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Alginate gels with a combination of calcium and chitosan oligomer mixtures as crosslinkers

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ABSTRACT

Alginates are polysaccharides that are widely used in relation to their ability to form gels. Recently we reported that alginates may also form gels with chitosan oligomers as crosslinkers (Khong, Aarstad, Skjåk-Bræk, Draget, & Vårum, 2013). The purpose of the present study was to characterize alginate gels crosslinked with calcium and chitosan oligomers. Using two different alginates of similar molecular weights but different chemical composition, i.e. guluronic acid content of 46 and 68%, we found that both alginates could form homogeneous gels with calcium and chitosan oligomers separately and without syneresis. Systematic combinations of calcium and chitosan oligomers as crosslinkers were tested, showing that up to 50% of the calcium could be substituted with chitosan oligomers without reduction in gel strength or increased syneresis for the alginate with the lowest guluronic acid content. Furthermore, the kinetics of the combined gels were different from pure calcium alginate gels.

1. Introduction

Chitosans form a family of linear polysaccharides consisting of (1 → 4)-β-linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc or A-unit) and its de-*N*-acetylated analogue (GlcN or D-unit). These polycationic derivatives of one of Nature's most abundant biopolymers (chitin) can be prepared with varying degree of polymerization (DP) and degree of acetylation (F_A) (Vårum & Smidsrød, 2005). The amine-group of the D-unit has a pK_a-value of ca. 6.5 (Anthonsen & Smidsrød, 1995; Strand, Tømmeraas, Vårum, & Østgaard, 2001; Tsukada & Inoue, 1981) and therefore the amount of charges also influences properties as their water-solubility as a function of pH (Vårum, Ottøy, & Smidsrød, 1994).

Chito-oligosaccharides (CHOS), i.e. shorter fragments of chitosans composed of the same building units and glycosidic linkages, can be prepared using both chemical and enzymatic methods. CHOS have attracted much attention in recent years as they have been suggested to exhibit numerous biological effects (Aam et al., 2010; Nilsen-Nygaard, Strand, Vårum, Draget, & Nordgård, 2015).

Alginates form a polysaccharide family (occurring in brown algae and bacteria) comprised of (1 → 4)-linked β-D-mannuronic acid (M-unit) in the $^4C_1$ conformation and its C5-epimer, α-L-guluronic acid (G-unit) in the $^1C_4$ conformation with a pK_a-value of ca. 3.5. These linear polyanionic block copolymers are composed of homopolymeric regions of M-units (M-blocks) or G-units (G-blocks), interspaced by regions of alternating epimers (MG-blocks) of different length (Draget, Moe, Skjåk-Bræk, & Smidsrød, 2006).

Hydrogels are cross-linked hydrophilic polymer chains able to capture large amounts water, defined by specific properties regarding their dynamic (Ross-Murphy, 1984). The cross-links in hydrogels can be established through covalent linkages (Desai, Koshy, Hilderbrand, Mooney, & Joshi, 2015; Li, 2010) or through ionic linkages (Mi et al., 1999). Ionically crosslinked hydrogels generally feature a higher swelling sensitivity to pH changes than their covalently crosslinked counterparts, which extends their potential applications since a further tuning of the hydrogel to their specific environment becomes possible (Cuan et al., 1996). Chitosan is known for its high biocompatibility, biodegradability (Nordtveit, Vårum, & Smidsrød, 1996; Vårum, Myhr, Hjerde, & Smidsrød, 1997) and low toxicity (Köping-Höggård et al., 2001; Strand, Danielsen, Christensen, & Vårum, 2005). Moreover, antimicrobial effects (Dutta, Tripathi, Mehrotra, & Dutta, 2009; Felt, Carrel, Baehni, Buri, & Gurny, 2000; Liu, Guan, Yang, Li, & Yao, 2000; Mellegård, Strand, Christensen, Granum, & Hardy, 2011) have been shown, which are beneficial for applications such as drug delivery, implants (Xia, Liu, Zhang, & Chen, 2011) and wound healing (Ong, Wu, Moochhala, Tan, & Lu, 2008; Ueno, Mori, & Fujinaga, 2001). Tailoring of chitosans with respect to DP, polydispersity, F_A and

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acetyl distribution is providing tools for controlling their function and properties in relation to their biological effects.

Alginates are able to form ionically crosslinked hydrogels in the presence of multivalent cations (e.g. Ca^{2+}, Sr^{2+} and Ba^{2+}). Calcium-alginate gels are intensively studied and widely used for cell immobilization and protection of the cells from the host's immune system (Draget & SkjåK-Braek, 2011; Leong et al., 2015; Strand, Mørch, & Skjåk-Bræk, 2000). These gels can be prepared through internal gelation or a diffusion method (Draget et al., 2006). The internal gelation provides a mechanism where insoluble calcium carbonate is mixed with an alginate solution, followed by a controlled lowering of the pH through a proton-donator such as the slowly hydrolyzing D-glucono-δ-lactone (GDL), causing a homogeneous release of the calcium ions and subsequent gel formation. The calcium ions show specific interactions with the G-units and form junctions zones specifically between G-blocks (Sikorski, Mo, Skjåk-Bræk, & Stokke, 2007). Khong, Aarstad, Skjåk-Bræk, Draget, and Vårum (2013) has shown the possibility of forming junction zones between consecutive M-units (using polymannuronic acid) crosslinked with fully deacetylated chitosan oligomers, and it was found that at the same conditions only very weak gels were formed using polyguluronic acid (poly-G). This new gelling system, also induced by the homogeneous lowering of the pH thereby charging the chitosan oligomers using the slowly hydrolyzing proton-donator GDL, provides a new possibility of crosslinking alginates. The suggested reason for this difference in the gelling properties between alginate composed of poly-M and poly-G is the match in charge distance between the chitosan and poly-M ($^4C_1$ conformation, diequatorial glycosidic linkage) of about 10.4 Å (Minke & Blackwell, 1978; Sørbotten, Horn, Eijsink, & Vårum, 2005) whereas the G-blocks ($^1C_4$ conformation, diaxial glycosidic linkage) exhibits a shorter charge distance of only 8.7 Å (Atkins, Mackie, & Smolko, 1970).

It seems then that Ca^{2+} and chitosan oligomers can provide crosslinkers for alginates where calcium ions are crosslinking through the G-blocks and the chitosan oligomers most effectively through the M-blocks. For certain cell lines immobilized in calcium alginate gels it has been found that the calcium ions can have negative effects (Chan & Mooney, 2013). Here we have undertaken a study of gelling properties of two different alginates of different composition using combinations of the two crosslinkers, where we have applied the internal gelation method (see Scheme 1) with the two different crosslinkers and measured the gel strength (Young's modulus) and syneresis as well as the gelling kinetics of the new gels.

2. Experimental

2.1. Materials

The mixture of chitosan oligomer mixture (MCO) was provided by Koyo Chem Co Ltd (lot number 121017WG). MCO was characterized by Size Exclusion Chromatography (see Fig. 1 in Supplementary material). The number-average degree of polymerization (DP_n) and the degree of acetylation (F_A) of the MCO as well as degree of and the individual oligomers (see Table 1 in Supplementary material) were determined by ^1H NMR (see Fig. 2 in Supplementary material) as described previously (Sørbotten et al., 2005). Two alginate samples (provided by FMC Biopolymer AS, Drammen, Norway) were isolated from stipe and from leaf of *Laminaria hyperborea*. Data for chemical composition and sequences and the molecular weight average are given in Table 1. Chemical composition and sequences in terms of diads, triads and average block length were determined by ^1H NMR and ^{13}C NMR as described previously (Grasdalen, Larsen, & Smidsrød, 1977). Weight and number average molecular weight were determined by SEC-MALLS. D-Glucono δ-lactone (GDL) was purchased from Sigma-Aldrich. $CaCO_3$ was (Reag. Ph Eur) was purchased from Merck. Other chemicals were of analytical grade and used without any further purification.

2.2. Gel preparation

Alginate samples were dissolved in distilled water at a concentration of 3% (30 mg/mL). MCO was dissolved in distilled water at a concentration of 10% and the pH was adjusted to 8.0 by 1 M NaOH. 1.0 g of the alginate solution was weighted into a glass vial and a maximum of 182 μL of the MCO (amount determined by syneresis experiments) was added together with distilled water to obtain a total volume of 2 mL. The mixture was stirred for 10 min and 26.6 mg of GDL were dissolved in 1 mL distilled water right before adding to mixture followed by intensive stirring. Ca^{2+} cross linked alginate gels were prepared by adding a suspension of a maximum of 3.6 mg $CaCO_3$ (amount determined by syneresis experiments) and 1 mL of distilled water to 1 mL alginate solution. The mixture was stirred for 10 min and 12.9 mg of GDL were dissolved in 1 mL distilled water right before adding to the mixture followed by intensive stirring. Determined amounts for MCO and $CaCO_3$ were set as 100%. Used amounts in the mixed gels (0–100%) are based these values (see details of calculations in Supplementary material).

Gel cylinders were made by placing aqueous solution of sodium alginate and solution of chitosan oligomers mixture, containing dispersed $CaCO_3$ and freshly dissolved GDL, in the cylinder wells (diameter = 16 mm, length = 18 mm) of the 24-well plate and covering the cylinders with a lid. After 24 h the gels were taken out and ready for the measurement.

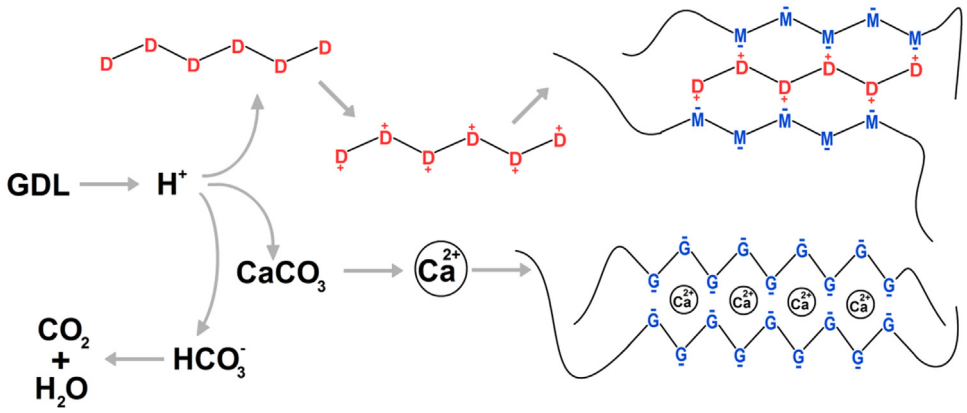
2.3. Syneresis and gel strength

Syneresis was measured as final gel weight after gentle wiping off excess water relative to the initial gel weight determined by the total volume of the gelling solution in each well. Gel strength was determined by compression measurements of the gel cylinder until rupture by using a texture analyzer (TA.XT.-Plus Texture Analyzer from Stable Micro Systems, Surrey, UK) equipped with a cylindrical probe (P/35, diameter = 35 mm) operated at a speed of 0.10 mm/s. A minimum of three parallels were determined. Young's modulus was calculated from the slope of the initial zone of the resulting stress-strain curve, defined as the ratio of tensile stress to tensile strain. Rupture strength was determined as the force when the gel ruptured. The height and diameter of each gel were manually measured by a digital caliper. A load cell with a capacity of 5 kg was used and the instrument was calibrated prior to the measurement.

2.4. Rheological measurement

The rheological characterization of the mixed alginate gels was performed using a Stresstech Rheometer (RheoLogica Instruments, Sweden) fitted with a cone-and-plate geometry (cone angle of 4° and diameter of 40 mm). To prevent drying of the samples during measurement, the sample was covered with a layer of low-viscosity silicon oil. A minimum of two parallels were performed for each kinetic gelling experiment.

The test methods employed were oscillatory time and stress sweep at a constant temperature of 20 °C. For time sweep, the experiments were performed at a low oscillation frequency (1 Hz) and a small strain (0.001) to ensure that the measuring conditions did not disrupt the gelation process. The strain sweep, at a constant frequency of 1 Hz, was used to determine the linear viscoelastic region (LVR) of the hydrogels. Frequency sweep experiments were performed in the linear viscoelastic region (0.01–10 Hz) with a con-



Scheme 1. Schematic illustration of the combined internal gelation of alginate crosslinked with calcium and MCO. D: glucosamine, M: mannuronate unit, G: guluronate unit, GDL: D-Glucono δ-lactone.

stant strain of 0.001 and a delay time of 0.033 min between the measurements to characterize the viscoelastic properties as a function of frequency.

3. Results and discussion

3.1. Characterization of chitosan oligomers

The size distribution of the mixed chitosan oligomers (MCO) was characterized (See Fig. 1 in Supplementary material), showing a dominance of oligomers with DP from 2 to 6. The higher DP oligomers (DP ≥ 7) are in lower amounts, although oligomers with DP up to 20 can be identified. Isolated fractions were collected and characterized by ^{1}H NMR spectroscopy to determine their degree of acetylation (F_A), see Table 1 in Supplementary material. The oligomers with DP 5 or less were all fully de-N-acetylated, while the hexamer had a F_A of 0.05 and then gradually increasing with increasing DP up to 0.28 (DP ≥ 19), as seen in the anomer region of the ^{1}H NMR spectra of isolated chitosan oligomer fractions (Fig. 2 in Supplementary material). Anomeric reducing end protons of a D-unit are found at 5.43 ppm (α-form) and at 4.92 ppm (β-form). The absence of α- and β-anomer of reducing end A-units (at 5.19 and 4.61 ppm) for oligomers with DP ≤ 5 shows that these chitosan oligomers fractions are fully de-N-acetylated (Ishiguro, Yoshie, Sakurai, & Inoue, 1992). The internal D-units resonate between 4.8 and 4.9 ppm while the internal A-units resonate at 4.6 ppm which can be seen in the spectra of oligomer fractions with DP ≥ 6 (Vårum, Anthonsen, Grasdalen, & Smidsrod, 1991). Also, the A-units seem are not found at the reducing end of any of the acetylated oligomers (see Fig. 2 in Supplementary material), as no resonances of reducing end A-units could be identified.

3.2. Characterization of alginates

Two alginate samples isolated from stipe and leaf of *L. hyperborea* with similar molecular weight were used for the gel experiments. The content of guluronate and mannuronate (F_G and F_M), diad sequences and selected triad sequences together with average block lengths of these alginates (Grasdalen, Larsen, & Smisrod, 1981; Grasdalen, 1983) were determined (see Table 1).

3.3. Gel strength and kinetics of gelation as function of added GDL

The two commercially available alginates with quite different composition (high and low G-content) but similar molecular weights (Table 1) were used for the gelling experiments. The pK_a-value of the carboxyl groups is about 3.5 (Haug, 1964), and therefore

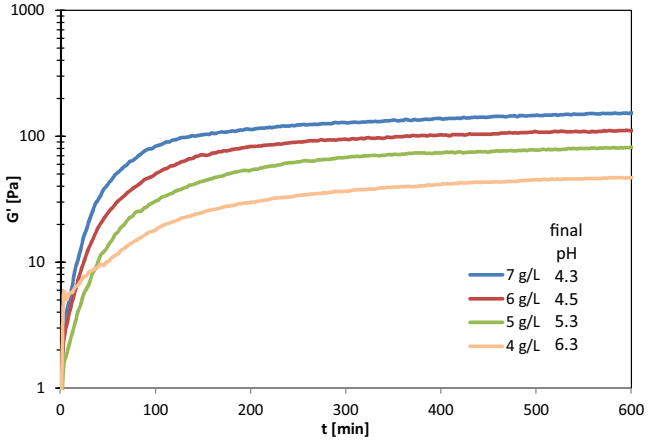


Fig. 1. Kinetics of gelation as a function of GDL concentration. To a leaf alginate solution (10 g/L) a fixed amount of MCO (7 g/L) was added, and then increasing an amount of GDL (4–7 g/L) was added and the G' was determined as a function of time. G" and delta are not shown in the Figure for reasons of clarity.

will be fully charged at neutral pH. The intrinsic pK_a-values of the amino-groups has been determined for fully de-*N*-acetylated MCO with increasing chain length (monomer to heptamer) and found to be ca. 6.7 for the higher DP oligomers whereas the dimer had a pK_a-value of 7.6 (Tsukada & Inoue, 1981), which means that the total MCO will be mainly uncharged at the pH 7.5 which is the pH where MCO and alginate is mixed (see Experimental for details). Even though the neutral solubility decreases with increasing chain length and decreasing F_A (Vårum et al., 1994), no precipitation was observed at pH 7.5. This can be explained by the increase in F_A with increasing chain length (Vårum et al., 1994). The gelation was performed as previously described (Khong et al., 2013). The kinetics of gelation were followed by measuring the storage modulus (G') and the loss modulus (G") as a function of the time. The pH at the apparent equilibrium was measured in addition. For all experiments a fixed MCO conc. of 7 g/L and alginate conc. of 10 g/L was used.

A frequency sweep control experiment without adding the proton donating substance D-glucono-δ-lactone (GDL) was performed (data not given) that showed liquid like properties of the alginate MCO solution at pH 7.5 (G" higher than G', phase angle close to 90°) at a broad frequency range (0.01–10 Hz).

Fig. 1 shows the time dependence of the storage modulus of a leaf alginate crosslinked with MCO with increasing amounts of GDL. For all different mixtures a phase transition from predominant viscous to predominant elastic properties, where G' exceeds G" and

Table 1
Chemical composition, block length (determined by ^{1}H NMR and ^{13}C NMR (*) spectroscopy), Number and Weight average Molecular Weights and Polydispersity Index (PI) (determined by SEC MALLS) of the two Alginates.

alginate source	F_G	F_M	F_{GG}	F_{MM}	F_{MG}	F_{GGG}	F_{MMM}	$N_{G>1}$	$N_{M>1}$	M_W (10^3 g mol^{-1})	M_n (10^3 g mol^{-1})	PI
L. hyperborea stipe	0.68	0.32	0.57	0.28*	0.10*	0.53	0.17*	14	3.2*	270	97.4	2.78
L. hyperborea leaf	0.46	0.54	0.36	0.43*	0.18*	0.20	0.19*	4.8	3.6*	220	105.4	2.09

the phase angle drops below 45°, occurring within the first minute, which indicates a fast gelling system (G" and δ are not depicted here). For the 7 g/L GDL mixture G' increases rapidly during the first 2 h of the measurement, starts to level off and prolongates into an apparent equilibrium after approx. 130 min. However, the ongoing small inclination indicates that some reorganization within the hydrogel network continues. For the remaining samples the apparent equilibrium approaches after approx. 180 min (6 g/L GDL), 210 min (5 g/L GDL) and 250 min (4 g/L GDL). These results show that the amount of GDL added to a solution with a fied concentration of alginate and MCO has direct influence on the gelling kinetics, the final pH and the final gel strength, as previously shown in alginates composed only of M-units (poly-M) (Khong et al., 2013).

3.4. Kinetics of gelation as function of the MCO concentration

Furthermore, the gelling kinetics of the two alginates were followed by measuring the storage modulus (G') and the loss modulus (G") as a function of the time, for a series of experiments with a fixed conc. of alginate (10 g/L), an increasing conc. of MCO (4–8 g/L) and a stoichiometrically adjusted amount of GDL corresponding to the number of introduced amino groups through MCO and a final pH of 4.4 (detailed calculations given in Supplementary information). Fig. 2 shows the time dependence of the storage modulus (G') with increasing amounts of MCO. It emerges that a higher concentration of MCO crosslinkers is leading to more rigid gels. A similar increase in G' with increasing chitosan oligomer mixture concentration was also observed for poly-M (Khong et al., 2013). The gelling kinetics are similar for all MCO and GDL concentration with a rapid increase of G' in the start, followed by an approaching equilibrium after 170 min for the leaf alginate (Fig. 2A) and 130 min for the stipe alginate (Fig. 2B). This is different from the kinetics of gelling with poly-M and MCO, where the rates of gelling was found to increase with increasing MCO concentrations, which could be attributed to a higher number of junctions in the gel network with increasing crosslinker concentration (Khong, 2013). However, care should be taken to directly compare the gelling kinetics described by Khong (Khong, 2013) with the current gelling system as the gelling systems are different both with respect to the MCO crosslinker that were used (different DP-distribution), and as the crosslinker in the gelling system described herein is a mixture of calcium and MCO.

The kinetics of gelling is influenced both by the concentration of the proton donator GDL (Fig. 1) and by the concentration of the proton acceptor and crosslinker MCO. This can be explained by the fact that the protons donated by GDL become equally distributed among the MCO. To form a stable junction zone it is estimated that the chitosan oligomer should have at least four positively charged D-units (with a strong increase of gel strength for every further charged D-unit) (Khong, 2013). If MCO concentration is increased and the GDL concentration is increased equally then the kinetics will be the same, which was also reflected by pH measurements during the experiment (data not given).

Fig. 2 shows that stipe alginate forms weaker gels than leaf alginate at the same MCO concentration, but reaches the equilibrium earlier. This difference in gel strength can be explained by the alginate composition (see Table 1). Stipe alginate has a similar mannuronic acid block length ($N_{M>1}$) and contains a small fraction of very long M-blocks (Aarstad, Tøndervik, Sletta, & Skjåk-Bræk,

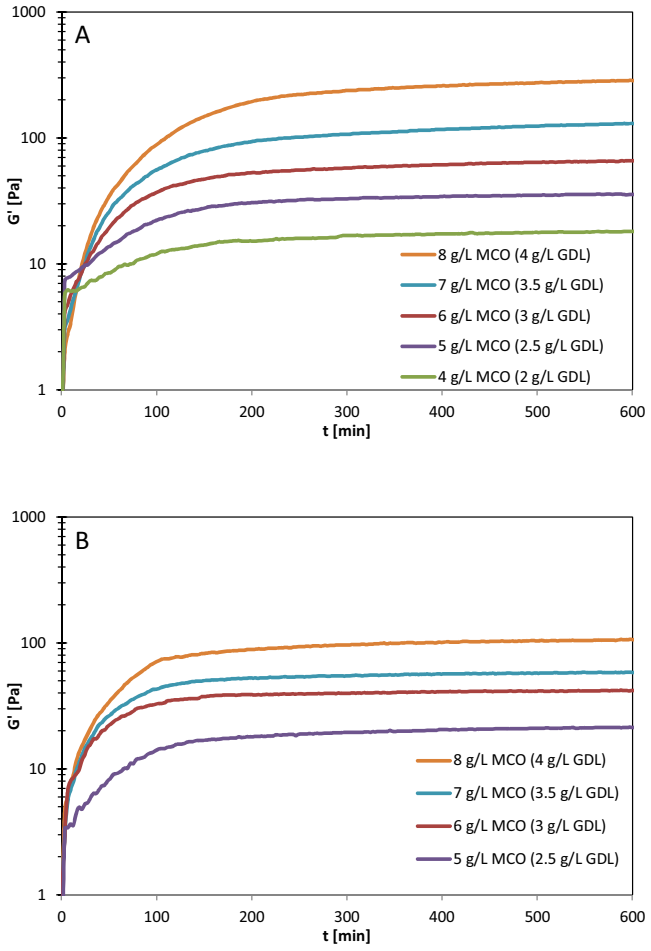


Fig. 2. Kinetics of gelation as a function of MCO added. (A) Leaf alginate. (B) Stipe alginate.

2012) but a lower fraction of mannuronic acid (F_M) compared to leaf alginate. However, the M-block length distribution of the leaf alginate has not been determined. It could be that the gelling kinetics could be important for the final gel strength, and that the difference between the two alginates of different chemical composition could be explained by differences in the possibility of reorganization of the crosslinkers in the gel, as has been suggested for alginate acid gels (Draget et al., 2000).

3.5. Gel strength and syneresis of mixed gels

The dependence of Young's modulus (E) and syneresis as a function of the concentration of MCO and Ca^{2+} was determined for the two alginates (see Figs. 3 and 4). A set of experiments were designed where the concentrations of MCO and calcium were systematically varied. For pure calcium-alginate gels, the maximum concentrations of calcium (100%) without significant syneresis were 15 and 16.5 mM for leaf and stipe alginate, respectively, while for chitosan oligomer alginate gels the maximum MCO (100%) concentration without significant syneresis was 25 mM (calculated as the molar

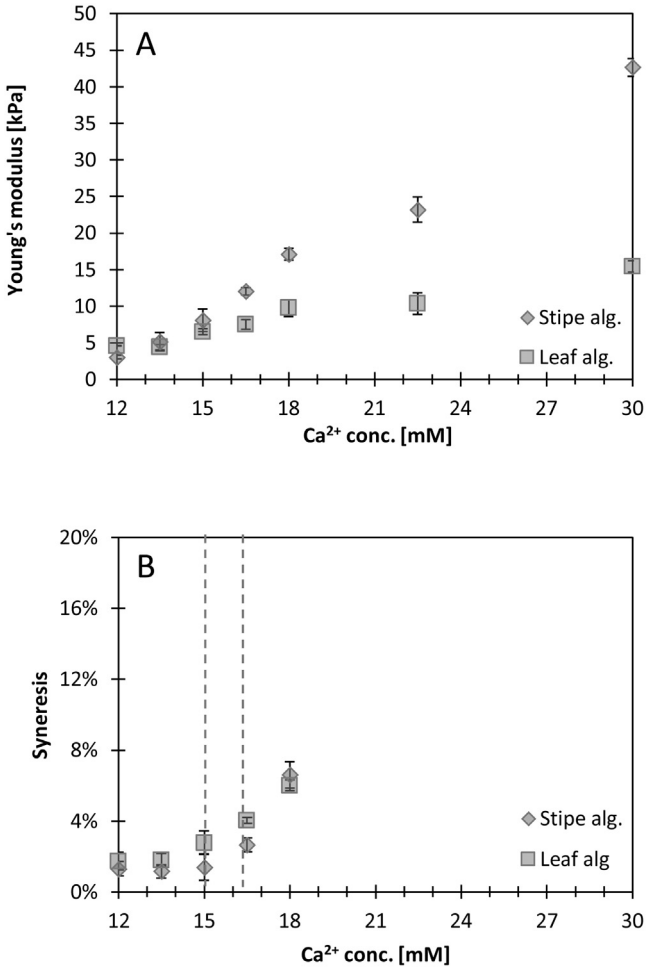


Fig. 3. Gel strength (Young's modulus) and syneresis of 1% alginate gels as a function of calcium concentration. (A) Gel strength as determined by the Young's modulus. (B) Syneresis, left dashed line: max. Ca^{2+} conc. before syneresis occurs in leaf alginate, right dashed line: max. Ca^{2+} conc. before syneresis occurs in stipe alginate.

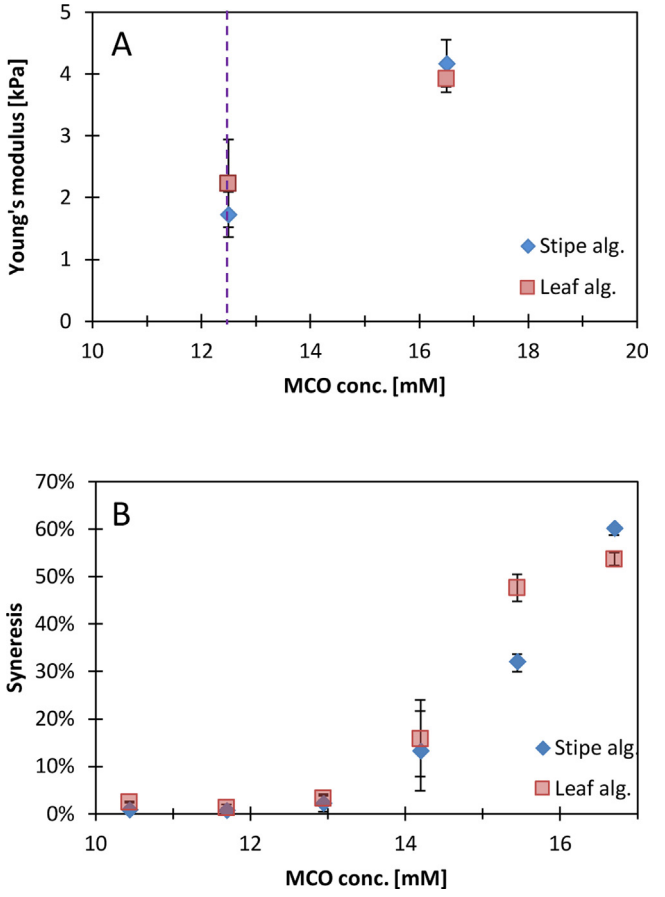


Fig. 4. Gel strength (Young's modulus) and syneresis of 1% alginate gels as a function of MCO concentration. (A) Gel strength as function of MCO concentration (conc. related to $(GlcN)_2$). (B) Syneresis, dashed line: max. chitosan conc. before syneresis occurs in (both) alginates.

concentration of the D-unit monomer) for both alginates. Accordingly, when preparing a leaf alginate with a mixture of 50% calcium and 50% MCO, the calcium concentration is reduced to 7.5 mM and the MCO concentration to 12.5 mM. Detailed values for the concentration of calcium, MCO and GDL are given in the Supplementary information.

In Fig. 5 is shown the results (Young's modulus and syneresis) with the leaf alginate when gels were prepared with only calcium as crosslinker, only MCO as crosslinker as well as mixtures of the two crosslinkers. It can be seen that the calcium-alginate gels are clearly stronger than the MCO-alginate gels with E of 6 and 3 kPa, respectively. However, mixed gels with half the calcium concentration can also be prepared without significant reduction in gel strength (E). Pure calcium-alginate gels with a 50% reduction in calcium concentration would result in a drastic reduction in E to less than 2 kPa, while a 25% reduction in the calcium concentration would result in a gel with a E of 2.8 kPa (yellow triangles in Fig. 5A). Clearly, mixed calcium-MCO alginate gels can be made with a drastic reduction in calcium-concentrations without reduction in the gel strength. These gels could be applied in systems where a reduced calcium concentration is preferred, e.g. for immobilization of calcium-sensitive cells. Such gels can also be made without significant syneresis (Fig. 5B).

In Fig. 6 is shown the results of similar gel experiments with the stipe alginate. For this alginate, the expected results of a stronger calcium-alginate gels was found, and a more drastic reduction in the gel strength was seen when using only MCO as cross-linker (E of 12 and 1.8 kPa, respectively; see Fig. 6A). However, for the mixed gels with stipe alginate, the calcium concentration could not be reduced as much as for the mixed gels with leaf alginate without a reduction in the gel strength (Fig. 6A). Pure calcium-alginate gels with a 25% reduction in calcium concentration would result in a drastic reduction in E to 3 kPa. With the mixed gels the gel strength can be maintained with a 25% reduction in calcium concentration. Pure calcium-alginate gels with a 25% reduction in calcium concentration would have a E of 3 kPa, i.e. a more than 70% reduction (yellow triangles in Fig. 6A). For a mixed stipe alginate gel with a 50% reduction of calcium-concentration, this gel would still maintain more than 70% of the gel strength (yellow triangles in Fig. 6A). Such stipe alginate gels can also be made without significant syneresis (Fig. 6B).

3.6. Kinetics of gelation of the mixed gels

We also measured the gelling kinetics of the mixed gels for the two alginates were followed by measuring the storage modulus (G') and the loss modulus (G") as a function of the time Fig. 7. The concentrations of all components are identical to the set of experiments previously described. In Fig. 7A is shown the kinetics of gelling of leaf alginate crosslinked with calcium (100%), MCO (100%) and the mixed gels (50% calcium and 50% MCO). Clearly, the fastest gel formation is in the gelling system with the MCO. The slowest gel formation is with the calcium that is released from the

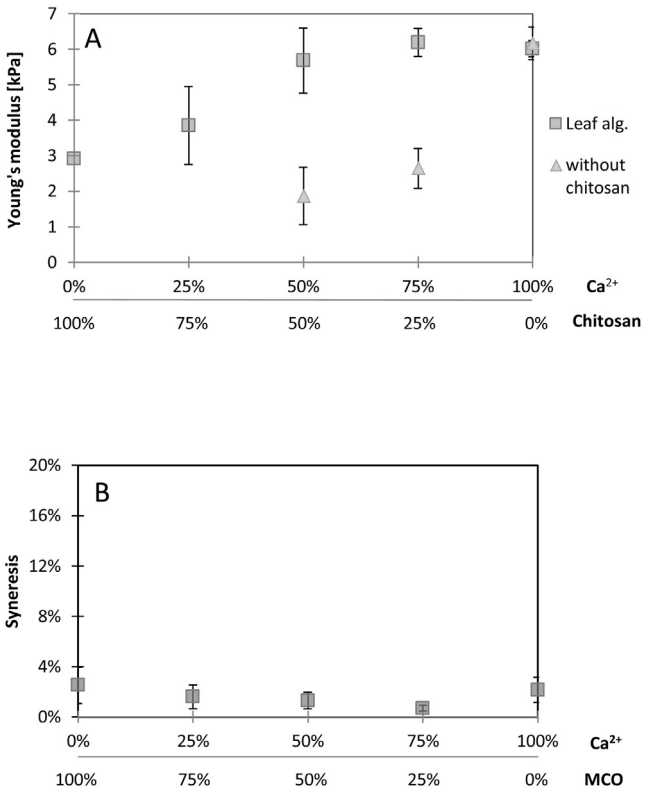


Fig. 5. Gel strength and syneresis of leaf alginate crosslinked with varying amounts of MCO and calcium (100% Ca^{2+}: 1.2 g/L, 100% MCO: 6 g/L). (A) Gel strength as Young's modulus, squares: mixture of Ca^{2+} and MCO. For comparison the gel strength of alginate when only calcium is added has been included (triangles). (B) Syneresis, squares: mixture of Ca^{2+} and MCO.)

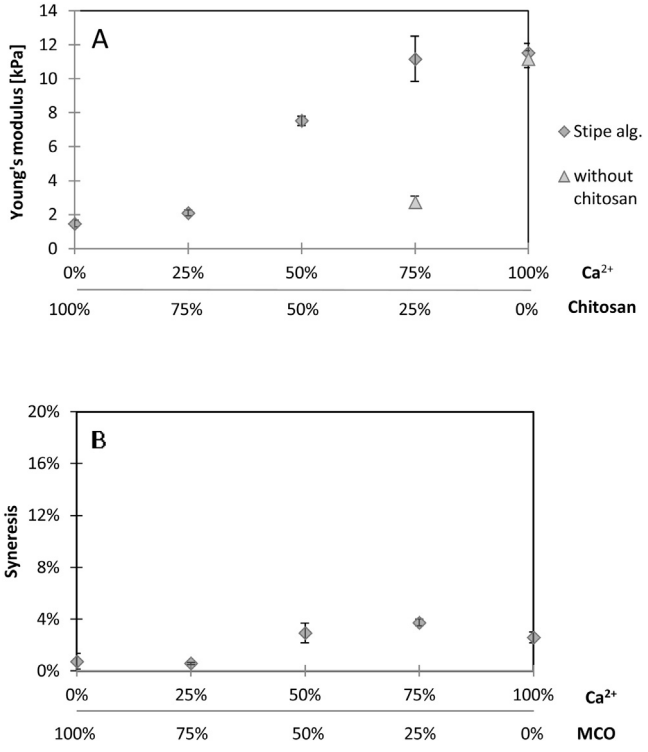


Fig. 6. Gel strength and syneresis of stipe alginate crosslinked with varying amounts of MCO and calcium (100% Ca^{2+}: 1.3 g/L, 100% Chit: 6 g/L). (A) Gel strength as Young's modulus, squares: mixture of Ca^{2+} and chitosan. For comparison the gel strength of alginate when only calcium is added has been included (triangles). (B) Syneresis, squares: mixture of Ca^{2+} and chitosan.

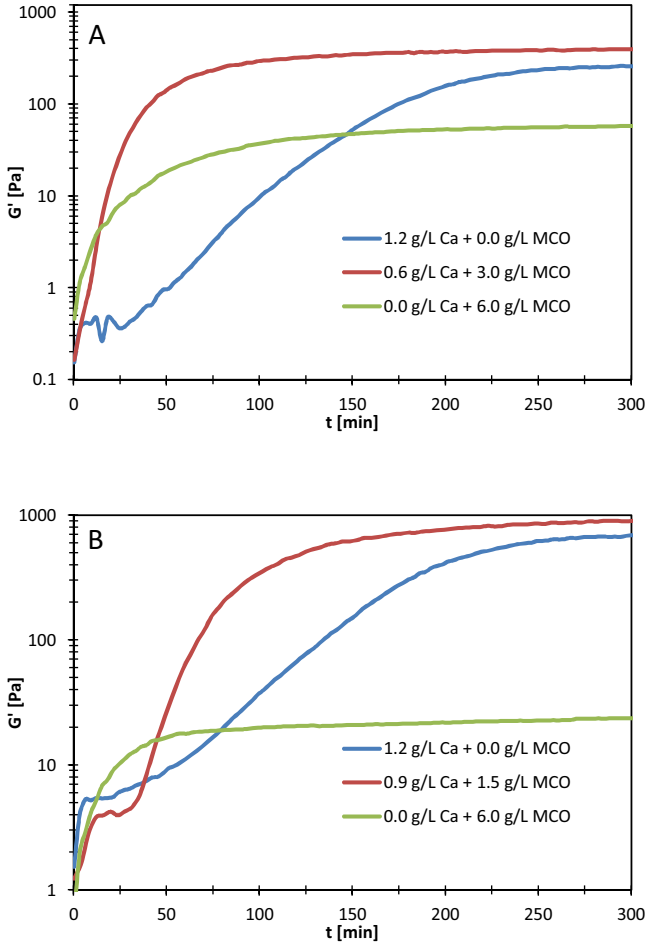


Fig. 7. Comparison of the kinetics of gelation when internal gelling was performed with only calcium (orange line), only MCO (green lines) and with a mixture of calcium and MCO (purple line). (A) Leaf alginate. (B) Stipe alginate.

solid calcium-carbonate. This is most probably because the rate-limiting step in the gel formation with calcium is the dissolution and release of calcium from the solid calcium-carbonate (Draget, Østgaard, & Smidsrød, 1990), while in the alginate-MCO gelling system the rate-limiting step is the release of protons from GDL (which in turn protonates the chitosan oligomers). The gelling kinetics of the mixed gels is in between the calcium and the MCO gelling systems. The mixed gel reveals an incorporation of both properties, the gelling kinetics of the MCO crosslinked gel and the gel strength of the calcium-alginate gel. A possible explanation could be that the MCO gets protonated faster because of the higher ratio of GDL to MCO (see Fig. 1) compared to the pure MCO crosslinked gel. For stipe alginate (Fig. 7B) the difference in gelling kinetics and in gel strength between the pure calcium-alginate gel and the pure MCO crosslinked gel is even more pronounced. Both can be explained by the alginate composition (see Table 1) as previously described for pure MCO crosslinked gels.

The mixed gel shows similar behavior for stipe and leaf alginates, with a gel strength similar to pure calcium-alginate gel but with faster gelling kinetics. The onset of gelation of the mixed gel is not as fast as for the pure MCO crosslinked gel. This can be explained by the lower concentration of MCO (1.5 g/L for 25%) which is not enough to form a gel on its own but the gelation proceeds faster for mixed gel (approached equilibrium after 250 min) as for the pure calcium-alginate gel (approached equilibrium after 400 min) as soon as enough Ca^{2+} is released to form a gel.

4. Conclusions

Homogeneous alginate gels crosslinked with both calcium and chitosan oligomers can be prepared by internal gelling using a proton donor to simultaneously release calcium ions and protonate chitosan oligomers. Up to 50% of the calcium in alginate gels could be substituted with chitosan oligomers without loss in gel strength or change in syneresis, which means that the new gels have potential new applications for e.g. immobilization of calcium-sensitive cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.09.006.

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