Mechanical Properties of C-5 Epimerized Alginates

ARTICLE *in* BIOMACROMOLECULES · OCTOBER 2008

Impact Factor: 5.75 · DOI: 10.1021/bm8003572 · Source: PubMed

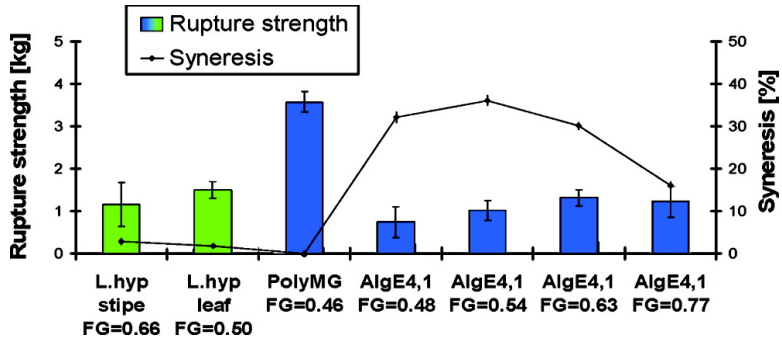
BioMACROMOLECULES

Article

Mechanical Properties of C-5 Epimerized Alginates

Y#. A. Mørch, S. Holtan, I. Donati, B. L. Strand, and G. Skjåk-Bræk

Biomacromolecules, **2008**, 9 (9), 2360-2368 • DOI: 10.1021/bm8003572 • Publication Date (Web): 15 August 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Biomacromolecules is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Mechanical Properties of C-5 Epimerized Alginates

Ý. A. Mørch,*,† S. Holtan,† I. Donati,‡ B. L. Strand,† and G. Skjåk-Bræk†

Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway, and Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Trieste, Italy

Received April 4, 2008; Revised Manuscript Received June 2, 2008

There is an increased need for alginate materials with both enhanced and controllable mechanical properties in the fields of food, pharmaceutical and specialty applications. In the present work, well-characterized algal polymers and mannuronan were enzymatically modified using C-5 epimerases converting mannuronic acid residues to guluronic acid in the polymer chain. Composition and sequential structure of controls and epimerized alginates were analyzed by ^{1}H NMR spectroscopy. Mechanical properties of Ca-alginate gels were further examined giving Young's modulus, syneresis, rupture strength, and elasticity of the gels. Both mechanical strength and elasticity of hydrogels could be improved and manipulated by epimerization. In particular, alternating sequences were found to play an important role for the final mechanical properties of alginate gels, and interestingly, a pure polyalternating sample resulted in gels with extremely high syneresis and rupture strength. In conclusion, enzymatic modification was shown to be a valuable tool in modifying the mechanical properties of alginates in a highly specific manner.

Introduction

Alginate is a family of polysaccharides found mainly in marine brown algae, but is also produced by certain bacteria like *Azotobacter vinelandii* and some *Pseudomonas* species.[1] It is a linear copolymer of 1,4-linked β-D-mannuronic acid (M) and its 5-epimere α-L-guluronic acid (G), where the monomers are arranged along the polymer chain as homopolymeric blocks of M, G, or MG. The exact composition and distribution of the blocks depends on the source from which the alginate has been isolated. In the presence of divalent cations like Ca^{2+}, Ba^{2+}, and Sr^{2+} alginates form stable hydrogels due to formation of junction zones involving several polysaccharide chains. This complexation with 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.[2,3] At high concentrations of divalent ions, the number of chain segments forming a junction zone can exceed two by lateral association.[4] As the G units in general have higher affinity for divalent ions than their M counterpart and due to the cooperative binding of ions to G-blocks,[5] the mechanical properties of alginate gels are highly dependent on the type and length of blocks as well as their overall distribution in the polysaccharide chains. In addition, the flexibility of the three types of blocks in alginate has been shown to vary, increasing in the order GG blocks < MM blocks < MG blocks.[6] Finally, the molecular weight and concentration of polymer will also be of importance for the mechanical properties of the gel.

As alginates have found broad application in a number of fields within both industry and medicine, the ability to control the various mechanical properties of these materials, including mechanical strength and stiffness, has been highly desired.[7] Various methods have been employed to control the mechanical properties of alginates, including the addition of short G-blocks,[8,9] chemical modification,[10] or careful choice of the alginate source.[7] As alginates rich in guluronic acid are generally considered to form rigid and mechanically strong gels,[11] these alginates are often chosen in systems that require high mechanical strength. Hence, there is a steadily growing demand for G-rich alginates both in the traditional applications of reformed food and pharmaceuticals and in a range of new applications. However, alginates from algae are heterogeneous with regard to composition and the combination of high gel strength with highly elastic consistency is hard to find among these alginates, as increased gel strength often is followed by increased brittleness.[7,12] Furthermore, the industrial demand for strong gelling alginates is higher than what can be produced from the natural sources of *Laminaria hyperborea* stipes. In recent years, much knowledge has been gained about the mannuronan C-5 epimerases, which convert mannuronic acid to guluronic acid in a postpolymerization step. A total of seven epimerases, termed AlgE1-AlgE7 have been isolated from *A. vinelandii* and cloned and expressed in *E. coli*.[13,14] The epimerases possess differences in their epimerization pattern, varying from the formation of contiguous G-blocks (...GGGGGG...) to elements of strictly alternating sequences (...MGMGMG...). Recently, mannuronan devoid of any G-residues has become available from C5-epimerase negative mutants of *Pseudomonas fluorescens*.[15,16] Hence, novel alginates may be produced by in vitro epimerization of mannuronan or algal alginates by one or several of the seven epimerases.

Treatment of alginates with mannuronan C-5 epimerases, thereby increasing the content and sequential arrangements of guluronic acid, allows for the alteration of both the flexibility of the chains and the ion-binding properties of the polymers. More specifically, by making alginates containing the Ca-binding GG and MG blocks solely, we have recently shown that we can control industrially important properties of alginate gels such as the mechanical strength, resistance to nongelling ions, syneresis, and porosity.[17] The direct involvement of long MG blocks in the gel network accounting for the syneresis of alginate gels upon calcium saturation has been proposed[18] and new methods allowing the determination of the real block

* To whom correspondence should be addressed. Tel.: (+47)73591689. Fax: (+47)73591283. E-mail: yrr.morch@biotech.ntnu.no.
† Norwegian University of Science and Technology.
‡ University of Trieste.

10.1021/bm8003572 CCC: $40.75
Published on Web 08/15/2008

lengths in alginate[19] adds knowledge to the relation between physical properties and molecular structure in these novel materials.

In the present study, we aimed at investigating the mechanical properties of these new materials and of enzymatically modified algal alginates. In particular, this is a further investigation of the role of alternating sequences on the mechanical properties of gels.

Materials and Methods

Materials. Sodium alginate samples from *Laminaria hyperborea* leaf and stipe and *Durvillea potatorum* were provided by FMC Biopolymer (Norway). High molecular mass mannuronan ($F_G < 0.001$) was produced by an epimerase-negative mutant (AlgG$^-$) of *Pseudomonas fluorescens*.[15] The recombinant mannuronan C-5 epimerases AlgE1, AlgE4, and AlgE6 were obtained as reported by Holtan et al.[19] D-Glucono-δ-lactone (GDL) was purchased from Sigma Chemical Co. (U.S.A.). Calcium carbonate (average particular size 4 μm) was purchased from Merck (Germany).

C-5 Epimerization. Alginates were dissolved in deionized (MQ) water overnight before concentrated stock solution of MOPS (3-[*N*-morpholino]-propanesulfonic acid) buffer, pH 6.9 with $CaCl_2$ and NaCl was added and the mixtures preheated at 37 °C. The respective enzymes were dissolved in MQ water and immediately added to the alginate solutions. Final concentrations of the reaction mixtures were 0.25% (w/v) alginate, 50 mM MOPS, 0.8 mM (AlgE1) or 2.5 mM $CaCl_2$ (AlgE6 and AlgE4), and 10 mM (AlgE4), 20 mM (AlgE1), or 75 mM NaCl (AlgE6). The G content in the final product was controlled by epimerization time and enzyme concentration. The mixtures were kept at 37 °C with constant stirring during epimerization. Reactions were terminated by removal of calcium with EDTA to 4 mM. The samples were purified by dialysis against 0.05 M NaCl and deionized water at 4 °C, and pH neutralized before freeze drying.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were recorded on a Bruker Advance DPX 300 spectrometer at 90 °C. Sample concentrations of 10 mg/mL (1.0% w/v) in deuterium oxide (^2H_2O) were used and the chemical shift was calculated with respect to 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP, Aldrich, U.S.A.) as the internal standard. Peaks were assigned according to Grasdalen.[20]

To reduce the viscosity of the high molecular mass polymers for NMR analysis, the samples were degraded by mild acid hydrolysis to a final DP_n of approximately 35. Aqueous solutions of 0.5 mg/mL alginate (0.05% (w/v)) at pH 5.6 were kept for 60 min in a water bath at 95 °C, before the pH was adjusted to 3.8, and the solutions kept for 40 min at 95 °C.

Preparation of Ca-Alginate Gel Cylinders and Gel Strength Measurements. Internally set, homogeneous Ca-alginate gels were made by the $CaCO_3$/D-glucono-δ-lactone (GDL) method[21] in 24-well tissue culture plates (16/18 mm, Costar, Cambridge, MA). Final concentrations were 1.0% (w/v) alginate, 15 mM $CaCO_3$, and 30 mM GDL. After allowing 20 h for the gels to set, they were removed from the wells and immersed in 50 mM $CaCl_2$ (in 0.2 M NaCl) solution for 48 h at 4 °C to saturate G binding sites with Ca^{2+}. Syneresis was determined as the weight reduction of the calcium-alginate gels with respect to the initial weight, calculated assuming a density value of 1. Gels were exposed to uniaxial compression to the point of rupture using a Stable Micro Systems TA-XT2 texture analyzer at 22 ± 1 °C with a compression rate of 0.1 mm/s. Young's modulus (E) was calculated from the initial slope of the force/deformation curve ($F/A = E \times \Delta l/l$).[22] For all gels exhibiting syneresis, the final alginate concentration was determined and E corrected adapting $E \propto c^2$.[11] For some gels, compression measurements were performed before soaking in 50 mM $CaCl_2$ solution.

Results

^{1}H NMR Analysis of Epimerized Alginates. Two native algal alginates and mannuronan were epimerized with the C5-epimerases AlgE1, AlgE4, and AlgE6. To be able to compare the epimerized samples, the goal was to achieve a similar M/G ratio using the different epimerases. Table 1 shows the chemical composition and sequence of the alginates analyzed by ^{1}H NMR. For the *Durvillea potatorum* alginate an increase in G content from 32 to approximately 60% was achieved, whereas for *Laminaria hyperborea* leaf alginate, an increase from 49 to approximately 70% G was obtained by epimerization with all three epimerases. It is further evident that AlgE1 and AlgE6 introduced guluronate as G blocks, while AlgE4 increased the guluronate content by introducing single G residues. In addition, the ratio F_{MGM}/F_{GGM}, which provides an evaluation of the length of alternating sequences,[23] was increased compared to controls upon AlgE4 epimerization. An increase in G blocks, F_{GGG}, was also observed for *L. hyp.* leaf and *D. pot.* samples upon AlgE4 epimerization.

Epimerization of mannuronan with the G block forming epimerases AlgE1 and AlgE6 resulted in samples of 80 and 88% G, respectively. However, the average length of G blocks ($N_{G>1}$) was greater for the AlgE1 treated sample. In addition, AlgE1 introduced single G residues to a larger extent than AlgE6.

Due to the processive mode of action of AlgE4,[24] introducing exclusively single G residues on a mannuronan backbone, only 50% of the mannuronic acid residues are available for epimerization. For the polyalternating (polyMG) sample, a maximum molar fraction of guluronic acid of 0.46 was obtained as the residual M blocks (4%) are not accessible to epimerization by being located at the end of the polymer chains.[24]

Alginates composed of alternating sequence and G blocks only were made by using a polyalternating sample as substrate for AlgE1 and AlgE6. By controlling the enzyme concentration as well as the epimerization time, the amount of guluronic acid introduced was controlled ending up with alginates of various G content. As seen from Table 1, the approximately same M/G ratio was achieved for both AlgE1 and AlgE6 epimerized polyMG samples (approximately 55, 65, and 75% G), making it possible to compare the functional properties of the alginate products directly. In addition, an alginate with 48% G composed of nearly pure polyalternating sequences interspersed with a very low amount of G blocks was made using AlgE1 on a polyalternating backbone. In contrast to the epimerized mannuronan samples, when having polyMG as substrate AlgE1 and AlgE6 gave very similar end products with respect to average G-block length.

Mechanical Properties of Ca-Alginate Gels. To study the mechanical properties of native and epimerized samples, homogeneous calcium-alginate gel cylinders were obtained by internal gelling with calcium carbonate before soaking in concentrated $CaCl_2$ solution to saturate all G binding sites with Ca^{2+}. Uniaxial compression measurements were performed on gel cylinders of native and epimerized alginate and the syneresis measured as percentage shrinkage upon gelation.

Young's Modulus and Syneresis of Epimerized Algal Polymers. Algal alginates from *Durvillea potatorum* ($F_G = 0.34$) and *Laminaria hyperbora* leaf ($F_G = 0.50$) were epimerized with one of the three epimerases AlgE1, AlgE4, and AlgE6 and Ca-saturated alginate gel cylinders prepared. Figure 1 and Table 2 give the Young's moduli and syneresis, respectively, of the resulting gels. A high-G alginate from the stipe of *Laminaria hyperborea* is included for comparison.

Table 1. Chemical Composition and Sequence for Alginate Samples Used in the Study as Measured by ^{1}H NMR[a]

sample	F_G	F_M	F_{GG}	$F_{GM,MG}$	F_{MM}	$F_{GGM,MGG}$	F_{MGM}	F_{GGG}	$N_{(G>1)}$	F_{MGM}/F_{GGM}	[η] mL/g
Controls											
L. hyp. stipe	0.66	0.34	0.55	0.12	0.22	0.05	0.09	0.50	13	1.8	590
L. hyp. leaf	0.50	0.50	0.33	0.17	0.33	0.06	0.14	0.28	6	2.3	1200
D. potatorum	0.34	0.66	0.17	0.17	0.49	0.06	0.14	0.12	4	1.4	1110
mannuronan	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0	0	870/ 2200
Epimerized Algal Samples											
L. hyp. leaf + AlgE4	0.64	0.36	0.38	0.26	0.10	0.08	0.22	0.31	5	2.8	1100
L. hyp. leaf + AlgE1	0.68	0.31	0.52	0.15	0.16	0.06	0.12	0.46	10	2.0	1020
L. hyp. leaf + AlgE6	0.67	0.33	0.52	0.14	0.20	0.06	0.08	0.46	10	1.3	1100
D. potatorum + AlgE4	0.58	0.42	0.30	0.29	0.13	0.11	0.21	0.19	3	1.9	950
D. potatorum + AlgE1	0.61	0.39	0.44	0.18	0.21	0.07	0.12	0.37	8	1.7	950
D. potatorum + AlgE6	0.62	0.38	0.45	0.17	0.21	0.10	0.09	0.36	6	0.9	1000
Epimerized Mannuronan											
mannuronan + AlgE4 (polyMG)	0.46	0.54	0	0.46	0.08	0	0.46	0	0		700
mannuronan + AlgE1	0.80	0.20	0.70	0.10	0.10	0.02	0.09	0.68	>40	4.5	1730
mannuronan + AlgE6	0.88	0.12	0.82	0.06	0.06	0.04	0.03	0.78	23	0.8	1150
Epimerized PolyMG											
polyMG + AlgE1	0.48	0.52	0.05	0.43	0.10	0.01	0.41	0.04	5	41	710
polyMG + AlgE1	0.54	0.46	0.17	0.37	0.09	0.04	0.37	0.13	5	9.3	670
polyMG + AlgE1	0.63	0.37	0.35	0.29	0.08	0.04	0.28	0.31	10	7.0	680
polyMG + AlgE1	0.77	0.23	0.59	0.18	0.05	0.03	0.16	0.56	21	5.3	670
polyMG + AlgE6	0.55	0.45	0.18	0.37	0.08	0.03	0.35	0.15	7	12	640
polyMG + AlgE6	0.63	0.37	0.33	0.30	0.07	0.03	0.29	0.29	10	10	630
polyMG + AlgE6	0.72	0.28	0.50	0.22	0.05	0.03	0.21	0.47	15	7.0	650

[a] F_G denotes the fraction of alginate consisting of guluronic acid. F_{GG} and F_{GGG} indicate the fraction of alginate consisting of guluronic acid in blocks of dimers and trimers, respectively, whereas F_{MM} indicates the fraction of alginate consisting of mannuronic diads. $F_{GGM,MGG}$ denotes the fraction of alginate which starts or ends with a block of guluronic acid. $F_{MG,GM}$ is the fraction of alginate consisting of mixed sequences of guluronic and mannuronic acid, with F_{MGM} denoting the fraction of alginate consisting of two mannuronic acids interspaced with guluronic acid. F_{MGM}/F_{GGM} provides an evaluation of the length of alternating sequences.[23] $N_{G>1}$ represents the average length of consecutive of guluronic acid residues (G blocks), excluding single G units from the average. Intrinsic viscosity [η] was measured at 20°C in 0.1 M NaCl aqueous solution in a micro Ubbelohde viscometer.

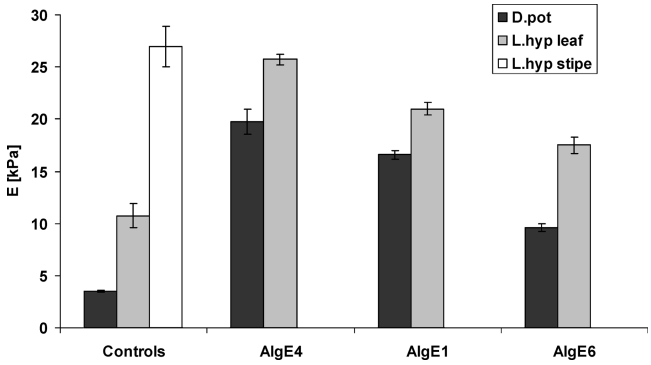


Figure 1. Young's moduli of calcium-saturated alginate gels. Controls are nonepimerized samples. *Laminaria hyperborea* stipe alginate (F_G = 0.66) is included for comparison. The G content for epimerized *D. pot.* and for *L. hyp.* leaf alginates was approx 60 and 70%, respectively (see Table 1). Values are given as means of six parallels ± SD.

From Figure 1, it is evident that epimerization in all cases led to an increase in Young's modulus. For both alginates, the highest values were achieved by introducing alternating blocks into the alginate backbone (AlgE4). Epimerization with AlgE1 gave higher values for Young's modulus than epimerization with AlgE6.

Upon gelation, all samples underwent a significant degree of syneresis (Table 2). However, increasing the amount of MG-blocks (AlgE4) led to similar or higher degree of syneresis than for controls, whereas introducing more G blocks (AlgE1 or AlgE6) resulted in the opposite.

Epimerization of Mannuronan. A pure mannuronan (F_M = 1.0) sample was used as substrate for the different epimerases to study the rheological properties of alginate gels with extreme composition.

Table 2. Syneresis of Ca-Saturated Alginate Samples before and after Epimerization[a]

sample	degree of syneresis (%)
L. hyperborea leaf	44 ± 1.0
L. hyperborea leaf + AlgE1	40 ± 1.8
L. hyperborea leaf + AlgE4	45 ± 0.1
L. hyperborea leaf + AlgE6	36 ± 1.4
D. potatorum	39 ± 0.6
D. potatorum + AlgE1	33 ± 0.3
D. potatorum + AlgE4	43 ± 2.1
D. potatorum + AlgE6	27 ± 1.8
L. hyperborea stipe	27 ± 0.6

[a] A *L. hyp* stipe sample (F_G = 0.66) is added for comparison. The values are given as means ± SD of six gels.

For Ca-saturated gel cylinders of these samples, the highest *E* was achieved for the AlgE1 epimerized alginate with 80% G (Figure 2). In comparison, the AlgE6 epimerized sample with a higher G content (88%) gave a lower Young's modulus. In agreement with our recent work,[18,25] the AlgE4 epimerized mannuronan sample of pure polyalternating structure formed a true gel but with a Young's modulus of only 6 kPa.

Epimerization of PolyMG. Alginates composed solely of MG and GG blocks, almost completely lacking the MM blocks found in algal alginates, were made in a two-step epimerization process; epimerization of mannuronan with AlgE4 forming a polyalternating (polyMG) alginate and a second epimerization of polyMG using the G block forming enzymes AlgE1 and AlgE6 (denoted AlgE4,1 and AlgE4,6, respectively). Young's modulus and rupture strength as well as syneresis of cylinders of both Ca-limited and Ca-saturated gels are given in Figure 3. Two algal alginates from *Laminaria hyperborea* are included for comparison.

As expected, all Ca-saturated gels had a higher Young's modulus and rupture strength than the nonsaturated gels.

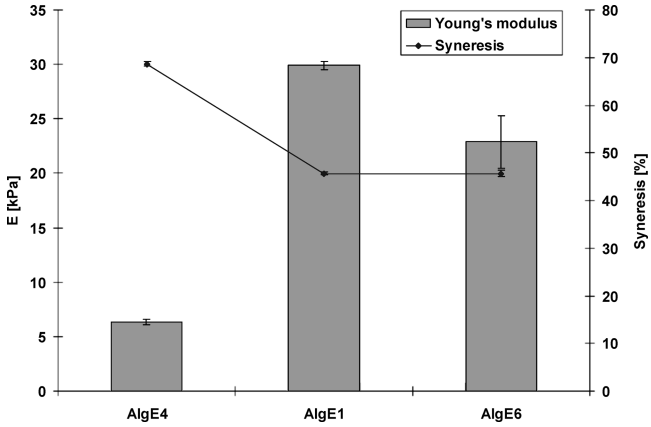


Figure 2. Young's moduli and syneresis of Ca-saturated gel cylinders of mannuronan epimerized with AlgE4, AlgE1, and AlgE6. G content: AlgE4, 46%; AlgE1, 80%; AlgE6, 88%. Values are means ± SD of 6−8 gels.

Interestingly, for the epimerized samples E was dependent on the G content (or G-block length) for the Ca-saturated gels only.

The very high degree of syneresis of epimerized Ca-saturated samples is also evident from Figure 3C. When two alginates with a G content of approximately 50% were compared, the syneresis was 46% and 72% for an algal and epimerized polyMG alginate, respectively. Further, rupture strength followed the syneresis for these gels, both decreasing with increasing G content. Gels containing large amounts of alternating structure, and few G blocks underwent a high degree of shrinkage and showed a very elastic behavior, whereas the presence of long G blocks gave less syneretic gels with a more brittle consistency (Table 3). For Ca-limited gels with a [mole Ca^{2+}]/[mole alginate uronic acid] ratio of 0.3, algal alginates underwent almost no syneresis whereas the volume reduction for AlgE1 epimerized polyMG reached 35% (Figure 3 B).

Surprisingly, a pure polyalternating sample gave gels of extremely high rupture strength compared to all other samples (Figure 3B,C). However, increasing the amount of guluronic acid in the form of G blocks by only 2% using AlgE1, the rupture strength was reduced by approximately 60 and 80% for the Ca-saturated and Ca-limited gels, respectively. Extreme differences in shrinkage were also observed between Ca-limited polyMG (46%) and AlgE1 epimerized polyMG (48%) samples with values of 0 and 32% syneresis, respectively. With undetectable shrinkage at Ca-limited conditions, the polyMG gel underwent almost 70% shrinkage upon Ca-saturation.

Figure 4 gives representative force-deformation curves for two algal and three AlgE4,1 epimerized alginates with various G content. The figure shows that upon uniaxial compression all alginates went through a linear region before entering a nonlinear region and finally rupture of the gel. Further, the length of the linear region decreased upon epimerization and with increasing G content. From Figure 4B it is further evident that all three epimerized samples go through a plastic region before entering the nonlinear region whereas this is not the case for the algal alginates.

For the epimerized polyMG alginates, Young's modulus, syneresis, and rupture point were quite similar for the gels with comparable guluronic acid content (Table 3). Hence, when working on a polyMG substrate, the two epimerases resulted in gels with similar properties.

Enhanced rupture strength and elasticity of the epimerized alginates compared to a seaweed alginate with $F_G = 0.66$ was also observed (Table 3).

Discussion

Mode of Action of Epimerases. The mode of action of the guluronan-block forming epimerases has been examined previously by our group.[19,26] For both AlgE1 and AlgE6 the preferred attack on a GGMM or GGMG sequence suggests that the majority of G block formation in alginates takes place as elongation of existing G blocks with only a minor contribution from the creation of new G blocks. In the current work, the considerable increase in F_{GGG} and $N_{G>1}$ upon treatment with both enzymes on algal alginates (*D. pot.* and *L. hyp.* leaf) compared to control (Table 1) confirms this. A slight increase in F_{GGM} representing the end of G blocks suggests a simultaneous creation of a few new guluronic acid blocks.

AlgE1 has been shown to be a bifunctional enzyme that also introduces single G residues in a similar manner to AlgE4.[27] The bifunctionality of AlgE1 was illustrated upon epimerization of a mannuronan backbone (Table 1). Although most of the G residues introduced were in the form of G-blocks, a minor portion was introduced between two M residues, resulting in a MGM fraction of 9%. AlgE6, however, introduced almost solely guluronic acid as blocks (only 3% MGM). However, when working on a polyMG substrate only one reaction can occur, leaving the end products of these two epimerases similar.

AlgE4 has been shown to perform a random initial attack[26,28] with a processive mode of action.[24] The sequential data in Table 1 illustrate that, upon AlgE4 epimerization, (i) the vast majority of guluronate residues were introduced into alternating sequences and (ii) the length of the alternating sequences increased. Further, the minor increase in F_{GG} and F_{GGG} upon AlgE4 epimerization suggests that this enzyme is able to introduce guluronate as a nearest neighbor to an existing G residue, either single or part of a block. This has been observed in a previous study for high concentrations of enzyme.[29] As there is no observed increase in F_{GG} on AlgE4 epimerized mannuronan (Table 1), this indicates that G residues must be present in the alginate for a G-block formation to take place.

Previously, the basic problem in relating the physical properties to the molecular structure has been the limited knowledge of the monomer sequence within the alginate molecule. By NMR spectroscopy, only average block lengths are obtained, and the block length and compositional distribution have been found by simulation only, assuming a statistical model for the monomer sequence.[30,31] Recently, the true G-block lengths and their distribution in epimerized alginates were determined by our group using a combination of methods, including NMR, mass spectrometry, and specific alginate degrading enzymes.[19] In this work, the differences between AlgE1 and AlgE6 were demonstrated with regard to both length and distribution of the G-blocks they introduce into a polyalternating alginate. The suggested higher processivity of AlgE1 results in the initial formation of extremely long guluronic acid blocks (52−58 G residues) in the polymer chain. AlgE6, however, forms shorter G blocks with a more heterogeneous chain-length distribution.[19] Upon increasing the total G content with increasing epimerization time, these differences will, however, gradually disappear as G blocks are "zipped", ending up with long stretches of G residues for both AlgE1 and AlgE6 epimerized samples.

In contrast to the AlgE1 epimerized polyM, the creation of very long G blocks in the AlgE1 epimerized polyMG in present work is not evident (Table 1). The average G-block length was calculated for all samples using $N_{G>1} = (F_G - F_{MGM})/F_{GGM}$. For alginates with very high proportions of alternating sequences the MG-5 M peak is dominating, making the integration of the GG-5 M peak from the ^{1}H NMR difficult. F_{GGM} values are,

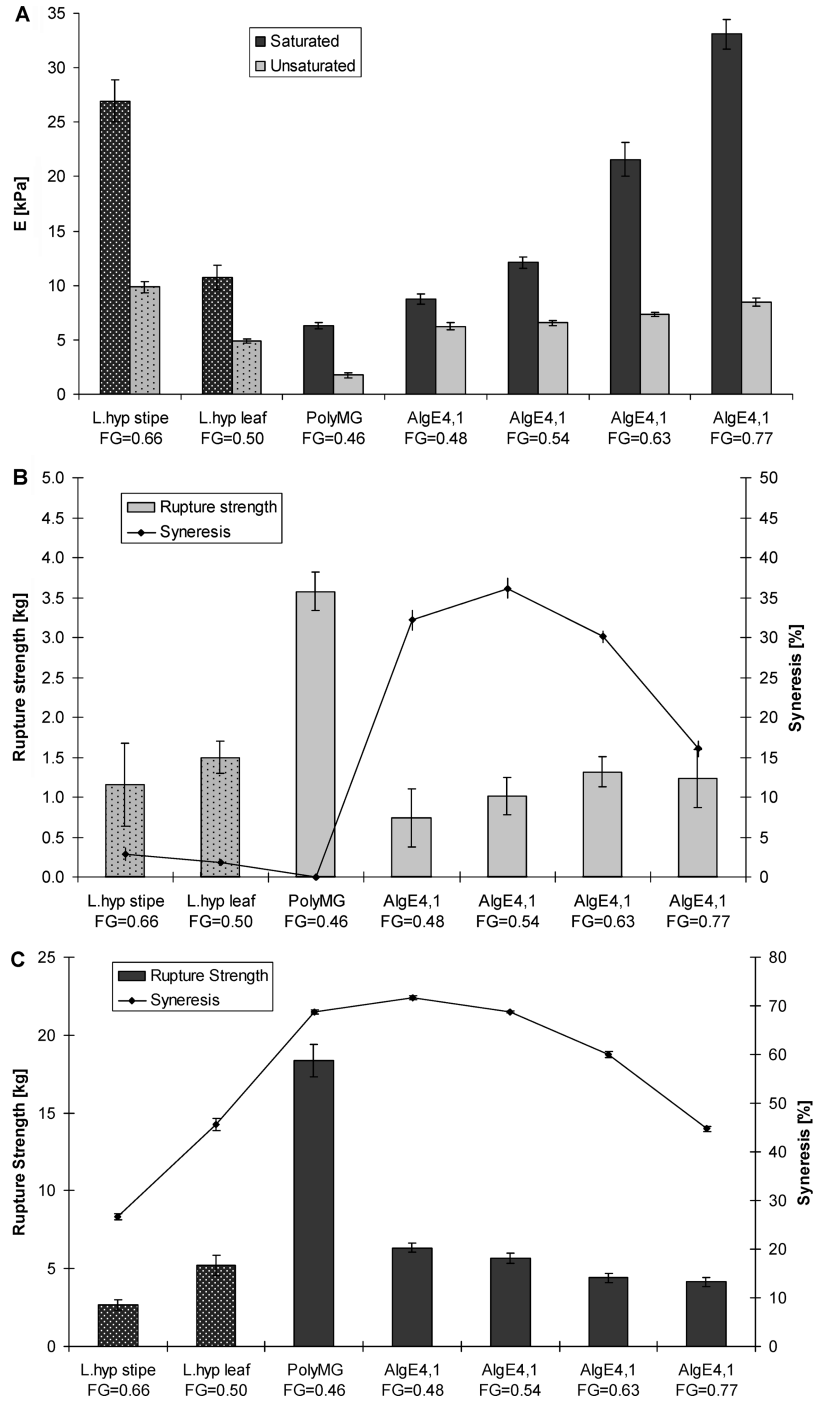


Figure 3. (A) Young's modulus of Ca-saturated (dark gray) and Ca-limited (light gray) gel cylinders of AlgE1 epimerized polyMG (AlgE4,1). Two seaweed alginates and a polyalternating alginate are included for comparison. The nonsaturated Ca-alginate gel of polyMG is from a different batch with same F_G but slightly higher M_w. (B) Rupture strength and syneresis of Ca-limited alginate gel cylinders. (C) Rupture strength and syneresis of Ca-saturated alginate gel cylinders. Values are means of 8 gels ± SD.

hence, probably estimated to be larger than the true values and as a result, the calculated $N_{G>1}$ values for AlgE4,1 and AlgE4,6 epimerized samples are most likely significantly lower than the actual G-block length in the alginates.

Young's Modulus and Syneresis of Epimerized Samples. The observed increase in Young's moduli for AlgE4 epimerized seaweed alginates could be attributed to the elongation of alternating sequences as described in our recent work[18] and, consequently, the formation of junctions involving long MG sequences. However, the increase in F_{GG} also observed in these samples, cannot be ruled out as a partial contributor to the elevated E values. In addition, because alternating blocks have been shown to possess the lowest extension and highest intrinsic flexibility of the three different building blocks in alginate,[6,16,32] an exchange of M blocks with MG blocks would give a more condensed alginate gel network with tight intermolecular cross-linking.[25] In contrast, for the AlgE1 and AlgE6 epimerized algal alginate samples the subsequent formation of a high number of interguluronate cross-links with elongated G blocks could be hampered due to topological restrictions, resulting in a smaller increase in E. This is also reflected in the degree of shrinkage for the Ca-alginate gels as the syneresis is higher for AlgE4 epimerized samples compared to samples epimerized with G block forming epimerases.

Table 3. Young's Modulus (*E*), Syneresis, Rupture Strength, and Elasticity for Ca-Saturated AlgE1 and AlgE6 Epimerized PolyMG Gels[a]

epimerase used on polyMG	F_G	*E* [kPa]	syneresis [%]	rupture strength [kg]	elasticity [%]
AlgE1	0.54	12 ± 0.5	69 ± 0.3	5.7 ± 0.3	71 ± 1.1
	0.63	22 ± 1.5	60 ± 0.6	4.4 ± 0.3	67 ± 0.7
	0.77	33 ± 1.4	45 ± 0.5	4.1 ± 0.3	62 ± 0.9
AlgE6	0.55	13 ± 0.5	67 ± 0.6	8.1 ± 0.8	77 ± 0.6
	0.63	19 ± 0.8	59 ± 0.6	5.3 ± 0.4	71 ± 1.1
	0.72	26 ± 1.2	46 ± 0.8	5.0 ± 0.4	68 ± 1.0
control (*L. hyp.* stipe)	0.66	27 ± 1.9	27 ± 0.6	2.7 ± 0.3	57 ± 0.9

[a] *Laminaria hyperborea* stipe alginate is given as a control. Elasticity is calculated as percentage deformation at gel rupture. Values are means ± SD of eight gels.

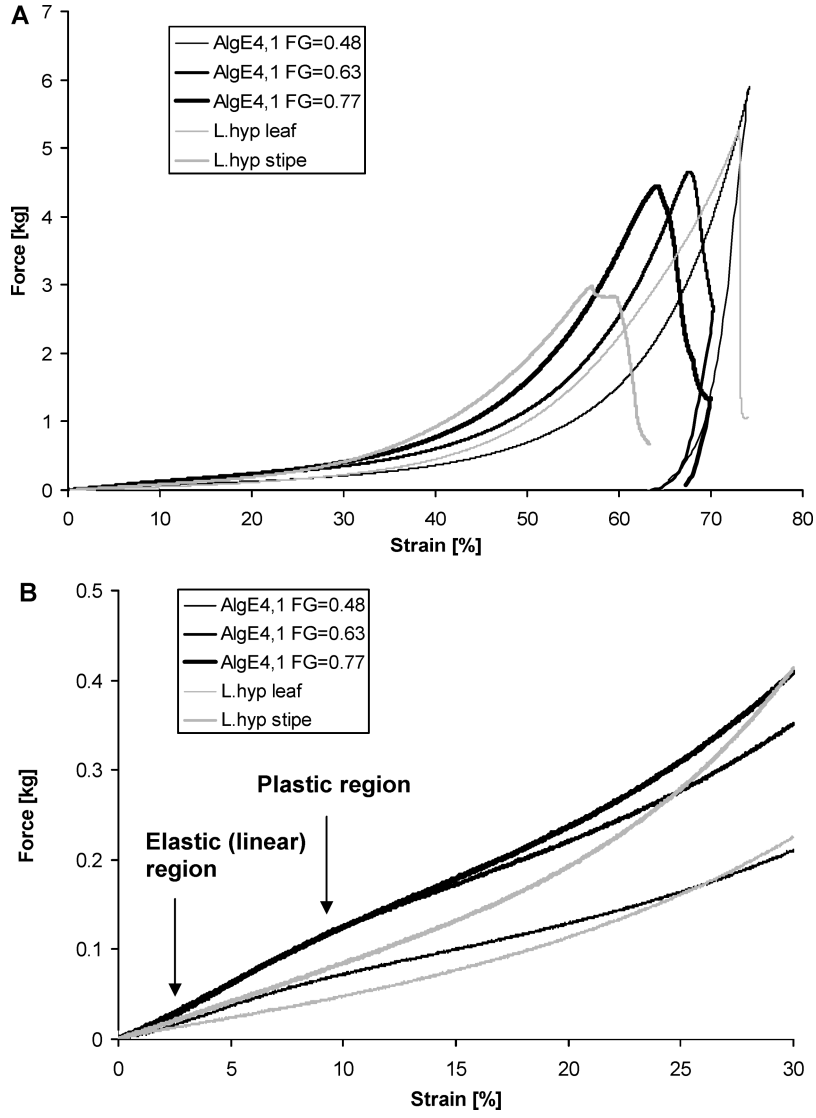


Figure 4. (A) Representative force vs strain curves for two algal (gray) and three AlgE4, AlgE1 epimerized (black) alginate samples by uniaxial compression measurements of Ca-saturated gel cylinders. Strain represents deformation/initial dimension. (B) The same curves up to only 30% strain, illustrating the plastic region of the epimerized samples.

The fact that the gels of seaweed alginates and mannuronan epimerized by AlgE1 displayed higher *E* values than those of the AlgE6 epimerized samples (Figure 1) deserves a further comment. It is likely that the bifunctionality of AlgE1 resulted in elongation of alternating sequences, thus explaining the higher Young's modulus of the AlgE1 epimerized gels (Table 1) by allowing the formation of mixed GG/MG and possibly MG/MG junctions upon calcium saturation,[18] in addition to allowing further intermolecular cross-linking of the elongated G blocks. For the same reasons the higher syneresis displayed by the AlgE1 compared to AlgE6 epimerized seaweed alginates (Table 2) might be explained.

Table 1 shows that alginates used in the present study were of different molar masses. It has previously been shown[11] that gel strength is independent of the molecular weight of alginate samples in the range of concentrations and intrinsic viscosities used in the present study. Variations in molecular weight and molecular weight distribution among these samples are, therefore, not expected to have an influence on their mechanical properties. A high M_w dependency on gel strength observed

for polyMG compared to the other alginates used in the study is, however, worth a comment. At low Ca^{2+} concentrations (15 mM $CaCO_3$, 1% (w/v) alginate), a polyalternating alginate sample with an intrinsic viscosity of 700 mL/g ($M_w \approx 130000$ g/mol; weight average molecular weight adapting values for a and K given by Vold et al.[33] ($a = 1.00$ and $K = 0.0054$) in the MHS equation for the correlation of intrinsic viscosity to M_w: $[\eta] = K \times M^a$) was not enough to form a stable gel, whereas using a sample with a slightly higher molecular weight (intrinsic viscosity 1040 mL/g, $M_w \approx 200000$; weight average molecular weight adapting values for a and K given by Vold et al.[33] ($a = 1.00$ and $K = 0.0054$) in the MHS equation for the correlation of intrinsic viscosity to M_w: $[\eta] = K \times M^a$) resulted in a stable gel under the same conditions. The high M_w dependency on gel strength for Ca-limited gels has previously been shown to be larger than for Ca-saturated gels,[34] probably due to the higher number of nonelastic chains as a result of a lower degree of cross-linking in the former. However, an alginate sample with intrinsic viscosity as high as 700 mL/g will probably contain chains much longer than the required length needed to form junctions. The reason for these observations is, therefore, at present not understood.

Large versus Small Deformation. An alginate gel is a complex network system. The gel strength will depend on the number and strength of junctions and on their potential lateral association, reflecting both entropic (rubber-like elasticity) and enthalpic contributions. Because these factors can only be estimated indirectly, a description of the mechanical properties of an alginate gel will necessarily include many approximations.[1,35] Hence, there are large discrepancies in the data found describing the rhelogical properties of these gels depending on the methods used and approximations taken.

Rheological studies of alginate gels are most commonly performed in the linear region of small deformation[36] where Young's modulus is typically used as a measure of gel strength. Although these data often give very reproducible results, nondestructive methods do not necessarily give the most correct picture of the mechanical properties important for the specific application of the gel. In fact, for many applications these samples will be exposed to stresses and strains well outside the linear viscoelastic region.[35] The method chosen to describe the rheological properties of the gel is thus of high importance. The present data show that for the epimerized samples containing a large amount of long MG sequences (Table 1) Young's modulus was lower than for algal alginates (Figure 3A). However, large deformation measurements showed the opposite with both higher rupture strength and elasticity for the epimerized samples (Figure 3C and Table 3).

We have previously proposed that long stretches of MG blocks present in the epimerized material may bring on a partial network collapse[18] that further might lead to lower E values due to the reduction in elastically active chains. When introducing higher amounts of G blocks into the polymer chain, stable junctions are introduced which hinder the collapse of the long MG blocks.[18] For materials with very high G content, the length of G blocks will be responsible for the high gel strength (higher E) as the contribution from MG blocks becomes small. This will result in a stiffer and less elastic (more brittle) network, reflected in Table 3 as a decrease in elasticity upon increasing G content.

With this in mind, it is important to underline that the E values presented in Figure 3A have been normalized with respect to gel shrinkage, applying $E/(c_{corr})^2$, where c_{corr} is the polymer concentration after syneresis.[37] Due to the highly syneretic behavior of Ca-saturated epimerized polyMG, these gels had high polymer density. The resulting higher cross-link density will also contribute to higher rupture strength. Compared to algal alginates, the functionality of these samples may consequently be greater for certain applications, as recently demonstrated,[17] with E, therefore, not being the parameter of choice for describing the mechanical properties of the system.

As both the modulus and the rupture strength will be correlated to the number of cross-links per unit volume, it might be expected that in general the rupture strength will increase with the apparent modulus.[38] However, as illustrated in Figure 3, this was not the case for alginate samples used in the present study. The negative correlation between Young's modulus and rupture force may be attributed to the nonlinearity of the force-deformation curves (Figure 4), which may be associated with permanent changes in the hydrogel network.[22,36,39,40] Hence, as Young's moduli values are based on the gradient in the linear region at small deformation, only involving network chain deformation, these values cannot be correlated with rupture.

The linear (elastic) region has been attributed to the deformation of network chains between junctions where the force is proportional to the corresponding deformation (Gaussian network theory).[36] In the present study, shorter linear regions were observed for epimerized alginates (Figure 4). Zhang et al.[36] have shown that the linear region decreases with both increasing alginate concentration and increasing the number and length of junctions. The high polymer concentration in the epimerized alginate gels (as a result of high degree of syneresis) and the likely higher number and length of cross-links (and, therefore, shorter network chains) in these alginates would hence explain these observations.

At the same time, the steepness of the nonlinear region increased with increasing G content but was lower for epimerized samples compared to algal samples with the same G content. When the deformation is large the junction zones are also deformed,[22,39,40] contributing to stress increase due to structure densification referred to as strain hardening.[36,41] As the stress will depend on both the strength and the total amount of junctions, increased stress upon increasing G content was expected.

Rupture strength is a reflection of the strength and number of junctions.[36] A junction experiencing the highest stress, normally the shortest one in length, fractures first. Short, stiff polymer chains between junctions will also contribute to the transmission of more energy to the junctions, thus facilitating rupture. The energy released from the fractured junctions will transfer to the neighboring chains, accelerating the break of other junctions. This cascading process will happen so fast that the gel fractures prior to achieving full extension of the network chains.[1,36]

The extremely high rupture strength for a polyMG sample (Figure 3B,C) was not expected as MG−MG junctions, which are the only junctions that can be formed in this sample, are considered to be both weak and few in number.[18] It is known that before gel rupture, segmental motions of the chain elements will lead to deformation and rearrangement of junctions in a process that dissipates energy. Unwinding these junctions will eventually lead to rupture, the strength depending on the nature of the junctions. Gels of polyMG alginate will exclusively consist of long homogeneous stretches of MG−MG junctions flanked by flexible MG network chains. Upon compression, the possibility that chains involved in the junctions are "sliding" in opposite directions without leading to rupture of the gel cannot be ruled out. This kind of mechanism would require large

amounts of energy and rupture of the gel would only occur by the unwinding of these long MG−MG junctions, reflecting the very high rupture strength of these gels. Introduction of small amounts of G blocks would break up the homogeneity not allowing the "sliding junction" mechanism, reflected in the steep reduction of rupture strength going from polyMG (46% G) to 48% G in Figure 3B,C.

Figure 4 demonstrated differences in force-strain curves between algal and epimerized alginates. Whereas gels of epimerized alginates went through a plastic deformation region upon compression, this was not observed for algal samples. Plasticity, characterized as a downward concavity in the force-deformation curve, was also observed for a polyalternating sample (data not shown). Hence, we hypothesize that the observed plasticity is connected to the length of alternating blocks and may be due to restructuring or "sliding" of the junctions involving MG sequences.

Ca-Saturated versus Ca-Limited Gels. In a previous study, Draget et al.[34] showed that the relationship between the gel strength and the Ca-concentration is not linear, indicating that upon increasing the Ca^{2+} concentration, the cations will fill up and elongate existing junctions before creating new, shorter G blocks. Figure 3A illustrates that the gel strength dependency on the G content seen for the calcium-saturated gels was absent for gels made under Ca-limited conditions ($[Ca^{2+}]/[\text{alginate sugar residue}] = 0.3$). This observation is probably a result of cooperative binding of Ca^{2+} into junction zones where long G blocks are filled before new junctions are formed, thereby not contributing to a large extent to the elastic modulus.

The absence of syneresis for the polyalternating sample at a concentration of 15 mM Ca^{2+} (shown as 0% syneresis in Figure 3C) indicated that more calcium is needed to induce collapse in the hydrogel network, which in the case of polyMG requires the formation of long MG/MG junctions.[18] Upon Ca-saturation, excess Ca^{2+} will contribute to long stretches of MG/MG junctions leading to partial collapse and extreme degree of shrinkage (approx 70%, Figure 3B) induced by zipping of long alternating blocks.[17,18] Introducing only very small amounts of GG blocks into the polyalternating sample (with an increase of only 2% G using AlgE1) resulted in a remarkable increase in syneresis for the Ca-limited sample. These findings indicate that at this specific Ca-concentration the presence of a few GG sequences is needed for the energetically driven collapse of the gel network with the concomitant formation of mixed GG/MG and probably MG/MG junctions in addition to GG/GG junctions.

Conclusions

Increased knowledge about the mode of action of the various C-5 epimerases has given more insight into the relationship between polymer structure and mechanical properties of alginate gels. The present study has shown that by utilizing the different epimerization patterns of the various epimerases, both native and bacterial alginates can be designed to possess specific mechanical properties which may be beneficial for numerous applications. The ability to control the rheological properties may be highly valuable for the application of alginates. The Young's modulus of high-G alginate gels from *L. hyperborea* stipe, which in general is considered to form strong gels, could be exceeded by a single epimerization reaction of mannuronan with AlgE1. When AlgE4 is used, Young's moduli values close to those of native *L. hyp.* stipe could be obtained by epimierization of *L. hyp.* leaf alginate. On the other hand, alginates with very homogeneous structure composed of only MG and GG blocks, resulted in gels of lower Young's modulus but were more resistant to rupture. Further, gels containing large amounts of alternating structure and few G blocks underwent a high degree of shrinkage and showed a very elastic behavior, whereas the presence of long G blocks gave less syneretic gels with a more brittle consistency. Hence, well characterized, tailor-made alginates with novel properties may replace less effective materials extracted from seaweeds.

Lastly, this study confirms our recent hypothesis that the role of alternating sequences must be taken into consideration when evaluating the functional properties of alginate hydrogels. Long sequences of alternating structure will lead to more elastic gels that undergo a high degree of syneresis with the concomitant increase in polymer density having great effect on the mechanical properties of the gel.

Acknowledgment. This work was funded by grants from the Norwegian Research Council (Y.A.M.) and EXTRA funds from the Norwegian Foundation for Health and Rehabilitation (B.L.S.). The authors wish to credit Wenche Iren Strand for all the excellent technical assistance. Dr. Ingvild Haug is thanked for valuable discussions on rheology.

References and Notes

(1) Moe, S. T.; Draget, K. I. ; Skjåk-Bræk, G. ; Smidsrød, O. Alginates *Food polysaccharides and their applications*; Marcel Decker, Inc.: New York, 1995; pp 245−286.
(2) Grant, G. T.; Morris, E. R.; Rees, D. A.; Smith, P. J. C.; Thom, D. *FEBS Lett.* **1973**, *32*, 195–198.
(3) Zimmet, P.; Alberti, K. G. M. M.; Shaw, J. *Nature* **2001**, *414*, 782–787.
(4) Stokke, B. T.; Draget, K. I.; Smidsrød, O.; Yuguchi, Y.; Urakawa, H.; Kajiwara, K. *Macromolecules* **2000**, *33*, 1853–1863.
(5) Smidsrød, O. *J. Chem. Soc., Faraday Trans.* **1974**, *57*, 263–274.
(6) Smidsrød, O.; Glover, R. M.; Whittington, S. G. *Carbohydr. Res.* **1973**, *27*, 107–118.
(7) Kong, H. J.; Wong, E.; Mooney, D. J. *Macromolecules* **2003**, *36*, 4582–4588.
(8) Draget, K. I. ; Smidsrød, O. Modification of gelling kinetics and elastic properties by nano structuring of alginate gels exploiting the properties of poly-guluronate *Gums and Stabilisers for the Food Industry 13*; RSC Publishing: Cambridge, 2006; pp 227−233.
(9) Jørgensen, T. E.; Sletmoen, M.; Draget, K. I.; Stokke, B. T. *Biomacromolecules* **2007**, *8*, 2388–2397.
(10) Draget, K. I.; Skjåk-Bræk, G.; Smidsrød, O. *Int. J. Biol. Macromol.* **1997**, *21*, 47–55.
(11) Martinsen, A.; Skjåk-Bræk, G.; Smidsrød, O. *Biotechnol. Bioeng.* **1989**, *33*, 79–89.
(12) Draget, K. I.; Moe, S. T.; Skjåk-Bræk, G.; Smidsrød, O., Alginates. In *Food Polysaccharides and Their Applications*, 2nd ed.; Stephen, A. M., Phillips, G. O., Williams, P. A., Eds.; CRC Press: Boca Raton, FL, 2006; pp 289−334.
(13) Ertesvåg, H.; Doseth, B.; Larsen, B.; Skjåk-Bræk, G.; Valla, S. *J. Bacteriol.* **1994**, *176*, 2846–2853.
(14) Svanem, B. I. G.; Skjåk-Bræk, G.; Ertesvåg, H.; Valla, S. *J. Bacteriol.* **1999**, *181*, 68–77.
(15) 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*, 3515–3523.
(16) Dentini, M.; Rinaldi, G.; Risica, D.; Barbetta, A.; Skjåk-Bræk, G. *Carbohydr. Polym.* **2005**, *59*, 489–499.
(17) Mørch, Y. A.; Donati, I.; Strand, B. L.; Skjåk-Bræk, G. *Biomacromolecules* **2007**, *8*, 2809–2814.
(18) Donati, I.; Holtan, S.; Mørch, Y. A.; Borgogna, M.; Dentini, M.; Skjåk-Bræk, G. *Biomacromolecules* **2005**, *6*, 1031–1040.
(19) Holtan, S.; Bruheim, P.; Skjåk-Bræk, G. *Biochem. J.* **2006**, *395*, 319–329.
(20) Grasdalen, H. *Carbohydr. Res.* **1983**, *118*, 255–260.
(21) Draget, K. I.; Østgaard, K.; Smidsrød, O. *Carbohydr. Polym.* **1990**, *14*, 159–178.
(22) Smidsrød, O.; Haug, A. *Acta Chem. Scand.* **1972**, *26*, 79–88.
(23) Grasdalen, H.; Larsen, B.; Smidsrød, O. *Carbohydr. Res.* **1981**, *89*, 179–191.

(24) Campa, C.; Holtan, S.; Nilsen, N.; Bjerkan, T. M.; Stokke, B. T.; Skjakbraek, G. *Biochem. J.* **2004**, *381*, 155–164.
(25) Draget, K. I.; Strand, B. L.; Hartmann, M.; Valla, S.; Smidsrød, O.; Skjåk-Bræk, G. *Int. J. Biol. Macromol.* **2000**, *27*, 117–122.
(26) Hartmann, M.; Holm, O. B.; Johansen, G. A. B.; Skjåk-Bræk, G.; Stokke, B. T. *Biopolymers* **2002**, *63*, 77–88.
(27) Ertesvåg, H.; Høidal, H. K.; Skjåk-Bræk, G.; Valla, S. *J. Biol. Chem.* **1998**, *273*, 30927–30932.
(28) Hartmann, M.; Duun, A. S.; Markussen, S.; Grasdalen, A.; Valla, S.; Skjåk-Bræk, G. *Biochim. Biophys. Acta* **2002**, *1570*, 104–112.
(29) Høidal, H. K.; Ertesvåg, H.; Skjåk-Bræk, G.; Stokke, B. T.; Valla, S. *J. Biol. Chem.* **1999**, *274*, 12316–12322.
(30) Larsen, B.; Painter, T.; Haug, A.; Smidsrod, O. *Acta Chem. Scand.* **1969**, *23*, 355–370.
(31) Smidsrod, O.; Whittington, S. G. *Macromolecules* **1969**, *2*, 42–44.
(32) Smidsrød, O. *Carbohydr. Res.* **1970**, *13*, 359–372.
(33) Vold, I. M. N.; Kristiansen, K. A.; Christensen, B. E. *Biomacromolecules* **2006**, *7*, 2136–2146.
(34) Draget, K. I.; Simensen, M. K.; Onsøyen, E.; Smidsrød, O. *Hydrobiologia* **1993**, *261*, 563–569.
(35) Kavanagh, G. M.; Ross-Murphy, S. B. *Prog. Polym. Sci.* **1998**, *23*, 533–562.
(36) Zhang, J. H.; Daubert, C. R.; Foegeding, E. A. *J. Food Eng.* **2007**, *80*, 157–165.
(37) Smidsrød, O.; Haug, A.; Lian, B. *Acta Chem. Scand.* **1972**, *26*, 71–78.
(38) Mitchell, J. R. *J. Texture Stud.* **1980**, *11*, 315–337.
(39) Andresen, I. L.; Smidsrød, O. *Carbohydr. Res.* **1977**, *58*, 271–279.
(40) Mitchell, J. R.; Blanshard, J. M. V. *J. Texture Stud.* **1976**, *7*, 219–234.
(41) Mancini, M.; Moresi, M.; Rancini, R. *J. Texture Stud.* **1999**, *30*, 639–657.

BM8003572