Alginate Oligoguluronates as a Tool for Tailoring Properties of Ca-Alginate Gels

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Summary: Alginates represent a family of structural polysaccharides derived from either seaweeds or bacteria. They readily form ionotropically-induced physcial gels in aqueous solution in the presence of multivalent ions. In this study, the homogeneous release of Ca^{2+} from Ca-ethylene glycol tetraacetic acid (Ca-EGTA) within an alginate solution is utilized to prepare homogeneous gelation conditions. The pH-dependence of the multiple equilibria involved, i.e. between Ca^{2+}, the various states of EGTA, the alginate and its protonation and ion binding, is modeled and used as a basis for designing the glucono-δ-lactone concentration to reduce the pH. This method is used for the perturbation of the Ca-induced gelation of alginates in the presence of oligoguluronate oligomers. These oligoguluronate oligomers are able to be involved in the binding of Ca^{2+}, but sufficiently short not to mediate connectivity through their chains. The results indicate that the oligoguluronate oligomers perturb the gelation of alginate differently in the Ca^{2+}-limited and non-Ca^{2+}-limited regimes. In the calcium limited regime, the oligoguluronate oligomers appear to sequester calcium either by binding to oligoguluronate sequences of the network, or between the free oligoguluronates, yielding an overall net effect of reduced gel strength. In the non-Ca^{2+} limited regime, the experimental data shows increased gel strength in the presence of oligoguluronate blocks. These results show that oligoguluronates can be used as modulators of gelation kinetics as well as local network structure formation and equilibrium properties in alginate gels.

Keywords: alginate; gel strength; in-situ ionotropic gelation; multiple equilibria; oligoguluronate

Introduction

Alginates constitute a family of naturally-occurring binary linear polysaccharides consisting of (1 → 4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. The sequential arrangement of their two constituting residues, M and G, varies, depending on the origin of the alginates.[1,2] The two most striking features with these macromolecules are that they undergo a sol/gel transition almost independent of temperature, provided that some multivalent cations are present, and that the distribution of the two monomers is not random but rather arranged in a block-wise fashion giving rise to G-blocks, M-blocks and blocks of a more alternating structure (MG-blocks). The property of a temperature-independent gel forms the basis for the greater popularity of alginates as an immobilization matrix for living cells.[3,4]

The basis for the gelling properties of alginates is their selectivity in ion-binding. It has been shown that the selective binding of certain alkaline earth metal ions (e.g. the strong and cooperative binding of Ca^{2+}

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relative to Mg^{2+}) increases markedly with increasing the content of the α-L-guluronate residues in the chains.[5,6] Polymannuronate blocks and alternating MG-blocks barely display any selectivity to bind Ca^{2+} relative to Mg^{2+}. However, there is a marked hysteresis in the binding of Ca^{2+}-ions to the G-blocks. The high selectivity between similar ions, such as those from the alkaline earth metals indicates that some ligand binding takes place as a result of structural features within the G-blocks. This phenomenon was initially attempted to be explained by the so-called ''egg-box'' model.[7,8] Although limited in its steric arrangement approach,[9–11] this simple ''egg-box'' model still persists, alluding to an intuitive understanding of the ion-binding properties of alginates. The limitation to dimerization only of alginate guluronic acid sequences within the molecules in the ''egg-box'' model is recently challenged by data obtained from small-angle X-ray scattering on alginate gels that suggest lateral association far beyond a pure dimerization with increasing $[Ca^{2+}]$ and the G-content of the alginate.[12,13]

The three characteristic block-like sequences of alginates, G, M and MG, can be isolated as separate oligomers by taking advantage of the different susceptibility towards acid hydrolysis of the different glycosidic linkages.[14] The subsequent necessary purification exploits the differences in the inherent acid solubility properties of the three types of the block-like sequences. G-blocks can, for example, be obtained well-above 90% purity, and with a number-average degree of polymerizaton, DP_n, of typically around 20. Once isolated and purified, these structures would not be able to give gel-like structures, but would rather form a precipitate in the presence of e.g. Ca^{2+} due to a complete lack of elastically active segments. Such isolated and purified G-blocks have been shown to be able to act as gelling modulators when mixed with a gelling alginate, both with respect to gelling kinetics and apparent equilibrium properties.

The scope of the present paper is to try to shed some further light into the understanding of the correlation between the binding of Ca^{2+} and the sol/gel transition of high molecular weight alginate gels in the presence of oligoguluronates.

Experimental Part

Alginate Samples

Alginate samples isolated from various sources were kindly provided by Pronova Biopolymer a.s., Drammen, Norway. The chemical composition and the fraction of the four possible next-neighbor residue arrangements, the diad sequences, were determined by high-field ^{1}H NMR spectroscopy according to Grasdalen.[15] The weight-average molecular weights of the samples, M_w, were determined from intrinsic viscosity, $[\eta]$, measurements, as previously outlined.[12]

The samples were classified according to their fraction of G-units, F_G, into three groups, each named with a three-letter acronym, as follows: LoG for samples with low F_G, InG for intermediate F_G , and HiG for highest F_G (Table 1). The subscripts added to these sample designations reflect the estimated M_w (in kDalton) of these samples. The G-block sample used

Table 1.
Properties of alginate samples.

Source	F_G	F_M	F_{GG}	F_{GM},	F_{MM}	F_{GGG}	F_{GGM}	F_{MGM}	$N_{G>1}$	M_W*	Acronym
L. hyperb.l.	0.50	0.50	0.30	0.23	0.24					155	InG_{155}
L. hyperb.s	0.62	0.38	0.55	0.31	0.07	0.51	0.04	0.03	14.8	310	HiG_{310}
L. hyperb.s	0.68	0.32	0.52	0.16	0.17					160	HiG_{160}
	0.94	0.06	0.83	0.11	0.05	0.8	0.03	0.08			G-block

*Weight-average molecular weight in kDalton.

had a guluronic acid content of 97% and a DP_n of 20, as determined by NMR spectroscopy.

Preparation of Ca-Alginate Gels

Alginate stock solutions with an alginate concentration $c_p = 20\,mg\,mL^{-1}$ were filtered through filters with pore-sizes of 1.2 and 0.7 μm (Millipore) and blended with solutions of an inactivated form of Ca^{2+}, followed by addition of the slowly hydrolyzing D-glucono-δ-lactone (GDL) to obtain homogeneous Ca-alginate gels. In this study, Ca^{2+} complexed with ethylene glycol tetraacetic acid (EGTA) was used, but we have previously also explored the use of Ca^{2+} in the form of an insoluble salt ($CaCO_3$).[12] The homogeneous alginate gels were prepared by blending an appropriate volume of the $20\,mg\,mL^{-1}$ alginate stock solution with the appropriate volume of a 100 mM Ca-EGTA solution of pH 7, followed by the addition of the required amount of solid GDL freshly dispersed in 0.2 mL H_2O. Finally, the total volume was adjusted to give the desired c_p and $[Ca^{2+}]$. The stock solution of 100 mM CaEGTA of pH 7 was prepared as outlined previously.[12] Based on the analysis of the multiple equilibria (see below), the concentration 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 hours following addition of GDL. The rate of change in pH and the release of Ca^{2+} from CaEGTA were monitored using a conventional pH electrode and a Ca^{2+} sensitive electrode in parallel experiments.

Rheological Characterization

Dynamic viscoelastic characterization of the alginate samples prior to gelation and 24 hours after inducing the gelation was carried out by determining the frequency dependence of the storage and loss moduli, $G'(\omega)$ and $G''(\omega)$, at $T = 20\,^{\circ}C$ employing a Rheologica Stress-Tech general purpose rheometer equipped with a 40 mm diameter serrated plate-plate measuring geometry, $\omega = 6.28\,s^{-1}$ and a 10 mPa stress. The samples were sealed with a low-density, low-viscosity silicon oil to avoid adverse effects associated with evaporation of solvent throughout the gelation experiments. Apparent equilibrium properties of the resulting gels were measured after 24 hours by calculating the Young's modulus (E) from the initial slope of the force/deformation curve obtained by longitudinal compression on a SMS TA–XT2 Texture Analyser at a compression rate of $0.1\,mm\,s^{-1}$.

Multiple Equilibria within the Calcium – EGTA – Alginate

The distribution of the total Ca^{2+} in the various association states with EGTA and alginate in the employed gelation method was estimated as a function of pH using the reported association constants for the various equilibria. The gelation method involves the slow hydrolysis of glucono-δ-lactone to lower the pH and thereby make the ionic form of Ca accessible to binding to alginate by reducing the stability of the CaEGTA complexes. The four ionization states of EGTA can be written:

$$H^+ + EGTA^{5-i} \rightleftharpoons EGTA^{4-i};$$
$$K_i = \frac{[EGTA^{4-i}]}{[H^+][EGTA^{5-i}]} \quad i = 1,..4; \tag{1}$$

where the notation used for EGTA implicitly indicates the number of hydrogens in the depicted net charge. The corresponding association constants are:[16] $\log K_1 = 9.46$, $\log K_2 = 8.85$, $\log K_3 = 2.68$ and $\log K_4 = 2.0$. The equilibria for binding of Ca^{2+} to EGTA are:

$$Ca^{2+} + EGTA^{5-j} \rightleftharpoons CaEGTA^{3-j};$$
$$K_{CaEj} = \frac{[CaEGTA^{3-j}]}{[Ca^{2+}][EGTA^{5-j}]}; \tag{2}$$
$$j = 1, 2;$$

The corresponding association constants are reported to $\log K_{CaE1} = 11.0$ and $\log K_{CaE2} = 5.33$. Introducing the shorthand notation E for EGTA, the

Equations for total amount of EGTA and Ca reads:

$$[E]_0 = \left[E^{4-}\right] + \left[E^{3-}\right] + \left[E^{2-}\right] + [E^-] + [E] + \left[CaE^{2-}\right] + [CaE^-] = \sum_{i=1}^{5}\left[E^{-(5-i)}\right] + \sum_{i=1}^{2}\left[CaE^{-(3-j)}\right] \quad (3)$$

$$[Ca]_0 = \left[Ca^{2+}\right] + \left[CaE^{2-}\right] + [CaE^-] \quad (4)$$

At a specified pH, total concentration of EGTA and Ca, the set of Equations 1-4 can be simultanously solved to provide the concentration of the various species using a matrix approach. Thus, Equations 1-4 combine to the following linear set of equations:

$$\begin{pmatrix} [H^+][E^{4-}] & -[E^{3-}]/K_1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & [H^+][E^{3-}] & -[E^{2-}]/K_2 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & [H^+][E^{2-}] & -[E^{1-}]/K_3 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & [H^+][E^{1-}] & -[E]/K_4 & 0 & 0 & 0 \\ [Ca^{2+}][E^{4-}] & 0 & 0 & 0 & 0 & -[CaE^{2-}]/K_{CaE1} & 0 & 0 \\ 0 & [Ca^{2+}][E^{3-}] & 0 & 0 & 0 & 0 & -[CaE^{1-}]/K_{CaE2} & 0 \\ [E^{4-}] & [E^{3-}] & [E^{2-}] & [E^{1-}] & [E] & [CaE^{2-}] & [CaE^{1-}] & 0 \\ 0 & 0 & 0 & 0 & 0 & [CaE^{2-}] & [CaE^{1-}] & [Ca^{2+}] \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ [E]_0 \\ [Ca]_0 \end{pmatrix} \quad (5)$$

Alginate is introduced into this system using the approximation of species with one association constant that is identified with the binding of Ca to α-L-GulpA residues. Thus, the set of Equations is extended by implementig the protonation of alginate:

$$H^+ + A\mathrm{lg}^- \rightleftharpoons HA\mathrm{lg}; \qquad K_a = \frac{[HA\mathrm{lg}]}{[H^+][A\mathrm{lg}^-]} \quad (6)$$

and the binding of Ca^{2+} to alginate:

$$Ca^{2+} + 2A\mathrm{lg}^- \rightleftharpoons CaA\mathrm{lg}_2; \qquad K_{A\mathrm{lg}} = \frac{[CaA\mathrm{lg}_2]}{[Ca^{2+}][A\mathrm{lg}^-]^2} \quad (7)$$

Introducing the Ca-alginate interaction extends the set of Equations (Eq. 5).

The set of Equations (Eq. 5) for a given set of conditions and as function of pH was solved using the Newton-Raphson method, generally applicable to nonlinear systems of Equations using the MNEWT routine.[17]

In addition to the constants specified above, the following constants were used when the calculations also included the alginate. The pK_a of the α-L-Gul and β-D-Man residues of alginate are reported to be 3.65 and 3.38, respectively.[18] Parameter values for the binding of Ca^{2+} to alginate can be obtained from the reported activity coefficient of Ca^{2+} in aqueous alginate solution, γ_{Ca},[19,20] or from the data reported based on NMR,[21] or more recent data obtained using isothermal titration calorimetry (ITC).[22,23] The actual values of the binding constants are $K_{Alg} = 3.3 \times 10^3\ M^{-1}$ from γ_{Ca}, $5 \times 10^3\ M^{-1}$ for the binding to α-L-Gul and $10 \times 10^3\ M^{-1}$ for the binding constant estimated in the second step of the ITC data.[23]

Results and Discussion

Mulitple Equilibria Between Calcium, EGTA and Alginate

The calibration of the amount of glucono-δ-lactone needed to reduce the pH sufficiently to reduce the association constant of CaEGTA to release Ca^{2+}, while at the same time not causing acid-induced gelation of alginate was carried out by adjusting the molar concentration of glucono-δ-lactone at the actual CaEGTA concentration. The kinetics of pH development and concentration of released Ca^{2+} was experimentally determined (Fig. 1), and the results compared to that calculated (Fig. 1b). The data (Fig. 1) indicate a reasonable qualitative agreement between the calculated release pofile of calcium from EGTA and that observed experimentally. Sample results from the calculations for the occurrence of the various species at different pH in the situation where Ca^{2+} is released from EGTA and

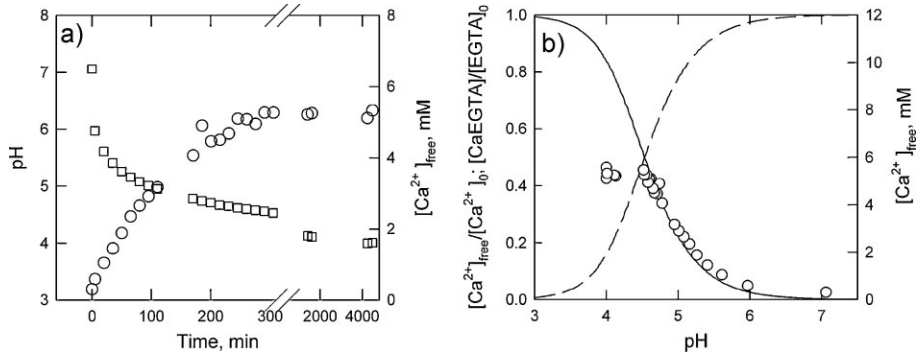

Figure 1.
(a) Experimentally determined pH (□) and free Ca^{2+} (○) versus time for an aqueous solution of 30 mM CaEGTA and 85 mM glucono-δ-lactone. (b) The experimentally determined relation between free Ca^{2+} and pH (○) and calculated molar fraction of free Ca^{2+} (continuous line) and CaEGTA to total EGTA (broken line) at the concentrations used in the experiments. The experimental data of free Ca^{2+} is adjusted to the relative scale.

made accessible to bind to alginate are shown in Figure 2. Such calculations indicate that reducing the pH significantly below 4 will result in increasing the abundance of the protonated (HAlg) form of alginate, and decreasing the fraction of the CaAlg form. Under such conditions, one can expect a blend of both iontropic induced gelation (by calcium) and a so-called acid-gelation mechanism. At around pH equal to 4, the situation appears to be optimal, in the sense that most alginate exists in the Ca-form, whereas the protonated alginate does not make up a significant fraction.

Influence of Alginate Concentration and Composition as well as Ca Concentration on the Strength of Alginate Gels

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 softer, more elastic gels.[1] The sample data in Figure 3 illustrate this for internal release of calcium for two alginates with total concentrations of 10 mM and 20 mM Ca^{2+}. Overall, the trends are, as exemplified by the data in Fig. 3, an increase of the equilibrium gel strength

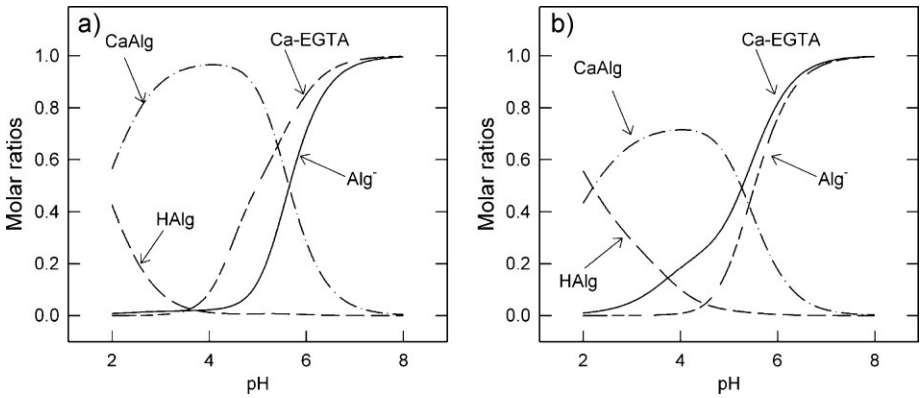

Figure 2.
Various molar ratios of CaAlg, protonated alginate Halg, Alg- and Ca-EGTA versus pH calculated as described in materials and methods. The calculations were carried out for (a) 15 mM Alg, 30 mM CaEGTA, and (b) 40 mM Alg, 30 mM CaEGTA.

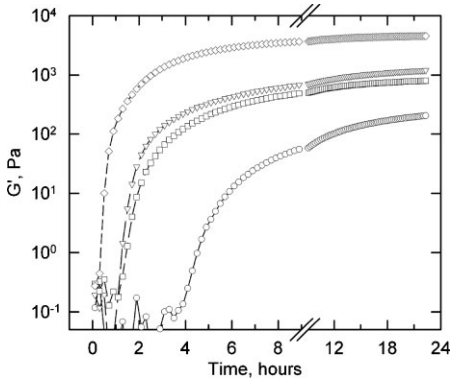

Figure 3.
Storage modulus G' versus time for Ca-induced gelation of alginate using release of Ca from CaEGTA within 10 mg mL^{-1} alginate solutions. The data are obtained for HiG_{160} (◇, □) and InG_{155} (▽, ○) alginates in total Ca^{2+} concentration of 10 mM (□, ○) and 20 mM (◇, ▽).

with increasing [Ca^{2+}] within the same alginate, and an increase in the gel strength at a given Ca-level when the G-content of the alginate increases. The temporal evolution in these cases, all being so-called Ca-limited, i.e. the ratio of [Ca^{2+}] to the binding sites of the alginates, assumed to be comprised of the G-fraction, is less than one, show that the compositions ultimately yielding the largest gel strengths, also are the ones that form gels most rapidly. Addition of G-block was previously shown to influence the kinetics of the gel formation process. A reduction in the gelation rate was also observed as a function of the quantity of G-blocks added. This effect 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. These observations open the way for the use of G-blocks instead of, for example, phosphates in kinetic control.

The effect of adding free G-blocks to gelling solutions of alginate was previously investigated.[14,24] The experiments revealed that the final *G*' value decreased upon addition of G-blocks. The effect has been found in experiments with Ca^{2+} concentrations equal to either 10 or 20 mM and has been seen to increase with increasing amount of G- blocks added. The gelation time t_c is on the other hand seen to increase with increasing amount of added G-blocks. This is explained by the increased total concentration of alginate strands in these solutions, increasing the difficulties associated with network formation.

In previous studies, the gel point and fractal dimensionalities at the gel point were determined for different alginate gels based on rheological characterization.[24] Both gels and pregel situations were additionally studied following dilution by ultramicroscopy (AFM). These experiments were performed to provide illustrations of the assembly modes of the alginates toward the gel state. The results revealed that the fractal dimension of the alginate gels increase when increasing the concentration of calcium ions from 10 to 20 mM. This increase was believed to reflect 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 develop in the solution. In situations of higher calcium saturation, lateral association of a high number of alginate chains occurs, giving ordered polysaccharide fiber-like structures.

In the presence of free G-blocks (G-block/ alginate = 1/1), the fractal dimension increased with increasing calcium concentration, accompanied with a simultaneous decrease in *G*'. These observations indicate that free G-blocks are involved in electrostatic calcium-mediated bonds. Thus, they compete with the alginate chains for the calcium ions. Free G-blocks may either compete with interchain G-sequences in the formation of junction zones and, therefore, do not lead to an increase in G' in the network formation, may mediate thicker junction zones by being "sandwiched" within laterally associated G-sequences, or may form interactions with other G-blocks. The rheology of an alginate-containing solution can therefore be changed by adding free G-blocks of mean chain length 20 units. This behavior is due to a different degree of Ca^{2+} saturation of the G components found in G-blocks in the alginate.

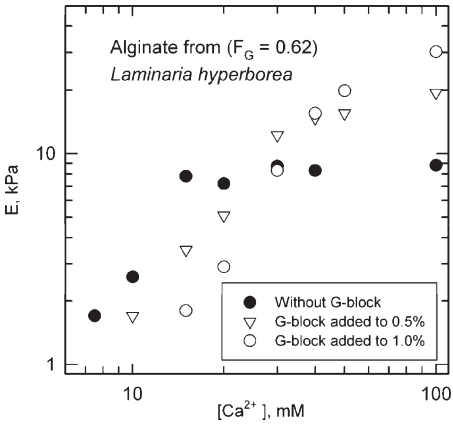

Figure 4.
Apparent equilibrium elastic properties in a system consisting of 10 mg mL^{-1} HiG$_{310}$ alginate with different additions of G-blocks (average DP$_n$ = 20) and increasing [Ca^{2+}].

Ca-Induced Gelation of Alginate - Non-Ca-Limited

The apparent equilibrium values of *G'* are not always reduced upon addition of free G-blocks to a geling alginate solution, but depends strongly on the amount of Ca^{2+} present. The data in Figure 4 show that whereas the apparent equilibrium elastic properties, given as Young's modulus as determined by longitudinal compression, are reduced with increasing addition of oligoguluronates at low [Ca^{2+}], there is a cross-over at around 30 mM Ca^{2+} above which the Young's modulus increases. The effect at low [Ca^{2+}] can be attributed to the ability of G-blocks to compete and bind Ca-ions, without participating in the network structure, and thereby preventing coherent network formation by the high M_w alginate.

Figure 5 shows the initial sol/gel transition in the system presented in Figure 4 where the [Ca^{2+}] is in the range where a general weakening of the apparent equilibrium elastic response is observed (15 mM) and above (40 mM). In both cases, an initial reduction in the gelling kinetics is observed.

A Possible Model for the Effect of Adding Oligoguluronates – Ca-Limited and Non-Ca-Limited Regimes

The various effects of alginate concentrations, fractional Ca-saturation and, in particular, the effect of adding oligoguluronates with respect to gelation and gel structure, are illustrated schematically in Figure 6. The observations of the gelling behavior of alginate solutions reveal that the gel strength increases whereas the gelation time decreases when reducing the alginate concentration (this is not explicitly included in the schematic illustration, Fig 6). The elastic modulus of an alginate gel depends on the number of cross-links as well as the length and stiffness of the chains connecting the cross-links. When Ca^{2+} is used as the cross-linker, the number of cross-links formed is determined by the relation between the available Ca^{2+} ions and the number of alginate chains. In

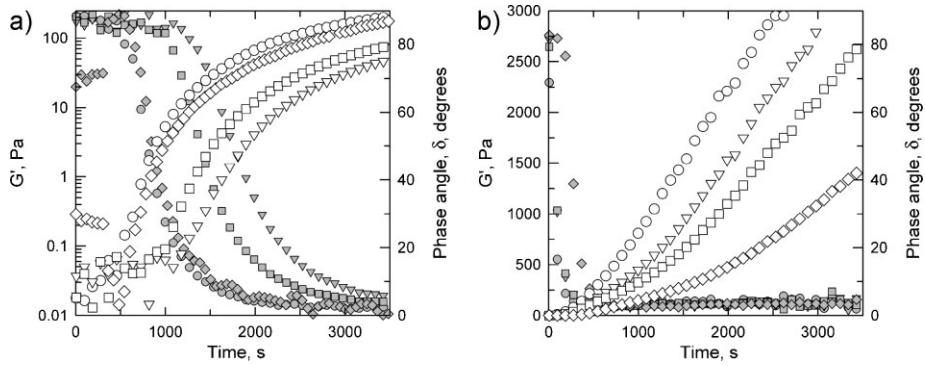

Figure 5.
Initial sol/gel transition of a system consisting of 10 mg mL^{-1} HiG$_{310}$ alginate with different additions of G-blocks (average DP$_n$ = 20) at (a) 15 and (b) 40 mM added Ca^{2+}.

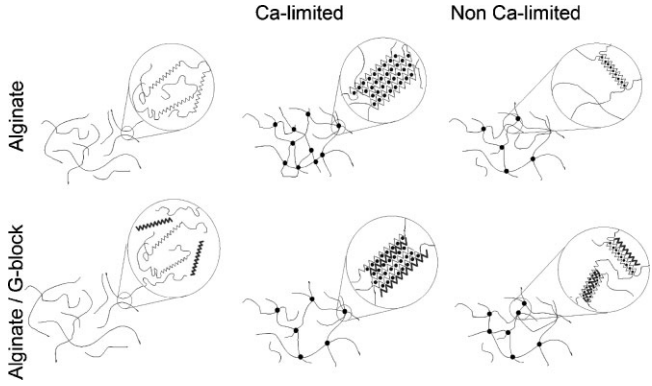

Figure 6.
Schematic illustration of relevant Ca-induced alginate chain associations for calcium limited and non-calcium limited situation. Possible effects of adding oligogulurontes in these cases are also included.

situations of low Ca^{2+} fractional saturation of the guluronic acid, a decreased gel strength might be observed due to a decrease in the number of cross-links formed. 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. When keeping the alginate concentration constant, *G'* increases with increasing $[Ca^{2+}]$. These two effects probably reflect an increased number of junction zones relative to the total number of active network chains and a decreased number of loose ends.

The decreased gel strength observed upon addition of G-blocks in the Ca-limited case 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 alginate chains but also between alginate chains and free G-blocks. 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 a reduced gel strength. The importance of this effect is expected to increase with decreasing divalent ion concentration. 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,[5] and the term ''nonequilibrium gel'' has been used to describe the alginate gel.[25] The difficulties associated with formation of the network increases with the density of alginate chains, i.e. with concentration, explaining an observed increased gelation time with increased alginate concentration.

The increase in Young's modulus at high Ca-concentrations, however, is not explained in a straightforward manner within the framework of the original ''egg-box'' model (dimerization of alginate junction zones). However, taking into consideration that further lateral association of alginate

chains within junction zones seems to be the general rule than an exception, it is a possibility that oligoguluronates may act between topologically restricted G-blocks in molecules of the gelling alginate and hence shorten the elastic segments of the resulting gel (Fig. 6). Furthermore, it has also been found that this increase in elastic properties at high [Ca^{2+}] increases with increasing G-block length in the high M_w alginate, thereby eliminating the possibility of oligoguluronates acting as inert fillers and suggests that specific interactions take place.

Conclusion

Addition of alginate oligomers to aqueous solutions of high molecular weight alginates represents a way to modulate gelation kinetics and final gel properties of ionitropically induced gels of alginates employing internal release of Ca^{2+}. The effect of oligoguluronate addition depends on the ratio between the total accessible Ca^{2+} and the Ca-binding sites made up both of the high molecular weight alginates and the oligoguluronates. Possible molecular mechanisms involving various modes of interaction between the oligoguluronates and the alginate chains are suggested. Thus, oligoguluronate addition can be used to modulate the rheological properties by affecting the gel kinetics, gel strength, viscosity, elasticity, and equilibrium properties in gelling alginate systems within various areas of application.

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