

Degradation kinetics of peptide-coupled alginates prepared via the periodate oxidation reductive amination route

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Abstract

Biomaterials based on peptide-coupled alginates must provide both optimal biological environments and tuneable stability/degradation profiles. The present work investigates the degradation pattern and kinetics of peptide-coupled alginates prepared via the periodate oxidation reductive amination route. Alginates degrade slowly (non-enzymatically) under physiological conditions by acid-catalysed hydrolysis and alkali-catalysed β -elimination, both operating simultaneously but dominated by the latter. While periodate oxidation alone largely increases the rate of β -elimination, substitution restores the susceptibility towards β -elimination to that of the parent alginate. For acid hydrolysis the rate of depolymerization is proportional to the degree of substitution, being approximately one order of magnitude larger than the parent alginate, but still lower than for the corresponding materials with fully reduced dialdehydes. The results also suggest a composition-dependent preference for substitution at C2 or C3. These results demonstrate how the various chemistries introduced by the coupling provide useful means to tune the biodegradability profiles.

Keywords

Alginate, SEC-MALS, periodate oxidation, reductive amination, acid hydrolysis, β -elimination

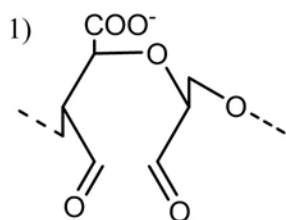
1 Introduction

The use of alginates as biomaterials is an area with rapid development. The materials are commonly calcium alginate hydrogels (homogeneous, heterogeneous), foams or fibres wherein cells, particles or therapeutic molecules are seeded (Andersen, Strand, Formo, Alsberg, & Christensen, 2012; Lee & Mooney, 2012). Different applications have different requirements for material properties, and the ability to tune these properties is gaining a lot of attention. In tissue engineering, for example, the material should create an unique microenvironment that offer mechanical support and provide various biological signals adapted to the specific cell types (Dvir, Timko, Kohane, & Langer, 2011).

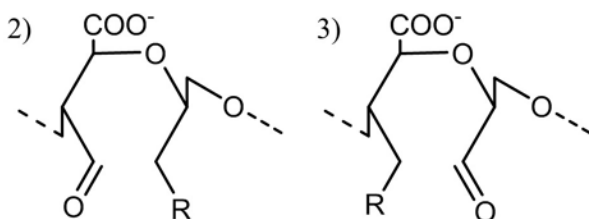
Alginates are unbranched polysaccharides that consist of 1,4-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) in regions of consecutive M residues (M-blocks), consecutive G residues (G-blocks) and alternating M and G residues (MG-blocks). Alginates are mainly found as structural components in brown algae and as capsular polysaccharides in some bacteria (e.g. *Azotobacter*- and *Pseudomonas* species). The polymer is synthesized *in vivo* as pure mannuronan and G residues are introduced at the polymer level by a series of processive C5 epimerases. Alginate hydrogels are formed by intermolecular ionic crosslinking of G-blocks, with some contributions from MG-blocks (Donati, Mørch, Strand, Skjåk-Bræk, & Paoletti, 2009), by certain divalent cations (e.g. Ca^{2+} , Ba^{2+} and Sr^{2+}). The structure therefore affects the gel forming ability and the mechanical properties of the gels (Aarstad, Strand, Klepp-Andersen, & Skjåk-Bræk, 2013; Mørch, Donati, Strand, & Skjåk-Bræk, 2007; Mørch, Holtan, Donati, Strand, & Skjåk-Bræk, 2008).

In recent years, bioactive peptide sequences have been chemically incorporated into alginates to induce cell interaction and stimulate cellular responses such as proliferation and differentiation into the desired cell phenotype. The peptides are attached either via the carboxyl groups using carbodiimide chemistry (Fonseca, Bidarra, Oliveira, Granja, & Barrias, 2011; Rowley, Madlambayan, & Mooney, 1999; Rowley & Mooney, 2002; Sandvig et al., 2015), or by reductive amination following partial oxidation with periodate (Dalheim et al., 2016). We recently showed that the latter approach has the advantage of more precise control of the degree of substitution (DS) and simpler access to higher DS values (Dalheim et al., 2016). The attachment of substituents via the periodate oxidation reductive amination route results in monosubstitution at either C2 or C3 (Kristiansen, Ballance, Pothast, & Christensen, 2009a). In addition small amounts of residual unreacted or reduced aldehydes may be present (Dalheim et al., 2016). The different possible structures are shown in Figure 1.

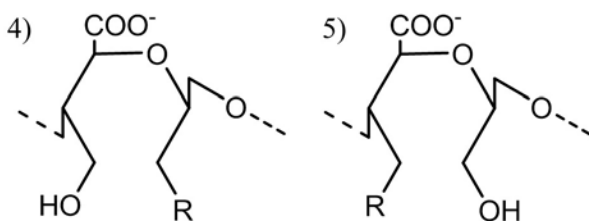
Oxidized



Oxidized and coupled



Oxidized, coupled and reduced



Oxidized and reduced

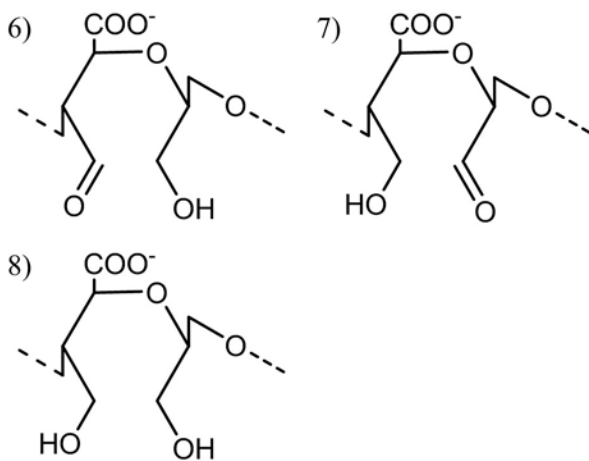


Figure 1: Possible structures (structure 2-8) after reductive amination of periodate oxidized alginates (structure 1), shown for an M residue. In aqueous solutions hydrated aldehydes (gem diols) or hemiacetals may form (not shown here according to common practice).

The performance of alginate biomaterials depends critically on the rate and extent of disintegration of the gels, which need to be tailored to the specific application. For example, in xenotransplantation to treat endocrine diseases such as diabetes (de Vos, Faas, Strand, & Calafiore, 2006) microencapsulation is used to shield the cells from a host immune response and little disintegration is therefore beneficial. In contrast, regeneration of tissue may benefit from a more degradable material for the cells to be able to proliferate and create their own extracellular matrix (Augst, Kong, & Mooney, 2006). Disintegration may occur by both exchange of the crosslinking divalent cations with monovalent cations (eg Na^+) (Shoichet, Li, White, & Winn, 1996) and degradation of the alginate chains.

The alginate chains degrade relatively slowly in the human body (Alshamkhani & Duncan, 1995), presumably due to the lack of alginate degrading enzymes. The *pseudo* first order rate constant for cleavage of glycosidic linkages under physiological conditions (pH 7.2, 37°C) has been estimated to $1.2 \times 10^{-6} \text{ h}^{-1}$ (Kristiansen, Tomren, & Christensen, 2011). The main mechanisms for alginate depolymerization under such conditions are acid catalysed hydrolysis and alkali catalysed β -elimination, which may operate simultaneously. It has been shown that even at neutral and slightly acidic conditions a significant part of the degradation occurs as β -elimination (Haug, Smidsrød, & Larsen, 1963). Chemical modification may drastically alter the stability properties of alginates. Periodate oxidation, which results in cleavage of C2-C3 bonds (ring opening) and introduction of aldehydes at C2 and C3 (dialdehydes) (Dryhurst, 1970), significantly increases the rate of β -elimination. For example, an 8% periodate oxidized alginate (POA) is degraded 6.4 times faster than the corresponding unoxidized alginate under physiological conditions (Kristiansen et al., 2011). Periodate oxidation has therefore been suggested as a strategy to increase the degradability of alginates (Boontheekul, Kong, &

Mooney, 2005; Bouhadir et al., 2001). The ring opening also increases the overall chain flexibility of the polymer (Lee, Bouhadir, & Mooney, 2002; Smidsrød & Painter, 1973; Vold, Kristiansen, & Christensen, 2006). Reduction of the dialdehydes to the corresponding alcohols, results in much enhanced susceptibility towards acids (the basis for Smith degradation in structural studies (Abdel-Akher, Hamilton, Montgomery, & Smith, 1952)). The number of modified residues, however, must be limited (0-5%) to avoid a significant reduction in the gel forming properties (Gomez, Rinaudo, & Villar, 2007; Kristiansen et al., 2009b).

Alginates that are substituted by peptides via periodate oxidation and reductive amination may in principle contain all structures shown in Figure 1. The relative distribution of the various types of uronate derivatives may significantly influence the stability properties. In the present work, we therefore analyse the stability of peptide-coupled alginates and compare to the corresponding (reduced or non-reduced) dialdehyde alginates, as well as parent (unmodified) alginates, including homopolymeric mannuronan. The amino acid derivative L-tyrosine methyl ester (MeOTyr) is used as a model for peptide coupling. Since degradation at physiological conditions involves two separate mechanisms operating simultaneously, the study was performed under conditions where either alkali-catalysed β -elimination (pH 10.4) or acid-catalysed hydrolysis (pH 4.0) dominate. Temperatures were identical or higher (50°C) than physiological conditions in order to accelerate the degradation. Extrapolation of results to physiological conditions is simple as activation energies often are known, and the rate constants of acid hydrolysis and alkaline β -elimination are proportional to $[H^+]$ and $[OH^-]$, respectively. The degradation was monitored by size exclusion chromatography with multi-angle light scattering detection (SEC-MALS), which provide accurate molecular weight data even for limited degrees of chain scission, as opposed to chemical methods or NMR.

2 Experimental

2.1 Materials

Mannuronan was isolated from an epimerase-negative mutant of *Pseudomonas fluorescense* (Gimmestad et al., 2003). Alginate from *Laminaria hyperborea* stipe was obtained from FMC Biopolymer, Drammen, Norway. Their compositions (determined by NMR according to Grasdalen et al. (Grasdalen, 1983; Grasdalen, Larsen, & Smidsrød, 1979)) and molecular weights (determined by SEC-MALS) are given in Table 1.

Table 1: Composition (sequence parameters, F_G = fraction of G residues) and molecular weights (M_w) of mannuronan and stipe alginate from *L. hyperborea* used in the present study.

Alginate	F_G	F_M	F_{GG}	F_{MG}	F_{MM}	F_{MGG}	F_{MGM}	F_{GGG}	$N_{G>1}$	M_w
				F_{GM}		F_{GGM}				(kDa)
Mannuronan	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0	900
<i>L. hyperborea</i> stipe alginate	0.65	0.35	0.53	0.12	0.23	0.05	0.10	0.48	11	110

2.2 Periodate oxidation

Alginate was dried overnight in a desiccator with phosphorus pentoxide (Sicapent, Merck) and dissolved in MQ water (deionised water purified with the MilliQ system from Millipore, Bedford, MA, USA) corresponding to a final concentration of 6.0 - 8.8 mg/ml depending on the viscosity of the solution. n-propanol (10 % v/v) was added as a radical scavenger to minimize

depolymerization in the subsequent oxidation step (Smidsrød, Haug, & Larsen, 1965). The solutions were then degassed for 10 min using nitrogen gas (N₂) and cooled down to 4 °C. 0.25 M sodium (meta)periodate (NaIO₄, Merck) in MQ water was prepared and bubbled with N₂ before added stoichiometrically to the alginate solutions according to the desired P₀ (periodate/monomer molar ratio). All handling was carried out in subdued light. The reaction was carried out for 96 hours at 4 °C and the samples were thereafter dialysed against MQ water until the conductivity was below 2 µS and finally freeze-dried.

2.3 Coupling of L-tyrosine methyl ester (MeOTyr) (reductive amination)

Periodate oxidized *L. hyperborea* stipe alginate (POA) and mannuronan (POM) were coupled to L-tyrosine methyl ester (MeOTyr) according to the previously published protocol (Dalheim et al., 2016). The alginate samples were dissolved in MQ-water corresponding to a final concentration of 3 mg/ml and added methanol (MeOH) corresponding to a final concentration of 12% (v/v). 0.25 M MeOTyr (Sigma-Aldrich) in MeOH and/or 0.25 M 2-methylpyridine borane complex (picoline borane, Sigma-Aldrich) in MeOH was then added to final concentrations of 2.42 mM and 24.24 mM, respectively. pH was adjusted to 5.8 using a 1 M acetate buffer pH 5 (final buffer concentration ~ 1-11 mM). The samples were incubated at room temperature for 48 hours and then dialysed against two shifts of 0.05 M NaCl and thereafter MQ water until the conductivity was below 2 µS. Finally, the samples were freeze-dried.

2.4 Borohydride reduction

Alginate samples (periodate oxidised) were dissolved in MQ water to a concentration of 4.4 mg/ml. Sodium borohydride (NaBH₄, Fluka) was added to 20% (w/v) and the samples were left for 2 hours on stirring at room temperature. The samples were then placed on ice and

concentrated acetic acid was added until all hydrogen gas had effervesced. pH was adjusted to ~7.0. The samples were then dialysed against MQ water until the conductivity was less than 2 μ S and freeze-dried.

2.5 NMR spectroscopy

1D ^1H NMR spectroscopy was used to analyse the degree of substitution for the MeOTyr-coupled alginate samples. The samples were subjected to mild acid hydrolysis as previously described (Ertesvåg & Skjåk-Bræk, 1999) to reduce the viscosity prior to the NMR analysis. 6-10 mg of the samples were dissolved in 600 μ l D_2O (99.9%). 5 μ l of 3-(Trimethylsilyl)propionic 2,2,3,3- d_4 acid (TSP, Sigma Aldrich) was added as an internal standard and 15 μ l Triethylenetetraamine-hexaacetic acid (TTHA, Sigma Aldrich) was routinely added as a chelating agent in case of residual divalent ions.

1D ^1H NMR spectra were recorded at 90 $^\circ\text{C}$ on a BRUKER Avance DPX 300 MHz spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) equipped with a 5 mm QNP probe. The spectra were recorded using TopSpin 1.3 software (Bruker BioSpin) and processed and analysed with TopSpin 3.0 software (Bruker BioSpin).

2.6 Alkaline degradation

The alginates were dried overnight in a desiccator with phosphorus pentoxide (Sicapent, Merck) and subsequently dissolved in MQ water to a stock concentration of 4.79 mg/ml. The solutions were degassed using nitrogen. The different alginate stock solutions were then distributed into sample tubes and preheated to 37 $^\circ\text{C}$. Preheated (37 $^\circ\text{C}$) and degassed 0.1 M carbonate buffer pH 10.4 was added to each sample to obtain a final alginate concentration of 3 mg/ml and an ionic strength (I) of 0.1 M. The samples were then incubated at 37 $^\circ\text{C}$ for 10 min – 24 hours. The

reactions were stopped by rapid cooling and neutralisation using HCl. Finally, the samples were freeze-dried.

2.7 Acidic degradation

Alginate stock solutions were prepared as described above (4.91 mg/ml), distributed into sample tubes, and preheated to 50 °C. Preheated (50 °C) and degassed 0.15 M citrate buffer pH 4.0 was added to each sample to obtain a final alginate concentration of 3 mg/ml and an ionic strength of 0.1 M. The samples were then incubated at 50 °C for 1 – 24 hours. The reactions were stopped by rapid cooling and neutralization using NaOH. The samples were freeze-dried.

2.8 SEC-MALS

Size exclusion chromatography with multi angle light scattering (SEC-MALS) was used to measure the molecular weight, essentially as previously described (Vold et al., 2006). The system consisted of a mobile phase reservoir, an on-line degasser, an HPLC isocratic pump (LC-10AD_{VP}, Shimadzu), an autoinjector (SCL-10A_{AV}, Shimadzu), a pre-column and two serially connected columns: TSK 6000 PWXL and TSK 5000 PWXL (Toso Haas) for high molecular weight samples and TSK 4000 PWXL and TSK 2500 PWXL (Toso Haas) for low molecular weight samples. The columns were followed by two serially connected detectors: a Dawn DSP laser light scattering photometer (Wyatt, USA) ($\lambda_0 = 0.633$ nm) and an Optilab DSP differential refractometer (P-10 cell) (Wyatt, USA). 0.15 M NaNO₃ with 0.01 M NaEDTA, pH 6 was used as the mobile phase. All samples were dissolved in the mobile phase (0.25-7.5 mg/ml) and filtered (pore size 0.8 μ m) prior to injection. The injection volume was adjusted to obtain an optimal light scattering signal and to avoid overloading. Astra software v. 6.1 (Wyatt, USA) was

used to collect and process the obtained data, using a refractive index increment (dn/dc_{μ}) of 0.150 ml/g for alginate samples (Vold et al., 2006).

3 Results and discussion

3.1 Preparation of materials

Periodate oxidized alginate from *L. hyperborea* stipe (POA, 65% G) and periodate oxidized mannuronan (POM, 0% G) with P_0 (periodate/monomer molar ratio) of 0.04 and 0.08 were prepared and further coupled to L-tyrosine methyl ester (MeOTyr) by reductive amination. As in our previous work (Dalheim et al., 2016), the amino acid derivative MeOTyr was used as a model compound for peptide coupling. The degree of coupling (D_{MeOTyr}) was analysed by NMR spectroscopy and defined/calculated as the molar fraction of uronic acid residues having a substituent according to previously established annotations (Dalheim et al., 2016; Kristiansen et al., 2009b). D_{MeOTyr} values close to P_0 were obtained (Table 2). Coupling to POM was, however, slightly more efficient than to POA, in accordance with our previous observations (Dalheim et al., 2016). Samples of *L. hyperborea* stipe alginate (hereafter referred to as alginate), mannuronan, POA and POM treated with picoline borane according to the reductive amination protocol (without addition of MeOTyr) and borohydride reduced POA and POM (POAr and POMr) were prepared as control materials for the degradation experiments.

3.2 Degradation, SEC-MALS analysis, and data processing

Using SEC-MALS to monitor the degradation introduces some methodical aspects as illustrated by typical examples of chromatograms and slice molecular weights after degradation of POA

($P_0 = 0.08$) and MeOTyr-coupled POA ($P_0 = 0.08$, $D_{\text{MeOTyr}} = 0.073$) at pH 10.4 and 37 °C in

Figure 2.

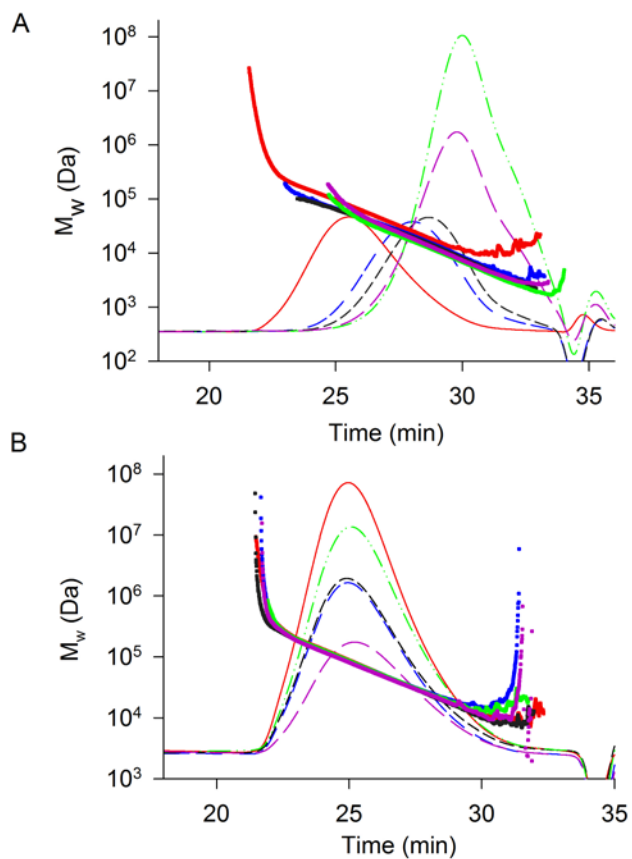


Figure 2: SEC-MALS data example after degradation at pH 10.4, 37 °C for A: POA ($P_0 = 0.08$), from left to right: 0min (red), 10min (blue), 30min (black), 6h (green) and 24h (purple) and B: MeOTyr-coupled POA ($P_0 = 0.08$, $D_{\text{MeOTyr}} = 0.073$), 0min (red), 10min (blue), 30min (black), 6h (green) and 24h (purple). Concentration profiles are represented with lines and slice molecular weights with dots.

Some of the samples contained detectable amounts of aggregates resulting in an upturn in the molecular weight at short elution times (Figure 2). The influence on the average molecular weights of these aggregates were minimized during processing of the data by defining the peak limits according to the RI profile and apply an exponential fit (first order) to the data. SEC-MALS provides in principle both M_w and M_n . However, M_w is here preferred in modelling of the degradation (see below). Although the SEC columns were selected according to the expected molar mass ranges throughout the degradation, the peak tails in some cases overlapped with the salt. In such cases M_n becomes overestimated to a larger extent than M_w . Since the materials in the first phase of the degradation generally had, or approached, molar mass dispersities ($D = M_w/M_n$) close to 2.0, the relation $M_n = M_w/2$ was used for modelling the degradation kinetics. A comparison of results obtained on the basis of M_n values obtained directly from SEC-MALS and as $M_w/2$ is elaborated in the Supporting Information. Despite some systematic differences the major conclusions described below are not changed.

3.3 Alkaline β -elimination - kinetics

MeOTyr-coupled alginate and mannuronan ($P_0 = 0.04$ and 0.08) and the corresponding control materials (parent unmodified and periodate oxidized polymers) were subjected to alkaline degradation at pH 10.4, $I = 0.1$ M and 37 °C. The degradation kinetics was analysed by monitoring changes in $1/M_w$ (Figure 3). The degradation was in most cases characterized by an initially rapid decrease in molar mass followed by a gradual decrease in degradation rate until a plateau value was obtained after approximately 3 hours. For further analysis, the data were fitted to the kinetic model described by Kristiansen et al. (2011). The model describes the degradation in terms of two separate rate constants, one for the labile bonds associated with oxidation (k_1) and one for unmodified residues (k_2), as well as the relative amounts of oxidized and unmodified

residues. The rapid consumption of labile bonds followed by slow (or experimentally undetectable) degradation of more stable bonds leads to the characteristic level-off behaviour (exemplified in Figure 3) The fraction of cleaved residues ($\alpha = 1/DP_n$) should, according to the model, asymptotically approach the fraction of labile dialdehyde residues. The level-off value of α (α_{l-o}) was therefore determined.

3.3.1 POM (unmodified)

For oxidized (unmodified) mannuronan (POM, $P_0 = 0.08$, Figure 3A) M_w levelled off at 4.8 kDa, corresponding to $DP_n = 12$. Hence, $\alpha_{l-o} = 0.081$, which imply quantitative cleavage of oxidized residues (101% of P_0 , see Table 2). In comparison, POM with $P_0 = 0.04$ (Figure 3B) levelled off somewhat below P_0 (83% of P_0), but in good agreement with the degree of substitution when substituted by MeOTyr (D_{MeOTyr} corresponding to 85% of P_0). This suggests substoichiometric oxidation for lower degrees of periodate oxidation, which is corroborated by an even larger difference described for 2% oxidized mannuronan (Kristiansen et al., 2011).

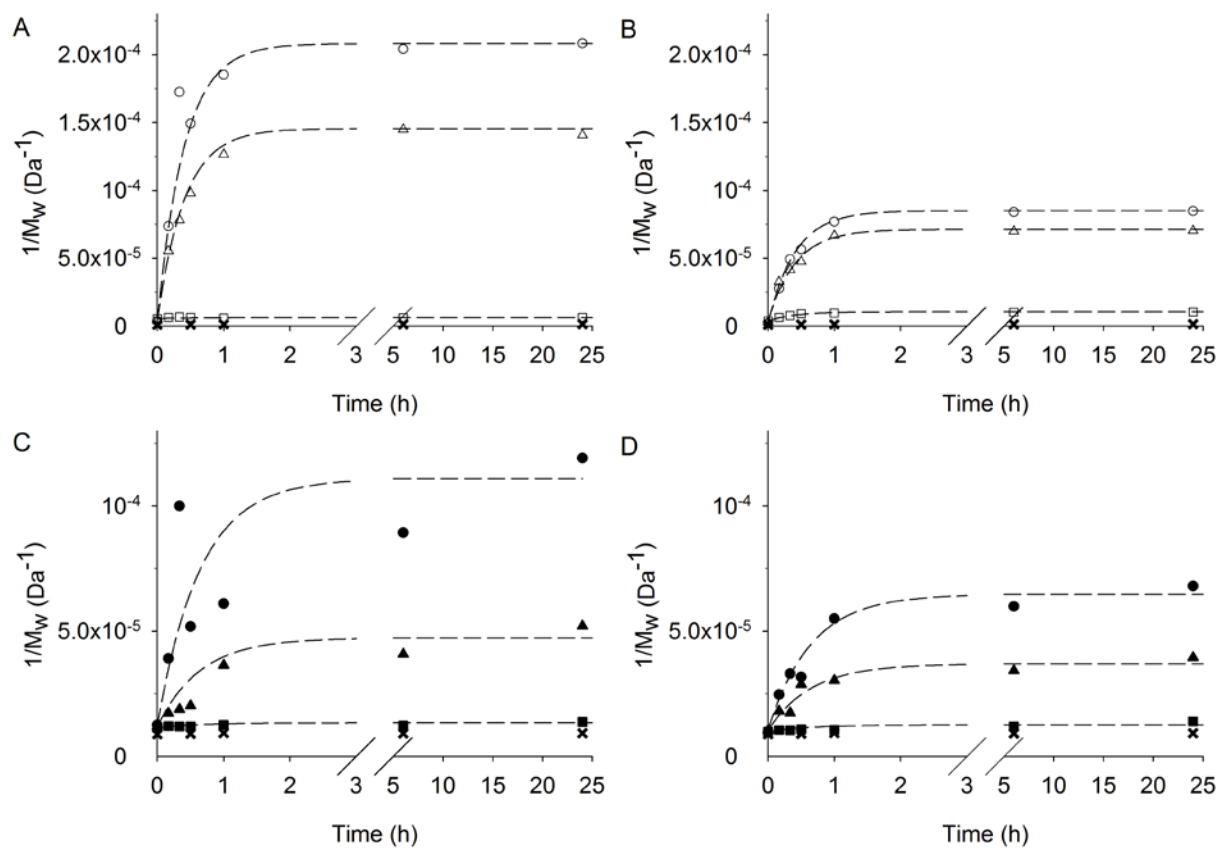


Figure 3: Degradation at pH 10.4 ($I = 0.1$ M), 37 °C. Experimental data (symbols) and fitted data (dotted lines) for A ($P_0 = 0.08$) and B ($P_0 = 0.04$): \circ : POM, Δ : POM reduced with picoline borane, \square : MeOTyr-coupled POM, and \times : mannuronan. C ($P_0 = 0.08$) and D ($P_0 = 0.04$): \bullet : POA, \blacktriangle : POA reduced with picoline borane, \blacksquare : MeOTyr-coupled POA, and \times : *L. hyperborea* stipe alginate (65% G).

3.3.2 Periodate oxidized alginate (POA)

Oxidized (unmodified) alginate (POA) similarly showed an initial rapid decrease in the molecular weight before it appeared to level off (Figures 3C and 3D). However, data for $P_0 = 0.08$ were scattered and prevented a reliable fit. The best fit suggested that 49.0% of the

oxidized residues were cleaved (Table 2). A better fit could be made to the data for POA with $P_0 = 0.04$ providing essentially the same level-off (plateau) value (54%). Such incomplete degradation has also previously been observed (Kristiansen et al., 2011). Since this happens only in alginate and not in mannuronan (at 4 – 8% oxidation) it is most likely associated with oxidized G residues. Periodate oxidation of G residues is known to be approximately 50% faster than of M residues (Painter & Larsen, 1970). For an alginate with 65% G a majority (roughly 74%) of the oxidized residues will therefore be derived from L-guluronic acid. Moreover, dialdehydes tend to form intramolecular hemiacetals with adjacent residues (Painter & Larsen, 1970). Hence, our findings may be explained by a tendency of oxidized G residues, but to a lesser extent M residues, to form alkali-stable hemiacetals. Due to the high content of G (and long G-blocks (Aarstad, Tøndervik, Sletta, & Skjåk-Bræk, 2012)) in the alginate, hemiacetals are most likely formed to adjacent (unoxidized) G residues.

3.3.3 *Picoline borane reduced POM and POA*

As additional controls, oxidized mannuronan and alginate (4 and 8% oxidation) were also treated with picoline borane in the absence of MeOTyr. In these cases the extent of alkaline degradation decreased markedly compared the purely oxidized samples, but was still well above the parent unoxidized materials (Figure 3), indicating partial reduction of aldehydes. Hence, reduction with picoline borane is not perfectly selective for Schiff bases, at least under the conditions used here. Picoline borane is often considered an efficient and safe choice for reductive aminations (Dalheim et al., 2016; Ruhaak, Steenvoorden, Koeleman, Deelder, & Wuhler, 2010; Sato, Sakamoto, Miyazawa, & Kikugawa, 2004; Unterrieser & Mischnick, 2011), but a certain carbonyl reduction has also been observed previously (Dalheim et al., 2016; Ruhaak et al., 2010). For POM treated with picoline borane (Figures 3A and 3B) the molecular weight levelled

off at α_{1-o} values approximately 70% of P_0 (Table 2). Consequently, around 30% of the original oxidized residues appear to be resistant to cleavage because of carbonyl reduction. In comparison, POA treated with picoline borane flattened out at a much lower level ($\alpha_{1-o} = 26\%$ and 18.0% for $P_0 = 0.04$ and $P_0 = 0.08$ respectively), suggesting that roughly 75-80% of the originally oxidized residues had become reduced and hence resistant to cleavage in alkali. On the other hand, degradation of POA (not reduced) showed that approximately 50% of the dialdehyde residues were resistant to cleavage in alkali, which complicates the interpretation of the degradation of POA treated with picoline borane. The 75-80% resistant residues could therefore be a mixture of reduced and hemiacetal-stabilized dialdehyde residues. Based on these results, it is therefore not possible to evaluate whether there is a difference in picoline borane reduction of M- and G residues with the current methodology.

Table 2: Overview of samples used in this study. P_0 : Periodate/monomer molar ratio during oxidation (oxidation degree), D_{MeOTyr} : Degree of substitution of MeOTyr (determined by NMR). α_{1-o} : Fraction of cleaved linkages after the degradation had levelled off. α_{1-o}/P_0 : Fraction of cleaved residues relative to the theoretical maximum (oxidation degree). k' : initial rate constant for degradation at pH 4.0 ($I = 0.1 \text{ M}$), $50 \text{ }^\circ\text{C}$.

Material	P_0	D_{MeOTyr}	Alkaline degradation		Acidic degradation
			α_{1-o}	α_{1-o}/P_0 (%)	$k' \times 10^{-3}$ (h^{-1})
Stipe alginate ($F_G = 0.65$)	-	-	-	-	0.03
Mannuronan ($F_G = 0$)	-	-	-	-	0.08
POM	0.04	-	0.033	83	0.09
POM (picoline borane reduced)	0.04	-	0.027	68	<
MeOTyr-coupled POM	0.04	0.034	0.003	7	0.70
POMr	0.04	-	-	-	1.30
POA	0.04	-	0.022	54	0.06
POA (picoline borane reduced)	0.04	-	0.011	26	-
MeOTyr-coupled POA	0.04	0.031	0.001	3	0.13
POAr	0.04	-	-	-	0.74
POM	0.08	-	0.081	101	0.15
POM (picoline borane reduced)	0.08	-	0.056	70	0.90
MeOTyr-coupled POM	0.08	0.086	0.000	0.4	1.40
POMr	0.08	-	-	-	2.80
POA	0.08	-	0.039	49	0.10
POA (picoline borane reduced)	0.08	-	0.014	18	0.60
MeOTyr-coupled POA	0.08	0.073	0.001	1	0.23
POAr	0.08	-	-	-	1.60

3.3.4 *MeOTyr-coupled mannuronan and alginate*

MeOTyr-coupled mannuronan and alginate showed a completely different behaviour compared to the oxidized samples. In both cases the degradation at pH 10.4 was very slow and essentially the same as for the parent unmodified mannuronan and alginate, respectively, indicating that attachment of substituents to the periodate oxidized materials by reductive amination almost completely eliminates the susceptibility to β -elimination. For example, coupled mannuronan ($P_0 = 0.08$, $D_{\text{MeOTyr}} = 0.086$) was only slightly degraded before levelling off at an M_w of 161.5 kDa (DP_n of 408). The corresponding fraction of cleaved linkages appeared to be only 0.4 % of P_0 (Table 2). Similarly, the degradation of MeOTyr-coupled alginate was minimal and indistinguishable from the parent alginate (Figures 3C and D). Fitting of the data suggested that only 0.1 % of the linkages were cleaved for both $P_0 = 0.04$ and 0.08 (Table 2).

3.3.5 *Structural aspects related to alkaline degradation*

The degradation results above also provide further structural information. The coupling reaction itself may result in different structures (Figure 1), each having quite different stability properties. Firstly, the substituent may be coupled either to C2 or to C3 in the oxidized residues, whereas double substitution does not seem to occur (Dalheim et al., 2016; Kristiansen et al., 2009a). Secondly, the remaining unsubstituted position can be either on the aldehyde form (structure 2-3 in Figure 1), or in the reduced form due to partial carbonyl reduction by picoline borane as described above (structure 4-5 in Figure 1). Partial carbonyl reduction is also supported by structural analysis by NMR (Dalheim et al., 2016). Thirdly, residual (unreduced) carbonyl functionalities could also be on the form of hemiacetals.

From the mechanism of β -elimination (Sharon, 1975) it can be hypothesized that it is preferentially the carbonyl functionality on C3 that is responsible for the increased rate of β -elimination in periodate oxidized alginates (Figure 5A). Our results therefore suggest either negligible amounts of structure 2 after coupling, or that structure 2 is stabilized by alkali-stable hemiacetals. The latter possibility cannot be excluded for the substituted stipe alginate. However, in the case of substituted mannuronan, the former possibility is supported by the observation of complete (stoichiometric) cleavage of oxidized residues. We have previously observed that MeOTyr coupling is less efficient on oxidized alginate with 65% G than on oxidized mannuronan (Dalheim et al., 2016). Residual unsubstituted oxidized residues occurring either as dialdehydes (structure 1, intact or as hemiacetals) or in the reduced form (structures 6 – 8) may therefore be present. No observable differences in the degradability between MeOTyr-coupled mannuronan and MeOTyr-coupled alginate were observed. In these cases only structures 2 and 6 would be degraded fast in alkali. The fraction of residual uncoupled residues might therefore not be reflected in the degradation data. Residual aldehydes after coupling can, if necessary, be completely removed by an extra reducing step using a strong reducing agent such as borohydride.

Since reduction of the dialdehydes to dialcohols eliminates the susceptibility to β -elimination, the extent of degradation of POM/POA treated with picoline borane becomes an indirect measure of the carbonyl reduction. Analogous to the coupling reaction, partial or incomplete aldehyde reduction can give different structures (Figure 1): A primary alcohol on either C2 (structure 6) or C3 (structure 7), or on both (structure 8). Using the same reasoning as above, only structure 7 and 8 will be resistant to β -elimination. Hence, the amount of reduced residues could be higher than the apparent amount of residues resistant to degradation due to structure 6.

3.4 Acid hydrolysis - kinetics

All materials were subjected to degradation at pH 4.0 and 50°C. Figure 4 shows plots of changes in $1/M_w$ for MeOTyr-coupled mannuronan and alginate, ($P_0 = 0.08$), including the corresponding control materials (analogous to the previous section). In this case only the initial phase of the degradation was observed within the timeframe of the experiments. No well-defined plateau values were therefore reached although some of the curves show a tendency for levelling off after approximately 6 hours. The stability was therefore characterized by the initial rate constant (k') rather than the fraction of cleaved residues (from plateau values of M_w or M_n). k' was estimated, according to the above mentioned reaction model (Kristiansen et al., 2011), setting the theoretical amount of modified residues to zero and k' therefore reflects the average degradability of the material as a whole. The same data set was also generated for $P_0 = 0.04$, which showed same trends as the samples with $P_0 = 0.08$ (See Supplementary Information). The obtained k' values are summarized in Table 2.

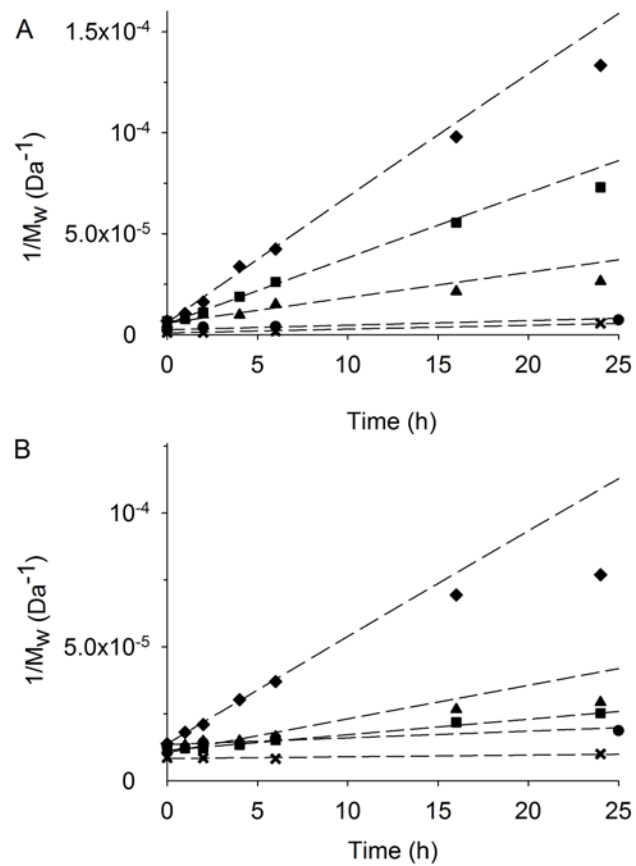


Figure 4: Degradation at pH 4.0 ($I = 0.1$ M), 50 °C. Experimental data (symbols) and fitted data (dotted lines) for A: \blacklozenge : POMr ($P_0 = 0.08$), \blacksquare : MeOTyr-coupled POM ($P_0 = 0.08$, $D_{\text{MeOTyr}} = 0.086$), \blacktriangle : POM treated with picoline borane ($P_0 = 0.08$), \bullet : POM ($P_0 = 0.08$) and \times : mannuronan. B: \blacklozenge : POAr ($P_0 = 0.08$), \blacksquare : MeOTyr-coupled POA ($P_0 = 0.08$, $D_{\text{MeOTyr}} = 0.073$), \blacktriangle : POA reduced with picoline borane ($P_0 = 0.08$), \bullet : POA ($P_0 = 0.08$) and \times : alginate ($F_G = 0.65$).

3.4.1 POM and POA (unmodified)

The rate of degradation of periodate oxidized mannuronan (POM) and periodate oxidized alginate (POA) is very slow under these conditions, and only slightly larger than mannuronan and *L. hyperborea* stipe alginate, respectively (shown for $P_0 = 0.08$ in Figure 4). The initial rate of degradation (Table 2) was roughly proportional to the degree of oxidation (P_0). Kristiansen et

al. (2011) observed that lowering the pH reduced the rate difference between alginates with different degrees of oxidation (decreasing influence of β -elimination). The rates were, however, not the same as for unoxidized alginates until pH 3.1, indicating that the degradation of POM/POA observed here at pH 4.0 to a certain extent can be attributed to β -elimination occurring simultaneously with acid hydrolysis.

3.4.2 Borohydride reduced POM and POA (POMr and POAr)

When the periodate oxidized mannuronan and alginate were reduced with borohydride to the corresponding dialcohols they became much more susceptible for acid hydrolysis, in agreement with the principles of Smith degradation (Abdel-Akher et al., 1952) (Figure 4). The rate constant (k') was roughly proportional to P_0 and approximately one order of magnitude larger than for the corresponding unreduced materials (POM/POA), respectively (Table 2). However, POMr (both $P_0 = 0.04$ and 0.08) was degraded 1.5 times faster than POAr, which suggests oxidized and reduced M residues are hydrolysed slightly faster than oxidized and reduced G residues.

3.4.3 MeOTyr-coupled POM and POA

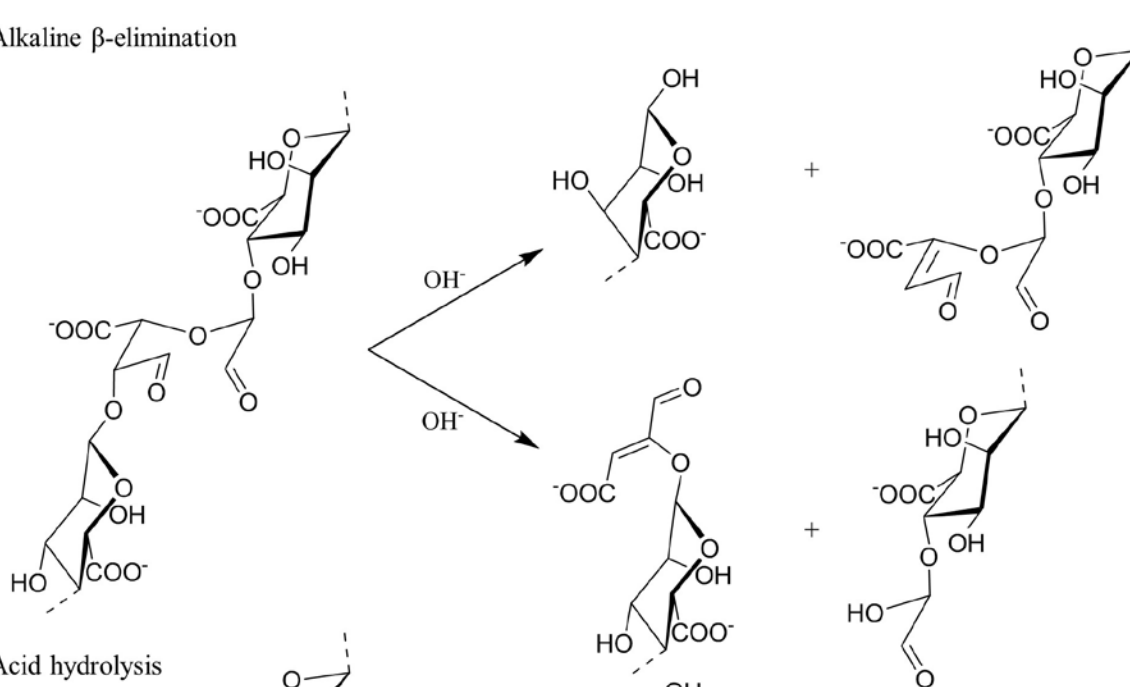
MeOTyr-coupled mannuronan and alginate were degraded with initial rates larger than those of the parent (unmodified) materials, but well below those of the corresponding dialcohols (POMr/POAr) (Figure 4). Specifically, MeOTyr-coupled POM ($P_0 = 0.08$) was degraded at approximately half the rate compared to POMr (Table 2). The degree of MeOTyr substitution obtained by NMR (D_{MeOTyr}) for this material was found to be 0.086, i.e. slightly above the theoretical value (0.080). The observed rate difference can therefore not be explained by 50% of the originally oxidized residues being intact. As described above, the coupling reaction might result in several structures (structures 2-5, Figure 1) that are possibly degraded with different

rates. Based on the chemistry of acid hydrolysis of periodate oxidized and borohydride reduced polysaccharides (Fransson & Carlstedt, 1974; Sharon, 1975) (shown for alginates in Figure 5B) it may be assumed that reduction of C2 is most essential for their increased acid lability. This implies that structures 2, 4 and 5 are degraded faster than structure 3. Structure 3 may (in aqueous conditions) exist as hemiacetals but this is not expected to influence the above mentioned assessments since they are considered relatively stable in dilute acid compared to the reduced form (Goldstein, Hay, Lewis, & Smith, 1965). Furthermore, the nature of the substituent may affect the rate of acid hydrolysis, however, various peptides are not expected to give very different degradation rates due to similar chemistries near the attachment point.

The residues that are oxidized and substituted with MeOTyr at C2 seem to be degraded slower than those that are oxidized and reduced at C2 ($k_{\text{structure 5}} < k_{\text{structures 2 and 4}}$). The data at pH 10.4 indicated a negligible amount of structure 2 in coupled mannuronan. The observed degradation rate at pH 4.0 can then be explained by the coupled residues having mainly structures 3 and 5, and further a preference for coupling to C3 in POM. Structure 4 cannot be completely ruled out, but the small amount of structure 2, and the fact that picoline borane reduction of residual aldehydes is incomplete, suggests the presence of only very small amounts of structure 4. For MeOTyr-coupled POA ($P_0 = 0.08$, $D_{\text{MeOTyr}} = 0.073$), the apparent rate of degradation ($k' = 0.23 \times 10^{-3} \text{ h}^{-1}$) was only 14 % of that of POAr, which is markedly less than coupled POM compared to POMr (50 %). Using a similar argumentation as for coupled POM this indicates a higher fraction of structure 3 and/or a higher fraction of structures 2 and 4 in coupled POA compared to in coupled POM.

3.4.4 Picoline borane reduced POM and POA

The degradation of POM treated with picoline borane was slower than for both POMr and MeOTyr-coupled POM (Figure 4) with a rate constant approximately one third of that of POMr (Table 2), indicating a partial reduction (ca 1/3) of aldehydes leading to acid-sensitive alcohols (as in Smith degradation). This is consistent with the results obtained at pH 10.4, where reduction led to stability. The possible structures of picoline borane reduced residues (structure 6-8, Figure 1) are, using similar argumentation as above, not equally susceptible to acid hydrolysis. In POM treated with picoline borane, about one third of the residues are consequently expected to have either structure 6 or 8. The extent of carbonyl reduction by picoline borane might therefore be slightly higher than what is reflected in the data due to structure 7. The rate of degradation of POA ($P_0 = 0.08$) treated with picoline borane relative to POAr was in the same range as POM treated with picoline borane relative to POMr, indicating that approximately 1/3 of the oxidized residues had structures 6 or 8 after picoline borane reduction. The relative amount of structure 7 cannot be extracted from the data. Nevertheless picoline borane seems to reduce POM and POA with similar efficiencies.

A) Alkaline β -elimination

B) Acid hydrolysis

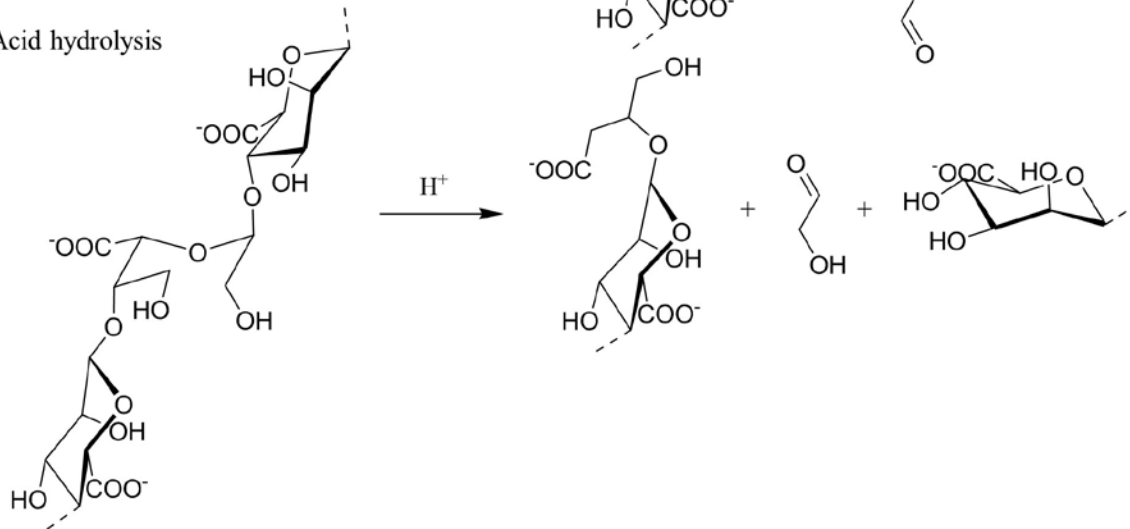


Figure 5: A: Putative chemistry of alkaline β -elimination of periodate oxidized alginates (shown for an $\text{MG}_{\text{ox}}\text{M}$ fragment) based on (Sharon, 1975). B: Chemistry of acid hydrolysis of periodate oxidized and borohydride reduced alginates (shown for an $\text{MG}_{\text{ox}}\text{M}$ fragment) according to (Sharon, 1975) and (Fransson & Carlstedt, 1974).

4 Concluding remarks

Peptide substitution of alginates via the ‘periodate oxidation - reductive amination’ route markedly changes the structure, and potentially, the stability properties of the modified residues. The rates and extents of both acid hydrolysis and alkaline β -elimination can be much affected, but also tuned to obtain the desired degradability.

Firstly, substitution largely eliminates the susceptibility to alkaline β -elimination transiently introduced by oxidation. Residual unreduced aldehydes on C3 may give some susceptibility to alkali. This may, however, be eliminated in an optionally second reduction step with a stronger reducing agent such as sodium borohydride. It follows that β -elimination will not contribute to any degradation at physiological conditions (pH 6-8, 37 °C) beyond that of the alginate itself. Secondly, substitution increases the susceptibility towards acid hydrolysis at pH 4, but the coupled alginate was degraded slower than the corresponding dialcohols. Some of the susceptibility at low pH can be attributed to partial aldehyde reduction by picoline borane during the coupling reaction, especially on C2. However, the contribution of acid hydrolysis to the overall stability at physiological conditions must be small or negligible. Hence, peptide-coupled alginates prepared by the ‘periodate oxidation reductive amination’ route have essentially the same degradation profiles *in vivo* as unmodified alginate.

Studies of the degradation kinetics of coupled alginates under alkaline and acidic conditions also reflect structural information related to the substitution protocol. The substitution of partially periodate oxidized alginates by reductive amination results in several possible reaction products with rather different stability properties, providing an additional means to tailor the stability.

We further observed a difference in the stability at pH 4.0 between MeOTyr-coupled mannuronan and alginate, respectively. These findings suggest that the preference for coupling at either C2 or C3 may be different for oxidized M and G residues. However, further structural analyzes by NMR or fragment analysis by mass spectrometry may be required for better understanding of these possible structural differences.

Various tissue engineering- and drug release strategies will have different requirements to the applied hydrogels. In tissue engineering attention has been drawn towards recreating the macrostructural properties as well as the nanostructure of the extracellular matrix (Dvir et al., 2011). The use of alginate hydrogels in different tissue engineering strategies will therefore have different requirements when it comes to for example peptide density (Dalheim et al., 2016; Ingber & Folkman, 1989; Mooney et al., 1992; Rowley & Mooney, 2002), mechanical properties (Drury & Mooney, 2003), and stability (Boontheekul et al., 2005; Bouhadir et al., 2001). The results obtained here indicate that the modified residues will not markedly change the overall degradability of alginate at physiological conditions. However, the toolbox of chemical modifications can be used to tune the material stabilities. A more degradable and yet peptide functionalized material can be obtained by additional periodate oxidation following the coupling reaction. This material is then expected to be less stable than unmodified alginates above pH 4 and have decreasing stability with increasing pH. A more acid labile material could further be made by oxidation and subsequent reduction after functionalization. In all cases, the total fraction of modified residues must be kept below 5-8% to retain gelling properties (Gomez et al., 2007; Kristiansen et al., 2009b).

Supporting information

A comparison of using M_n from SEC-MALS directly or M_n calculated as $M_w/2$ in the characterization of the materials' degradability.

Plots of changes in $1/M_w$ at pH 4.0 and 50 °C for MeOTyr-coupled POM and POA ($P_0 = 0.04$) and their corresponding control materials (mannuronan/alginate, POM/POA, POMr/POAr and POM/POA treated with picoline borane).

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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