

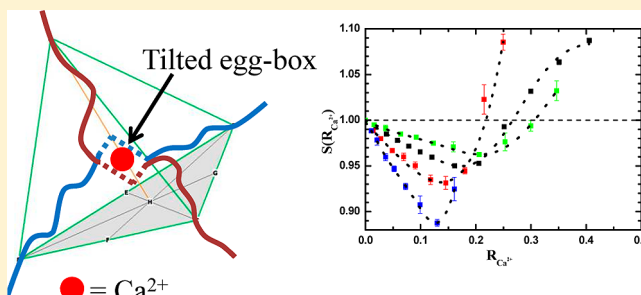
# On the Initial Binding of Alginate by Calcium Ions. The Tilted Egg-Box Hypothesis

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**ABSTRACT:** The initial binding of calcium ions by alginate chains was investigated in dilute solution. The use of viscometry, light scattering, circular dichroism, and fluorescence quenching performed on both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  alginate systems allowed collecting new experimental data in addition to those already reported in the literature. This led us to propose an ion multicomplex binding modality and to disprove the  $\text{Ca}^{2+}$ –alginate monocomplex formation. The first mode of bonding was proposed to be formed by four G residues from facing chains in a conformation which, albeit ordered, is different from the well-known “egg box” one. This was indicated as a locally tilted conformation (tilted egg-box). The addition of further bonding ions caused the well-known cooperative egg-box conformation (strong bonding) with notable variation in the physical–chemical properties of the dilute solution.



## 1. INTRODUCTION

Alginate is a collective term indicating a family of polysaccharides derived from brown algae and bacteria. From the chemical standpoint, they are linear polymers of 1→4 linked  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G). Along the chain, the two types of constituting saccharides are arranged in blockwise patterns, composing homopolymeric regions indicated as M-blocks and G-blocks, respectively, and copolymeric regions of MG-blocks. The relevance of alginate as gel forming agent has been largely exploited for low added value applications (food) as well as in advanced and demanding fields such as biomedicine. The latter have sprout renovated interest in the elucidation of the gel formation process that led to partially redeposit the model of junctions holding together alginate chains.<sup>1</sup>

Although numerous advancements in the description of alginate gels have been accomplished,<sup>2</sup> the initial binding of calcium ions by the polysaccharide chains still remains only partially explored. Commonly used terms such as length of the gel junctions and minimum and average length of the G-blocks seized the specific molecular discontinuity between nongelled and gelled states, but in all likelihood they are not appropriate to picture the initial binding of the gelling ion by the alginate chains, especially in dilute solution. While the so-called egg-box model for  $\text{Ca}^{2+}$ –alginate has been confirmed by recent results on X-ray scattering on G-blocks treated with excess of calcium,<sup>3</sup> its existence in the initial step of the ion binding process has been recently questioned and a monomolecular process has been proposed.<sup>4</sup> Specifically, in view of some variation of physical–chemical properties of alginate solutions, it has been

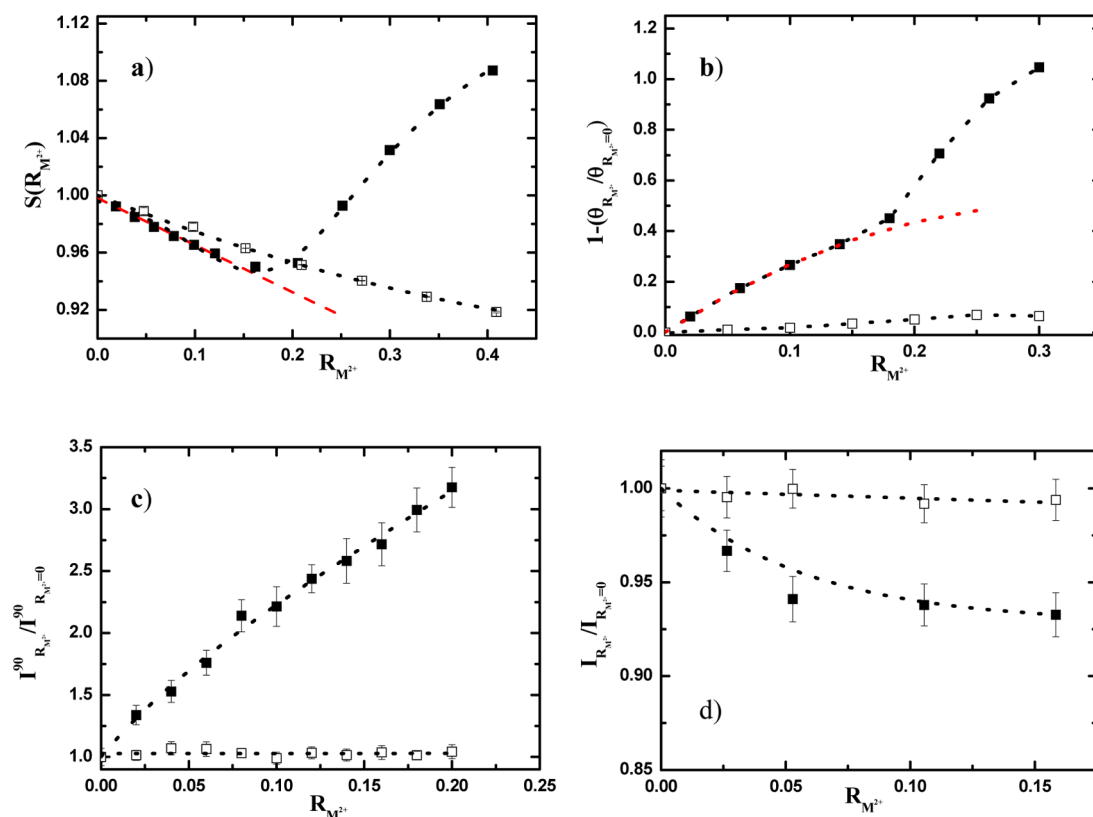
suggested that, provided the concentration of calcium ions is sufficiently low, the ion bonding occurs on a single alginate chain causing charge annihilation. Only in a later step does the merging of two alginate–calcium single arrays lead to the egg-box formation.

In the present paper, we challenged this interpretation and, in view of additional experimental data, reconciled the initial binding of calcium by alginate chains with a multicomplex formation. More specifically, calcium ions are explicitly considered as linkers holding two chains together by a short-range attraction. This scenario comprises calcium ion-induced contact points which differ from the “classical” arrangement of the egg-box (although they are formed by four G residues) mostly because they lack of physical contiguity along the chain: we describe this conformation as *tilted egg-box*. Moreover, we speculate over the concept of chain-mediated ion-clusterization for alginate as described in the literature for different biopolymers.<sup>5–9</sup>

The present contribution follows a series of papers by some of the authors that have focused on nonspecific affinity<sup>10,11</sup> in the alginate– $\text{Mg}^{2+}$  system and on the  $\text{Ca}^{2+}$ –pectate ion bonding.<sup>12,13</sup> Here we turn our attention to the study of the mechanism of calcium binding by alginate chains in its initial stage, i.e., for  $R_{\text{Ca}^{2+}} \ll 1$  (where  $R_{\text{Ca}^{2+}}$  stands for the ratio between the moles of calcium and the moles of monomeric units). In the present manuscript, we will focus on an alginate

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**Figure 1.** Relative viscosity variation,  $S(R_{M^{2+}})$  (a), ellipticity variation (b), and relative increase of intensity of the light scattered at  $90^\circ$  (c) for *L. hyperborea* upon addition of calcium (■) or magnesium (□), respectively. Conditions: polymer concentration 0.7 g/L,  $T = 25^\circ\text{C}$ . (d) Relative variation of the fluorescence intensity of DAR-labeled alginate from *L. hyperborea* at 1 g/L upon addition of  $\text{Mg}^{2+}$  ions (□) and  $\text{Ca}^{2+}$  ions (■) respectively. Excitation: 520 nm; Emission: 576 nm. Lines are drawn to guide the eye. In all cases, aqueous NaCl 0.05 M was used. In parts a and b, red dashed lines represent the fitting of the experimental data point for  $R_{\text{Ca}^{2+}} < 0.15$ .

from *Laminaria hyperborea*, deferring an extended analysis on other alginate samples or specific sequences (guluronan, mannuronan and polyalternating) to forthcoming works.

## 2. MATERIALS AND METHODS

Sodium alginate isolated from *L. hyperborea* stipe (compositional fraction,  $F$ , as  $F_G = 0.65$ ,  $F_{GG} = 0.53$ , and  $F_{GGG} = 0.49$ ; relative molecular mass,  $\text{MW} \sim 130\,000$ ) was provided by FMC Biopolymers (Norway). Sodium chloride, calcium chloride, magnesium chloride, 5,6-diammino- $N,N,N',N'$ -tetraethylrhodamine B (DAR), 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride, and  $N$ -hydroxysuccinimide were purchased from Sigma-Aldrich (St. Louis, MO).

**Viscosity measurements.** The variation of the relative viscosity of the sodium form of alginate from *L. hyperborea* was measured at  $25^\circ\text{C}$  by means of a Schott-Geräte AVS/G automatic measuring apparatus and an Ubbelohde type viscometer upon addition of  $\text{CaCl}_2$  and  $\text{MgCl}_2$  at different values of the ratio,  $R_{M^{2+}}$ , between moles of divalent ions and moles of monomeric units (eq 1)

$$R_{M^{2+}} = [\text{M}^{2+}]/[\text{Polym}]_{\text{RU}} \quad (1)$$

Measurements were performed at different total polymer concentration and in the presence of different amounts of aqueous sodium chloride,  $R_{\text{Na}^+}$  (eq 2)

$$R_{\text{Na}^+} = [\text{Na}^+]/[\text{Polym}]_{\text{RU}} \quad (2)$$

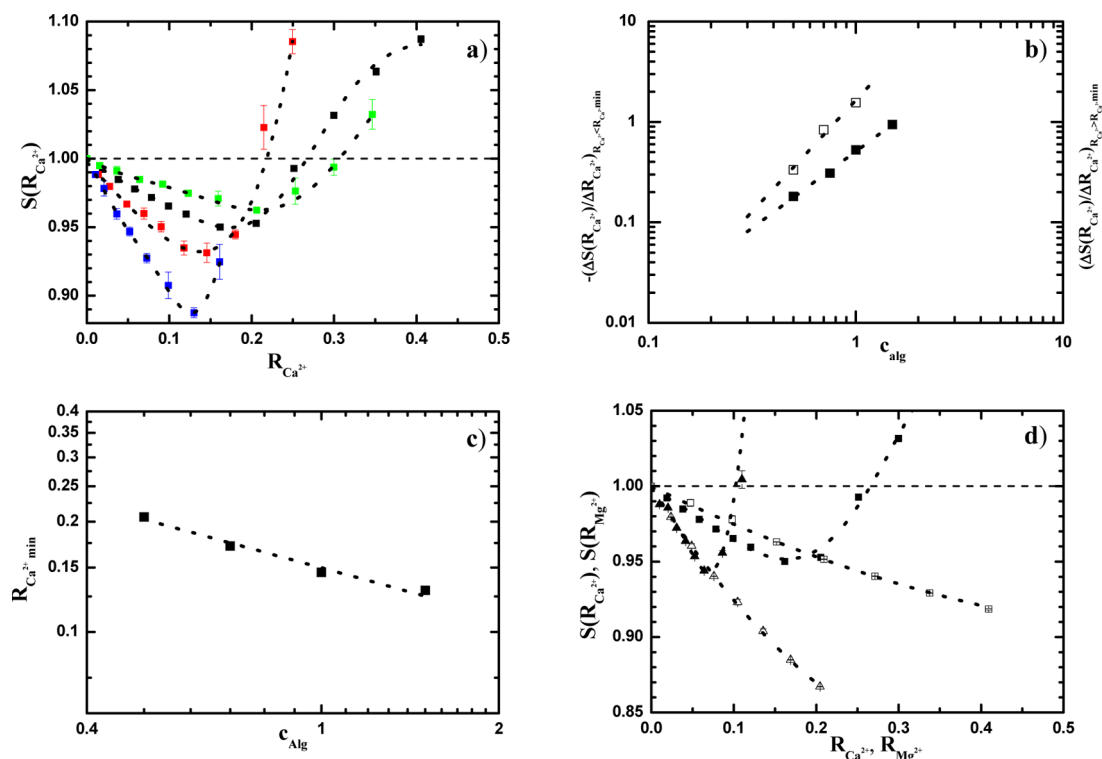
The results were reported as the ratio of the relative viscosity after and prior to the addition of the divalent ions,  $S(R_{M^{2+}})$ , defined as (eq 3)

$$S(R_{M^{2+}}) = \frac{(\eta_r)_{R_{M^{2+}}}}{(\eta_r)_{R_{M^{2+}}=0}} \quad (3)$$

where the relative viscosity,  $\eta_r$ , is defined as the ratio between the flow time in the capillary for the polymer solution and that of the solvent.

**Circular Dichroism Spectroscopy.** Circular dichroic (CD) spectra of the sodium form of alginate from *L. hyperborea* were recorded in the presence of 0.05 M aqueous  $\text{NaClO}_4$  (polymer concentration 0.7 g/L) with a JASCO J-700 spectropolarimeter. A quartz cell of 1-cm optical path and the following setup were used throughout the measurements: bandwidth, 1 nm; time constant, 2s; scan rate, 20 nm/min. Four spectra, corrected for the background, were averaged for each sample. The spectrum of each sample was recorded before and after the addition of  $\text{MgCl}_2$  and  $\text{CaCl}_2$ , respectively, at different values of  $R_{M^{2+}}$  (eq 1).

**Light Scattering.** A Perkin-Elmer LS50B spectrofluorimeter was used to record the intensity of the scattered light ( $90^\circ$ ;  $\lambda = 550\text{ nm}$ ) by solutions of the sodium form of alginate from *L. hyperborea* in aqueous NaCl 0.05 M upon irradiation at 550 nm incident light ( $T = 25^\circ\text{C}$ ). Eight spectra were averaged for each sample. The spectrum of each sample was recorded before and after the addition of  $\text{MgCl}_2$  and  $\text{CaCl}_2$ , respectively, at different  $R_{M^{2+}}$  (eq 1) values.



**Figure 2.** a) Dependence of the relative viscosity ratio ( $S(R_{Ca^{2+}})$ ) from  $R_{Ca^{2+}}$  for alginate from *L. hyperborea* in aqueous NaCl 0.05 M at a polymer concentration of 0.5 g/L (green), 0.7 g/L (black), 1 g/L (red), and 1.4 g/L (blue), respectively. (b) Dependence of the slope  $-(\Delta S(R_{Ca^{2+}})/\Delta R_{Ca^{2+}})_{R_{Ca^{2+}} < R_{Ca^{2+}}^{min}}$  from alginate concentration before ( $R_{Ca^{2+}} < R_{Ca^{2+}}^{min}$ , left y-axis,  $\blacksquare$ ) and after ( $R_{Ca^{2+}} > R_{Ca^{2+}}^{min}$ , right y-axis,  $\square$ ) the minimum ( $R_{Ca^{2+}}^{min}$ ) in part a. Lines represent the best fit of the experimental data points obtained with  $-(\Delta S(R_{Ca^{2+}})/\Delta R_{Ca^{2+}})_{R_{Ca^{2+}} < R_{Ca^{2+}}^{min}} \propto c_{alg}^{1.5 \pm 0.1}$  for  $\blacksquare$  ( $R^2 = 0.98$ ) and  $(\Delta S(R_{Ca^{2+}})/\Delta R_{Ca^{2+}})_{R_{Ca^{2+}} > R_{Ca^{2+}}^{min}} \propto c_{alg}^{2.2 \pm 0.2}$  for  $\square$  ( $R^2 = 0.99$ ). (c) Dependence of  $R_{Ca^{2+}}^{min}$  from alginate concentration. Line represents the fitting of the data points according to  $R_{Ca^{2+}}^{min} \propto 1/(c_{alg})^{1/2}$  ( $R^2 = 0.97$ ). (d) Dependence of  $S(R_{Ca^{2+}})$  and  $S(R_{Mg^{2+}})$  from  $R_{Ca^{2+}}$  (full symbols) and  $R_{Mg^{2+}}$  (open symbols), respectively, for *L. hyperborea* at 0.7 g/L (squares) and 1.4 g/L (triangles). In both cases,  $R_{Na^+} = 14.1$ . In parts a and d, lines are drawn to guide the eye.

**Quenching Experiments.** For fluorescence quenching measurements, alginate was labeled with DAR as previously reported.<sup>14</sup> Measurements were performed on DAR-labeled alginate from *L. hyperborea* in the presence of aqueous NaCl 0.05 M (polysaccharide concentration = 1 g/L) before and after the addition of  $CaCl_2$  and  $MgCl_2$  at different  $R_{M^{2+}}$  values. An excitation wavelength of 520 nm was used and the intensity of the emitted light was measured at 577 nm using a Perkin-Elmer LS50B spectrofluorimeter operating at 25 °C.

### 3. RESULTS AND DISCUSSION

The present contribution aimed at revisiting some recently drawn conclusions on the binding of calcium ions by alginate. Specifically, the mode of the initial binding of calcium ions to the polysaccharide chain will be the main target of the present work. First of all, we tried to reproduce the experimental results reported by Fang et al.<sup>4</sup> on the variation of the relative viscosity of alginate upon addition of divalent ions (hereafter indicated as  $S(R_{M^{2+}})$ , eq 3)), as a function of the amount of divalent cations added ( $R_{M^{2+}}$ , eq 1) (Figure 1a). In the presence of  $Mg^{2+}$  ions, a monotonic decrease in  $S(R_{Mg^{2+}})$  was detected. This is a consequence of the affinity driven condensation of these divalent ions onto the polyuronate, thereby decreasing its effective linear charge density, intrachain repulsion and overall dimensions.<sup>11</sup> On the other hand, the addition of same (small) amounts of  $Ca^{2+}$  ions caused a decrease in  $S(R_{Ca^{2+}})$  which was more marked than the one recorded for  $Mg^{2+}$  ions; however, when  $R_{Ca^{2+}}$  exceeded  $\sim 0.17$ , an upturn in the experimental curve was found with a rapid increase of  $S(R_{Ca^{2+}})$  upon

increasing  $R_{Ca^{2+}}$ . The value at which  $S(R_{Ca^{2+}})$  reaches the minimum is hereafter indicated as  $R_{Ca^{2+}}^{min}$ .

The discrepancy of behavior between calcium and magnesium toward alginate over the whole range of  $R_{M^{2+}}$  cannot be taken into account considering basic polyelectrolyte aspects only; instead, it points to additional implications of molecular origin.

Although our results are consistent with the work of Fang and co-workers,<sup>4</sup> it was decided to perform additional measurements using different techniques to be able to test in a more thorough way the model proposed by those Authors. In essence, this envisaged a monocomplex mechanism for alginate-calcium bonding, in the  $R_{Ca^{2+}}$  range from 0 to  $R_{Ca^{2+}}^{min}$ , with divalent ions linked to one single polysaccharide chain.

Figure 1b summarizes the CD data obtained from a *L. hyperborea* alginate solution upon treatment with different divalent ions. As already reported, the addition of  $Mg^{2+}$  ions, being governed by nonbonding affinity, brings about a negligible variation on ellipticity without any profound conformational effect on the sugar residues.<sup>10,11</sup> At variance, the addition of calcium ions, from the very beginning, caused a modification of the electronic environment of the chromophore, i.e., the carboxylate anion, resulting in a net variation of the ellipticity of the system under analysis. As previously reported for the case of galacturonan, this effect was traced back to ion bonding by the polysaccharide and change of the conformation of the glycosidic linkages. Nevertheless, looking in detail to the experimental curve of Figure 1b, two binding modes seemed to be present in the alginate- $Ca^{2+}$  system: the

first one took place approximately in the range  $0 < R_{Ca^{2+}} \leq 0.17$  while a second one was detected for  $R_{Ca^{2+}} > 0.17$ . The two binding modes basically differ as to the relative variation of the physical quantity upon addition of the binding ion. The biphasic behavior suggests that the conformational effect which takes place in parallel with the sharp increase in the relative viscosity roughly doubles the observed chiroptical effect of the preceding bonding mode. Although informative on the distinction between “affinity” and “bonding”, CD data *per se* cannot provide an insight on the chain molecularity of the process (monocomplex, i.e. intramolecular binding, or multi-complex, i.e. intermolecular binding, formation). As to this aspect, a conclusive evidence can be provided by light scattering. Figure 1c shows that the very initial  $Ca^{2+}$ -bonding mode is already associated with a substantial increase of the intensity of the scattered light, which is a sensitive indicator of mass of the scattering elements. This result was clearly not consistent with an intramolecular calcium ion-alginate monocomplex formation for  $R_{Ca^{2+}} < R_{Ca^{2+}min}$  but rather pointed to an ion-induced chain–chain association even for initial additions of calcium ions. The same effect was not detected upon addition of  $Mg^{2+}$  ions to the alginate solution, in agreement with the concept of affinity-driven interaction, ruled basically by Manning’s theory derived for mixtures of ions,<sup>15</sup> between the ion and the nonassociated polysaccharide chain.

This latter aspect was explored further by means of fluorescence quenching measurements using alginate labeled with DAR-2 (Figure 1d). The addition of  $Mg^{2+}$  ions brought about just a slight quenching of the emitted light, in agreement with the hypothesis of a minor shrinking of the polymer coil following a reduction of the net chain charge density, due to bivalent ion condensation. At variance, when calcium ions were added, a much larger decrease of the emitted light was detected; this was likely caused both by a more marked coiling due to a higher fraction of divalent condensed ions resulting from a higher affinity and by some calcium-induced approach of alginate coils (point-like local dimerization).

Further analyses were performed by measuring  $S(R_{Ca^{2+}})$  as a function of polysaccharide concentration (Figure 2a). It was seen that the slope of the curve  $S(R_{Ca^{2+}})$  vs  $R_{Ca^{2+}}$  increased upon increasing the polysaccharide concentration. In particular, Figure 2b showed that when  $R_{Ca^{2+}} < R_{Ca^{2+}min}$ , the slope (changed in sign) of the linear part of Figure 2a scaled with polysaccharide concentration according to  $-(\Delta S(R_{Ca^{2+}})/\Delta R_{Ca^{2+}}) \propto c_{alg}^{1.5 \pm 0.1}$ . This power law dependence correlated nicely with the one found for the elastic response of half-staggered double stranded (unligated) fibrin clog as a function of fibrinogen concentration in the presence of thrombin,<sup>16</sup> a well known example of biopolymer chain association. This result strengthened the conclusion that even for the very first additions of calcium ions the bonding process to alginate is not compatible with a monomolecular process, but, rather, with intermolecular association driven by loosely bound cross-linking agents. This was also the case discussed by Greenfield et al.,<sup>17</sup> where the elastic response of peptide amphiphile molecules treated with HCl showed a power law dependence from the concentration with an exponent of 1.51 which was traced back to the acid-induced weak cross-links. The analysis on the dependence of the slope of  $S(R_{Ca^{2+}})$  from alginate concentration was performed also for the linear part of the second half of the curves in Figure 2a, i.e., for  $R_{Ca^{2+}} > R_{Ca^{2+}min}$  (Figure 2b). The precipitation of the alginate sample at 1.5 g/L prevented including this sample in the analysis. It can be seen

that the experimental data correlated with a power law  $(\Delta S(R_{Ca^{2+}})/\Delta R_{Ca^{2+}}) \propto c_{alg}^{2.2 \pm 0.2}$ . This modification of the power law dependence from biopolymer concentration was also found by Greenfield et al.<sup>17</sup> on peptide amphiphiles molecules upon going from a weak to strong cross-links.

Another important aspect of Figure 2a is that, at a constant aqueous NaCl concentration of 0.05 M,  $R_{Ca^{2+}min}$  is a function of alginate concentration. Indeed, it scales with the polysaccharide concentration according to  $R_{Ca^{2+}min} \propto 1/(c_{alg})^{1/2}$  (Figure 2c). Again, this is in contrast with the mechanism predicting the formation of calcium-alginate monocomplex which dimerizes only when the  $Ca^{2+}/G$  stoichiometry reaches 0.25, as proposed by Fang and co-workers.<sup>4</sup>

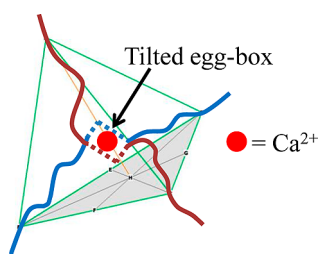
Additional evidence supporting the multichain process and disproving the monocomplex formations for the initial calcium binding by alginate is reported in Figure 2d, where the variation of  $S(R_{Ca^{2+}})$  was compared with that of  $S(R_{Mg^{2+}})$  at two different alginate concentrations. In this case, a constant  $R_{Na^+}$  was maintained. The first consideration is that, focusing on the addition of  $Mg^{2+}$  ions, the decrease of  $S(R_{Mg^{2+}})$  is more marked upon increasing alginate concentration. This is expected on the basis of theoretical polyelectrolyte grounds, since the actual concentration of  $Me^{2+}$  ions (at the same  $R_{Me^{2+}}$  value) increases with the polymer concentration. This brings about two converging effects: (i) an increase of the ionic strength and then a shielding of intrachain repulsion; and (ii) a relative increase of the fraction of condensed divalent counterions, with a decrease of the total number of unshielded charges. Those effects contribute to a shrinkage of the coil dimensions. However, it is noteworthy that the difference between  $S(R_{Mg^{2+}})$  and  $S(R_{Ca^{2+}})$  in the range  $0 < R_{Ca^{2+}} < R_{Ca^{2+}min}$  is reduced upon increasing alginate concentration (Figure 2d). The traditional interchain model of  $Ca^{2+}$  bonding by alginate can easily interpret this effect as due to a more marked chain–chain association, from the very beginning of divalent ion addition, simply stemming from the obvious effect of polymer concentration increase over an association process. At variance, this specific result cannot be reconciled, in our opinion, with the  $Ca^{2+}$ –alginate monocomplex model proposed by Fang and co-workers. In fact, the accepted (much) higher affinity of the alginate chain for  $Ca^{2+}$  ions, if potentiated by bonding to single-chain sites, would predict even larger decrease with respect to  $Mg^{2+}$  ions, thereby leading to an increase of the difference between  $S(R_{Mg^{2+}})$  and  $S(R_{Ca^{2+}})$  upon increasing alginate concentration.

The discrepancies between our experimental data and the model by Fang et al.<sup>4</sup> led us to propose a different model depicting the initial calcium ion bonding as an association process involving one calcium ion and monomeric units from two alginate chains. The coordination of the guluronate units around the ion resembles an egg-box structure, although with a different geometrical order, and it is indicated as the *tilted egg-box* (Scheme 1).

The bonding of a calcium ion by two GG sequences on two facing alginate chains led to the formation of chain–chain dimers which are not parallel with respect to each other but, because of the repulsion between the carboxylic groups, they tend to be placed at an angle probably not far from 90°. This tilted orientation will be even more approaching a totally diverging one by considering the four chain stretches before and after the GG sequences (actually, the geometrical configuration providing the absolute energy minimum would be that of a tetrahedron, with the  $\langle GG-Ca^{2+}-GG \rangle$  unit in the



**Scheme 1. Graphical Representation of the Initial Binding of Calcium (Red Circle) by Facing GG Dimers (Dotted Bars) in the Initial Tilted Conformation**



center and the four chain branches pointing to the four vertices). The calcium ions coordinated in the tilted egg-box structures parallel the weak linkers described by Geenfield and co-workers<sup>17</sup> and the molecular arrangement resembled the half-staggered fibrin association nicely depicted by Falvo et al.,<sup>18</sup> thus explaining the power law dependence found, in the case  $R_{Ca^{2+}} < R_{Ca^{2+}min}$ , for the slopes of the curves reported in Figure 2a.

The hypothesis of the tilted egg-box results from the interpretation of the experimental results reported in the present manuscript and frames the beginning of the alginate chain bonding by calcium ions. It is, however, expected that, when a certain amount of bound calcium ions is exceeded, the formation of the well-known  $(GGG)_n$  junctions described by Smidsrød and Morris<sup>19,20</sup> takes place.

In this sense, it is tempting to speculate on a mechanism driving junction formation from the initial bonding process; this could be inspired by the model already proposed by Borukhov et al. for biopolymers giving rise to polymer bundle formation in a cooperative fashion.<sup>5,6</sup> In particular, the clusterization into a (comparatively) long ordered sequence, passing from  $n$  point-like linkers to a strong cross-link stretch formed by  $n$  ions, that Borukhov calls the “railway track”,<sup>8</sup> might find applicability also in the case of alginate. Along this line, the formation of a rigid and ordered array of calcium mediated alginate chains could represent the onset of the upturn of the experimental curve of  $S(R_{Ca^{2+}})$  (Figures 1a and 2a). This latter consideration seems to be sustained also by the power law dependence of  $(\Delta S(R_{Ca^{2+}})/\Delta R_{Ca^{2+}}) \text{ vs } c_{alg}$  for  $R_{Ca^{2+}} > R_{Ca^{2+}min}$ . However, while the Borukhov model has been theoretically and experimentally proven plausible for highly charged biopolymers with a high value of the persistence length,  $l_p$  (as an example actin has a Manning–Oosawa linear charge density  $\xi^{21,22}$  of approximately 3.4 at 20 °C and an estimated  $l_p$  of  $2 \div 18 \mu\text{m}$ ), its applicability to the case of polyuronates, alginate in the specific case ( $\xi = 1.53$ ,<sup>11</sup>  $l_p \sim 15 \text{ nm}^{23,24}$ ), has to be taken with caution and an extended analysis along this line could be of notable interest.

#### 4. CONCLUSIONS

The present manuscript focused on the initial binding of calcium ions by alginate. The authors have reported evidence that cast doubts on the monocomplex mechanism proposed by Fang et al. and, at the same time, have provided a novel model that implies, even for small amount of  $\text{Ca}^{2+}$  ions added, the dimerization of alginate chains. Because of the geometrical and electrostatic (like-charge repulsion) considerations, for the first additions of calcium ions, a chelation, indicated as *tilted egg-box*, was proposed. Although the elucidation of the mechanism

originating egg-box junctions needs further analyses, similarly to the description of bundles formation by other biopolymers, the present paper speculates over a mechanism resembling the so-called “railway track” formation, where the junction formation took place upon clusterization of separate cross-linking ions.

The present work should be considered as the starting point for the elucidation of the initial calcium ions binding by alginate, while the process of clusterization of the ions, with the obvious implications on the transport properties of the solution, deserves a more detailed analysis, deferred to forthcoming work. A similar analysis will also need to be directed toward alginates of different compositions, in particular on those containing exclusively (or largely) alternating (MG) sequences and G-blocks.

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##### Notes

The authors declare no competing financial interest.

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