Influence of Oligoguluronates on Alginate Gelation, Kinetics, and Polymer Organization

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Structural polysaccharides of the alginate family form gels in aqueous Ca^{2+}-containing solutions by lateral association of chain segments. The effect of adding oligomers of α-L-guluronic acid (G blocks) to gelling solutions of alginate was investigated using rheology and atomic force microscopy (AFM). Ca−alginate gels were prepared by in situ release of Ca^{2+}. The gel strength increased with increasing level of calcium saturation of the alginate and decreased with increasing amount of free G blocks. The presence of free G blocks also led to an increased gelation time. The gel point and fractal dimensionalities of the gels were determined based on the rheological characterization. Without added free G blocks the fractal dimension of the gels increased from $d_f = 2.14$ to $d_f = 2.46$ when increasing $[Ca^{2+}]$ from 10 to 20 mM. This increase was suggested to arise from an increased junction zone multiplicity induced by the increased concentration of calcium ions. In the presence of free G blocks (G block/alginate = 1/1) the fractal dimension increased from 2.14 to 2.29 at 10 mM Ca^{2+}, whereas there was no significant change associated with addition of G blocks at 20 mM Ca^{2+}. These observations indicate that free G blocks are involved in calcium-mediated bonds formed between guluronic acid sequences within the polymeric alginates. Thus, the added oligoguluronate competes with the alginate chains for the calcium ions. The gels and pregel situations close to the gel point were also studied using AFM. The AFM topographs indicated that in situations of low calcium saturation microgels a few hundred nanometers in diameter develop in solution. In situations of higher calcium saturation lateral association of a number of alginate chains are occurring, giving ordered fiber-like structures. These results show that G blocks can be used as modulators of gelation kinetics as well as local network structure formation and equilibrium properties in alginate gels.

Introduction

Alginates are salts of alginic acid, a linear heteropolysaccharide consisting of (1→4) linked β-D-mannuronic acid, designated M, and α-L-guluronic acid, designated G. The polymers are made up of homopolymer sequences of mannuronic acid, referred to as M blocks, homopolymer guluronic acid sequences, G blocks, and mixed sequences of mannuronic acid and guluronic acid units, so-called MG or alternating blocks. The distribution of blocks along the alginate molecules depends on the type of algae from which the alginate is isolated as well as the age and part of the algae. Alginate from the stem may have a different sequence and block composition compared to alginate isolated from the leaves. The season in which the algae are harvested also affects the block composition and sequence.[1,2]

Alkali-metal, magnesium, and ammonium salts of alginates are water soluble. By adding multivalent cations, for example, Ca^{2+}, Sr^{2+}, Ba^{2+}, Fe^{3+}, or Al^{3+} ions, to an alginate solution a gel is formed due to formation of junction zones involving several polysaccharide chains. The probable chelation of ions by G blocks has been described by the "egg-box" model[3,4] in which each divalent ion interacts with two adjacent G residues as well as with two G residues in an opposing chain.[5] A number of 8 ± 2 subsequent G residues are required for formation of stable junction zones by complexation with Ca^{2+}.[6]

At intermediate to high pH conditions the electron-donating moieties of the alginates, including all of the negatively charged as well as negative dipole oxygen atoms in the disaccharides (i.e., hydroxyl oxygens, ether oxygens, and carboxylate oxygens), contribute to chelating aqueous cations such as Ca^{2+} (Figure 1). The carboxylate functional groups of subsequent G units in the alginate chains have appropriate spacing and geometry for cation binding,[10] and G units have a higher affinity for divalent cation binding than their M counterparts.[11,12] The gel strength of alginate gels therefore depends on various parameters such as the G content of the alginate, the calcium concentration, and the molecular weight and concentration of the polysaccharides. At high Ca^{2+} saturation of the guluronic acid the junction zone multiplicity, i.e., the number of chain segments laterally associated to form a junction zone, can increase beyond two chains.[13] In such situations, the effective junction zone functionality, i.e., the number of alginate chains extending from a junction zone, can exceed four.

Addition of gel-inducing ions to high molecular weight alginate in aqueous solution results in macroscopically inhomogeneous structures due to the rapid ion binding. In the preparation of more homogeneous gels it has, therefore, been common to use either dialysis or in situ release of ions to induce gel formation. In dialysis the cross-linking ion, usually calcium, diffuses into the alginate solution producing a gel which is strongest near the surface.[14−16] In in situ gelling the calcium ions are released from dispersed particles or other less soluble forms of Ca present in the alginate solution. A homogeneous

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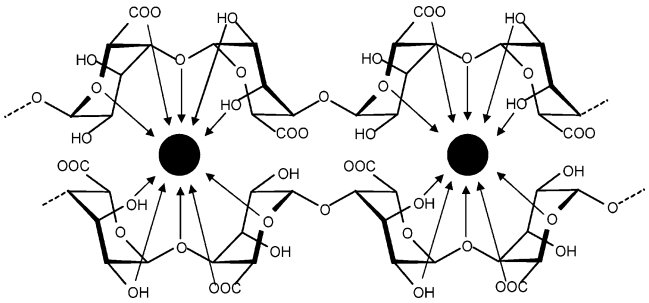

Figure 1. Schematic illustration of chelate formation between paired α-L-guluronic acid sequences of the alginate and divalent metal ions involving both carboxyl and hydroxyl groups. Gel formation at the polymer level is an irreversible, cooperative process where gel-forming ions are selectively bound in long G-block sequences, forming kinetically stable interchain bridges between chains.[7,8] The arrows indicate possible coordinating oxygens of alginate.[9]

gel is formed and the gelling speed controlled by adding an insoluble form of calcium together with an agent who ensures slow release of the ion. In order to obtain this slow ion release it has been common to use sequestering agents such as citrate, phosphate, EDTA, or EGTA to achieve controlled release of the cross-linking ion.[17,18] Alternatively, insoluble salts or salts which are hard to dissolve such as phosphates, sulfates, citrates, or tartrates and sequestering agents, which are often phosphates, as well as possibly other acids or buffers, have been used.[19,20] When applying an agent which induces a slow release of protons and thus a slow release of calcium ions the gelling speed can be controlled. An example of such an agent is D-glucono-δ-lactone (GDL).

In order to be able to utilize the valuable properties of polysaccharides it is of importance to control parameters such as gelling kinetics, gel strength, and syneresis, i.e., the rheological properties. Such control is of importance when applying these polymers as gelling or film-formation agents, viscosity producers, or stabilizers of emulsions and suspensions in various types of products. This is valid for application within food but also to other products such as use of alginate as a thickener for paint paste for textile printing. Addition of phosphate is often used in this connection.[19,20] However, for a number of years concern has been expressed regarding use of additives, in particular use of phosphate, in alginate-containing food products. The amount of phosphate has reached a high level in foods in industrialized parts of the world, a fact which is regarded as problematic since a high intake of phosphate disturbs the ion equilibrium in the body which have unfortunate health-related consequences.[21] In addition, phosphates and the other additives may produce undesired effects such as poor taste and a different texture to that desired. This applies, for example, to production of restructured meat products and baking creams.

On this background there is, therefore, a need for a system which can utilize the valuable properties of alginates in a controlled manner without the need to use many additives or such large quantities of additives, in particular phosphate. It has previously been found that G blocks, i.e., blocks of guluronic acid, can be used as modulators for rheology in gelling alginate systems within various applications.[22] Addition of such blocks thus makes it possible to control the gelling kinetics, gel strength, viscosity, elasticity, equilibrium properties, and syneresis. One aim of the experiments presented in the following was to investigate the effect of adding G blocks to alginate solutions on the structure and properties of the gels formed.

Networks made from partially cross-linked polymers near their gel point exhibit self-similar behavior, as expressed by power law relaxations.[23,24] The range of self-similarity is defined by two limiting length scales. The upper limit is the correlation length, defined by the linear size of the typical cluster, and a lower limit is roughly given by the size of one preformed linear chain, i.e., the mean distance between cross-links. The correlation length increases with the approach to the gel point and diverges at the critical extent of reaction, i.e., the gel point where the infinite cluster is formed. Above the gel point it decreases again with further cross linking. Dynamic mechanical measurements of the complex modulus at the gel point show a power law in the frequency dependence over the entire frequency range investigated, monitoring self-similarity.[25,26]

We investigated different alginate gels obtained in the absence or presence of added free G blocks. For each gel the storage, $G'(\omega)$, and loss, $G''(\omega)$, moduli were continuously recorded for a range of ω throughout the gelation process. The gel point and fractal dimensionalities at the gel point were determined based on rheological characterizaton. The gels (or pregel situations being close to the gel point) were also studied following dilution by ultramicroscopy methods (AFM). These experiments were performed to provide illustrations of the assembly modes of the alginates toward the gel state.

Materials and Methods

Alginate Samples. Alginate samples isolated from various sources were employed. The chemical composition, fraction of diad sequences F_{GG}, F_{MG}, and F_{MM}, determined by high-field ^{1}H NMR,[27] and intrinsic viscosity, $[\eta]$, in 0.1 M NaCl at $T = 20$ °C determined in a capillary viscometer are summarized in Table 1. Low molecular weight G block alginates were produced by means of acid hydrolysis as previously described.[29,30] Chemical composition and sequence as well as the number-average degree of polymerization (DP_n) of the G-block sample used is given in Table 2. Alginate stock solutions with polymer concentration C_p equal to 16−32 mg/mL were prepared by dissolving alginate powder in distilled water. In a previous study of the samples investigated here a three-letter acronym was used to depict the sample with low F_G, LoG, intermediate F_G, InG, and high F_G, HiG.[13] In the present study only samples with high and intermediate F_G were studied, and the terms InG and HiG are used (Table 1). The subscripts added to these sample designations reflect the M_w (in 10^3 g/mol) of the samples. M_w was determined based on the intrinsic viscosity $[\eta]$, determined in a capillary viscometer, and the Mark−Houwink equation $[\eta]$ (mL/g) $= 4.85 \times 10^{-3} M_w^{0.97}$ as reported.[13] Apparent small deviations between the given M_w (Table 1) and that directly obtainable using the Mark−Houwink equation is due to truncation to the number of significant figures following calculation.

Preparation of Samples for Atomic Force Microscopy. The alginate solutions were prepared for atomic force microscopy (AFM) following a procedure reported elsewhere.[31] Aliquots of the samples were mixed with 60% aqueous glycerol to a final polysaccharide concentration of 2−4 μg/mL and a final weight fraction of glycerol equal to 50%. The ionic strength of the aqueous glycerol solution was adjusted by addition of NH_4Ac to a final concentration of 0.1 M. Finally, 50 μL of these solutions was sprayed on freshly cleaved mica discs and vacuum dried at a pressure of 1.3×10^{-3} Pa for at least 2 h prior to AFM imaging.

Homogeneous Ca−alginate gels or solutions containing alginate clusters were prepared for AFM from alginate solutions (1 or 0.1 mg/mL) upon addition of Ca−EGTA (50 or 100 mM, pH 7) and slowly hydrolyzing D-glucono-δ-lactone (GDL). The 100 mM Ca−EGTA, pH 7, stock solution was prepared by adding 3.8 g of EGTA and 1.47 g of $CaCl_2 \cdot 2H_2O$ to 50 mL of deionized H_2O, the pH was adjusted to 7 with 1 M NaOH, and the total volume was finally adjusted to 100 mL. The 50 mM Ca−EGTA stock solution was prepared using the same procedure by reducing the amounts of materials added. The concentra-

Table 1. Chemical Composition and Intrinsic Viscosity of the Alginate Samples Studied[a]

alginate source	F_G	$F_{MG/GM}$	F_{MM}	F_{GG}	F_{GGG}	$[\eta]$, mL g^{-1}	$M_w/10^3$ g mol^{-1}	sample designation
L. hyperborea leaf	0.50	0.19	0.31	0.31		1420	455	InG_{455}
L. hyperborea stipe	0.69			0.59	0.52	748	235	HiG_{235}
L. hyperborea stipe	0.66	0.12	0.22	0.54		1440	465	HiG_{465}

[a] The chemical composition is specified in terms of the molar fraction of guluronic acid (F_G), diad frequencies ($F_{MG/GM}$, F_{MM}, F_{GG}), as well as the triad fraction F_{GGG}. More extensive sets of fractional contents of the triad sequences of alginates have been reported elsewhere.[28]

Table 2. Chemical Composition and Sequence as Well as the Number-Average Degree of Polymerization (DP_n) of the G-Block Sample Used in this Study[a]

F_G	F_M	F_{GG}	F_{MM}	$F_{GM,\,MG}$	F_{GGG}	F_{GGM}	F_{MGM}	DP_n
0.94	0.06	0.83	0.05	0.11	0.8	0.03	0.08	19

[a] The molar fraction of guluronic acid (F_G), mannuronic acid (F_M), diad frequencies ($F_{MG/GM}$, F_{MM}, F_{GG}), as well as the triad fractions of F_{GGG}, F_{GGM}, and F_{MGM}.

tion of GDL required for each concentration of Ca^{2+} when using Ca−EGTA as the Ca^{2+} source was calibrated to yield pH 4 after equilibration for about 24 h after addition of GDL. The rate of change in pH and release of Ca^{2+} from Ca−EGTA were previously determined using a conventional pH electrode and a Ca^{2+}-sensitive electrode in parallel experiments without alginate.[13] The amount of GDL needed in order to obtain a decrease in pH from pH 7 to pH 4 within 24 h after addition of GDL was determined for solutions containing 10 and 20 mM Ca^{2+}, respectively. In some experimental series G blocks were added to the alginate solution, giving a mass fraction of alginate G blocks relative to alginate equal to 1:2 or 1:1.

Samples taken from the mixtures containing homogeneous Ca−alginate gels, prepared as described above, were added to glycerol/water solutions and sprayed onto mica as described for the alginate solutions. Before inspection the samples were vacuum dried at a pressure of 1.3×10^{-3} Pa overnight. An alternative technique used for preparation of gel samples for AFM included use of zinc ions. This procedure does not involve use of glycerol during sample preparation. Zn^{2+} is one out of several transition-metal cations that effectively bind macromolecules to mica.[32] The abilities of the transition-metal cations to immobilize negative charged macromolecules onto mica are related to their size, which allows them to fit into the cavities above the recessed hydroxyl groups in the mica lattice. Zinc nitrate, $Zn(NO_3)_2$, was added to the solutions containing the alginate gels to give a zinc concentration equal to 0.1 mM. Aliquots of this sample were prepared for AFM imaging by depositing a small volume onto a freshly cleaved 5 mm diameter mica disc, and after 10 min of incubation the sample was dried in a stream of N_2 followed by vacuum drying at a pressure of 1.3×10^{-3} Pa overnight. No systematic differences were observed in the AFM topographs depending on the preparation procedure.

Atomic Force Microscopy. AFM topographs of alginate or Ca−alginate gels were obtained using a Digital Instruments Multimode IIIa atomic force microscope equipped with an E-scanner. The instrument was operated in tapping mode using NCH-W cantilevers (NANOSENSORS, Neuchatel, Switzerland) with nominal resonance frequencies in the interval 306−366 kHz. The topographs obtained at scan sizes in the range from 1 μm × 1 μm to 5 μm × 5 μm (512 × 512 pixels) were flattened line by line prior to further analysis.

Rheology. The rheometer used was a Paar Physica Rheometer MCR 300. A LAUDA Ecoline low-temperature thermostat RE 104 was used for cooling of the peltier element of the rheometer. The temperature was adjusted to 20 °C. The frequency dependence of the storage and loss modules of the samples were determined prior to, during, and after the gelation process. A parallel plate geometry with serrated plate surfaces (PP50 serrated plate, diameter = 50 mm) which provide minimal wall slip was used. The samples were sealed using low-viscosity silicon oil to avoid effects due to solvent evaporation. The loss and storage modules were measured at 10 different frequencies in the interval 0.1−10 Hz. The amplitude was set to 1 mrad, and the measuring gap was set to 1 mm.

Preparation of Samples for Rheological Studies. When preparing alginate solutions for rheological studies the alginate stock solutions were diluted to a concentration observed to give a suitable starting viscosity for the gelation studies. The concentrations chosen were in the interval 15−40 mg/mL and depended on the intrinsic viscosity of the alginate solutions. The Ca−alginate gels prepared for rheological studies were prepared using the same procedure as described above for samples intended for AFM investigations. The measurements were started 1 min after addition of GDL. In experiments where G blocks were added these were added to the alginate solution prior to addition of Ca−EGTA and GDL.

Results and Discussion

AFM Imaging of the Gelation Process. AFM was used to obtain information concerning the gelation process by revealing the structure of the aggregates at different stages as well as at the end of the gelation process. Figure 2A presents an AFM tapping-mode topograph showing free alginate molecules on a mica surface. When adding Ca−EGTA to a solution of alginate molecules a gel is formed. The image presented in Figure 2B shows structures present in the gelling solution containing InG_{455} alginate and 10 mM Ca−EGTA 50 min after addition of GDL. At this time $G''/G' \approx 1.8$. The image reveals the existence of microgels a few hundred nanometers in diameter. The image presented in Figure 2C is a magnification of a section of image 2B. Image D reveals the structures existing in a gelling solution containing InG_{455} alginate and G blocks (G block/alginate 1:1) and 20 mM Ca−EGTA 85 min after addition of GDL. The bright linear/branched structures in the image correspond to structures extending several nanometers above the mica surface. Analysis of several AFM topographs indicated that under the present preparation conditions the structures extended up to 5 nm above the mica surface. These structures are most probably formed by lateral alignment of alginate chain segments. The existence of a fine polymer network surrounding the thicker elongated structures is clearly visible. Formation of junction zones occurring on ionotropic gelation of alginate has previously been investigated using small-angle X-ray scattering.[13] In that study it was concluded that homoguluronic acid sequences associate laterally to various multiplicities depending on the fractional Ca saturation of the guluronic acid, the alginate source, and the overall degree of polymerization. For *Laminara hyperborea* leaf alginate, i.e., alginate of the type InG_{455}, in the presence of 20 mM Ca^{2+}, a high amount of lateral association was observed. This is in accordance with the observations presented in Figure 2.

Figure 3 shows the coexisting association multiplicities of the alginate, presumably between the G sequences, of a gel network developed from a solution containing InG_{455} alginate, free G blocks (G block/alginate 1:1), and 20 mM Ca^{2+}. The image reveals the structure of the gel 170 min after addition of GDL. At this time $G''/G' = 0.037$ at $\omega = 0.628$ rad s^{-1} and increases to 0.087 at $\omega = 62.8$ rad s^{-1}. On the nanometer scale the network appears to be inhomogeneous with areas of high polymer density interspersed with areas of low polymer density

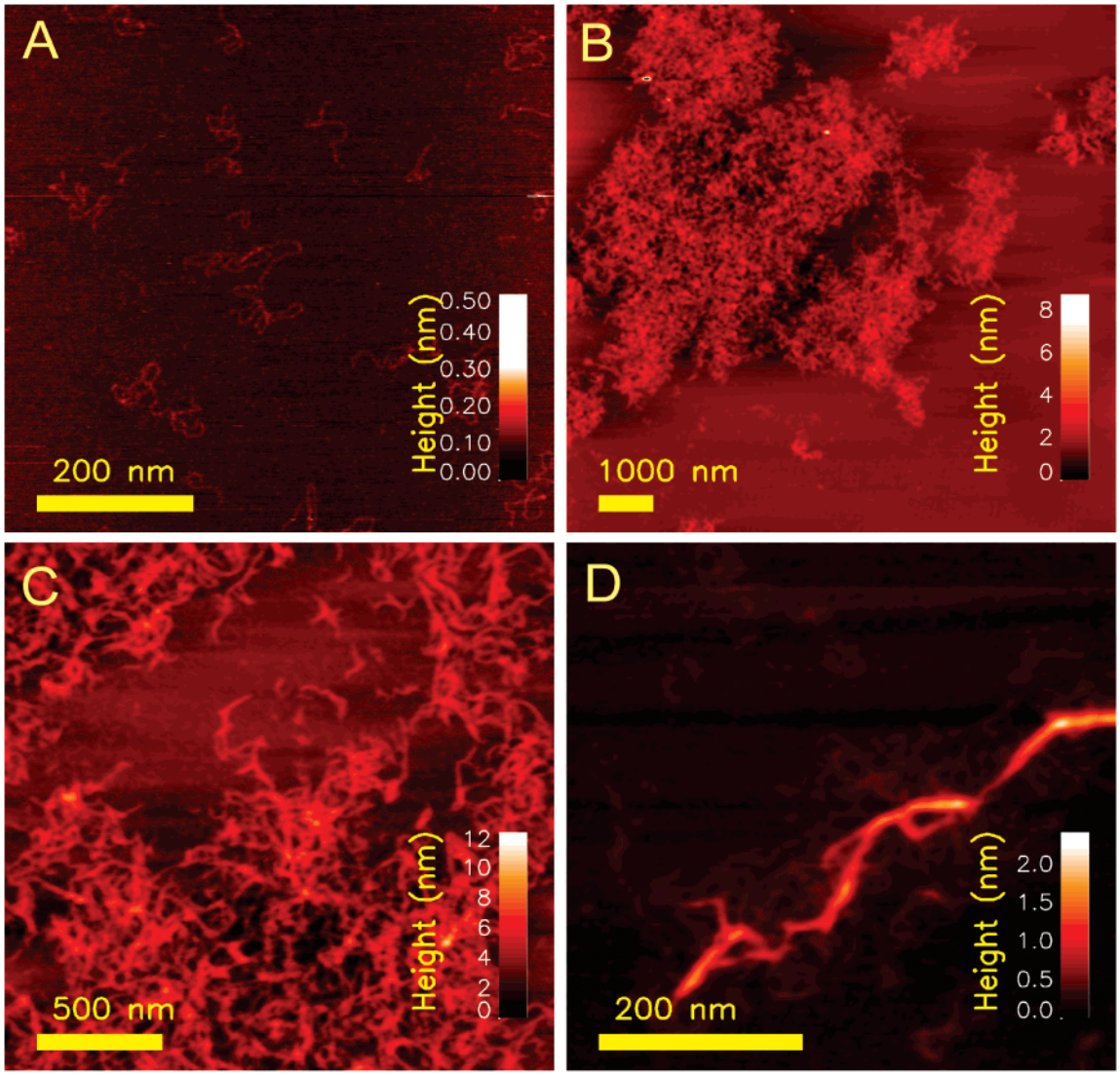

Figure 2. Tapping-mode AFM topographs of alginate and alginate clusters developed under gelation of alginate. (A) InG_{455} alginate in 0.1 M NH_4Ac (scan size, 700 nm) prepared using the glycerol method. (B) Clusters developing in a solution containing InG_{455} alginate and 10 mM Ca^{2+}. The image reveals the structures existing in the solution 3000 s after addition of GDL. (C) Magnification of an area of image B. (D) Gel developed from a solution containing InG_{455} alginate 20 mM Ca^{2+} and free G blocks (G block/alginate 1:1). The topograph reveals the structure of gel 5 100 s after addition of GDL. The AFM topographs B−D were obtained on samples prepared by adding 0.1 mM $Zn(NO_3)_2$.

and open pores (Figure 3). The structures visible in the image are also consistent with the existence of different association modes. Among these different modes some involve lateral association of a high number of G sequences within the alginate chains, giving ordered polysaccharide fiber-like structures extending high above the mica surface. Others involve fewer chain segments, visible as bundles of smaller diameter. On the basis of such images some information about relevant sizes of pores in the gel network can, in principle, be determined. In this study determination of the pore size was complicated by the inhomogeneity of the network, resulting in large fluctuations in the determined pore size.

Viscoelasticity of Aqueous Homogeneous Ca−Alginate Gels during Sol−Gel Transition. Figure 4 presents rheological data obtained for InG_{455} alginate solutions (16 mg/mL, 10 or 20 mM Ca^{2+}, 12.5 mg/mL GDL) as a function of increasing time after addition of GDL. Before attaining the gel point G'' is higher than G' in the whole frequency range investigated without any plateau appearing in the G' vs ω curve. This is the characteristic of a viscoelastic fluid. Determination of G' and G'' for 10 different frequencies in the interval 0.1−10 Hz as a function of time (Figure 4A) reveals that G' changes only slightly during the first few minutes. The hydrolyzation of GDL leads to a decrease in pH, which subsequently leads to release of Ca from Ca−EGTA due to a reduced association constant between Ca and EGTA at low pH.[18] These are the two main processes in the current method for inducing gelation of alginate. The steep increase in G' vs time observed after approximately 60 min is due to a rapid release of Ca^{2+} at this time due to pH-induced dissociation of the Ca−EGTA complex. The less steep increase observed after longer times may be due to either the lack of free Ca^{2+} ions or the lack of free G blocks not involved in cross-links. This behavior was observed for all the alginate samples studied. After passing through the gel point G' becomes higher than G''(Figure 4B), and a plateau appears in the G' vs ω curve in the low-frequency range. This indicates formation of a viscoelastic gel.

Effect of Alginate Concentration on Gel Strength and Gelation Time. Table 3 gives an overview of G' and G'' at the start and end of the gelation process obtained for different alginate samples. The modulus of Ca−alginate gels depends strongly on the composition and sequence of the monomers in the alginate molecules. In general, alginates rich in guluronate residues form strong, brittle gels, while M-rich alginates form

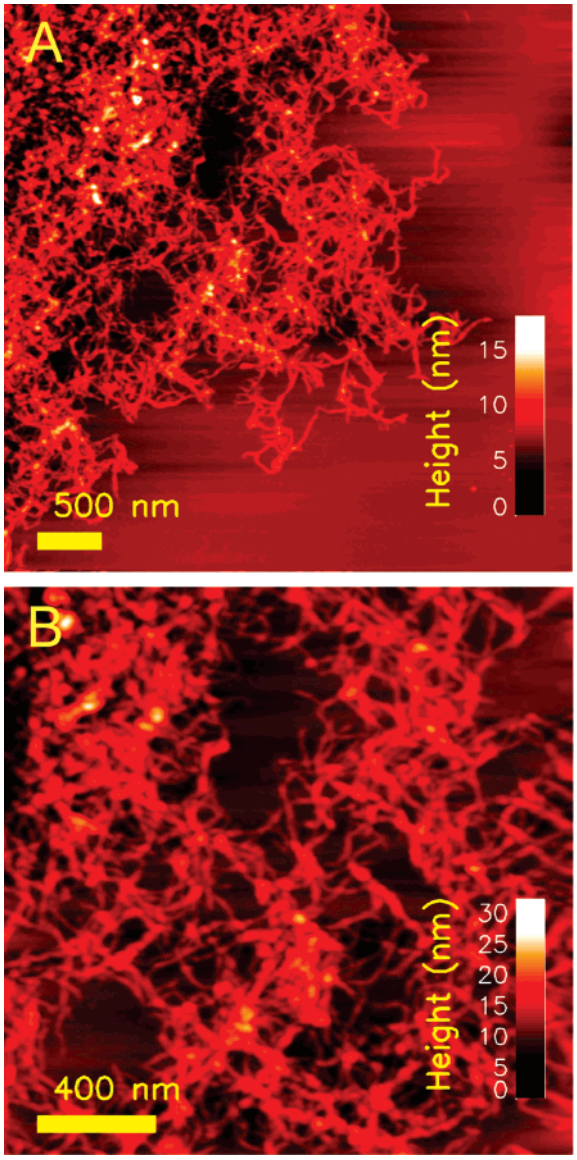

Figure 3. Tapping-mode AFM topographs of an alginate gel. (A) Gel developed from a solution containing InG_{455} alginate and free G blocks (G block/alginate 1:1) and 20 mM Ca^{2+}. The image reveals the structure of the gel 170 min after addition of GDL. The AFM topograph was obtained on a sample prepared by adding 0.1 mM $Zn(NO_3)_2$. (B) Magnification of a section of image A.

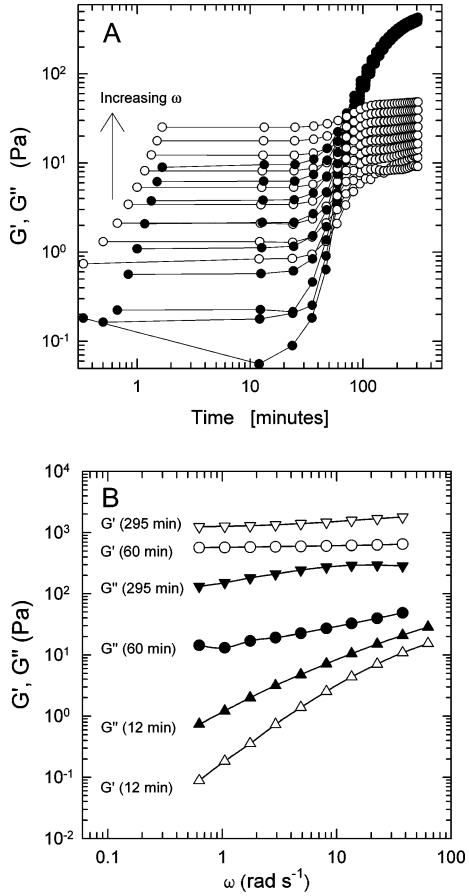

Figure 4. Rheological data obtained for InG_{455} alginate solutions (16 mg/mL, 10 mM Ca^{2+}, 12.5 mg GDL/mL). (A) Storage (G', filled circles) and loss (G'', open circles) modulus as a function of time after addition of GDL. The data are obtained for $\omega = 0.63$, 1.05, 1.75, 2.92, 4.86, 8.12, 13.5, 22.6, and 37.7 rad s^{-1}. (B) G' and G'' as a function of angular frequency (ω) at 12, 60, and 295 min after addition of GDL.

softer, more elastic gels.[33] The current data (Table 3) confirm this trend in the sense that the HiG_{465} alginate gives rise to a stronger gel than the InG_{455} alginate for gels obtained from alginate solutions of the same concentration.

The observations of the gelling behavior of the InG_{455} alginate solutions reveal that the gel strength increases whereas the gelation time decreases when reducing the alginate concentration (Table 3). This behavior is due to a different degree of Ca^{2+} saturation of the G components found in G blocks in the alginate. In general, the elastic modulus of an alginate gel depends on the number of cross-links and length and stiffness of the chains between cross-links. In the present system Ca is used as the cross linker in all experiments, so the affinity between the cross linker and the polymers is constant. The reason for the decreased gel strength might therefore be a decrease in the number of cross-links formed. The number of cross-links formed is in this system determined by the relation between the available Ca^{2+} ions and the number of alginate chains. When keeping the calcium concentration constant this relation will be maximized at low alginate concentration, but under these conditions the number of loose polymer ends is also expected to be high. Furthermore, for a certain alginate concentration G' increases with increasing $[Ca^{2+}]$. This behavior is in both cases due to an increased number of junction zones relative to the total number of active network chains and a decreased number of loose ends. This effect also explains the increased gelation time observed when increasing the alginate concentration. Whereas binding of multivalent cations to a binding site on the alginate chain is rapid, building a network structure to form the maximum possible number of interguluronate sites is limited by diffusion of the polymer chains. In order to form a network of perfectly aligned blocks in the network, some dissociation of junctions must take place following the first cation-induced random dimerization. This dissociation is kinetically unfavorable,[34] and the term "non-equilibrium gel" has been used to describe the alginate gel.[35] The difficulties associated with formation of the network increases with the density of alginate chains, i.e., with concentration, explaining the observed increased gelation time with increased alginate concentration.

Effect of Addition of Free G Blocks on Alginate Gel Strength. The effect of adding free G blocks to gelling solutions of InG_{455} alginate was investigated. Free G blocks were added to the alginate stock solution with a G block/alginate mass fraction equal to 1:2 and 1:1. The results obtained when studying the solutions containing these solutions using rheology are presented in Table 4. The experiments revealed that the final

Table 3. Summary of Rheological Parameters Obtained for Ca^{2+}-Induced Gelation of Alginates[a]

				start of gelation		end of gelation				
alginate	C_p (g/L)	$[Ca^{2+}]$ (mM)	$([Ca^{2+}]Z_G/[G]Z_{Ca})$	G' (Pa)	G'' (Pa)	G' (Pa)	G'' (Pa)	t_c (min)	$\tan\delta_c = (G'/G'')_c$	d_f
InG_{455}	12	10	0.59	0.024−0.72	0.59−14.2	502−541	14.9−34.8	38.5	1.7	2.14
	12	20	1.18	0.026−1.26	0.59−14.2	1640−1840	82.2−128	16	1.0	2.48
	16	10	0.44	0.11−8.44	1.29−25.7	398−445	11.4−48.7	56.5	1.6	2.14
	16	20	0.88	0.081−9.14	1.22−25.1	1210−1750	153−301	18.5	1.1	2.46
HiG_{465}	16	10	0.33	0.114−8.87	1.43−29.2	290−355	15.8−62.9	61.5	1.5	2.19
	16	20	0.67	0.084−9.62	1.41−28.8	3060−3550	201−299	21	1.3	2.29
HiG_{235}	32	10	0.16	0.101−5.96	1.46−42.8	68.6−155	17.1−105	149	2.9	1.78
	32	20	0.32	0.035−0.91	1.64−48.7	1830−2120	109−231	39	1.9	2.02

[a] The storage, G', and loss, G'', moduli at $\omega = 1.75$ rad s^{-1} for Ca−alginate gels at the start ($t = 0$ min) and end ($t = 305-320$ min) of the gelation process, as well as critical values for Ca−alginate gels are presented.

Table 4. Summary of Rheological Parameters Obtained when Adding α-L-Guluronate Oligomers to Alginate[a]

					start of gelation		end of gelation				
alginate	G block/ alginate	C_p (g/L)	$[Ca^{2+}]$ (mM)	$([Ca^{2+}]Z_G/[G]Z_{Ca})$	G' (Pa)	G'' (Pa)	G' (Pa)	G'' (Pa)	t_c (min)	$\tan\delta_c = (G'/G'')_c$	d_f
InG_{455}		16	10	0.44	0.114−8.44	1.29 -25.7	398−445	11.4−48.7	56.5	1.6	2.14
	1:2	16	10	0.22	0.171−11.2	1.59−28.9	207−252	10.3-48.9	61.5	1.4	2.24
	1:1	16	10	0.15	0.244−12.9	1.71−30.3	141−194	10.4−53.5	72	1.3	2.29
		16	20	0.88	0.081−9.14	1.22−25.1	1210−1750	153−301	18.5	1.05	2.46
	1:2	16	20	0.44	0.126−11.5	1.62−29.2	1170−1280	35.0−93.0	22.5	1.1	2.43
	1:1	16	20	0.29	0.201−15.2	1.97−32.5	750−838	26.7−73.8	24.5	1.1	2.43

[a] Storage, G', and loss, G'', moduli at $\omega = 1.75$ rad s^{-1} for Ca−alginate gels at the start ($t = 0$ min) and end ($t = 305-320$ min) of the gelation process, as well as critical values for Ca−alginate gels are presented. The fraction G block/alginate indicates the relative mass fraction of the two molecules.

G' value decreased upon addition of G blocks (Figure 5). This effect was significant for addition of G blocks to the mass faction G block/alginate 1:2 and 1:1 and found to increase with increasing amount of G blocks added. This effect was found in experiments with $[Ca^{2+}]$ concentrations equal to either 10 or 20 mM. At this stage it is worth mentioning that the apparent equilibrium values of G' do not always exhibit a decrease in such mixed G block/alginate systems but that it depends strongly on the amount of Ca^{2+} present. It has been shown[22] that at a mixing ratio of 1:1 (total C_p 20 mg/mL) there is a crossover at approximately 40 mM Ca^{2+} above where the mixed system exhibits higher moduli compared to the nonmixed, pure 10 mg/mL alginate sample. At 100 mM Ca^{2+} Young's modulus (E) has been determined to be approximately 3 times higher for the mixed system compared to the pure alginate gel.[22]

The decreased gel strength observed upon addition of G blocks can in part be explained from the dependence of G' on the number of cross-links in the gel. The free G blocks compete with the G blocks existing as part of the alginate chains for the Ca^{2+} ions. The calcium-mediated bonds will therefore form not only between the InG_{455} alginate chains but also between alginate chains and free G blocks. This situation is illustrated schematically in Figure 6. Due to its ability to bind calcium, the presence of the G blocks reduces the degree of fractional calcium saturation of the alginate. Due to the short length of the G blocks, being on average about 20 guluronic acid residues long, links involving these blocks will not contribute to the elasticity of the polymer network. Formation of ionic cross-links between alginate and free G blocks is therefore expected to result in a reduction in the density of functional cross-links in the network and therefore reduced gel strength. The importance of this effect is expected to increase with decreasing divalent ion concentration. The gelation time t_c was also seen to increase with increasing amount of added G blocks. This can be explained by the increased total concentration of alginate strands in these solutions, increasing the difficulties associated with network formation.

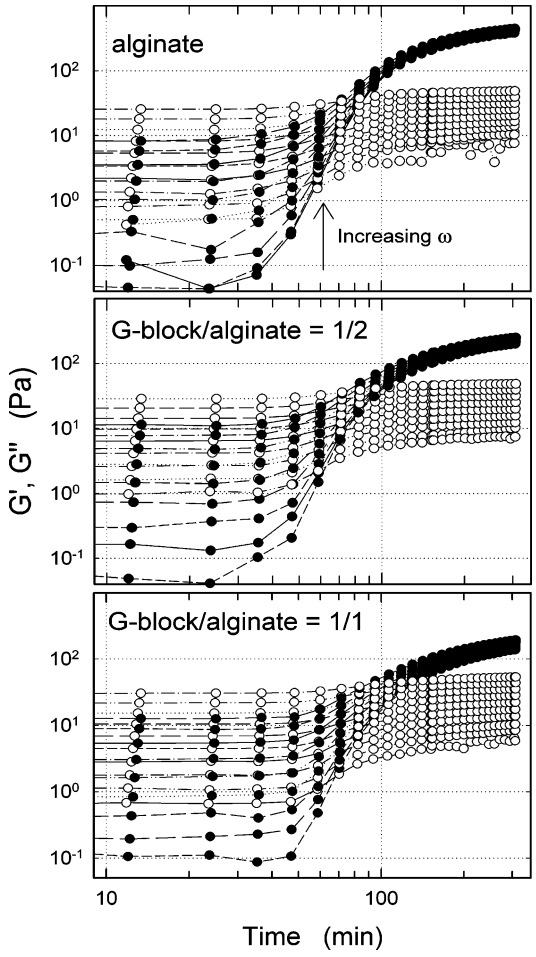

Figure 5. Rheological data obtained for InG_{455} alginate solutions (16 mg/mL, 10 mM Ca^{2+}, 12.5 mg GDL/mL) with different amounts of added G blocks. The data shown are G' (filled circles) and G'' (open circles) as a function of increasing time after addition of GDL. The data are obtained for $\omega = 0.63-37.7$ rad s^{-1} as in Figure 4A.

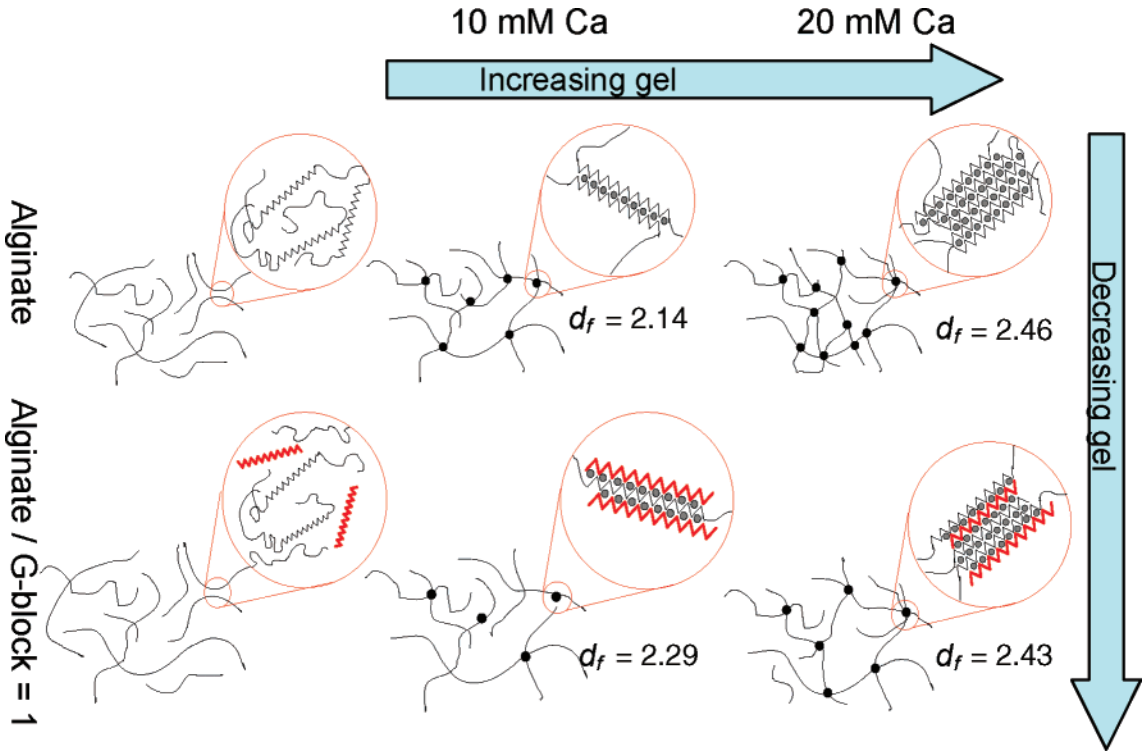

Figure 6. Schematic illustration of possible structures formed in alginate solutions in the presence of different calcium concentrations and different relative amounts of free G blocks (red lines). Under low calcium concentration the alginate strands associate to a dimeric junction zone with a $d_f = 2.14$. The junction zone multiplicity increases with increasing calcium concentration giving $d_f = 2.46$ at 20 mM Ca^{2+}. The gel strength decreases upon addition of free G blocks, whereas d_f increases slightly at low calcium concentration (10 mM) compared to the situation without G blocks.

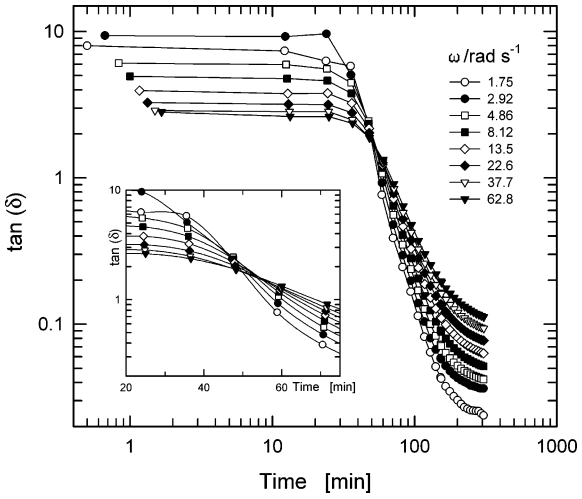

Figure 7. Loss tangent (tan δ) versus time for 16 mg/mL InG_{455} in 10 mM Ca^{2+}. (Insert) Magnification of part of the figure revealing the behavior close to the critical point. On the basis of the point of interception the gel point was determined. The critical exponent n was determined using tan δ at the gel point and eq 1.

Kinetic Effects Observed Upon Addition of Free G Blocks. Figure 5 shows the effect on the kinetics in the gel formation process, measured as the change in dynamic storage modulus (G'), with addition of different quantities of G block with 10 and 20 mM Ca^{2+}. A reduction in gelling rate as a function of the quantity of G blocks added is shown in both cases. This essential result regarding use of G block instead of, for example, phosphates in kinetic control can be attributed to the G blocks' ability to bind Ca^{2+} in the early phase of gel formation without entering into the network structures.

Critical Exponents for the Sol−Gel Transition. According to Chambon and Winter[25] the gel point is characterized by the loss angle, δ, given by $\tan \delta = G''/G'$, being independent of the frequency of the oscillating excitation, ω. Thus, the gel point is obtained by determination of $G'(\omega)$ and $G''(\omega)$ for a range of ω. The crossover between the low and high ω data in a graph of $\delta(t)$ using ω as a parameter will reflect the gel point. The gel point is thus determined by interpolation between data obtained for the pregel and postgel situation and is not directly dependent on determination of G' and G'' at the gel point. The critical phase angle for the sol−gel transition, δ_G, can be used to deduce the fractal dimension at the gel point, d_f, using the relaxation exponent, n, given by

$$\tan \delta_G = \tan\left(\frac{n\pi}{2}\right) \quad (1)$$

In this work the gel point is reached after a time t_c, after inducing gelation. At t_c the viscosity approaches infinity and a network spreading the whole sample space begins to appear in the system.

The values of t_c and n for the sol−gel transition in aqueous alginate solutions induced by initiating release of Ca^{2+} from dispersed Ca−EGTA have been determined in the following way using the Winter's criterion. By presenting tan δ, obtained from $G'(\omega)$ and $G''(\omega)$ as a function of time (Figure 7), the critical time t_c needed for gel formation to occur was determined from the intersecting point, where tan δ became independent of ω (Figure 8). When determining t_c, data obtained at 3−4 chosen frequencies were used. On the basis of tan δ measured at the gel point and eq 1, the relaxation exponent n was determined.

The fractal dimension d_f of a gel is a measure of the compactness of the clusters in the intermediate or fractal length scale regime. The fractal dimension is expected to depend on weather the developing structures tend to grow preferentially

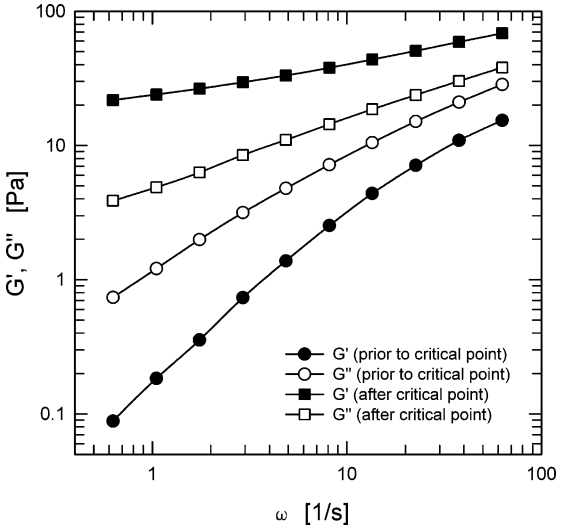

Figure 8. *G′* and *G″* as a function of angular frequency for 16 mg/mL InG_{455} alginate exposed to 20 mM Ca^{2+}. Two chosen measurements are shown: one describing the behavior of the system prior to the gel point, and one describing the system after the gel point has been attained.

in one dimension or by more branch-like structures. The parameter d_f is in these extreme cases expected to be $d_f = 1$ or $d_f = 3$. The critical phase angle for the sol−gel transition, δ_G, can be used to deduce the fractal dimension at the gel point, d_f, using the relaxation exponent, n. However, based on the fractal behavior of the critical gel, several relationships between the critical exponent n and the fractal dimension d_f have been proposed.[36−38] When the hydrodynamic interaction is completely screened out and the excluded volume effect is dominant in the cluster the following expression describes the relation between d_f and n[37]

$$d_f = \frac{2n}{1-n} \tag{2}$$

If the excluded volume effect as well as the hydrodynamic interaction can be completely screened out, n is expressed by the following equation

$$n = \frac{d}{\overline{d_f} + 2} = \frac{d(d+2-2d_f)}{2(d+2-d_f)} \tag{3}$$

where d_f is the fractal dimension of the polymer when the excluded volume effect is fully screened and d is the space dimension. If only partial screening exists, the fractal dimension takes a value between $\underline{d_f}$ and $\overline{d_f}$.[39]

In this study d_f and $\overline{d_f}$ were calculated from n using eqs 2 and 3, respectively. Unreasonable values of d_f beyond three were obtained from eq 2. The reason for this may be that at high alginate concentrations the excluded volume was screened within the correlation length.[40] Consequently, in further discussion we rely on the d_f values evaluated from eq 3. The tan δ_c and d_f values determined for alginate systems with mass fraction G block/alginate 1:2 and 1:1 are listed in Table 4. The data indicate that the gelation time, t_c, increases with increasing added amount of G block whereas it decreases with increasing $[Ca^{2+}]$. tan δ_c decreases with increasing $[Ca^{2+}]$. Whereas the increase in the fractal dimension at the gel point was clearly visible upon addition of G blocks at low calcium concentration, the dimension remained the same within experimental error when inspecting the results obtained for solutions at higher calcium concentration. On the basis of d_f and the gel strength determined in the presence or absence of G blocks it can be concluded that the added G blocks contribute to an increased junction zone multiplicity at low calcium concentration but not to the junction zone functionality.

Relation between Fractional Ca Saturation of the Guluronic Acid Unit and Gel Structure. Homopolymers of mannuronic acid and guluronic acid both bind Ca^{2+} with dissociation constants equal to 2×10^{-4} and 1×10^{-3} M, respectively.[12] Due to this significantly larger dissociation constant determined for Ca−guluronic acid complexes compared to Ca−mannuronic acid complexes binding of Ca^{2+} to mannuronic acid is not considered in the following discussion. The fractional saturation f_{sat} of the G units can be calculated using the following equation, where $[Ca^{2+}]$ and [G] is are concentration of calcium and guluronic acid residues, respectively, and Z_{Ca} and Z_G is their valence.

$$f_{sat} = \left(\frac{[Ca^{2+}]Z_G}{[G]Z_{Ca}}\right) \tag{4}$$

The fractional Ca saturations (eq 4) is included in Tables 3 and 4 for varying alginates present at varying concentration and varying calcium concentrations.

The d_f values presented above indicate the importance of the fractional Ca saturation of the guluronic acid for the distribution of junction zones multiplicities in alginate gels. The results show that f_{sat} to a large extent determines the distribution of junction zone multiplicity. This conclusion is strengthened by the AFM observations and is also consistent with the results obtained in a previous study of Ca−alginate gels where SAXS was applied.[13] In the latter an increasing $[Ca^{2+}]$ was reported to yield an increasing curvature in cross-sectional Guinier plots. On the basis of those data it was concluded that in these gels the lateral condensation into junction zones is not limited to one association mode but controlled by the concentration of Ca^{2+} for a given C_p and alginate type. A d_f equal to 2.48, which is the highest fractal dimension observed in these systems, can be obtained also for the lowest alginate concentration studied, equal to 12 g/L, provided a high f_{sat} (Table 3). The results also reveal that f_{sat} alone does not determine d_f. The content and distributions of G blocks also influences the gelation behavior through its influence on the junction zone structure. HiG_{465} alginate, which has a higher G content than InG_{455} alginate (Table 1), forms structures with a higher d_f than what is observed for InG_{455} alginate. This holds for both 10 and 20 mM calcium solutions even though the f_{sat} values for these solutions is lower in the HiG_{465} system compared to the InG_{455} system (Table 3). This difference in behavior may result from the cooperative binding of Ca^{2+}, which is known to increase with increasing G block length up to a G block with more than 20 residues.[41] The calcium ions are therefore expected to have a higher affinity for binding sites existing between long G blocks (which exist in higher number in HiG_{465} than in InG_{455}) than for binding sites existing between shorter G blocks. This cooperativity of the binding process may thus explain the observed differences in behavior between these two alginates.

The f_{sat} value appears also to be a useful parameter in assessing the alginate gel behavior in presence of G blocks. In situations where G blocks were added f_{sat} was calculated based on the total number of units of α-L-guluronic acid present in alginate chains or in the added G blocks. A schematic presentation of the structures formed in the presence of varying amounts of G blocks and calcium is presented in Figure 6. In the absence

of added G blocks the fractional dimension at the sol−gel transition point of the gels increases with increasing concentration of calcium from $d_f = 2.1$ at $f_{sat} = 0.44$ to $d_f = 2.46$ at $f_{sat} = 0.88$ (Table 4). This increase can be explained by an increased junction zone multiplicity made possible by an increased availability of calcium ions. The storage modulus also increases with increasing f_{sat}. If looking at the values obtained at $\omega = 63$ rad s^{-1} it increases from 445 Pa at $f_{sat} = 0.44$ to 1750 Pa at $f_{sat} = 0.88$ (Table 4). This increase reflects an increase in the number of active network chains with increasing f_{sat} ratio.

At low calcium saturation the increase in d_f resulting from the presence of G blocks is increasing with increasing amount of G blocks added increasing from $d_f = 2.14$ in the absence of G blocks to $d_f = 2.24$ for a G block/alginate ratio equal to 1/2 and further to $d_f = 2.29$ for a G block/alginate ratio equal to 1 (Table 4). Concomitant with the increase in d_f a decrease in G' is observed to occur. An interpretation of these observations is that free G blocks are binding to other free G blocks or to G blocks existing in the alginate chains through electrostatic calcium-mediated bonds. This situation is schematically illustrated in Figure 6. The free G blocks are competing with the alginate chains for the calcium ions. The bonds formed between free G blocks and another G block being part of the alginate network or not is not contributing to network formation and is thus not leading to a increase in G'. Due to the short chain length of the free G blocks the steric hindrance possibly limiting junction zone multiplicity is decreased in these systems, explaining the observed high d_f.

If inspecting the behavior of the gels formed in the presence of higher calcium saturation the same tendency of decreased storage modulus with increased concentration of free G block is observed, decreasing from $G' = 1750$ Pa in the absence of free G blocks, via 1280 Pa at G block/alginate = 1/2 to 838 Pa at G block/alginate = 1 (values obtained for $\omega = 63$ rad s^{-1}). This can again be explained by the decreased f_{sat} brought about by the increased amount of G units relative to calcium ions in these systems. At this high calcium concentration the effect of the G block concentration on d_f is however not observed. For the solutions containing 20 mM Ca^{2+}, d_f remains constant within experimental error. The reason for this may be that $d_f = 2.46$, which is in between the value expected for a ideal random coil ($d_f = 2$) and the value expected for a globule ($d_f = 3$), is the maximum value this parameter can take for the fiber-like junction zones formed by the alignment of multiple G blocks occurring under these experimental conditions. The reason for this is probably related to steric crowding of the chains emanating from the junctions, which is restraining the alignment of the strands and therefore limiting the maximum attainable mass density in this system.

Conclusions

The experiments presented in this paper reveal that the strength of the alginate gels decreases upon addition of G blocks whereas the gelation time increases. This behavior was observed in gelling solutions containing 10 and 20 mM Ca^{2+}. The fractal dimension of the alginate gels increased from $d_f = 2.14$ to $d_f = 2.46$ when increasing the concentration of calcium ions from 10 to 20 mM, reflecting an increased junction zone multiplicity resulting from an increased availability of calcium ions. The AFM topographs indicated that in situations of low calcium saturation microgels are developing in the solution. In situations of higher calcium saturation lateral association of a high number of alginate chains are occurring, giving ordered polysaccharide fiber-like structures. In the presence of free G blocks (G block/alginate = 1/1) the fractal dimension increased from 2.29 at 10 mM calcium to 2.43 at 20 mM calcium accompanied with a simultaneous decrease in G'. The observations indicate that free G blocks are involved in electrostatic calcium-mediated bonds. They are thus competing with the alginate chains for the calcium ions. The bonds formed involving free G blocks may either compete with other G sequences in formation of junction zones and are thus not leading to an increase in G′ in the network formation, may mediate thicker junction zones by being "sandwiched" within laterally associated G sequences, or form interactions with other G blocks. The results reveal that the rheology of an alginate-containing solution can be changed by adding free G blocks of mean chain length 20 units. Such G blocks can thus be used as modulators for rheological properties in the sense that they control the kinetics, gel strength, viscosity, elasticity, and equilibrium properties in gelling alginate systems within various areas of application.

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