Molecular Engineering as an Approach to Design New Functional Properties of Alginate

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Through enzymatic modification, we are now able to manipulate the composition and sequential nanostructures of alginate, one of the most versatile gelling polymers found in nature. Here we report the application of a set of processive polymer-modifying epimerases for the preparation of novel alginates with highly improved functional properties essential for numerous applications as gel matrices. Gels of enzymatically engineered alginate were found to be more elastic and compact, less permeable, and extremely stable under physiological conditions, offering significant advantages over native alginates. As a result, this study shows that, by controlling alginate nanostructure, its macroscopic properties can be highly controlled. The ability to tailor alginate has a great impact on the wide use of this biomaterial in industry and medicine. More importantly, this adds more knowledge to the link between polymer nanostructure and macroscopic properties and may serve as a model system for other polymer-based materials.

Introduction

Alginate, a collective term for a family of polysaccharides synthesized by seaweed and bacteria, is used in a wide range of food, pharmaceutical, and specialty applications for thickening, stabilizing, gelling, and film forming.[1] Since these polymers have the unique ability to form strong gels under physiological conditions, seaweed alginate has become the most extensively used material for immobilizing living cells[2] as well as for tissue engineering.[3] The successful use of alginates as an immobilization material has been hampered by their mechanical instability, osmotic swelling under physiological conditions, high porosity, and wide pore size distribution. Moreover, as nature-derived polymers, alginates are compositionally heterogeneous, thereby hampering many of their applications.

In molecular terms, alginates are binary linear polysaccharides composed of the two monomers—α-L-guluronic (G) and β-D-mannuronic (M) acid, which form M blocks, G blocks, and blocks of alternating sequence (MG).[4] In nature, alginates are found to exhibit great variation in composition and arrangement of the two monomers in the polymer chain. Blocks of repeating G units (G blocks) form cavities that bind divalent cations that cross-link G blocks of other alginate chains.[5] Hence, G-block sequences are required for the alginate to form a strong gel with divalent ions such as Ca^{2+}, Ba^{2+}, and Sr^{2+}. A strong correlation therefore exists between the sequential structure and functional properties of alginates.

Biosynthesis of alginates proceeds by polymerization of a mannuronan homopolymer, followed by a post-polymerization (C-5 epimerization) in which some of the M residues are converted to G units.[6] This is an analogous reaction to the conversion of D-glucuronic acid into L-iduronic acid in dermatan[7] and heparin/heparin sulfate.[8] Various recombinantly produced mannuronan C-5 epimerases, based on genes from *Azotobacter vinelandii*, are now available[9] and were used in the present study to modify the nanostructures of alginates in vitro. Our goal was to characterize these structures and test the macroscopic properties of the resultant materials. As alginate microbeads are widely used as immune barriers for cell transplantation, specific properties were studied with respect to their function as an entrapment material for cells.

Materials and Methods

Alginates and Epimerases. High molecular weight sodium alginate samples isolated from *Macrocystis pyrifera* (high-M alginate) and *Laminaria hyperborea* stipe (high-G alginate) were obtained from Sigma Chemicals and FMC Biopolymer, respectively. High molecular weight mannuronan was isolated from an epimerase-negative mutant ($AlgG^-$) of *Pseudomonas fluorescens*.[10] Polyalternating alginate (polyMG, 46% G) was prepared from mannuronan using the epimerase AlgE4. G blocks were introduced into the alginate chain by a second epimerization with the AlgE1 epimerase, ending up with alginate samples without M blocks and with G content from 50 to 91%. Specifications for the epimerization reaction are given elsewhere.[11] The total G content in the final product was controlled by varying the epimerization time and enzyme concentration.

The mannuronan epimerases AlgE1 and AlgE4 were produced by fermentation of recombinant *Escherichia coli* strains as described previously.[11]

NMR Spectroscopy. ^1H NMR spectra were recorded on a Bruker Advance DPX 300 spectrometer at 90 °C. Spectra were obtained using a pulse repetition time of 5.6 s and a 30° pulse angle. 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 (Aldrich) as the internal standard.

Mechanical Properties. Alginate gel cylinders were made by internal gelling using the $CaCO_3$/glucono-δ-lactone method[12] followed by dialysis against 50 mM $CaCl_2$ in 0.2 M NaCl overnight at 4 °C to form Ca-saturated gels. Syneresis of the gels was determined as the

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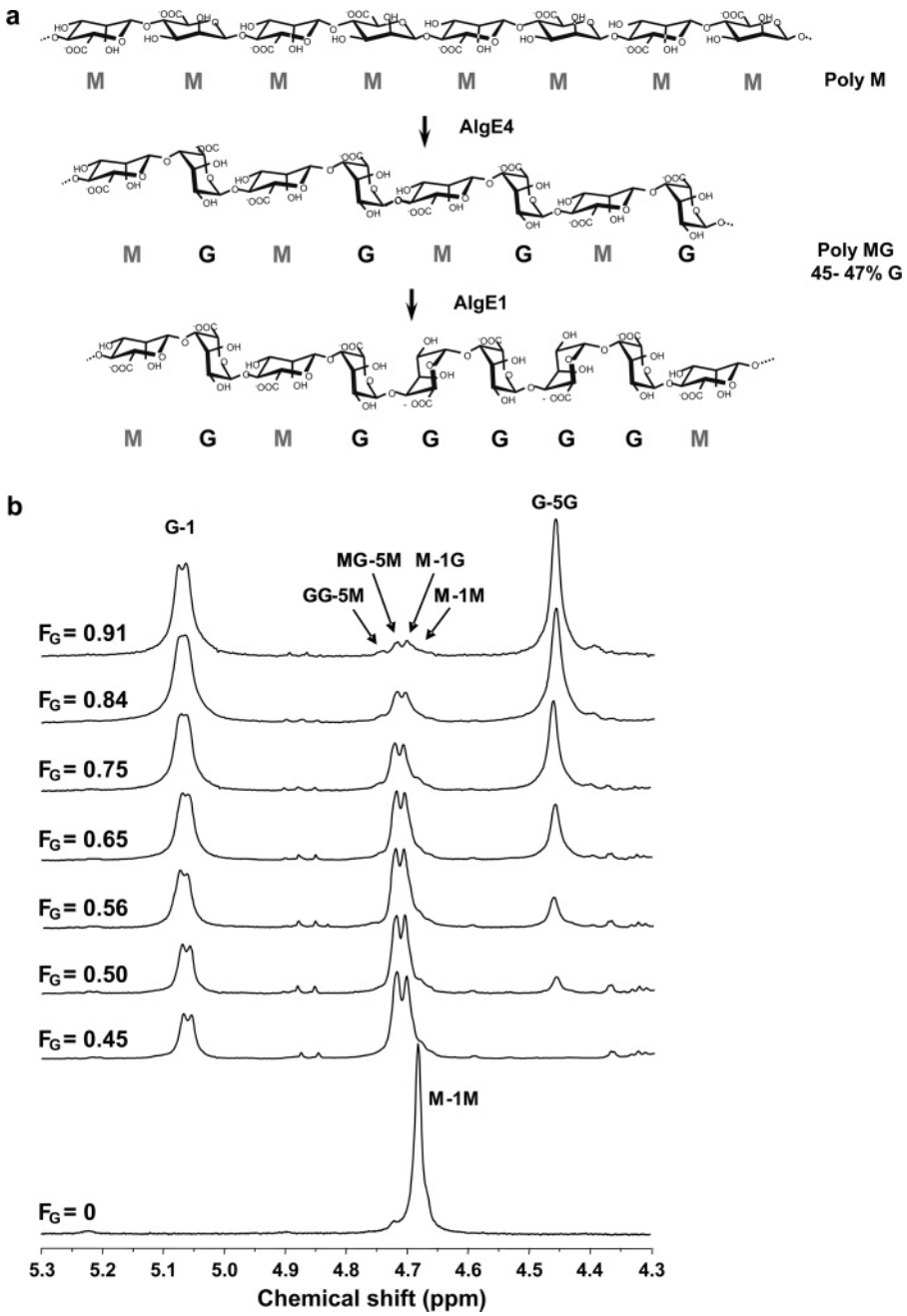

Figure 1. (a) Illustration of the epimerization of mannuronan (polyM) with two C-5 epimerases to form an alginate containing G blocks and MG blocks solely (no M blocks). The first step using AlgE4 introduces single guluronic acid residues, resulting in an alginate with alternating structure (polyMG) containing 45−47% G. The second step, involving AlgE1, introduces G blocks into the alginate chain. (b) ^{1}H NMR spectra of epimerized alginates starting from mannuronan ($F_G = 0$). PolyMG ($F_G = 0.45$) is formed by epimerization of mannuronan with the C-5 epimerase AlgE4, followed by epimerization with epimerase AlgE1, introducing G blocks in various amounts into the alginate chain ($F_G = 0.5-0.91$). The total G content in the end product was controlled by the time of epimerization.

weight reduction of gel cylinders with respect to the initial weight, assuming a density value of 1. Gels were compressed 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.

Fabrication of Microbeads. Microbeads were made by dripping a 1.8% (w/v) sterile filtered alginate solution (in 0.3 M mannitol, pH 7.4) into a solution of 50 mM $CaCl_2$ (in 0.15 M mannitol, pH 7.4 with 0.1% Tween20) using an electrostatic bead generator (7 kV, 10 mL/h, steel needle with outer diameter 0.4 mm, 1.7 cm distance from the needle to the gelling solution).

Alginate Distribution in the Gel. A fraction (1/2500) of the carboxylic groups in alginate were labeled with fluoresceinamine ($C_{20}H_{13}NO_3$, mixed isomers, Sigma) as previously described.[13] Alginate gel beads were visualized using a confocal laser scanning microscope (CLSM) (Zeiss). Images were obtained by a mean of eight scans through an equatorial slice of the beads. For visualization of alginate, a 488 nm Argon laser was used, and the fluorescence was detected with a 500−550 nm bypass filter.

Stability Measurements. Gel beads were made as described above and kept in the gelling solution overnight. A 3 mL portion of physiological saline solution (0.9% w/v) was added to 0.5 mL of gel beads after removal of the gelling solution. The sample was kept on a turn-over stirrer for 1 h before the saline was removed and renewed. The saline solution was exchanged seven times. The diameter of beads was determined before each change of saline solution.

Ion Binding. A 100 μL portion of alginate (2% w/v in water) was dripped into a 50 mM $CaCl_2$ solution and kept overnight. The Ca^{2+} solution was removed, and 10 mL of saline was added. After 1 h, the

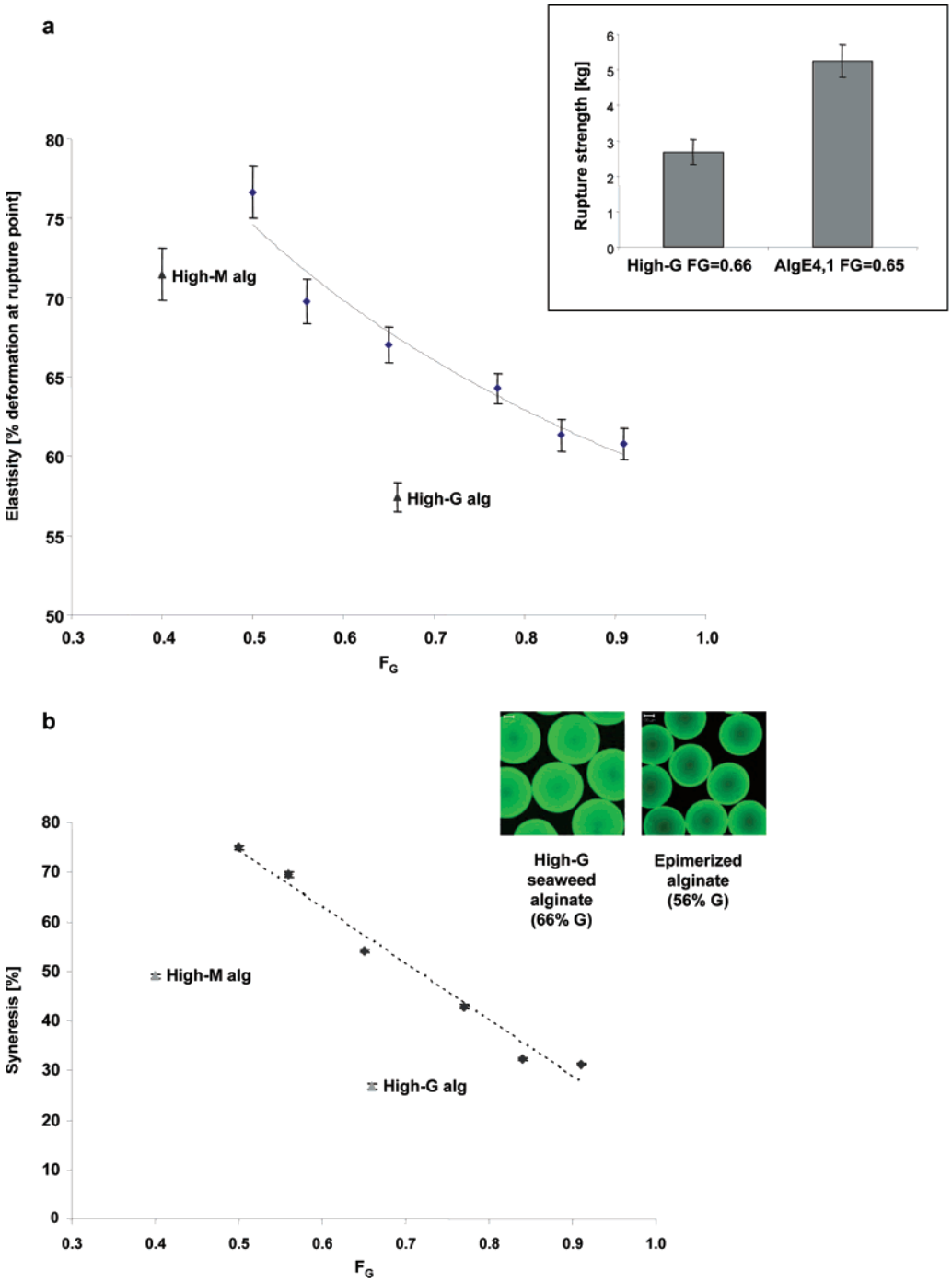

Figure 2. Elasticity and syneresis (shrinkage) of gel cylinders of two seaweed alginates (high-G, $F_G = 0.66$, and high-M, $F_G = 0.40$) and AlgE1-epimerized polyMG alginates with different G contents. The gels were made from 1% (w/v) alginate by internal gelling in calcium followed by dialysis in 50 mM CaCl2. (a) Elasticity as a percentage of deformation of gel cylinders at the gel rupture point. The rupture strength of an epimerized alginate with a G content similar to that of a native alginate is also given to illustrate the difference in rupture strength. (b) Syneresis (shrinkage) of gel cylinders as a function of G content in the alginate. Values are means ± standard deviation (SD) of eight parallels. The lines are drawn to guide the eye. The inset shows CLSM images of gel beads of fluorescence-labeled alginate (green), showing the distribution of polymer in the beads. Images are taken as an optical slice through the bead equator.

NaCl was renewed. The procedure was repeated twice before the alginate was placed in 5 mL of 50 mM Na−citrate until the gel dissolved. The concentration of Ca^{2+} in the NaCl and Na−citrate solutions was determined using inductively coupled plasma mass spectroscopy (ICP-MS).

Permeability Measurements. The permeability of radio-labeled immunoglobulin G (IgG) into alginate gel beads was studied using encapsulated paramagnetic mono-sized polymer particles (Dynabeads M-450 Tosylactivated, Dynal).[14] The Dynabeads were coupled with monoclonal mouse anti-human tumor necrosis factor (TNF) antibodies.[15] Briefly, the Dynabeads were encapsulated into alginate beads by mixing a Dynabead suspension with Na−alginate to a final concentration of 0.4 mg Dynabeads/mL alginate, and beads were made from exactly 1.5 mL of alginate solution using the procedure described above. The alginate beads with encapsulated Dynabeads were washed three times in 5 mL of saline and kept in 0.01 M Tris buffer, 0.9% (w/v) NaCl, 0.1% (w/v) bovine serum albumin, and 0.1% (w/v) Na−azide before adding 100 μL of [^{125}I]-labeled anti-mouse IgG from sheep (Amersham Biosciences). The mixture was kept on a turn-over stirrer for 20 h, and the capsules were washed three times before the amount of radio-labeled

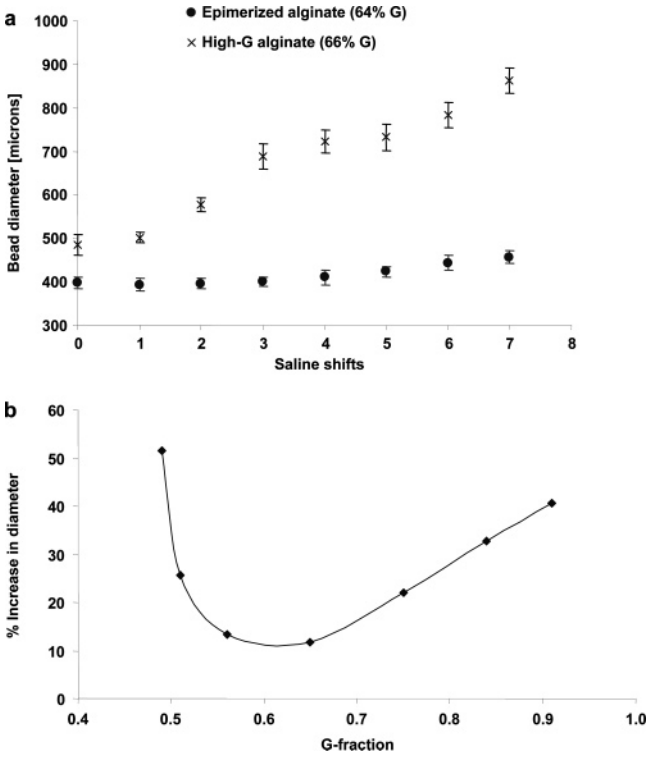

Figure 3. Swelling of alginate beads in physiological saline solution measured as an increase in diameter. The NaCl solution was exchanged every hour for 7 h. (a) Increase in bead diameter as a function of the number saline exchanges for epimerized alginate beads ($F_G = 0.65$) and natural alginate ($F_G = 0.66$). Values are given as the means ± SD of 30 beads. (b) Swelling of beads of AlgE1-epimerized polyMG alginate as a function of G content after seven shifts in saline solution.

IgG bound to encapsulated Dynabeads was measured in a gamma counter.

Results and Discussion

Enzymatic Modification. The structural modification of alginate is based on a two-step process outlined in Figure 1a. Starting with a homopolymeric mannuronan of high molecular weight derived from an epimerase-negative *Pseudomonas fluorescence* mutant,[10] an epimerization reaction was carried out with the C-5 epimerase AlgE4. This enzyme epimerizes every second residue, converting the entire polysaccharide backbone into a strictly polyalternating sequence without breaking the macromolecular chain.[16] The resulting polyMG alginate was further processed by a second C-5 epimerase, AlgE1. This enzyme acts processively on polyMG by converting M residues flanked by G or by condensing G blocks, thus resulting in initially very long blocks of G residues (>40 residues)[11] (Figure 1a,b). The total G content in the end product was controlled by the time of epimerization. Characterization of novel alginates with variable G contents was performed using ^{1}H NMR spectroscopy (see Appendix) and intrinsic viscosity measurements (Table 1). The ^{1}H NMR spectra of alginates used in the present study (Figure 1b) show how the signal from two neighboring M residues (M−1M) in the starting mannuronan material disappears upon "full" epimerization with AlgE4, which introduces single G residues in the alginate chain. Introduction of G blocks by AlgE1 is evident from an intensity increase for the signal from neighboring G residues (G−5G) upon epimerization time, with a simultaneous decrease in the M signal (G−5M, M−1G). The low signal from the end of the G blocks (G blocks neighboring an M, GG−5M) suggests long homopolymeric stretches of G residues in epimerized materials. Thus, the most important structural features distinguishing these engineered polymers from seaweed or bacterial alginates are the *absence* of M blocks and the high content of long G blocks. Consequently, they are compositionally more homogeneous than wild-type materials.

Mechanical Properties of Alginate Hydrogels. The novel alginates were tested for their macroscopic properties. The elasticity of epimerized Ca−alginate gels, determined by compression measurements of gel cylinders to their point of rupture (Figure 2a), was found to be highly dependent on their G content. Furthermore, these gels displayed a considerable increase in elasticity compared to seaweed alginates with comparable G content. As a result, these gels will be less prone to rupture, as demonstrated in the same figure showing the rupture strength of a seaweed alginate compared to an epimerized alginate of similar G content.

Syneresis, the shrinkage of hydrogels with concomitant release of water, will, because of the different flexibilities of the three blocks in alginate,[2,17] depend on the composition of monomers in the polymer chain. The result of syneresis is smaller gels with a higher total concentration of alginate, leading to a more compact and stronger hydrogel network. Figure 2b shows how epimerization led to a high degree of syneresis compared to that found in seaweed alginates, probably as a result of denser packing of the alginate chains due to the high amount of elastic (flexible) MG blocks in the enzymatically modified alginate. In addition, we have previously proposed that syneresis of hydrogels can be related to the length of alternating sequences[18] where long stretches of MG blocks present in the epimerized material may induce a partial network collapse. The

Table 1. Composition, Sequence, and Intrinsic Viscosity [η] of all Samples Used in the Study[a]

alginate	abbreviation	F_G	F_M	F_{GG}	F_{MM}	$F_{GM=MG}$	[η][b] (mL/g)
Laminaria hyperborea stipe	high-G	0.66	0.34	0.55	0.22	0.12	619
Macrocystis pyrifera	high-M	0.40	0.60	0.21	0.40	0.20	823
polyalternating	polyMG	0.45	0.55	0	0.11	0.45	1154
AlgE1-epimerized polyMG	AlgE4,1 $F_G = 0.50$	0.50	0.50	0.10	0.11	0.40	595
AlgE1-epimerized polyMG	AlgE4,1 $F_G = 0.56$	0.56	0.44	0.21	0.09	0.35	699
AlgE1-epimerized polyMG	AlgE4,1 $F_G = 0.65$	0.65	0.35	0.36	0.06	0.29	780
AlgE1-epimerized polyMG	AlgE4,1 $F_G = 0.67$	0.67	0.33	0.36	0.03	0.30	951
AlgE1-epimerized polyMG	AlgE4,1 $F_G = 0.75$	0.75	0.25	0.56	0.06	0.19	498
AlgE1-epimerized polyMG	AlgE4,1 $F_G = 0.77$	0.77	0.23	0.65	0.10	0.13	758
AlgE1-epimerized polyMG	AlgE4,1 $F_G = 0.84$	0.84	0.16	0.73	0.04	0.12	895
AlgE1-epimerized polyMG	AlgE4,1 $F_G = 0.91$	0.91	0.09	0.84	0.02	0.07	1002

[a] *F* denotes the fraction of monomers and monomer sequences found in these samples. [b] The intrinsic viscosity was measured at 20 °C in 0.1 M NaCl aqueous solution in a Micro Ubbelohde viscometer.

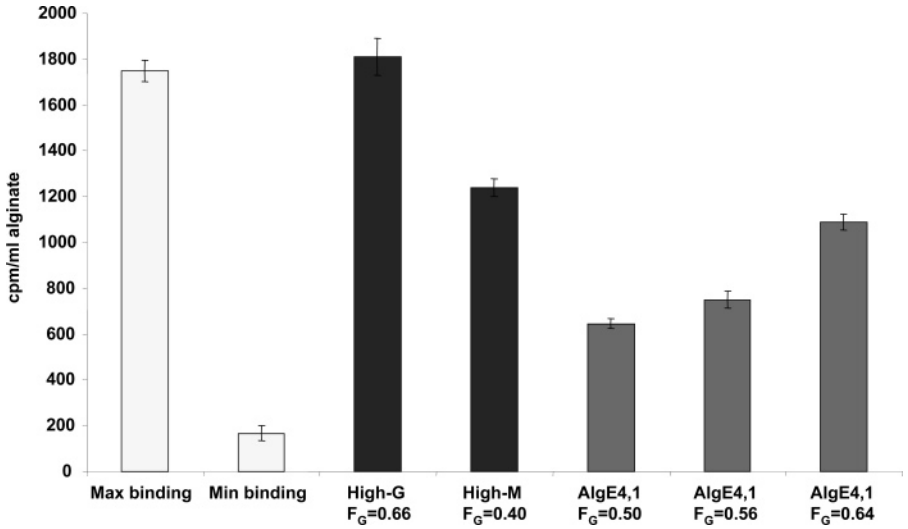

Figure 4. Permeability of [^{125}I]-IgG into beads of AlgE1-epimerized polyMG alginate given as counts per minute (cpm) per milliliter of alginate. Controls are two natural alginates: High-G ($F_G = 0.66$) and high-M ($F_G = 0.40$). Positive and negative controls are measured by the binding of IgG to free Dynabeads in solution and empty beads, respectively. Values are means ± SD of three parallels.

Table 2. Ca Content in 100 μL of Alginate as Measured with ICP-MS[a]

	amount of Ca^{2+} [μg]				
sample	in 1st NaCl solution	in 2nd NaCl solution	in 3rd NaCl solution	in Na−citrate	total in Ca−alginate gel
polyMG $F_G = 0.45$	150 ± 7	48 ± 10	26 ± 2	1 ± 0.1	225
high-G $F_G = 0.66$	263 ± 34	52 ± 15	36 ± 18	3 ± 0.3	354
AlgE4,1 $F_G = 0.65$	258 ± 19	36 ± 5	19 ± 2	72 ± 8	385
blank	0.2	0.2	0.2	0.9	1.5

[a] The values are means of three parallels ± SD.

effect of syneresis can also be visualized by CLSM of fluorescently labeled alginate gel beads (Figure 2b, inset). Microbeads became smaller upon epimerization, leading to a higher degree of inhomogeneity in polymer distribution in gel beads, with the concentration of alginate being higher at the bead surface than at its center.

Stability of Microcapsules. An alginate gel can be viewed as an osmotic swelling system in which the osmotic contribution to swelling is dominated by dissociable counterions in the network. This swelling is counterbalanced by the elasticity of the network, represented by the number and strength of junctions.[19] The stability of alginate gels was evaluated in physiological saline solution. This allows the exchange of cross-linking Ca^{2+} with non-cross-linking Na^+ ions, leading to both destabilization of the junctions in the gel network and a higher concentration of dissociable counterions in the alginate bead with concomitant influx of water. The high osmotic pressure in the gel and the removal of stabilizing Ca^{2+} ions will result in swelling and dissolution of the gel beads. Figure 3a shows the increase in diameter of alginate beads as a function of saline solution changes, illustrating the effect of epimerization on gel bead stability. Surprisingly, after seven shifts in saline, the bead diameter of epimerized alginate with a G content of 64% had increased by only 15%. In comparison, a seaweed alginate with similar G content (66%) had swelled by 78% after seven shifts. The swelling of epimerized alginate beads was also found to be highly dependent on G content (Figure 3b), with a minimum at approximately 60% G. Interestingly, the introduction of only a small percentage of G blocks is enough to stabilize the gel network significantly.

In order to elucidate the very high stability of the enzymatically modified material, the binding of Ca^{2+} to different alginate materials was quantitatively measured using ICP-MS. Table 2 shows that the leakage of Ca^{2+} out of the gel was lower for epimerized material compared to seaweed alginate with a similar total G content, indicating that Ca^{2+} binds with higher affinity to alginates strictly composed of MG and GG blocks. The direct involvement of MG blocks in an alginate gel in forming both mixed GG/MG and MG/MG junctions with Ca^{2+} has recently been proposed.[18] Because of the conversion of M blocks into MGM sequences in the epimerized alginate samples, a larger amount of Ca^{2+} ions will be bound in junction zones in the gel network. In the case of alginate, only the free counterions will contribute to osmotic pressure.[19] Hence, the epimerized material will be less prone to swelling. In addition, the elasticity term in the swelling equation will be larger due to a higher number of cross-linked chains (higher concentration of alginate), probably also counteracting the swelling.

We have recently shown that polyMG can form stable hydrogels in the presence of calcium.[18] The binding of Ca^{2+} in this alginate will, however, be easily exchanged with Na^+ because of low selectivity binding compared to the cooperative binding in junctions between G blocks.[2] In addition, Table 2 shows that the total amount of Ca^{2+} bound is lower in polyMG compared to alginates containing G blocks, illustrating the necessity of introducing G blocks into the polymer chain for the formation of a stable gel network and ideal binding. However, when the G content exceeded 70% in enzymatically modified alginate, the stability of the gel decreased. This is probably due to the presence of large amounts of long, stiff G blocks hindering ideal packing of the polymer chains.

Permeability of Microcapsules. Increased syneresis and polymer concentration is expected to influence gel pore size. As IgG (150 kDa) has been found to be at the exclusion limit

of alginate microbeads composed of naturally occurring alginates,[20] the ingress of IgG was used as a measure of permeability. IgG is indeed of great interest since it is the smallest in the immunoglobulin family and also an important immunomolecule that may be essential to exclude during immunoisolation. All microbeads of enzymatically modified alginate were less permeable to IgG compared to the two seaweed alginates (Figure 4). Comparison of an epimerized alginate to a seaweed alginate with comparable amounts of guluronic acid (64 and 66% G, respectively) revealed that epimerization led to a significant reduction in permeability (62% bound IgG compared to 104% for seaweed alginate). The high degree of syneresis, the high concentration of alginate at the surface, and the nonswelling behavior of enzymatically modified alginate gel beads are probably responsible for these differences.

Conclusions

This study shows that the utilization of highly specific epimerases gives us the opportunity to modify polymer nanostructure, resulting in completely new biomaterials with highly improved functional properties. By controlling the composition, sequential structure, and compositional heterogeneity of alginates, the final polymers are designed to optimize calcium binding and to enhance flexibility in the interjunction regions. Thus we are using biotechnology to tailor biopolymer properties essential for new applications as gel matrices. More importantly, by controlling the composition using enzymatic modification, macroscopic properties can be tailored to meet the requirements of any desired system involving alginate.

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Supporting Information Available. Information about the determination of monad, diad, and triad frequencies in alginate is available free of charge via the Internet at http://pubs.acs.org.

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