Effect of Elongation of Alternating Sequences on Swelling Behavior and Large Deformation Properties of Natural Alginate Gels

Ivan Donati,*,† Yrr A. Mørch,‡ Berit L. Strand,‡ Gudmund Skjåk-Bræk,‡ and Sergio Paoletti†

Department of Life Sciences, University of Trieste, Via Licio Giorgieri 1, 34127 - Trieste, Italy, Institute of Biotechnology, Norwegian University of Science and Technology, Sem Sælands v. 6/8, 7491 Trondheim, Norway

Received: June 11, 2009; Revised Manuscript Received: August 7, 2009

The physical properties of alginate gels correlate with alginate composition. Blocks of guluronic acid (G) strongly contribute to gel formation. Recently, the role of alternating sequences in calcium−alginate gels has been elucidated. The present contribution aimed at extending the analysis already reported (Donati, I.; Holtan, S.; Mørch, Y. A.; Borgogna, M.; Dentini, M.; Skjåk-Bræk, G. *Biomacromolecules* **2005**, *6*, 1031) and at explaining some apparent mismatch of experimental data. In the present work, calcium hydrogels from different alginate samples have been analyzed by means of uniaxial compression and puncture tests to evaluate their Young's modulus and work at break. The role of long MG blocks in mechanical deformations (small and large domains) as well as in swelling experiments was investigated with natural and MG-enriched (AlgE4 epimerized) alginate samples. Alginates with elongated alternating sequences displayed, upon treatment with saline solution, a notable increase in swelling behavior, which was not paralleled by increased mechanical properties (Young's modulus). This behavior was traced back to the disentanglement of MG/MG junctions, which increased the local charge density, reducing the osmotic contribution to hydrogel swelling. The analyses of the large deformation curves for natural and epimerized alginates revealed an increase in the energy to breakage in the latter case caused by the dissipation effect of "sliding" MG/MG junctions.

Introduction

Carbohydrate-based biopolymers constitute very important raw materials for both industrial and biotechnological applications. The food industry has largely exploited various polysaccharides and their peculiar properties in several applications ranging from semisolid formulations to nutraceuticals.[1] In addition, polysaccharides constitute a major component in semisolid biological environments, commonly termed as extracellular matrix (ECM), where they provide for both recognition phenomena (heparin and heparan sulfate, hyaluronic acid to name a few) and suitable mechanical properties (resistance to compression and shear) within a highly swollen environment. Therefore, several applications in bioreactor design and tissue engineering have been proposed for polysaccharide-based capsules and scaffolds.[2−6] As an example, alginate capsules were used to entrap human Langerhans islets for the treatment of type-I diabetes.[5,7] Moreover, alginate-based scaffolds, loaded with hydroxyapatite, have been recently proposed for bone tissue engineering.[8] In the above-mentioned applications, the elucidation of the relationship between mechanical performances and polysaccharide compositional and physical−chemical features is of paramount importance.

Alginate is a collective term for a family of polysaccharides produced by brown algae and bacteria. Chemically, they are copolymers of 1 → 4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) arranged in a blockwise pattern along the chain with homopolymeric regions of M (M blocks) and G (G blocks) residues interspersed with regions of alternating structure (MG blocks). The ion-binding properties of the G blocks composing the polysaccharide chain have been largely studied, and they were described by using the so-called "egg-box" model, which implies that junction zones are formed by two facing helical G sequences: each cross-linking ion (i.e., Ca^{2+}) interacts with two adjacent G residues as well as two with G residues in the opposing chain. Simdsrød[9] and Morris and Rees[10] agreed in describing the junctions formed by G blocks as composed by dimers or by a few laterally associated polymer strands. Overall, this led to a description of the junctions in the gel as crystallites flooded by the "sticky sea" of the elastically active chains. As preliminary as this description might have been, no better model from the point of view of the clarity, simplicity, and adherence to experimental evidence has so far been presented in these authors' opinion. Over the years, several attempts have been performed to disclose the molecular features of the ion-induced gelation mechanism for alginate, revealing that the model originally proposed has to be regarded as substantially correct.[11] In particular, the 2/1 helical geometry of the G-blocks involved in calcium binding in the junctions, after being questioned,[12] has been recently confirmed.[13] In addition, theoretical investigations on ion-induced junction formation for polyuronates, carried out by using computational methods[14] and an adaptation of the Manning's counterion condensation theory,[15,16] were found to be in substantial agreement with the "egg-box" model.

The vast majority of the mechanical determinations focused on the properties displayed by the gel in the so-called small deformation range (up to approximately 10%). For example, the viscoelastic behavior of calcium−alginate gels has been investigated,[17] and a correlation between the Young's modulus (E) and the polysaccharide characteristics[18] has been proposed. However, interesting and intriguing studies in the large defor-

* Corresponding author. Phone: +39 040 558 3681. Fax: +39 040 558 3692. E-mail: idonati@units.it.
† University of Trieste.
‡ Norwegian University of Science and Technology.

10.1021/jp905488u CCC: $40.75
Published on Web 09/04/2009

TABLE 1: Chemical Composition[a] and Intrinsic Viscosity of the Natural and AlgE4 Epimerized Alginate Samples Used

sample	F_G	F_M	F_{GG}	$F_{GM,MG}$	F_{MM}	F_{GGG}	$F_{GGM,MGG}$	F_{MGM}	$N_{G>1}$	F_{MGM}/F_{GGM}	$[\eta]$ (dL/g)[b]
L. hyp.	0.65	0.35	0.53	0.12	0.23	0.49	0.04	0.07	14.5	1.75	6.41 ± 0.02
LhypE4	0.73	0.27	0.56	0.17	0.10	0.52	0.05	0.10	12.6	2	6.05 ± 0.03
M. pyr.	0.42	0.58	0.21	0.21	0.37	0.17	0.04	0.17	6.3	4.25	8.87 ± 0.04
MpyrE4	0.56	0.44	0.23	0.32	0.13	0.20	0.04	0.27	7.3	6.75	7.91 ± 0.01
A. nod.	0.41	0.59	0.20	0.21	0.38	0.13	0.07	0.14	3.9	2	8.33 ± 0.02
AnodE4	0.54	0.46	0.22	0.31	0.16	0.15	0.07	0.24	4.3	3.43	7.90 ± 0.03

[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. $F_{GGM,MGG}$ indicates the fraction of alginate that starts or ends with a block of guluronic acid. $F_{GM,MG}$ indicates 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. $N_{G>1}$ denotes the average length of G-blocks as $N_{G>1} = (F_G - F_{MGM})/F_{GGM}$. [b] Solvent: NaCl 0.1M, 20 °C.

mation range have also been recently performed.[1,19,20] The comprehension of the large deformation behavior certainly has a deep impact also in the development of alginate-based materials to be used as bioreactors for regenerative medicine, since the forces exerted in vivo onto the gel implants are likely to belong to this category.

In this scenario, the hypothesis of an involvement of alternating sequences in alginate gel formation opened up the possibility of new insight into the molecular details of the system.[21,22] The availability of pure alginates resembling separately the three extreme block sequences present in the natural polysaccharide (namely, G-blocks, M-blocks, and alternating (MG)-blocks) was found to be a fundamental tool to point out their specific role in the final hydrogel. The ability of a "polyalternating" alginate, thus composed exclusively of MG sequences and completely devoid of G-block, to form stable hydrogels represented not only the most striking evidence of the calcium binding properties of long MG junctions but also the cornerstone to propose the involvement under certain circumstances of such sequences in alginate gels. It should be stressed, however, that the mechanical properties of alginate gels composed exclusively of MG sequences are very poor and that the presence of G-blocks is required to prepare networks of measurable strength and of biotechnological significance. In addition, a thorough analysis carried out to compare the properties of alginates in the presence of different gel-forming divalent cations[23] sketched an overall picture of the ion-induced gelation process, which turned out to be more complicated than expected.

It has been reported that the enzymatic elongation of MG sequences brings about a notable increase in the dimensional stability of alginate hydrogels.[24] These observations have interesting consequences when alginate is considered as material for cell encapsulation and cell therapy, for which the requirement of stability in body fluids is mandatory. Despite the obvious implications of the swelling behavior of alginate gels for biomedical and industrial applications, to date, an interpretation of such phenomenon is still lacking.

The aim of the present contribution is to further disclose the role of alternating sequences on the swelling behavior and mechanical properties of alginate gels. The availability of the AlgE4 epimerase,[25−27] which converts M-blocks in strictly alternating sequences, is expected to bring about a notable contribution in explaining the effect of MG-blocks' elongation[21] within a G-block containing alginate. In view of the above-reported considerations, we will focus our attention on natural and MG-enriched alginate samples.

Materials and Methods

Commercial samples of sodium alginate isolated from *Laminaria hyperborea* stipe (*L. hyp.*), *Macrocystis pyrifera* (*M. pyr.*), and *Ascophyllum nodosum* (*A. nod.*) were provided by FMC Biopolymers (Drammen, Norway). The main structural and compositional features of the samples are reported in Table 1. D-Glucono-δ-lactone (GDL) was purchased from Sigma Chemical Co. (St. Louis, MO). Calcium carbonate (average particle size, 4 μm) was purchased from Merck (Darmstadt, Germany). Recombinant Mannuronan C-5 epimerase AlgE4 was produced and purified as previously reported.[25,28] Epimerization with AlgE4 of the natural alginate samples was performed according to the procedure reported elsewhere[21] (molar ratio of enzyme/uronic groups was 1:60 000).[29]

Viscosity Measurements. Reduced capillary viscosity of the sodium form of samples listed in Table 1 was measured in 0.1 M NaCl at 20 °C by using a Schott-Geräte AVS/G automatic apparatus and an Ubbelohde type viscometer. Intrinsic viscosity values were determined by analyzing the concentration dependence of the reduced specific viscosity (η_{sp}/c) and of the reduced logarithm of the relative viscosity ($\ln \eta_{rel}/c$).

NMR Spectroscopy. ^{1}H NMR spectra were recorded at 90 °C with a Bruker DPX 400 spectrometer (9.4 T) operating at 400 MHz for proton, and samples were prepared as described by Grasdalen et al.[30] The chemical shifts are expressed in parts per million downfield from the signal for 3-(trimethylsilyl)propanesulfonate. The integration of the ^{1}H NMR signals allowed determination of the composition of the different alginate samples reported in Table 1.

Hydrogel Formation in Calcium Limited Conditions. Homogeneous calcium gels from alginate samples listed in Table 1 were prepared by blending the aqueous polymer solution with an inactivated form of Ca^{2+} ($CaCO_3$) at different concentrations (ranging from 10 to 40 mM) followed by the addition of the slowly hydrolyzing GDL. A constant molar ratio, $[GDL]/[Ca^{2+}] = 2$, was used in all the experiments. The final, nominal concentration of polymer was 1% (w/V) in all cases. Aliquots of Ca−polymer gelling solutions were cured for 24 h at room temperature in 24-well tissue culture plates having a diameter of 16 mm and height of 18 mm (Costar, Cambridge, MA).

Hydrogel Formation in Calcium Saturated Conditions (Dialysis Gels). Homogeneous calcium gels from alginate samples listed in Table 1 were prepared by blending the aqueous polymer solution with $CaCO_3$ (15 mM) and GDL (30 mM) according to the experimental procedure reported in the previous section. After 24 h of curing, the gel cylinders were transferred to a gelling bath containing $CaCl_2$ (50 mM) and NaCl (200 mM) and maintained at 4 °C for 48 h prior to measurements. For the swelling experiments, calcium hydrogel cylinders obtained after the dialysis procedure were rapidly rinsed, immersed in a 150 mM NaCl solution (for each specimen, 20 mL of saline solution was used), and kept at 4 °C under gentle stirring for 24 h prior to measurements.

Syneresis of the calcium−alginate gels was determined as the weight reduction of the hydrogels with respect to the initial weight, calculated assuming a density value of 1. The alginate gels were gently dried off before the weighing procedure. The syneresis was calculated as $(1 - W/W_0) \times 100$, where W and W_0 are the final and initial weight of the gel cylinders, respectively.

Uniaxial Compression and Penetration To Break Measurements. A stable Micro System TA-XT2 texture analyzer (Stable Micro Systems, Godalming, U.K.), operating at 22 ± 1 °C and with a load cell of 50 N was used to measure the force/deformation curve of the hydrogels obtained from samples listed in Table 1. A constant compression speed of 0.1 mm/s was maintained in all cases. A parallel plate geometry with a 50-mm-diameter upper plate was used for the compression measurements.

The plot of force (F) applied in the compression tests versus the displacement recorded were converted into engineering stress (σ_E, Pa) vs engineering strain (ε_E) according to the following definitions (eq 1):[20]

$$\sigma_E = \frac{F(t)}{S_0}, \quad \varepsilon_E = \frac{H_0 - H(t)}{H_0} \tag{1}$$

where S_0 and H_0 are the initial cross-sectional area and height, respectively, of each specimen, and $H(t)$ is its instantaneous height. The Young's modulus (E, Pa) of the hydrogels obtained in the different conditions was calculated from the initial slope of the linear curve $\sigma_E - \varepsilon_E$.

In the penetration-to-failure tests, the deformation until breakage[31] was performed by means of a parallel plate geometry with a 2-mm-diameter upper plate, and the engineering strain at failure (ε_{Eb}) was evaluated. This allowed calculation of the work at break (J m^{-3}), $W_{E,b}$, as[20]

$$W_{E,b} = \int_0^{\varepsilon_{E,b}} \sigma_E \, d\varepsilon_E \tag{2}$$

Results and Discussion

(a) Effect of the Alternating Sequences on Swelling Behavior and Young's Modulus of Alginate Gels. Three different natural alginates from *L. hyperborea*, *M. pyrifera*, and *A. nodosum* have been considered together with the corresponding AlgE4-epimerized samples: LhypE4, MpyrE4, and AnodE4, respectively (Table 1). For the sake of clarity, the present section will resume some considerations and conclusions drawn on a previous paper.[21] It can be noticed that the ratio F_{MGM}/F_{GGM}, which is correlated with the length of the alternating sequences,[32] is significantly enhanced upon enzyme treatment. This statement is particularly evident when natural alginate samples containing significant amounts of M-block are considered (MpyrE4, +58%; AnodE4, +71%; whereas LhypE4 shows a bare 14% increase). Table 1 reveals, in addition, that the AlgE4 treatment of the natural alginate samples brings about but a negligible variation on both the average length of the G-blocks ($N_{G>1}$) and the intrinsic viscosity (i.e., overall molecular dimensions). The evaluation of the latter parameter and, hence, of the molecular weight of the alginate samples was performed, since this has been shown to notably affect the mechanical properties of the calcium hydrogels.[18]

Samples listed in Table 1 were used to prepare calcium-saturated gel cylinders, and the variations in their dimensions as well as in their mechanical properties in the linear viscoelastic range (i.e., Young's modulus) were recorded upon saline solution replacements. Figure 1a−c shows that although the hydrogels from *L. hyp.* display just a 5% increase in volume after two saline changes (Figure 1a), increases of ∼41% and 59% were found for cylinders obtained from *M. pyr.* and *A. nod.*, respectively (Figure 1b−c). This result can be largely expected in view of the notable differences in the average length of the G blocks (see Table 1)[35] that are considered as responsible for the more "crystallite" component of the gels. It is also noteworthy that gels obtained from *M. pyr.* are able to withstand a higher number of saline solution changes than those obtained from *L. hyp.*, although being characterized by a higher swelling. When AlgE4-treated alginates (i.e., LhypE4, MpyrE4, and AnodE4) are considered, a decrease in the swelling with respect to the natural samples is detected. This is particularly evident from the comparison between the epimerized and natural

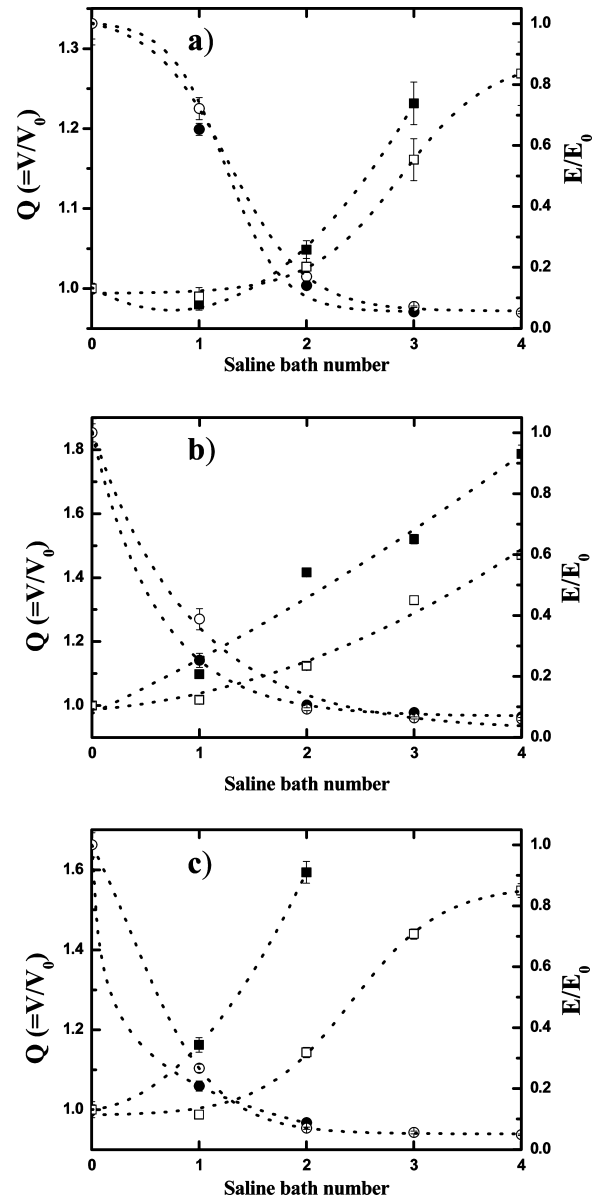


Figure 1. Dependence of the relative increase in volume (squares, left-end y-scale) and of the relative variation of the Young's modulus (circles, right-end y-scale) from the saline (NaCl 0.15M) bath number for (a) *L. hyp.* (solid symbols) and LhypE4 (open symbols), (b) *M. pyr.* (solid symbols) and MpyrE4 (open symbols), and (c) *A. nod.* (full symbols) and AnodE4 (open symbols). Lines are drawn to guide the eye. Numerical values are reported in Table 1 of the Supporting Information section. Data are reported as mean ± SD ($n = 5$).

alginate samples from *M. pyr.* and *A. nod.* (Figure 1b and c). In fact, increases of 7% and 16% relative dimensions are detected for gel cylinders from *M. pyr.* and *A. nod.*, respectively, after only 1 saline bath change. At variance, cylinders obtained from the AlgE4-treated samples (i.e., MpyrE4 and AnodE4) did not display any significant variation in volume after one change of NaCl solution. In view of the minor variations on $N_{G>1}$ detected for these alginates after AlgE4 epimerization, the enhanced swelling resistance achieved by epimerization necessarily stems from the elongation of the alternating sequences. It is important to note that these findings qualitatively parallel all previous reports on the stability of calcium gel beads obtained from natural and AlgE4-epimerized alginates.[24,34]

Figure 1a−c reports also the relative variation, at each change of the saline bath, of the Young's modulus (E) of the calcium gel cylinders obtained from natural and AlgE4-epimerized samples reported in Table 1. In all the cases analyzed, the replacement of the saline solution leads to a marked decrease in E, which can be safely traced back to the disruption of the gel junction zones caused by the displacement of the Ca^{2+} ions by the competing Na^+ ions (see the Supporting Information for an extended discussion). Surprisingly, the relative decrease in E of the natural alginate samples from *L. hyp.*, *M. pyr.*, and *A. nod.* is similar to that of their respective AlgE4-treated samples; that is, LhypE4, MpyrE4, and AnodE4. Thus, it seems that the difference in the equilibrium volume displayed by the AlgE4-epimerized samples, in particular by MpyrE4 and AnodE4, with respect to the natural samples is not caused by a higher resistance of the junctions themselves toward calcium displacement by competing sodium ions.

The swelling of alginate gels is a consequence of the balance between osmotic pressure in the gel (due to the Donnan effect of the free counterions) and the elastic reaction of the network (due to the elastically active chains) (see the Supporting Information). The osmotic pressure difference, Q_e, between the inside and outside of a polyelectrolyte hydrogel at constant ionic strength can be written, according to Candau et al., as[35,36]

$$Q_e \propto \alpha^{6/5} \cdot N_k^{4/5} \tag{3}$$

and the elastic modulus, E, as

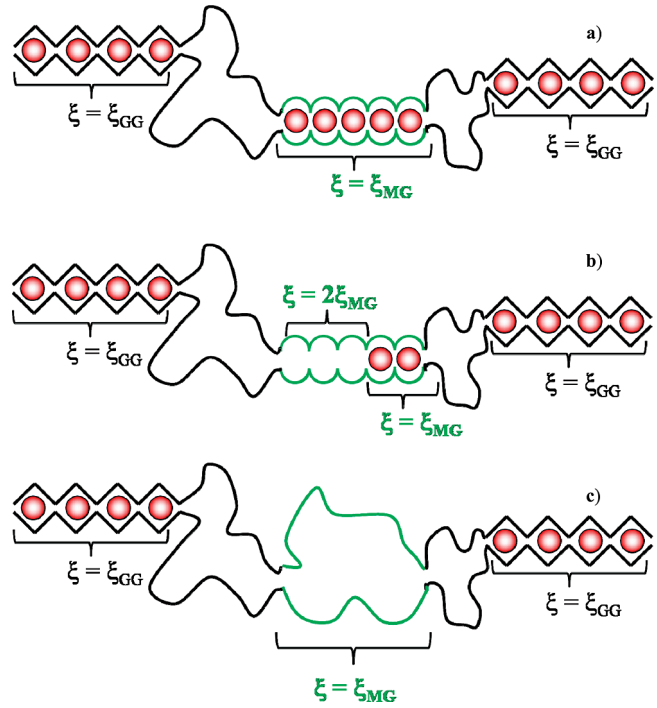
$$E \propto N_k^{-8/5} \cdot \alpha^{-2/5} \tag{4}$$

where N_k is the number of statistical chain units between cross-links and α is the ionization degree, thus reflecting the counterions of the polyelectrolyte contributing to the osmotic pressure.

Hence, in the case of the calcium−alginate system (a polyelectrolyte gel), it should be expected that a decrease in number of cross-links (i.e., an increase in the number of conformationally free chain segments) should be paralleled by the relative increase in the volume of the hydrogel. This is very well shown in Figures 9, 10, and 17 in the paper by Candau and co-workers.[36]

In the case of epimerized alginate gels, this theoretical prediction is not supported by the results in Figure 1a−c. Hence, a paradox is represented by the fact that, despite a similar relative decrease in the mechanical properties, the natural and the AlgE4-treated samples from *M. pyr.* and *A. nod.* do not show a parallel hydrogel volume increase upon saline bath treatment.

SCHEME 1: Graphical Representation of the MG-Rich Alginate Gels in Calcium Saturated Conditions (a) and after Treatments with Saline Solution (b and c)[a]



[a] MG junctions are represented in green.

It has been shown that the junction formation process between a cross-linking ion and two polyuronate strands can be efficiently described by using an extension of the Manning's counterion condensation theory, which assumes a complete charge annihilation between the positive charges of the coordinated cation and the negatively charged uronic groups.[15,16] It follows that the linear charge density of the equivalent GG/GG and MG/MG calcium-coordinated junctions can be considered to be equal to the one displayed by the isolated single polysaccharide chain (i.e., 1.6 and 1.5, respectively) (Scheme 1a). The fraction of ions noncontributing to the osmotic pressure (per mole of polymer fixed charge) for the gel-junction, f_{no}^J, can be calculated as follows:

$$f_{no}^J = \frac{1}{2} \cdot r^J + \frac{1}{2} \tag{5}$$

where the value 1/2 stems from the assumed stoichiometry of the egg-box-bonded calcium ion, and r^J, the total fraction of condensed counterions, reads[37]

$$r^J = \frac{1}{z_{Na^+} \cdot x_{Na^+} + z_{Ca^{2+}} \cdot (1 - x_{Na^+})} \cdot \left[1 - \frac{1}{\xi \cdot (z_{Na^+} \cdot x_{Na^+} + z_{Ca^{2+}} \cdot (1 - x_{Na^+}))}\right] \tag{6}$$

where z_{Na^+} and $z_{Ca^{2+}}$ are the valences of the Na^+ and Ca^{2+} ions, respectively, and x_{Na^+} and $x_{Ca^{2+}} = (1 - x_{Na^+})$ are the mole fractions of the Na^+ ions and of the Ca^{2+} ions of the whole of condensed counterions, respectively. Under the experimental conditions reported in the Materials and Methods section, f_{no}^J equals 0.68.

Once the treatment with the competing Na^+ ions takes place, some of the MG/MG junctions, which are likely to be less resistant than the ones involving the GG sequences,[22] start to unravel. This opening leads to the loss of stringent requirements for cation coordination and to a partial separation of some of the MG junctions. Due to the limited flexibility of the polysaccharide, the chains (each characterized by a value, ξ, of charge density) are close-by and will likely be sensed by a probe ion as a single equivalent line-of-charge with a doubled charge density (i.e., 2ξ) (Scheme 1b). An additional element favoring such a close arrangement might reside in the condensed ions-mediated attraction of the two like-charged polysaccharide chains, as originally proposed by Ray and Manning[38−40] and recently reformulated by Perico and co-workers.[41]

Upon removal of calcium ions, the fraction of ions not contributing to the osmotic pressure (per mole of polymer fixed charge) of the reeled off alginate chains, $f_{no}^{2\xi}$, reads

$$f_{no}^{2\xi} = r^{2\xi} \tag{7}$$

where

$$r^{2\xi} = \frac{1}{z_{Na^+}\cdot x_{Na^+} + z_{Ca^{2+}}\cdot(1 - x_{Na^+})}\cdot \left[1 - \frac{1}{2\cdot\xi\cdot(z_{Na^+}\cdot x_{Na^+} + z_{Ca^{2+}}\cdot(1 - x_{Na^+}))}\right] \tag{8}$$

In the present case, $f_{no}^{2\xi}$ equals 0.67. Hence, such a lateral dimer will not bring any additional contribution to the osmotic pressure once the calcium ions have been removed. However, at the same time, the number of segments, N_k, has increased, affecting much more E (eq 4: power of the scaling law −8/5) than Q_e (eq 3: power of the scaling law 4/5).

Overall, the partial opening of the MG/MG junctions will (i) reduce the elastic retractive force of the hydrogel by increasing N_k and, at the same time, (ii) not contribute to the osmotic force determining swelling of the hydrogel. This effect will be more marked when longer MG-blocks are present. Thus, this hypothesis explains reasonably well both the remarkable reduction in swelling of *M. pyr.* as compared to *A. nod.* (which has a similar dyadic composition but a much lower value of the F_{MGM}/F_{GGM} ratio) and their decrease in swelling upon AlgE4 treatment (Figure 1).

Eventually, the progressive saline change leads to a complete opening of the MG junctions (and of many of the GG-involving junctions as well). On this length scale, the resulting MG sequences are likely to be sensed by a probe ion as separate chains with their typical charge density (Scheme 1c). In these terms, the fraction of ions not contributing to the osmotic pressure (per mole of polymer fixed charge), $f_{no}^{\xi} = 1 - \xi^{-1}$, equals 0.33, and the osmotic force significantly contributes to the swelling of the hydrogel.

(b) Effect of the Alternating Sequences on Stress−Strain Curves at Large Deformations. It is commonly known that the stress−strain curve of alginate gels displays a linear region that extends approximately up to a deformation of 10−12% of the specimen, followed by a nonlinear trend described as "strain-hardening". The latter is a rather general phenomenon for biopolymer gels and consists of an upturn in the stress−strain curve once the linear response is exceeded. Additionally, a plastic region can also be present in the stress−strain curve, depending on the composition of the samples.[42] The strain-hardening behavior of alginate hydrogels has been traced back to the disruptive deformation of rigid junction zones rather than to the stretching of the network chain. It follows that the fracture or failure of the gel above a certain deformation can be safely attributed to the stress-induced opening of the primary junctions composing the hydrogel.[43]

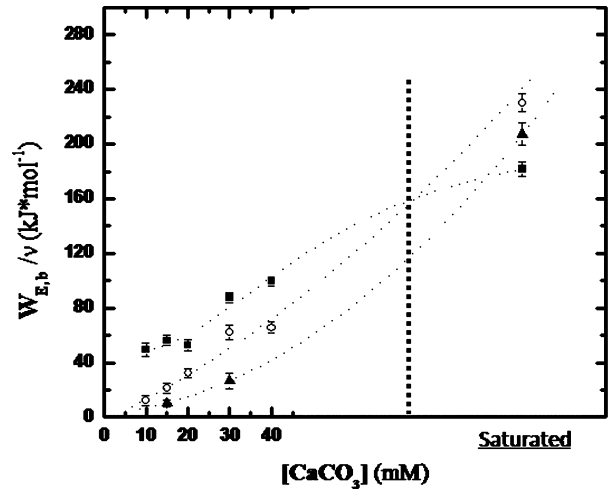


Figure 2. Dependence of the work at break per mole of junction ($W_{E,b}/\nu$) on the calcium concentration for (■) *L. hyp,* (○) *M. pyr.* and (▲) *A. nod.* Data are reported as mean ± SD (n = 8). Lines are drawn to guide the eye.

Alginate gels from the samples listed in Table 1 have been prepared by using different amounts of calcium and their compression until breakage was recorded. Once the work at break ($W_{E,b}$) (per unit volume)[20] and the number of equivalent junctions (per unit volume, ν) are estimated (see eq 2 in the Supporting Information and discussion therein on the front factor, ρ), the ratio $W_{E,b}/\nu$ (i.e., the energy required to break a mole of equivalent junction in the gel) has been calculated for the different natural alginate samples; namely, *L. hyp.*, *M. pyr.*, and *A. nod.* (Figure 2).

It can be noticed that when a low calcium concentration is used and, thus, a barely detectable syneresis is displayed,[21] the work at break per mole of equivalent junction ($W_{E,b}/\nu$) decreases along the series *L. hyp.* > *M. pyr.* > *A. nod.* This result nicely parallels the average length of the G-block of the different natural alginates (Table 1), leading us to conclude that the longer the GG/GG-junction formed in the gel, the higher the energy required to break it. Moreover, the trend of $W_{E,b}/\nu$ for the natural alginate samples at low calcium concentration points to the fact that the addition of calcium ions induces the formation of few but complete (rather than numerous but incomplete) junctions. This is in agreement with the concept of cooperative binding already proposed for alginate gels[18] and contradicts what has been previously suggested.[1]

When the calcium concentration used in the natural alginate samples is increased, a different trend $W_{E,b}$ is noticed. In particular, for saturated gels, $W_{E,b}$ decreases along the series *M. pyr.* > *A. nod.* > *L. hyp.* It is not realistic to think that upon calcium saturation, the resistance of the (shorter) junctions formed by *M. pyr.* is higher than those (longer) from *L. hyp.*; thus, it can be safely concluded that an additional mechanism could contribute to dissipate some of the energy applied during the large deformation (puncture) test and to enhance the elasticity of the MG-rich alginate samples. In this sense, there is a striking similarity between the trend found for $W_{E,b}/\nu$ and both the length of the MG sequences of the natural alginate

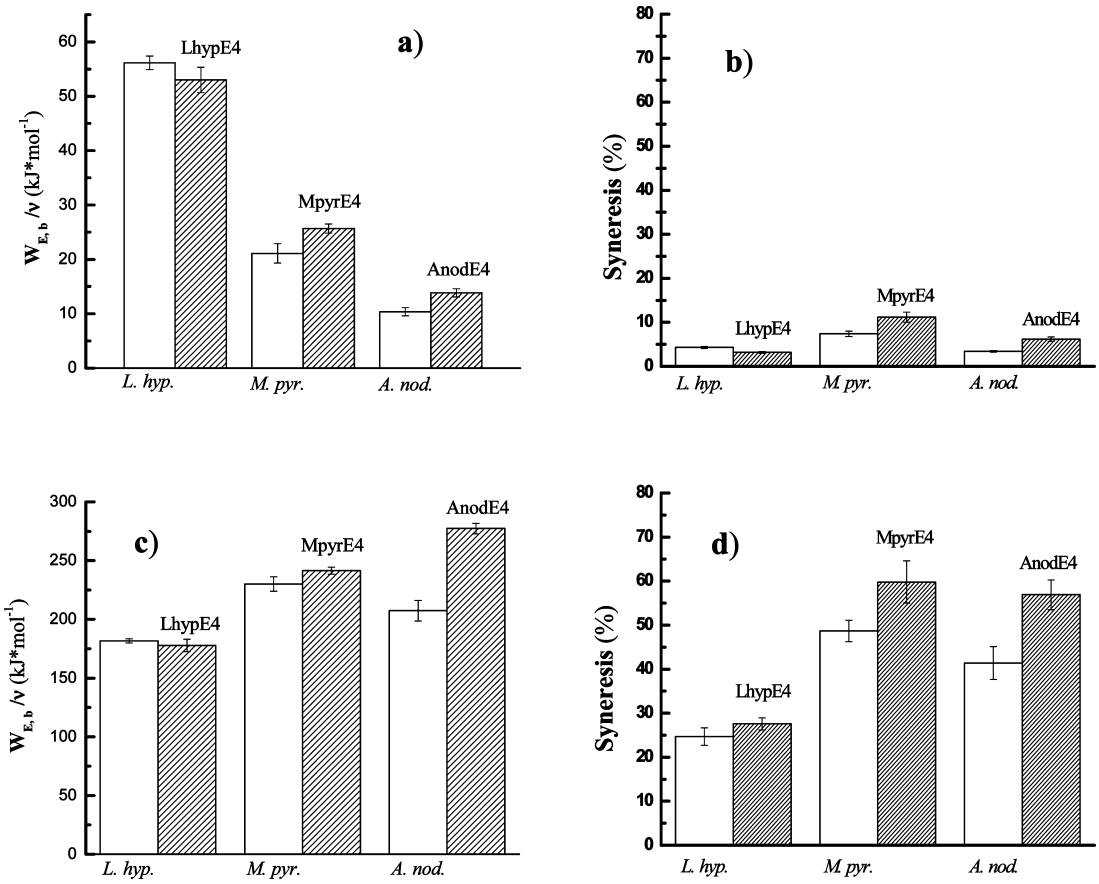


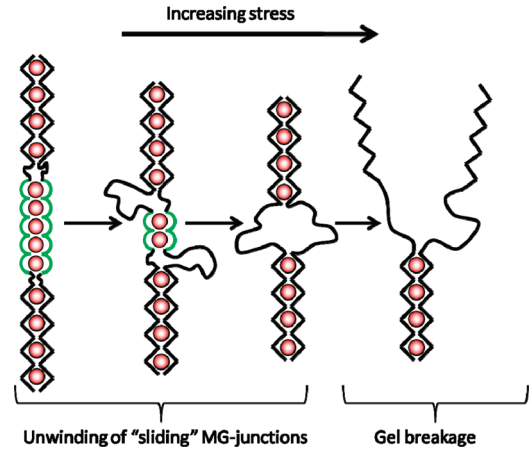
Figure 3. Work at break per mole of junction (a and c) and syneresis (b and d) for the natural and AlgE4-epimerized alginates reported in Table 1 in calcium-limited conditions (15 mM $CaCO_3$, a and b) and in calcium-saturated conditions (c and d). Data are reported as mean ± SD ($n = 8$).

samples (correlated with the F_{MGM}/F_{GGM} ratio in Table 1) and the syneresis measured for their calcium hydrogels (see Figure 3d).

From these considerations, one can propose MG/MG junctions to act as weak "reels" embedded within the hydrogel network that dissipate the stress applied by sliding over one other.[21,42] It follows that the longer the alternating sequences involved in MG/MG junctions, the higher the amount of energy that can be dissipated. The complete separation of the MG sequences will lead to an accumulation of the stress on the GG-containing junction, which will eventually open up, leading to gel breakage. The overall picture of the effect of long, alternating sequences in the dissipation of the energy can be depicted as in Scheme 2, where the "sliding" behavior of the MG/MG junctions is underlined.

To add further evidence to the present hypothesis, the analyses of the stress−strain curves at large deformations were performed on the epimerized samples listed in Table 1; that is, LhypE4, MpyrE4, and AnodE4 (Figure 3a and c). The minor elongation of alternating sequences achieved after treatment with the AlgE4 epimerase on *L. hyp.* brings about but a small variation in the work at break per mole of equivalent junctions. At variance, in the case of MpyrE4 and AnodE4, an increase in $W_{E,b}/v$ was detected with respect to the hydrogels obtained from natural alginate samples of *M. pyr.* and *A. nod.*, respectively, both for the calcium limited conditions and for the saturated gels (Figure 3a and c). This trend is paralleled also by the syneresis of the gel (Figure 3b and d), which was previously correlated with the possible formation of MG/MG junctions. Overall, these results point to a relevant role of the "unzipping" process of elongated alternating sequences in the dissipation of the applied stress.

SCHEME 2: Graphical Representation of the "Sliding" MG/MG Junctions upon Application of a Stress[a]



[a] MG junctions are represented in green.

Conclusions

Alginate gels represent very challenging semisolid structures with applications in biotechnological and industrial fields. The ability to control and tune the mechanical properties of alginate gels represents a key feature for improving the applications of this biomaterial. The cornerstone of this work is the availability

of the powerful tool represented by AlgE4 epimerase, which by elongating the MG sequences allows monitoring the effect of compositional modifications on the mechanical properties of the alginate gels. Recently, the importance of the alternating sequences in alginate gels has been pointed out, and their direct involvement in the network architecture of the polyuronate hydrogel has been proposed. The present communication extends this view and aims at allocating different peculiar aspects of the behavior of alginate gels to the presence of MG/MG junctions. In particular, the swelling behavior of the hydrogels toward competing monovalent ions and the work at break per mole of junctions have been considered. Since both of these characteristics are affected by MG elongation, we propose the following hypotheses to describe their role in alginate gels:

(i) The addition of Ca^{2+} ions induces the formations of the GG-containing junctions (it is not the scope of the present article to discriminate between GG/GG and GG/MG junctions).

(ii) The addition of an excess of cross-linking ions induces the formation of MG/MG junctions, leading to a collapsed network. In view of the dependence of the syneresis on the length of the alternating sequences,[21,44] this "zipping" mechanism might also be responsible for this ion-induced water release (shrinkage) from the gel.

(iii) The MG/MG junctions represent "reels" inside the alginate hydrogel that, in response to the presence of competing ions or to the application of a stress, can be opened up, accounting for a lower swelling behavior and a higher elasticity of the gel.

(iv) In the case of the competing ions, the partial disentangling of the MG/MG junctions leads to the formation of polymer threads with a doubled linear charge density. This induces a decrease in the mechanical properties without affecting the osmotic pressure within the hydrogel.

(v) As to the elasticity of the hydrogels, the application of a stress induces a "sliding" of the MG/MG junctions, which dissipates the energy applied. Experimentally, this is detected as a higher work at break per mole of junctions in AlgE4-treated alginate samples.

Acknowledgment. This work was supported by a grant to I.D. (Progetto Giovani Ricercatori 2006) from the University of Trieste. The authors would like to thank the reviewers of the manuscript for their suggestions.

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

References and Notes

(1) Zhang, J.; Daubert, C. R.; Foegeding, E. A. *J. Food Eng.* **2007**, *80* (1), 157–165.
(2) Kvam, B. J.; Fragonas, E.; Degrassi, A.; Kvam, C.; Matulova, M.; Pollesello, P.; Zanetti, F.; Vittur, F. *Exp. Cell Res.* **1995**, *218* (1), 79–86.
(3) Williams, J. M.; Zhang, J.; Kang, H.; Ummadi, V.; Homandberg, G. A. *Osteoarthr. Cartilage* **2003**, *11* (1), 44–49.
(4) Brun, P.; Cortivo, R.; Zavan, B.; Vecchiato, N.; Abatangelo, G. *J. Mater. Sci.: Mater. Med.* **1999**, *10* (10), 683–688.
(5) de Vos, P.; Faas, M. M.; Strand, B.; Calafiore, R. *Biomaterials* **2006**, *27* (32), 5603–5617.
(6) Donati, I.; Stredanska, S.; Silvestrini, G.; Vetere, A.; Marcon, P.; Marsich, E.; Mozetic, P.; Gamini, A.; Paoletti, S.; Vittur, F. *Biomaterials* **2005**, *26* (9), 987–998.
(7) Soon-Shiong, P.; Heintz, R. E.; Merideth, N.; Yao, Q. X.; Yao, Z.; Zheng, T.; Murphy, M.; Moloney, M. K.; Schmehl, M.; Harris, M.; Mendez, R.; Mendez, R.; Sandford, P. A. *Lancet* **1994**, *343* (8903), 950–951.
(8) Turco, G.; Marsich, E.; Bellomo, F.; Semeraro, S.; Donati, I.; Brun, F.; Grandolfo, M.; Accardo, A.; Paoletti, S. *Biomacromolecules* **2009**, *10* (6), 1575–1583.
(9) Smidsrød, O. *Faraday Discuss.* **1974**, *57*, 263–274.
(10) Morris, E. R.; Rees, D. A.; Thom, D.; Boyd, J. *Carbohydr. Res.* **1978**, *66* (1), 145–154.
(11) Donati, I.; Paoletti, S. Material Properties of Alginates. In *Alginates; Biology and Applications*; Rhem, B. H. A., Ed.; Springer-Verlag: Berlin, Heidelberg, 2009; in press.
(12) Li, L.; Fang, Y.; Vreeker, R.; Appelqvist, I.; Mendes, E. *Biomacromolecules* **2007**, *8* (2), 464–468.
(13) Sikorski, P.; Mo, F.; Skjåk-Bræk, G.; Stokke, B. T. *Biomacromolecules* **2007**, *8* (7), 2098–2103.
(14) Braccini, I.; Pérez, S. *Biomacromolecules* **2001**, *2* (4), 1089–1096.
(15) Donati, I.; Benegas, J. C.; Cesaro, A.; Paoletti, S. *Biomacromolecules* **2006**, *7* (5), 1587–1596.
(16) Donati, I.; Benegas, J. C.; Paoletti, S. *Biomacromolecules* **2006**, *7* (12), 3439–3447.
(17) Mitchell, J. R.; Blanshard, J. M. V. *J. Texture Stud.* **1976**, *7* (2), 219–234.
(18) Draget, K. I.; Simensen, M. K.; Onsøyen, E.; Smidsrød, O. *Hydrobiologia* **1993**, *260−261* (1), 563–565.
(19) Zhang, J.; Daubert, C. R.; Foegeding, E. A. *J. Food Sci.* **2005**, *70* (7), e425–e431.
(20) Mancini, M.; Moresi, M.; Rancini, R. *J. Food Eng.* **1999**, *39* (4), 369–378.
(21) Donati, I.; Holtan, S.; Mørch, Y. A.; Borgogna, M.; Dentini, M.; Skjåk-Bræk, G. *Biomacromolecules* **2005**, *6* (2), 1031–1040.
(22) Dentini, M.; Rinaldi, G.; Barbetta, A.; Risica, D.; Anselmi, C.; Skjåk-Bræk, G. *Carbohydr. Polym.* **2007**, *67* (4), 465–473.
(23) Mørch, Y. A.; Donati, I.; Strand, B. L.; Skjåk-Bræk, G. *Biomacromolecules* **2006**, *7* (5), 1471–1480.
(24) Mørch, Y. A.; Donati, I.; Strand, B. L.; Skjåk-Bræk, G. *Biomacromolecules* **2007**, *8* (9), 2809–2814.
(25) Ertesvåg, H.; Doseth, B.; Larsen, B.; Skjåk-Bræk, G.; Valla, S. *J. Bacteriol.* **1994**, *176* (10), 2846–2853.
(26) Ertesvåg, H.; Høidal, H. K.; Skjåk-Bræk, G.; Valla, S. *J. Biol. Chem.* **1998**, *273* (47), 30927–30932.
(27) Svanem, B. I. G.; Skjåk-Bræk, G.; Ertesvåg, H.; Valla, S. *J. Bacteriol.* **1999**, *181* (1), 68–77.
(28) Ertesvåg, H.; Skjåk-Bræk, G. Modification of alginates using mannuronan C-5 epimerases. In *Methods in Biotechnology*, 10th ed.; Bucke, C., Ed.; Humana Press: Totowa, NJ, 1999; pp 71−78.
(29) Holtan, S.; Zhang, Q.; Strand, W. I.; Skjåk-Bræk, G. *Biomacromolecules* **2006**, *7* (7), 2108–2121.
(30) Grasdalen, H.; Larsen, B.; Smidsrød, O. *Carbohydr. Res.* **1979**, *68* (1), 23–31.
(31) Pons, M.; Fiszman, S. M. *J. Texture Stud.* **1996**, *27* (6), 597–624.
(32) Grasdalen, H.; Larsen, B.; Smisrød, O. *Carbohydr. Res.* **1981**, *89* (2), 179–191.
(33) Skjåk-Bræk, G.; Smidsrød, O.; Larsen, B. *Int. J. Biol. Macromol.* **1986**, *8* (6), 330–336.
(34) Strand, B. L.; Mørch, Y. A.; Syvertsen, K. R.; Espevik, T.; Skjåk-Bræk, G. *J. Biomed. Mater. Res., Part A* **2003**, *64* (3), 540–550.
(35) Nisato, G.; Schosseler, F.; Candau, S. J. *Polym. Gels Networks* **1996**, *4*, 481–498.
(36) Skouri, R.; Schosseler, F.; Munch, J. P.; Candau, S. J. *Macromolecules* **1995**, *28* (1), 197–210.
(37) Paoletti, S.; Benegas, J.; Cesàro, A.; Manzini, G.; Fogolari, F.; Crescenzi, V. *Biophys. Chem.* **1991**, *41* (1), 73–80.
(38) Ray, J.; Manning, G. S. *Langmuir* **1994**, *10* (7), 2450–2461.
(39) Ray, J.; Manning, G. S. *Macromolecules* **1997**, *30* (19), 5739–5744.
(40) Ray, J.; Manning, G. S. *Macromolecules* **2000**, *33* (8), 2901–2908.
(41) Pietronave, S.; Arcesi, L.; D'Arrigo, C.; Perico, A. *J. Phys. Chem. B* **2008**, *112* (50), 15991–15998.
(42) Mørch, Y. A.; Holtan, S.; Donati, I.; Strand, B. L.; Strand, W. I.; Skjåk-Bræk, G. *Biomacromolecules* **2008**, *9*, 2360–2368.
(43) Kong, H. J.; Wong, E.; Mooney, D. J. *Macromolecules* **2003**, *36* (12), 4582–4588.
(44) Draget, K. I.; Gåserød, O.; Aune, I.; Andersen, P. O.; Storbakken, B.; Stokke, B. T.; Smidsrød, O. *Food Hydrocolloids* **2001**, *15* (4−6), 485–490.

JP905488U