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Polyelectrolyte layer interpenetration and swelling of alginate–chitosan multilayers studied by dual wavelength reflection interference contrast microscopy

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Abstract

Polyelectrolyte multilayers of alginate and either poly-L-lysine or chitosan have been studied with dual wavelength reflection interference contrast microscopy (DW-RICM). Alginates with different ratios of the two monomer residues, β-D-mannuronic acid (M) and α-L-guluronic acid (G) were included to study possible effects of specific divalent ions selected from their ability to influence gelation of alginate. Measurements of multilayer thickness revealed the importance of the preparation conditions. The multilayer thickness was reduced with increasing ionic strength following preparation, suggesting a dominance of an ordinary screening of the alginate component. The results indicate that the interaction between alginate and chitosan are different from that between alginate and poly-L-lysine, with the latter appearing to be more of a "hit-and-stick" reaction while rearrangements during the adsorption process is occurring to a larger extent in the preparation of the chitosan–alginate multilayers.

Keywords: Alginate; Chitosan; Multilayers; Layer interpenetration; DW-RICM

1. Introduction

Alginates are structural polysaccharides extracted from brown algae where they constitute part of the intercellular matrix as a gel induced by the presence of divalent cations. Their function is skeletal, giving both strength and flexibility to the plant. Additionally, alginates are produced as an exocellular polymer in bacteria such as *Azotobacter vinelandii* and several *Pseudomonas* species (Moe, Draget, Skjåk-Bræk, & Smidsrød, 1995). Alginate is composed of guluronic (G) and mannuronic (M) acid units forming regions of M-blocks, G-blocks and blocks of alternating sequence (MG-blocks), where the relative proportions of these sequential organizations depends on the source (Haug, Larsen, & Smidsrød, 1974; Moe et al., 1995). The heterogeneity in both the content and sequence of uronic acids leads to alginates differing in their functional properties. The distributions of G-residues in alginates are determined by C-5-epimerases acting at the polymer level. For *A. vinelandii* a family of alginate epimerases (AlgE's) is identified, each epimerase introducing different residue sequences in the alginate (Ertesvåg et al., 1995; Svanem, Skjåk-Bræk, Ertesvåg, & Valla, 1999). These enzymes open the possibility of producing alginates that can be tailored for their application, in addition to production of alginates with a more uniform distribution in composition and sequence. By production of mannuronan C-5-epimerases and application of them to modify the residue sequence of the mature alginates, a more detailed basis for the structure–function relationship of alginates can be investigated than that offered by alginates isolated from natural resources.

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Numerous applications of alginates exploit their ability to form gels as well as their biocompatibility. Upon introduction of divalent ions such as Ca^{2+}-ions, junction zones between consecutive G-residues are formed, conventionally described in terms of the egg-box model as chelation of the Ca^{2+}-ions (Grant, Morris, Rees, Smith, & Thom, 1973). The gel strength depends on the composition and sequence of the units in the alginate chains. The selectivity for certain divalent ions such as Ca^{2+} increases with the increasing content of G-residues. Recently, alginates with long stretches of alternating G/M residue sequences obtained by application of AlgE4 enzymes acting on mannuronan, are also reported to form gels in the presence of Ca^{2+} (Dentini et al., 2007; Donati et al., 2005).

Alginate has previously been employed for multilayers in combination with poly-L-lysine (PLL) to suppress the interaction between surfaces and cells (Elbert, Herbert, & Hubbell, 1999). Furthermore, due to the biocompatibility and the rapid gel-formation by addition of divalent cations, alginate beads are widely employed for microencapsulation purposes (Orive et al., 2004; Strand, Mørch, & Skjåk-Bræk, 2000). The subsequent adsorption of coating layers onto these beads is to some extent analogous to multilayer formation using the gel bead as a substrate. Both chitosan and PLL have been employed as the stabilizing polycation layer of alginate gel beads (Gåserød, Smidsrød, & Skjåk-Bræk, 1998; Thu et al., 1996).

In this study, multilayers of well-defined alginates with different composition obtained from natural resources or mannuronan produced by genetically altered bacteria and subsequently modified by epimerases, are included. Such a selection of material affords investigation of possible effects of compositional details of the polyanion. Multilayers of the various alginates are made with either chitosan or PLL. The results obtained here suggest dominance of different reaction modes between alginate and the two polycations during the build-up of the multilayers, i.e., the PLL–alginate combination appear to form multilayers by a mechanism approaching ''hit-and-stick'', whereas rearrangements between the polyanion–polycation layers to a larger extent appear to take place in the alginate–chitosan case. Additionally, the subsequent swelling responses of the prepared multilayers appear mainly to be governed by a polyelectrolyte response of the alginate component, also preserving some of the specific interactions between alginates and various types of divalent cations. This shows that multilayers including alginates offer a certain versatility controlled by the molecular features of the actual alginates.

2. Materials and methods

2.1. Alginate samples

Alginate from *Laminara hyperborea* stipe with a G-fraction equal to 0.68 (FMC Biopolymers, Drammen, Norway) (referred to as F_G 0.68), was used without further purification. Additionally the following alginate samples were employed: mannuronan (polyM) produced by an epimerase negative mutant of *Pseudomonas fluorescences* (Gimmestad et al., 2003), this mannuronan was epimerized either with the G-block forming epimerase AlgE1 to a G-fraction of 0.8 (F_G 0.8) or with the MG-block forming epimerase AlgE4 to a polyalternating alginate with a G-fraction of 0.47 (polyMG). The epimerases AlgE4 and AlgE1 were produced by fermentation of recombinant *Escherichia coli* strains and purified (Ertesvåg, Høidal, Schjerven, Glærum Svanem, & Valla, 1999; Høidal, Ertesvåg, Skjåk-Bræk, Stokke, & Valla, 1999). The epimerization was carried out by incubation of mannuronan with the epimerase in 3-(*N*-morpholino)propane sulfonic acid (MOPS) buffer (50 mM, pH 6.9) in the presence of Ca^{2+} and Na^{+} (2.5 mM $CaCl_2$ and 10 mM NaCl for AlgE4 epimerization; 0.8 mM $CaCl_2$ and 20 mM NaCl for AlgE1 epimerization). The alginates were further characterized by ^{1}H NMR spectroscopy (Grasdalen, 1983). Before use, the alginates were dissolved in MQ-water overnight. The intrinsic viscosities of the alginates were determined in aqueous solutions of 0.1 M NaCl at 20 °C (Harding, Vårum, Stokke, & Smidsrød, 1991). The molecular weights of the employed alginates were estimated based on the experimentally determined correlation between the molecular mass and intrinsic viscosity (Vold, Kristiansen, & Christensen, 2006). In that study, the hydrodynamic properties of the alginates, including epimerized mannuronan, conformed to the same Mark–Houwink–Kuhn–Sakurada (MHKS) relationship. The radius of gyration, R_g of the corresponding M_w was also estimated based on the experimental data reported by Vold and coworkers

Table 1
Alginate samples used in this study, described with their intrinsic viscosity [η], molecular weight (M_w) and the fraction of G-blocks (F_G), the fraction of G-diades (F_{GG}) and the average G-block length larger than one residue ($N_{G>1}$)

Alginate	Alginate source	[η] (ml/g)[a]	M_w[b] (g/mol)	R_g[c]	F_G[d]	F_{GG}[d]	$N_{G>1}$[d]
PolyM	Produced from *Pseudomonas fluorescences* mutant	2250	695	110	–	–	–
PolyMG	PolyM epimerized with AlgE4	1720	465	88	0.47	–	–
F_G 0.68	Extracted from *Laminara hyperborea* stipe	1440	345	75	0.68	0.56	12
F_G 0.8	PolyM epimerized with AlgE1	1730	470	90	0.8	0.70	44

[a] Determined in 0.1 M NaCl (Harding et al., 1991).
[b] Estimated using the experimentally determined relationship between the molecular weight and intrinsic viscosity of alginates, with the epimerized alginates included in the determination (Vold et al., 2006).
[c] Estimated radius of gyration at $I = 0.1$ M at the given M_w using the experimentally determined relationship between M and R_g (Vold et al., 2006).
[d] Determined by ^{1}H NMR (Grasdalen, 1983).

(2006). The properties of the employed alginates are listed in Table 1.

2.2. Chitosan sample

The employed chitosan was kindly provided by Dr. K.M. Vårum, Department of Biotechnology, NTNU. The chitosan is characterized by its degree of acetylation ($F_A = 0.1$), determined by ^{1}H NMR (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991). The intrinsic viscosity ($[\eta] = 210$ ml/g) was determined as described previously in a solution of 0.02 M acetate buffer and 0.1 M NaCl (Draget, Vårum, Moen, Gynnild, & Smidsrød, 1992). The average molar mass ($M_w = 33 \times 103$ g/mol) was estimated from the intrinsic viscosity using the MHKS-equation, where the constants a and K were estimated according to the F_A (Anthonsen, Vårum, & Smidsrød, 1993). An apparent radius of gyration was estimated to $R_g = 8$ nm ($I = 0.1$ M) at the given M_w based on the experimentally determined relation between R_g and M_w for a series of chitosans (Lamarque, Lucas, Viton, & Domard, 2005). Additionally, a chitosan with $F_A = 0.49$, $[\eta] = 450$ ml/g, $M_w = 102 \times 10^3$ g/mol and estimated $R_g = 35$ nm were also used in a few experiments. The chitosans were dissolved in acetic acid (1%), to a concentration of 1 mg/ml.

2.3. Polylysine sample

For the coating of alginate capsules, poly-L-lysine (PLL) $M_w = 22 \times 10^3$ g/mol (as provided by the manufacturer) has been found to be optimal based on capsule stability and permeability (Thu et al., 1996), and this M_w of PLL was therefore used to prepare multilayers. The PLL (Sigma–Aldrich) was dissolved in MQ-water.

2.4. Preparation of polyelectrolyte multilayers

Before preparation of multilayers the substrates (glass cover slides) were cleaned by sonication for 30 min, twice in 2% detergent solution (Hellmanex, Hellma, Germany) and twice in deionized water, each step followed by extensively rinsing in deionized water. This yields a surface with reported charge density -0.7 mCm^{-2} (Grünberg, Helden, Leiderer, & Bechinger, 2001) that is highly hydrophilic as characterized by aqueous droplets readily spreading to a very flat shape.

The samples were prepared manually by immersing the substrate alternately in polycation and polyanion solutions (50 μg/ml), for 5 min. Between each polyelectrolyte exposure, the sample was rinsed three times in aqueous salt of the same ionic strength as used in the polymer solution. The ionic strength was kept constant during the rinsing steps to avoid exposing the multilayers for osmotic shocks during the preparation. For multilayers with poly-L-lysine the pH was adjusted to physiological conditions (pH 7.4), but for the (chitosan_alginate) films the pH was selected to 5.5 to be approximately intermediate between the pK_a of chitosan ~6.5 (Anthonsen & Smidsrød, 1995; Strand, Tømmeraas, Vårum, & Østgaard, 2001) and alginate (pK_a of 3.4 for M-residues and 3.65 for G-residues (Haug, 1964)). The notation (alginate_polyanion)$_n$ used in the following depict a multilayer with the actual alginate (polyM, polyMG, F_G 0.68, or F_G 0.8) in combination with the depicted polycation in a total of n-bilayers.

2.5. Dual wavelength reflection interference contrast microscopy

Reflection interference contrast microscopy (RICM) utilizes the interference of monochromatic light reflected from two interfaces, the glass-buffer interface and the buffer-probe interface. Here, a spherical polystyrene bead is used (detailed description below). This yields a characteristic interference pattern of Newton fringes from which the height of the bead above the glass (i.e., the thickness of the multilayer sample) can be deduced. Dual wavelength reflection interference contrast microscopy (DW-RICM) is employed (Schilling, Sengupta, Goennenwein, Bausch, & Sackmann, 2004) to be able to determine absolute heights of the bead above the glass (Fig. 1).

The interference patterns obtained using the spherical bead, at two wavelengths ($\lambda = 486$ nm and $\lambda = 546.1$ nm) were analyzed to obtain the bead height (i.e., the film thickness), using a routine previously described (Schilling, 2003; Schilling, Sackmann, & Bausch, 2004; Schilling et al.,

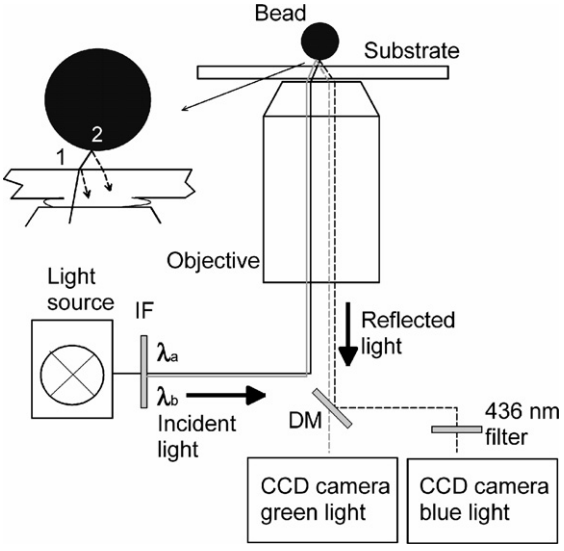

Fig. 1. Schematic overview of the dual wavelength reflection interference contrast microscope set-up adapted from Schilling and coworkers (2004). Two wavelengths ($\lambda = 436$ nm and $\lambda = 546$ nm) were selected from the incident light using an interference filter (IF) and sent through the objective lens (Zeiss Antiflex, 63×, oil immersion, with numerical apperture 1.3). The beams reflected from the substrate – multilayer (1) and multilayer – bead (2) (upper left) interface generate the interference patterns for both wavelengths. The reflected light were separated in their two wavelength components using a dichroic mirror (DM) and the two interference patterns were determined using two separate CCD-cameras and analyzed.

2004). The refractive index of water was used when the height was calculated. For typical standard refractive index increments of polyelectrolytes ($dn/dc \sim 0.15$ ml/g), this procedure introduces only minor errors. The reported height measurements are mean values with standard deviations obtained by several observations within a given multilayer preparation as well as independently prepared samples. The sample-to-sample variation in height is of the same order as the variation within a sample.

Carboxyl-modified polystyrene beads of diameter 10.15 ± 0.06 μm (Duke Scientific, Palo Alta, USA) were used as probes for the RICM-measurements. The beads were coated with bovine serum albumin (BSA) to reduce hydrophobic attraction to the sample surface. Furthermore, it is not expected that any van der Waals interactions will contribute to the potential experienced by the polystyrene beads from the substrate and thus the measured heights. This is because the van der Waals interactions will be retarded and screened at thicknesses larger than the Debye screening length, which holds in this case (Bevan & Prieve, 1999; Picart et al., 2004). The coating of the beads was carried out by incubation with BSA-solution (1 mg/ml in MQ-water) for 30 min. Excess BSA was removed by centrifugation (4000 rpm, 4 min) followed by resuspension of the beads in water. These steps in this washing procedure were repeated four times. The BSA-coated polystyrene beads maintain significant fluctuations when hovering on top of the multilayer films suggesting that there are no strong attractive interaction between these beads and the multilayer. This is in contrast to other coatings of the beads that were reported to yield attractive interaction between coated beads and the multilayers that suppresses bead fluctuations, no measured multilayer growth and probably embedding of the beads inside the multilayers (Picart et al., 2004).

3. Results

3.1. Swelling of chitosan–alginate multilayers

The multilayer thickness of alginate–chitosan layers, prepared at two ionic strengths (NH_4Ac, $I = 5$ mM, and 150 mM), using the alginate F_G 0.68, increased with the number of polycation/polyanion pairs (bilayers) (Fig. 2a and b). This holds for both ionic strengths when the thickness was determined at the same ionic strength as the preparation conditions. When multilayers prepared at different ionic strengths are equilibrated and measured in the same ionic strength, the multilayers prepared at 150 mM were found to be slightly thicker than those prepared at 5 mM (compare Fig. 2a and b). For all measurements, the standard deviations are based on averaging of several measurements, and thus reflect both the inherent resolution of the instrument, as well as the height variations in the sample.

The swelling response of the multilayers was investigated by changing the ionic strength and incubating the samples for at least 4 h before measuring the height. Comparing the

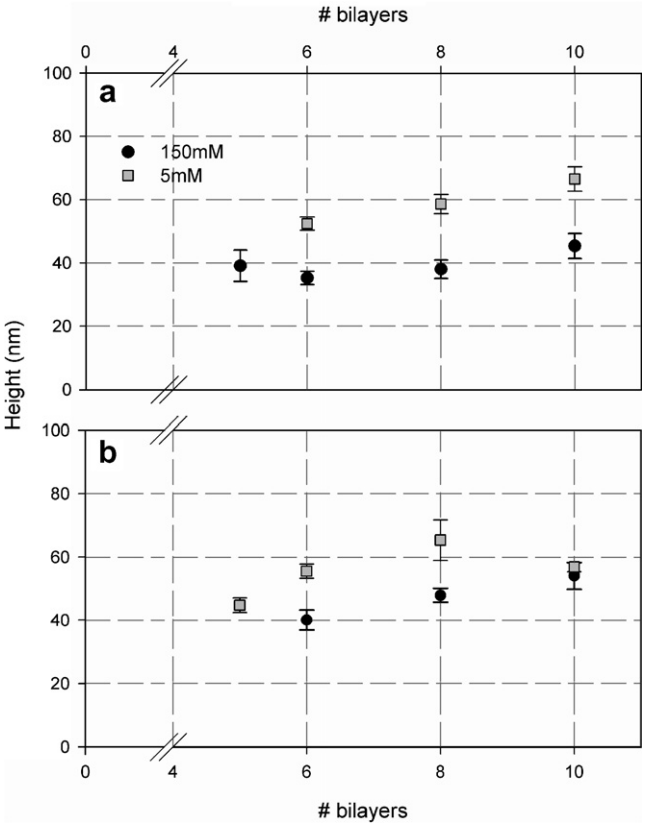

Fig. 2. The thickness of chitosan–alginate (F_G 0.68) multilayers measured with DW-RICM. (a) multilayers prepared at 5 mM NH_4Ac and (b) multilayers prepared at 150 mM NH_4Ac. The swelling of the multilayers was studied by changing the ionic strength after preparation, and measuring the multilayers at 5 mM (squares) and 150 mM (circles). Mean values and error bars are based on 8–12 measurements on at least two different samples.

measurement in different ionic strength for each preparation condition, the layers were found to contract upon increase in ionic strength for measurement (Fig. 2). The changes in thickness of the multilayers were found to be reversible (data not shown). No further change in swelling ratio was observed by increasing the equilibration time to 24 h. The thickness swelling ratio, calculated as the ratio between the thickness measured at 5 mM and the thickness measured at 150 mM was 1.4–1.5 for multilayers prepared at both ionic strengths, and independent of the number of bilayers. The exception is the measurements of 5 bilayers, where the ionic strength of the solution does not seem to influence the thickness. These data points also deviate by being thicker than expected compared to the multilayers with more layer pairs.

3.2. Multilayers with alginate as the polyanion: different salts

To investigate the response of the multilayers not only to changes in ionic strength but also to different valencies of the salt, 10 bilayers of alginate F_G 0.68 in combination with either of the two polycations (chitosan or PLL) was prepared in three different salts (NaCl, $MgCl_2$, Na_2SO_4

all $I = 0.15$ M). The error bars (standard deviation) represent the heterogeneity in thickness both within a given multilayer preparation and between independently prepared multilayers. The data show that the multilayer thickness increases somewhat when the preparations and measurements were carried out using Na_2SO_4 as compared to NaCl (Fig. 3). However, the thickness observed for preparation and measurement in Na_2SO_4 is still within the range of the height variations determined for the multilayers determined at the same ionic strength of NaCl. For both chitosan and PLL, thicker multilayers were observed when prepared at $I = 0.15$ M of $MgCl_2$. These multilayers were approximately twice as thick as those prepared in 0.15 M NaCl (Fig. 3). A similar variation has been found for other multilayer systems when the thickness has been measured by DW-RICM (Maurstad, Bausch, Sikorski, & Stokke, 2005; Picart et al., 2004). Furthermore, AFM topographs of chitosan–alginate multilayers have shown that the sample surfaces are not smooth, and that the surface roughness varies with the particular alginate employed in the build-up process (data not shown).

Alternatively, the thickness of multilayers ((chit_F_G 0.68)$_{10}$ and (PLL_F_G 0.68)$_{10}$) originally prepared in 0.15 M NaCl were determined in these three different aqueous salt solutions (NaCl, $MgCl_2$, Na_2SO_4; all $I = 0.15$ M, incubated for at least 4 h). For each polyelectrolyte combination, the data are all within the same range of variations in thickness upon exchange of the salt subsequent to the layer-by-layer deposition (Fig. 3).

3.3. Effect of the gelling ion Ca^{2+} on PLL–alginate multilayers

Preservation of the well-known Ca^{2+}-dependent gelling capacity of alginate within the multilayers was tested using (PLL_alginate)$_{10}$ films prepared in 0.15 M NaCl. This was carried out using the four different alginates with various content and sequential arrangement of the α-L-GulA (Table 1) representing various Ca^{2+}-dependent gelling capacity. The multilayers prepared in 0.15 M NaCl were subsequently incubated in a gelling solution corresponding to that employed for alginate beads (Strand, Mørch, Espevik, & Skjåk-Bræk, 2003), consisting of $CaCl_2$ (50 mM) in mannitol (0.15 M), and left overnight before rinsing in 0.15 M NaCl. The thickness of the multilayers using alginate F_G 0.68, was found to decrease after incubation with the gelling solution, while no significant change was observed when the other alginates were employed (Fig. 4).

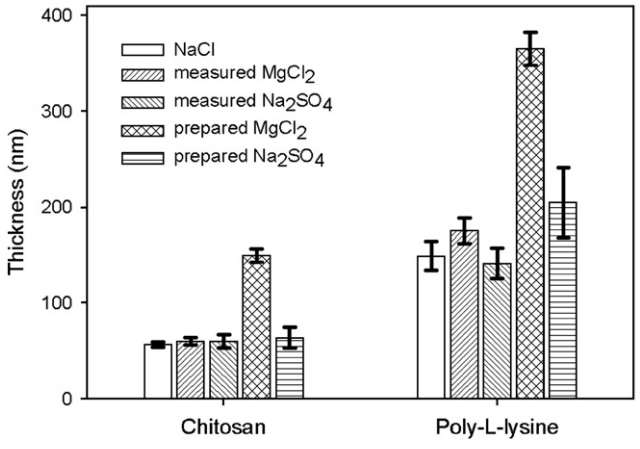

Fig. 3. The influence of different salts (NaCl, $MgCl_2$, Na_2SO_4) during and after preparation on the thickness of 10 bilayers of alginate employing either chitosan or poly-L-lysine as the polycation was measured employing DW-RICM. The multilayers were either prepared in $I = 0.15$ M NaCl followed by changing the salt type (labelled "measured") or prepared in different salts (labelled "prepared"). The ionic strength was kept constant at 0.15 M in all cases.

3.4. Degree of interpenetration between alginate and polycation

The apparent degree of interpenetration between the polyelectrolyte layers, as well as the influence of different residue composition and sequences in the alginate, was determined using DW-RICM (Fig. 5). Alginate was physisorbed to the polystyrene beads using a procedure similar to that described for the BSA-coating of the beads. The thickness of this alginate layer was determined from the increased height of the alginate beads above a glass surface as compared to BSA-coated beads. Polycation was subsequently adsorbed to glass, and the height of the polycation layer determined to be 16 nm for the PLL layer, 17 nm (chitosan $F_A = 0.1$) for the chitosan layer. The determined height of 28 nm for adsorbed chitosan ($F_A = 0.49$; $R_g = 35$ nm, a chitosan not used further for the multilayers) indicate that the height of the adsorbed polycation layer depend on molecular properties of the polycation. When the alginate coated beads are put on top of the polycation layer, the reduction in height as compared to the additive height of the polycation and alginate layer reflect the degree of interpenetration. The measurements were car-

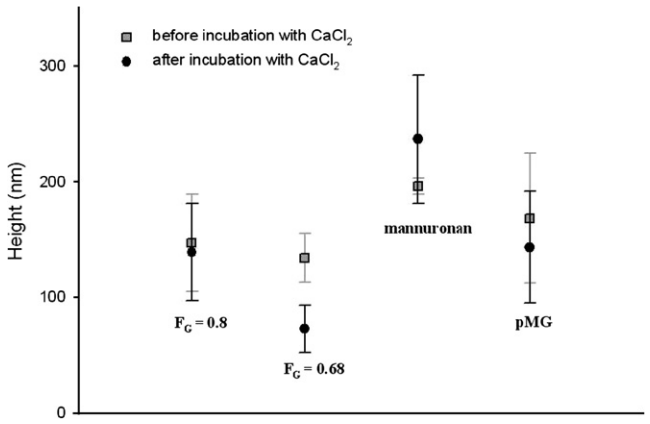

Fig. 4. The thickness of 10 bilayer poly-L-lysine–alginate multilayers prepared in 0.15 M NaCl and determined in this solvent (squares) and after (circles) incubation in aqueous 50 mM $CaCl_2$ in 0.15 M mannitol followed by rinsing in 0.15 M NaCl. Data were obtained for multilayers using four alginates differing in their G-fraction.

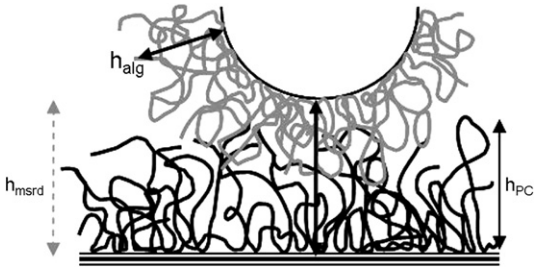

Fig. 5. Schematic illustration of the 10 μm bead coated with alginate hovering over a polycation layer used to determine degree of interpenetration between the polyanion/polycation pair using DW-RICM. Please note that the ratio of the radius of curvatures between the bead and the size of the polymers are not to scale.

ried out at $I = 0.15$ M NaCl. Even though BSA-coated polystyrene bead was used for one of these series, the approach provide some information about the relative differences reflecting alginate composition, as well as differences between the polycations for the degree of interpenetration between the adsorbed polyelectrolytes.

The degree of interpenetration was determined to be larger for the chitosan–alginate multilayers than the PLL–alginate multilayers, for all three alginates employed (a ratio of 1.3 times for polyM, 1.9 times for polyMG and 1.4 times for F_G 0.68; Table 2). Furthermore, for both polycations, the order of the relative degree of interpenetration was found to depend on the G-content of the alginate used, with increasing degree of interpenetration in the order of decreasing G-content (Table 2).

4. Discussion

The results obtained here on alginate–chitosan and alginate–PLL multilayer films display features that in some respects are similar to other multilayer films. There are, however, several observed properties that cannot be explained analogously to that suggested for multilayers of more flexible (synthetic) polymers. These differences will, in the following, be suggested to arise from firstly, different mode of reactions between alginates and chitosan on one hand and alginates and PLL on the other yielding differences in multilayer structure of these two pairs; secondly, differences in sensitivity to post-preparation incubation salt solutions, possibly due to differences in chain lengths and flexibility of the polyelectrolytes, and thirdly, polymer-specific properties of alginate.

The experimental data show that alginate–PLL multilayers are thicker than those prepared of alginate–chitosan (Fig. 3). The apparent thickness of the first polycation layer deposited on the glass substrate was 16 nm and 17 nm for PLL and chitosan ($F_A = 0.1$), respectively (Table 2). For chitosan, this is close to the estimated R_g thus indicating significant flattening of the adsorbed conformation compared to the solution state. The thickness of the first layer of PLL is of the same order of magnitude as the coil dimensions reported for PLL ($M_w = 28 \times 10^3$ g/mol) (Chittchang et al., 2002). The latter comparison indicates that PLL is not to the same extent brought in close proximity to the glass surface in the adsorption process as chitosan. This is despite the fact that PLL can be expected to more pliable than chitosan as indicated by their persistence lengths, L_p, L_p being ~6–12 nm (Cölfen, Berth, & Dautzenberg, 2001) for chitosan and $L_p \sim 2$ nm for PLL (Brant & Flory, 1965). The observed differences in thickness of the multilayer films are therefore suggested to arise from differences in degree of interpenetration between alginate and respective polycation layers in the deposition (Table 2). These differences in degree of interpenetration can, in turn, be rationalized in terms of differences in mobility of the chains due to differences in interaction strengths between the polyelectrolytes in the two different pairs.

This can be explained from different modes of reaction, with PLL interacting with alginate that to some extents sticks on first contact (diffusion limited mode of interaction) but allow some rearrangement, while the interaction between chitosan and alginate to a larger extent is reaction limited, allowing more rearrangements subsequent to first contact. Thus, the observed differences in thickness for the two multilayer-combinations are suggested to arise from the different modes of reaction, with chitosan being able to diffuse more into the multilayers. The multilayers with PLL are therefore expected to be thicker and rougher, and the differences will be amplified with the number of bilayers, increasing the difference between the two polyanion–polycation pairs (Fig. 6). This idea is substantiated in the RICM-measurements of the degree of interpenetration in different alginate/polycation systems (Table 2). An overall layer thickness of 40–60 nm for chitosan–alginate multilayers (Fig. 2) and 135–210 nm for PLL–alginate

Table 2
Degree of interpenetration of adsorbed polycation–polyanion pairs extracted from RICM-measurements of alginate-coated beads over a polycation layer

Alginate	h_{alg} (nm)	Additive heights (nm)		h_{msrd} (nm)		Degree of interpenetration	
		$h_{chit} + h_{alg}$	$h_{PLL} + h_{alg}$	(chit + alg)	(PLL + alg)	(chit − alg)	(PLL − alg)
F_G 0.68	6	23	22	17	18	0.26	0.18
PolyM	5	22	21	8	11	0.64	0.48
PolyMG	10	27	26	13	19	0.52	0.27

The degree of interpenetration is here defined as $h_{int} = (h_{PC} + h_{alg} - h_{msrd})/(h_{PC} + h_{alg})$ where h_{PC}, PC = chit or PLL, h_{alg} is the thickness of the polycation layer and alginate layer, respectively, and h_{msrd} the measured height when using the alginate bead over the polycation layer (Fig. 5). The degree of interpenetration was determined in 0.15 M NaCl. The height of the polycation layer was measured to be 16 nm for PLL and 17 nm for chitosan ($F_A = 0.1$, $M_w = 33 \times 10^3$ g/mol).

multilayers (Fig. 3) following 10 polycation–polyanion deposition cycles indicate an average thickness increase per deposition cycle much below that of the R_g of the polysaccharides. This possibly arises from interpenetration between the layers that depend on the polycation. The larger degree of interpenetration for the chitosan–alginate pair is in support for a reaction-limited interaction as opposed to a mode of interaction closer to a diffusion-limited behavior for the PLL–alginate pair. A build-up mechanism has been suggested for other polyelectrolyte multilayers, where studies have shown that one component can diffuse through a number of layers within the system (Picart et al., 2002). This is in accordance with the present investigations of the chitosan–alginate multilayer system. However, in other systems, the added polyelectrolyte is suggested only to interact with the outer layer of the multilayers (Lavalle et al., 2002), which is an extreme in the diffusion limited mode of interaction (hit and stick) to describe certain features of the interaction between polyanions and polycations.

Earlier investigations have shown that when PLL or chitosan is interacting with alginate gels, PLL binds stronger to the alginate than chitosan. The studies also showed that chitosan penetrates further into the alginate gel beads as compared to PLL (Ottøy, 1996), which is analogous to the suggested hypothesis.

The increase in thickness of the (chit_F_G 0.68)$_n$ multilayers with increasing ionic strength in the deposition solution (Fig. 2) is in accordance with observations of other polyelectrolyte multilayers (Blomberg, Poptoshev, & Caruso, 2006; Dubas & Schlenoff, 1999; Steitz, Leiner, Siebrecht, & Klitzing, 2000). With increasing ionic strength, the polyelectrolytes will adopt a more coiled conformation in solution due to increased screening of the charges. The adsorption of the coiled conformation as compared to extended molecules at low ionic strength will result in thicker layers. Additionally, increased salt concentration will reduce the electrostatic repulsion between the adsorbed polymers on the surface, allowing for an increased adsorbed amount of polymer (Schönhoff, 2003).

In addition to displaying a dependence on the ionic strength in the adsorption solution, the multilayers responded to changes in the ionic strength after preparation (Fig. 2). The swelling behavior, assumed to be constrained to one dimension (height), can be expected to be driven by expansion/contraction of the polyelectrolytes within the multilayers. Swelling of multilayers depends on the polyelectrolyte pair (Dubas & Schlenoff, 2001; Steitz et al., 2000; Sukhorukov, Schmitt, & Decher, 1996), and has been suggested to originate in differences in hydrophobicity of the polyelectrolyte complex (Dubas & Schlenoff, 2001). However, while some multilayers have been found to swell on increasing salt concentration (Dubas & Schlenoff, 2001), it is also reported multilayers with swelling response dominated by screening of the polyanion component dominating for I up to 5 mM, followed by a transition to increasing swelling with increase in ionic strength beyond 50 mM (Burke & Barrett, 2005). The (chit_F_G 0.68) multilayers display a decreased thickness upon increased ionic strength. The volume swelling ratio of covalently crosslinked alginate gel beads when equilibrated in 5 and 150 mM NaCl, respectively, is ~2.5 (Moe, Skjåk-Bræk, Elgsaeter, & Smidsrød, 1993). This corresponds to a linear swelling ratio of 1.4, which is comparable to the observed change in thickness for the chitosan–alginate multilayers (1.4–1.5). Hence, the post-preparation swelling behavior of the (chitosan_F_G 0.68) multilayers is found to closely resemble that observed both for polyelectrolytes in solution and alginate gels. The observed swelling is therefore suggested to arise from an alginate "core" of the multilayer structure intermediate between the chitosan deposition interfaces susceptible to changes in electrolyte concentration (Fig. 6). In addition, the magnitude of the swelling can be expected to be influenced by the chain flexibility of the polyelectrolytes participating in the multilayers. While chitosan–alginate multilayers display a swelling

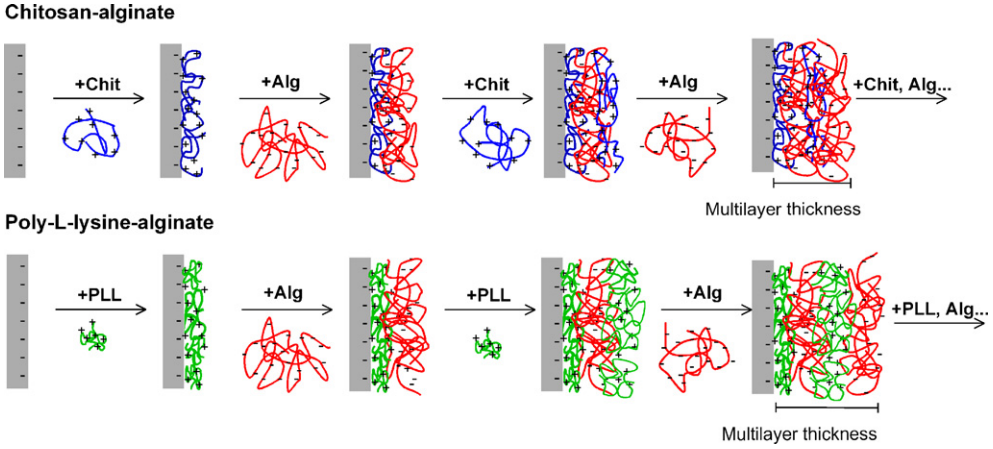

Fig. 6. Schematic illustration of growth of multilayer structures of chitosan–alginate and poly-L-lysine–alginate multilayers with differences in chain lengths and chain stiffness of the polycations. The compression of the various chains when deposited, as well as larger degree of interpenetration within the chitosan–alginate than the poly-L-lysine pair is qualitatively illustrated.

behavior, chitosan–xanthan multilayers do not (Maurstad et al., 2005). Alginate with a persistence length, L_p, of about 5–17 nm (Smidsrød & Haug, 1968; Strand, Bøe, Dalberg, Sikkeland, & Smidsrød, 1982; Vold et al., 2006) is more flexible than xanthan ($L_p \sim 120$ nm (Sato, Norisuye, & Fujita, 1984)) and thus displays larger relative changes in hydrodynamic radius as response to changes in ionic strength.

Preparation of multilayer films using divalent salts instead of NaCl yielded an increase in thickness of the multilayers for $MgCl_2$ but not for Na_2SO_4. The lack of response when using Na_2SO_4 indicates that the change in salt counterions mainly influences the alginate and not the polycation. This might be due to the lower molecular weight of the polycation as compared to alginate, giving rise to a system where the thickness of the multilayers is found to be closely linked to the response of the alginate component on different electrolyte types and concentrations. Mg^{2+} is one of the divalent ions with low selectivity to the carboxylate group on the mannuronic and guluronic acid residues of alginate (Moe et al., 1995) and the response to this cation should mostly be due to electrostatic interaction with alginate. Ca^{2+} on the other hand, displays a high selectivity for the G-residues compared to the M-residues (Steginsky, Beale, Floss, & Mayer, 1992), and in alginate gels Ca^{2+}-ions function as bridges between G-blocks on different chains or different parts within the same chain, often represented by the egg-box model (Grant et al., 1973). A recent study has shown that the presence of calcium influenced the incremental adsorption of alginate (61% G) to a surface attributed to a complexation between alginate and calcium as in the egg-box model (de Kerchove & Elimelech, 2007). Assessment of effects of Ca^{2+} can only be carried out in post-preparation studies of the multilayers involving alginates since addition of this cation to the alginate deposition solution will induce aggregation/gelation of the material to be deposited.

Using $CaCl_2$ in the post-preparation treatment, addition of Ca^{2+} may potentially induce crosslinking junctions between pair of accessible alginate segments. Such accessible segments may exist as part of the core of the alginate layer not penetrated by the polycations, as suggested above, or made accessible by competition between Ca^{2+} and the polycations. Alginate segments existing in loops with respect to ionic bonds with PLL, similar to conformational classification of polymers adsorbed to surfaces (Fleer, Cohen Stuart, Scheutjens, Cosgrove, & Vincent, 1993) may also constitute a possible state accessible for such interactions. This Ca^{2+} crosslinking – "gelation" – will then collapse these parts of the alginate and thus reduce the height of the multilayers. Such a "gelation" was observed only for F_G 0.68. Comparing the alginates, F_G 0.8 has much longer G-blocks than F_G 0.68 (Table 1). The reason that a similar expected response to Ca^{2+} incubation in the (PLL_F_G 0.8)$_{10}$ multilayer is lacking, remains to be fully understood.

5. Conclusion

In this study the response of multilayers employing alginates as the polyanion and either chitosan or poly-L-lysine as the polycation to different salt conditions has been studied employing DW-RICM. The results indicate that a smaller fraction of the ionic groups on alginate compared to the polycation is participating in ionic bonds between the alginate and the polycation. The results presented here are in favor of different interaction mechanisms between chitosan–alginate and poly-L-lysine–alginate during the assembly process, with the latter being more of a "hit-and-stick" process (diffusion limited reaction). The study furthermore show that the specific chemical composition of alginates is of importance in determining their interaction with a polycation and hence when assembled into multilayer structures.

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References

Anthonsen, M. W., & Smidsrød, O. (1995). Hydrogen ion titration of chitosans with varying degrees of N-acetylation by monitoring induced ^{1}H-NMR chemical shifts. *Carbohydrate Polymers, 26*, 303–305.

Anthonsen, M. W., Vårum, K. M., & Smidsrød, O. (1993). Solution properties of chitosans: Conformation and chain stiffness of chitosans with different degrees of N-acetylation. *Carbohydrate Polymers, 22*, 193–201.

Bevan, M. A., & Prieve, D. C. (1999). Direct measurement of retarded van der Waals attraction. *Langmuir, 15*, 7925–7936.

Blomberg, E., Poptoshev, E., & Caruso, F. (2006). Surface interactions during polyelectrolyte multilayer build-up. 2. The effect of ionic strength on the structure of preformed multilayers. *Langmuir, 22*, 4153–4157.

Brant, D. A., & Flory, P. J. (1965). The configuration of random polypeptide chains. *Journal of the American Chemical Society, 87*, 2788–2800.

Burke, S. E., & Barrett, C. J. (2005). Swelling behavior of hyaluronic acid/polyallylamine hydrochloride multilayer films. *Biomacromolecules, 6*, 1419–1428.

Chittchang, M., Salamat-Miller, N., Alur, H. H., Velde, D. G. V., Mitra, A. K., & Johnston, T. P. (2002). Poly(L-lysine) as a model drug macromolecule with which to investigate secondary structure and microporous membrane transport, part 2: Diffusion studies. *Journal of Pharmacy and Pharmacology, 54*, 1497–1505.

Cölfen, H., Berth, G., & Dautzenberg, H. (2001). Hydrodynamic studies on chitosans in aqueous solution. *Carbohydrate Polymers, 45*, 373–383.

de Kerchove, A. J., & Elimelech, M. (2007). Formation of polysaccharide gel layers in the presence of Ca^{2+} and K^+ ions: Measurements and mechanisms. *Biomacromolecules, 8*, 113–121.

Dentini, M., Rinaldi, G., Barbetta, A., Risica, D., Anselmi, C., & Skjåk-Bræk, G. (2007). Ionic gel formation of a (pseudo)alginate character-

ised by an alternating MG sequence produced by epimerising mannuronan with AlgE4. *Carbohydrate Polymers, 67*, 465–473.

Donati, I., Holtan, S., Mørch, Y. A., Borgogna, M., Dentini, M., & Skjåk-Bræk, G. (2005). New hypothesis on the role of alternating sequences in calcium-alginate gels. *Biomacromolecules, 6*, 1031–1040.

Draget, K. I., Vårum, K. M., Moen, E., Gynnild, H., & Smidsrød, O. (1992). Chitosan cross-linked with Mo(VI) polyoxyanoins: A new gelling system. *Biomaterials, 13*, 635–638.

Dubas, S. T., & Schlenoff, J. B. (1999). Factors controlling the growth of polyelectrolyte multilayers. *Macromolecules, 32*, 8153–8160.

Dubas, S. T., & Schlenoff, J. B. (2001). Swelling and smoothing of polyelectrolyte multilayers by salt. *Langmuir, 17*, 7725–7727.

Elbert, D. L., Herbert, C. B., & Hubbell, J. A. (1999). Thin polymer layers formed by polyelectrolyte multilayer techniques on biological surfaces. *Langmuir, 15*, 5355–5362.

Ertesvåg, H., Høidal, H. K., Hals, I. K., Rian, A., Doseth, B., & Valla, S. (1995). A family of modular type mannuronan C-5-epimerase genes controls alginate structure in Azotobacter vinelandi. *Molecular Microbiology, 16*, 719–731.

Ertesvåg, H., Høidal, H. K., Schjerven, H., Glærum Svanem, B. I., & Valla, S. (1999). Mannuronan C-5-epimerases and their application for in vitro and *in vivo* design of new alginates useful in biotechnology. *Metabolic Engineering, 1*, 262–269.

Fleer, G. J., Cohen Stuart, M. A., Scheutjens, J. M. H. M., Cosgrove, T., & Vincent, B. (1993). *Polymers at interfaces*. London: Chapmann & Hall.

Gåserød, O., Smidsrød, O., & Skjåk-Bræk, G. (1998). Microcapsules of alginate–chitosan – I A quantitative study of the interaction between alginate and chitosan. *Biomaterials, 19*, 1815–1825.

Gimmestad, M., Sletta, H., Ertesvåg, H., Bakkevik, K., Jain, S., Suh, S., et al. (2003). The *Pseudomonas* fluorescens AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. *Journal of Bacteriology, 185*, 3515–3523.

Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. C., & Thom, D. (1973). Biological interactions between polysaccharides and divalent cations: The egg-box model. *FEBS Letters, 32*, 195–198.

Grasdalen, H. (1983). High-field, 1H n.m.r. spectroscopy of alginate: Sequential structure and linkage conformation. *Carbohydrate Research, 118*, 255–260.

Grünberg, H. H. V., Helden, L., Leiderer, P., & Bechinger, C. (2001). Measurements of surface charge densities on Brownian particles using total internal reflection microscopy. *Journal of Chemical Physics, 114*, 10094–10104.

Harding, S. E., Vårum, K. M., Stokke, B. T., & Smidsrød, O. (1991). Molecular weight determination of polysaccharides. In C. A. White (Ed.), *Advances in Carbohydrate Analysis* (pp. 63–144). Birmingham, C & A: JAI Press.

Haug, A. (1964). *Composition and properties of alginates*. Thesis. Trondheim: Norwegian Institute of Technology.

Haug, A., Larsen, B., & Smidsrød, O. (1974). Uronic acid sequence in alginate from different sources. *Carbohydrate Research, 32*, 217–225.

Høidal, H. K., Ertesvåg, H., Skjåk-Bræk, G., Stokke, B. T., & Valla, S. (1999). The recombinant *Azotobacter vinelandii* mannuronan C-5-epimerase AlgE4 epimerizes alginate by a nonrandom attack mechanism. *Journal of Biological Chemistry, 274*, 12316–12322.

Lamarque, G., Lucas, J.-M., Viton, C., & Domard, A. (2005). Physicochemical behaviour of homogenous series of acetylated chitosans in aqueous solution: Role of various structural parameters. *Biomacromolecules, 6*, 131–142.

Lavalle, P., Gergely, C., Cuisinier, F. J. G., Decher, G., Schaaf, P., Voegel, J. C., et al. (2002). Comparison of the structure of polyelectrolyte multilayer films exhibiting a linear and exponential growth regime: An in situ atomic force microscopy study. *Macromolecules, 35*, 4458–4465.

Maurstad, G., Bausch, A. R., Sikorski, P, & Stokke, B. T. (2005). Electrostatically self-assembled multilayers of chitosan and xanthan studied by atomic force microscopy and micro-interferometry. *Macromolecular Symposia, 227*, 161–172.

Moe, S. T., Draget, K. I., Skjåk-Bræk, G., & Smidsrød, O. (1995). Alginate. In *Food polysaccharides and their applications* (pp. 245–286). NY, New York: Stephen, A. Marcel Dekker.

Moe, S. T., Skjåk-Bræk, G., Elgsaeter, A., & Smidsrød, O. (1993). Swelling of covalently crosslinked alginate gels: Influence of ionic solutes and nonpolar solvents. *Macromolecules, 26*, 3589–3597.

Orive, G., Hernandez, R. M., Gascon, A. R., Calafiore, R., Chang, T. M. S., de Vos, P., et al. (2004). History, challenges and perspectives of cell microencapsulation. *Trends in Biotechnology, 22*, 87–92.

Ottøy, M.H. (1996). *Chemical and physical characterization of chitosans*. Ph.D. Thesis. Trondheim.

Picart, C., Mutterer, J., Richert, L., Luo, Y., Prestwich, G. D., Schaaf, P., et al. (2002). Molecular basis for the explanation of the exponential growth of polyelectrolyte multilayers. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 12531–12535.

Picart, C., Sengupta, K., Schilling, J., Maurstad, G., Ladam, G., Bausch, A. R., et al. (2004). Micro-interferometric study of the structure, interfacial potential and viscoelastic properties of polyelectrolyte multilayer films on a planar substrate. *Journal of Physical Chemistry B, 108*, 7196–7205.

Sato, T., Norisuye, T., & Fujita, H. (1984). Double-stranded helix of xanthan in dilute solution: Evidence from light scattering. *Polymer Journal, 16*, 341–350.

Schilling, J. (2003). *Entwicklung einer schnellen digitalen Bildverarbeitungstechnik und einer interferometrischen 2λ – Mikroskopietechnik: Anwendung in der Zell-Biophysik*. Ph.D. Thesis. Munich: E22 Technische Universität München.

Schilling, J., Sackmann, E., & Bausch, A. R. (2004). Digital imaging processing for biophysical applications. *Review of Scientific Instruments, 75*, 2822–2827.

Schilling, J., Sengupta, K., Goennenwein, S., Bausch, A. R., & Sackmann, E. (2004). Absolute interfacial distance measurements by dual-wavelength reflection interference contrast microscopy. *Physical Review E, 69*, 021901-1–021901-9.

Schönhoff, M. (2003). Layered polyelectrolyte complexes: Physics of formation and molecular properties. *Journal of Physics: Condensed Matter, 15*, R1781–R1808.

Smidsrød, O., & Haug, A. (1968). A light scattering study of alginate. *Acta Chemica Scandinavica, 22*, 797–810.

Steginsky, C. A., Beale, J. M., Floss, H. G., & Mayer, R. M. (1992). Structural determination of alginic acid and the effects of calcium binding as determined by high-field n.m.r. *Carbohydrate Research, 225*, 11–26.

Steitz, R., Leiner, V., Siebrecht, R., & Klitzing, R. (2000). Influence of the ionic strength on the structure of polyelectrolyte films at the solid/liquid interface. *Colloids and Surfaces A, 163*, 63–70.

Strand, B. L., Mørch, Y. A., Espevik, T., & Skjåk-Bræk, G. (2003). Visualization of alginate–poly-L-lysine–alginate microcapsules by confocal laser scanning microscopy. *Biotechnology and Bioengineering, 82*, 386–394.

Strand, B. L., Mørch, Y. A., & Skjåk-Bræk, G. (2000). Alginate as immobilization matrix for cells. *Minerva Biotechnologica, 12*, 223–233.

Strand, K. A., Bøe, A., Dalberg, P. S., Sikkeland, T., & Smidsrød, O. (1982). Dynamic and static light scattering on aqueous solutions of sodium alginate. *Macromolecules, 15*, 570–579.

Strand, S. P., Tømmeraas, K., Vårum, K. M., & Østgaard, K. (2001). Electrophoretic light scattering studies of chitosans with different degrees of N-acetylation. *Biomacromolecules, 2*, 1310–1314.

Sukhorukov, G. B., Schmitt, J., & Decher, G. (1996). Reversible swelling of polyanion/polycation multilayer films in solutions of different ionic strength. *Berichte der Bunsen-Gesellschaft Physical Chemistry Chemical Physics, 100*, 948–953.

Svanem, B. I. G., Skjåk-Bræk, G., Ertesvåg, H., & Valla, S. (1999). Cloning and expression of three new *Azotobacter vinelandii* genes closely related to a previously described gene family encoding mannuronan C-5-epimerases. *Journal of Bacteriology, 181*, 68–77.

Thu, B., Bruheim, P., Espevik, T., Smidsrød, O., Soon-Shiong, P., & Skjåk-Bræk, G. (1996). Alginate polycation microcapsules I. Interaction between alginate and polycation. *Biomaterials, 17*, 1031–1040.

Vårum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidsrød, O. (1991). Determination of the degree of N-acetylation and the distribution of N-acetyl groups in partially N-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. *Carbohydrate Research, 211*, 17–23.

Vold, I. M. N., Kristiansen, K. A., & Christensen, B. E. (2006). A study of the chain stiffness and extension of alginates, in vitro epimerized alginates, and periodate-oxidized alginates using size-exclusion chromatography combined with light scattering and viscosity detectors. *Biomacromolecules, 7*, 2136–2146.