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Ionic gel formation of a (pseudo)alginate characterised by an alternating MG sequence produced by epimerising mannuronan with AlgE4

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Abstract

The scope of this paper is the characterization, in terms of viscoelastic and mechanical properties, of ionic gels obtained from solutions of pseudoalginates characterized by a strictly alternating MG sequence (ALG(47%, 0%GG)) in the presence of calcium ions. ALG(47%, 0%GG) was obtained using a recombinantly produced mannuronan C-5 epimerase, named AlgE4 which catalyses the conversion of mannuronic residues into guluronic–mannuronic (GM) blocks. It was established that the kinetics of gelation as well as the mechanical properties and degree of syneresis of the ensuing gels are markedly dependent on both polymer concentration and Ca^{2+} content.

The molecular dynamic investigation was carried out on a comparative basis between poly(MG) vs. poly(G) and demonstrated that in the case of poly(MG) the structural unit composed by two calcium paired MG oligomer enjoy a higher degree of flexibility in comparison to the equivalent structure based on GG sequence. Furthermore, the electrostatic interaction between Ca^{2+} ions and carboxylate groups of M and G units is the main driving force to gel formation.

Keywords: Pseudo(alginates)/calcium gels; Alternating mannuronic–guluronic sequences; Rheology; Circular dichroism; Molecular dynamics

1. Introduction

Alginate is a binary co-polymer of β-D-mannuronate (M) and α-L-guluronate (G) arranged in a block-wise pattern along the linear chain. This polymer has wide industrial applications due to its viscosifying, gelling, suspending and ion-binding abilities. (Smidsrød & Draget, 1996).

The physical properties of alginates depend not only upon the uronate composition, i.e. the M/G ratio, but also upon the monomer sequence and distribution in the copolymer. The proportion and sequential arrangement of the uronic acid in alginates depends upon the species of algae and the kind of algae tissue from which the polysaccharide is extracted, and vary widely from species to species. This compositional heterogeneity represents a limitation in advanced technological applications in which is of vital importance the availability of alginates with a specific and constant primary structure. The lack of these requisites makes it difficult to obtain materials with the desired and reproducible characteristics. For instance, alginates beads from *Laminaria hyperborea* and *Macrocystis pyrifera* characterized by a different content of guluronate, were used as an immobilization matrix of hepatocytes (Murtas et al., 2005). The two kinds of beads exhibited a different permeability with respect to high molecular weight blue-dextrans, used as the probe macromolecules mimicking proteins and antibodies. Furthermore, alginate composition seems to affect also the metabolic activity of the entrapped hepatocytes: the secretion of albumin was better in the case of high mannuronic alginates than high guluronic alginates (Khalil et al., 2001). Thus, the primary structure of the alginates employed affects both the beads porous structure and the

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physiological response of the entrapped hepatocytes. Also the investigation of the relationship between the properties of alginates both in solution and in the gel state and their M/G ratio and residues sequential arrangement suffers of the variability and lack of control on the primary structure of natural occurring alginates. This drawback was well known to the scientific community involved in alginate research and efforts have been directed towards biosynthetic pathways capable of achieving control over the proportion and distribution of M and G in alginates. This work originated from the discovery that alginate-producing bacterium *Azotobacter vinelandii* encodes at least seven different mannuronan C-5 epimerases. These genes have been sequenced and cloned and expressed in *Escherichia coli*; the enzymes thus produced have been designed AlgE1–AlgE7 (Ertesvåg, Doset, Larsen, Skjåk-Bræk, & Valla, 1994; Svanem, Skjåk-Bræk, Ertesvåg, & Valla, 1999). These enzymes are capable of converting M residues into G residues in the polymer chain with different patterns of epimerisation (Draget, Skjåk-Bræk, & Smidsrød, 1997), and can therefore be used to modify alginates *in vitro* to obtain new alginates with the desired content and distribution of G residues (Ertesvåg et al., 1995). For instance, the C-5 epimerase AlgE4 forms alginates with long strictly alternating sequences (Hartmann, Holm, Johansen, Skjåk-Bræk, & Stokke, 2002), while AlgE1 introduces stretches of G sequences. Thus, an alginate of whatever residue composition and sequence can be converted to an alginate with a composition close to the desired one by employing the appropriate single or blend of C-5 epimerases. Nevertheless, the compositional heterogeneity in natural alginates substrates still represents a limitation as far as the degree of control on the primary structure of the final product is involved. In this respect, a further improvement was represented by the microbial production of a homopolymeric mannuronan, using a C-5 epimerase negative mutant of recombinant *Pseudomonas fluorescens* (Gimmestad et al., 2003).

By using this polymer as substrates for a single or a blend of recombinant epimerises we are able to generate alginates with specific composition and sequential structures. This has opened the possibility to study the effect of increasing the content of G blocks and/or MG blocks on the mechanical properties of epimerised mannuronan both in solution and in the gel phase (Dentini, Rinaldi, Barbetta, Risica, & Skjåk-Bræk, 2006; Dentini, Rinaldi, Risica, Barbetta, & Skjåk-Bræk, 2005) and to make the important discovery of the ability of alternating MG alginate to undergo gelation in the presence of Ca^{2+} ions (Donati, Holtan, Mørch, Dentini, & Skjåk-Bræk, 2005) a behavior which was attributed, before the advent of the C-5 epimerases, to alginates rich in G stretches only.

In the present article, we focus on the rheological study of both the gelling kinetics and the viscoelastic properties of the ionic gels obtained from mannuronan epimerised with AlgE4 in the presence of calcium ions. The involvement of MG sequence in the formation of the junction zones induced by calcium ions was investigated by molecular dynamics and a new molecular model for poly(MG)–Ca^{2+} structure is proposed.

2. Experimental

2.1. Materials

High molecular weight mannuronan was isolated from the fermentation of fructose in the presence of an epimerase-negative $AlgG^-$ strain of *P. fluorescens* (Gimmestad et al., 2003). Purification and deacetylation were carried out as described earlier (Ertesvåg & Skjåk-Bræk, 1999). Pure mannuronan was epimerized by AlgE4 epimerase as described earlier. (Ertesvåg & Skjåk-Bræk, 1999).

The mole fraction of guluronate (G) residues, F_G, of GM (MG) diad sequences, F_{MG}, as determined by ^1H-NMR (Grasdalen, 1983; Grasdalen, Larsen, & Smidsrød, 1979) in D_2O at 90 °C was 47%. The average molecular weights of mannuronan (496000) and mannuronan epimerised by AlgE4 (533000) (hereinafter indicated as ALG(0%G/0%GG) and ALG(47%G/0%GG), respectively) were determined by HPSEC-RI-MALLS using three serially connected columns TSK gel G 6000/5000/4000 PWXL. Detectors were refractive index (RI), UV monitor LKB UV II Amersham Pharmacia Biotech and multiple angle light scattering (MALLS) Dawn DSP equipped with a He–Ne laser operating at 632.8 nm, Wyatt Technology Corp.

2.2. Circular dichroism

The circular dichroism spectra were recorded using a spectropolarimeter Jasco J715-A in the wavelength range 190–300 nm with four repetition scans and a sensitivity of five *mdeg*. All measurements were performed at room temperature using 0.5 cm quartz cells.

In studies of polymer–Ca^{2+} interactions, a starting stock of a polymer solution at a concentration of 0.1% w/v was prepared in a 50 mM Tris/HCl, 50 mM $NaClO_4$ (pH 6.9) buffer solution. CD spectra were recorded as a function of the ratio R (Ca^{2+} equivalents/polymer equivalents). Values of R were obtained by using a polymer solution at a concentration of 0.02% w/v and adjusting the amount of Ca^{2+}. To this end, standard $CaCl_2$ solutions in buffer were used.

2.3. Preparation of ALG(47%G/0%GG) Ca^{2+}-induced gels

All experimental polymer solutions were prepared by dilution of stock polymer solutions. The formation of Ca^{2+}-gels was obtained through the *in situ*, controlled release of Ca^{2+} ions from a Ca–EDTA complex induced by the protons generated by the slow hydrolysis of GDL according to a well established procedure (Draget, Østgaard, & Smidsrød, 1989; Draget, Østgaard, & Smidsrød, 1990; Skjåk-Bræk, Smidsrød, & Larsen, 1986).

2.4. Rheological characterization

The dynamic viscoelastic characterization of ALG (47% G/0%GG) was carried out using a concentric cylinder cell of a Bohlin "CS" Version 4.033 (Couette geometry "C14", ratio of inner and outer cylinders, $r_1/r_2 = 0.909$). A few drops of mineral oil were added on top of the polymer solution in order to minimize solvent evaporation.

The gelling kinetics of ALG(47%G/0%GG) in the presence of the Ca–EDTA/GDL system was studied at $T = 20 \pm 0.1$ °C and $\omega = 0.1$ Hz. The target strain was 1% or 0.2% (according to R) since strain sweeps on a few selected gels demonstrated that these strains produced working deformations well within the linear viscoelastic region. For the dynamic experiments carried out by varying T, the heating rate was set at 0.5 or 1 °C/min.

The degree of syneresis was determined at different times by weighting the final gel after a gentle wiping off of the excess water and relating this value to the original gelling volume.

2.5. Molecular dynamics simulations

The charge distributions of the α-L-(1 → 4)polyguluronic acid (α-L-Gul*p*A) and the β-D-(1 → 4)mannuronic acid (β-D-Man*p*A) repeating units, negatively charged, were calculated by means of GAUSSIAN 98 program (Frisch et al., 1998), using the 6-31G* basis set and restrained electrostatic potential (RESP) fitting (Fig. 1) (Bayly, Cieplak, Cornell, & Kollman, 1993). The parameters for the other interactions were adopted from the PARM99 set Cornell (Cieplak et al., 1995), and the GLYCAM set for glycosides (Woods, Dwek, Edge, & Fraser-Reid, 1995). The initial structure of GG diad was constructed in the 2_1 conformation, as indicated by Braccini, Grasso, and Pérez (1999).

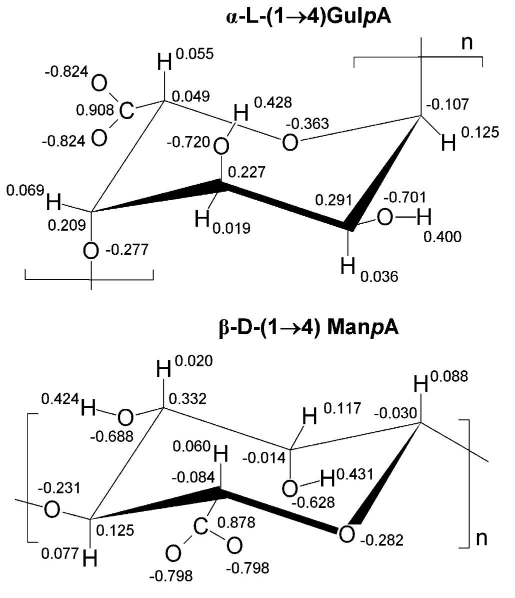

Fig. 1. Point atomic charges in α-L-GulpA and β-D-ManpA repeating units, evaluated with GAUSSIAN 98 program and, eventually, RESP fitting.

Two oligomers of guluronate with seven repeating units each, and four calcium ions were arranged in antiparallel pairing, according to the structural parameters indicated by Braccini and Pérez (2001) for the egg-box model. The initial structure was immersed in a (≈38 × 33 × 61 Å^3) box containing ≈1700 TIP3P water molecules (Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983) and six Na^+ ions were introduced to neutralize the total charge. Initially, the system was subjected to energy minimization on the water molecules and the sodium ions, followed by a second minimization on the whole system.

The MD simulations were carried out as follows. During the first 25 ps harmonic positional restraints were applied (force constant = 500 kcal/(mol Å^2)) on the homo G oligomers and the calcium ions. The temperature was increased from 100 to 300 K over the first picosecond. Then, three simulations of 25 ps at 300 K were performed, scaling the positional restraint force constant to 25, 15 and 5 kcal/(mol Å^2), respectively. In the last 25 ps, calcium ions were left free to move.

Finally, 9.5 ns of simulation at 300 K were performed, until solute positional RMSD remained lower than 1 Å over the final 5 ns-trajectory.

Starting from the final structure of the simulation, the chirality of C-5 carbons of alternating G residues was flipped to obtain M residues with diaxial links. After a slight minimization and a MD simulation of 25 ps, with the alginate backbone and the calcium ions positionally restrained, the M residue conformations were continuously changed over 100 ps towards the most stable conformations with di-equatorial links.

As before, 9.5 ns of simulation at 300 K were performed, with the solute positional RMSD being lower than 1 Å over the final 5 ns-trajectory.

All simulations were carried out with the Amber 7.0 program (Case et al., 2002). Periodic boundary conditions, constant pressure, an integration time step of 2 fs, and restraint of all hydrogen-containing bonds through the SHAKE algorithm (Ryckaert, Cicciotti, & Berendsen, 1977) were employed in all the simulations, as well as PME with a cut off of 9 Å for evaluating all non-bonded interactions.

3. Results and discussion

3.1. Study of the interaction ALG(47%G/0%GG)–Ca^{2+} ions in dilute aqueous solution by means of circular dichroism (CD) spectra

CD is a particularly valuable and sensitive technique able to reveal the existence and the extent of the interaction between a polymer and a potential ligand. The presence of molecules or ions able to induce conformational changes in the polymeric chain under appropriate physical conditions, leads to a more or less pronounced change in the specific ellipticity trace vs. wavelength.

It is well known that concentrated solutions of alginates are able to form solid-like structures (gels) in the presence of bivalent ions at suitable values of the ratio R (Ca^{2+} equivalents/polymer equivalents) (Smidsrød & Draget, 1996).

At lower polymer concentrations, the interaction between Ca^{2+} and the chain segments gives rise to conformational modifications that can be easily identified by the CD technique (Morris, Rees, Thom, & Boyd, 1978; Morris, Rees, & Thom, 1980).

CD spectra of ALG(0%G/0%GG) and ALG(47%G/0%GG), were recorded at several values of the ratio R. In the case of mannuronan (ALG(0%G/0%GG)), no variation in specific ellipticity is observed upon a variation of R. This means that there is no specific polymer–ion interaction and hence no conformational change occurs. This result is in agreement with the egg-box model, which predicts interactions between bivalent ions and G blocks solely. On the other hand, if the egg-box model was assumed as unconditionally "valid", the possibility of gel formation from relatively concentrated solutions of pseudoalginates characterised by a strictly alternate MG sequence and hence of conformational variations in dilute solutions induced by Ca^{2+} ions would be ruled out. On the contrary, it turned out that the CD spectrum of ALG(47%G/0%GG) exhibited an influence from R (Fig. 2(a)).

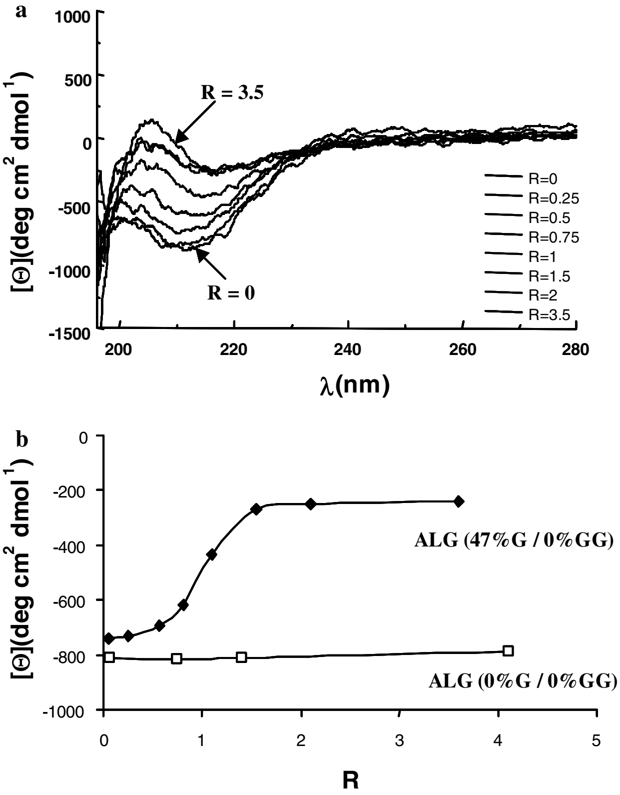

Fig. 2. (a) CD spectra of ALG(47%G/0%GG) as a function of R (Ca^{2+} equivalents/polymer equivalents). (b) Dependence of the specific ellipticity measured at 212 nm on R (Ca^{2+} equivalents/polymer equivalents) for solutions of ALG(47%G/0%GG) and ALG(0%G/0%GG). Polymer concentrations: 0.02% w/v.

Such experimental evidence represents, at the molecular level, the premise for the formation of the macroscopic Ca^{2+}-induced gel that occur at higher polymer concentrations.

Results shown in Fig. 2(a) are conveniently summarised in Fig. 2(b) where the specific ellipticity recorded at $\lambda = 212$ nm is reported as a function of R for the two samples. A sigmoidal trace in the case of ALG(47%G/0%GG), suggests that the modification in its conformation follows a cooperative mechanism.

3.2. Rheology of Ca^{2+}-induced ALG(47%G/0%GG) gels

The egg-box model for ionotropic gelation of alginates describes divalent ions such as Ca^{2+} embedded in the cavities formed by contiguous G-residues. In the 70s, several studies established that junction zones consist of two or more associated chains (Morris et al., 1978; Smidsrød, 1974).

More recently, small-angle X-ray scattering (SAXS) and rheological measurements yielded additional information regarding the cross-sectional radius of gyration of the gels which is related to the junction zone multiplicity and to the size of their domains (Stokke et al., 2000; Yuguchi, Urakawa, Kajiwara, Draget, & Stokke, 2000). These studies proved that homoguluronic sequences can associate laterally according to different multiplicities. The dimensions of the "bundles" depends on the bivalent ion concentration and on the molecular weight and chemical composition of the sample. It was found that lateral association is promoted by high levels of Ca^{2+} in solution. In this case, a somewhat remarkable degree of syneresis may occur. Whilst most of the previous published literature focusing on alginates has stressed the predominant role played by G blocks in the process of gel formation, some experimental evidence for a role of MG sequences in the interaction with Ca^{2+} ions has also been reported (Haug, Larsen, & Smidsrød, 1974; Morris et al., 1980).

The availability of more definite MG alternating sequences offers the possibility of unequivocally demonstrating that MG-blocks can also interact in the presence of Ca^{2+} yielding true gels.

The dependence of storage modulus as a function of time at $R = 0.4$ is shown in Fig. 3 for two different polymer concentrations. In the case of the 0.5% w/v solution a longer induction time is required to allow the gel structure to form. The transition time decreases with increasing polymer concentration and for the 0.7% w/v solution it is close to 15 min. The mechanical spectra of these gels are shown in Fig. 4. A weak frequency dependence of G' can be observed. Although less strong than the corresponding acid "versions", ALG(47%G/0%GG)/Ca^{2+} (Dentini et al., 2005) gels are mechanically endowed with characteristics typical of stable, non-flowing polymeric solid-like structures. To give a measure of the strength of the gels described in this section it is useful to compare the storage modulus of the gels obtained from ALG(44%G/32%GG)/Ca^{2+} at the same

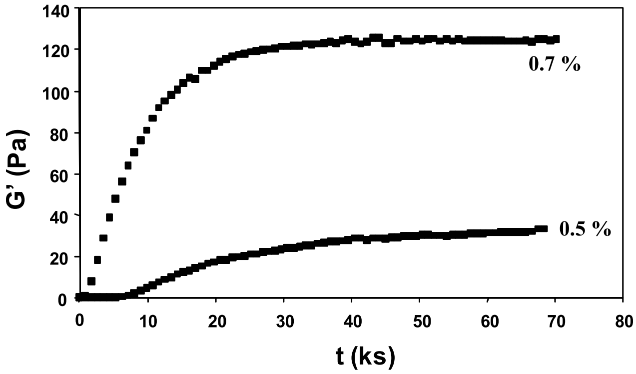

Fig. 3. Time dependence of the storage modulus for solutions of ALG(47%G/0%GG) at different polymeric concentrations (% w/v) in the presence of Ca–EDTA/GDL ($R=\text{Ca}^{2+}$ equivalents/polymer equivalents = 0.4). $\omega=0.1$ Hz, $\gamma=1\%$ and $T=20$ °C.

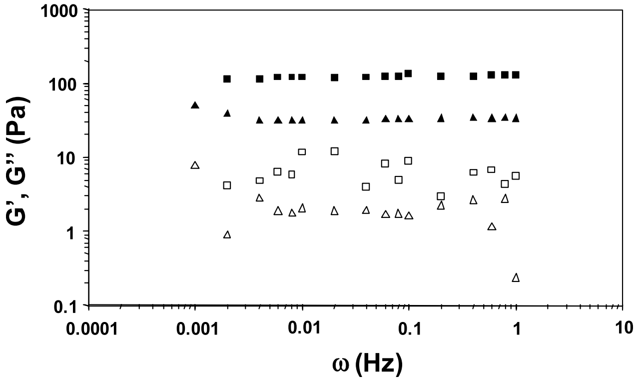

Fig. 4. Mechanical spectra of gels obtained from solutions at different polymeric concentrations of ALG(47%G/0%GG) in the presence of Ca–EDTA/GDL ($R=0.4$). Full symbols: G′. Empty symbols: G″. Polymer concentrations (% w/v): ■/□, 0.7; ▲/△, 0.5. $T=20$ °C; $\gamma=1\%$.

condition of polymer concentration and with $R=0.8$. In these gels G′ was approximately an order of magnitude higher and the gels were rather brittle (data not shown). Similar G′ values for alginates with a comparable composition in G and GG blocks are reported in the literature (Stokke et al., 2000).

The response to thermal treatment of the gel formed starting from the 0.7% w/v solution is shown in Fig. 5. Both G′ and G″ gradually decrease with increasing T, but the extent of the decrease in G′ is more pronounced than that for G″. This leads to an intersection of the two traces. The phase angle, δ, whose value increases with increasing T, indicates a progressive loss of the initial predominant elastic properties. At T values close to 50 °C, the system is still endowed with gel characteristics. Upon cooling of the polymeric network down to the initial T, the experimental points retrace the heating curves. The absence of hysteresis is a clear indication of thermo-reversibility. It is worth pointing out the slight degree of sineresys recorded for these gels ($R=0.4$) over a period of several days, Fig. 7(a). This seems to indicate that at this relatively low value of R, the rearrangement of paired MG sequences to give rise to extended lateral association aggregates occurs to a negligible extent (Fig. 8(a)).

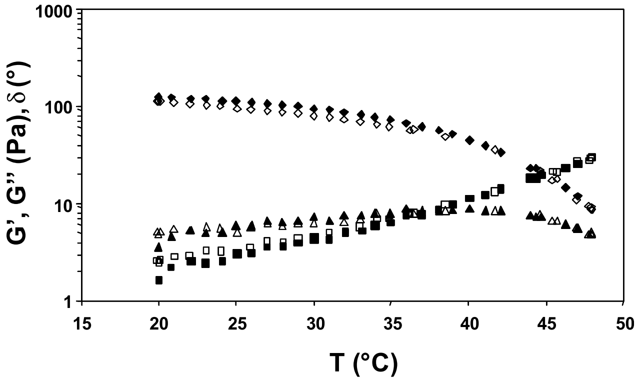

Fig. 5. Thermal response of the gel made from a 0.7% w/v solution of ALG(47%G/0%GG) in the presence of Ca–EDTA/GDL ($R=0.4$). Full symbols: G′, G″ and δ recorded during heating. Empty symbols: G′, G″ and δ recorded during cooling. ◆/◇, G′; ▲/△, G″; ■/□, δ.

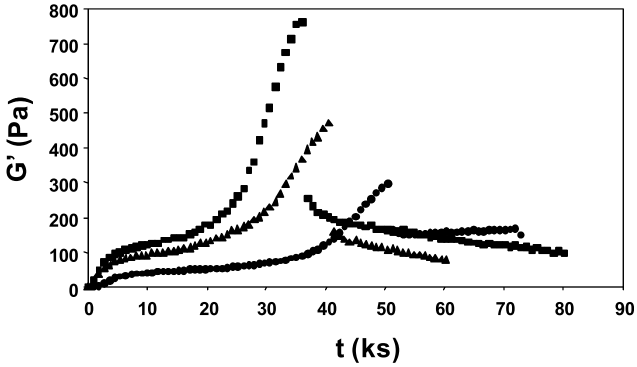

Fig. 6. Time dependence of the storage modulus for solutions of ALG(47%G/0%GG) at different concentrations (■, 0.7%, ▲, 0.6% and ●, 0.5% w/v) in the presence of Ca–EDTA/GDL ($R=1.4$). $\omega=0.1$ Hz, $\gamma=0.2\%$, $T=20$ °C.

When the value of R is set at 1.4 the kinetic of gelation of solutions of ALG(47%G/0%GG) follow a completely different behaviour (Fig. 6). The sol → gel transition occurs within 30 min from the introduction of GDL. The increase in G′ shows a sharp primary gelling phase culminating at about 5 ks, followed by a plateau phase (temporal range between 5 and 23 ks at a concentration of 0.7% w/v) and a secondary more pronounced increase in the storage modulus (23–35 ks at 0.7% w/v). The extension of this pseudo-intermediate plateau decreases with the increase in polymer concentration. The storage modulus finally undergoes a sudden "collapse" followed by a gradual continuous decrease of its value (35–80 ks at a polymer concentration of 0.7% w/v).

The peculiar behaviour of ALG(47%G/0%GG)/Ca^{2+} gels at high R can be interpreted on the basis of the physico-chemical properties of alternate sequences.

In particular, the first kinetic stage may be the result of the Ca^{2+}-induced interactions involving chain segments and leading to an extended network characterised by a high degree of swelling. This is consistent with the observation

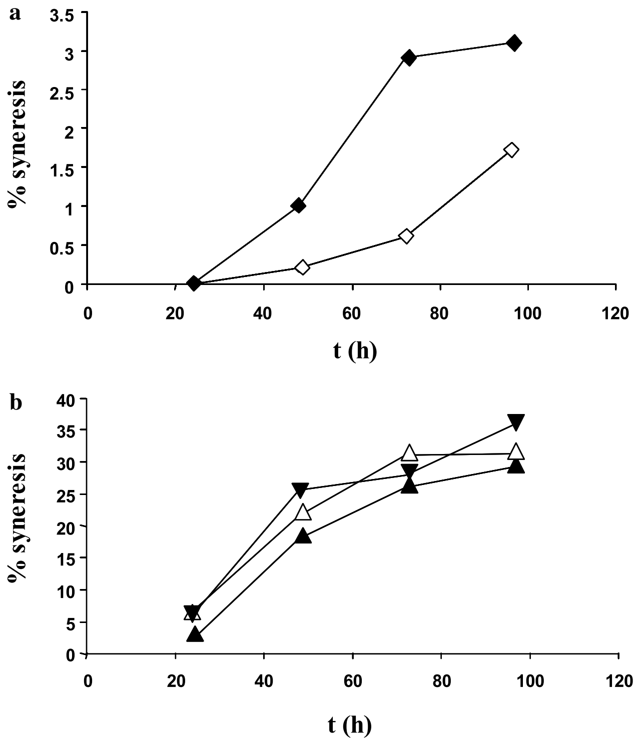

Fig. 7. Time course of the degree of syneresis of gels obtained form solutions of different concentrations of ALG(47%G/0%GG) (C_p) and R values. (a) C_p: ◆ 0.7%, ◇ 0.5% w/v. $R = 0.4$. (b) ▲ 0.7%, ▲ 0.5%, △ 0.6% w/v. $R = 1.4$.

that the value of the storage modulus of the gel obtained from a polymer solution at a concentration of 0.7% w/v in correspondence to the plateau region is very similar in value to that of the terminal plateau of Fig. 3 for the same polymer concentration but with a $R = 0.4$. This implies that in both the gel structures, the amount of calcium ions incorporated in the complex is similar. It is likely that already at $R = 0.4$ not all the calcium ions are involved in chains bridging, but a fraction of them act as additional ionic strength. This picture is supported by the experimental evidence that the gel obtained at such value of R (0.4) and polymer concentration (0.7% w/v), when left to stand still undergoes syneresis (even though to a small extent) (Fig. 7a). Thus, it is reasonable to assume that when a high amount of calcium ions is used ($R = 1.4$), the excess of Ca^{2+} ions will give rise to lateral association of chains to form "bundles" and this may be the origin of the incremented stiffness of the material (salt induced gel shrinkage) (Fig. 8(b)).

The following behaviour of the system depends on the stability of the complex $[(R–COO)_xCa]^{(x-2)+}$ (Nottelmann & Kulicke, 1991). As the affinity of alternate sequences towards bivalent cations increases with increasing the extension, (Stokke, Smidsrød, Bruheim, & Skjåk-Bræk, 1991) of MG sequences it is reasonable to predict a high stability for the above-said complex.

Furthermore, at high value of the ionic strength, the chain charges are more and more screened and the solution quality is reduced. Not only lateral association of the double helices is favoured but the solvent quality tends to be decreased. Finally, a theta state and phase separation (collapse) of the gel is caused resulting in the pronounced syneresis (Fig. 7(b)). Water is expelled out of the matrix and this may cause the bob to slip on the gel surface, which translates into an abrupt decrease of G′. From this point on, data must be considered to be invalidated by the heterogeneity of the system. Analogous slipping phenomena occurring as the result of syneresis have been observed elsewhere for other polymers (Chronakis, Piculell, & Brogström, 1996). The occurrence of time dependent syneresis to a significant extent is put into evidence in Fig. 7(b). Note that the onset of syneresis is considerably delayed in comparison to that recorded by rheological experiments. This is probably to be accounted to the different experimental conditions experienced by the gels in the two cases: equilibrium and dynamic conditions.

To have an idea of the ability of the gels to retain their strength on heating, the gel obtained from a polymeric solution of 0.7% w/v and with a $R = 1.4$ was heated to 90 °C inside the rheometer couvette and then removed from it. A picture of it was taken (Fig. 8(2-a)). It is evident how the specimen maintained the typical appearance of gels. Its better tolerance to heating treatment in comparison to corresponding acid gels (Dentini et al., 2006) may be the discriminating factor in those applications in which relatively high temperatures are required. The gel removed from the rheometer couvette was subsequently soaked in a concentrated aqueous solution of $CaCl_2$. Twenty-four hours later the gel was removed from the solution and a picture of it was taken again. A notable shrinkage can be observed (Fig. 8(2-b)).

3.3. Molecular dynamics

Molecular dynamics has been used in the past to predict the stability and the structural details of the calcium mediated association among guluronic chains. These gelling properties of alginates have so far been ascribed to the presence of G blocks within the polymer primary structure while poly(MG) has been considered unable to form gels. A remarkable finding of the present paper and of that of Donati et al. (2005) is that mannuronan epimerized by AlgE4 epimerase is also able, under appropriate experimental conditions, to undergo gelation and form strong gels. It is of interest to verify if this unexpected behaviour has a theoretical counterpart and to compare a poly(G)–Ca^{2+} gel model to that obtainable for poly(MG)–Ca^{2+} gel so that similarities in behaviour can be attributed to the structural features relevant for gel formation.

Fig. 9(A) and (B) shows the average structures of a gel unit made of poly(G)–Ca^{2+} (A) and poly(MG)–Ca^{2+} (B) oligomers that are representative of gel structures.

The average helical twist is 19° in both cases. In other words, for a complete helical turn 19 residues are necessary

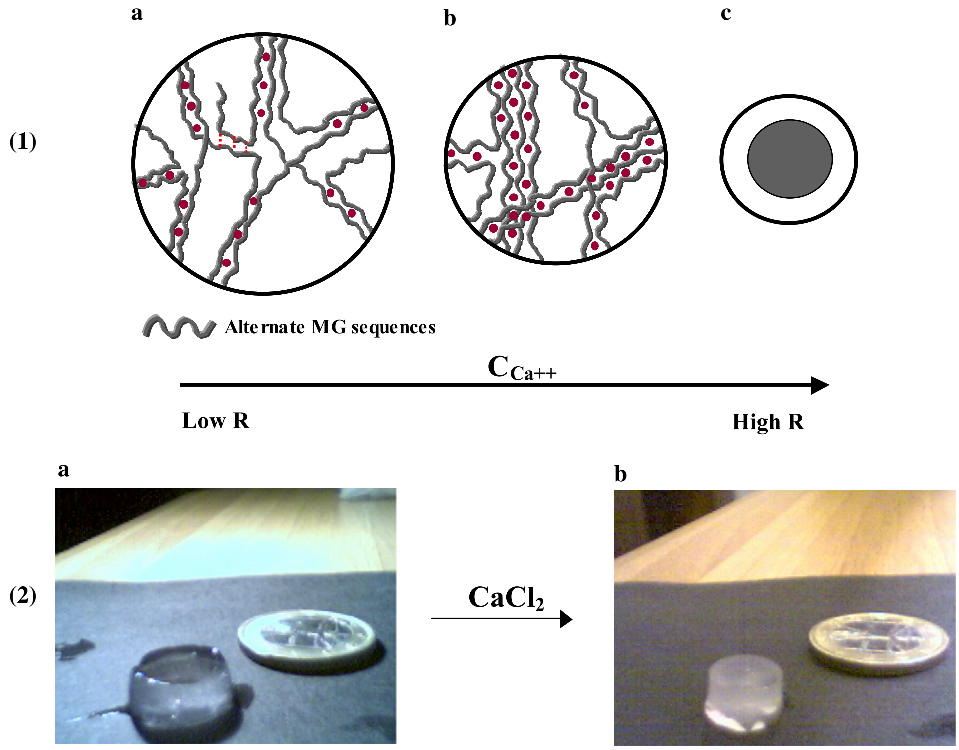

Fig. 8. Sketch representation (1) and actual gel appearance (2) illustrating the influence of the concentration of calcium ions on the behaviour of a ALG(47%G/0%GG)/Ca^{2+} gel. (1-a) swollen gel; (1-b) progressive shrinking induced by additional Ca^{2+} ions; (1-c) collapse of the structure. The gel shown in (2-a) was photographed after being heated at 90 °C. The same gel was then dialysed against a concentrated solution of $CaCl_2$ for 24 h (2-b). $C_p = 0.7\%$ w/v, $R = 1.4$.

in both cases. The main difference between the two structures is the size of the fluctuations around the average helical twist (19°) which turned out to be ±4° for (A) and ±12° for (B). This seems to point to a more flexible structure in the case of poly(MG) as compared to that of poly(G).

This is better viewed on examination of Fig. 10 which shows residue fluctuations expressed in Å around average positions at different locations along the oligomer. It can be seen that the amplitude of bars corresponding to atoms of poly(MG) (black bars) are larger than those corresponding to poly(G) (white bars). The first and last residues of all oligomers exhibit larger fluctuations and this has to be ascribed to end effects. The origin of the difference in flexibility between the two different kind of sequences here examined

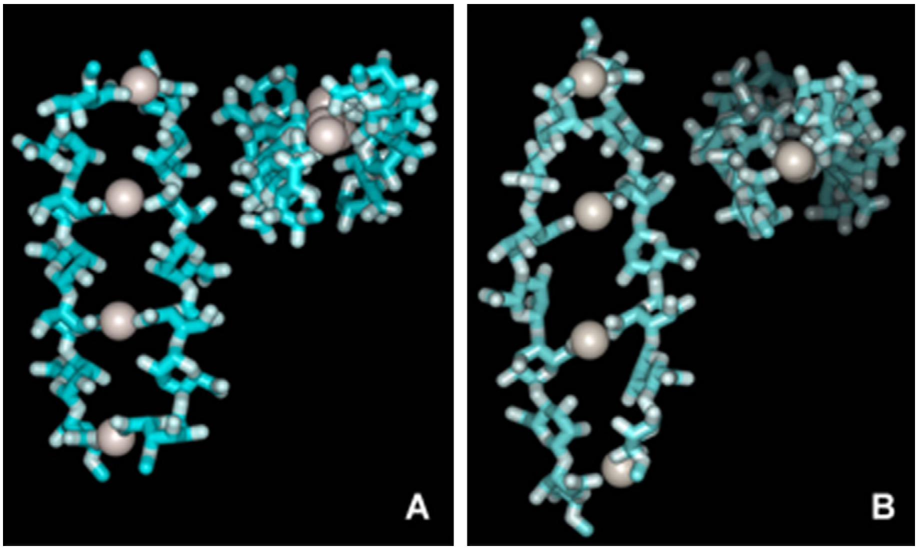

Fig. 9. Representation of the egg-box models for homo G (A) and MG (B) oligomers. Structures correspond to the average conformations of the final 5-ns trajectories, sampled every 10 ps.

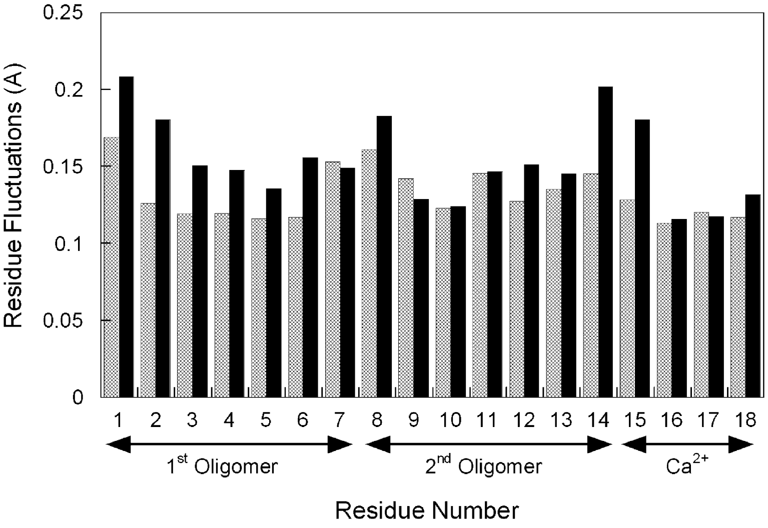

Fig. 10. Histograms relative to the atomic positional fluctuations for all the residues. Values were calculated over the final 5-ns trajectories for G (shaded bars) and MG (solid bars) oligomers. For each simulated system, bars from 1 to 7 correspond to the first oligomer, bars from 8 to 14 to the second oligomer in antiparallel conformation, bars from 15 to 18 to the four calcium ions.

may be the following: in the poly(G) model, guluronates form intramolecular hydrogen bonding between the carboxyl groups and the 2-OH groups of the preceding residues. In the poly(MG) model, at the MG steps there are hydrogen bonds between the carboxyl groups of the mannuronates and the 2-OH groups of the subsequent guluronates, as well as between the 3-OH groups of the mannuronates and O5 of the subsequent guluronates. On the contrary, at the GM steps, no hydrogen bonds are present between consecutive residues, conferring to the chain greater overall flexibility than poly(G) chains.

There is no differences as far as calcium fluctuations are involved apart from one in the case of the MG oligomer simulations. This case could also be due to end effects. It is worth noting that, as the systems have twofold symmetry, mechanical properties of residues at symmetric positions (e.g. 1 and 8, 2 and 7, 15 and 18 and so on) should be equal for large simulation time.

However, this is often beyond the scope of the available calculation power. In addition, it is not possible to simulate an infinite chain, which would be the most accurate way to evaluate the mechanical properties of the real polymers. Apart of the aforementioned ends effects, oligomer simulations should be considered representative of the actual systems and are able to predict the relative flexibility of the different molecules at least.

Contrary to previous proposals (Braccini & Pérez, 2001), where dipolar interactions between hydroxyl groups at O(2) and O(3) of poly(G) and calcium ions seem to play a role in stabilizing the complex, in our simulations the interactions between charged carboxylic groups of both G and M residues and Ca^{2+} ions predominate. It is important to underline that in the case of poly(MG) short-range interactions between calcium ions and hydroxyl groups are prevented by the intrinsic configurational characteristics of the MG building blocks. The gel forming ability of poly(MG) in the presence of Ca^{2+} seems to indicate that electrostatic interaction between Ca^{2+} ions and carboxylic groups are the main driving force to gel formation.

4. Conclusions

The availability of pseudoalginates with a strictly alternating sequence rendered possible a detailed characterization of its gelling behaviour. Both the gelling behaviour as well as the mechanical properties and the degree of syneresis strongly depend on the value of *R*. At relatively low value of *R*, gels of ALG(47%G/0%GG) even though endowed with characteristics typical of strong gels are relatively elastic. The increase in temperature enhances such feature. The degree of syneresis for these gels is negligible. The kinetic of gelation follows a completely different pattern at relatively high values of *R* and is indicative of a molecular reorganization occurring inside the gels. This is also evidenced by a significant degree of syneresis.

Molecular dynamics simulations were carried out on two types of gel building blocks: poly(G) and poly(MG) (in association with Ca^{2+}). Two main consequences resulted: gel made of poly(MG) seemed to be more flexible than that derived from poly(G) and gel formation is driven predominantly by electrostatic forces.

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