An analysis of G-block distributions and their impact on gel properties of *in vitro* epimerized mannuronan

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Olav Aarstad[*], *Berit Løkensgard Strand, Lise Mari Klepp-Andersen and Gudmund Skjåk-Bræk*

Department of Biotechnology, Norwegian University of Science and Technology, NTNU

Sem Sælands vei 6-8, N-7491 Trondheim, Norway

* Corresponding author:

Mailing address: Department of Biotechnology, Sem Sælandsvg 6-8, NTNU, N-7491 Trondheim, Norway

Phone: (+47)73593317

Email: olava@nt.ntnu.no

This paper reports a study of the distribution and function of homopolymeric guluronic acid blocks (G-blocks) in enzymatically modified alginate. High molecular weight mannuronan was incubated with one native (AlgE6) and two engineered G-block generating mannuronan C-5 epimerases (AlgE64, and EM1). These samples were found to contain G-blocks with a DP ranging from 20 to approximately 50, lacking the extremely long G-blocks (DP>100) found in algal alginates Calcium gels from epimerised materials were highly compressible and exhibited higher syneresis and rupture strength, but lower Youngs modulus than gels made from algal polymers of similar G-content. Addition of extremely long G-blocks to the epimerized alginate resulted in decreased syneresis and rupture strength, and an increased Young's modulus that can be explained by reinforcement of the crosslinking zones at the cost of length and/or numbers of elastic segments. The presence and impact of these extremely long G-blocks found in natural alginates suggest that alginate gels can be viewed as a nano-composite material.

Keywords: alginate, G-block length, mannuronan C-5 epimerases, syneresis, Youngs modulus, nanocomposites

INTRODUCTION

Alginates are linear glycuronans composed of (1→4) linked residues of β-D-mannuronic acid (M) and α-L-guluronic acid (G). The residues are arranged in a block-wise fashion along the polymer chain forming homopolymeric (M-blocks or G-blocks) or heteropolymeric regions (e.g., MMG, GGM, GMG). They are highly soluble in water and form gels with cations. The gel-forming capacity correlates with the content and average length of the G-blocks.[1,2] The nonrandom, non-repeating structure in alginates has been attributed to its unique biosynthesis in which the G-residues are introduced in a postpolymerization step

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catalyzed by mannuronan C-5 epimerases.[3,4] In a recent paper we described a new strategy for analysis of block length distribution in alginates based on specific enzyme degradation combined with chromatography and NMR.[5]

When this analysis was performed on native alginates from a range of brown algae, we found to our surprise that all samples, independent of their composition, contained a fraction of 10-15 % of homopolymeric G-blocks comprising more than 100 consecutive residues. The biosynthesis and functional role of these long G-blocks in calcium alginate gels is still elusive.

The alginate producing bacterium, *Azotobacter vinelandii,* expresses seven AlgE isoenzymes. These epimerases are modular enzymes consisting of one or two catalytic A-modules and one to seven regulatory R-modules.[4] The seven AlgE enzymes yield different residue sequences in the polymer products[6-9], but the nature of the structure-function relationship is not fully understood. In this study we were using epimerases that generate G-blocks, acting either in a processive mode where the enzyme slides along the polymer, carrying out repetitive epimerisations without dissociating, or in a preferred attack mode where the affinity for the substrate increases with epimerisation. Since mannuronan C-5 epimerases from the alginate producing bacteria are available in our laboratory, the aim of this work was to make alginate with less compositional heterogeneity by modifying homopolymeric mannuronan with specific epimerases, analyse the G-block length and distribution and use the material to elucidate the role of long G-blocks in alginate gels.

MATERIALS AND METHODS

Alginates

L. hyperborea alginate (F_G = 0.67 and M_w ≈2.0x10^5 Da) was provided by FMC Biopolymer. A high molecular weight mannuronan (F_G = 0.0 and M_w ≈3.4x10^5 Da) was isolated from an

epimerase negative AlgG⁻ mutant of *Pseudomonas fluorescens*.[10] Purification and deacetylation was done as described elsewhere.[11]

Enzymes

Three mannuronan C-5 epimerases were used in this study: the wild type epimerase AlgE6 from *A. vinelandii* and the two genetically engineered epimerases AlgE64 and EM1.

AlgE6 was produced by fermentation of the recombinant *E. coli* strain SURE and partially purified by ion-exchange chromatography on Q-Sepharose FF as previously described.[6]

The hybrid enzyme AlgE64 was made by combining the A-module from AlgE6 with the R-module from the highly processive AlgE4 (unpublished results).

EM1 was selected from high throughput screening of a library of mutant epimerases based on six wild type enzymes from *A. vinelandii*, constructed by error prone PCR technology.[12]

Alginate lyase from *Haliotis tuberculata* was purified from abalones as previously described.[13]

C-5 epimerization

Alginates with F_G ranging from 0.01 to 0.88 were prepared by epimerization of mannuronan with the mannuronan C-5 epimerases. Mannuronan (0.25% w/v) was dissolved in 50 mM MOPS buffer, pH 6.9 with 2.5 mM $CaCl_2$ and 70 mM NaCl. The respective enzymes were added to a final mannuronan/enzyme ratio of 300 and incubated at 37 °C. Aliquots were withdrawn after 0.5-24 hours and the reaction was quenched by addition of EDTA to a final concentration of 4 mM. The solutions were dialysed against 50 mM NaCl followed by dialysis against MQ water until the conductivity was below 4 µS at 4 °C. The pH was adjusted to 7.0 before freezedrying.

Lyase degradation

L. hyperborea alginate and epimerized samples were degraded by adding M-lyase (0,016 U/mg substrate) to a 0.25% solution of substrate in 200 mM NH_4Ac, pH 6.9 with 50 mM

NaCl, followed by incubation at 30 °C for 24 hours. The reaction was terminated by heating the solution at 95 °C for 10 minutes.

NMR Spectroscopy

Undegraded samples were acid hydrolyzed to a final DP_n between 30 and 50 prior to analysis in order to reduce viscosity. Samples (5-10 mg) were dissolved in 600 μl D_2O with 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (Aldrich) as an internal standard and triethylenetetraamine-hexaacetate (Sigma) as a calcium chelator. ^{1}H-NMR spectra were recorded on a Bruker DPX 400 spectrometer at 90 °C, using a 30° pulseflip-angle, a spectral width of 4789 Hz and a 32 K data block size. Signal assignment and calculation of molar frequencies of diads and triads and thereby average G-block length ($N_G > 1$), was carried out as described previously [14, 15].

HPAEC-PAD.

Lyase degraded alginates (1-1.5 mg/ml, 25 μl) were analysed on a Dionex BioLC system (Dionex Corp, Sunnyvale, CA) consisting of an AS50 autosampler, an ED40 Electrochemical Detector, a GP50 Gradient Pump and an Ionpac AS4A column as previously reported.[16]

SEC-MALLS

Starting materials and lyase degraded samples fractionated on preparative SEC (1-5 mg/ml, 100-200 μl, 0,2 μm filtered) were analysed on a HPLC system with 50 mM Na_2SO_4/ 10mM EDTA buffer, pH 6. The system consisted of an online degasser, HPLA isocratic pump, autoinjector, pre-column and serially connected Tosoh Biosep TSK 6000, 5000 and 4000 (PWXL) columns. The outlet was connected to a Dawn Helios II multi-angle laser light scattering photometer (Wyatt, USA) followed by an Optilab T-rEX differential refractometer. Data were collected and processed with ASTRA 5.0.[17]

SEC

Fractionation of lyase degraded samples (50-100 mg) by preparative size exclusion chromatography was performed on three serially connected Superdex-30 columns (2.6 x 60 cm) as described previously.[18] The material which eluted in the void volume was pooled and dialysed against MQ-water before freeze drying.

Preparation of Ca-alginate gels

Internally set gel cylinders were made by mixing alginate solutions with a dispersion of $CaCO_3$ particles. The mixture was degassed for 20 minutes, using vacuum suction, before the gel formation process was initiated by addition of a freshly made solution of D-glucono-δ-lactone (GDL). Final concentrations were 1.0% w/v alginate, 15 mM $CaCO_3$ and 30 mM GDL. The blend was immediately transferred to 24-well tissue culture plates (16/18 mm, Costar, Cambridge, MA) and left on a levelled bench for curing at room temperature for 24 hours. Calcium saturation was achieved by immersing the gel cylinders in a solution of 50 mM $CaCl_2$ and 200 mM NaCl (100 ml for each gel) for 24 hours at 4 °C.

Syneresis and gel strength measurements

Syneresis and deformation was determined by weighing the gels and measuring height and diameter with a vernier caliper. Assuming no significant change in density, syneresis was calculated as $S = ((W_0-W)/ W_0) \times 100$ where W_0 and W are the initial and final weights respectively.

Force/deformation curves were recorded at 22 °C on a Stable Micro Systems TA-XT2 texture analyzer equipped with a P/35 probe and a compression rate of 0.1 mm/s. The Youngs modulus ($E = (F/A)/\Delta l/l$) was calculated from the initial slope of the curve (0.1-0.3 mm) using a 5 kg load cell. In order to compare the mechanical strength of gels with different degrees of syneresis, the final alginate concentration was calculated and E was corrected using the semiempirical relationship: $E_{corrected} = E_{measured} \cdot (C_{initial}/C_{final})^2$.[19] Rupture strength and

penetration-to-break was measured in a separate experiment using a 30 kg load cell. All data was collected and processed with the "Texture Expert Exponent 32" software.

RESULTS AND DISCUSSION

In vitro epimerisation of mannuronan.

High molecular weight mannuronan was epimerized with AlgE6, AlgE64 and EM1 for 1-48 hours. These enzymes act on mannuronic acid blocks of all sizes either in a processive or preferred attack mode of action. Characterization of the polymers was performed with ^{1}H-NMR and intrinsic viscosity measurements. From the NMR spectra in Figure 1 and the calculated sequential parameters given in Table S1 in the Supporting Information, it is evident that mannuronan is converted into polymers with a G content and average G-block length ($N_{G>1}$) matching or even surpassing what is found in the high-G alginate extracted from *Laminaria hyperborea* stipes. The fraction of MG-blocks is also slightly lower than in the algal polymer. Viscometry suggests that no significant depolymerisation took place during epimerization (data not shown).
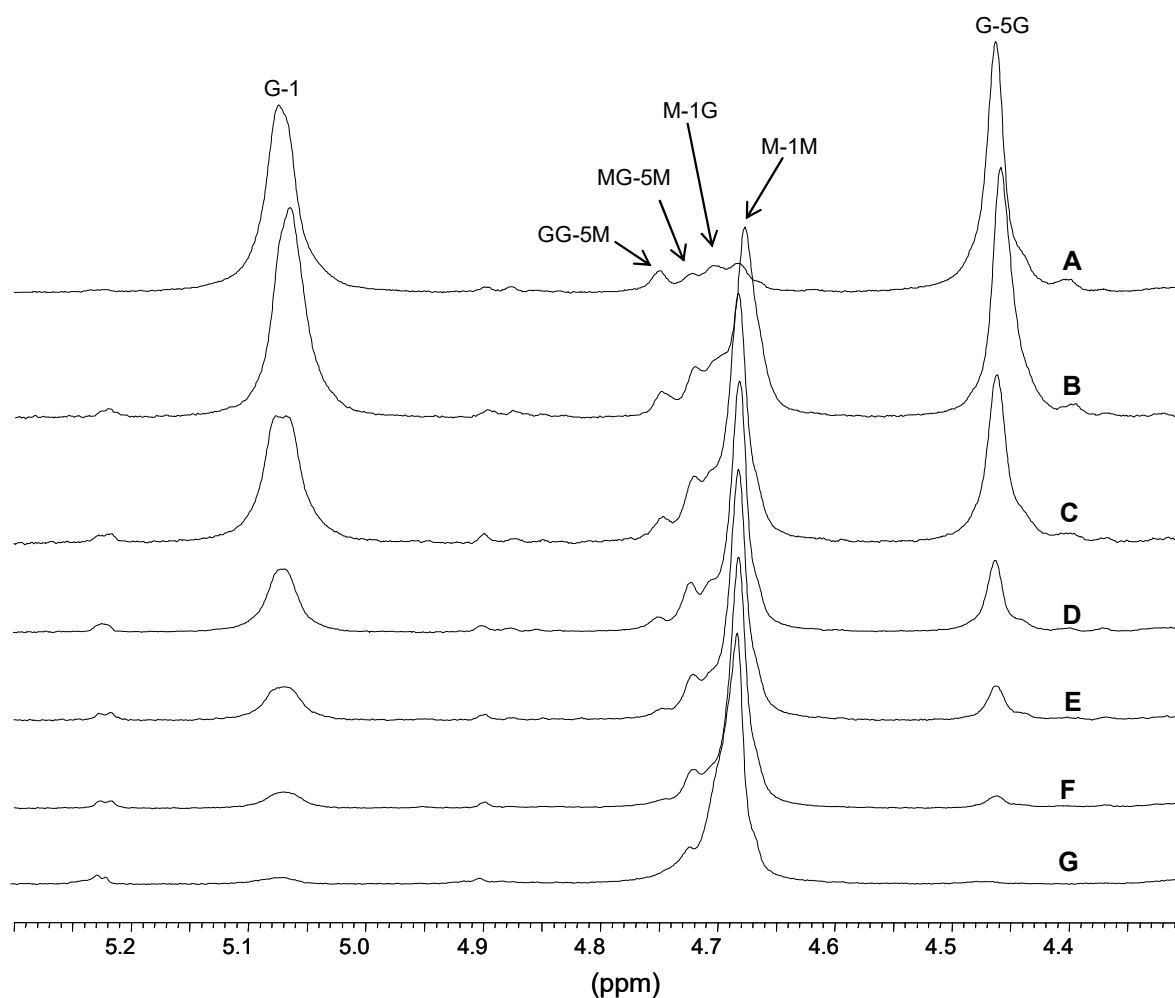
Figure 1. Overlaid ^{1}H-NMR spectra (400MHz) of the anomeric region of mannuronan epimerized with AlgE64 for 1-48 hours. (**A**) $F_G = 0.84$, (**B**) $F_G = 0.58$, (**C**) $F_G = 0.45$, (**D**) $F_G = 0.29$, (**E**) $F_G = 0.18$, (**F**) $F_G = 0.09$ and (**G**) $F_G = 0.04$.

AB-nC denotes the resonance from proton n in uronic acid B with neighbouring uronic acids A and C.

G-block distribution analysis

In order to study the evolution of G-blocks as a function of the degree of epimerization, samples degraded with alginate lyase from *H. tuberculata,* specific for M-M and G-M glycosidic linkages, were analysed by HPAEC-PAD. The chromatograms of the digest of

Poly-M epimerized with AlgE64 are given in Figure 2. Most of the resolution is lost for oligomers with DP>15. This is probably caused by heterogeneous regions with a few

M-residues near the ends that either are too short or do not have the right sequence to be recognized as a substrate by the M-lyase, which results in several oligomers with the same DP but different sequences, each with slightly different retention times. Chain lengths had to be estimated in an indirect manner since long G-oligomers with an unsaturated 4-5 bond on the non reducing end (ΔG- oligomers), introduced after the lyase catalysed β-elimination reaction, were not available. The difference in retention time between short ΔG-oligomers (DP 2-8) and standards of corresponding M, G and ΔM-oligomers was plotted as a function of chain length. Extrapolation of these curves together with retention times of M, G and ΔM oligomers with DP $\leq$ 50 can then be used to calculate approximate chain lengths in figure 2.

The chromatograms provide information on the development of G-blocks, although it is not possible to determine the amount of each oligomer. G-blocks with DP>15 appear even at 4% epimerization and gradually increases up to a DP between 50 and 60 for the sample with 84% G. The bulk of G-blocks are however somewhat shorter. Chromatograms of samples epimerized with AlgE6 and EM1 (data not shown) have a similar chain-length distribution as a function of G-content, which indicates that these epimerases are processive enzymes. The chromatogram of *L. hyperborea* alginate. (Figure 2A) is markedly different from the others as it contains a significant fraction that elutes much later than the G-blocks produced in vitro. This region is outside the "HPAEC-PAD realm" and had to be studied further by NMR and MALLS after partial fractionation on SEC.
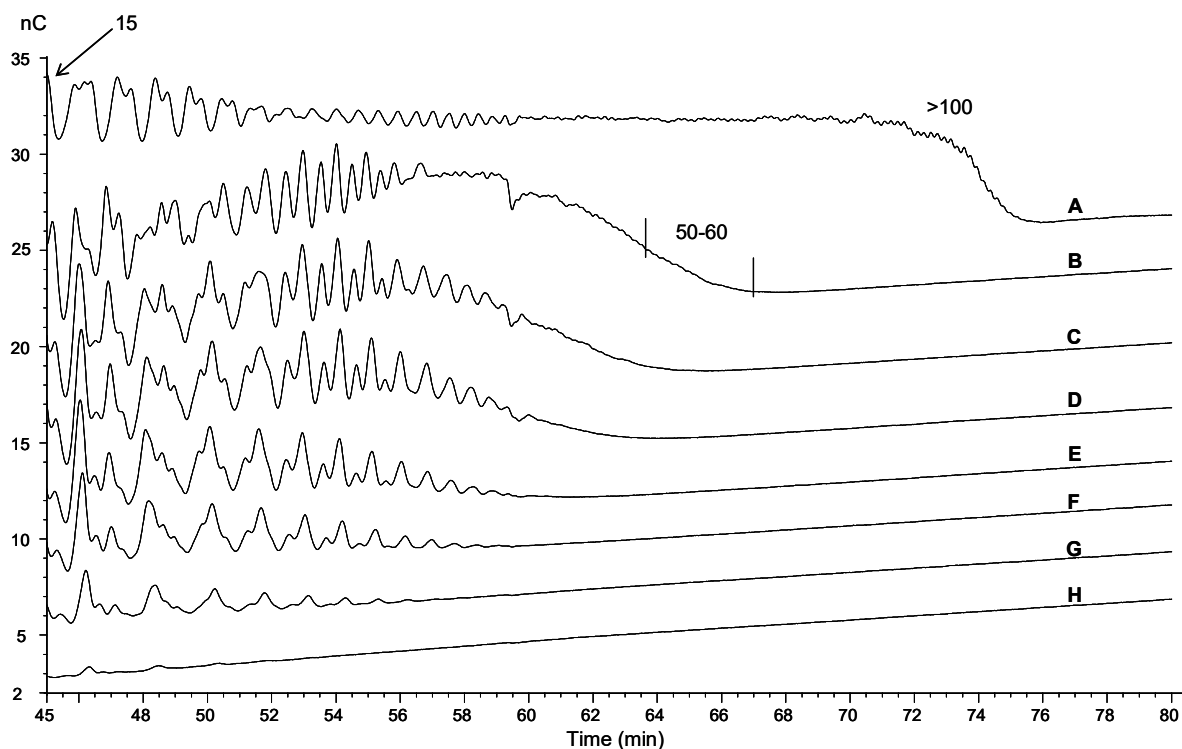
Figure 2. Overlaid sections of HPAEC-PAD chromatograms of alginates degraded with M-lyase (0,016U/mg) for 24 hours at 30 °C. (**A**) *L. hyperborea* F_G=0.67 and mannuronan epimerized with AlgE64; (**B**) F_G = 0.84, (**C**) F_G = 0.58, (**D**) F_G = 0.45, (**E**) F_G = 0.29, (**F**) F_G = 0.18, (**G**) F_G = 0.09 and (**H**) F_G = 0.04. Estimated chain lengths are given in the chromatogram. The lysates were separated on a Dionex IonPac AS4A column with a linear gradient of 8.75 mM sodium acetate/min in 100 mM sodium hydroxide at a flow rate of 1ml/min.

The lyase digests of the highest epimerized samples from each epimerization series were separated on Superdex 30 SEC columns with the purpose of getting more information on purity and chain length for the longest G-blocks. The chromatograms are shown in figure 3. The shortest fractions (DP 2-4) consist mainly of ΔM oligomers with minor amounts of ΔG and ΔGMG, as confirmed by HPAEC-PAD (data not shown), which decreases with

increasing G-content as expected. The G-block distribution in epimerized samples is very similar, despite differences in G content up to 16%. Most of the G-blocks have a DP between 20 and 50, with a maximum around DP 35.

A small shoulder in the beginning of the chromatogram is seen for both the AlgE64 and EM1 epimerized samples. These peaks are too large as to be explained by protein residues from the epimerization. A slow condensation of intermediate long G-blocks which result in G-blocks comparable in size with those found in algal alginate could explain an abrupt change in retention time, although the amount is too small to be confirmed by HPAEC-PAD.

The most notable difference between chromatograms of *L. hyperborea* alginate and epimerized samples is a more narrow and pronounced void fraction in the former. Although the asymmetric peak indicates overload and a deviation from the actual distribution, the peak width at half height is very reproducible. It should be noted that it is important to inject approximately the same amount of sample when comparing block distributions. An increase in concentration will cause some peak widening, although the slightly larger void fractions (up to 37% in the region 380-550 minutes) in epimerized samples does not account for the large difference in peak width.
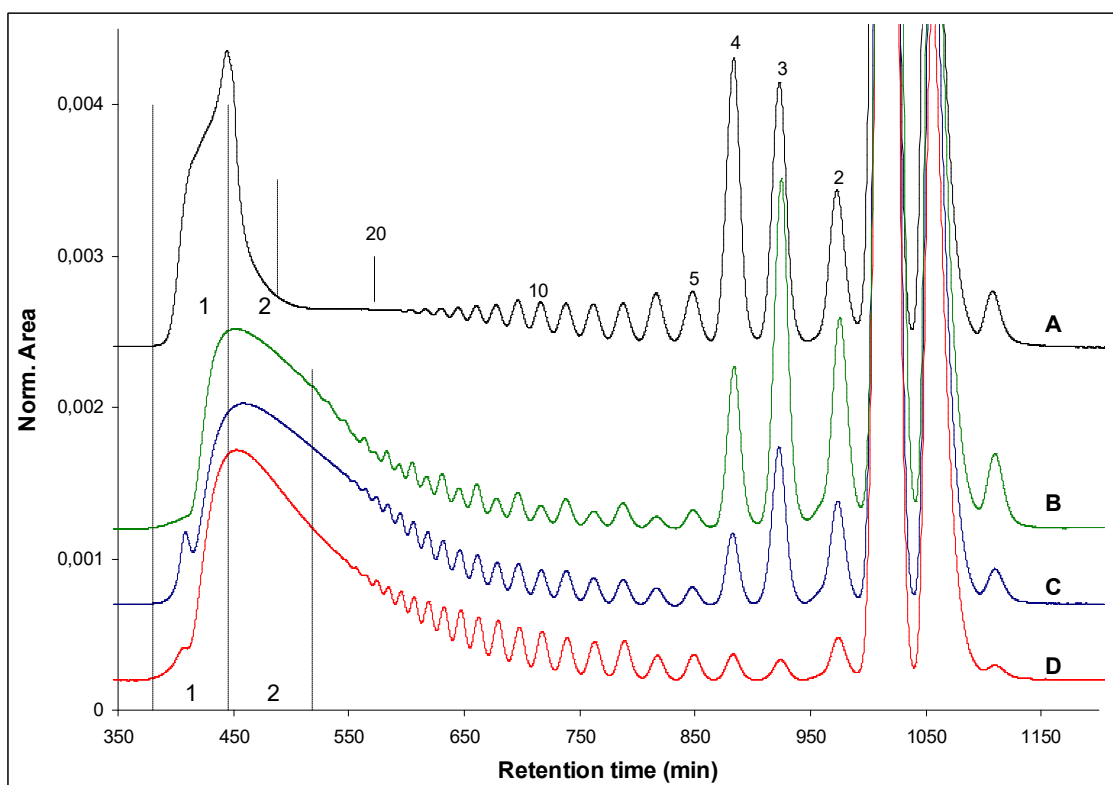
Figure 3. SEC chromatograms of alginates (50mg, 10mg/ml) degraded with M-lyase (0,016U/mg) for 24 hours at 30 °C. (**A**) *L. hyperborea*, and mannuronan epimerized with: (**B**), AlgE6, F_G = 0.68 (**C**) EM1, F_G = 0.77 and (**D**) AlgE64, F_G = 0.84. The lysates were separated on three Superdex 30 columns serially connected using 0.1 M NH$_4$Ac as mobile phase and a flow rate of 0.8 ml/min. All chromatograms were baseline corrected and normalized in order to adjust for differences in injection volumes. Collected void fractions are indicated with dotted lines.

Void fractions were collected as indicated in figure 3 and analysed with ^{1}H-NMR and SEC-MALLS. Chain length and purity is given in Table S2 in the Supporting information and confirms that *L. hyperborea* alginate indeed contains a fraction of G-blocks much longer than what can be made by in vitro epimerization of mannuronan. Furthermore the differences in block lengths between epimerized samples are relatively small. Void fraction 2 was collected over a larger interval for the epimerized samples in order to cover more of the unresolved part

of these chromatograms, so only Void 1 can be directly compared. It can be difficult to achieve precise measurements of DP_n for long oligomers as ^{1}H-NMR signals from the unsaturated non-reducing end (Δ-4G and Δ-4M) are small, and at the same time the M_w region (~6-30 kD) approaches the lower limit regarding both separation and detection on SEC-MALLS.

Even though the trends are the same for both methods, the discrepancy is rather large in some cases. Some aggregates were observed in all samples. This will contribute much to the M_w determined from MALLS if included in the calculation, but has a much smaller influence on NMR data.

The purity is higher than 93% in all cases and in addition is probably underestimated due to a low signal/noise ratio. It is not possible to exclude the presence of a few internal M residues, but the fact that F_G is lower for the shorter void fractions suggests that M residues are situated close to the ends as mentioned earlier. The relative contribution to F_G will then be higher for the shorter void fractions.

Analysis of gels made from epimerized materials and the effect of long G-blocks

The most obvious structural difference between the native algal alginate and the highest epimerized mannuronan seems to be the fraction of extremely long G-blocks in the former. We have previously suggested that such long G-blocks might function as reinforcement bars in the ionic gel network [5]. To test this hypothesis, we studied how addition of long G-blocks into in vitro epimerized alginates affected gel properties. G-blocks (240mg, G> 98%, DP_n >100) were obtained by collecting void fractions of dialysed *L. hyperborea* alginate lysate from 22 consecutive SEC separations, each with a yield of about 20 %. The highly reproducible separations ($\Delta t_{w0.5}$ < 7%) shown in Figure 4 allows a systematic study on how

gel properties are affected by addition of G-blocks of varying length, although only one fraction was collected in this study.
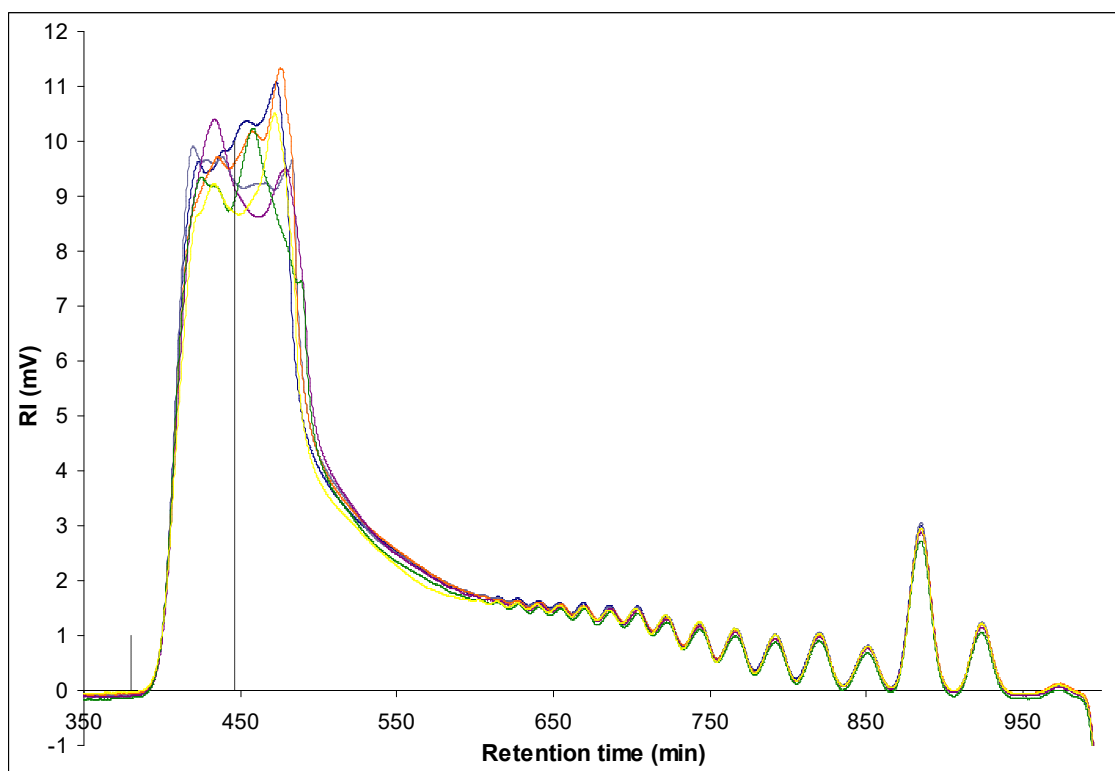

Figure 4. Retention time reproducibility. Overlaid chromatograms of 6 consecutive SEC separations of G-blocks from *L. hyperborea* lysate (50 mg, 10 mg/ml). The conditions were the same as in figure 3, except that the lysate was dialysed in order to get a higher yield of G-blocks. The G-blocks were collected as indicated in the chromatogram.

The gels compared in this study were made from *L. hyperborea* alginate (F_G=0.67) and mannuronan epimerized with AlgE6 (F_G = 0.51 and 0.68). In order to study the effect of long G-blocks on gel properties, a gel was also made from a solution of Poly-M epimerized with AlgE6 (F_G = 0.51) with added G-blocks as to give a total F_G = 0.67. The final alginate concentration was 1.0 % w/v in all cases.

Macroscopic properties of saturated Ca-alginate gel cylinders were measured in terms of syneresis, Youngs modulus, compressibility and rupture strength (Figure 5 A-D).
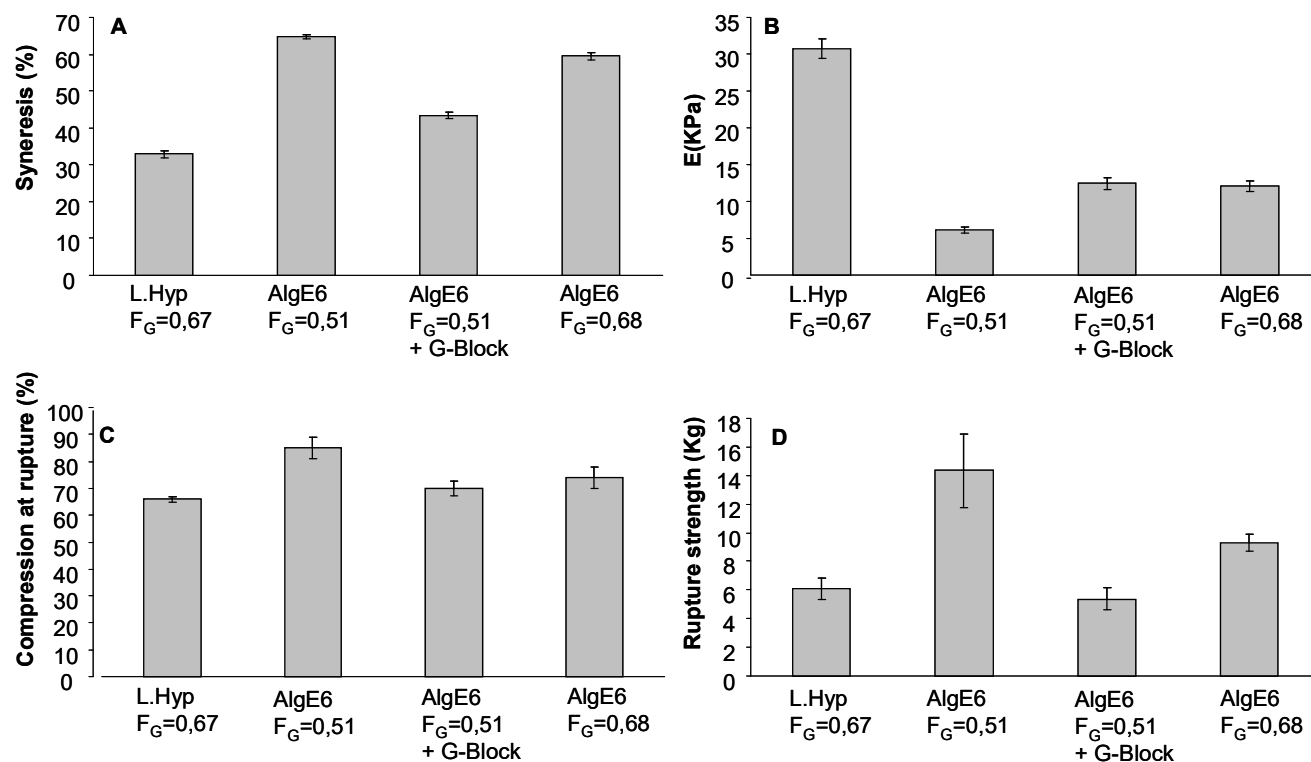

Figure 5. Characterization of alginate gels.(**A**) Syneresis, (**B**) Youngs moduls, (**C**) compression at rupture and (**D**) Rupture strength. Values are means of 12 gels +/- StDev for A and B, and means of 4 gels for C and D.

The gels made from mannuronan epimerized with AlgE6 both displayed a much higher degree of syneresis than what was found in the algal alginate (60 - 65% syneresis vs. 33% respectively) (Figure 5A). The addition of long G-blocks to the alginate consisting of M- and intermediate long G-blocks reduced the syneresis to a level comparable with the natural alginate (43% syneresis). Alginate from *L. hyperborea* leaf with $F_G = 0.50$ has been reported earlier with 44% syneresis.[20] Hence, it seems clear that very long G-blocks, found in a variety of natural alginates [5] has a profound impact on equilibrium properties of alginate gels since

the lack of these results in highly syneretic Ca-alginate gels and addition of G-blocks caused a strong decrease in syneresis.

The high syneresis in the gels of epimerised alginate was not expected since these alginates are almost devoid of MG sequences. It is generally agreed that syneresis is correlated to the amount and length of MG-blocks. Syneresis of 45 – 70% are reported for saturated Ca-gels of MG- and G-blocks, the latter corresponding to the lowest content of G-blocks.[20] Also, several studies have shown that introduction and elongation of MG sequences in alginates from brown algae by in vitro epimerization with AlgE4 leads to increased syneresis.[21,22] This is thought to be a consequence of increased elastic segment flexibility[23] and formation of secondary MG/MG junctions through a calcium dependent zipping mechanism.[24]

There was a big difference in the syneresis in Ca-limited gels and Ca-saturated gels in this study. No syneresis could be observed in the Ca-limited gels (data not shown) hence the syneresis was due to increased crosslinking following increased calcium concentrations. This is in discrepancy with Ca-alginate gels made of MG-and G-blocks where syneresis is observed also under Ca-limited conditions.[20] A more extensive study of syneresis as a function of fractional calcium saturation would be needed in order to try to explain the observed syneresis in gels made from AlgE6 epimerized alginates on a molecular basis.

Another atypical feature of the gels made from epimerized mannuronan was their low Youngs moduli (Figure 5B) relative to the high content of G. Despite a higher $N_{G>1}$, generally used to describe the length of the G-blocks in the alginate, than *L. hyperborea* alginate (Table 1), the Youngs modulus was reduced by a factor of 2.5 and 5 for gels made from epimerized material with $F_G = 0.68$ and 0.51, respectively. To our knowledge, this is the first example of

an alginate where the positive relationship between rigidity and $N_{G>1}$ is not obeyed. Since the epimerized alginates also have a higher M_w than *L. hyperborea* alginate, the number of loose ends cannot explain this discrepancy, and it seems like the effect is solely attributed to the lack of long G-blocks. This is at present the strongest support for our reinforcement bar hypothesis.

Although the Youngs modulus was doubled when long G-blocks were added to AlgE6 (F_G = 0.51) as to give a final F_G = 0.67, it was not significantly larger than the gels made of AlgE6 epimerized mannuronan (F_G = 0.68). An average junction zone functionality, $f \geq 3$ is required in order to form a continuous network, and this condition is perhaps not satisfied for most of the long G-blocks. The observed increase in Youngs modulus may therefore be caused by lateral association of long G-blocks to primary junction zones, in a similar manner to what has been reported for shorter G oligomers.[25]

The effect of the syneresis on the distribution of alginate in the gel cylinders and the subsequent effect on Youngs modulus has not been elucidated. The gels were made to be homogenous with a constant distribution of alginate throughout the gel, however previous results from AlgE4/AlgE6 epimerised materials may indicate that increased syneresis results in more inhomogeneous gels [26]. Fluorescent labelling of the alginate and subsequent analyses by confocal microscopy could reveal the distribution of alginate in the gel cylinders.[27]

Further compression of the Ca-alginate gels showed that the alginates containing intermediate long G-blocks could be compressed to a higher extent before rupture than the natural alginate, shown as both elevated deformation and force at rupture (Figure 5C and D). Indeed, the enzymatically modified alginates could withhold about 50 to 150 % higher compression force before rupture than the natural alginate. As for the syneresis and Youngs modulus, we were again surprised by the behaviour of the epimerised alginates displaying

now high degree of elasticity despite the high G content, and even more, the high $N_{G>1}$. The brittleness previously positively correlated to F_G[20,28] seems to more correctly depend on the content of the very long G-blocks in the alginate. Indeed, the M- and G-block alginates could withhold 9 – 15 kg compression in this study (Figure 5D that have previously only been exceeded by poly-MG alginate.[20] One limitation with the rupture measurements is the change of the gel shape upon compression that is not taken into account. The change in gel shape (e.g. an increase in surface contacting the probe and the table) will result in reduced effective stress (F/A). Again, the addition of long G-blocks to the enzymatically modified alginate resulted in Ca-gels with similar properties to the natural alginate. The addition of long and rigid G-blocks acting as reinforcement bars seen as increase in Youngs modulus, will not contribute to the elastic properties of the gel. Indirectly, they probably reduce the elastic properties by a shortening and/or reduced number of elastic regions between the crosslinks and by this reduce the ability of the chain to extend upon deformation of the gel. This leads to breakage of the gel at lower compression upon addition of the G-blocks, generally recognized as the formation of a more brittle gel, and can also explain the reduction in syneresis upon the addition of long G-blocks (Figure 5A).

CONCLUDING REMARKS

Analysis of the G-block distribution of *in vitro* epimerized mannuronan has shown that the molecules can be converted to more than 85% with a broad distribution of block length ranging up to approximately 60 consecutive G residues. Even in samples with the highest conversion, the longest homopolymeric G-blocks are still much shorter than what we find in alginate from algae. We have shown earlier[18] that the G-block forming AlgE epimerases

effectively introduce G-blocks into poly-MG and are thus able to convert an M-residue flanked by G. It might still be that the condensation of G-blocks by epimerizing an M residue flanked by long G sequences i.e. (-GGGGG-M-GGGGG- → -GGGGG-G-GGGGG-) is a very slow process. Since the binding cleft in even the smallest enzyme AlgE64, comprising one A and one R module, can accommodate more than 10 sugar residues[29,30], low subsite specificities for G residues could explain the low condensation rate. Moreover an increase in G content could result in aggregation or pre-gelling of alginate both depleting the calcium level necessary for enzyme activity or render the substrate less accessible for the enzyme.

Alginate contributes to strength and flexibility of algal tissue and acts as a re-swelling protective barrier in metabolically dormant *Azotobacter* cysts. Whether the difference in G-block length between algae and epimerised samples reflects the different role of alginate in brown algae and bacteria is an open question. The information about alginate turnover and properties of the epimerases in brown algae is limited. Brown algae apparently have a number of genes coding for epimerases[31], but no single enzyme has yet been expressed and characterized. It is however established that the G-content increase associated with the age of algal tissue, suggests that long G-blocks are synthesized over an extended period of time.[32]

We have previously shown that upgrading of low gelling alginates into material that forms mechanically strong gels can be achieved by treating algal alginate either with AlgE4, which converts the remaining MM block into MGM sequences or by AlgE1 and AlgE6, which elongate the existing G-blocks.[20,21] The alginates epimerised with AlgE4 were more syneretic than the natural alginate whereas the alginates epimerised with AlgE1 or AlgE6 were less syneretic than the respective natural alginate.

In the present paper where homopolymeric mannuronan was used as a substrate for AlgE6, the resulting material forms calcium gels that are much more syneretic and compressible than gels made from algal alginate. This difference is probably due to the lack of the fraction of

long G-blocks in the epimerized samples. As AlgE6 was used for increasing the G-blocks in both mannuronan (this study) and the natural alginates[20], this may indicate an ability of AlgE6 to prolong G-blocks in natural alginates in contrast to when mannuronan is used as substrate. Further block analyses of these materials may reveal potential differences in the enzymatic product depending on the substrate. Moreover, differences in gel properties between in vitro epimerized and seaweed alginates with comparable average block length ($N_G>1$) and G content (F_G), demonstrates that there are functional features of alginate gels which cannot be explained solely on the basis of triad frequencies obtained from NMR.

Supporting information

S1: Molecular weight and sequence parameters in alginates used in this study.

S2: Chain length and purity of G-blocks in alginate from *L. hyperborea* stipe and *in vitro* epimerized mannuronan.

This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

(1)   Smidsrød, O., *Acta Chem Scand* **1972,** 26, (1), 79-88.

(2)   Skjåk-Bræk, G.; Smidsrød, O.; Larsen, B., *Int J Biol Macromol* **1986,** 8, (6), 330-336.

(3)   Haug, A.; Larsen, B., *Carbohyd Res* **1971,** 17, (2), 297-308.

(4)   Ertesvåg, H.; Høidal, H. K.; Hals, I. K.; Rian, A.; Doseth, B.; Valla, S., *Mol Microbiol* **1995,** 16, 719-731.

(5)   Aarstad, O. A.; Tøndervik, A.; Sletta, H.; Skjåk-Bræk, G., *Biomacromolecules* **2012,** 13, (1), 106-116.

(6)   Svanem, B. I. G.; Skjåk-Bræk, G.; Ertesvåg, H.; Valla, S., *J Bacteriol* **1999,** 181, 68-77.

(7)   Høidal, H. K.; Ertesvåg, H.; Skjåk-Bræk, G.; Stokke, B. T.; Valla, S., *J Biol Chem* **1999,** 274, 12316-12322.

(8)   Ramstad, M. V.; Ellingsen, T. E.; Josefsen, K. D.; Høidal, H. K.; Valla, S.; Skjåk-Bræk, G.; Levine, D. W., *Enzyme Microb Tech* **1999,** 24, (10), 636-646.

(9)   Ertesvåg, H.; Høidal, H. K.; Schjerven, H.; Glærum Svanem, B. I.; Valla, S., *Metab Eng.* **1999,** 1, 262-269.

(10)   Gimmestad, M.; Sletta, H.; Ertesvåg, H.; Bakkevig, K.; Jain, S.; Suh, S.; Skjåk-Bræk, G.; Ellingsen, T. E.; Ohman, D. E.; Valla, S., *J Bacteriol* **2003,** 185, (12), 3515-3523.

(11)   Ertesvåg, H.; Skjåk-Bræk, G., Modification of alginate using Mannuronan C-5-epimerases. In *Methods in Biotechnology*, Bucke, C., Ed. Carbohydrate Biotechnology Protocols: 1999; Vol. 10, pp 71-78.

(12)   Tøndervik, A.; Klinkenberg, G.; Aachmann, F. L.; Svanem, B. I. G.; Ellingsen, T. E.; Valla, S.; Skjåk-Bræk, G.; Sletta, H., **2012**.

(13)   Boyen, C.; Kloareg, B.; Polnefuller, M.; Gibor, A., *Phycologia* **1990,** 29, (2), 173-181.

(14)   Grasdalen, H., *Carbohyd Res* **1983,** 118, (Jul), 255-260.

(15)   Campa, C.; Holtan, S.; Nilsen, N.; Bjerkan, T. M.; Stokke, B. T.; Skjåk-Bræk, G., *Biochem J* **2004,** 381, (1), 155-164.

(16)   Tøndervik, A.; Klinkenberg, G.; Aarstad, O. A.; Drabløs, F.; Ertesvåg, H.; Ellingsen, T. E.; Skjåk-Bræk, G.; Valla, S.; Sletta, H., *J Biol Chem* **2010,** 285, (46), 35284-35292.

(17)   Vold, I. M. N.; Kristiansen, K. A.; Christensen, B. E., *Biomacromolecules* **2006,** 7, 2136-2146.

(18)   Holtan, S.; Bruheim, P.; Skjåk-Bræk, G., *Biochem J* **2006,** 395, 319-329.

(19)   Martinsen, A.; Skjåk-Bræk, G.; Smidsrød, O., *Biotechnol Bioeng* **1989,** 33, (1), 79-89.

(20)   Mørch, Y. A.; Holtan, S.; Donati, I.; Strand, B. L.; Skjåk-Bræk, G., *Biomacromolecules* **2008,** 9, (9), 2360-2368.

(21)   Draget, K. I.; Strand, B.; Hartmann, M.; Valla, S.; Smidsrød, O.; Skjåk-Bræk, G., *Int J Biol Macromol* **2000,** 27, (2), 117-122.

(22)   Strand, B. L.; Mørch, Y. A.; Syvertsen, K. R.; Espevik, T.; Skjåk-Bræk, G., *Journal of Biomedical Materials Research, Part A* **2003,** 64A, (3), 540-550.

(23)   Draget, K. I.; Gåserød, O.; Aune, I.; Andersen, P. O.; Storbakken, B.; Stokke, B. T.; Smidsrød, O., *Food Hydrocolloid* **2001,** 15, (4-6), 485-490.

(24)	Donati, I.; Holtan, S.; Mørch, Y. A.; Borgogna, M.; Dentini, M.; Skjåk-Bræk, G., *Biomacromolecules* **2005,** 6, (2), 1031-1040.

(25)	Jørgensen, T. E.; Sletmoen, M.; Draget, K. I.; Stokke, B. T., *Biomacromolecules* **2007,** 8, 2388-2397.

(26)	Mørch, Y. A.; Donati, I.; Strand, B. L.; Skjåk-Bræk, G., *Biomacromolecules* **2007,** 8, (9), 2809-2814.

(27)	Strand, B. L.; Morch, Y. A.; Espevik, T.; Skjak-Braek, G., *Biotechnol. Bioeng.* **2003,** 82, (4), 386-394.

(28)	Donati, I.; Mørch, Y. A.; Strand, B. L.; Skjåk-Bræk, G.; Paoletti, S., *J. Phys. Chem. B* **2009,** 113, (39), 12916-12922.

(29)	Rozeboom, H. J.; Bjerkan, T. M.; Kalk, K. H.; Ertesvåg, H.; Holtan, S.; Aachmann, F. L.; Valla, S.; Dijkstra, B. W., *The Journal of Biological Chemistry* **2008,** 283, (35), 23819–23828.

(30)	Aachmann, F. L.; Glærum Svanem, B. I.; Valla, S.; Petersen, S. B.; Wimmer, R., *J Biomol Nmr* **2005,** 31, 259-260.

(31)	Nyvall, P.; Corre, E.; Boisset, C.; Barbeyron, T.; Rousvoal, S.; Scornet, D.; Kloareg, B.; Boyen, C., *Plant Physiol.* **2003,** 133, (2), 726-735.

(32)	Haug, A.; Larsen, B.; Baardseth, E. In *Comparison of the constitution of alginates from different sources.*, Proc. Int. Seaweed Symp., 6th 1968, Margalef, R., Ed. Direction General de Pesca Maritima: Madrid 1969; pp 443-451.

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Olav Aarstad, Berit Løkensgard Strand, Lise Mari Klepp-Andersen and Gudmund Skjåk-Bræk

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