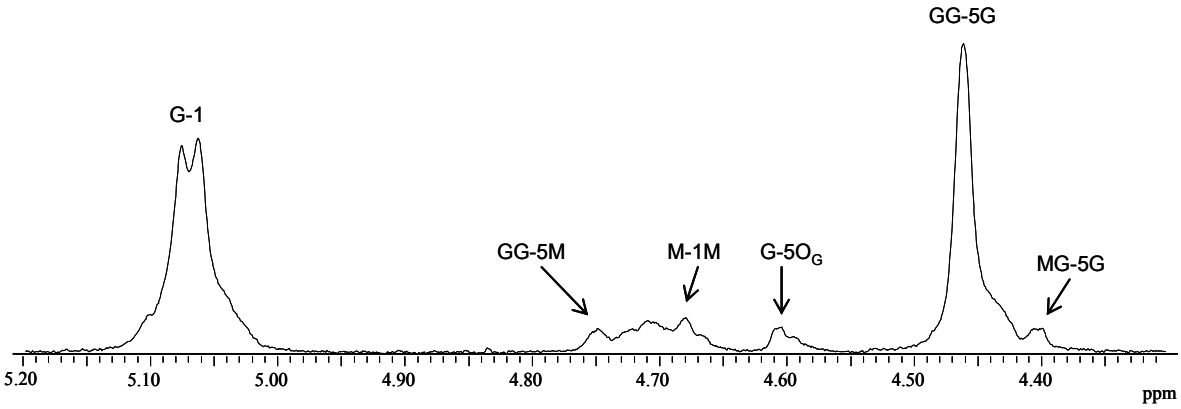

Figure 22: The ^{1}H-NMR spectrum of mannuronan epimerized to F_G = 0.90 followed by periodate oxidation to P_0 = 0.08. All samples are reduced with 20% (w/v) borohydride after oxidation and acid degraded (60 min pH 5.6 followed by 45 min pH 3.8, 90°C) before NMR analysis. Peaks were assigned according to Grasdalen (1983) except for the peaks and G-5O$_G$ and (see text).

Table 4 shows the G-block length calculated using this expression ($N_{G>1}$*) and it was observed that $N_{G>1}$* rapidly decreased as the degree of oxidation increased.

Table 4: Mannuronan was oxidized/reduced before (Strategy I) or after (Strategy II) epimerization with AlgE6. The fraction of G-residues (F_G), G-block length ($N_{G>1}$) and calculated P_0 (calc P_0) from ^{1}H-NMR spectra are shown for the different samples. 3 natural occurring alginates are shown for comparison.

Sample	**P_0**	**F_G**[1]	**$N_{G>1}$**[2]	**Calc P_0**	**M_w [kDa]**	**M_w/M_n**	**G' [Pa]**[3]
Mannuronan	0.00	0	-	-	161	2.5	-
I	0.02	0.75	16	0.02	96	2.2	1237
I	0.04	0.64	12	0.04	94	2.5	85
I	0.06	0.54	11	0.06	88	2.2	<1
I	0.08	0.43	9	0.07	71	2.1	<1
II	0.00	0.90	22	-	156	2.7	149
II	0.02	0.88	17	0.02	115	2.3	133
II	0.04	0.86	12	0.04	107	2.6	37
II	0.06	0.84	9	0.05	104	2.3	12
II	0.08	0.82	8	0.07	98	2.1	4
Durvilla antarctica[4]	0.00	0.34	5	-	146	2.0	108
Laminaria hyperborea leaf[4]	0.00	0.51	11	-	129	2.1	243
Laminaria hyperborea stipe[4]	0.00	0.65	12	-	117	2.3	308

[1]Assuming that the probability of oxidizing an M-residue is equal to oxidizing a G-residue (random oxidation)

[2]for samples II $N_{G>1}=F_G/(F_{GGM}+F_{GGG*})$, see text.

[3]after 24 h. Samples I were prepared using 20 mM Ca^{2+}, while 10 mM Ca^{2+} were used for samples II.

[4]acid hydrolysed.

4.4.3 Molecular weight and chain stiffness analysis

The SMV analysis of both materials (Strategies I and II) showed a decrease in molecular weight compared to the parent mannuronan (Table 4). Depolymerisation during periodate oxidation of alginate is well-known and has been reported previously (Painter & Larsen, 1970, Vold et al., 2006). In this study, interestingly, M_w was almost independent of P_0 in the range 0.02-0.08. Table 4 also shows that the mannuronan samples oxidized/reduced before epimerization had a lower M_w compared to the samples oxidized/reduced after epimerization. This difference was most likely due to degradation of the oxidized/reduced mannuronans during epimerization. In a separate experiment, epimerization of oxidized (non-reduced) mannuronan was carried out, showing that the degradation was even more severe. Since the conditions for epimerization (pH 6.9, 37 °C, 48 h) are relatively mild, this describes the instability of the oxidized/reduced mannuronan residues and the even more unstable oxidized/non-reduced residues under these conditions. This observation is in accordance with a previous study describing the degradation of oxidized alginates (Bouhadir et al., 2001).

The exponent (a) and constant (K) in the Mark-Houwink-Sakurada (MHS) relationship, $[\eta] = K \cdot M^a$), is a measure of the shape and extension of polysaccharide chains. No large differences could be observed for these parameters for the oxidized/reduced samples (Figure 23). The persistence length (q) is another measure of polymer flexibility. In section 4.1, q of a broad range of oxidized/reduced alginate samples were determined using the Bohdanecký wormlike chain model. A pronounced decrease in q with increasing P_0 was observed, for instance q decreased from 12 nm for the unoxidized sample to 4 nm for a sample with P_0 = 0.44. In this case q was calculated in the same way (ionic strength: 0.17 M), and a value of 12-11 nm was obtained for the samples with P_0 = 0.00-0.04. The samples with P_0 = 0.06 and 0.08 showed a q equal to 9 nm and 8 nm, respectively. This indicates that the overall flexibility of the samples increased as P_0 increased, which is in agreement with previous studies (Lee et al., 2002; Smidsrød & Painter, 1973).

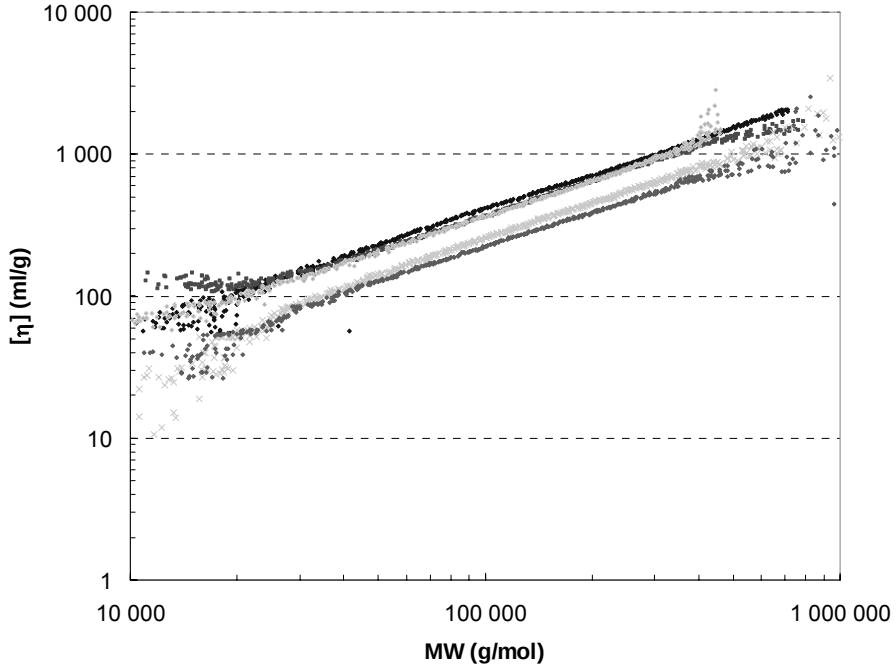

Figure 23: MHS plot of mannuronan oxidized/reduced after epimerization (Strategy II). P_0 = 0.02, 0.04, 0.06 and 0.08.

4.5 Gelation of mannuronan oxidized/reduced before or after epimerization

Mannuronan oxidized/reduced before or after epimerization were studied by small-strain oscillatory measurements. The hydrogels were made by the 'internal setting' method as described by Draget et al. (1993). In this method a solution of alginate and $CaCO_3$ is acidified, when D-glucono-δ-lactone (GDL) slowly hydrolyses. This process releases free Ca^{2+} ions from $CaCO_3$, which allows junction zones to be formed between the alginate polymers, leading to homogenous gel formation. The small-strain oscillatory technique was regarded the best option to study these gels, since it gave information about the gelling kinetics and required relatively small sample amounts compared to other rheological tests.

Several parameters can influence the gel elasticity and kinetics. In this particular study the physical parameters were kept constant, while chemical parameters such as F_G, $N_{G>1}$ and molecular weight distribution (MWD) was varied. The main purpose of this investigation was to study the impact of periodate oxidation upon the gel elasticity. The temperature was set to 20 °C in all experiments and the gelling kinetics was studied within a timeframe of 24 h.

Mannuronan oxidized/reduced before epimerization (Strategy I) was studied by small-strain oscillatory measurements, and 20 mM Ca^{2+} and 40 mM GDL was used. In Figure 24A, the

dynamic storage modulus (G') as a function of time is shown. It can be observed that periodate oxidation has a pronounced effect upon G', and only samples with $P_0 = 0.02$ and $P_0 = 0.04$ formed true gels by the definition G' > G'' (G''; dynamic loss modulus) over a wide range of frequencies (Kavanagh & Ross-Murphy, 1998). The two samples that do form true gels start the sol/gel transition at approximately 1.7 h, as observed by a significant rise in the dynamic storage modulus (G'). Even though the gelling started at the same time, the kinetics were different for the two samples. The $P_0 = 0.02$ sample exhibited a steeper increase in G' than the $P_0 = 0.04$ sample. The terminal G' value after 24 h decreased dramatically when the degree of oxidation increased, as can be observed in Figure 24A and Table 4. The molecular weight and $N_{G>1}$ has been pointed out to be the most influential parameters on alginate hydrogel elasticity (Draget et al., 1993a, b, 2000). Even though the samples were degraded during periodate oxidation, the difference in molecular weight was not sufficient to explain the drop in G' between the different oxidized samples. It was therefore concluded that the reason could be found in other consequences of periodate oxidation and two such consequences might explain the observation. First, the distribution of G-blocks might be different in the oxidized/epimerized samples compared to native alginates. Secondly, an increase in <u>local</u> chain flexibility may result in a higher probability for intramolecular cross-links, where the alginate molecule partly folds back on itself instead of forming intermolecular neighbouring cross-links, which is a pre-requisite for the formation of functional elastic segments. In order to gain further understanding of this phenomenon, the sample with $P_0 = 0.06$ and alginate from *L. hyperborea* leaf with similar F_G, $N_{G>1}$ and polydispersity index (M_w/M_n) was compared with regard to the final G' obtained after 24 h (Table 4). It must be noted that the molecular weight differs by approximately 30 %. The G' after 24 h of the sample $P_0 = 0.06$ was less than 1 Pa, while the unoxidized alginate sample mentioned above was 243 Pa (Table 4). Even though there was a difference in molecular weight, this cannot account for the large decrease in G', and it is suggested that the increased flexibility was the main reason for the large decrease.

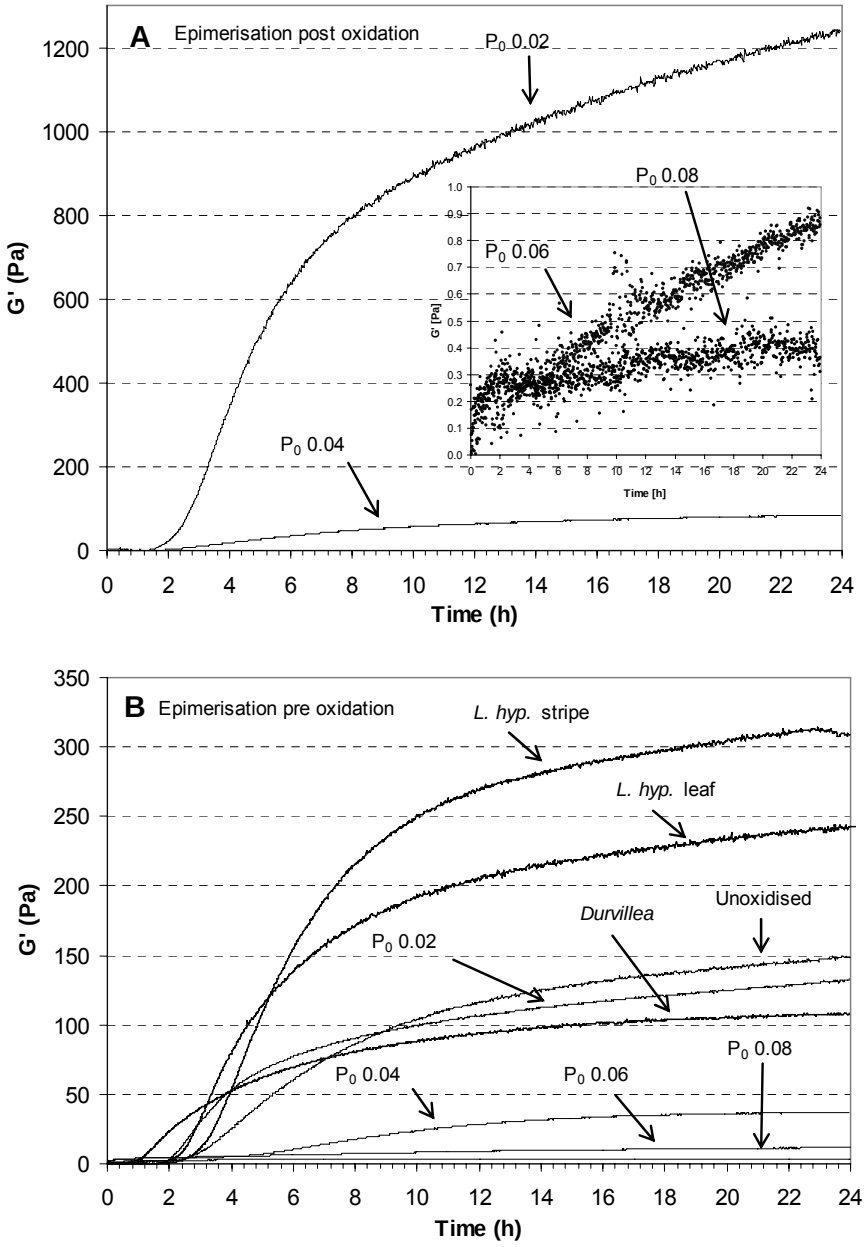

Figure 24: A: Dynamic storage modulus (G') versus time (h) for mannuronan oxidized/reduced before epimerization with AlgE6 (Strategy I). B: G' versus time (h) for mannuronan oxidized/reduced after epimerization with AlgE6 (Strategy II). Alginate from *Laminaria hyperborea* (leaf and stipe) and *Durvillea antarctica* are shown for comparison. The gels were prepared by 'internal setting' (A: 20 mM $CaCO_3$/40 mM GDL. B: 10 mM $CaCO_3$/20 mM GDL).

Mannuronan oxidized/reduced after epimerization (Strategy II) was also studied by small-strain oscillatory measurements. Since the Ca^{2+} concentration used for the samples categorized as Strategy I lead to syneresis of the gel for the unoxidized sample with $F_G = 0.90$, half the concentration of Ca^{2+} and GDL were used (10 mM Ca^{2+} and 20 mM GDL) for the samples belonging to Strategy II and for the native alginates. Alginate from *D. antarctica* and *L. hyperborea* stipe/leaf with varying F_G, $N_{G>1}$ and molecular weight were also included for

comparison (Table 4). The final gel elasticity decreased in much the same way as the samples described above (Strategy I) (Figure 24B). Alginate from *L. hyperborea* stipe and the sample $P_0 = 0.04$ (Strategy II), which have similar $N_{G>1}$ and molecular weight, can be compared. The latter have a G' of 308 Pa, while the first have a G' of 37 Pa, suggesting that it was the increased local flexibility and the different distributions of G-blocks that was the cause of the difference in G' values. Figure 24B also shows that for the sample $P_0 = 0.02$, the sol/gel transition started approximately 30 min before the unoxidized sample (Strategy II). The reason for this is not clear, but can be tentatively explained by a faster organization of the gelling zones due to increased local flexibility. This theory is partly supported by the observations made by Draget et al. (2000). They investigated Ca^{2+}-gels of alginates with different sequential arrangements of M-blocks and G-blocks following epimerization with AlgE4 leading to the introduction of MG sequences at the expense of M-blocks. It has been reported that the local flexibility within alginate molecules differs depending on their composition of M and G (Smidsrød et al., 1973; Stokke et al., 1991). Thus it was observed that alginates containing more flexible regions initiated gelling at an earlier stage than alginates possessing less local flexibility.

When the two set of samples (Strategy I and II) are compared, it can be observed that G' (24 h) of the samples with $P_0 = 0.06$ and $P_0 = 0.08$ for the series oxidized/reduced before epimerization was marginally lower than the same samples oxidized/reduced after epimerization (Table 2). This might seem surprising since the gels of the samples oxidized/reduced after epimerization was prepared using half the amount of Ca^{2+} and GDL. However, this difference could be explained by the fact that the M_w difference between the two set of samples were approximately 20-30%. This M_w difference could affect the G' to some extent (Draget et al., 1993), but the main reason was probably the low accuracy of the measurement, when G' got close to, or below, 10 Pa.

Finally, it can be stated that periodate oxidation is a useful tool to control gel strength/elasticity of alginate hydrogels. Despite the decrease in molecular weight upon oxidation, it seems clear that it is mainly the increased flexibility, and not only an overall decrease in G-block length that cause the altered gel characteristics.

5. GENERAL DISCUSSION

5.1 Carbonyl detection in polysaccharides

The carbonyl profile detection method developed in this study can be regarded as a general method for the analysis of polysaccharides dissolved in an aqueous solvent. The motivation for establishing such a method is wide. Polysaccharides are used in several applications, and we are relying on their structure to give products certain characteristics. If their structure is changed as a consequence of the introduction of carbonyl groups, for instance making them more prone to degradation, a precise method for carbonyl detection will be of great importance. Further, some potential specific areas of application can be pointed out. First, there is an increasing demand for well characterized polysaccharides to be used as standard/reference polymers, and this method can contribute to the validation of such standards. Secondly, polysaccharides are sometimes exposed to chemical reactions during extraction/purification or modification, which may introduce small amounts of carbonyls. This method can then be of interest in the study of the final product. Finally, the natural aging of polysaccharides in the presence of light and oxygen might lead to oxidation, which introduce aldehyde/or keto groups into the polymer. The latter is a well known phenomenon, particularly in cellulose (Henniges et al., 2006), and is known to affect the stability of the polysaccharide structure. The sensitive carbonyl assay may detect signs of aging at an early stage.

It should be emphasised that the carbonyl detection method described as one method in fact involves three different labelling approaches. The appropriate approach should be selected depending on the sample. This means that not all approaches/strategies can be applied for all samples. The criteria for selection are mainly the polysaccharides pH stability, and (if known) some knowledge regarding which range the carbonyl content is likely to be within. If one wishes to separate between aldehydes and ketones the samples must be analyzed by both the tritium (or the CCOA approach), and the 2-AB approach. The difference in carbonyl content can then be ascribed to keto groups, since NaB^3H_4 reduces both aldehydes and ketones, while 2-AB only reacts with aldehydes.

5.2 A re-investigation of the reactivity of sphagnan towards amino compounds

The re-investigation of sphagnan revealed that its reactivity with amino substances was much lower than previously reported and offered further evidence for the non-existence of 5-KMA. This might seem disappointing considering the promised applications of sphagnan in biotechnology as a tanning agent (and possibly many other uses), but also because its role in nature (binding nitrogen) is probably not as significant as first anticipated. However, it is important that these questions now have a definite answer. That being said, sphagnan might still be an interesting polysaccharide for further studies. The method developed, as a part of this thesis, to detect carbonyl group profiles reveals that the high MW fraction contains a substantial amount of carbonyl groups, up to 22-24 per polymer molecule. Although this is a very small part of the sample, it is a structural feature that separate sphagnan from other pectins. Analysing this fraction in greater detail using other methods (e.g. acid hydrolysis followed by NMR/MS) might show new structural features and possibly give further information on how sphagnan is linked to the hyaline cell wall. In the chemical sense this part of the sample is indeed reactive with amino compounds, such as 2-aminobenzamide (2-AB), and this reactivity is free to be exploited in a commercial context.

5.3 Alginate chain stiffness and extensions

The alginate chain stiffness and extensions were studied. The data obtained for the exponent in the MHS relationship, along with persistence lengths from Bohdanecký analysis were generally in accordance with other literature data. However, literature data do vary somewhat, possibly as a consequence of particulate material in the sample and/or the presence of aggregates. It is believed, that this study contributes to clarify the picture of the conformation of alginate in solution given the big selection of pure alginate samples studied.

It should be mentioned that the similarity of the MHS exponent between alginate with different chemical composition (both naturally occurring and in vitro epimerized) contrasts some earlier studies (Smidsrød et al., 1973; Stokke et al., 1993). Since it is not possible to re-investigate the samples analyzed by Smidsrød et al. (1973), it is difficult to find the reason why they detect different chain stiffness depending on the alginate chemical composition. It is possible that the result is due to impurities in or aggregation of the samples. Such aggregates might be hard to spot, when MALLS is not coupled with SEC. The advances in instrumentation, especially the option of including an on-line viscometry detector, make the

analysis of a broad range of samples easier and more precise than in some earlier studies. The fact that no differences in chain flexibility was observed is further at variance with theoretical calculations of alginate chain extension based on the local chemistry of alginate monomers and the geometry of the four possible glycosidic linkages (MM, MG, GM, GG) (Stokke et al., 1993). On the other hand they are collaborated by the studies of Dentini et al. (2005) showing that the B parameter of *in vitro* epimerized alginates was largely independent of the chemical composition. It could also be that the difference in overall chain stiffness is so small that it cannot be detected by the methods considered above. Even though the overall chain stiffness was similar for alginates with different content and organisation of M and G, it seems clear that on the local scale the M-G and G-M linkages do give rise to 'kinks' in the chains, thereby changing the local directionality. This is particularly important information in the study of alginate gels.

5.4 Gelation of mannuronan oxidized/reduced before or after epimerization

The use of oxidized/reduced mannuronan as a substrate for the C-5 epimerases AlgE4 and AlgE6 was studied. It was found that the epimerases could act upon the stretches of M that was not affected by the oxidation. This means that alginates with stretches of poly-G or poly-MG separated by oxidized residues could be tailored. These materials separate from unmodified alginate in both chemical and physical sense.

Chemically, it means that the oxidized residues can be degraded much faster than the glycosidic linkages between intact G and M residues, thus offering a way of controlled degradation under mild acidic conditions. If P_0 is known, the sample can be exposed to mild acid hydrolysis and the resulting DP_n will correspond to the number of intact residues present between the oxidized units. This can be important in situations where alginate is used as an encapsulation material for drugs and it is crucial that the drug is released in a controlled way under acidic conditions.

Physically, the oxidation has an effect upon alginate chain stiffness and extension, and if hydrogels are formed the behaviour is different compared to gels made of unoxidized alginates. The difference in chain flexibility is due to the oxidized monosaccharides, which have almost unrestricted rotation. Gels made of such materials can lead to several effects as discussed earlier (see section 4.5). Gels made of alginate with low P_0 initiate gelling at a stage

prior to the unoxidized samples. Generally the materials form weaker gels (lower dynamic storage modulus (G')) as the P_0 increase. The P_0 also affect the G-block length ($N_{G>1}$). By comparing naturally occurring alginates from *Durvillea antarctica* and *Laminaria hyperborea* (stipe and leaf) with similar MW and $N_{G>1}$, it is suggested that it was mainly the increased flexibility and a different G-block distribution that caused the gels to have different physical properties. Without undermining the importance of a different G-block distribution in the oxidized/reduced samples, the differences in G' between these and naturally occurring alginates was so large that the increased flexibility was likely to be the main reason for the different physical properties. Like the difference in chemical properties, the difference in physical properties might also find various applications. As shown, obtaining high P_0 was an efficient way to 'knock out' the gelling properties of alginate. In some applications one could imagine that this might be convenient, even though no such application exist at the present. However, the addition of alginate to for instance dairy products might lead to gelling or aggregation, due to interactions with divalent ions, such aggregation/gelling could be unwanted. In this case oxidized/reduced alginate might be preferred.

Finally, it should be mentioned that even though the periodate oxidation so far has been described as a good strategy to modify alginate materials, the degradation accompanying it restricts its use to some extent.

5.5 Future studies

The carbonyl detection method developed could potentially be applied in various cases in the future as mentioned in section 5.1.

The original interest in sphagnan has been lost as a consequence of the non-existence of 5-KMA. However, its carbonyl content seems to differ from other polysaccharides possessing only the reducing end carbonyl. Further studies of different MW fractions of sphagnan might give more insight into the nature of these carbonyl groups and why they arise, but at the present this merely seems to be of theoretical interest.

The results presented of tailored alginate materials with increased flexibility are initial results. More studies should be carried out with a larger selection of samples. It would also be extremely useful to have samples with generally higher molecular weight (> 150kDa), since

G’ is influenced by this parameter, especially if M_w is below 70-80 kDa. In order to obtain such samples one has to start with mannuronan with higher molecular weight or find a way to diminish the degradation during periodate oxidation. If larger amounts of sample can be obtained, other rheological tests, such as measuring Young’s modulus might reveal more information. Although some lose thoughts has been put forward in this thesis regarding the potential application of such materials, it should be emphasized that a substantial amount of research must be carried out in order to turn these materials into some form of commercial product.

6. CONCLUDING REMARKS

This thesis deals with carbonyl groups in polysaccharides, their detection, and impact on the polysaccharide chemical and physical structure. The main findings are summarised below.

The ability of the pectic polysaccharide sphagnan to bind amino containing substances was re-investigated, mainly because of its previously described ability to bind nitrogen compounds in nature. It was found that its reactivity was much lower than previously reported. The presence of a new monosaccharide unit, named 5-keto mannuronic acid (KMA), was also reported previously. No evidence for 5-KMA was found, which is in accordance with recent literature, and instead a flaw in the colorimetric assay used as one of several methods to detect 5-KMA was discovered. Collagen treated with sphagnan revealed that it did not meet the demands for a 'true' tanning agent and that any interaction was merely electrostatic. During this work one became aware of the lack of a proper method for carbonyl profile detection in polysaccharides dissolved in an aqueous solvent.

A method for detecting carbonyl group profiles, within polysaccharides, based on three different labelling approaches/strategies was developed. The method was applied to sphagnan and the result revealed an abnormally high carbonyl content at high molecular weights, due to aldehydes. The aldehyde structures are most likely introduced in nature and not during extraction. This conclusion is reached because sphagnan extracted with only mild acid hydrolysis, which is not known to introduce intramolecular carbonyl groups, give similar results as sphagnan extracted the conventional way (using chlorite). The analysis also indirectly proves that 5-KMA does not exist in sphagnan.

The chain stiffness and extensions of alginates with different chemical composition and periodate oxidized and borohydride reduced alginate was analysed using SMV. No difference in the exponent (a) in the Mark-Houwink-Sakurada (MHS) relationship, or persistence length (q) based on the Bohdanecký analysis, was detected for different natural and in vitro epimerized alginates. However, a big difference was detected in oxidized/reduced alginate and the MHS exponent decreased from 1.00 before oxidation to 0.65 for 44% oxidation. Even though the oxidized samples were mainly a positive control of the method for probing differences in chain stiffness, they also demonstrated that periodate oxidation could be an

interesting way to modify alginates and potentially give them new properties. This initiated the work studying the effect of periodate oxidation upon alginate further.

The NMR spectrum of oxidized/reduced alginate was investigated and it was found that the fraction of oxidized units (P_0) could be estimated from the ^{1}H-NMR spectrum for low P_0. Further, it was found that oxidized mannuronan could act as a substrate for the C-5 epimerases AlgE4 and AlgE6. With this as a pre-requisite, two new alginate materials were designed. Mannuronan was oxidized/reduced before (Strategy I) or after (Strategy II) epimerization with AlgE6. After NMR and SMV characterization, hydrogels were made by 'internal setting'. By comparing gels of oxidized/reduced materials with gels made of naturally occurring alginates, it was found that the oxidation affected the dynamic storage modulus (G') to a large extent and that this mainly was due to the increased flexibility rather than a reduction in average G-block length ($N_{G>1}$). In the samples with low P_0 it was also found that the oxidation affected the gelling kinetics, resulting in gelling at an earlier stage than unoxidized samples.

Finally, the results shows that periodate oxidation can be used as a 'tool' to control gel strength, however the degradation accompanying oxidation restricts its use to some extent.

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An evaluation of tritium and fluorescence labelling combined with multi-detector SEC for the detection of carbonyl groups in polysaccharides

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ABSTRACT

The carbonyl content of a pectic polysaccharide from *Sphagnum papillosum* (sphagnan) and periodate oxidised alginates was investigated using three different carbonyl labelling strategies combined with size-exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) and on-line fluorescence or off-line tritium detection. The labelling strategies were tritium incorporation via NaB^3H_4 reduction, and fluorescent labelling with carbazole carbonyl oxyamine (CCOA), or 2-aminobenzamide (2-AB), respectively. Carbonyl quantification was based on labelled pullulan, dextran and alginate standards possessing only the reducing end carbonyl group. As a result the carbonyl distribution in the polysaccharides could be determined. In sphagnan it was found that the carbonyl content increased with increasing molecular weight, whereas in periodate oxidised alginate the carbonyl content was as expected independent of the molecular weight. The methods proved useful for carbonyl detection in water soluble polysaccharides in general. The tritium incorporation method was preferred for alkali stable polysaccharides, while the CCOA method was most suitable for acid stable polysaccharides with low carbonyl content. The 2-AB method is applicable for all polysaccharides tested with varying carbonyl content; however, it lacks the ability to detect ketone functionalities.

1. Introduction

The quantification of carbonyl groups in populations of polysaccharide chains in solution is important because of their impact on structural and reactive properties. All polysaccharides contain one carbonyl group at their reducing end which is in equilibrium with a hemi-acetyl group, while the main cause of intramolecular carbonyl group formation in polysaccharides are by chemical oxidation either intentionally or non-intentionally. Oxidation may lead to altered chain extensions (Christensen, Vold, & Vårum, 2008; Vold, Kristiansen, & Christensen, 2006) increased reactivity and hence new possible applications (Bouhadir et al., 2001). In other cases the oxidation may be regarded as a drawback foremost since the oxidised unit is a 'hot-spot' for degradation, cellulose being the most prominent example (Potthast, Rosenau, & Kosma, 2006), but also if further reactivity is unwanted.

Intramolecular carbonyl groups introduced by oxidation are capable of existing in a variety of forms, and equilibrium occurs between the various forms in solution. In water they may exist hydrated, as acyclic aldehydes, hemi-acetals or hemi-aldals, or as combinations of these. In polysaccharides hemiacetal formation may be intermolecular as well as intramolecular (Gutherie, 1961).

Several different traditional techniques exist for measuring aldehyde and keto groups in oligo and polysaccharides (Mclean, Werner, & Aminoff, 1973; Potthast et al., 2006; Richards & Whelan, 1973). The most important traditional methods for this purpose are summarized in Table 1. The majority of these offer an estimate of the average number of carbonyl groups (weight basis). One of the most common strategies is incorporation of tritium utilizing NaB^3H_4 reduction for carbonyl detection in oligosaccharides. Borohydride reduces carbonyl compounds including aldoses and ketoses. This reaction is in general 'stoichiometric' (Mclean et al., 1973). The first step is a nucleophilic addition reaction, where $NaBH_4$ acts as a donor of a hydride ion that is attracted to the partial positive charge of the carbonyl carbon. In the second step, water protonates the tetrahedral alkoxide intermediate and yields the alcohol product. In the case of reaction with NaB^3H_4 at most one tritium atom can be bound to one carbon per carbonyl group reduction (Tinnacher & Honeyman, 2007). To our knowledge there has been only one attempt to use this method to quantify carbonyl groups in a polysaccharide (Kongruang & Penner, 2004). The main challenge using this method is potential alkaline depolymerisation because of the alkaline reaction conditions needed for borohydride stability. This is mainly an issue in polyuronides were 'internal' β-elimination can occur. Degradation due to the 'peeling reaction'

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Table 1
Common methods for determining carbonyl content in oligosaccharides/polysaccharides.

Method	Advantages/drawbacks	Refs. (method)
Colorimetric methods; e.g., copper number (Cu^{2+} (blue) → Cu^{+} (red))	+Simple ÷Sensitivity problems (det. limit = 0.3 mg ± 2%), back-oxidation. Averages only	Hodge & Hofreiter (1962).
Schiff base reactions of C=O groups with hydrazines or *O*-substituted hydroxylamines followed by; • C/N analysis • fluorescence detection	+Simple ÷Sensitivity problems with C/N analysis, fluorescence sometimes dependent on where the label is situated. Averages only	Maekawa & Koshijima (1991) Ramsay et al. (2001) Shilova & Bovin (2003)
Reduction using NaB^3H_4	+Sensitive ÷Rarly applied to polysaccharides because of possible alkaline degradation. Averages only	Mclean et al. (1973) Richards & Whelan (1973) Takeda et al. (1992)
Fluorescence/absorbance labels in combination with a separation technique; e.g., Fmoc-hydrazine, 2-aminopyridine etc.	+Data for the whole molecular weight distribution ÷Some is highly toxic, low yield, long reaction times	Praznik & Huber (2005) Röhrling et al. (2001)
Direct physical techniques; IR, NMR, etc.	+Simple ÷Poor sensitivity. Averages only	Calvini et al. (2004) Gomez, Rinaudo, & Villar (2006)

is hampered by reduction at the reducing end (Sharon, 1975). A purification step is also needed after reduction to dispose of background activity which is possibly a result of impurities in the NaB^3H_4 (Mclean et al., 1973).

Recently, a variety of fluorescence labels have been introduced for measuring carbonyl groups/reducing ends in oligosaccharides, e.g., 2-aminobenzamide (2-AB) or 9-fluorenyl-methoxycarbonylhydrazine (Fmoc-hydrazine) (Bigge et al., 2002; Zhang, Cao, & Hearn, 1991). These labels are often hydrazines or substituted hydroxylamines which bear a hydrophobic fluorescent group and can be detected at the picomolar level. The labels are connected via reductive amination (Fig. 1) or as for hydroxylamines directly via imine formation without the reduction step.

By combining carbonyl detection with size exclusion chromatography (SEC) followed by multi-angle laser light scattering (MALLS) the distribution of carbonyl groups may be obtained. In addition, estimates of the molecular weight directly provide the number of reducing ends, enabling a possibility for correction when the goal is to analyse intramolecular carbonyl groups in the polysaccharide chain. If SEC-MALLS is used to separate the labelled compounds some specific requirements are needed with respect to the label (Röhrling et al., 2002b). First it is important that the emission wavelength does not interfere with the working wavelength of the laser. Secondly the fluorescence emission wavelength should be independent on where the label is located on the polysaccharide. Finally it is important that the label does not interact significantly with the column material affecting separation and/or destroy columns after long-term use.

Overcoming the limitations mentioned above the fluorescent label carbazole carbonyl oxyamine (CCOA) has been used in combination with SEC-MALLS and on-line fluorescence detection. This strategy has been developed for measuring small amounts of carbonyl functionalities in cellulose as a function of molecular weight (Potthast et al., 2003; Röhrling et al., 2002a, 2002b). The CCOA label has the advantage of being an *O*-substituted hydroxylamine, having an increased reactivity towards carbonyl groups compared to a hydrazine and thus reduction of the double bond formed upon imine formation is not necessary. The label is however, synthesized especially for the detection of carbonyl groups in cellulose dissolved in *N,N*-dimethylacetamide (DMAc)/LiCl and is not yet commercially available (Röhrling et al., 2001). β-glucans labelled with 2-aminopyridine (2-AP) at their reducing end have also been separated with SEC in combination with RI and on-line fluorescence detection (Praznik & Huber, 2005). The method applying 2-AP however, uses a great excess of the highly toxic reagent (~2000 times carbonyl content) in order to get an acceptable conversion and the labelling procedure is not suitable for acid labile polysaccharides.

2-aminobenzamide (2-AB) represents an alternative label widely used for labelling of carbohydrates and is compatible with several chromatographic means of separation including Bio Gel P4, high-performance anion-exchange chromatography and a variety of HPLC procedures (Bigge et al., 2002; France, Cumpstey, Butters, Fairbanks, & Wormald, 2000).

The purpose of this study was to compare and evaluate three different labelling techniques in combination with SEC-MALLS in order to estimate the carbonyl content of both neutral and charged polysaccharides as a function of their molecular weight. The approaches used are labelling with tritium utilizing NaB^3H_4 reduction and labelling with the fluorescent labels CCOA and 2-AB. The 2-AB is connected via the direct reductive amination approach (Fig. 1). Both the NaB^3H_4 and 2-AB have the advantage of being commercially available.

The methods are evaluated using pullulan, dextran and alginate standards. Carbonyl groups were introduced by partial (2–8%) periodate oxidation (Aalmo & Painter, 1981). The resulting dialdehydes can in principle react with two amino-containing labels. To study the stoichiometry of the reaction with 2-AB, oligomers of alginate ($DP_n = 20$) were prepared and subjected to oxidation and labelling, and the products were studied by ^1H NMR.

These methods are also applied to determine the average amount of carbonyl groups in a pectic polysaccharide, named sphagnan, released by mild acid hydrolysis from *Sphagnum papillosum* (Ballance, Børsheim, Inngjerdingen, Paulsen, & Christensen, 2007). It has been claimed that sphagnan contains ~25 M% of a novel keto-uronic acid residue in the form of 5-keto-D-mannuronic acid (5-KMA) which could exist as either a pyranose (5-KMA*p*) or furanose (5-KMA*f*), of which the latter contains a α-keto-carboxylic acid group (Painter, 1983, 1991). 5-KMA in sphagnan was claimed to be responsible for giving *Sphagnum* moss special properties in preserving organic material. The existence of 5-KMA has been rejected (Ballance et al., 2007), but to our knowledge no exact measure of the carbonyl group distribution in sphagnan exists.

2. Materials and methods

2.1. Standards and chemicals

The pectic polysaccharide sphagnan was extracted according to the method previously described (Ballance et al., 2007). Dextran was obtained from Polymer Standard Service (PSS), Germany. Pullulan standards were supplied from Hayashibara, Japan. Alginate (LF 10/60) containing 40% guluronic acid and mannuronan were obtained from FMC Biopolymer, Drammen, Norway. Alginate containing 90% guluronic acid was prepared by epimerising polym-

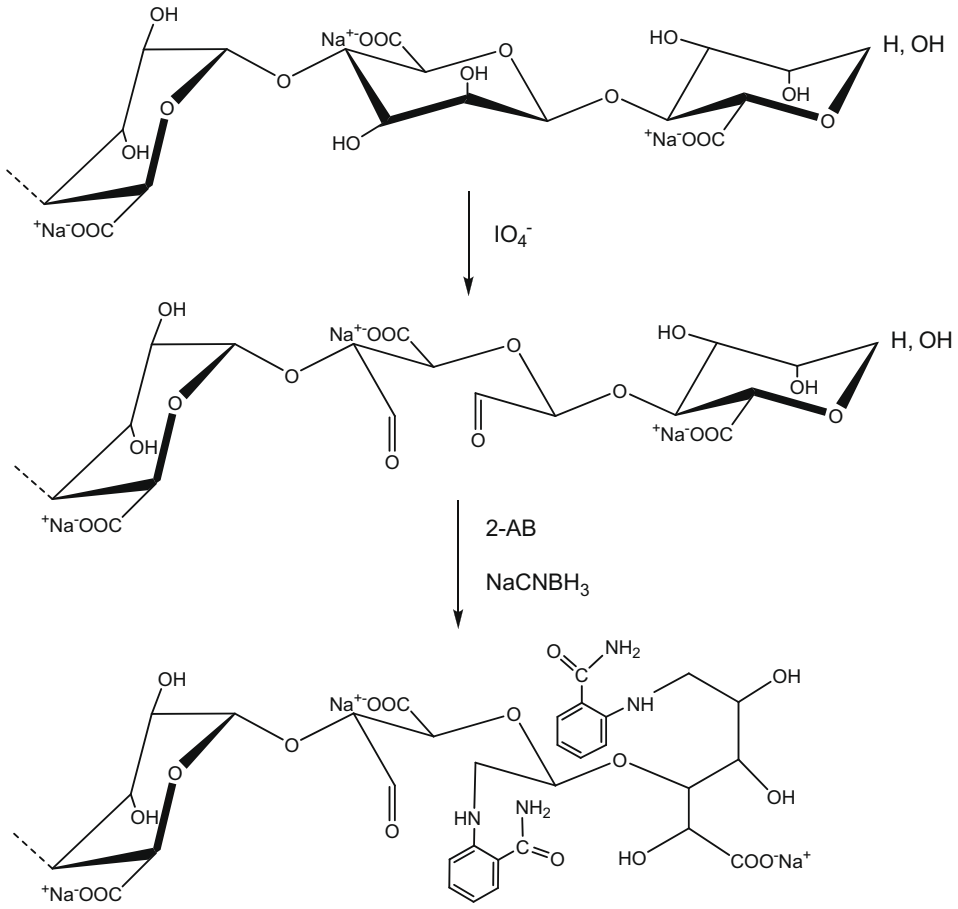

Fig. 1. Chemical structure of an alginate end fragment (. . .GMM) treated with periodate (oxidation assumed to occur randomly) followed by reaction with 2-aminobenzamide (2-AB) and $NaCNBH_3$ (direct reductive amination). Only one of the two aldehydes formed upon periodate oxidation reacts with 2-AB (see text). Abbreviations: M, β-D-mannuronic acid; G, α-L-guluronic acid.

annuronic acid with the alginate epimerase AlgE6 according to Holtan, Bruheim, and Skjåk-Bræk (2006). 100 mCi NaB^3H_4 (12.1 Ci/mmol) was obtained from Amersham Bioscience. Carbazole-9-carboxylic acid [2-(2-aminooxyethoxy)ethoxy)]amide (CCOA) was synthesized according to Röhrling et al. (2001). All other chemicals were obtained from commercial sources and were of analytical grade.

2.2. Periodate oxidation of pullulan and alginate

Pullulan and alginate were partially oxidised using sodium meta periodate. The polysaccharides were dissolved in MQ-water [deionized water purified with the MilliQ system from Millipore (Bedford, MA, USA)] to a concentration of 8.89 mg/mL. The solution was then made up with 10% (v/v) *n*-propanol (free radical scavenger (Painter & Larsen, 1973)) and MQ-water and degassed prior to the addition of 0.25 M sodium meta periodate in order to create a theoretical degree of oxidation equal to 2%, 4%, 6% and 8%. The final polysaccharide concentration was 4.45 mg/mL and the weight was corrected for 10% (w/w) water content. All pipetting and weighing was done in subdued light. The reaction was carried out at 20 °C. The monomer weight was taken to be 162 and 198 g/mol for pullulan and alginate, respectively.

2.3. Labelling with NaB^3H_4

0.3 M NaB^3H_4 (specific activity 2.5 mCi/mmol) in 0.1 M NaOH was added to ten milligram of polysaccharide dissolved in 2 mL MQ-water. The samples were set to react in the fume hood for 24 h at room temperature on a shaking devise. The reaction was then stopped by cooling the samples on ice and adding 200 μL of concentrated acetic acid and left for 1 h until all hydrogen/tritium gas had effervesced. The samples were dialysed (Medicell International Ltd., Size 4 Inf Dia 22/32″ – M.W.C.O. 12-14 kDa) against two shifts of 0.05 M NaCl and MQ-water until the conductivity was <2 μS/cm and the tritium count was equal to background (50–100 CPM). Dialysis was followed by freeze-drying.

2.4. Labelling with CCOA

2.5 mg of CCOA in 100 mM acetate buffer (pH 4) was added to ten milligram of polysaccharide dissolved in 1 mL of MQ-water. The samples containing a polyelectrolyte were pre-adjusted to pH 4. The samples were then set to react on a shaking devise for 168 h at 40 °C followed by dialysis and freeze-drying in the same way as the tritium labelled samples.

2.5. Labelling with 2-AB

Ten milligram of polysaccharide was dissolved in 3 mL of MQ-grade water, 0.1 mL 1 M 2-AB in 100% MeOH and 0.1 mL of 5 M $NaCNBH_3$ in 1 M NaOH was added in the fumecupboard. The pH was then adjusted by adding approx. 0.3 mL of 1 M acetate buffer, pH 5, to give a final pH 5.8 in the reaction solution. Addition of buffered acetate was necessary to prevent the pH from falling below five and thus preventing the undesirable formation of HCN gas. The samples were then set to react on a shaking devise for 48 h at RT. To remove excess label the samples were either dialysed in

the same way as for the tritiated samples or precipitated with 50% (v/v) isopropanol. The precipitate was centrifuged at 2559*g* for 5 min and washed with 100% isopropanol three times. The pellet were left for 24 h in the fumehood and re-dissolved in mobile phase for SEC-MALLS with fluorescence detection.

2.6. ^{1}H NMR of 2-AB labelled derivatives

^{1}H NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer at 90 °C. The oxidised alginate samples for ^{1}H NMR was prepared by degrading the alginate by mild acid hydrolysis. The samples were then reduced using 0.5 M $NaBH_4$, oxidised and labelled as described above. The pullulan oligomers were obtained directly from Hayashibara, Japan. The mannuronan oligomer ($DP_n = 20$) was prepared by partial acid hydrolysis and purified using SEC. After labelling with 2-AB and removal of excess label the oligosaccharides were dissolved in D_2O to a concentration of 10 mg/mL followed by the addition of 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (Aldrich, Milwaukee, WI, USA) as internal standard for the chemical shift. In the case of analysing alginate 0.3 M triethylenetetramine-hexaacetic acid (TTHA) was added in order to chelate any Ca^{2+}-ions present.

2.7. Size-exclusion chromatography with multi-angular laser light scattering (SEC-MALLS) and fluorescence or tritium detection

A part of the sample was dissolved in MQ-water and mobile phase was added so that the concentration was equal to the mobile phase on the SEC setup. Mobile phase was 0.05 M Na_2SO_4/0.01 M EDTA, pH 6. In the case were CCOA or 2-AB labelled samples were analysed the mobile phase was made up by adding 20% (v/v) acetonitrile. The flow rate was set to either 0.4 mL/min or 0.5 mL/min.

The samples were filtered through a 0.2 μm filter and injected on different combinations of Tosoh Biosep TSK 6000, 5000, 4000 and 3000 PWXL columns connected to a Dawn DSP multi-angle laser light scattering photometer (Wyatt, USA) ($\lambda_0 = 633$ nm) followed by an Optilab DSP differential refractometer (P-10 cell). In the case of fluorescence detection a Shimadzu fluorescence monitor (model RF-530) was inserted between the MALLS and the RI detector. The excitation and emission wavelengths were set to 286 nm and 340 nm, respectively, for CCOA. While for 2-AB the respective excitation and emission wavelength were set to 340 nm and 450 nm. Tritium was detected by collecting fractions after the RI detection (fraction size: 600 μL). Each fraction (500 μL) was transferred to a scintillation vial, 10 mL of scintillation cocktail (HiSafe OptiPhase 3) was added and finally the samples were counted for 5 min each on a liquid scintillation counter (Wallac 1410). Data was collected and processed using Astra version 4.90 and transferred into an excel spreadsheet for further processing. For alginate and sphagnan a specific refractive index increment (dn/dc) of 0.150 mL/g was used, while for dextran and pullulan 0.148 mL/g was used.

3. Results and discussion

3.1. Carbonyl detection using tritium labelling

The carbonyl functional groups in the polysaccharide were reduced to alcohols with NaB^3H_4 in order to incorporate one tritium atom per. aldehyde and/or keto group initially present. The reduction of oligosaccharides and polysaccharides with $NaBH_4$ is a *pseudo* first order reaction when carried out with an excess of borohydride, typically 0.5–5 M, to ensure a quick reduction and prevent alkaline degradation (Painter & Larsen, 1973). When tritiated borohydride is used the sensitivity of the assay is dependent on the ratio between tritiated to non-tritiated borohydride. A total concentration of 0.1 M NaB^3H_4 (spec. activity 2.5 mCi/mmol). in the reaction mixture was chosen since this gave sufficient sensitivity (without exceeding the limits for general radioactive disposal in Norway) and is previously shown to completely convert glucose to glucitol within 24 h at room temperature (Hansson, Hartler, Szabo, & Teder, 1969). The pH was kept equal to 12.5 in order to prevent decomposition of borohydride (Mochalov, Khain, & Gil'manshin, 1965).

The incorporation of tritium was initially measured for pullulan and sphagnan showing that reduction was nearly complete after just a few hours upon addition of borohydride. After 24 h no more tritium could be incorporated and this reaction time was chosen in subsequent experiments.

The possibilities for alkaline degradation were assayed with SEC-MALLS. However, no significant degradation of alginate (unoxidised), dextran, pectin or pullulan was detected under these conditions.

In order to determine the relative carbonyl content in an unknown sample one or more references/standards with known carbonyl content are needed. Pullulan, dextran and alginate were chosen. ^{3}H labelled samples were analysed using SEC-MALLS, and fractions (600 μL) were collected for off-line tritium counting. The chromatograms were divided into slices corresponding to the fraction size, and M_n (or DP_n) was calculated for each fraction (Fig. 2). The specific incorporation of ^{3}H (activity per μg) was inversely proportional to DP_n across the DP range, independent of the type of polysaccharide (Fig. 2B). Thus, any of these polysaccharides (and possibly many others) may serve as standards. A standard prepared at the same time as the samples must be included in all experiments since the activity of the tritiated borohydride changes both as a consequence of disintegration of the isotope and decomposition of the borohydride itself. The method is also very much dependent on a precise determination of the delay volume between the different detectors and the fraction collector.

The method was first applied to a pectic polysaccharide from *S. papillosum*, named sphagnan. According to fig. 2, the carbonyl content of sphagnan is generally higher than the standards. For example, at DP = 100 each chain contains on average one carbonyl group in addition to the reducing end, whereas at DP = 400 the corresponding number is nine (Fig. 2B). The average carbonyl content is 2.9 carbonyl groups per 100 monomers. During the extraction of sphagnan the *Sphagnum* holocellulose is treated with chlorite in order to remove lignins. It was suspected that this could introduce carbonyl groups into sphagnan. Therefore sphagnan was prepared without the chlorite bleaching step (method given by Ballance, Kristiansen, Holt, & Christensen, 2008) and measured using this method. The data in fig. 2 shows no significant difference between the two methods for preparing sphagnan, indicating that no significant amount of carbonyl functionalities was introduced during the chlorite bleaching step. Thus, a small amount of carbonyl groups, in addition to the reducing end, are indeed present in sphagnan.

The method was subsequently applied to periodate oxidised alginate and pullulan (2–8%). However, these samples were significantly depolymerised during labelling and the degree of oxidation was highly underestimated compared to the theoretical values. Although this may give interesting information about the degradation of such samples in alkali this was outside the scope of this work and further investigation of these samples were abandoned at this point.

3.2. Carbonyl detection using the fluorescent label CCOA

The fluorescent label carbonyl carbazole oxyamine (CCOA) was previously used for carbonyl detection in cellulose. Cellulose was either dissolved in 9% (w/v) DMAc/LiCl (homogenous procedure) or suspended in 20 mM zinc acetate buffer, pH 4, (heterogeneous

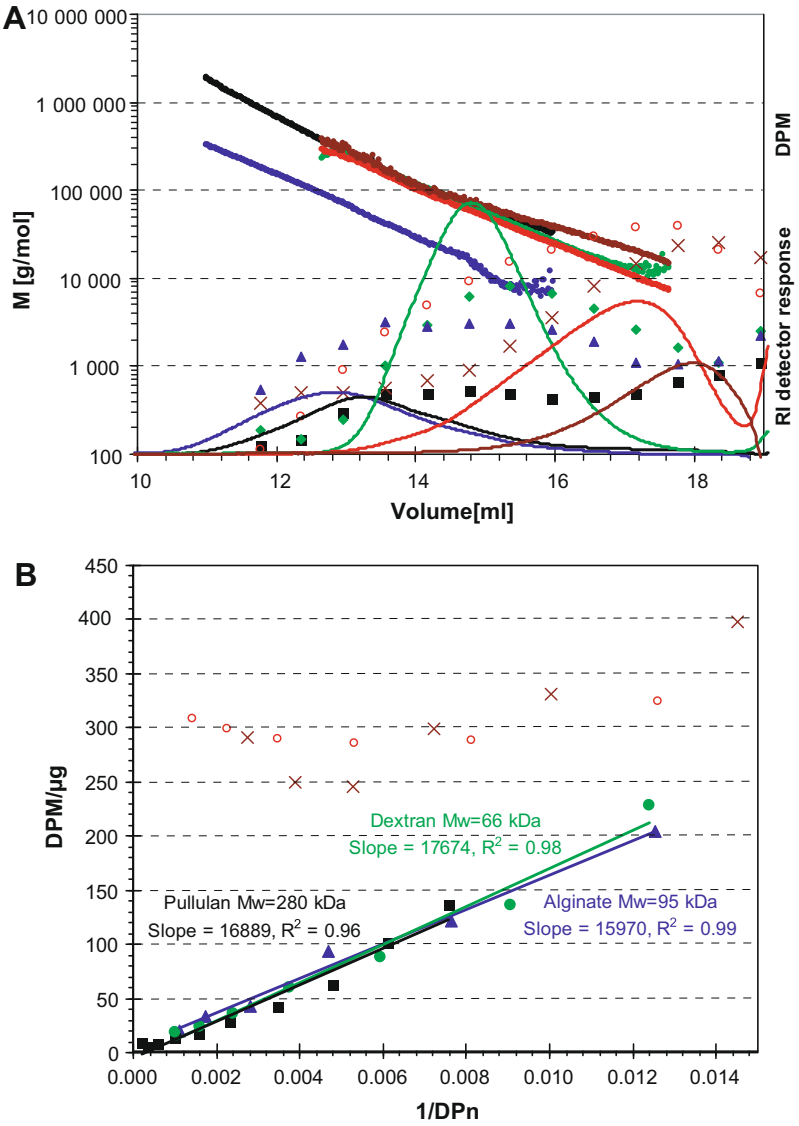

Fig. 2. Carbonyl detection using the tritium incorporation (via NaB^3H_4 reduction) method applied to alginate (90% guluronic acid) (▲), dextran (●), pullulan (■), sphagnan (X) and sphagnan not treated with chlorite (○). Upper figure (A): molecular weight – volume plot (dots), refractive index (RI) (thin lines) and tritium activity (DPM – disintegrations per minute) profiles. For tritium detection fractions (600 µL) was collected throughout the chromatographic profile and counted off-line. Lower figure (B): number average DP is calculated for the fractions demonstrated as spacing between the DPM data points in the upper figure. The monomer weight of pullulan/dextran, alginate and sphagnan were taken as 162, 198 and 200 g/mol, respectively. For colours, see on-line version.

procedure) in both cases followed by SEC-MALLS with fluorescence detection using 0.9% (w/v) DMAc/LiCl as mobile phase (Röhrling et al., 2002b). We wanted to adapt this method to polysaccharides labelled and analysed in an aqueous system. Pullulan, alginate, dextran and sphagnan were labelled based on the protocol for homogenous labelling of cellulose (the procedure being heterogeneous for water soluble polysaccharides), however, the zinc salt could not be included in the acetate buffer since alginate forms gels with divalent cations. Zinc was included in the buffer when assaying cellulose because it has the ability to hydrolyse lactones (Röhrling et al., 2001). Pullulan, dextran or alginate are not known to contain a significant amount of lactone structures. The structure of sphagnan, not being known in detail, might contain lactone structures. If such structures were to be present they would lead to an overestimation of the carbonyl content using this method.

The labelling of dextran and pullulan was successful, but alginate was severely degraded. It was observed, analogous to the NaB^3H_4 method, that the pullulan and dextran standards gave overlapping and near linear functions in a plot of fluorescence/RI vs. $1/DP_n$ as shown in Fig. 3B.

The separation of the CCOA labelled polysaccharides was successful although unreacted CCOA was absorbed onto the column material. This phenomenon was also described by Röhrling et al. (2002b) when DMAc/LiCl was used as mobile phase, but the interaction is likely to be stronger using an aqueous mobile phase. It is assumed that this interaction is due to the fact that unbound CCOA penetrates deep into the pores of the polyhydroxy methacrylate co-polymer network and weakly sticks to it by hydrophobic interactions. Different modifiers were tested including 20% (v/v) 2-propanol, methanol and acetonitrile to avoid this interaction. A mobile phase consisting of 10% (v/v) dimethylsulfoxide (DMSO), 0.05 M Na_2HPO_4 and 0.1% (w/v) sodium dodecyl sulfate adjusted to pH 6, previously used to successfully separate lignosulfonates at pH 10.5, (Fredheim, Braaten, & Christensen, 2002) was also tested. The best result was obtained with 0.05 M Na_2SO_4/0.01 M EDTA mobile phase containing 20% (v/v) acetonitrile. 2-propanol was equally good, but acetonitrile was preferred because of its lower

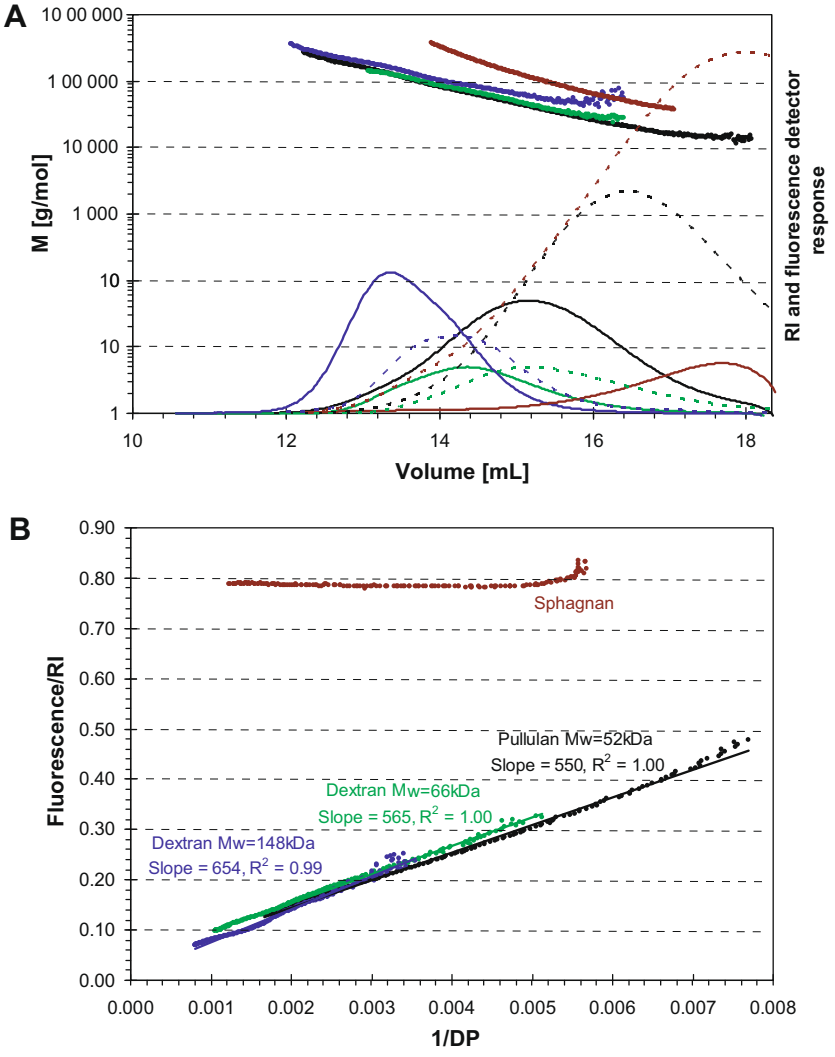

Fig. 3. Carbonyl detection using the carbazole carbonyl oxyamine (CCOA) method applied to sphagnan, pullulan and dextran. Upper figure (A): molecular weight – volume plot (dots), refractive index (RI) (thin lines) and fluorescence (dotted lines) profiles. Lower figure (B): monomer weight of pullulan/dextran and sphagnan are taken as 162 and 200 g/mol, respectively. For colours, see on-line version.

viscosity. The CCOA did, even with modifier present in the mobile phase, elute from the column some time after the salt peak leading to extended runtimes. The amount of unbound CCOA in the samples was however, very low since dialysis removed almost all of it.

The CCOA method was applied to sphagnan (Fig. 3). However, the carbonyl content was underestimated compared to the tritium approach resulting in an average carbonyl content of 1.4 carbonyl groups per 100 monomers. The result was corrected for a 2.6% emission background originating from the sphagnan itself. The background emission was evenly distributed at all molecular weights. It was also observed that the molecular weight was increased in the high molecular weight region of the sample when sphagnan was reacted with CCOA, reflected in an approximately 25% higher M_w and 8% higher M_n value. This phenomenon is due to partial aggregation of the CCOA labelled sphagnan sample.

Alginate and pullulan partially oxidised in the range 2–8% with periodate were also analysed, but all samples formed insoluble particles during labelling with CCOA. This is probably due to the hydrophobic character of the label which makes the polymer amphiphilic and hence difficult to dissolve in water. Since unoxidised alginate also was degraded during labelling it is reasonable to believe that the oxidised samples were degraded even more severely. The aggregates were dialysed and freeze dried, but showed no sign of dissolving in the mobile phase containing 20% (v/v) acetonitrile.

3.3. Carbonyl detection applying the fluorescent label 2-aminobenzamide (2-AB)

A third strategy was tested in order to find a method that could be used for acid or alkaline labile polysaccharides. A reference method was also needed to ensure the validity of the tritium labelling approach. 2-aminobenzamide (2-AB) is a common fluorescence label attached for reducing oligosaccharides in quantitative yields using direct reductive amination, e.g., pectic oligosaccharides (Bigge et al., 2002; Ishii, Ichita, Matsue, Ono, & Maeda, 2002). In addition it was found that the label fulfilled the demands given in the introduction and could be used in combination with SEC-MALLS. To ensure that the labelling were complete the reaction kinetics were investigated for all polysaccharides involved, see Fig. 4. The reactions could be fitted to a pseudo first order reaction with R^2 = 0.99–0.96. The figure shows that the labelling was complete after 24 h.

Oligomers of alginate and pullulan (unoxidised and periodate oxidised) were studied by ^{1}H NMR to reveal whether they were fully reactive/substituted with 2-AB or not. Fig. 5 shows the ^{1}H

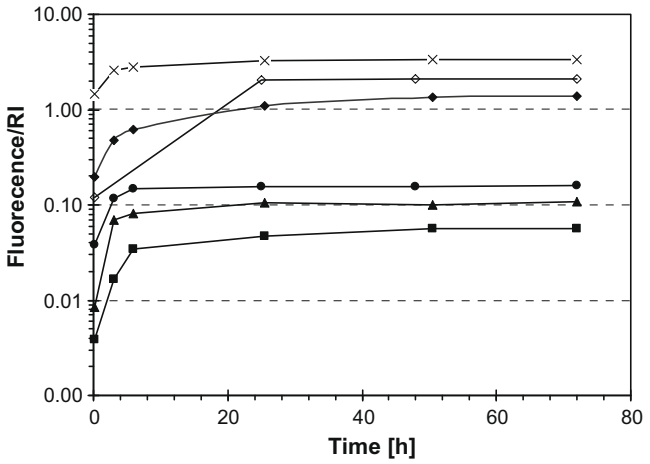

Fig. 4. Reaction kinetics for 2-AB labelling of pullulan (■), alginate (▲), mannuronan (●), sphagnan (♦), mannuronan 6% periodate oxidised (◇) and alginate (40% guluronic acid) 8% periodate oxidised (x).

NMR spectra of unlabelled mannuronan (A) and mannuronan labelled for 24 h (B). Signals from the reducing ends at 5.22 and 4.88 ppm disappear after labelling, indicating complete substitution of the reducing ends. The four protons from 2-AB appear as doublets and triplets in the 6.8–7.7 ppm region. This is analogous to the observations made by Ishii et al., 2002 for pectin oligomers. Comparing the signals from the four protons on 2-AB with the internal H-1 proton on mannuronic acid also confirmed that the oligomer was fully substituted given $DP_n = 20$. The DP_n of the mannuronan oligomer was estimated from the ^{1}H NMR spectrum by the following expression; $DP_n = (I_{M-1} + I_{M-1red\alpha} + I_{M-1red\beta})/(I_{M-1red\alpha} + I_{M-1red\beta})$ (Grasdalen, 1983) and confirmed by SEC-MALLS analysis.

Alginate oligomers reduced with 0.5 M $NaBH_4$ prior to 4% and 8% periodate oxidation were also prepared, labelled (24 h) and analysed with ^{1}H NMR, see Fig. 5C and D. The percentage of oxidation refers to oxidised residues, one residue yielding two potentially reactive carbonyl groups. The signal from one of the four detected protons on 2-AB should therefore comprise 8% and 16% of the signal intensity of the H-1 signal for mannuronic acid units, assuming that maximum one of the two potential sites on the end residues was attacked by periodate, however, it only comprised exactly half the theoretical amount. The experiment were also repeated using pullulan with $DP_n = 51$, in this case assuming no double oxidation at the α-(1-6) linkage, giving the same result (data not shown).

Since the kinetic data suggest that the labelling reaction was complete we take this as indirect evidence that the oxidised units are monosubstituted and that 2-AB is only able to react with one of the two vicinal aldehydes formed upon periodate oxidation. It is not clear whether this is due to the fact that the 2-AB cannot react with an aldehyde group when an other 2-AB molecule is located in its close vicinity or if one of the aldehydes formed upon periodate oxidation is simply not reactive towards 2-AB. When substituted hydroxylamines was reacted with cellulose with low degrees of oxidation (~1%) both aldehydes formed seems to be reactive (Potthast, Kostic, Schiehser, Kosma, & Rosenau, 2007). On the other hand when periodate oxidised alginate was substituted with a polyether via direct reductive amination only 0.12 mol polyether was incorporated per mol of uronic acid as opposed to 0.40 mol if 100% conversion was to be obtained (Carré, Delestre, Hubert, & Dellacherie, 1991).

The 2-AB labelled standards were analysed on SEC-MALLS with fluorescence detection and no degradation was observed for any of the samples analysed. Unreacted 2-AB interacts with the column material similar to CCOA, but in this case the interaction was weaker and using the buffer described earlier containing 20% (v/v) acetonitrile, 2-AB elutes off the column just after the salt peak. In an attempt to avoid this interaction completely the samples were labelled with 2-aminobenzoic acid (2-AA) as an alternative to 2-AB. It was suspected that the charge on 2-AA would minimize the interaction with the column material. However, no significant

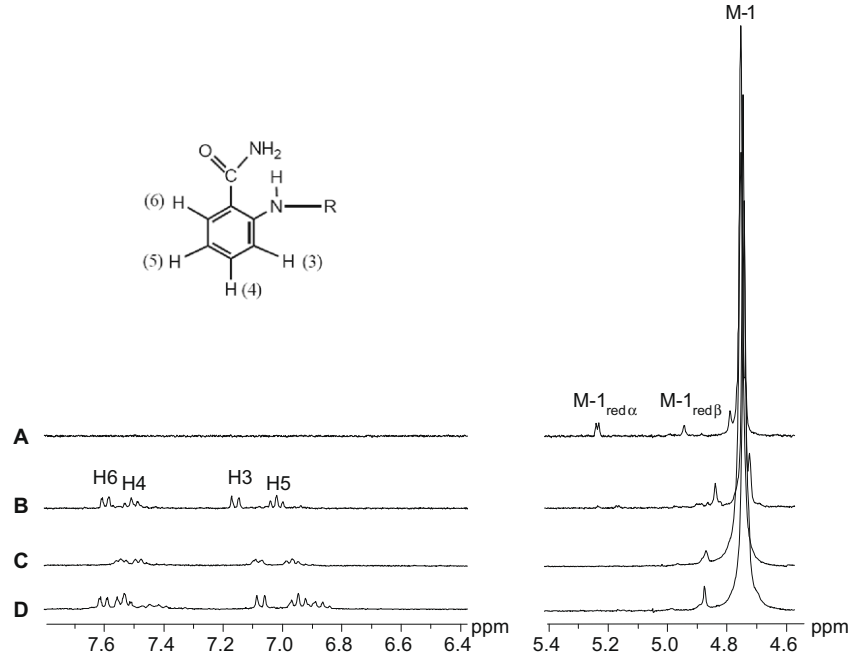

Fig. 5. ^{1}H NMR spectra of a mannuronan oligomer are shown before (A) and after (B) labelling with 2-aminobenzamide (2-AB) at the reducing end. C and D shows a 4% and 8% periodate oxidised mannuronan oligomer (borohydride reduced before oxidation), respectively, after labelling with 2-AB. All samples were labelled for 24 h. The peaks appearing in the 6.8–7.7 ppm region belongs to the four protons on 2-AB as indicated. The chemical shifts of the protons on 2-AB are assigned according to Ishii et al. (2002) and modelling using ChemBioDraw Ultra version 11.0. Abbreviation; *R* = anchor point for carbonyl group.

improvement was observed, but instead it was found that the 2-AA violated the second criteria mentioned earlier resulting in a small shift of the emission wavelength depending on where the label was located on the polysaccharide.

As for the two previous methods tested the method was able to detect the reducing end of pullulan, dextran and alginate. The additional carbonyls in periodate oxidised alginate up to 8% oxidation were also detected. Taken into account that 2-AB is only reactive towards one of the two aldehydes formed per residue upon periodate oxidation, the plot fluorescence/RI introduced earlier can be described by:

Fluorescence $\propto n_e + n_{ox}$

$$\text{Fluorescence} = A(n_e + n_{ox}) = A\left(\frac{n_0}{DP} + n_0 D_{ox}\right) = An_0\left(\frac{1}{DP} + D_{ox}\right) \quad (1)$$

RI = mass = $M_0 n_0$

$$\frac{\text{Fluorescence}}{RI} = \frac{An_0}{M_0 n_0} \cdot \left(\frac{1}{DP} + D_{ox}\right) = \frac{A}{M_0} \cdot \left(\frac{1}{DP} + D_{ox}\right) \quad (2)$$

A = constant, n_e = number of reducing end residues, n_{ox} = number of oxidised residues, n_0 = number of monomers, M_0 = monomer weight, D_{ox} = degree of oxidation (= n_{ox}/n_0).

The constant A, expressing the relationship between fluorescence intensity and number of carbonyl groups, can be found from the slope of the linear plot (A = slope M_o), while the degree of oxidation can be estimated from the intercept (D_{ox} = Intercept/(A/M_0)). A plot of the detected degree of oxidation versus theoretical degree of oxidation yielded $R^2 = 0.99$. Fig. 6 show that the linearity of the plots was reduced as the degree of oxidation was increasing. This is partly due to degradation during oxidation, but foremost the presence of small aggregates in these samples. The aggregates were present in both labelled and unlabelled oxidised samples possibly due to intermolecular cross-linking (Gutherie, 1961), since they disappear upon borohydride reduction. Although the aggregates constitute a very small part of the sample (<1%), they affect the light scattering signal to a significant extent in the high molecular weight region. Depolymerisation during periodate oxidation of alginate has been previously described and the shift in the RI-profile (Fig. 6) is mainly due to this and cannot be attributed to conformational change alone (Vold et al., 2006).

Sphagnan was also analysed using the 2-AB method resulting in an average carbonyl content that was identical to the one obtained using tritium labelling. The fluorescence signal was corrected for a background emission of 3.8% of the total intensity, originating from unlabelled sphagnan.

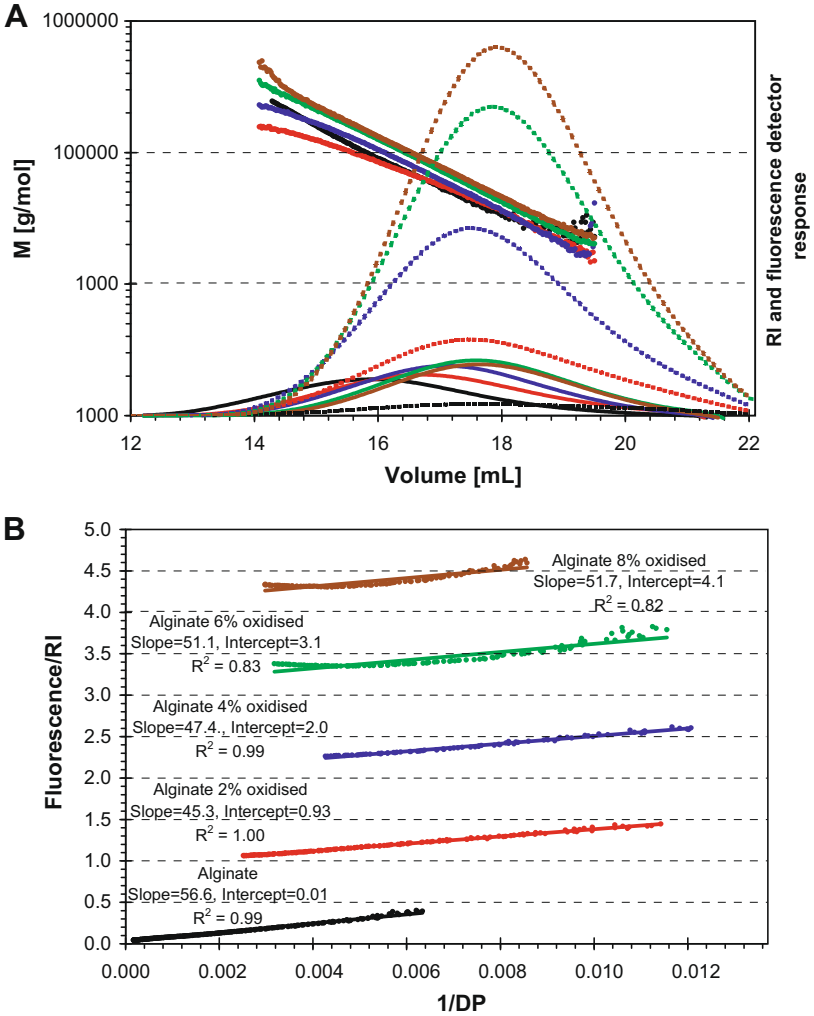

Fig. 6. Carbonyl detection using the 2-aminobenzamide (2-AB) method applied to alginate (40% guluronic acid) partially oxidised 2%, 4%, 6% and 8%. Upper figure (A): molecular weight – volume plot (dots), refractive index (RI) (thin lines) and fluorescence (dotted lines) profiles. Lower figure (B): monomer weight is taken as 198 g/mol for both oxidised and unoxidised alginate residues. For colours, see on-line version.

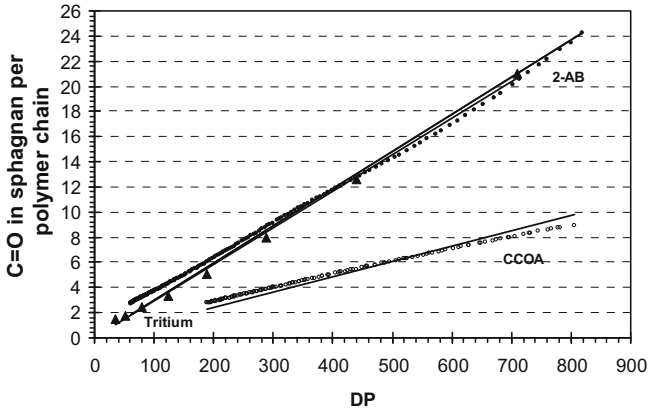

Fig. 7. The carbonyl content in sphagnan relative to a pullulan standard using the tritium (▲), 2-AB (●) and CCOA (○) approach. The data in the figure can be fitted to linear functions; slope 2AB and tritium method = 0.03 (R^2 = 0.99), slope CCOA method = 0.012 (R^2 = 0.96). The 2-AB method cannot detect ketones.

3.4. Comparison of the tritium, CCOA and 2-AB methods for determining the carbonyl distribution in sphagnan

Applying both the NaB^3H_4 and the 2-AB method to the pectic polysaccharide sphagnan an average carbonyl content of 2.9 carbonyl groups per 100 monomers was detected. Fig. 7 also shows that the two methods give the same carbonyl distribution. The CCOA method however, estimates an average content of 1.4 carbonyl groups per 100 monomers. The carbonyl profile is not only lower on average, but underestimates the carbonyl content at higher molecular weights compared to the two other methods. The reason for this could probably be found in a combination of different factors. The sample aggregates upon labelling and thus the molecular weight is overestimated. Aggregation might also affect the reaction rate between the label and the carbonyl functionalities in the polymer. If labels are closely located in space this might also lead to fluorescence quenching resulting in a lower fluorescence signal (Miller, 2005). All in all the CCOA method does not seem to be the appropriate method of choice for this sample.

The nature of the carbonyl groups in sphagnan is not known. However, it is widely accepted that aromatic amines can only react with aldehyde groups and not with ketones (Borch, Bernstein, & Durst, 1970). Since the tritium and 2-AB approach gives the same result it seems acceptable to assume that all the carbonyl groups in sphagnan are in fact aldehydes.

A novel monosaccharide, named 5-keto-D-mannuronic acid (5-KMA), was claimed to be present in sphagnan (Painter, 1983, 1991). The 5-KMA contains a keto group that should, if present, be detected by the method using NaB^3H_4, but not by the 2-AB method. The fact that the two methods give identical results implies that 5-KMA does not exist in sphagnan, which is in accordance with the recent observations made by Ballance et al. (2007, 2008).

The average carbonyl content of sphagnan was estimated by Ballance et al. (2008). Sphagnan extracted with or without the use of chlorite was reacted with either hydroxylamine or phenylhydrazine at pH 4–4.5 and the total nitrogen content was measured. The results were all similar and the *N* content of sphagnan treated with hydroxylamine was 0.63% ± 0.085 (n = 4). Given an *N* content in untreated sphagnan of 0.2% the carbonyl content was estimated to an upper limit of 5.6 carbonyl groups per 100 monomers. It was however, emphasised that the nitrogen analysis method used was on the borderline of both the sensitivity and resolution of the instrument. We believe that the methods presented here give a more precise determination of the carbonyl content in sphagnan.

Acknowledgement

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Novel alginates prepared by independent control of chain stiffness and distribution of G-residues: Structure and gelling properties.

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Abstract

The study of alginate hydrogels is of increasing interest, given their potential applications as biomaterials for tissue engineering and for encapsulating drugs and living cells. In this study we present a new strategy for tailoring alginates on the basis of homopolymeric mannuronan, where the chain stiffness and the content of G-residues could be varied independently. Partial periodate oxidation (0-8%), introducing flexible linkages through C2-C3 cleavage and ring opening, was combined with *in vitro* epimerization, introducing either alternating (MG) sequences (in the case of enzyme AlgE4) or G-blocks (in the case of enzyme AlgE6). Both enzymes are recombinantly expressed from *Azotobacter vinelandii.* Two strategies were followed: a) Oxidation followed by epimerization b) Epimerization to 90% followed by oxidation. The resulting alginates were characterised by NMR spectroscopy and size exclusion chromatography (SEC) with multi angular laser light scattering (MALLS) and viscosity detectors. Gels were prepared using the 'internal setting' method with either 10 mM or 20 mM Ca^{2+} present, and studied by small-strain oscillatory measurements. It was found that periodate oxidation, in the range P_0 = 0.02-0.06, had a pronounced influence on the gelling properties. The decrease in dynamic storage modulus (G') could mainly be attributed to increased flexibility and not only a decrease in G-block lengths as a consequence of oxidation. The new alginate gels are easily degradable in a mild acidic environment and the degradation is easier to control than gels made of unoxidized alginate.

Keywords: Mannuronan, alginate epimerases, hydrogels, periodate oxidation, acid hydrolysis, NMR & small-strain oscillatory measurement.

Introduction

Alginate is a family of unbranched polysaccharides consisting of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers forming regions of M-blocks (MM), G-blocks (GG) or alternating structures (MG). The guluronic acid residues are introduced *post* polymerisation by a series of C-5 epimerases. A family of seven C-5 epimerase genes, the AlgE genes, has been isolated from *Azotobacter vinelandii* and recombinantly expressed and sequenced in *Escherichia coli* (Ertesvåg et al., 1999; Ertesvåg et al., 1994; Svanem et al., 1999). The possibility of obtaining pure mannuronan and utilizing the C-5 epimerases *in vitro* (Mørch et al., 2007), in particular AlgE4 and AlgE6, is part of the background for the new alginate materials discussed in this study. AlgE4 strictly converts MM to MG blocks in the polymer, while AlgE6 introduces G-blocks of various lengths (Campa et al., 2004; Holtan et al., 2006). When additional G-blocks are introduced by AlgE6 they can merge, and long stretches of guluronic acid are created (Mørch et al., 2008).

The structure of alginate allows the polymer chains to ionically interact with each other through divalent cations, in particular Ca^{2+}, Ba^{2+} or Sr^{2+}, hereby forming hydrogels. The complexation of alginate with divalent ions has been described by the "egg-box" model in which each divalent ion interacts with two adjacent residues as well as with two G-residues in an opposing chain (Grant et al., 1973). Such alginate hydrogels have been intensively studied as tissue engineering scaffolds or encapsulation materials for living cells or other biologically active compounds. One of the most studied systems is the encapsulation of insulin producing cells in Ca^{2+}-alginate microcapsules for transplantation into patients to treat Type 1 diabetes (Strand et al., 2003). Alginate hydrogels have also been tested in drug delivery systems. The physical properties of the gel are vital because it is important that the drug is released in a controllable manner. If it is released above or below some critical level, the drug could potentially be either toxic or inefficient (Gomez et al., 2007). The properties of the alginate hydrogels can be controlled by parameters like Ca^{2+} concentration, pH, molecular weight and the chemical composition of the alginate (Draget et al., 1993). In this study we also suggest that chemical modification through periodate oxidation can be used to control gel strength.

Periodate oxidation of alginate is highly specific and results in the cleavage of the C2-C3 bond in the alginate monosaccharide units (Fig. 1A). The oxidation is assumed to act randomly upon homogenous substrates (Painter & Larsen, 1970). It is generally considered that the cleavage reaction involves reversible formation of a cyclic complex or intermediate, via an acyclic ester, and that the intermediate decomposes via a cyclic transition state to the

products (Perlin, 2006). In polysaccharides the carbonyl groups formed may exist hydrated, as acyclic aldehydes, hemiacetals or hemialdals, or as combinations of these. The hemiacetal formation may be intermolecular as well as intramolecular (Gutherie, 1961; Painter & Larsen, 1970). If oxidation is followed by borohydride reduction the reactive dialdehydes are reduced to diols. Periodate oxidation of alginate also leads to some depolymerisation. The mechanism involved is presumably a free radical mediated mechanism (Balakrishnan et al., 2005; Painter & Larsen, 1970), however conclusive proof of such a mechanism is still lacking. When a polysaccharide is partially oxidized by periodate the mol fraction of oxidized units compared to unoxidized units can be expressed as P_0, hence 4 mol% oxidation can be expressed as $P_0 = 0.04$.

Recently, a commercial alginate (M/G-ratio: 0.47) was periodate oxidized as a means to make alginate hydrogels with new properties (Gomez et al., 2007). It is known that the periodate oxidation of alginate has a pronounced effect upon chain extension and flexibility (Smidsrød & Painter, 1973; Lee et al., 2002; Vold et al., 2006), thus this should influence the gelling ability of alginate. This is confirmed in the study of Gomez et al. (2007). In this study it is concluded that the gel strength is rapidly decreasing with increasing P_0 and reaching $P_0 = 0.10$ no gelling takes place in an excess of calcium. However, in the experiment of Gomez et al. (2007) some of the oxidation sites were within G-blocks and these consequently split the G-block. The G-block length ($N_{G>1}$) is important in controlling gel strength (Draget et al., 1993). Performing periodate oxidation therefore introduces both increased flexibility and a reduction in G-block length, thus it is not concluded whether the increased flexibility alone affects the gel strength. It is indeed pointed out that the molecular weight is rapidly decreasing during periodate oxidation, also being an important factor in gel formation (Draget et al., 1993a, b, 2000).

The degradation of partially oxidized alginate and its potential application for tissue engineering has also been investigated (Bouhadir et al., 2001). By partially oxidizing alginate the hydrogels formed can be degraded in a more controlled fashion, since the oxidized residues are much more susceptible to acid hydrolysis than the unoxidized residues (Bouhadir et al., 2001). The increased susceptibility to acid hydrolysis of periodate oxidized and borohydride reduced oligo- and polysaccharides is well known and often applied in Smith degradation (Abdelakher et al., 1952). Periodate oxidized polysaccharides in general are also more susceptible to alkaline degradation, demonstrated with both periodate oxidized dermatan sulphate and cellulose (Calvini et al., 2004; Fransson & Carlstedt, 1974).

In this study we first investigated the structural outcome of periodate oxidized mannuronan. Periodate oxidized and subsequently borohydride reduced mannuronan was further used as a substrate for the C-5 epimerases AlgE4 and AlgE6 (Fig. 1A). The gel strength and gelling kinetics of hydrogels formed of mannuronan epimerized with AlgE6 after or before periodate oxidation (P_0 = 0.02-0.08) and reduction were studied performing small-strain oscillatory measurements (Fig. 1B). The mannuronan epimerized after oxidation results in a new alginate material with long G-blocks that were interspersed by oxidized mannuronan residues possessing increased flexibility. Assuming random oxidation the G-block length was dictated by the number of mannuronan residues between the oxidized units, but limited by the minimum number of residues needed for the enzyme to attack. It was assumed that the enzyme was specific and did not attack oxidized residues. Mannuronan epimerized prior to oxidation had extremely long G-blocks interspersed by oxidized units. These materials were compared with alginate from *Laminaria hyperborea* and *Durvillea antarctica* having comparable G-block lengths which made it possible to study whether it was the increased flexibility introduced by the periodate oxidation or a reduction in G-block length accompanying the oxidation that caused the drop in the dynamic storage modulus (G'). Finally, it was investigated whether periodate oxidation could be used to 'tune' gel strength, potentially resulting in new easily degradable alginate gels.

2. Materials and methods

2.1 Materials and chemicals

Mannuronan and alginate from *Durvillea antarctica* and *Laminaria hyperborea* stipe/leaf were obtained from FMC Biopolymer, Drammen, Norway. The mannuronan was further purified by precipitation in 50% ethanol, filtration and two cycles of washing with 98% ethanol. The other alginates used in this study were obtained by epimerizing mannuronan with the C-5 epimerases AlgE4 and AlgE6, obtained as described earlier (Campa et al., 2004; Holtan, Bruheim & Skjåk-Bræk, 2006). All chemicals were obtained from commercial sources and were of analytical grade.

2.2 Periodate oxidation and borohydride reduction of mannuronan/alginate

Mannuronan/alginate was dissolved in MQ-water (deionized water purified with the MilliQ system from Millipore (Bedford, MA, USA.)) to a concentration of 8.89 mg/mL. The solution was then made up with 10 % (v/v) n-propanol (free radical scavenger) and MQ-water. Degassing (nitrogen) was preformed prior to the addition of 0.25 M sodium meta periodate in order to obtain mannuronan/alginate with P_0 (periodate/monomer ratio) of 0.02-0.08. The final polysaccharide concentration was 4.45 mg/mL. All pipetting and weighing were performed in subdued light and the reaction was carried out at 20ºC.

2.3 Epimerization

In the first part of the study periodate oxidized mannuronan (P_0 = 0.05-0.20) was used as the substrate for the epimerases AlgE4 and AlgE6. The substrate was dissolved in MQ-water overnight before a concentrated stock solution of MOPS buffer (pH 6.9) with $CaCl_2$ monohydrate and NaCl were added, and the mixtures were pre-heated at 37 °C. The respective enzymes were dissolved in MQ-water and immediately added to the substrate solutions. Final concentrations of the reaction mixtures were 0.25% (w/v) substrate, 50 mM MOPS, 2.5 mM $CaCl_2$ and 10 mM NaCl. The epimerization reaction was left for 20 h and stopped by lowering the pH to 3 with cold HCl.

In the second part of the study, mannuronan was epimerized with AlgE6 after or prior to periodate oxidation and reduction. The epimerization was carried out as above, but with some important exceptions. The NaCl concentration in the reaction solution was 75 mM and extra

Ca^{2+} and enzyme equalling half of the starting amount were added after 24 h. The total reaction time was extended to 48 h and the enzyme:substrate ratio was 1:20.

In both parts of the study the alginate was dialysed against 0.05 M HCl and finally against MQ-water at 4 °C until the conductivity was below 4 μS. The pH was then adjusted to 6.8, followed by freeze drying. The AlgE6 epimerized samples for small-strain oscillatory measurements were further purified to remove residual enzyme by precipitation with 50% (v/v) ethanol, filtration and 2 cycles of washing with 98% (v/v) ethanol.

2.4 NMR spectroscopy

All homonuclear experiments were carried out on a BRUKER Avance DPX 300 or 400 spectrometer equipped with a 5 mm QNP and 5mm z-gradient DUL (C/H) probe, respectively. Heteronuclear experiments were performed on a BRUKER Avance 600 spectrometer equipped with a 5 mm z-gradient CRTXI (H/C/N) probe. The NMR data were processed and analysed with BRUKER XwinNMR Ver. 3.6 software.

Prior to NMR analysis, the viscosity of the high-molecular-weight alginate were reduced by mild acid hydrolysis according to Ertesvåg & Skjåk-Bræk (1999). The alginates were then neutralized and freeze-dried and prepared by dissolution in 99.9% D_2O (Chiron, Trondheim, Norway). 5μL 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (Aldrich, Milwaukee, WI, U.S.A.) was used as internal standard for the chemical shift. For samples containing guluronic acid 20 μL of 0.3 M triethylenetetra-amine hexa-acetate (TTHA) (Sigma) was added to chelate any remaining Ca^{2+} from the epimerization.

Homonuclear 1D, 2D correlation spectroscopy (COSY) in magnitude mode and 2D rotating frame Overhauser effect spectroscopy (ROESY) with mixing time 200 ms were recorded at 90 °C for the assignment of the alginate oligomers. The ^{13}C heteronuclear single quantum coherence (HSQC) experiment was recorded at 40 °C.

2.5 Small-strain oscillatory measurements

The small-strain oscillatory measurements were performed using a StressTech Rheometer from Reologica, Lund, Sweden.

75 mg of alginate was dissolved in 5 mL of MQ-water. $CaCO_3$ was added to a concentration of either 10 mM or 20 mM. The gelling was initiated by adding D-glucono-δ-lactone (GDL) dissolved in MQ-water to a concentration of 20 mM or 40 mM. The final polysaccharide concentration was 10 mg/mL.

All experiments were carried out on 40 mm serrated plate/plate geometry with 1 mm gap. A constant temperature of 20 °C was used. The experiment was preformed with a controlled strain of 0.005 Pa. A sample of approximately 2.5 mL was applied to the rheometer, and the sample was covered with low viscosity silicone oil (BDH Silicone Products, KeboLab-10cSt at 20 °C) to prevent evaporation.

3. Results and Discussion

3.1 Structure studies of periodate oxidized mannuronan

Partially oxidized mannuronan (P_0 = 0.05-0.20) was characterised by ^{1}H-NMR spectroscopy before using this material as a substrate for C-5 epimerization. The main aim was to study the structural outcome before and after mild acid hydrolysis.

Figure 2 shows the ^{1}H-NMR spectra of a mannuronan oligomer (Fig. 2A) and a mannuronan oligomer with P_0 = 0.10 (Fig. 2B), both with DP_n ~ 30. Multiple peaks can be seen in the anomeric region of oxidized mannuronan compared to unoxidized mannuronan (4.8–5.2 ppm). The additional peaks must originate from protons of the oxidized mannuronan unit (O_M*) and the mannuronan residue neighbouring the oxidized unit (M-O_M*) (Fig. 1A). The complex peak pattern could tentatively be attributed to inter and intra residue hemiacetal formation. When the oxidized oligomer was reduced by sodium borohydride the pattern in the anomeric region was simplified, and two new peaks located at 4.75 and 4.85 ppm arise in addition to the always present H-1 proton from mannuronic acid residues (M-1) (Fig. 2C). The peak at 4.75 ppm appears as a shoulder on the M-1 peak. One of the two new peaks may originate from the H-1 proton of an unoxidized residue having an oxidized and reduced unit (O_M) as a neighbour in the polymer chain ($\underline{M}$-1O_M). The other peak, at 4.85 ppm, may originate from the H-1 proton in an oxidized and reduced unit having an unoxidized residue as a neighbour in the chain ($\underline{O_M}$-1M). In order to assign these two peaks the oxidized and reduced oligomer was exposed to mild acid hydrolysis (Smith degradation). The Smith technique results in a splitting of the oxidized unit (Fig. 3) and hence the peak originating from the H-1 proton on this unit would shift or disappear from the anomeric region of the spectrum. After hydrolysis the peak at 4.75 ppm did indeed disappear and at the same time new peaks appeared representing the formation of reducing ends (M-$1_{red\alpha}$ and M-$1_{red\beta}$) along with a triplet at 5.05 ppm (Fig. 2D). The weak reducing end signals are most likely a result of the hydrolysis of glycosidic linkages between unoxidized residues. Based on these observations the peak at 4.75 ppm was assigned to the signal from $\underline{O_M}$-1M. The signal at 4.85 ppm however was still intact after hydrolysis and was assigned to the $\underline{M}$-1O_M sequence. It was thus concluded that the integral of the latter signal could be taken as a direct measure of the degree of oxidation, when compared against the total signal from mannuronic acid (M-1). The reason why the M-1O_M signal was intact after hydrolysis of the oxidized unit, and not

simply appears as a new reducing end signal, is presumably that the oxidized unit is hydrolysed in such a way that a fragment of it is still attached to its neighbouring residue (Fig. 3). This scheme fits with the predictions reported by Sharon (1975) for the weak acid hydrolysis of periodate oxidized and borohydride reduced polysaccharide nigeran. According to Sharon (1975) the remains of the oxidized unit can be cleaved off, yielding a new reducing end using more acidic conditions. However, the exact nature of the remains of the split oxidized unit, along with the conditions needed for further cleavage is to our knowledge not well known for polyuronic acids.

The degradation of oxidized mannuronan was studied in the NMR tube (90 °C and pD 4.5) in an attempt to assign the origin of the triplet signal at 5.05 ppm appearing upon acid hydrolysis. Signs of degradation could be observed already after 15-30 minutes (Fig. 4). In addition to a weak signal from M-$1_{red\alpha}$ the triplet signal at 5.05 ppm was observed. It was formed almost instantly and it was constant, while the reducing ends were increasing as the hydrolysis proceeded. It has been previously reported that the oxidized and reduced units of methyl α-glucoside are hydrolysed at a rate 10^5 times faster than the unoxidized residues (Sharon, 1975). It was therefore assumed that the triplet signal was either due to the protons on the remains of the oxidized unit or the formation of glycolaldehyde, the latter being a 'biproduct' of the acid hydrolysis as shown in figure 3. A ^{1}H 2D COSY experiment was conducted in order to clarify this and the result is shown in figure 4. The figure show that the proton giving rise to the triplet at 5.05 ppm only had a cross peak with the doublet peak at 3.51 ppm. No other cross peaks could be indentified to protons from the oligomer. This observation therefore points towards the existence of glycolaldehyde. To further verify this, a ROESY experiment was recorded, and no cross peaks were found for any of the sugar signals. This indicates that signals at 3.52 ppm and 5.05 ppm were due to a single compound. Finally, to prove the existence of glycolaldehyde, a ^{13}C HSQC was recorded. In this experiment the protons at 5.05 ppm coupled to a carbon atom with chemical shift of 90.54 ppm, which could be assigned directly to the hemiacetal group, while the doublet peak at 3.51 ppm coupled to a carbon atom with chemical shift of 65.29 ppm, which fits with the expected chemical shift for the alcohol group of glycolaldehyde. The peak assignment also fits with the listed spectrum (accession # bmse000258) deposited in the Biological Magnetic Resonance Databank (http://www.bmrb.wisc.edu/) for glycolaldehyde in D_2O, and is consistent with previous literature describing glycolaldehyde on its hemiacetal form (Amyes & Richard, 2007; Glushonok et al., 2000).

3.2 Periodate oxidized and reduced mannuronan as a substrate for AlgE4 and AlgE6

In an attempt to 'tailor' alginate with increased chain flexibility and long stretches of MG or GG sequences, mannuronan was oxidized and borohydride reduced followed by epimerization with AlgE4 (i) or AlgE6 (ii). Using this strategy only the M residues are oxidized and the G-blocks or MG sequences, which are the main structural elements contributing to gel formation, are introduced in the segments between the oxidized residues (Fig. 1).

The results of AlgE4 and AlgE6 acting upon oxidized and reduced mannuronan are summarized in table 1, showing that the epimerases indeed could work upon these substrates. Since the C-5 epimerases are known to be highly substrate specific it was assumed that only unoxidized residues were epimerized, which seems to be in agreement with the observation that the conversion to guluronic acid decreased when the degree of oxidation increased.

The decrease in L-guluronic acid was expected since sufficiently long and uninterrupted mannuronan sequences, upon which the epimerases could act, become less abundant. Assuming random oxidation, the distribution of blocks consisting of contiguous unoxidized M-residues can be calculated by directly applying the theory for random depolymerisation (or polymerisation) of linear polymers (Tanford, 1961). In this case the degree of oxidation (P_0) replaces the degree of chain scission (α) used in the original formula. We assume this approach is valid for $P_0 < 0.5$, which is the well-known oxidation limit of alginate (Larsen & Painter, 1969). The weight fraction of blocks with *n* contiguous M-residues ($W(n_M)$) for an indefinitely long chain then follows the expression:

$$W(n_M) = n_M P_0^2 (1-P_0)^{(n_M-1)} \qquad (1)$$

The action of AlgE4 and AlgE6 upon mannuronan oligomers has previously been investigated. It was found that AlgE4 needs a minimum of 6 consecutive M-residues to start epimerization, while 8 residues are needed for AlgE6 (Campa et al., 2004; Holtan et al., 2006). At $P_0 = 0.10$ $W(n_M \geq 8)$ equals 0.81 (81% of the M residues are found in blocks with $\geq$ 8 M residues), indicating that the enzymes have several M-stretches to attach. When P_0 increases to 0.20, $W(n \geq 8)$ is reduced to 0.50 (Fig. 5). Since no information exists stating how many M residues are epimerized as n_M increases, it seems difficult to predict accurately the expected (theoretical) F_G value for periodate oxidized mannuronan. However, it can partly explain why no epimerization was observed at $P_0 = 0.20$ for neither AlgE4 nor AlgE6. In

addition to the limitations in n_M, the M-residues neighbouring the oxidized unit might also be unavailable for epimerization, causing the stretches upon which the enzymes can act to be even fewer. The increased flexibility of the polymer may also affect the epimerization to some extent. The details concerning this are still not completely elucidated and await further investigation.

3.3 Mannuronan epimerized with AlgE6 before or after oxidation/reduction

Two different strategies were applied to engineer chemically modified alginates that could serve as building blocks for novel hydrogels. First, mannuronan was partially oxidized in the range P_0 = 0.02-0.08, reduced by borohydride, and subsequently epimerized using AlgE6 (strategy I). The conditions used for epimerization introduce 90% guluronic acid in unoxidized mannuronan. Secondly, pure mannuronan was first epimerized with AlgE6 to obtain nearly poly-guluronic acid (90% G, $N_{G>1}$ = 22), and then periodate oxidized (P_0 = 0.02-0.08), followed by borohydride reduction (strategy II).

The samples were initially investigated by size-exclusion chromatography (SEC) combined with multiangle laser light scattering (MALLS) and viscosity detectors (VISC). ^{1}H-NMR was also applied in order to determine the content and distribution of guluronic acid and the structural outcome of the periodate oxidation.

The SEC-MALLS-VISC analysis of the oxidized alginates (Strategies I and II) showed a decrease in molecular weight compared to the parent mannuronan (Fig. 6 and Table 2). Depolymerisation during periodate oxidation of alginate is well-known and has been reported previously (Painter & Larsen, 1970, Vold et al., 2006). In this study, interestingly, M_w was almost independent of P_0 in the range 0.02-0.08. Table 2 also shows that the mannuronan samples epimerized after oxidation/reduction had a lower M_w compared to the mannuronan samples epimerized prior to oxidation. This difference is most likely due to degradation of the oxidized/reduced mannuronans during epimerization. In a separate experiment, epimerization of oxidized (non-reduced) mannuronan was carried out, showing that the degradation was even more severe. Since the conditions for epimerization (pH 6.9, 37 °C, 48 h) are relatively mild, this describes the instability of the oxidized/reduced mannuronan residues and the even more unstable oxidized/non-reduced residues under these conditions. This observation is in accordance with a previous study of the degradation of oxidized alginates (Bouhadir et al., 2001).

The exponent (*a*) and constant (*K*) in the Mark-Houwink-Sakurada (MHS) relationship, $[\eta] = K \cdot M^a$), is a measure of the shape and extension of polysaccharide chains. No large differences could be observed for these parameters for the oxidized/reduced samples. The persistence length (*q*) is another and more direct measure of the polymer flexibility. Vold et al. (2006) estimated *q* based on the Bohdanecký wormlike chain model). They investigated a broad range of oxidized/reduced alginate samples and found, for instance, that *q* decreased from 12 nm for the unoxidised sample to 4 nm for a sample with $P_0 = 0.44$. In this study *q* was calculated in the same way (ionic strength: 0.17 M), and a value of 12-11 nm was obtained for the samples with $P_0 = 0.00$-0.04. The samples with $P_0 = 0.06$ and 0.08 showed a *q* equal to 9 nm and 8 nm, respectively. This shows that the flexibility of the samples did indeed increase as P_0 increase, which is in agreement previous studies (Vold et al., 2006; Lee et al., 2002).

Figure 7A and 7B show the ^{1}H-NMR spectrum of mannuronan epimerized with AlgE6 following oxidation and reduction (strategy I), with P_0 equal to 0.04 and 0.08, respectively. The peaks in the spectrum were assigned according to Grasdalen et al. (1983) and Holtan et al. (2006). The signal M-1O_M can also be observed as for oxidized mannuronan. The signal originating from the ^{1}H-proton of the oxidized unit ($\underline{O_M}$-1M) was no longer present since the sample was partially hydrolysed prior to NMR analysis to improve the quality of the spectrum. The fraction of guluronic acid residues (F_G) was calculated according to Grasdalen (1983). It should be mentioned that the triplet originating from glycolaldehyde observed at 5.05 ppm in the spectra of acid hydrolysed oxidized and reduced mannuronan should also be present, but is overlapping with the G-1 signal. Since the triplet comprises < 2% of the G-1 signal in samples with $P_0 < 0.10$ and $F_G \geq 0.40$, its presence was ignored in the sequence calculations. The average G-block length can be calculated from the expressions:

$$N_{G>1} = \frac{n_G - n_{MGM}}{n_{GGM}} = \frac{F_G - F_{MGM}}{F_{GGM}} \qquad (1)$$

n represents the number and F the fraction of the monad or triad sequences, respectively (Grasdalen, 1983). The degree of oxidation could be estimated directly from the spectrum in the same way as described for oxidized and reduced mannuronan (Table 2). Excellent agreement between the observed degree of oxidation and P_0 indicates that all periodate was consumed in the P_0 range studied, and that any over-oxidation in these cases can be neglected. As the degree of oxidation increases, a decrease in the fraction of G-residues can be observed along with a reduction in G-block length (Table 2). The main reason for this was that the

enzyme had fewer sites for attack, and a minimum of 8 intact mannuronate residues are required for AlgE6.

The mannuronan epimerized with AlgE6 prior to oxidation and reduction (strategy II) was also studied by ^{1}H-NMR, and figure 7C and 7D shows the ^{1}H-NMR spectrum for samples with P_0 equal to 0.04 and 0.08, respectively. The spectrum is qualitatively similar to the spectra discussed above, except for the appearance of a peak assigned as G-5O_G, analogous to the M-1O_M peak at 4.60 ppm. This peak can be taken as a measure of P_0 in the same way as stated above for the M-1O_M peak. The M-1O_M is not seen, which is expected since F_M is only 0.10. This means that if P_0 is 0.08, only 0.008% of the oxidized residues originate from mannuronic acid residues, assuming random oxidation (Painter & Larsen, 1970). This argument also justifies the use of the G-1O_G peak as a direct measure of the degree of oxidation for low P_0. The peak attributed to the H-5 proton on an oxidized guluronic acid residue (O_G-5G) cannot be observed, since this unit is cleaved during acid hydrolysis analogous to the material described above. The average G-block length can be calculated from the NMR-spectrum, but since the oxidation takes place predominantly within G-residues and thus splitting the G-blocks some modifications must be made to the original expression given by Grasdalen (1983). The following expression is suggested, when an oxidized G-residue (G*) is taken to terminate a G-block in the same way as an M-residue:

$$N^*_{G>1} = \frac{n_G - n_{MGM}}{n_{GGM} + n_{MGG^*} + n_{GGG^*}} \qquad (2)$$

Due to low abundance and/or poor resolution of the signals some assumptions have to be made in order to simplify this expression.

a) n_{MGM} can be neglected since $n_{MGM} << n_G$. AlgE6 is a G-block forming enzyme and few single G-units are present.
b) All the oxidized guluronan residues (G*) are mainly within a G-block and the probability of the sequence MGG* is low, hence $n_{MGG^*} \sim 0$.
c) The signal from glycolaldehyde overlapping with the signal for H-1 on guluronic acid can be ignored on the same grounds as described above.

Applying these assumptions to expression 2 yields:

$$N^*_{G>1} = \frac{n_G}{n_{GGM} + n_{GGG^*}} = \frac{F_G}{F_{GGM} + F_{GGG^*}} \qquad (3)$$

Table 2 show the G-block length calculated using this expression ($N_{G>1}$*) and it is evident that $N_{G>1}$* is rapidly decreasing as the degree of oxidation is increasing.

3.4 Gelation of mannuronan epimerized after or prior to oxidation and reduction

Mannuronans epimerized after or prior to periodate oxidation and borohydride reduction with AlgE6 (strategy I and II, P_0 = 0.02-0.08) were studied using small-strain oscillatory measurements. This method was considered appropriate since it gives information about gelling kinetics and requires low sample amounts compared to other rheological methods. Gelling was initiated using the 'internal setting method' described by Draget et al. (1993) to make homogenous alginate hydrogels. The principle of the 'internal setting method' is that $CaCO_3$ (powder) is added to the sample followed by addition of by D-glucono-δ-lactone (GDL), which gradually makes the mixture acidic, thus slowly releasing Ca^{2+}-ions in order for the gel to set homogeneously.

Various parameters, both physical and chemical, might influence the gel elasticity and kinetics. In this study the physical parameters were kept constant, implying that the chemical parameters like F_G, $N_{G>1}$ and the molecular weight distribution (MWD) control the final gel elasticity. In addition it was expected that the flexible segments introduced through periodate oxidation would have considerable influence on the characteristics of the formed gels. All gels were formed at 20 °C within a timeframe of 24 h.

Gelation of mannuronan epimerized after oxidation and reduction (strategy I) was studied, and the results are given in figure 8A. An alginate concentration of 10 mg/mL, 20 mM Ca^{2+} and 40 mM GDL were used. For the samples P_0 = 0.02 and P_0 = 0.04 the sol/gel transition started at approximately 1.7 h, as observed by a significant rise in the dynamic storage modulus (G') and a drop in the phase angle (fig. 8A and 8B). The kinetics however, was different since the sample with P_0 = 0.02 exhibited a steeper increase in G' than the sample with P_0 = 0.04. The terminal G' value after 24 h decreased dramatically when the degree of oxidation increased, as can be observed in figure 8A and table 2. Only the samples with P_0 = 0.02 and P_0 = 0.04 formed true gels according to the criterion G'>G'' (G''; dynamic loss modulus) over a wide range of frequencies (Kavanagh & Ross-Murphy, 1998). As discussed above F_G, $N_{G>1}$ and the molecular weight decreased as the degree of oxidation increased (Table 2). $N_{G>1}$ and the molecular weight have previously been pointed out as the most critical parameters for efficient gel formation (Draget et al., 1993). However, the

decrease in molecular weight among the different oxidized samples was not sufficiently pronounced to explain the observed decrease in G' (Draget et al., 1993). The most likely explanations can be found in other consequences of periodate oxidation. First, the distribution of G-blocks might be different in the oxidized/epimerized samples compared to native alginates. Secondly, an increase in chain flexibility may result in a higher probability for intramolecular cross-links, where the alginate molecule partly folds back on itself instead of forming intermolecular neighbouring cross-links, which is a pre-requisite for the formation of functional elastic segments. In order to gain some further understanding of the rapid decrease in G', the sample with P_0 = 0.06 and alginate from *L. hyperborea* leaf with similar composition (F_G and $N_{G>1}$), and polydispersity index (M_w/M_n) were compared with regard to the final G' obtained after 24 h (Table 2). The molecular weight (M_w) differs for the two samples by approximately 30%. While the alginate from *L. hyperborea* leaf had a G' value of 243 Pa, the sample with P_0 = 0.06 had a value less than 1 Pa. The gel made from *L. hyperborea* leaf is also prepared using half the amount of Ca^{2+} and GDL (10 mM Ca^{2+} and 20 mM GDL) compared to the oxidized sample. Although there is a difference of 30% in molecular weight, it seems unlikely that this factor alone would result in a 243 times lower G'. Without undermining the importance of a different G-block distribution in the two samples, it seems fair to conclude that the increased flexibility is the main reason for this huge difference in G'.

The mannuronan samples epimerized with AlgE6 before oxidation and reduction (strategy II), thus having a constant F_G of 0.90, were also studied by small-strain oscillatory measurements as shown in figure 8C. Alginate from *D. antarctica*, *L. hyperborea* stipe and leaf with varying F_G, $N_{G>1}$ and molecular weights, are also included for comparison. For these samples the alginate concentration was kept equal to the one above (10 mg/mL), but 10mM Ca^{2+} and 20 mM GDL were used in order to avoid syneresis for the unoxidized material (F_G = 0.90). The same concentrations were used for the oxidized samples to allow direct comparison. From figure 8C and 8D it can be observed that for the sample P_0 = 0.02 the sol/gel transition starts approximately 30 min prior to the unoxidized sample (strategy II). The reason for this is not obvious, but might be explained by a higher probability for intermolecular cross-links to occur, when the chains are more flexible. An apparently similar observation was made by Draget et al. (2000), when investigating Ca^{2+}-gels of alginates with different sequential arrangements of M-blocks and G-blocks following epimerization with AlgE4 leading to the introduction of the MG sequences on the expense of M-blocks. It has been reported that the local flexibility within alginate molecules differs depending on their

composition of M and G (Smidsrød et al., 1973; Stokke et al., 1991). Thus it was observed that alginates containing more flexible regions initiated gelling at an earlier stage than alginates possessing less local flexibility. The final G' (24 h) of the oxidized samples (strategy II) decreased as the degree of oxidation increased in the same way as described for the samples above (strategy I). Alginate from *L. hyperborea* stipe has lower guluronic acid content, but similar $N_{G>1}$ and molecular weight as the sample $P_0 = 0.04$ (Table 2), the G' values being 308 Pa and 37 Pa, respectively. Again this suggests that the increased flexibility is the main reason for the rapid drop in G' as the content of oxidized units increase.

Comparing the two sets of samples, it can be observed that the samples with $P_0 > 0.04$ epimerized prior to oxidation (strategy II) had slightly higher G' values than the ones epimerized after oxidation (strategy I). This might seem surprising since the Ca^{2+} content was higher (factor 2) for the latter samples and the $N_{G>1}$ was marginally higher. This result can possibly be explained by the fact that the molecular weight of the samples epimerized prior to oxidation (strategy I) is 20-30% lower than the samples epimerized after oxidation (strategy II). Since the molecular weight of these samples ($P_0 > 0.04$) are in a range where a difference of 20% might result in a significant change in G', this can be one reason for this observation. Another reason might be that the accuracy (signal to noise ratio) of the G' measurements are low, when measuring G' values less than 10 Pa.

In general, the data in figure 8 suggests that periodate oxidation is a useful tool to control gel strength in the same way as varying the content and distribution of guluronic acid and molecular weight. Under the conditions used in this study a $P_0 \geq 0.06$ 'knocks out' the materials gelling ability resulting in a viscous liquid. Although this initially can be interpreted as a drawback it might be beneficial in an application where gelling of alginate is unwanted.

Conclusion

In this study we found that periodate oxidized and borohydride reduced mannuronan could serve as substrate for the C-5 epimerases AlgE4 and AlgE6, when P_0 is below 0.10. The effective degree of oxidation could be directly estimated from ^{1}H-NMR, suggesting a stoichiometric relationship between added periodate and oxidized units. Gels made of mannuronan epimerized by AlgE6 before or after oxidation/reduction show that gel properties can be tuned by periodate oxidation for P_0 less than 0.06. The decrease in G' with increasing P_0 was most likely mainly attributed to increased flexibility in the alginate chains. The

resulting gels are easily degraded in a mild acidic environment and the degradation is easier to control than in gels made of unoxidized alginate (Bouhadir et al., 2001).

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Figure and table captions

Figure 1.

A: Structure of a mannuronan fragment (…MMM…) followed by periodate oxidation, borohydride reduction and epimerization (…GMM…). Abbreviations: M, β-D-mannuronic acid; G, α-L-guluronic acid. B: Schematic illustration of two different strategies for making alginate materials.

Figure 2.

The ^{1}H-NMR spectra of mannuronan oligomers (DP_n~30); untreated (A), P_0 = 0.10 (B), P_0 = 0.10, borohydride reduced (C), P_0 = 0.10, borohydride reduced and acid degraded (D). Peaks were assigned according to Grasdalen (1983) except for the peaks M-1O and O_M-1M (see text).

Figure 3.

Mannuronan treated with sodium periodate, sodium borohydride and degraded by mild acid hydrolysis. Suggested reaction products based upon Fransson & Carlstedt (1974) and Sharon (1975).

Figure 4.

2D COSY (magni mode) experiment of a mannuronan sample with P_0 = 0.10, borohydride reduced and acid degraded (pD 4.5, 12 h, 90 °C).

Figure 5.

The weight fraction of M-blocks (n_M) larger or equal to 4 (black), 6 (blue), 8 (red), 10 (green) or 12 (brown), from the top and down, are shown as a function of the fraction of oxidized units (P_0) within the polymer. For colours, see on-line version.

Figure 6.

Mannuronan epimerized with AlgE6 to 90% guluronic acid and periodate oxidized in the range P_0 = 0.02-0.08 and reduced by borohydride (strategy II) was analysed with size exclusion chromatography (SEC) combined with multiangular laser light scattering (MALLS) and viscosity (VISC) detectors.

Figure 7.

The ^{1}H-NMR spectrum of mannuronan oxidized to P_0 = 0.04 (A) and P_0 = 0.08 (B) and epimerized with AlgE6 to 64% and 43% guluronic acid, respectively. Mannuronan epimerized to 90% guluronic acid followed by oxidation to P_0 = 0.04 (C) and P_0 = 0.08 (D) is also shown. All samples are reduced with borohydride after oxidation and hydrolysed (60 min pH 5.6 followed by 45 min pH 3.8 90 °C) before NMR analysis. Peaks were assigned according to Grasdalen (1983) except for the peaks G-5O_G and M-1O_M (see text).

Figure 8.

Upper figures: Dynamic storage modulus (G') (A) or phase angle (B) versus time (h) for mannuronan epimerized after oxidation with AlgE6 (strategy I). Lower figures: G' (C) or phase angle (D) versus time (h) for mannuronan epimerized prior to oxidation with AlgE6 (strategy II). Alginate from *Laminaria hyperborea* (leaf and stipe) and *Durvillea antarctica* are shown for comparison. The gels were prepared by 'internal setting' (A and B: 20 mM $CaCO_3$/40 mM GDL. C and D: 10 mM $CaCO_3$/20 mM GDL).

Table 1.

The fraction of guluronic acid (F_G) and G-block length ($N_{G>1}$) are shown for periodate oxidized mannuronan epimerized with AlgE4 (i) and AlgE6 (ii).

Table 2.

The fraction of G-residues (F_G), G-block length ($N_{G>1}$), calculated degree of oxidation (CDO), weight average molecular weight (M_w), polydispersity (M_w/M_n) and dynamic storage modulus (G') after 24 h are shown for the different samples epimerized with AlgE6, after (strategy I) or prior (strategy II) to periodate oxidation/reduction. Three natural occurring alginates are shown for comparison.

Figures and Tables

Figure 1

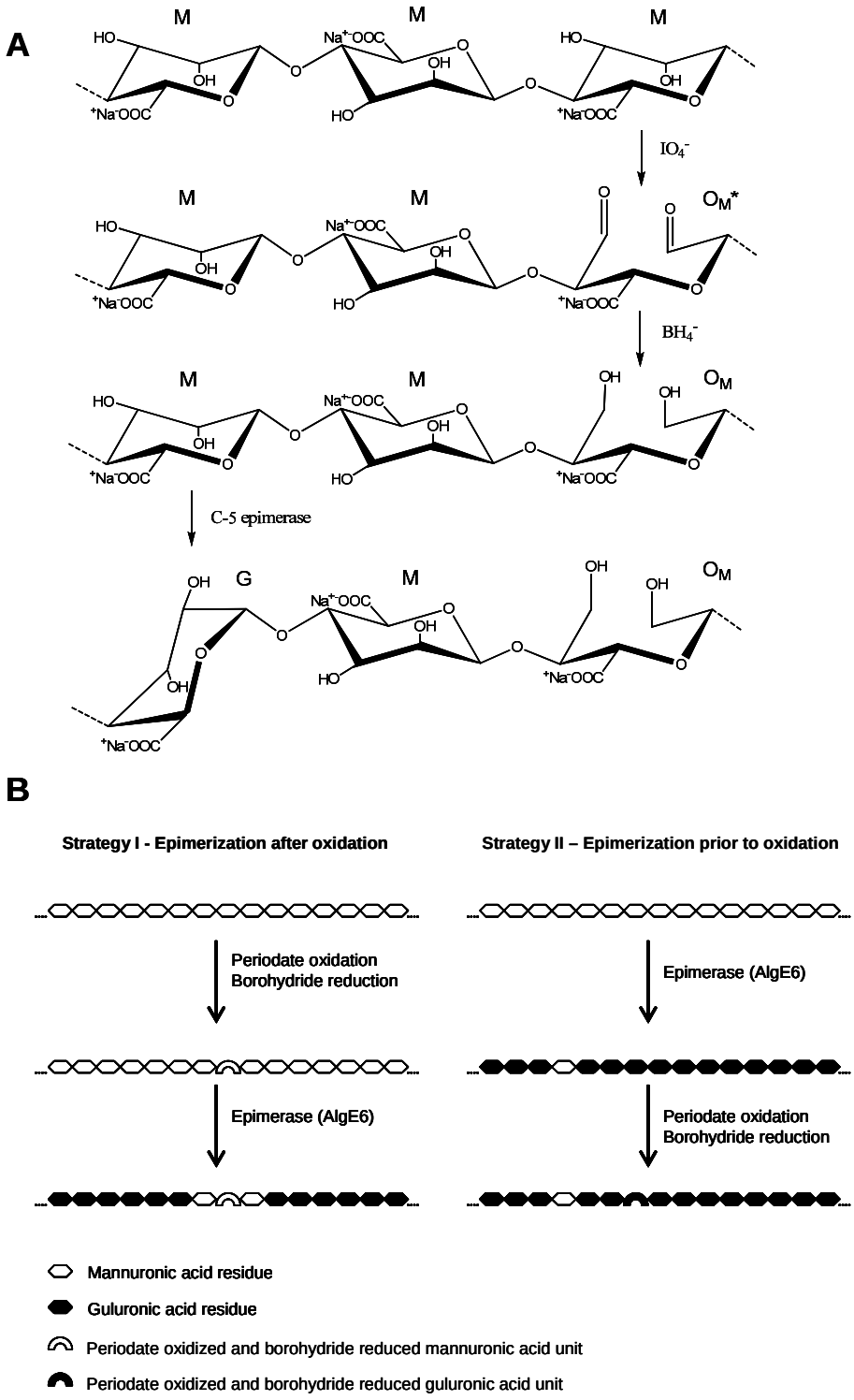
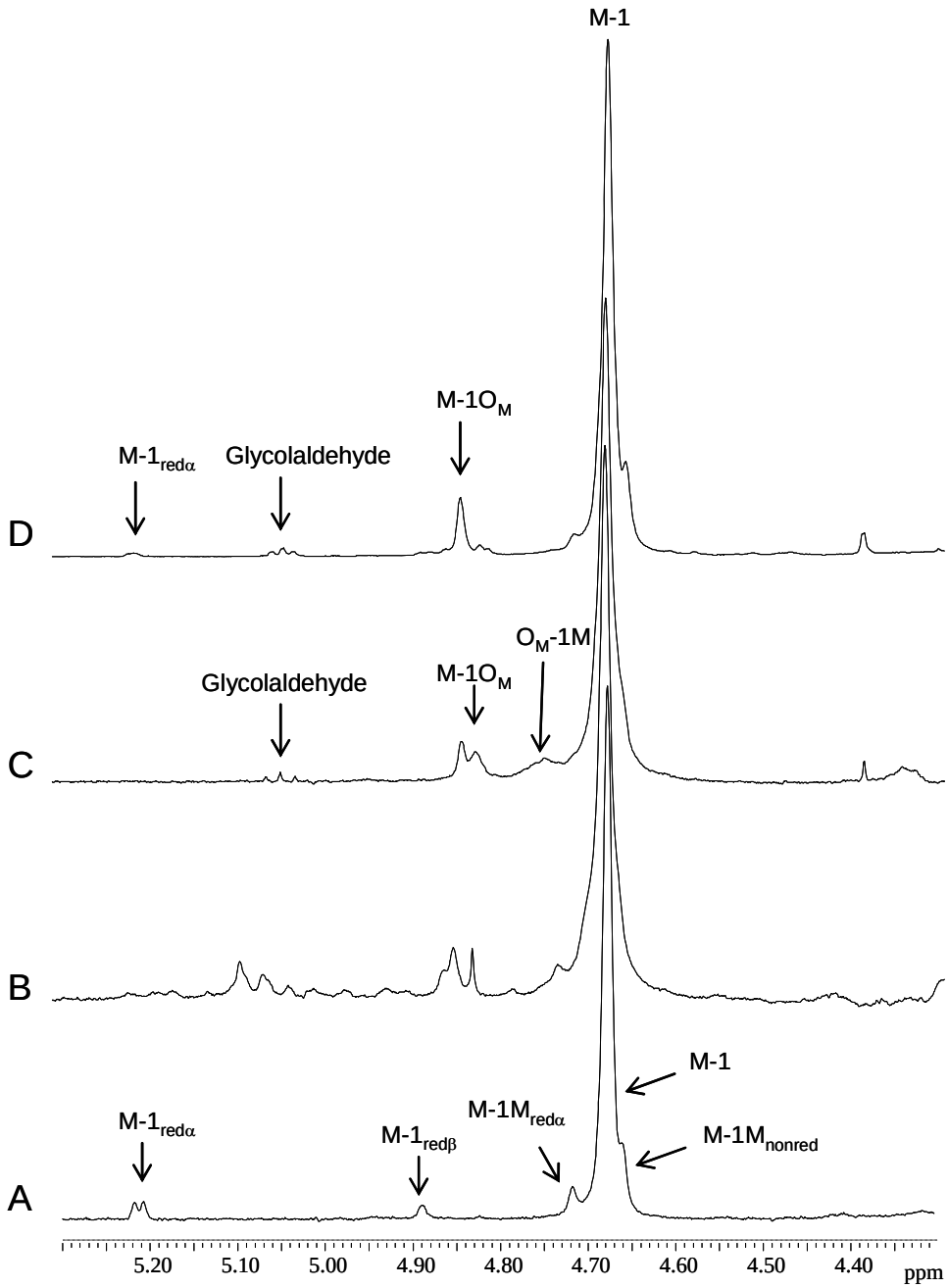


Figure 2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M-1O$_M$
M-1$_{red\alpha}$
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D
O$_M$-1M
Glycolaldehyde
M-1O$_M$
C
B
M-1
M-1M$_{red\alpha}$
M-1$_{red\alpha}$
M-1$_{red\beta}$
M-1M$_{nonred}$
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5.00
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4.80
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4.40
ppm

Figure 3

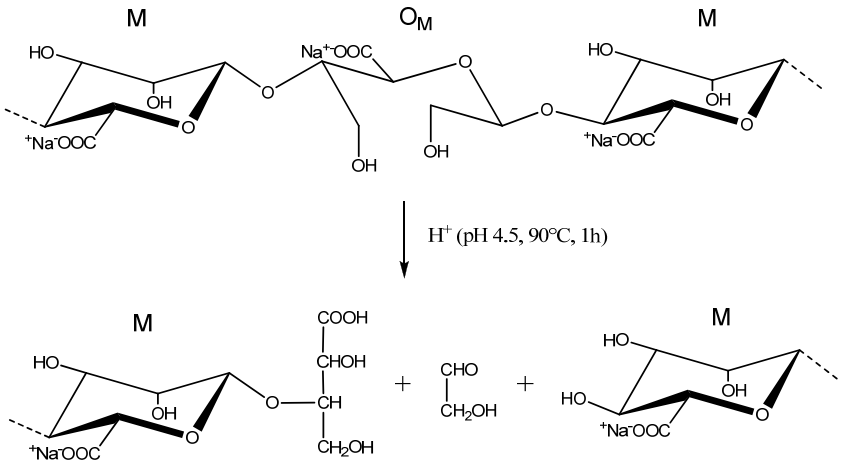

Figure 4

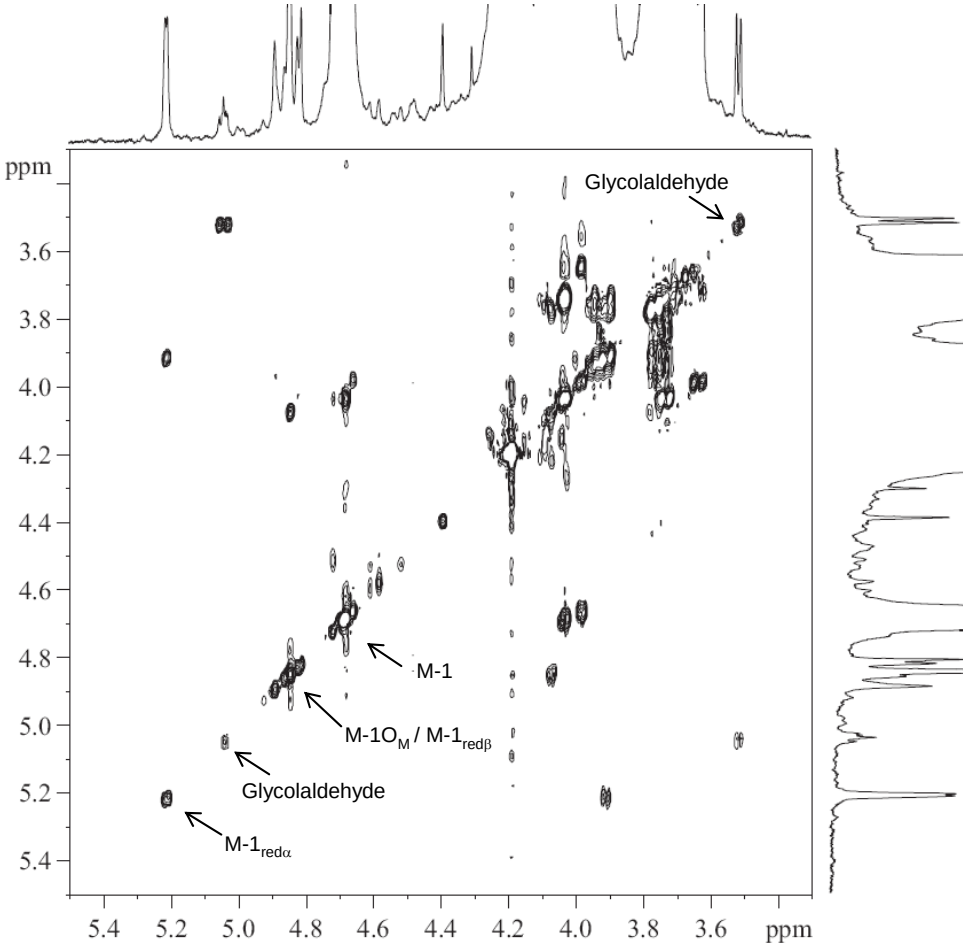

Figure 5

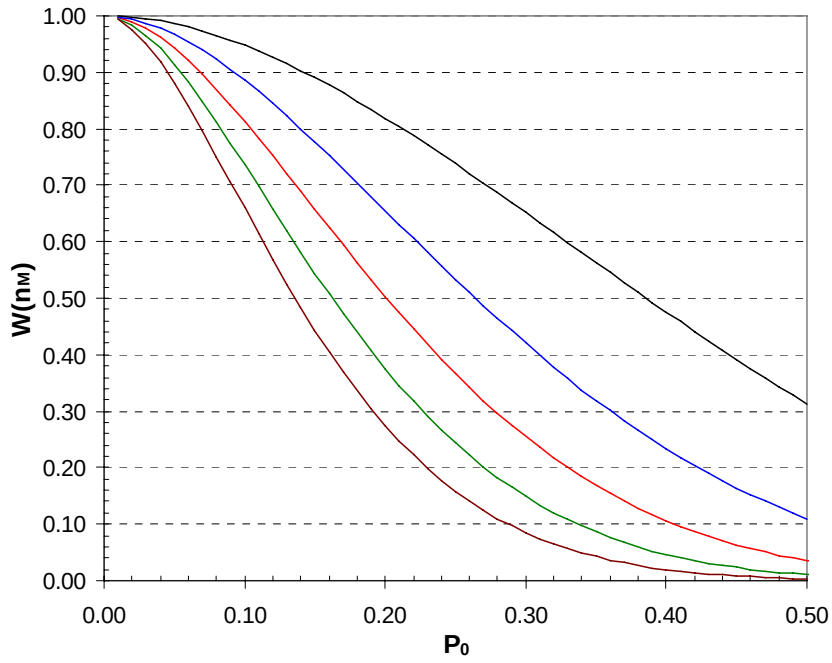

Figure 6

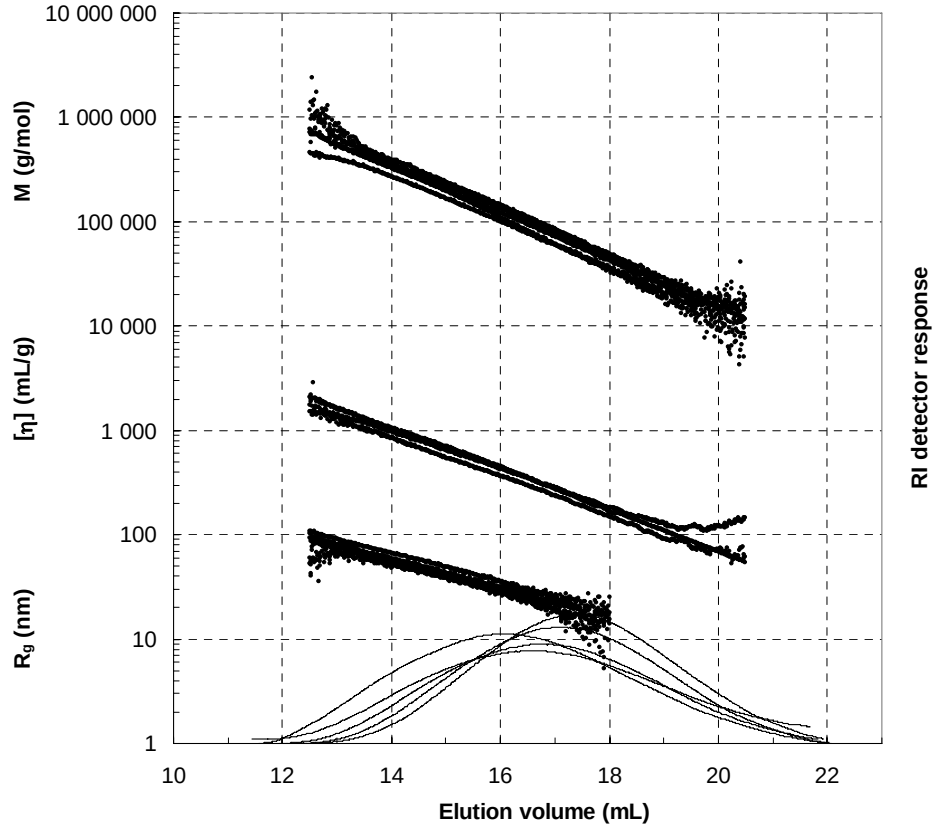

Figure 7

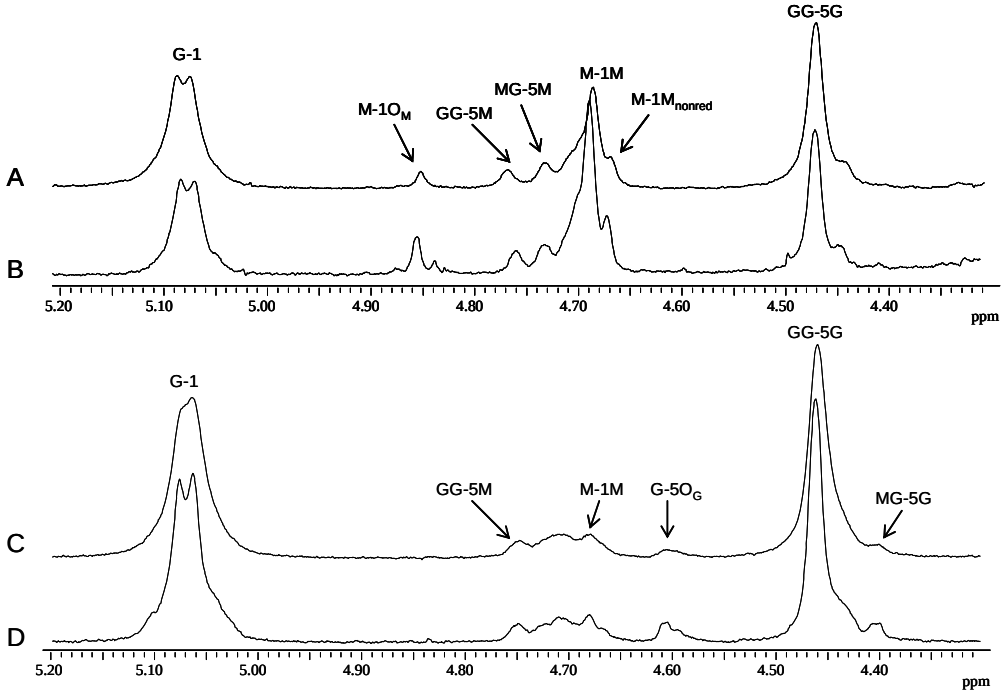

Figure 8

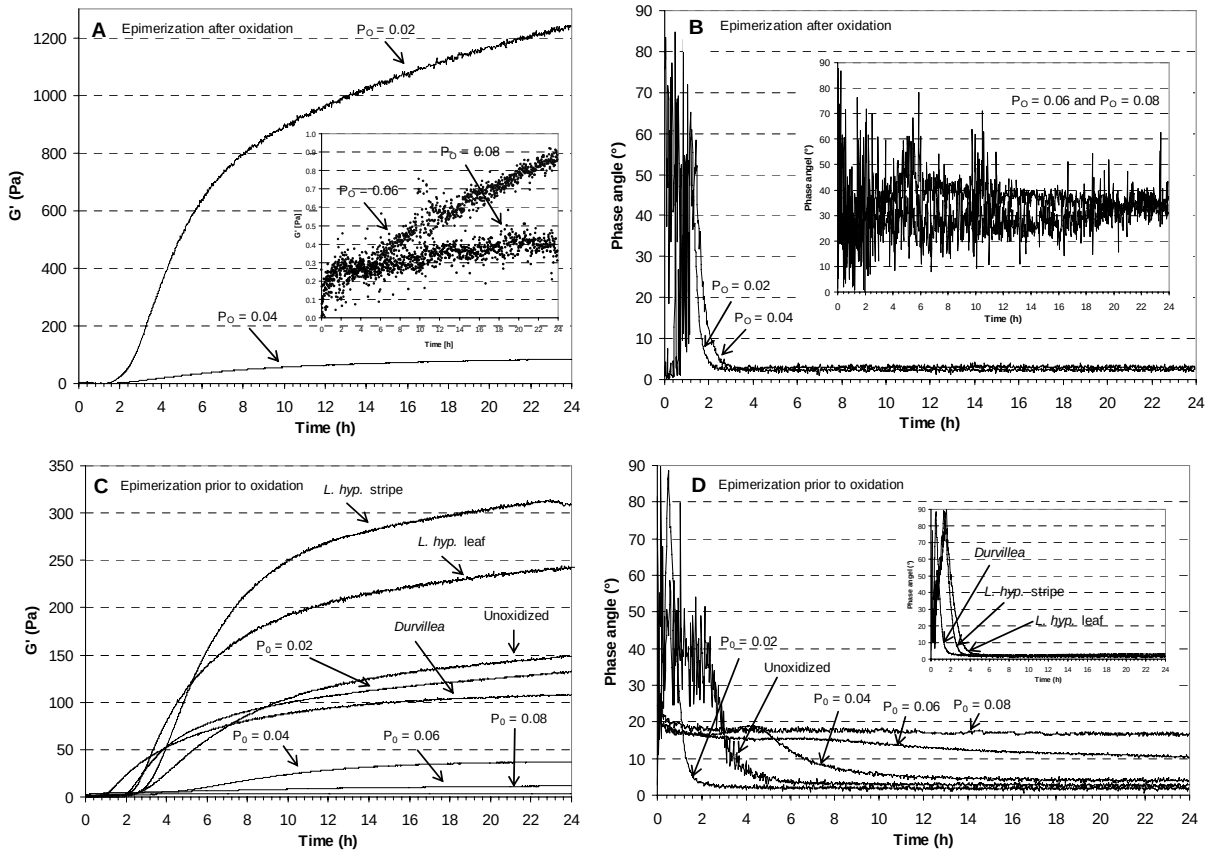

Table 1

Sample	P_0	F_G	$N_{G>1}$
i	0.00	0.46	n.d.
i	0.05	0.26	n.d.
i	0.10	0.14	n.d.
i	0.20	n.d.	n.d.
ii	0.00	0.40	5.80
ii	0.05	0.34	4.80
ii	0.10	0.29	3.00
ii	0.20	n.d.	n.d.

n.d. - not detected

Table 2

Sample	P_0	F_G	$N_{G>1}$*	Calc P_0	M_w [kDa]	M_w/M_n	G' [Pa]**
Mannuronan	0.00	0	-	-	161	2.5	-
I	0.02	0.75	16	0.02	96	2.2	1237
I	0.04	0.64	12	0.04	94	2.5	85
I	0.06	0.54	11	0.06	88	2.2	<1
I	0.08	0.43	9	0.07	71	2.1	<1
II	0.00	0.90	22	-	156	2.7	149
II	0.02	0.90	17	0.02	115	2.3	133
II	0.04	0.90	12	0.04	107	2.6	37
II	0.06	0.90	9	0.05	104	2.3	12
II	0.08	0.90	8	0.07	98	2.1	4
*Durvilla antarctica****	0.00	0.34	5	-	146	2.0	108
Laminaria hyperborea leaf***	0.00	0.51	11	-	129	2.1	243
Laminaria hyperborea stipe***	0.00	0.65	12	-	117	2.3	308

*for samples II $N_{G>1}=F_G/(F_{GGM}+F_{GGG*})$, see text.

**after 24 h. Samples I were prepared using 20 mM Ca^{2+}, while 10 mM Ca^{2+} were used for samples II.

***acid hydrolysed.

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Interactions of polysaccharides extracted by mild acid hydrolysis from the leaves of *Sphagnum papillosum* with either phenylhydrazine, *o*-phenylenediamine and its oxidation products or collagen

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Abstract

The purpose of this research was to evaluate if pectin-like polysaccharides, collectively known as sphagnan, extracted by acid hydrolysis from the leaves of *Sphagnum* moss have a unique ability to react with phenylhydrazine, *o*-phenylenediamine or collagen. A previous assay for determination of carbonyl groups in sphagnan by reaction with phenylhydrazine was disproved due to spectrophotometric interference from furans. The actual carbonyl content of sphagnan is estimated to be much less than previously thought. NMR spectroscopy showed that small amounts of *o*-phenylenediamine and/or its oxidation products bind to sphagnan probably via imine formation, but evidence of quinoxaline formation was inconclusive. Sphagnan–gelatin mixtures formed complex coacervates at pH 2.0–4.8 at low ionic strength, which is typical of electrostatic polyelectrolyte interactions, rather than covalent carbonyl-amine reactions. Measurements of hydrothermal stability and collagenase-degradation of sphagnan-treated hide powder collagen suggest that sphagnan is a poor tanning agent. The results indicate the suggested preservative properties of sphagnan are not related to tanning.

Keywords: Gelatin; Tanning; Bog body; Preservation; Maillard, 5-KMA

1. Introduction

It has been claimed that pectin-like polysaccharides in the leaves of *Sphagnum* moss, known collectively as sphagnan, have a unique potential to react with certain amines (Børsheim, Painter, & Christensen, 2001; Painter, 1991). This notion stems from the alleged presence in sphagnan of ~25 M % novel ketouronic acid residues in the form of 5-keto-D-mannuronic acid (5-KMA) which could exist in the form of either pyranose (5-KMA*p*) or furanose (5-KMA*f*), of which the latter contains an α-keto-carboxylic acid group (Painter, 1983; Painter, 1991). Two predominant reactions between amines and 5-KMA have been proposed (Painter, 1991). The first is mild acid-catalysed imine (C=NR) formation via nucleophilic addition of an amine to the α-keto group of 5-KMA*f* to form a carbinolamine, followed by acid-catalysed elimination of water. The second is glycosylamine formation by reaction of a semi-protonated diamine with 5-KMA*p* (Painter, 1991). Glycosylamine formation is promoted by electrostatic interaction between the deprotonated carboxyl group in 5-KMA and the protonated amine. The amine then forms a hydrogen bond with the hemiketal hydroxyl group of 5-KMA*p*, followed by spontaneous elimination of water to yield a glycosylamine (Painter, 1991). The hypothesis of imine formation was based on observations that sphagnan readily reacts with aqueous phenylhydrazine (calculated pK_a 5.29) to form orange-coloured polymeric phenylhydrazones, whereas sphagnan treated with $NaBH_4$, a carbonyl-reducing agent, is inert (Painter, 1983). Hydrolysis of the orange-coloured complexes with concentrated

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HCl followed by filtration and dilution led to the indirect determination of 5-KMA by measuring the absorbance of the released phenylhydrazine (Painter, 1983; Smidsrød & Painter, 1984). Observation of a fluorescent quinoxalinol formed when the free acid-form of sphagnan was mixed with *o*-phenylenediamine (OPD) (calculated pK_a 4.46) was presented as further evidence for imine formation (Painter, 1991).

The hypothesis of glycosylamine formation was based on observations that lower concentrations ($\leqslant$20 mM) of diamines (e.g. lysine, arginine or guanidine) than of simple amines (>100 mM) inhibits the precipitation of a gelatin–sphagnan complex (Painter, 1991). Furthermore, prior treatment of sphagnan with 20% $NaBH_4$ rendered this polymer ineffective to precipitate gelatin (Painter, 1991).

The practical significance of these various amine–sphagnan interactions is the potential for sphagnan, via its 5-KMA residues, to cross-link and ultimately tan, via Maillard browning, the surface of proteinaceous materials (Børsheim et al., 2001; Painter, 1991). It was also suggested that the same reactions lead to the immobilisation and inactivation of saprogenic enzymes (Painter, 2003). Analogy was made with the tanning abilities of various periodate-oxidised polysaccharides such as dialdehyde starch (Fein & Filachione, 1957; Nayudamma, Joseph, Rao, & Hemalatha, 1967), and other carbonyl-tanning agents such as formaldehyde and glutaraldehyde (Painter, 1991). It was proposed that tanning by sphagnan could explain the preservation of proteinaceous materials and their appearance. This includes the brown leathered skin on human bodies preserved in *Sphagnum*-dominated peat bogs (Painter, 1991; Painter, 1995), the browning of the dermis and yellowing of the scales of small fresh fish embedded in *Sphagnum* leaves (Børsheim et al., 2001), and the browning and bio-resistance of strips of pig and mackerel skin repeatedly exposed to sphagnan in a series of 'tanning', washing and drying cycles (Børsheim et al., 2001). The wound healing properties of *Sphagnum* mosses were also attributed to their tanning effect (Painter, 2003). Given the potentially high environmental and biotechnological significance of these claims, we deemed them to warrant further investigation.

In two recent studies (Ballance, Børsheim, & Christensen, 2004; Ballance, Børsheim, Inngjerdingen, Paulsen, & Christensen, 2007) we set out on the quest to verify or refute previous claims regarding the structure of sphagnan with particular emphasis on obtaining direct evidence of 5-KMA in *Sphagnum* moss. In the first study, a review of the literature, we only found indirect evidence to support the existence of 5-KMA in *Sphagnum* (Ballance et al., 2004). In the second follow-up study we then found this indirect evidence to be irreproducible (Ballance et al., 2007). In this study we aim to further examine the reactions between the free acid-form of sphagnan and either phenylhydrazine, OPD or collagen. Sphagnan was extracted by mild acid hydrolysis from acetone/methanol- or chlorite-treated *Sphagnum papillosum* leaves, of which the latter has been characterised in a previous study (Ballance et al., 2007). First we examined the interaction between sphagnan and phenylhydrazine, which was previously used as the basis of an assay to quantify the content of carbonyl groups in sphagnan (Painter, 1983; Smidsrød & Painter, 1984). Second we examine the interaction between OPD and its oxidation products with sphagnan in aqueous solution and look for evidence of quinoxaline formation. Finally we study the interaction of sphagnan with collagen in the form of gelatin or hide powder. Complex coacervate formation in sphagnan–gelatin mixtures were studied under a range of pH conditions. The tanning ability of sphagnan was assessed by measuring the hydrothermal stability and collagenase-degradation of hide powder collagen treated with sphagnan.

2. Materials and methods

2.1. Preparation of acetone/methanol-treated leaves

Sphagnum papillosum plants were collected, identified, processed and treated with acetone/methanol as previously described (Ballance et al., 2007).

2.2. Extraction of sphagnan from acetone/methanol-treated leaves

Twenty-five grams of acetone/methanol-treated *Sphagnum* leaves (H^+-form) were suspended in 1.5 l degassed water and autohydrolysed at 95 °C for 24 h. At daily intervals over 3 days the liquid was separated from the residual solid by vacuum filtration through a Whatman GF/D microfibre glass filter. The residual solid was then suspended in new degassed water and the autohydrolysis repeated in two more cycles. The liquid solutions were pooled (4.5 l) and concentrated by rotary evaporation at 30 °C to ca. 200 ml prior to further filtration through 0.22 μm membranes. The filtrate was then repeatedly dialysed against distilled water. Finally the dialysate was sterile filtered through 0.22 μm membrane and freeze-dried. The yield of light brown crude solid in its acid-form was 1.2 g. This material was characterised by some of the methods used to investigate the structure of sphagnan extracted from chlorite-treated leaves (Ballance et al., 2007).

2.3. Extraction of sphagnan from chlorite-treated leaves

Sphagnan was extracted by mild acid hydrolysis from chlorite-treated leaves as previously described (Ballance et al., 2007) and stored in the fridge in its acid-form.

2.4. Reaction with $NaBH_4$

Polysaccharides were treated with $NaBH_4$ as previously described (Ballance et al., 2007). In some cases the $NaBH_4$ concentration was increased to 20% (w/v) with a reaction time of 11 days. These samples were converted into their

H^+-form by dialysis against cold 0.02 M HCl followed by distilled water and freeze-dried.

2.5. Preparation of phenylhydrazine and o-phenylenediamine and hydroxylamine derivatives

Phenylhydrazine derivatives were prepared by reaction of 100 mg polysaccharide with 20 mg phenylhydrazine hydrochloride in 10 ml distilled water at a pH between 4 and 4.5 for 2 h at 60 °C. OPD derivative was prepared by reaction of 250 mg sphagnan (H^+-form) with 50 mg OPD in 50 ml distilled water for 24 h at 22 °C (Painter, 1991). Hydroxylamine derivative was prepared by reaction of 20 mg sphagnan with 8 mg hydroxylamine hydrochloride at pH 4–4.5 in 3 ml distilled water for 24 h at 22 °C (Calvini, Conio, Princi, Vicini, & Pedemonte, 2006; Kim & Kuga, 2000). All samples were dialysed first against 0.5 M NaCl and then against water until the conductivity was <2 μS cm^{-1}, and finally freeze-dried. Samples were also prepared where sphagnan was substituted by 2% or 20% w/v $NaBH_4$-treated sphagnan. All samples were stored in the dark at 4 °C until analysed.

2.6. Preparation of N-acetylneuraminic acid-quinoxalinol derivative

Fifty milligrams of *N*-acetylneuraminic acid (NANA) Type IV from *Escherichia coli* was dissolved in 50 ml 1 mg/ml OPD, and stirred in the dark at 22 °C for 24 h. The solution was then concentrated by rotary evaporation, and applied to a 10 g Sep-Pak C^{18} solid-phase extraction column. Bound material was eluted with one column volume of 40% methanol (v/v) in water. The methanol was then removed by distillation and the sample freeze-dried to yield about 45 mg solid off-white material. The product was stored in the dark at 4 °C until analysed.

2.7. Phenylhydrazine assay for determination of carbonyl groups

Native and 20%-$NaBH_4$-treated sphagnan from chlorite-treated leaves (acid-form) were reacted with phenylhydrazine hydrochloride as described above with incubation for 2 h at 60 °C followed by dialysis and freeze-drying. Prior to spectrophotometric analysis 2 mg of these samples were dissolved in 2 ml distilled water, or dissolved in 2 ml concentrated HCl, incubated for 24 h and then diluted to 4 ml with distilled water. As controls, native polysaccharide samples not exposed to phenylhydrazine were dissolved in concentrated HCl, incubated and diluted in the same way. The UV spectrum of 200–400 nm was obtained in a quartz cell in a calibrated Perkin-Elmer Lambda 25 UV/VIS spectrometer. In a separate analysis, the absorbances of each sample at 250, 275 and 300 nm were recorded. These values were then used to calculate the absorbance at 275 nm, assuming a linear baseline fit between 250 and 300 nm. Matrix matched phenylhydrazine standards ($n = 3$) of 0–5 mM were used to determine the fraction of incorporated phenylhydrazine per monosaccharide in the polymer. In our calculations the average molecular weight of sugar monomers was assumed to be 200.

2.8. Total nitrogen and carbon analysis

Total elemental nitrogen and carbon in freeze-dried samples (5–10 mg) was analysed by the flash combustion method (Kirsten, 1979) on a Thermo Finnigan FlashEA 1112 automatic elemental analyser (detection limit 100 ppm N). Phenanthrene was used as a calibration standard to yield linear fits ($R^2 = 0.98$–1).

2.9. Infrared spectroscopy

Samples were placed in KBr discs and analysed in a Nicolet 20SXC FT-IR spectrophotometer.

2.10. NMR spectroscopy

Samples were proton-exchanged with D_2O in two cycles prior to further preparation. Thirty miiligrams of NANA-OPD derivative was dissolved in 600 μl methyl alcohol-d_6, and spiked with 5 μl tetramethylsilane (TMS) as internal standard. Approximately 30 mg sphagnan-OPD was dissolved in 600 μl D_2O, and 5 μl 1% 3-(trimethylsilyl)propionic-2,2,3,3,-d_4 Na salt (TSP) in D_2O was added as internal standard. 1D ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 300 or 400 MHz spectrometer at room temperature (NANA-OPD derivatives) or 90 °C (polysaccharide samples).

2.11. Electrophoretic mobility and diffusion measurements of sphagnan–gelatin mixtures

Type A gelatin from porcine skin (approximately 300 Bloom, p*I* 7.4) or Type B gelatin from bovine skin (approximately 225 Bloom, p*I* 4.5) was purchased from Sigma. The p*I* was determined as described below for sphagnan–gelatin mixtures. Gelatin was soaked in distilled water for 2–3 h at room temperature, followed by mechanical stirring for 10–15 min at ~70 °C (Gilsenan, Richardson, & Morris, 2003) to give a dissolved gelatin concentration of 2 mg/ml. After cooling, some solutions were supplied with 40 mM arginine-, lysine- or guanidine hydrochloride. 2.5 ml gelatin solution was pipetted into a tube, followed by 2.5 ml 1 mg/ml sphagnan, 20% $NaBH_4$-treated sphagnan (both H^+-form), polygalacturonic acid (Na^+-form) or oximated-sphagnan dissolved in a weak phosphate stock solution. The final solutions comprised a 2:1 (w/w) mixture of gelatin and polysaccharide, and in some samples 20 mM amino acid, or just gelatin, all with a pH of 6.0–7.0 in 0.01 M phosphate. Ionic strength was prior adjusted with NaCl in the buffer stock solutions to give an added *I* of 0.03 (0.05 with amino acids) in all gelatin–polysaccharide mixtures. The electrophoretic mobility (an estimate of net

charge through the calculated zeta potential) and diffusion coefficient of the mixtures were then measured by laser Doppler velocimetry and dynamic light scattering, respectively, at pH intervals of 0.5 (within an interval threshold of ±0.2) from 6.0 to 2.0 at 25 °C using a Malvern Nano-ZS Zetasizer equipped with an acid/base autotitrator. Conductivity was also measured at the same time, and related to the corresponding ionic strength of a series of NaCl solutions. The calculated diffusion coefficient is only a qualitative measure of complex coacervate formation since the raw data (correlation function versus time) obtained from such polydisperse and sedimenting samples are inaccurate (Malvern Instruments, 2006).

2.12. Tanning assay

One hundred milligrams of standard bovine hide powder made from alkali-extracted hide (BLC Leather Technology Centre, UK) was soaked in 2 ml distilled water for 30 min at 30 °C. The sample was then adjusted to 0.01 M acetate ($I = 0.03$ with NaCl) prepared at pH 4.2 with a 10× stock solution, and incubated a further 30 min at room temperature. The measured pH of the mixtures was 4.0–4.5. Thirty milligrams of solid test tannage was then added over a period of 2 h while the sample was rolled. Mixing was continued for a further 5 h, followed by incubation overnight at room temperature. All samples were then rolled for a further 1 h and washed in distilled water, followed by drying overnight in a weighing boat. 10–15 mg dried sample was soaked for 72 h in 1 ml distilled water, and excess water was carefully removed by blotting the sample on tissue paper. The hydrated material was then sealed into an aluminium pan, and the denaturation temperature (onset of triple helix to random coil transition temperature) of the hide powder collagen was determined by differential scanning calorimetry (DSC) in a Perkin-Elmer DSC 7 instrument, using a punctured empty pan as reference. Samples ($n = 3$) were heated from 20 to 110 °C at a scan rate of 5 °C min^{-1} (Covington, Hancock, & Ioannidis, 1989). Commercial vegetable tannins (polyphenols) used as positive controls were seta-sun mimosa-extract from Brazil (72.5% tanning content) and unsweetened/normal chestnut-extract (75–77% tanning content).

2.13. Collagenase assay

Collagenase Type I from *Clostridium histolyticum* (Sigma) was incubated with 15 mg hide powder in 0.05 M Tris–HCl (pH 7.0) and 0.04 M $CaCl_2$ at a maintained ratio of 1:50 enzyme to substrate at 37 °C. Hydroxyproline content in the supernatant was measured after 7 days incubation by a colorimetric assay (Woessner, 1961).

3. Results and discussion

An explicit step-by-step procedure for the assay previously used to determine carbonyl groups in sphagnan is lacking. For example, in the assay described by Painter (1983) and Smidsrød and Painter (1984) it states that polymeric orange-coloured complexes (phenylhydrazones) are formed upon reaction of sphagnan with excess 0.1% phenylhydrazine for 2 h at 60 °C, but does not state the reaction pH or how they are isolated. Although it is mentioned in one paper (Smidsrød & Painter, 1984) that orange-coloured complexes were recovered by centrifugation, this was probably only in the case of insoluble holocellulose–phenylhydrazine complexes. In our experiments, no precipitation or flocculation was observed upon reaction of sphagnan with phenylhydrazine under mildly acidic conditions, either before or after centrifugation. Polymeric orange-coloured complexes were, however, recovered by dialysis against 0.5 M NaCl and water, followed by freeze-drying. Mildly acidly conditions were chosen as optimum as to have enough acid to catalyse the reaction and enough free amine for the nucleophilic attack (Kim & Kuga, 2000; McMurry, 1988).

In the next step of Painter's assay, the orange-coloured complexes were hydrolysed with concentrated HCl, followed by 'filtration and appropriate dilution', and determination of released phenylhydrazine by spectrophotometric absorbance at 275 nm (Painter, 1983; Smidsrød & Painter, 1984). In our experiments, such treated samples had absorbance bands at 210–230 and 260–300 nm irrespective of whether they contained orange-coloured complexes or not (Fig. 1). These bands are diagnostic of furfural, hydroxymethylfurfural and other furans (Martinez, Rodriguez, York, Preston, & Ingram, 2000; Taher & Cates, 1974), which are readily formed by dehydration of pentose and hexose monomers even under mildly acidic conditions (Popoff & Theander, 1972). The furans therefore make a substantial contribution to the absorbance measured at 275 nm. According to Painter's assay, sphagnan from chlorite-treated leaves (not treated with phenylhydrazine) seemingly contained 0.17 carbonyl groups per monosaccharide, while sphagnan from acetone/methanol-treated leaves were calculated to be only marginally higher at 0.21 carbonyl groups per monosaccharide. Further analysis of biopolymer preparations not exposed to concentrated acid revealed no significant absorbance above background at either 210–230 or 260–300 nm (Fig. 1). Taken together, these results cast serious doubt over the validity of Painter's assay of carbonyl groups in sphagnan, but may explain how the figure of 27 M % 5-KMA in sphagnan (Painter, 1983; Smidsrød & Painter, 1984) was reached.

An assay for the determination of carbonyl groups has been developed for neutral polysaccharides such as cellulose via oxime formation which makes use of fluorescence detection (Röhrling et al., 2002) or total N analysis (Maekawa & Koshijima, 1991). Since at present, and to our knowledge, there are no such corresponding assays in existence using fluorescent labels to detect carbonyls for anionic polysaccharides we opted for the total nitrogen analysis approach. After reaction of sphagnan with hydroxylamine at pH 4–4.5 the N content was 0.63% ± 0.085 ($n = 4$).

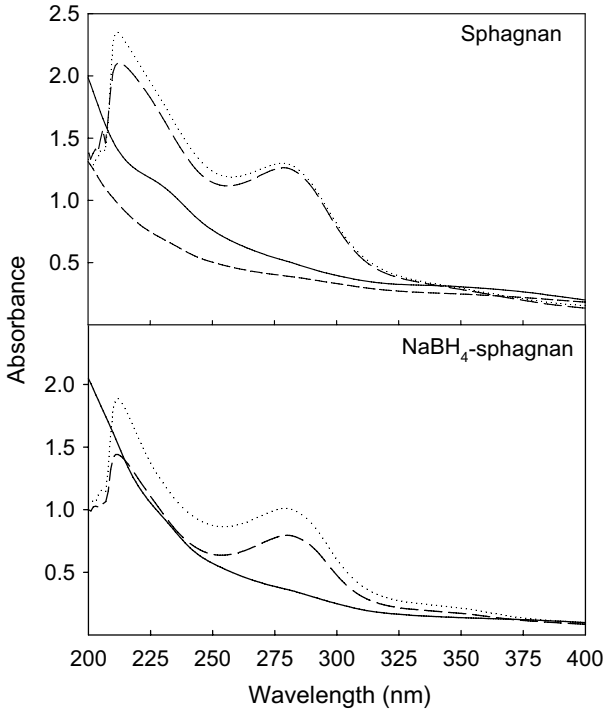

Fig. 1. UV spectra (200–400 nm): of native and phenylhydrazine derivatives of sphagnan and 20%-$NaBH_4$ sphagnan from chlorite-treated leaves. Dotted lines are samples incubated with phenylhydrazine at 60 °C for 2 h, hydrolysed in concentrated HCl for 24 h, and diluted to 6 M HCl. Solid lines are samples treated with phenylhydrazine, but not with acid, and dissolved in water. Long dashed lines are native samples hydrolysed in concentrated HCl for 24 h, and diluted to 6 M HCl. Short dashed lines represent spectra of native samples dissolved in water.

Similar values were also obtained for the sphagnan extracted from acetone/methanol-treated leaves and reacted with hydroxylamine or from corresponding samples reacted with phenylhydrazine. Since the N content of untreated sphagnan was about 0.2% this means that <1 in 18 sugars contain a reactive carbonyl group in addition to the reducing end rather than about 1 in 4 as suggested earlier (Painter, 1983; Smidsrød & Painter, 1984). As of yet, however, a more accurate measurement of the carbonyl content of sphagnan cannot be made with confidence simply because the nitrogen analysis method is not sensitive enough and its resolution is too low.

It has been suggested that sphagnan in its free acid-form reacts with OPD to form a quinoxaline (Painter, 1991), which in turn was considered diagnostic of an α-keto acid group in sphagnan. NMR analysis lends some weight to suggest that OPD or its common oxidation products such as 2,3-diaminophenazine binds to sphagnan from chlorite- or acetone/methanol-treated *Sphagnum* (Fig. 2a). The two broad resonances at 7.3–7.9 ppm and at 118/128 ppm in the ^{1}H and ^{13}C NMR spectra, respectively, are all characteristic of an aromatic compound coupled to a polymer (Fig. 2a, b and d). There are no aromatics in sphagnan extracted from chlorite-treated leaves because any originally present are destroyed by the chlorite treatment (Ballance et al., 2007). The aromatics present in the native sphagnan extracted from acetone/methanol-treated leaves resonate at 6.5–7 ppm (Fig. 2c) and the identity of these is discussed below. In addition since any free OPD or its oxidation products remaining in the sample would have appeared in the same chemical shift range as a sharp resonance rather than a broad one. This observation indicates OPD or its oxidation products with a free amino group are bound to sphagnan. No characteristic aromatic resonances from OPD or its oxidation products were observed in the NMR spectra of sphagnan prior treated with the carbonyl-reducing agent $NaBH_4$ (not shown). These results therefore suggest that OPD or its oxidation products reacted with the free acid-form of sphagnan via nucleophilic addition to form an imine (C=NR). Nitrogen analysis of OPD reacted samples also supports this contention. Attempts by 2D NMR, IR and fluorescence spectroscopy to identify any quinoxalines or vicinal diones in sphagnan though were inconclusive. This is possibly because they either comprise just a minor fraction and hence their signals were too weak, or the resonances were masked by others from the carbohydrate polymer. Quinoxalines were formed as expected when OPD was reacted with sialic acid under the same conditions as sphagnan to give characteristic signals in both NMR and IR spectroscopy (Table 1).

Dynamic light scattering measurements at low ionic strength of freshly prepared 1:2 (w/w) sphagnan (from chlorite-treated leaves)–gelatin mixtures ($I = 0.03$) titrated from pH 6.0 to 2.0 indicated complex coacervation (as the inverse of the calculated diffusion coefficient) between the two polymers at pH 2.0–4.8 (Fig. 3a). No complex formation was detected at any pH for single polymer solutions (results not shown). These patterns of complex coacervation are typical for polyelectrolyte complexes formed by electrostatic interaction of oppositely charged polymers rather than a covalent reaction between carbonyl groups in sphagnan and amino groups in gelatin. Below pH 5 all the basic amino acids in gelatin are fully charged, and the association of gelatin and sphagnan is increasingly favoured as the net positive charge density along the gelatin chain increases with protonation of the carboxyl groups down to the pH where maximum coacervation occurs at the so called electrical equivalence pH (3.5 for a 2:1 [w/w] gelatin–sphagnan mixture). Here the total charges on gelatin and sphagnan are opposite and equal (Fig. 3b).

Dissolution of the coacervate starts to occur as the majority of carboxyl groups in sphagnan become protonated as the pH drops from 3.5 to 2.5 (Fig. 3). This results in charge screening with a consequent loss of negative charge (Fig. 3b). Titration with NaCl has a similar dissociating effect (not shown) but is rather driven by the counterion-dictated entropy of mixing (de Kruif, Weinbreck, & de Vries, 2004; Piculell, Bergfeldt, & Nilsson, 1995). Similar results have been obtained for gelatin–acacia mixtures (Burgess & Carless, 1984).

It is interesting to note that coacervation of Type A gelatin–sphagnan occurs over a wider pH range than that with Type B gelatin (Fig. 3a). This difference in pH profiles can

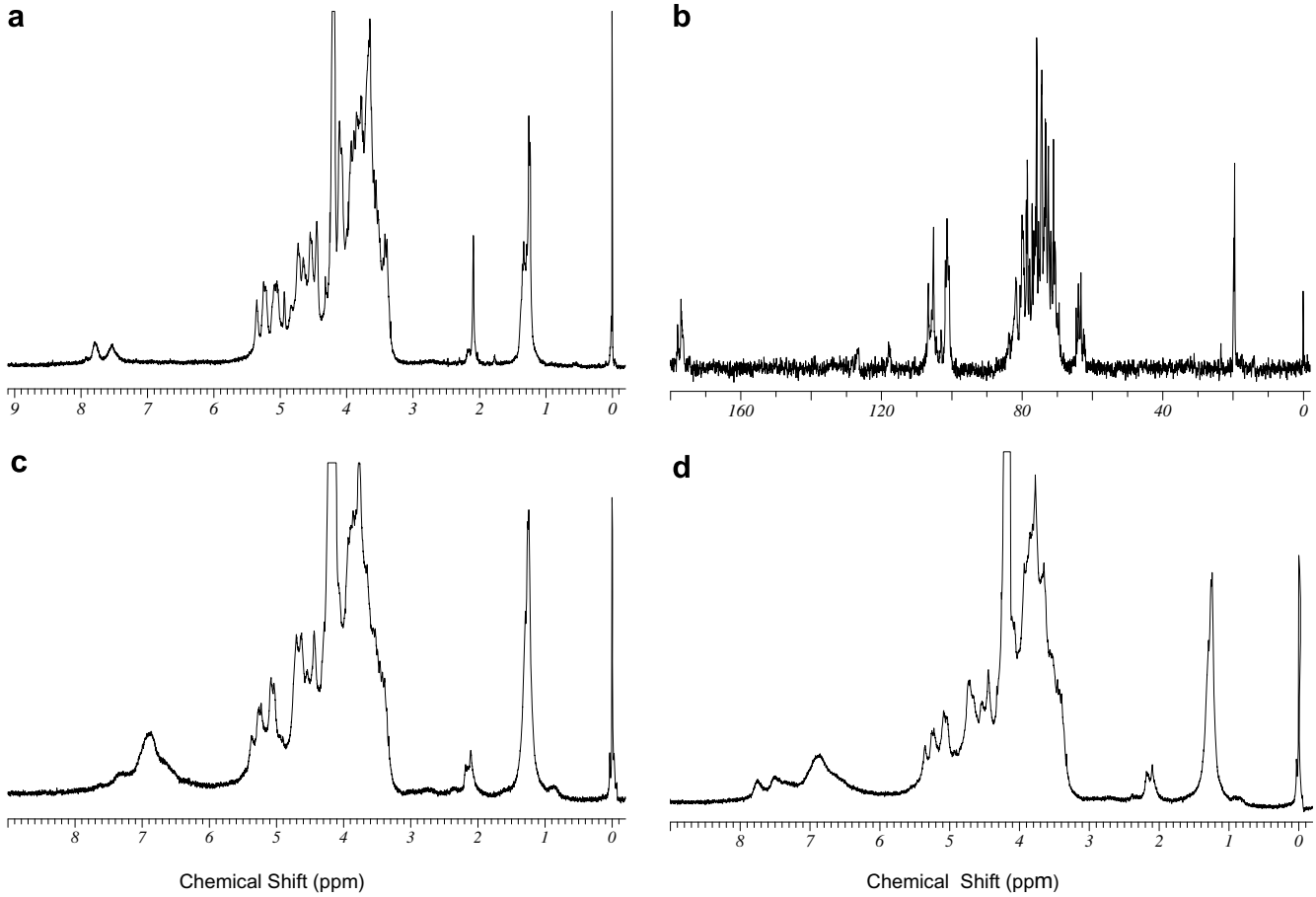

Fig. 2. Three hundred MHz ^{1}H (a) and 75 MHz ^{1}H-decoupled ^{13}C (b) NMR spectra of sphagnan from chlorite-treated leaves after reaction with OPD, and 300 MHz ^{1}H NMR of sphagnan from acetone/methanol-treated leaves before (c) and after (d) reaction with OPD with TSP as reference. In ^{1}H-spectra the residual water peak is at 4.2 ppm.

Table 1
Spectroscopic data for quinoxaline of sialic acid

^{1}H NMR (d_4-MeOD): 1.98 (s, 3H), 2.03 (br, 1H), 2.97–3.12 (m, 1H), 3.43 (d, 1H, $J = 8.8$ Hz), 3.61 (q, 1H, $J = 5.8$ and 10.8 Hz), 3.68–3.75 (m, 1H), 3.77–3.81 (dd, 1H, $J = 3.0$ and 8.8 Hz), 3.98 (q, 2H, $J = 8.8$ and 10.3 Hz), 4.64 (t, 1H, $J = 6.3$ and 13.4 Hz), 7.18 (m, 2H), 7.48 (m, 2H)

^{13}C NMR (d_4-MeOD): 23.0 (CH_3), 35.7, 55.7, 65.6, 69.2, 70.1, 71.7, 72.9, 103.5 (phenyl), 118.2 (phenyl), 121.2 (phenyl), 123.6 (2× phenyl), 128.5 (phenyl), 129.0 (quinoxaline), 154.5 (quinoxaline), 175.0 (C=O)

IR (KBr disc): 3315 (s), 1728 (w), 1650 (s) (quinoxaline, C=N), 1504 (s), 1434 (s), 1375 (m), 1336 (m), 1274 (m), 1225 (m), 1073 (m), 1035 (m), 746 (s) cm^{-1}

s, singlet; m, multiplet; br, broad; d, doublet; q, quartet; t, triplet; dd, doublet of doublets.

be explained by the different positive charge densities of the gelatins. Since alkali is used in the production of Type B gelatin, all glutamine and asparagine residues are deamidated to form glutamic and aspartic acid, respectively. Consequently, there are fewer carboxyl groups in Type A gelatin, and its p*I* is higher (Fig. 3b). Type A gelatin therefore has a higher positive charge density at pH 3.8–5.0 than Type B gelatin, and can thus form complex coacervates with sphagnan at a slightly higher pH (Fig. 3). Dissolution of Type B gelatin–sphagnan complexes occurs at a slightly higher pH because there are more carboxyl groups which are protonated in this gelatin at a given pH below its p*I*. As mentioned in the methods, the diffusion coefficients obtained from the dynamic light scattering correlogram are not quantitative, but sufficient for qualitative comparative purposes. The results are also consistent with previous studies of mixtures of Type A and B gelatin and acacia (see Figs. 1 and 2 in Burgess & Carless, 1984). Polygalacturonic acid, 20% $NaBH_4$-treated sphagnan and sphagnan in the presence of 20 mM arginine, lysine or guanidine all formed complex coacervates when mixed with Type B gelatin at low ionic strength. We could therefore not substantiate the earlier claims made by Painter (1991) that $NaBH_4$ treatment, or the addition of basic amines at low concentration, inhibits gelatin precipitation. There was neither any evidence to suggest that the coacervates that form with gelatin and sphagnan are denser than with gelatin and other acidic polysaccharides.

A true tannage elevates hydrothermal stability with a consequent rise in shrinkage temperature, whereas 'leathering', typical of oil, brains and smoke-treated collagen, just renders a material non-putrescible and changes its physical appearance. Measurements of hydrothermal stability as

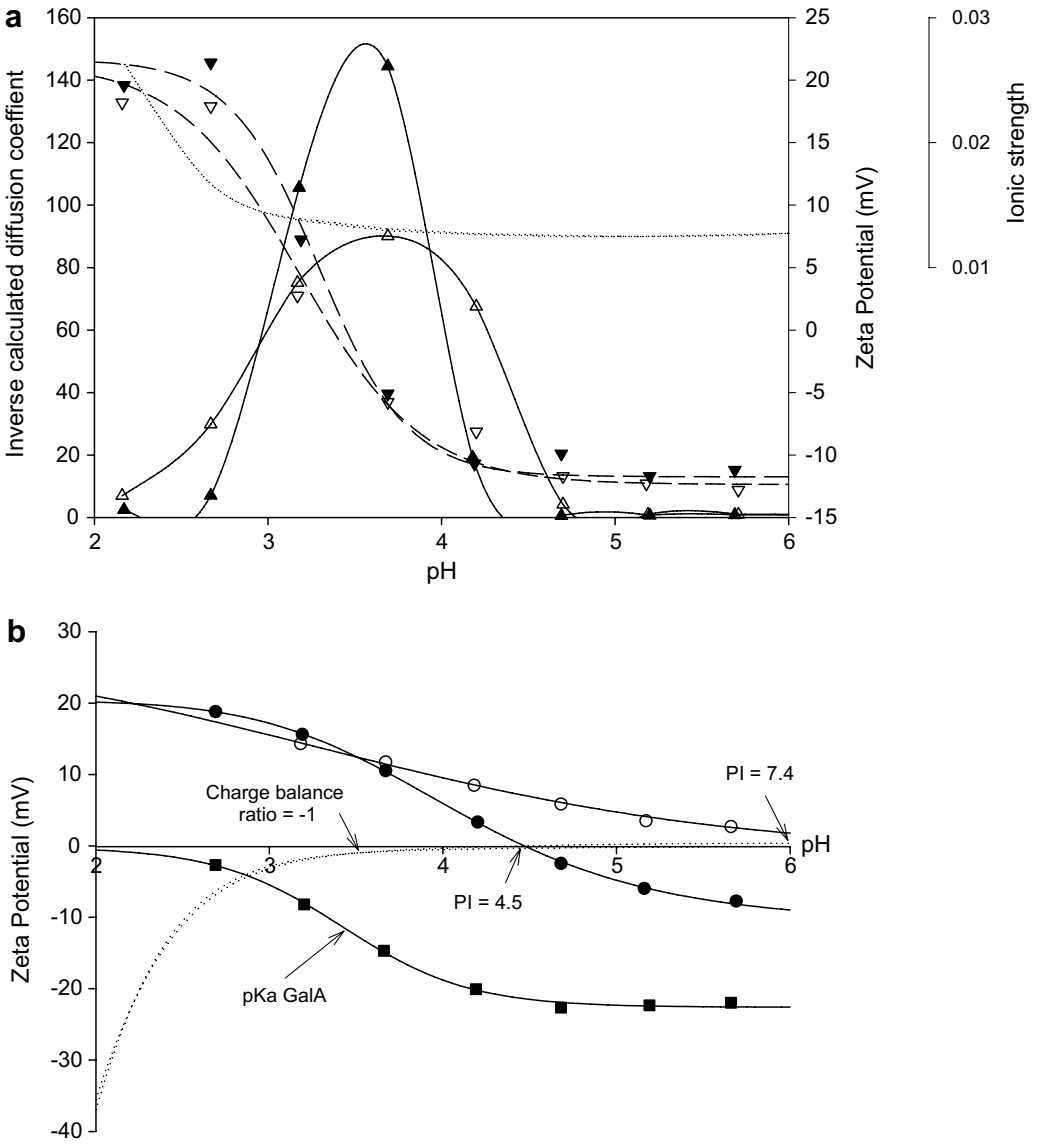

Fig. 3. Electrophoretic mobility and diffusion measurements of 2:1 (w/w) gelatin–sphagnan mixtures from pH 6.0 to 2.0. (a) Open and solid triangles represent Type A gelatin–sphagnan and Type B gelatin–sphagnan mixtures, respectively. Up triangles and fitted solid lines represent the inverse of the calculated diffusion coefficient, while down triangles and the fitted dashed lines represent the electrophoretic mobility measurements expressed as the calculated zeta potential. The dotted line is the calculated ionic strength determined from *in situ* conductivity measurements. (b) Electrophoretic mobility measurements of 0.1% (w/w) solutions of Type A gelatin (open circles), Type B gelatin (solid circles) and sphagnan (solid squares). The dotted lines represent the gelatin/sphagnan charge ratio.

denaturation temperature of bovine hide powder collagen treated with sphagnan (from chlorite-treated leaves) at pH 4.0–4.5 for 24 h showed little difference from an untreated control (Table 2). Furthermore, the sphagnan-treated collagen was readily degraded by collagenase (Fig. 4). On the other hand, positive control polyphenolic tannins, chestnut- and mimosa-extracts, increased the hydrothermal stability of collagen by 10–15 °C (Table 2) as previously found (Covington, 2001). The control tannins also imparted resistance to degradation by collagenase (Fig. 4). In earlier calorimetric analysis of mackerel skin treated repeatedly with sphagnan, the release of water at

Table 2
Denaturation temperatures ($n = 3$) of untreated hide powder collagen and hide powder collagen treated with sphagnan from chlorite-treated leaves (A), sphagnan from acetone/methanol-treated leaves (B) and two polyphenolic vegetable tannins

Hide powder treatment	Denaturation temp. (°C)
Untreated	44.0 (42.0)
Sphagnan A	46.0 (44.0)
Sphagnan B	54.0 (48.0)
Chestnut-extract	57.5 (57.5)
Mimosa-extract	63.5 (63.5)

Corresponding denaturation temperatures after 4 wetting and drying cycles are given in brackets.

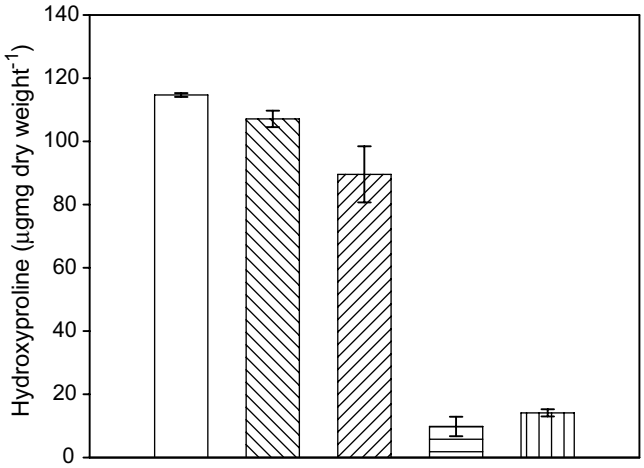

Fig. 4. The amount of hydroxyproline (µg/mg dry weight) released by collagenase after 1 week incubation at 37 °C from hide powder previously incubated for 24 h with either no added substrate (open), sphagnan from chlorite-treated leaves (negative diagonals), sphagnan from acetone/methanol-treated leaves (positive diagonals), chestnut-extract (horizontal lines) and mimosa-extract (vertical lines).

104–108°C versus 98–104 °C for various controls was observed as an endothermal peak (Fig. 4 in Børsheim et al., 2001), and cited as evidence that sphagnan is a tannin. This physical parameter, however, falls outside current definitions of what comprises tanning (Covington, 2001), and therefore cannot be used as evidence for such a process. Interestingly, sphagnan extracted from acetone/methanol-treated leaves did, after one wetting and drying cycle, increase the hydrothermal stability of the hide powder to a similar degree to that observed for the hydrolysable chestnut polyphenol extract (Table 2). In contrast, after four wetting and drying cycles, the increase in hydrothermal stability of this sphagnan-treated hide powder was lost. Many common plant-derived vegetable tannages are polyphenols which fall into a defined molecular weight range of 500–3000 Da (Covington, 1997). Whereas chestnut-extract is a low molecular weight sugar derivative based on glucose, sphagnan extracted from acetone/methanol-treated leaves is a complex carbohydrate polymer with an average molecular weight of 2.7×10^4 Da. As already mentioned it also contains some covalently linked aromatic compounds, as observed in ^{1}H NMR at a chemical shift of 6.5–7.0 ppm, whose fine structure is not known (Fig. 2c and d). It is possible that these aromatic compounds interact with collagen via hydrogen bonding in a similar way as polyphenols, but fail to tan the hide powder in these experiments either because they are too scarce, or the high molecular weight polymer is unable to penetrate sufficiently into the collagen. In the traditional practice of tanning with vegetable extracts, the hide was placed in pits and treated with the tannin for a 'year and a day' (Covington, 1997) to ensure sufficient penetration into the hide. In accordance with hydrothermal measurements, hide powder collagen treated with sphagnans for 24 h were readily degraded by collagenase (Fig. 4). Whether longer or repeated exposure to sphagnan would have reduced its degradation by collagenase and increased its hydrothermal stability has so far not been tested. However, given the results presented here, and the fact that no other plant-derived polyanionic polysaccharides are shown to be tannins, it seems unlikely that the carbohydrate residues in sphagnan will prove to be good tannins.

Specimens of tanned skin samples taken from three different bog bodies and analysed by pyrolysis GC–MS (Stankiewicz, Hutchins, Thomson, Briggs & Evershed) did not reveal the presence of any rhamnose- or hexose-derived components indicative of the presence of sphagnan, and which have previously been detected in subfossil *Sphagnum* leaves (van Smeerdijk & Boon, 1987). In fact, the only *Sphagnum*-derived compound identified in the bog body skin samples was 4-isopropenylphenol, which originated from sphagnum acid (a phenolic component unique to *Sphagnum*) tightly bound to collagen amino acid residues (Stankiewicz, Hutchins, Thomson, Briggs, & Evershed, 1997).

It seems that pectin-like polysaccharides released from *Sphagnum* moss or its holocellulose by acid hydrolysis, either in the lab or naturally in the environment, forms associations with proteins as polyelectrolyte complexes. If such complexes make any contribution towards the preservation of the protein it is probably not because carbohydrate directly participates in the process of tanning.

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Paper IV

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