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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%) followed by borohydride reduction, 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/reduction followed by epimerization (b) epimerization to 90% G followed by oxidation/reduction. 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 local 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.

1. 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, Doseth, Larsen, Skjåk-Bræk, & Valla, 1994; Ertesvåg et al., 1999; Svanem, Skjåk-Bræk, Ertesvåg, & Valla, 1999). The possibility of obtaining pure mannuronan and utilizing the C-5 epimerases *in vitro* (Mørch, Donati, Strand, & Skjåk-Bræk, 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, Bruheim, & Skjåk-Bræk, 2006). When additional G-blocks are introduced by AlgE6 they can merge, and long stretches of guluronic acid are created (Mørch, Holtan, Donati, Strand, & Skjåk-Bræk, 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 G-residues as well as with two G-residues in an opposing chain (Grant, Morris, Rees, Smith, & Thom, 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, Mørch, Syvertsen, Espevik, & Skjåk-Bræk, 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, Rinaudo, & Villar, 2007). The properties of the

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alginate hydrogels can be controlled by parameters like Ca^{2+} concentration, pH, molecular weight and the chemical composition of the alginate (Draget, Simensen, Onsøyen, & Smidsrød, 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, Lesieur, Labarre, & Jayakrishnan, 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 (Lee, Bouhadir, & Mooney, 2002; Smidsrød & Painter, 1973; Vold, Kristiansen, & Christensen, 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 local 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., 1993; Draget et al., 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 degradation 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, Hamilton, Montgomery, & Smith, 1952). Periodate oxidized polysaccharides in general are also more susceptible to alkaline degradation, demonstrated with both periodate oxidized dermatan sulphate and cellulose (Calvini, Conio, Lorenzoni, & Pedemonte, 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 local 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 alginates from *D. antarctica* and *L. hyperborea* 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 et al., 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 20 °C.

After periodate oxidation, sodium borohydride was added to a final concentration of 20% (w/v) and left for 2 h at RT. The samples were then placed on ice and concentrated acetic acid was added until all hydrogen gas had effervesced. The samples were further adjusted to pH 7 and dialysed against MQ-water at 4 °C until the conductivity was below 4 μS. Finally the samples were freeze–dried.

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.

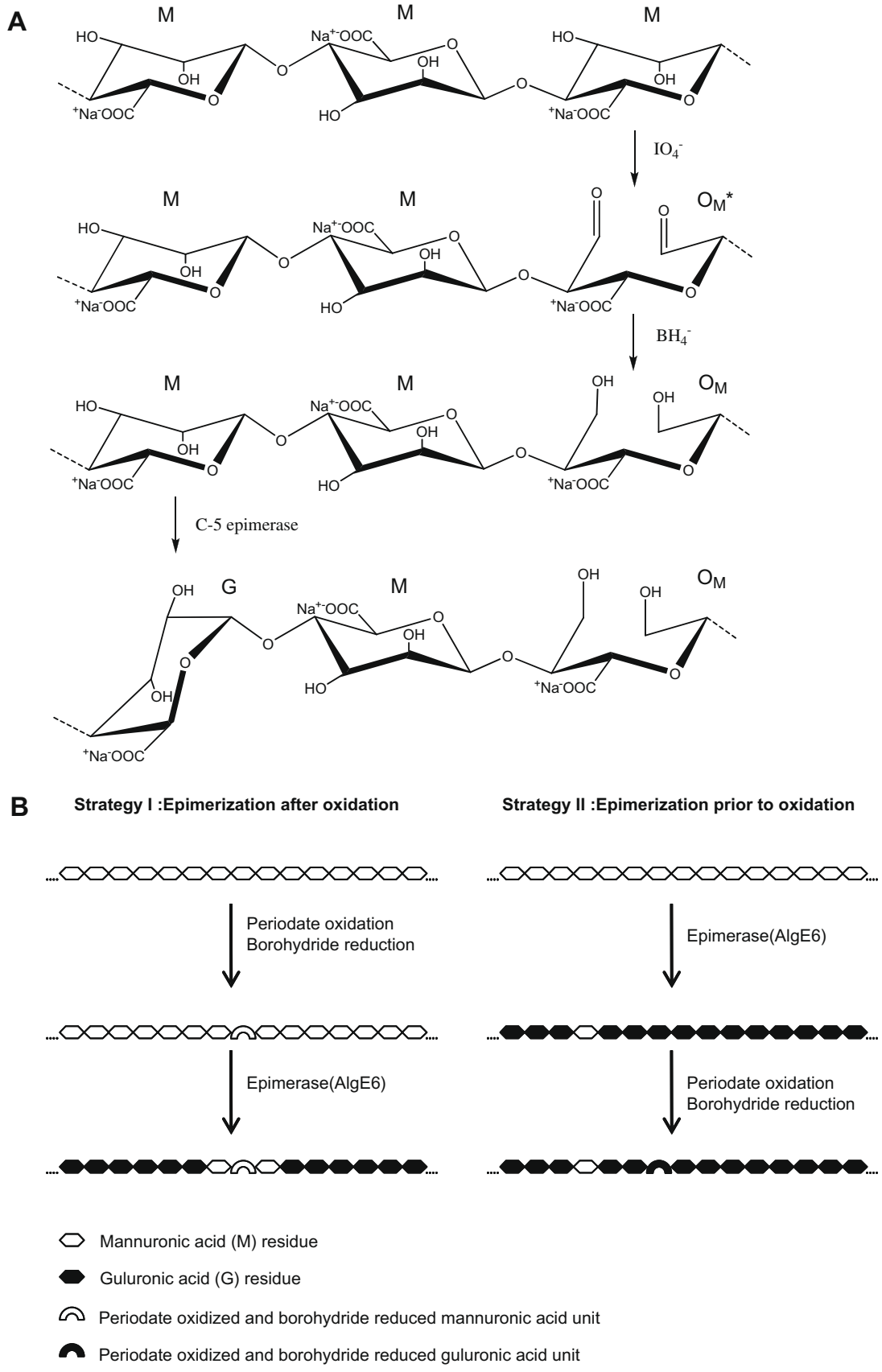

Fig. 1. (A) Structure of a mannuronan fragment (...MMM...) followed by periodate oxidation, borohydride reduction and epimerization (...GMM...). *Abbreviations:* M, β-D-mannuronic acid; G, α-L-guluronic acid. (B) Schematic illustration of two different strategies for making alginate materials.

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 5 mm 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 and Skjåk-Bræk (1999). The alginates were then neutralized and freeze–dried and prepared by dissolution in 99.9% D_2O (Chiron, Trondheim, Norway). Five microliters 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (Aldrich, Milwaukee, WI, USA) 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.

Seventy-five milligrams 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 for 24 h. 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.

2.6. SEC-MALLS-VISC measurements

Measurements were carried out at ambient temperature on an HPLC system consisting of a solvent reservoir, on-line degasser, HPLA isocratic pump, autoinjector, pre-column, and three columns (serially connected) of TSK G-6000PWXL, 5000 PWXL, and 4000 PWXL. In some experiments, only one or two of these columns were used. The column outlet was connected to a Dawn DSP multiangle laser light scattering photometer (Wyatt, USA) (λ_0 = 633 nm) followed by Optilab DSP differential refractometer (P-10 cell) and finally a Viscotek TDA 301 viscosity detector. The mobile phase used was 0.05 M Na_2SO_4/0.01 M EDTA, pH 6. The flow rate was 0.4 mL/min when the viscometer was connected; otherwise, it was 0.5 mL/min. The injection volume was 100–250 μL, and the sample concentration was adjusted to obtain the best possible light scattering signal without influencing the RI profile (overloading). Samples were filtered (pore size 0.22 μm) prior to injection. Data from the light scattering and the differential refractometers were collected and processed using Astra (v. 4.90) software (Wyatt, USA), whereas data from the viscometer were collected and processed by the TriSec (v. 3.0) software. Data from both software packages (slice results) were exported to an Excel spreadsheet for further processing.

3. Results and discussion

3.1. Structure studies of periodate oxidized mannuronan

Partially oxidized mannuronan (P_0 = 0.05–0.20) was characterised by ^1H 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.

Fig. 2 shows the ^1H NMR spectra of a mannuronan oligomer (Fig. 2A) and a mannuronan oligomer with P_0 = 0.10 (Fig. 2B), both with $DP_n \sim 30$. 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 res-

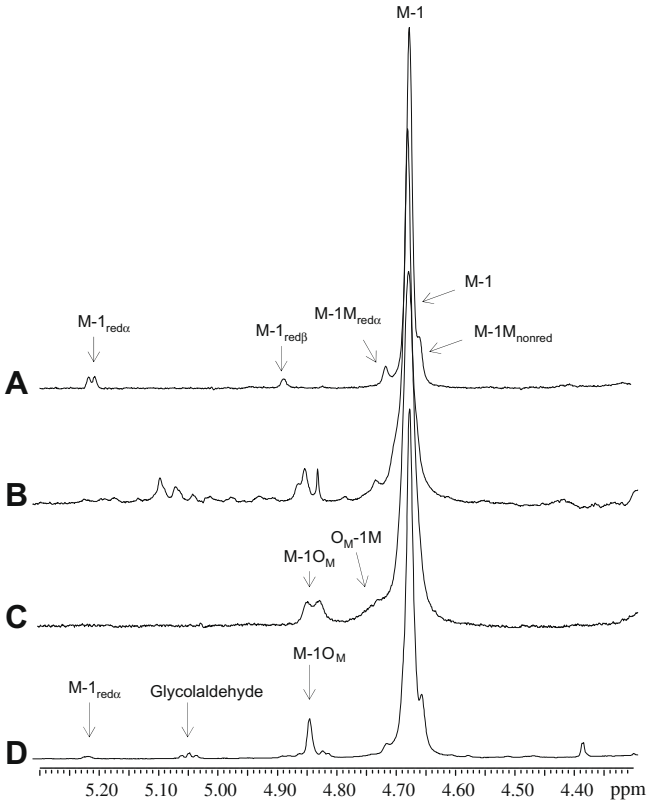

Fig. 2. ^1H NMR spectra of mannuronan oligomers ($DP_n \sim 30$); untreated (A), P_0 = 0.10 (B), P_0 = 0.10, borohydride reduced (C), P_0 = 0.10, borohydride reduced and acid degraded (D). Peaks were assigned according to Grasdalen (1983) except for the peaks M-1O and O_M-1M (see text).

idue 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 (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 (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 O_M-1M. The signal at 4.85 ppm however was still intact after hydrolysis and was assigned to the 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 min (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 Fig. 3. A ^{1}H 2D COSY experiment was conducted in order to clarify this and the result is shown in Fig. 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 3.51 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 gem-diol 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 in its hydrated form (Amyes & Richard, 2007; Glushonok, Glushonok, & Shadyro, 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. 1A).

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

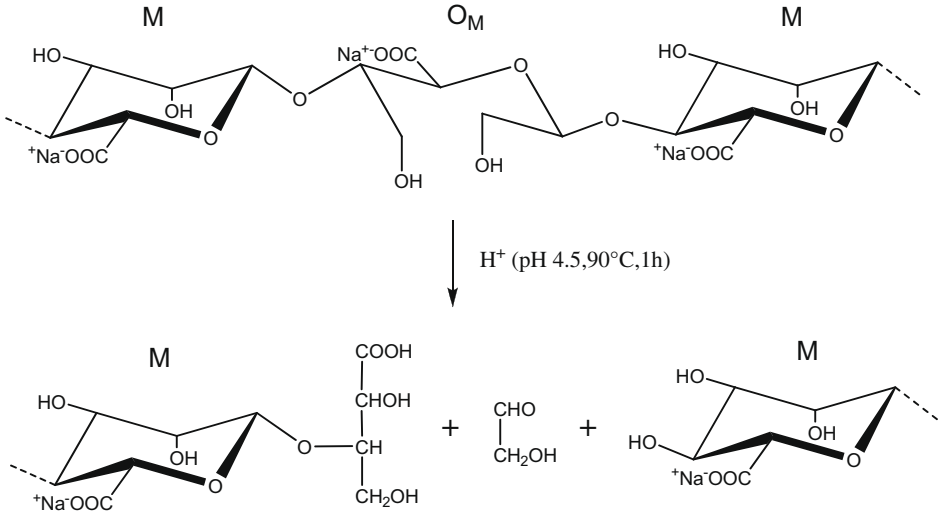

Fig. 3. Mannuronan treated with sodium periodate, sodium borohydride and degraded by mild acid hydrolysis. Suggested reaction products based upon Fransson and Carlstedt (1974) and Sharon (1975).

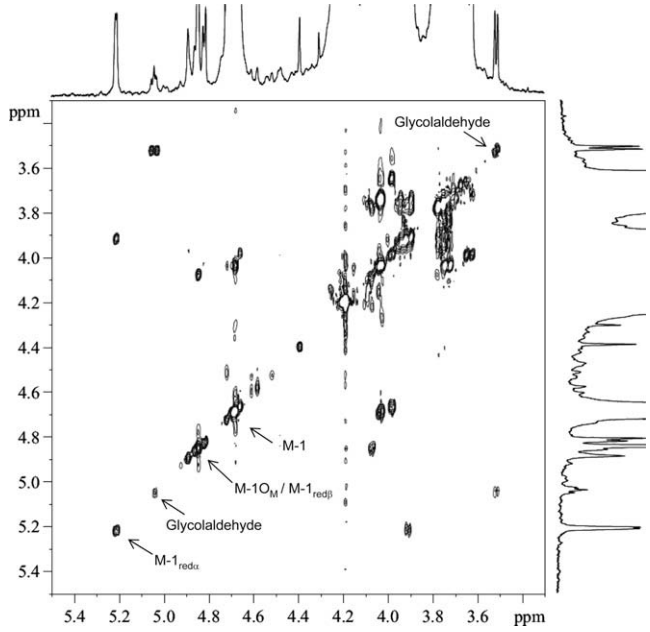

Fig. 4. 2D COSY (magni mode) experiment of a mannuronan sample with $P_0 = 0.10$, borohydride reduced and acid degraded (pD 4.5, 12 h, 90 °C).

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:

$$W(n_M) = n_M P_0^2 (1 - P_0)(n_M - 1) \quad (1)$$

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 $P_0 = 0.10$ $W(n_M \geqslant 8)$ equals 0.81 (81% of the M residues are found in blocks with $\geqslant$8M residues), indicating that the enzymes have several M-stretches to attach. When P_0 increases to 0.20, $W(n \geqslant 8)$ 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.

Table 1
The fraction of guluronic acid (F_G), the diades MM (F_{MM}), GG (F_{GG}) and MG/GM (F_{MG}/F_{GM}) together with G-block length ($N_{G>1}$) are shown for periodate oxidized mannuronan epimerized with AlgE4 (i) and AlgE6 (ii).

Sample	P_0	F_G	F_{MM}	F_{GG}	F_{MG}/F_{GM}	$N_{G>1}$
i	0.00	0.46	0.04	n.d.	0.48	–
i	0.05	0.26	0.47	n.d.	0.26	–
i	0.10	0.14	0.68	n.d.	0.17	–
i	0.20	0.00	0.80	n.d.	n.d.	–
ii	0.00	0.40	0.50	0.28	0.11	5.80
ii	0.05	0.34	0.58	0.21	0.11	4.80
ii	0.10	0.29	0.60	0.14	0.13	3.00
ii	0.20	n.d.	0.80	n.d.	n.d	–

n.d., not detectable.

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

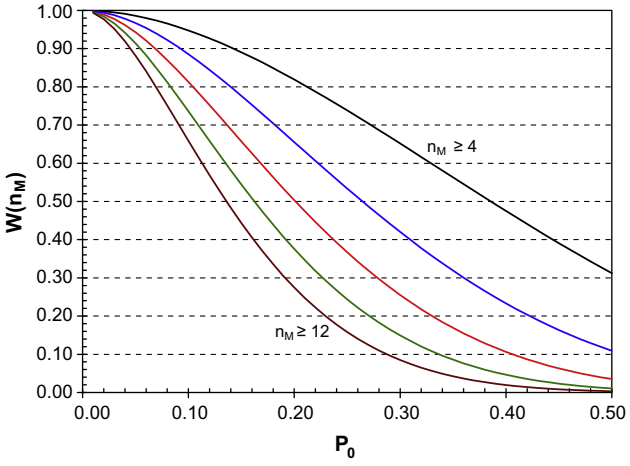

Fig. 5. The weight fraction of M-blocks (n_M) larger or equal to 4 (black), 6 (blue), 8 (red), 10 (green) or 12 (brown), from the top and down, are shown as a function of the fraction of oxidized units (P_0) within the polymer. (For interpretation of colour mentioned in this figure the reader is referred to the web version of the article.)

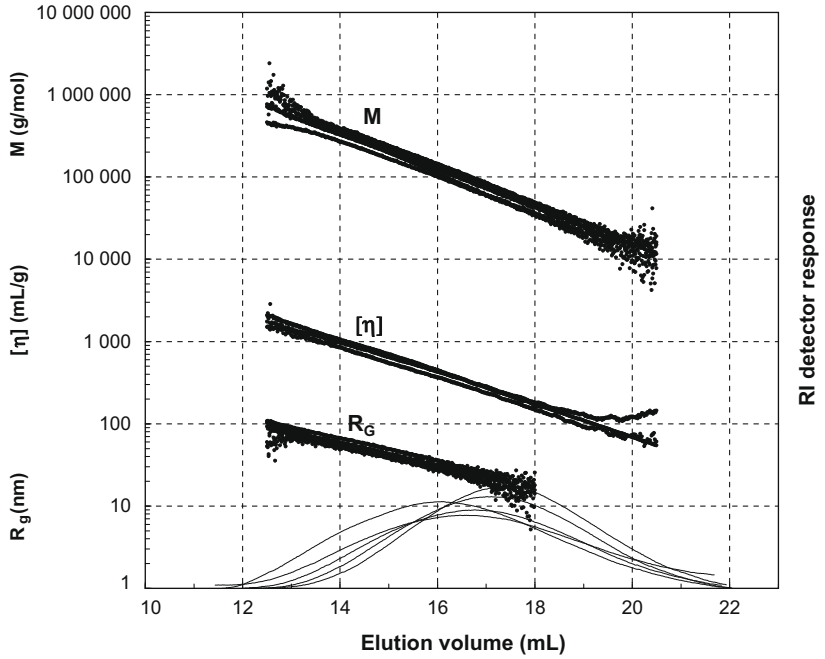

Fig. 6. Mannuronan epimerized with AlgE6 to 90% guluronic acid and periodate oxidized in the range P_0 = 0.02–0.08 and reduced by borohydride (strategy II) was analysed with size-exclusion chromatography (SEC) combined with multiangular laser light scattering (MALLS) and viscosity (VISC) detectors.

Table 2
The fraction of G-residues (F_G) together with F_{MM}, F_{GG}, F_{MG}/F_{GM}, 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.

Sample	P_0	F_G[a]	F_{MM}	F_{GG}	F_{MG}/F_{GM}	$N^*_{G>1}$[b]	Calc. P_0	M_w [kDa]	M_w/M_n	G'[c] [Pa]
Mannuronan	0.00	0	1.00	n.d.	n.d.	–	–	161	2.5	–
I	0.02	0.75	0.05	0.62	0.16	16	0.02	96	2.2	1237
I	0.04	0.64	0.29	0.54	0.08	12	0.04	94	2.5	85
I	0.06	0.54	0.42	0.44	0.07	11	0.06	88	2.2	<1
I	0.08	0.43	0.39	0.36	0.12	9	0.07	71	2.1	<1
II	0.00	0.90	0.03	0.42	n.d.	22	–	156	2.7	149
II	0.02	0.88	0.04	0.43	n.d.	17	0.02	115	2.3	133
II	0.04	0.86	0.03	0.41	n.d.	12	0.04	107	2.6	37
II	0.06	0.84	0.04	0.41	n.d.	9	0.05	104	2.3	12
II	0.08	0.82	0.04	0.40	n.d.	8	0.07	98	2.1	4
Durvilla antarctica[d]	0.00	0.34	0.45	0.20	0.18	5	–	146	2.0	108
Laminaria hyperborea leaf[d]	0.00	0.51	0.33	0.33	0.17	11	–	129	2.1	243
Laminaria hyperborea stipe[d]	0.00	0.65	0.23	0.53	0.12	12	–	117	2.3	308

n.d., not detectable due to very small and insufficiently resolved peaks.
[a] Assuming that the probability of oxidizing an M-residue is equal to oxidizing a G-residue (random oxidation).
[b] For samples II $N^*_{G>1} = F_G/(F_{GGM} + F^*_{GGG})$, see text.
[c] After 24 h. Samples I were prepared using 20 mM Ca^{2+}, while 10 mM Ca^{2+} were used for samples II.
[d] Acid hydrolysed.

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 overall polymer extensions. 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 overall extension of the samples did indeed decrease as P_0 increase, which is in agreement previous studies (Lee et al., 2002; Vold et al., 2006).

Fig. 7A and B 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 (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 (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 $F_G \geqslant 0.40$, its presence was ignored in the sequence calculations. The average G-block length can be calculated from the expressions:

$$N_{G>1} = \frac{n_G - n_{MGM}}{n_{GGM}} = \frac{F_G - F_{MGM}}{F_{GGM}} \quad (2)$$

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 Fig. 7C and D 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:

$$N^*_{G>1} = \frac{n_G - n_{MGM}}{n_{GGM} + n_{MGG^*} + n_{GGG^*}} \quad (3)$$

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 $n_{MGG^*} \sim 0$.

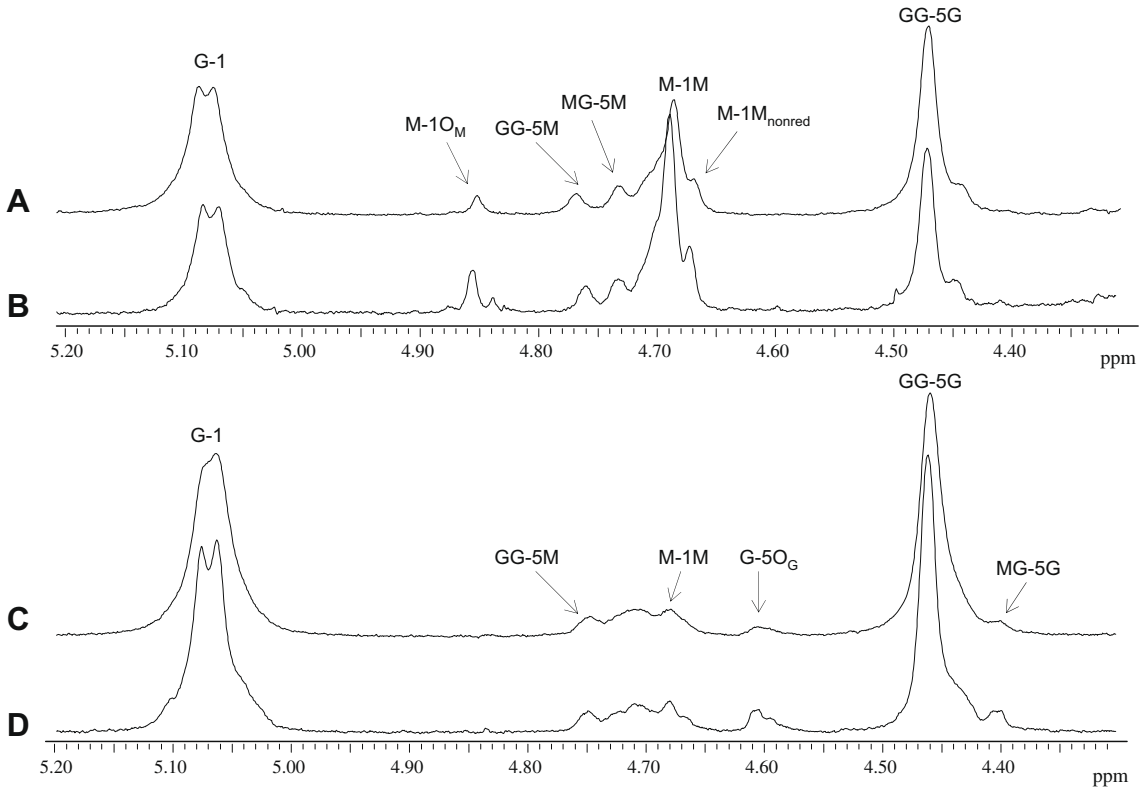

Fig. 7. The ^{1}H NMR spectrum of mannuronan oxidized to P_0 = 0.04 (A) and P_0 = 0.08 (B) and epimerized with AlgE6 to 64% and 43% guluronic acid, respectively. Mannuronan epimerized to 90% guluronic acid followed by oxidation to P_0 = 0.04 (C) and P_0 = 0.08 (D) is also shown. All samples are reduced 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).

(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:

$$N^{*}_{G>1} = \frac{n_G}{n_{GGM} + n_{GGG^{*}}} = \frac{F_G}{F_{GGM} + F_{GGG^{*}}} \quad (4)$$

Table 2 shows 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 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.

Due to the acid lability of the oxidized/reduced units a control experiment was carried out, under identical conditions as all other samples in question, to investigate whether any degradation took place during the gelling experiment. The molecular weight of an oxidized/reduced sample of *Laminaria hyperboera* alginate with $P_0 = 0.06$ (three replicates) was measured, before and after gelling, using SEC-MALLS (data not shown). The molecular weight remained unchanged and it was concluded that no degradation of the polymer took place during gelling.

Gelation of mannuronan epimerized after oxidation and reduction (strategy I) was studied, and the results are given in Fig. 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 B). 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 Fig. 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, the large increase in local chain flexibility at the oxidized residues may result in 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 local flexibility is the main reason for the big difference in G'.

The mannuronan samples epimerized with AlgE6 before oxidation and reduction (strategy II), were also studied by small-strain oscillatory measurements as shown in Fig. 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 10 mM 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 Fig. 8A and D it can be observed that for the sample $P_0 = 0.02$ the sol/gel transition starts earlier, and is transiently more rapid compared to the unoxidized sample (strategy II). The reason for this is not obvious, but can be tentatively explained by a faster organization of the gelling zones due to increased local flexibility. This effect cannot be easily observed at higher P_0 due to the weak gels formed. An apparently similar observation was also 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, Glover, & Whittington, 1973; Stokke, Smidsrød, Bruheim, & Skjåk-Bræk, 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 local 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

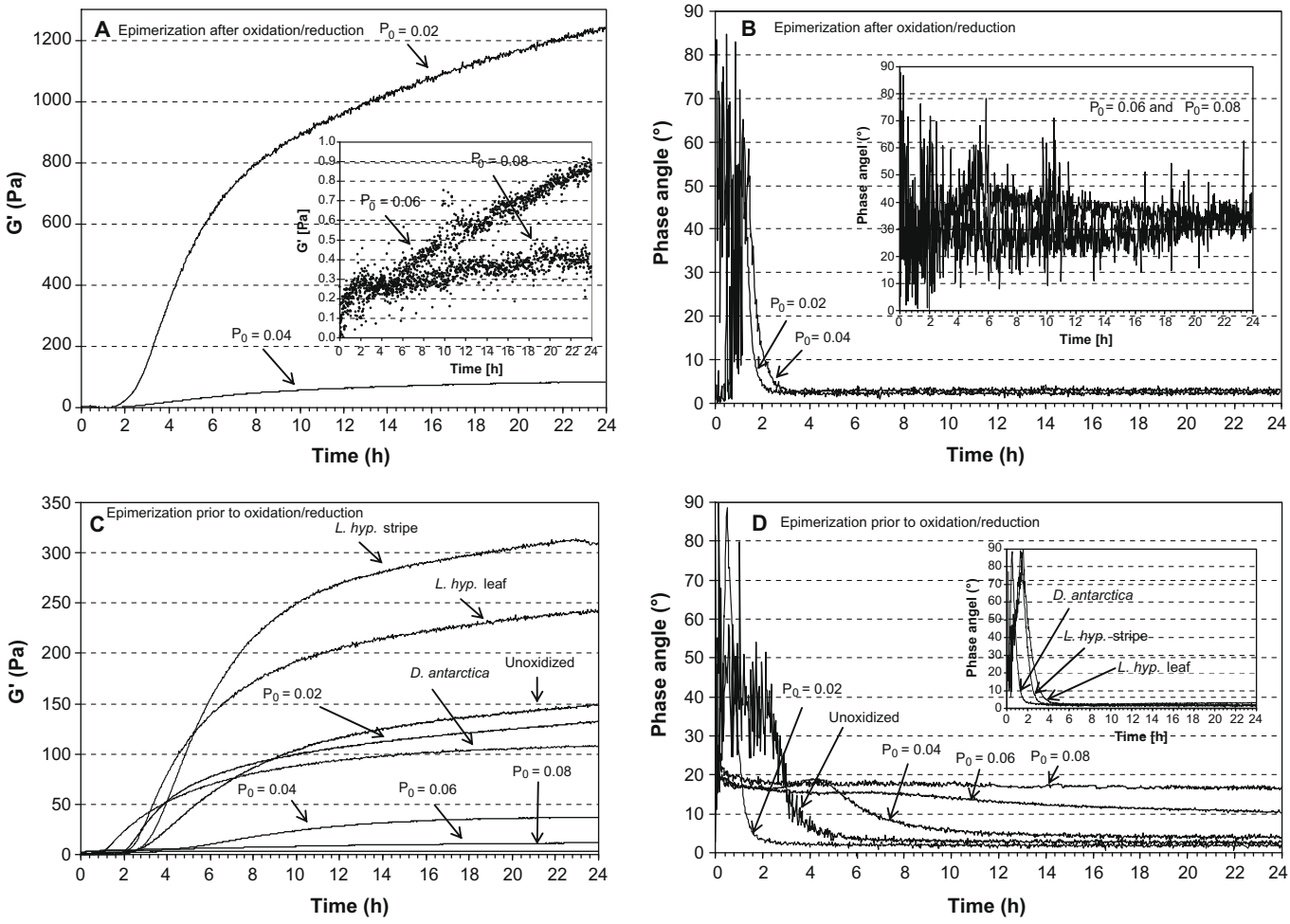

Fig. 8. Upper figures: Dynamic storage modulus (G') (A) or phase angle (B) versus time (h) for mannuronan epimerized after oxidation with AlgE6 (strategy I). Lower figures: G' (C) or phase angle (D) versus time (h) for mannuronan epimerized prior to oxidation with AlgE6 (strategy II). Alginate from *Laminaria 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).

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 Fig. 8 suggests that periodate oxidation followed by borohydride reduction 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 $P_0 \geqslant 0.06$ '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.

4. 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 local 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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