



Polyelectrolyte complex formation using alginate and chitosan

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ARTICLE INFO

Article history:

Received 2 January 2008

Received in revised form 21 April 2008

Accepted 29 April 2008

Available online 15 May 2008

Keywords:

Alginate

Chitosan

Polyelectrolyte

Complexes

Zeta potential

Particle size

ABSTRACT

Polyelectrolyte complexes (PECs) of alginate and chitosan were formed by addition of 0.1% alginate solution (pH ~6.5) to 0.1% chitosan solution (pH ~4.0), and by adding the chitosan solution to the alginate solution under high shearing conditions. Variations in the properties of the polymers and the preparation procedure were studied, and the resultant PEC size, zeta potential (Zp), and pH were determined using dynamic light scattering (DLS), electrophoresis and by measuring turbidity and pH. Tapping mode atomic force microscopy (AFM) was used to examine some of the complexes. The particle size was decreased as the speed and diameter of the dispersing element of the homogenizer was increased. The net charge ratio between chitosan and alginate, and the molecular weights (M_w) of both the alginate and chitosan samples were the most significant parameters that influenced the particle size, Zp, and pH. The mixing order also influenced the size of the PECs, however, the Zp and pH were not affected by the mixing order. The stability of the complexes was investigated by incubation at an elevated temperature (37 °C), storage for one month at 4 °C, alteration of the pH of the PEC mixture, and addition of salt to physiological ionic strength (0.15 M NaCl). The properties of the PEC could be affected according to the molecular properties of the polyelectrolytes selected and the preparation procedures used. The resultant PEC sizes and properties of the complex were rationalised using a core-shell model for the structure of the complexes.

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1. Introduction

Oppositely charged polysaccharides in aqueous solutions interact spontaneously to form polyelectrolyte complexes (PECs) when they are mixed. The entropy gain associated with the release of counter-ions is one of the major driving forces for PEC formation (Thünemann, Müller, Dautzenberg, Joanny, & Löwen, 2004). Polysaccharides are of particular interest due to their desirable biocompatible, biodegradable, hydrophilic and protective properties (Barichello, Morishita, Takayama, & Nagai, 1999; Chellat et al., 2000). Interactions between cationic chitosan and anionic alginate leads to PEC formation, and these PECs have potential applications such as drug or gene delivery systems in biomedicine. It has been demonstrated that linking bioactive alginate to particles increases biological stimulation (Berntzen et al., 1998). The alginate–chitosan PECs may also present an immune stimulating property arising from the immunogenic properties of the constituting polymers that can be affected by the form of presentation analogously to conjugation of alginates to particles.

Chitosan is a natural cationic polysaccharide that is formed by the *N*-deacetylation of chitin, which is a product found in crusta-

cean shells. Chitosan is a linear binary copolymer that consists of β (1 → 4)-linked 2-acetoamido-2-deoxy- β -D-glucopyranose (GlcNAc; A-unit) and 2-amino-2-deoxy- β -D-glucopyranose (GlcN; D-unit). The A- and D-type residues are randomly distributed along the chitosan chain (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991a, 1991b). The average linear charge density of chitosans is determined by the degree of acetylation (F_A) and also by the pH of the solution. The degree of acetylation determines the density of the amino groups that can carry a charge, and the pH determines the degree of protonization of the amino groups. Chitosans of different chemical compositions (F_A values in the range of 0.01–0.49) have similar dissociation constants (pK_a) of 6.5–6.6 (Strand, Tømmeraas, Vårum, & Østgaard, 2001).

Alginate is a natural anionic polysaccharide and can be extracted from marine brown algae. Alginate is a linear binary copolymer that consists of (1 → 4)-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues. The relative amount of the two uronic acid monomers and their sequential arrangement along the polymer chain vary widely, depending on the origin of the alginate. The uronic acid residues are distributed along the polymer chain in a pattern of blocks, where homopolymeric blocks of G residues (G-blocks), homopolymeric blocks of M residues (M-blocks), and blocks with an alternating sequence of M and G units (MG-blocks) can co-exist [for a review see Moe, Draget, Skjåk-Bræk, & Smidsrød, 1995]. Alginates may be designed with other

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monomeric sequences and can be produced using biotechnological methods (Bjerkkan et al., 2004). The polyelectrolyte properties of the monomer residues of alginates have previously been reported upon (Haug, 1964), and the pK_a -values for mannuronic and guluronic acid monomers were found to be 3.38 and 3.65, respectively.

Polyelectrolyte complexes may be formed from alginate and chitosan using different methods. In this study, we used a one-stage process, where one polymer solution is slowly added in a dropwise manner into the other solution (Daly & Knorr, 1988). Alternatively, a two-stage procedure may be used to form PECs. The first step in this process is the preparation of Ca-alginate gel beads (Hackel, Klein, Megenet, & Wagner, 1975; Skjåk-Bræk & Martinsen, 1991). The beads are then transferred into a chitosan solution, and a PEC membrane is formed on the surface of the beads (Douglas & Tabrizian, 2005; Gåserød, Smidsrød, & Skjåk-Bræk, 1998).

A more molecular based structure-function relationship for the formation of PECs from alginate and chitosan and the parameters that influence the process are needed in order to produce high-quality, safe products that can be used in biomedical applications (Skaugrud, Hagen, Borgersen, & Dornish, 1999). PEC formation has previously been reported to be affected by the ratio of alginate to chitosan, the molecular weight of the polysaccharides and the pH of the solutions (Douglas & Tabrizian, 2005). The fraction of acetylated monomers, F_A , in the chitosan has also been shown to be a determining parameter in PEC formation (Lee, Park, & Ha, 1997). It is necessary to study PECs in physiological environments in order to assess their suitability for use in pharmaceutical and biomedical applications. In this study, we investigated variations in preparation procedures and polymer characteristics in order to study their influence on PEC particle size and particle surface charge (zeta potential, Z_p), and the final pH of the PEC solution in more detail than has previously been revealed. The influence of biopolymeric properties such as charge ratio [alginate/chitosan ratio, $K(-/+)$], the average molecular weights (M_w) of chitosan and alginate, and chemical composition (F_A of chitosan or fraction of guluronic acid, F_G of alginate) were studied, and some of the particles were visualized by AFM. The stability of the PECs was also investigated with respect to storage time, physiological pH and temperature. Salt (NaCl) was added to the PEC solutions in order to obtain physiological conditions, and the resulting particle sizes and zeta potentials were studied.

This study focused on PEC formation from alginate and chitosan using a one-stage process. A sodium alginate solution was added to a chitosan chloride solution under high shearing conditions, or chitosan chloride was added to sodium alginate. We describe how the PEC particle sizes and surface charge can be controlled by varying preparation procedures and polymer characteristics.

2. Materials and methods

2.1. Biopolymer samples

The sodium alginate and chitosan chloride samples were kindly provided by FMC BioPolymer AS/NovaMatrix (Sandvika, Norway). The chitosan samples (Table 1) varied in their degree of acetylation (F_A) and in their M_w . The chitosan F_A was determined by NMR spectroscopy (Vårum et al., 1991a), and the chitosan M_w was determined by SEC-MALLS (Size Exclusion Chromatography Multi Angle Laser Light Scattering detector) (Holme, Lindmo, Kristiansen, & Smidsrød, 2003) using the mobile phase (0.2 M $\text{CH}_3\text{COONH}_4$, pH 4.5), and also from the apparent viscosity (η/mPas) of a 1% chitosan aqueous solution (w/w) (Brookfield rotational viscometer 20 rpm, at 20 °C) using a previously derived relationship between apparent viscosity and molecular

Table 1

Chitosans used for the formation of alginate–chitosan complexes

F_A^a	App. viscosity ^b η (mPas)	M_w^c (kg/mol)	Identification term
0.14	160	400	C _{0.14,400}
0.14	88	343	C _{0.14,343}
0.14	16	117	C _{0.14,117}
0.15	4	47	C _{0.15,47}
0.47	5	~50	C _{0.47,50}
0.61	n.d. ^d	115	C _{0.61,115}
0.02	7	~50	C _{0.02,50}

^a F_A determined by NMR spectroscopy (Vårum et al., 1991a).

^b Apparent viscosity η (mPas) measured in a 1% solution (w/w), Brookfield rotational viscosimeter, water bath at 20 °C and 20 rpm.

^c M_w determined by SEC-MALLS (Size Exclusion Chromatography Multi Angle Laser Light Scattering). The M_w of the chitosans C_{0.47,50} and C_{0.02,50} are calculated from viscosity measurements.

^d Not determined.

weight (Holme, Davidsen, Kristiansen, & Smidsrød, 2008). The sample notation C _{F_A , M_w} was used for the chitosans, where the first subscript represents the F_A , and the second, the weight average molecular weight (M_w) in kg/mol. Alginates with various weight average molecular weight, M_w , and various fractions of guluronic acid (G) and mannuronic acid (M) units were used to investigate the effects on the PECs (Table 2). The fraction of G in the alginates, F_G , was determined by NMR spectroscopy (Grasdalen, 1983). The M_w of the alginates was determined by SEC-MALLS (Holme et al., 2003). The sample notation Alg _{F_G , M_w} was adapted for the alginates, and the first subscript was used to describe the fraction of G-units (F_G) and the second subscript referred to the M_w in kg/mol.

2.2. Preparation of polyelectrolyte complexes

Aqueous stock solutions (0.1% w/v) of sodium alginate and chitosan chloride were prepared by dissolving the polymer in MQ-water and magnetically stirring (~100 rpm) the solution overnight at 4 °C. The pH of the alginate solution was ~6.5, and the pH of the chitosan chloride solution was ~4.0 after the dissolution. These pHs ensured that the alginate was fully deprotonated and that the chitosan was fully protonated. The polyelectrolyte complexes (PECs) were prepared at room temperature. The PEC solutions were mixed using an Ultra-turrax homogenizer with high shearing conditions. The alginate solution was added in a dropwise manner to the chitosan solution at a flow rate of 1 ml/s, thus forming alginate in chitosan PECs (AiC PECs). The PECs were prepared in order to give a final charge ratio $K = q_{\text{alginate}}/q_{\text{chitosan}} \in (0.01, 17)$ for complex formation, under the assumption that the chitosan was fully protonated, using the calculation described by Danielsen, Vårum, and Stokke (2004). The order in which the solutions were added was also investigated in order to form chitosan in alginate PECs (CiA PECs) in addition to the AiC PECs. To examine the influence of chitosan and alginate molecular parameters, PECs from different alginates and chitosans were prepared. The notation (Alg _{F_G , M_w} C _{F_A , M_w}) _{K} was adapted in order to describe the PECs, and the subscript K represents the net polyanion–polycation

Table 2

Alginates used for the formation of alginate–chitosan complexes

F_G^a	App. Viscosity ^b η (mPas)	M_w^c (kg/mol)	Identification term
0.46	127	170	Alg _{0.46,170}
0.69	138	219	Alg _{0.69,219}
0.69	5	41	Alg _{0.69,41}

^a F_G determined by NMR spectroscopy (Grasdalen, 1983).

^b Apparent viscosity η measured in a 1% solution (w/w), Brookfield rotational viscosimeter, water bath at 20 °C.

^c M_w determined by SEC-MALLS (Size Exclusion Chromatography Multi Angle Laser Light Scattering).

charge ratio (–/+) in the final PEC dispersion, $\text{Alg}_{\text{FG,MW}}$ and $\text{C}_{\text{FA,MW}}$ reflect the alginate and chitosan used for PEC formation, respectively, and the order of $\text{Alg}_{\text{FG,MW}}$ and $\text{C}_{\text{FA,MW}}$ in the brackets represents the mixing order [alginate into chitosan ($\text{Alg}_{\text{FG,MW}} \text{C}_{\text{FA,MW}}$) or chitosan into alginate ($\text{C}_{\text{FA,MW}} \text{Alg}_{\text{FG,MW}}$)]. Using this notation, ($\text{Alg}_{0.46,170} \text{C}_{0.14,400}$)_{0.3} complexes were prepared by adding alginate solution into chitosan solution to yield an average charge ratio (–/+) of 0.3, and the use of an alginate with $F_G = 0.46$ and a M_W of 170 kg/mol, and a chitosan with an $F_A = 0.14$ and a M_W of 400 kg/mol.

2.3. Homogenization

The PECs were generally prepared by mixing polyelectrolyte solutions with an Ultra-turrax homogenizer (Ika Werke) at 11,000 rpm, using a dispersing element with a 25 mm diameter. However, the influence of the blending conditions on the properties of the AiC PECs (mass ratio = 1, corresponding to $K = 1.2$) was investigated, using dispersing elements that varied in size (diameters of the dispersing element = 5, 17 and 25 mm) and speed (11, 000–24,000 rpm). The details of the different homogenizers are shown in Table 3.

2.4. Characterization of the complexes

In order to characterize the complexes, the z-average diameter (s_z), zeta potential (Zp), pH and transmittance of the PEC solutions were measured 1–3 h after the complexes were prepared. The pH was measured (Radiometer Copenhagen, PHM 92 Lab pH meter) while the PEC solution was magnetically stirred. In order to obtain a qualitative measure of the sizes of the PECs, the transmittance of the PEC solutions was measured at $\lambda = 800$ nm (UV-1201, Shimadzu). At this wavelength, neither alginate nor chitosan absorb light, therefore, the size and number of particles is the only variables that affect the degree of transmittance. MQ-water was used as a standard, and was set to 100% transmittance. A dynamic light scattering (DLS) measurement was made using a Zetasizer Nano instrument (Zetasizer Nano, Malvern instrument, USA), and used to determine s_z . Based on measurements of the Brownian motion of the particles in the solution, the z-average diameters (s_z) of the particles were determined using the Stokes–Einstein equation. The Zp of the solutions was measured by laser Doppler electrophoresis (Zetasizer Nano, Malvern instrument, USA). The s_z and Zp values are the mean values with standard deviations from three to six replicate measurements of each sample. For $K \sim 1$, most PEC diameters had values of $s_z > 6 \mu\text{m}$. This is beyond the range of the Nano sizer instruments (range 0.6 nm–6 μm according to the manufacturer), therefore these measurements were only used in a qualitative manner, and the 6 μm level was marked on the figures.

2.5. AFM imaging

Samples for AFM imaging were prepared by transferring 10 μL PEC solution to a freshly cleaved 5 mm diameter mica disk, and

after 1–2 min of incubation, the samples were dried in a stream of N_2 gas. An AFM (Digital Instrument Multimode IIIa) was used for imaging. The instrument was operated in tapping mode, as previously described (Maurstad, Danielsen, & Stokke, 2003; Stokke, Falch, & Dentini, 2001) using silicon nitride cantilevers (PPP-NCH-W, Pointprobe plus, Nanosensors) with nominal spring constants of 10–130 N/m. The topographs were obtained at scan sizes in a range of $0.5 \mu\text{m} \times 0.5 \mu\text{m}$ to $15 \mu\text{m} \times 15 \mu\text{m}$ (512×512 pixels). The topographs were flattened line by line excluding the polyelectrolyte complexes to correct for the tube-like scanner movement on a small sector of a sphere in presenting the height over the supporting surface, and use only the determined height of the supporting mica as the basis for this procedure.

2.6. PEC stability upon changes in pH, temperature and storage time

In order to investigate PEC stability with respect to pH, the solutions of the complexes were characterized by following changes in pH. After the ($\text{Alg}_{0.69,5} \text{C}_{0.47,50}$)_{0.2} complexes were prepared, the pH of the PEC solution was adjusted (pH 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 6.4, 6.7, 7.0, 7.4, 8.0) by addition of 0.1 M HCl or 0.1 M NaOH while the solution was magnetically stirred. Experiments were also carried out in order to examine the effects of temperature and storage time upon PEC stability. PEC ($\text{Alg}_{0.69,41} \text{C}_{0.47,50}$)_{0.2} (pH not adjusted) was incubated at 37 °C using a water bath, and the PECs were characterized after 30 min, 4 h and 8 h incubation periods. PEC ($\text{Alg}_{0.69,41} \text{C}_{0.14,177}$)_{0.3}, ($\text{Alg}_{0.69,41} \text{C}_{0.15,47}$)_{0.3} and ($\text{Alg}_{0.69,41} \text{C}_{0.47,50}$)_{0.3} were characterized immediately after formation, and also after 1 month storage at 4 °C.

3. Results

3.1. Influence of homogenization parameters

The polyelectrolyte complexes were prepared using different homogenizers, and the speed and diameter of the dispersing elements of the homogenizers were varied in order to examine the effects of homogenizer parameters upon the PECs obtained (Table 3). PEC ($\text{Alg}_{0.69,41} \text{C}_{0.15,50}$)_{1.2} was prepared at a mass ratio of 1, which corresponded to a charge ratio, K (–/+) ~ 1.2 . The particle size distributions showed that the PEC diameters decreased as homogenizer speed was increased (Fig. 1). At a homogenizer speed of 24,000 rpm, particles with an average diameter (s_n) of ~ 100 nm were formed, whereas at 11,000 rpm, particles of $s_n \sim 1000$ nm were formed. Use of a homogenizer speed of 11,000 rpm led to a more narrow particle diameter distribution than when a homogenizer speed of 16,000 rpm (data not shown) or 24,000 rpm (Fig. 1) was used. Furthermore, the diameter of the dispersing element was also shown to influence the particle size. The smallest diameter of the dispersing element [T8 (5 mm, 25,000 rpm)] resulted in the production of large particles $s_n \sim 6 \mu\text{m}$, whereas the largest diameter of the dispersing element [T25 (25 mm, 24,000 rpm)] led to the production of smaller particles $s_n \sim 100$ nm (Fig. 1).

3.2. Influence of charge ratio K (–/+) and mixing order

Fig. 2 shows the influence of variations in charge ratio (K ranging from 0.01 to 17) on PEC formation by adjusting the amount of alginate and chitosan. The effect of altering the order of polyelectrolytes mixing is also shown in Fig. 2. Significant alterations in s_z (Fig. 2a), transmittance (Fig. 2b), Zp and pH (Fig. 2c) values occur at a stoichiometric charge ratio between the polyanion and polycation. A significant increase in s_z and pH, and a concomitant reduction of transmittance and Zp was observed when $K \sim 1$ (Fig. 2). The s_z increased from 470 nm for ($\text{Alg}_{0.69,41} \text{C}_{0.47,50}$)_{0.3} to $s_z > 6 \mu\text{m}$ for

Table 3
Ultra-turrax homogenizers used in the experiments

Homogenizer	Diameter, dispersing element (mm)	Speed ($\times 1000$ rpm)	Term used for identification ^a
T8 (IKA Werke)	5	5–25	T8(5 rpm)
Janke & Kunkel (IKA Werke)	17	20	JK(17 rpm)
T25 (IKA Werke)	25	11–24	T25(25 rpm)

^a To identify the Ultra-turrax homogenizers, the first number in the parenthesis indicates the diameter of the dispersing element, while the second number indicates the speed ($\times 1000$ rpm) used to mix the polyelectrolyte solutions.

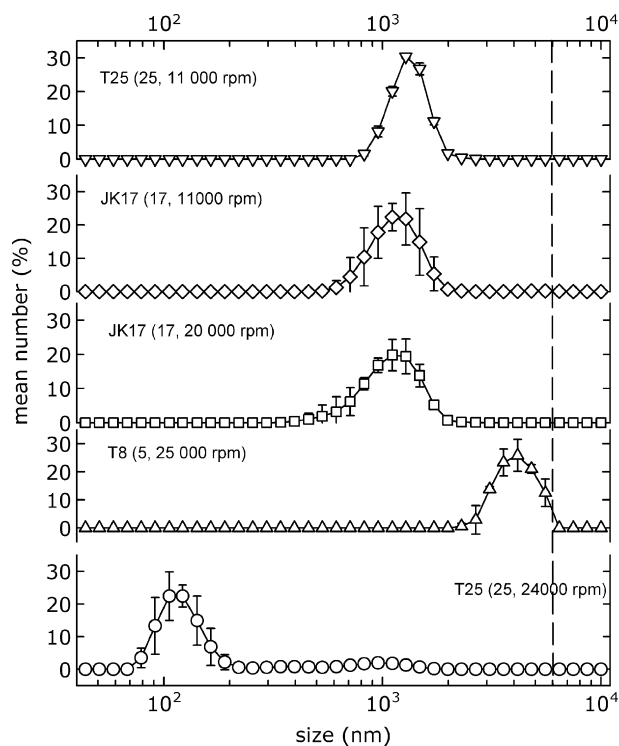


Fig. 1. The effect of homogenizer size and speed on the size-distribution of the alginate in the chitosan polyelectrolyte complex (AiC PEC) formed at a mass ratio of 1 ($K = 1.2$), using (Alg_{69,41} C_{0,15,50}). The mean number (%) vs. size (diameter) distributions when using homogenizers with dispersing elements with diameters of 25 mm (T25), 17 mm (JK17) or 5 mm (T8) and homogenizer speeds ranging between 11,000 and 24,000 rpm is shown. The nominal upper limit for the determination of a particle size of 6 μm is shown as a dotted line.

(Alg_{0,69,41} C_{0,47,50})_{1,0} (Fig. 2a). When K was increased from 0.3 to 1.0, the transmittance of these complexes at 800 nm decreased from over 0.98 to below 0.88 (Fig. 2b), the pH increased from 4.0–4.5 to 6.0–6.5, and the Zp decreased from +40 to –40 mV (Fig. 2c).

Changing the order of polyelectrolyte mixing from polyanion in a polycation solution to polycation in a polyanion solution affected the properties of the PECs obtained. For cationic PECs ($K < 1$), the s_z for (C_{0,15,47} Alg_{0,69,41}) was larger than the s_z for (Alg_{0,69,41} C_{0,15,47}). For anionic PECs (hence $K > 1$, an excess of alginate), $s_z(\text{C}_{0,15,47} \text{Alg}_{0,69,41}) < s_z(\text{Alg}_{0,69,41} \text{C}_{0,15,47})$. These observations indicated that the order of polymer addition affects the size of the PEC particles produced.

3.3. Influence of alginate and chitosan molecular parameters

The preparation of alginate in chitosan polyelectrolyte complexes using alginates and chitosans that had different molecular parameters and K values of 0.3, 1 and 3 indicated that the most important parameter that influences PEC properties is the charge ratio K (–/+) (Fig. 3). For $K \sim 1$, the complexes were unstable, which was indicated by fluctuations in Zp, pH (Fig. 3c) and transmittance measurements (Fig. 3b). The variations of s_z , Zp and pH with respect to F_A are small. The smallest PECs were made when chitosan was in excess, and when the alginate solution was added to the chitosan solution. At $K \sim 0.3$, the s_z values of (Alg_{0,69,41} C_{0,02,50})_{0,3}, (Alg_{0,69,41} C_{0,15,47})_{0,3} and (Alg_{0,69,41} C_{0,47,50})_{0,3} were 748 nm, 526 nm and 467 nm, respectively. In an excess of alginate, and $K = 3$, the s_z values for (Alg_{0,69,41} C_{0,02,50})₃, (Alg_{0,69,41} C_{0,15,47})₃ and (Alg_{0,69,41} C_{0,47,50})₃ were 2.4 μm , 1.2 μm and 1.5 μm , respectively. Altering the F_A of the chitosan did not affect the pH or the Zp of the PEC formed (Fig. 3c). The Zp decreased from +40 mV at

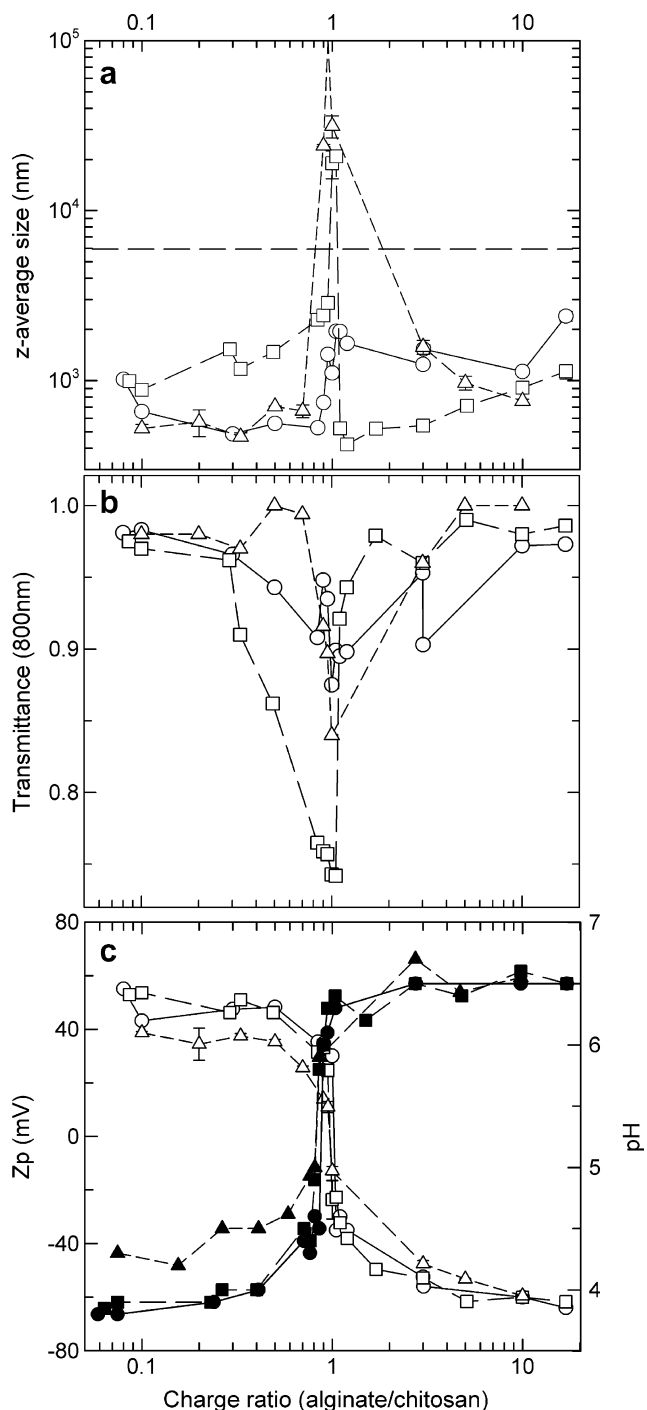


Fig. 2. Parameters of the alginate–chitosan polyelectrolyte complexes (PEC) obtained at charge ratios K from 0.01 to 17. (a) The s_z -average diameter s_z , (open symbols), (b) the transmittance (open symbols) and (c) Zp (open symbols) and pH (filled symbols) for AiC and CiA PECs vs. the alginate to chitosan charge ratio. AiC PECs were (Alg_{0,69,41} C_{0,15,47}) (○, ●) and (Alg_{0,69,41} C_{0,47,50}) (△, ▲), whereas the CiA PEC was (C_{0,15,47} Alg_{0,69,41}) (□, ■).

$K = 0.3$ to 0 mV at $K = 1$, and a further decrease in Zp < -40 mV was observed when $K = 3$. The pH of the PEC dispersion was 4.0–5.0 at $K = 0.3$, and increased to pH 6.5–7.5 when $K = 3$. The size of complexes at $K \sim 1$ was $s_z > 6 \mu\text{m}$ (Fig. 3a) and the transmittance (800 nm) < 0.88 (Fig. 3b).

Selection of polymers with various M_w values influenced the resulting s_z of the complexes (Figs. 4 and 5). Increasing the M_w of the polymer in excess, either alginate ($K \sim 3$) or chitosan ($K \sim 0.3$), led to an evident increase in PEC size. This effect was

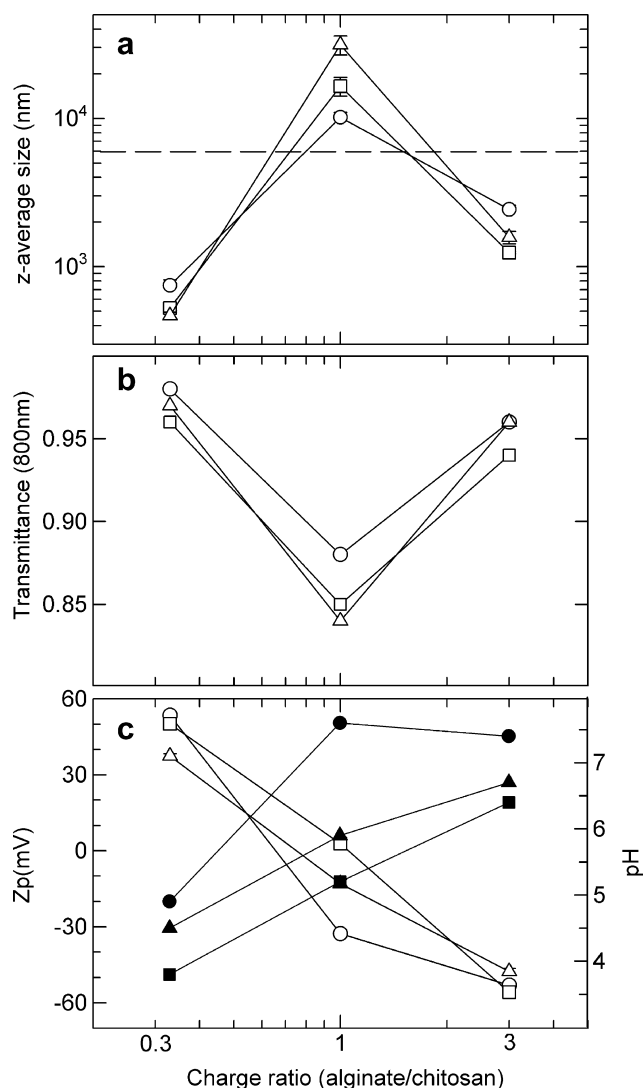


Fig. 3. (a) The z-average diameter s_z (open symbols), (b) transmittance (800 nm, open symbols), (c) Zp (open symbols) and pH (filled symbols) for different alginate to chitosan charge ratios ($K = 0.3$, 1 and 3) for AIC PEC made from $C_{0.02,50}$ (\circ), $C_{0.15,47}$ (\triangle) and $C_{0.47,50}$ (\square). The alginate was constant $Alg_{0.69,41}$.

not as obvious when the M_w of the deficient polymer was increased. Alginates with different F_G values were used to prepare complexes, and the chitosan ($C_{0.15,47}$) was kept the same. There was no systematic variation in the properties of the complexes according to the G-content of the alginate (Fig. 5). An overall assessment of these data (Figs. 3–5) indicates that chitosans and alginates with low M_w values give the smallest complexes, however, altering the molecular parameters F_G and F_A does not significantly change the PEC properties.

3.4. AFM imaging

The PECs were visualized by AFM. Three samples prepared at different conditions and with varying scan sizes are shown in Fig. 6. Complexes exist in variable shapes and forms, such as round spherical particles, linear chains, segments and aggregating flocks (Fig. 6). Fig. 6a shows PEC ($Alg_{0.69,41} C_{0.47,50}$) $_{0.3}$ (excess of chitosan) appears as small segments with spherical, round and linear shapes. The sizes of these complexes were determined to be $s_z \sim 500$ nm using DLS. For PEC ($Alg_{0.69,41} C_{0.14,343}$) $_1$, i.e. at $K \sim 1$, $s_z > 6 \mu m$, it was not possible to observe clearly distinguishable isolated

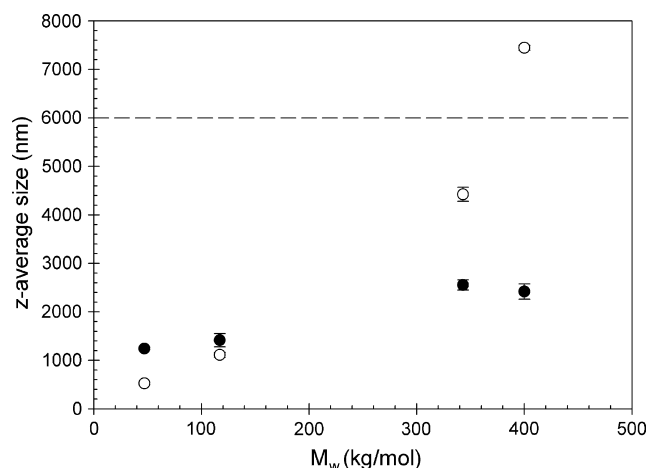


Fig. 4. The z-average diameter for alginate in chitosan polyelectrolyte complexes vs. the molecular weight of the chitosan ($F_A = 0.14$) used to prepare the complexes. The alginate $Alg_{0.69,41}$ was used to prepare the polyelectrolyte complexes using alginate to chitosan charge ratios of 0.3 (open symbols) and 3 (filled symbols).

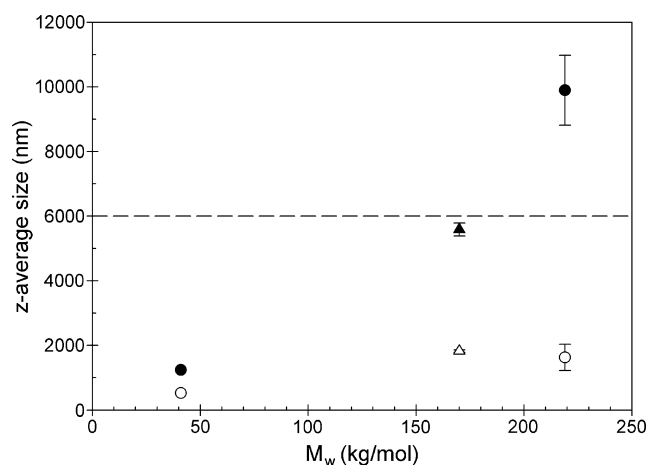


Fig. 5. The z-average diameter for alginate in chitosan polyelectrolyte complexes vs. the molecular weight of alginate when a chitosan $C_{0.15,47}$ was used to prepare the complexes. Complexes were prepared using alginates of two different G-fractions, $F_G = 0.69$ (circle) and $F_G = 0.46$ (triangle), and alginate to chitosan charge ratios of 0.3 (open symbols) and 3 (filled symbols).

structures, and instead larger complexes with various shapes, flocks, and aggregates were observed (Fig. 6b). In an excess of alginate, i.e. for PEC ($Alg_{0.69,41} C_{0.14,343}$) $_3$, both complexes and aggregates were observed, and a chequered layer was also observed in the background (Fig. 6c).

3.5. Stability of the PECs

The PECs were subjected to changes in pH, temperature and were also stored for one month at 4 °C. One month storage at 4 °C or incubation at 37 °C for 8 h did not cause any changes in PECs diameter or Zp (Fig. 7).

When the pH in the PEC ($Alg_{0.69,41} C_{0.47,50}$) $_{0.2}$ solution was adjusted within an interval of pH 3–6.4 by addition of HCl or NaOH, the particle diameter stayed almost constant (Fig. 8). As the pH was increased between 6.4 and 6.7, the diameter of the PEC particles significantly increased. As shown in Fig. 8, the observed particle diameter at pH 7.0 is about 50 times the size observed at pH 6. The particle size levels out at pH 7.0. A decrease in the Zp of the complexes was observed as the size of the particles increased.

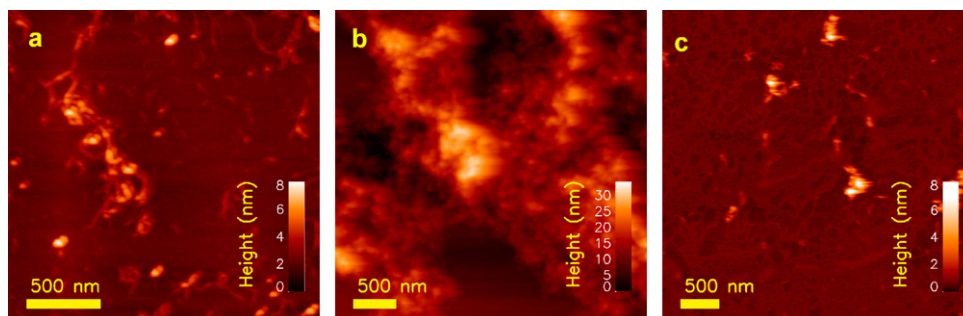


Fig. 6. AFM height topographs obtained from three different PECs (a–c). (a) PEC (Alg_{0.69,41} C_{0.47,50})_{0.3}, (b) PEC (Alg_{0.69,41} C_{0.14,343})₁ and (c) PEC (Alg_{0.69,41} C_{0.14,343})₃.

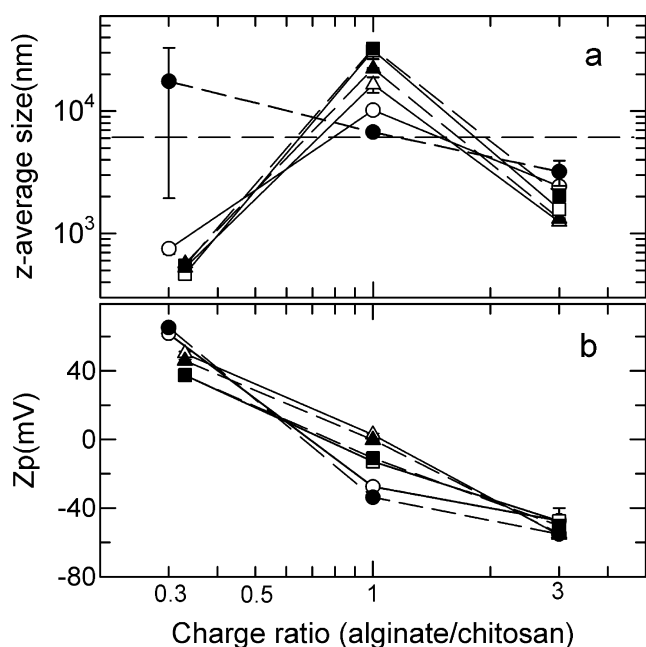


Fig. 7. The z-average diameter s_z (a), and zeta potential Z_p (b) for alginate in chitosan polyelectrolyte complexes prepared using alginate Alg_{0.69,41} and chitosans C_{0.14,117} (○, ●), C_{0.15,47} (△, ▲) and C_{0.47,0.50} (□, ■), respectively. The measurements were performed immediately after the PEC was formed (solid line, open symbols) and after one month storage at 4 °C (dashed line, filled symbols).

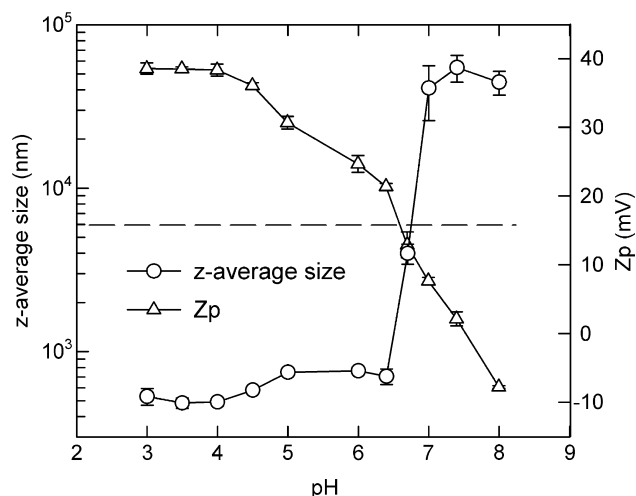


Fig. 8. The z-average diameter s_z (○) and zeta potential Z_p (△) for polyelectrolyte complexes (Alg_{0.69,41} C_{0.47,50})_{0.2} vs. pH. The pH was adjusted in the PEC solution.

Table 4

Comparison of 0 M NaCl and 0.15 M NaCl in PEC solution

	0 M NaCl		0.15 M NaCl in PEC solution	
	Size (nm)	Zp (mV)	Size (nm)	Zp (mV)
(Alg _{0.69,41} C _{0.15,47}) _{0.2}	1458 ± 120	58 ± 1	501 ± 7	26 ± 1.1
(Alg _{0.69,41} C _{0.47,50}) _{0.2}	567 ± 22	43 ± 0.3	1538 ± 161	16 ± 1.4
(Alg _{0.69,41} C _{0.61,115}) _{0.2}	1755 ± 194	39 ± 1	1090 ± 18	12 ± 0.8
(Alg _{0.69,41} C _{0.15,47}) _{1.8}	1781 ± 56	−49 ± 0.8	>6 μm (18390 ± 1168)	−24 ± 2.4

Table 4 shows that the absolute value of the Z_p of the PECs is reduced from 40–50 mV to 15–25 mV after salt is added to a final concentration of 0.15 M NaCl. No systematic variation in particle size was observed when salt (0.15 M NaCl) was added to the PEC mixture.

4. Discussion

4.1. The influence of homogenizer parameters

The homogenization parameters used when blending the poly-anions and polycations together appeared to be an important factor in controlling the particle size of the resulting polyelectrolyte complexes. The data (Fig. 1) show that the resulting sizes of the polyelectrolyte complexes prepared from alginate and chitosan are influenced by both the size- and speed parameters of the homogenizer. Homogenization at a high speed and use of a dispersing element with a large diameter produces the smallest particles. These data are in agreement with the size of the particles that are described by the Ultra-turrax supplier when different dispersing elements are used; a large dispersing element diameter should produce smaller particles. These observations are also in accordance with effects on the size of polycaprolactone-coated and chitosan-coated epichlorohydrin-crosslinked alginate microspheres (Lillard, Palaniappan, & Koritala, 2004). High shear mixers create an intense, concentrated energy input that can disperse particles significantly quicker than traditional mixing methods. The size of the PECs indicates that several molecules are involved in each complex, and therefore, polymer degradation is not expected to contribute to the size of the PECs at the highest shear rates. Based on these initial observations, a constant set of preparation conditions were selected for further studies. Importantly, we found that experiments carried out at stoichiometric charge ratio ($K \sim 1$) led to the production of PECs that were out of the range of the particle size characterization instruments, and the data under these conditions was not reproducible from experiment to experiment.

4.2. DLS measurements and intensity size distribution s_z

The polyelectrolytes used in this study are polydisperse, therefore, the given size always represents an average value of the par-

ticles in the solution. The aim of the current study was to select a few representative parameters that conveyed the essential features of the particles prepared under various conditions. The size distributions of the complexes were mostly monomodal. In cases with more than one peak, the largest peak (i.e. most of the particles) generally occurred at the largest particle diameter. Selection of the average particle size ignores the possible presence of smaller PECs within the distribution. The PEC particle size is an important parameter when determining locations for drug and gene delivery and entrapment (Douglas & Tabrizian, 2005). Therefore, accurate measurements of the particle size and the homogeneous PEC fractions are valuable for biological, medical and industrial applications. The AFM images (Fig. 6) and the standard deviations of s_n (Fig. 1) and s_z (Figs. 2a–5) indicate that there are variations in the sizes and shapes of PECs within one sample. The AFM images indicate that a variety of particle shapes, which range from spherical and round segments via linear chains to larger aggregates, may occur. The AFM images in a previous study that visualized PECs of alginate and chitosan synthesized at lower polyelectrolyte concentrations indicated that the PECs were mostly present as globular species, and there was possibly also a poorly-resolved internal structure (Danielsen, Maurstad, & Stokke, 2005). Despite the shortcomings associated with only selecting the mean value and the associated standard deviation for discussion of the major trends in the data, this provide an indication from the DLS measurements and the associated physical appearance within the distributions as obtained from the AFM topographs. The concerns of possible effects of preparation procedures, size of analysed ensemble, and artefacts (Stokke & Brant, 1990; Stokke, Elgsaeter, Hara, Kitamura, & Takeo, 1993) should also be included when judging the correspondence between the data obtained by AFM and other physical measurements, as DLS applied for the present samples.

4.3. A core-shell polyelectrolyte complex model

We suggest that a core-shell model can account for some main features of the alginate–chitosan complexes obtained using different blending conditions with respect to the molar ratio in the final blend and the polymer properties (see Fig. 9). This model is analogous to the model proposed for polyelectrolyte complex formation for chitosan–dextran sulphate (Schatz, Domard, Viton, Pichot, & Delair, 2004). In the case of chitosan–dextran sulphate, PEC formation resulted in particle flocculation at balanced charge ratios of the components, whereas in an excess of cations (chitosan) or anions (dextran sulphate), dense and small non-aggregating particles were formed (Schatz et al., 2004). When the component in excess acts as a host and can accommodate several molecules of the com-

ponent that is titrated into the mixture, the segregation of hydrophobic segments lead to the production of particles which are electrostatically stabilized by a large hydrophilic corona of the excess component. However, when the component in excess is smaller than the component that is titrated in, the outcome of the process depends on the capacity of the excess polymer to neutralize charges. The “scrambled” egg model (Michaels & Miekka, 1961; Philipp, Dautzenberg, Linow, Kötz, & Dawydoff, 1989) may describe some essential morphological features of polyelectrolyte complexes made from flexible polymers. Within the “scrambled” egg model a possible interaction model where alignment of charges yielding aligned (–/+) charge neutralization in a lattice-like type of interaction, is not realized due to various type of mismatch arising from e.g. structural (charge spacing and distributions) or differences in polymer flexibility. Basically, all of the charges on the molecules are not accessible for electrostatic interactions for steric reasons within the “scrambled” egg model. Such features may also occur locally within a core-shell model, where the focus is more on the net local charge balance in various regions of the complexes. The recently reported persistence length of 12 nm alginates in an unperturbed state (Vold, Kristiansen, & Christensen, 2006), and in the order of 5 nm for chitosans (Rinaudo, Milas, & Le Dung, 1993; Schatz et al., 2004), along with a report that alginate–chitosan PECs do not adapt regular rod-like or toroidal morphologies (Maurstad et al., 2003) all support the notion that “scrambled” egg regions also may exist in the complexes described in this study. This is also in line with the reported fraction of carboxylic acid of alginate being involved in complexation with chitosan being much less than one (Becherán-Marón, Peniche, & Argüelles-Monal, 2004).

In line with the basic features of the core-shell model, we observed that the zeta potentials of the alginate–chitosan PECs reflect the excess component, and therefore the value of the Z_p changes sign at $K = 1$. In an excess of either chitosan or alginate, the Z_p has a large positive or negative value and small, non-aggregating particles are formed. The smallest particles are obtained in an excess of chitosan at $K = 0.3$ (AiC) and in an excess of alginate at $K = 1.2$ (CiA). This non-aggregating behaviour is suggested to arise due to an excess of the major component, which forms a stabilizing shell around the particles.

At the crossover from a negative to positive Z_p (which occurs at $K \sim 1$), the data indicate that there is an increase in the mean complex size ($s_z > 6 \mu\text{m}$; Figs. 2a,b, 3a,b, 7a and 8), and there are also relatively large differences in size between parallel preparations. These observations probably reflect the formation of flocks and aggregates, as indicated in the AFM images (Fig. 6b). These findings are in line with similar findings that were observed near a net charge neutrality for DNA and cationic polymers (Tang & Szoka, 1997), chitosan and dextran sulfate (Drogoz, David, Rochas, Domard, & Delair, 2007) or the aggregation of poly(diallyldimethylammonium)chloride and sodium poly(ethane sulfonate) at $K = 1.1$ (Kekkonen, Lattu, & Stenius, 2001), and other similar results (Dautzenberg, 2000; Schatz et al., 2004). The aggregation behaviour observed around $K \sim 1$ can be rationalized within the core-shell model, since local patches that have a net charge that is different from zero can participate in electrostatic-mediated interactions with oppositely charged patches on other clusters. At an increasing distance from $K \sim 1$, the probability of having local patches of opposite net charge on different clusters is expected to diminish. The capability to build larger aggregates/agglomerates therefore mainly occurs close to $K \sim 1$. Although, the core-shell model description of alginate–chitosan, as suggested here, accommodates some main global trends of the PECs, it is expected that it needs to be elaborated when even more detailed characterization of the PECs will be available, e.g., similar to that already reported by Drogoz et al. (2007).

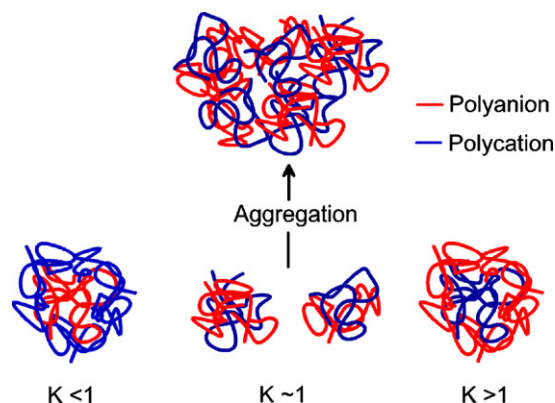


Fig. 9. Schematic illustration of a core-shell model for polyelectrolyte complexes of alginate (polyanion) and chitosan (polycation) at net charge ratios less than one, equal to one and larger than one.

In this study, altering the chitosan F_A did not significantly influence the properties of the resultant complexes. When the chitosan F_A is large, the mass ratio of chitosan/alginate is larger than when chitosans with a low F_A for a given K are used. However, our results still indicate that the particle sizes are not affected by F_A . In this context, chitosans with an F_A value between 0.02 and 0.15 when fully protonated will have the same effective charge density due to counter-ion condensation. The chitosan with an $F_A = 0.47$ has a lower maximum charge density. The fact that the charge density of the chitosan does not appear to affect the properties of the polyelectrolyte complexes may indicate that not all of the charges are accessible to interact with the polyanions, which may be caused by conformational features.

Similarly, the data presented in Fig. 5 indicates that there is no systematic influence of the G-content of the alginate on the properties of the polyelectrolyte complexes. This result was expected since the linear charge densities of alginates are almost independent of the G-content of the alginates. Previous reports have suggested that there are some differences in the molecular chain flexibility between the alginates (Moe et al., 1995), but more recent reports do not support an increase in the chain stiffness of alginates that is associated with increasing G-content (Vold et al., 2006). Therefore, a correlation between alginate G-content and the properties of the polyelectrolyte complexes mediated by variation in chain stiffness is also not expected to exist.

The data (Figs. 4 and 5) show that PEC made from chitosans and alginates with low M_W form smaller complexes when compared to chitosans and alginates with high molecular weights. The Zp and pH curves follow almost equal trends, and are independent of variations in the polymer M_W . It has been reported that the molecular weight of the polymers influences PEC formation. The smallest particles were found using a combination of low M_W alginate and low M_W chitosan and a two-stage process (Douglas & Tabrizian, 2005). A possible scenario that could account for the observed effects of molecular weight is that an increased M_W supports interactions with accessible chain segments that are not engaged in other interactions (analogously to tail and loop segments) to a larger extent, and therefore facilitate the formation of PECs with larger sizes. This is also in agreement with the observation that an increase in the M_W of the polymer in excess resulted in the largest increase in PEC size.

4.4. Stability

The AiC PEC stability was tested with respect to changes in pH, ionic strength and exposure to elevated temperatures. The most significant changes were observed between pH 6.4 and 7.0, where the PEC size increased 50 times and the Zp changed from +30 mV at pH 6 to ± 2.1 mV at pH 7.4 (Fig. 8). At pH > pK_a , the amine groups of the chitosan are less protonated and the remaining protonated groups available for interaction would result in overall weaker electrostatic interactions (Fig. 8), leading to the observed changes in the particle size and zeta potential. A similar trend has been reported for alginate–chitosan PEC prepared by gelling (Douglas & Tabrizian, 2005). Another study showed that there were strong plasmid–polymer [poly{(2-dimethylamino)ethyl methacrylate}–plasmid] interactions, and this resulted in the formation of smaller complexes at pH 5. Increasing the pH close to the pK_a value (~ 7.5) of the polymer resulted in increased particle size and formation of aggregates at pH 8 (Cherng, Talsma, Verrijck, Crommelin, & Hennink, 1999). This is in agreement with our observations that increasing the pH close to the pK_a value of chitosan of 6.5 (Strand et al., 2001) resulted in an increased particle size. It has been suggested that strong polymer interactions occur when most side groups of the polymer are protonated, resulting in a complex of high charge density where the formation of small particles are favoured

(Cherng et al., 1999). Moreover, a lower degree of protonation also resulted in particles with lower absolute value of Zp, which appears to cause aggregate formation.

The effect of adding salt into the PEC solution is shown in Table 4. As expected, the increase in ionic strength due to 0.15 M NaCl resulted in a decrease of Zp (15–25 mV), clearly demonstrating the screening effect of counter-ions. Furthermore, reducing the charge of the surfaces should increase aggregate formation. Variations in the resulting PEC sizes were found, and this reflects an unstable system. When the resulting Zp values (15–25 mV) are compared with the results presented in Fig. 8, it is clear that the PECs are in a state where aggregate formation begins. Incubation at 37 °C for 8 h (data not shown) did not affect the particle size, transmittance, Zp or pH, and one month storage at 4 °C also did not affect particle size or Zp (Fig. 7). Storage and temperature effects on PEC particles have been studied by several groups, and different results have been obtained from different studies (Danielsen et al., 2005; Maurstad & Stokke, 2004; Romøren, Aaberge, Smistad, Thu, & Evensen, 2004; Vijayanathan, Lyall, Thomas, Shirahata, & Thomas, 2005). Chitosan-based polyplexes were stored for one year at 4 °C, 25 °C and 45 °C (Romøren et al., 2004). No changes were observed in the polyplexes stored at 4 °C, and minor changes were observed in the polyplex kept at 25 °C. The polyplex stored at 45 °C changed within a short period of time. Another study reported that incubation at 50 °C for 5.5 h led to annealing and the formation of a denser chitosan–DNA complex (Danielsen et al., 2005), whereas xanthan–chitosan PECs have been shown to increase in size when heated (Maurstad & Stokke, 2004). Stable DNA–polyamine particles were observed at temperatures <50 °C, while particle size significantly increased for temperatures >60 °C (Vijayanathan et al., 2005). From these reports, it is likely that storage beyond the period reported in these studies may affect the properties of PECs.

5. Conclusions

This study demonstrated that the rotational speed and diameter of the dispersion element of the homogenizer, and molecular properties of the polyelectrolytes should be carefully chosen in order to optimize the particle size and the particle surface charge (Zp) of alginate–chitosan complexes. The charge ratio and the molecular weight were shown to be parameters that affected particle size, Zp and pH in the mixture. At charge ratios close to one (neutral), the sizes of the largest particles were less reproducible. The smallest particles were obtained by adding one of the two polymer solutions into an excess amount of the other solution and by using forms of the component polymers with low molecular weights. Increasing the pH of the PEC mixture above 7 led to a significant increase in particle size, while the PECs were very stable over a temperature range of 4–37 °C. Increasing the ionic strength in the PEC mixture (0.15 M NaCl) led to PECs with less reproducible structures, and the PECs had a reduced zeta potential.

Acknowledgements

This work was supported by The Norwegian Research Council, Grant No. 166794/V30 (GM) and FMC BioPolymer AS/NovaMatrix.

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