Scientific paper

Self-assembled Polyelectrolyte Nanocomplexes of Alginate, Chitosan and Ovalbumin

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Abstract

Polyelectrolyte complex (PEC) nanoparticles for delivering model protein drug ovalbumin were prepared from two polysaccharide polymers, alginate and chitosan. The parameters influencing the complex formation were characterised using colloid titration in combination with dynamic light scattering. The polyelectrolyte interactions and morphology of the formed complexes were verified by differential scanning calorimetry and scanning electron microscopy, respectively. The PEC formation was predominantly pH- and concentration-dependent. The complexation of ovalbumin with a negatively charged alginate occurred only at a pH below the isoelectric point of the ovalbumin. After the complexation, negatively charged complexes of alginate and ovalbumin were further coated with chitosan. The optimal composition of the PEC, yielding 280 nm sized particles having a zeta potential –40 mV, was determined for alginate:ovalbumin:chitosan in a mass ratio 1 : 1 : 0.1, respectively, giving their final concentration 0.5 : 0.5 : 0.05 mg/ml. The loading of ovalbumin in the PEC depended on the initial amount of ovalbumin used to produce the PEC, and ranged from 7–38% for different formulations, however, the association efficiency remained pretty similar for all formulations, i.e. 80–85%. Mild formulation conditions, nanometre-sized particles, and a high protein association efficiency are promising factors towards the development of a delivery system for proteins.

Keywords: Nanoparticles, polyelectrolyte complexation, colloid titration, protein delivery, ovalbumin

1. Introduction

Nanoparticles (NPs) have become a focus of attention in the field of biomedicine owing to their capacity to deliver various drugs. The interest of NPs as drug carriers relies primary on the integration of a drug and a carrier into a new entity, a nanomedicine, which often possesses improved biopharmaceutical properties compared with the drug in its conventional formulation.[1] A category of drugs that has been particularly studied for their association with the nanocarrier is the one represented by macromolecules, such as peptides and proteins, which could enable an alternative route for their delivery. The widespread use of proteins is however limited because of their specific nature dictating only an invasive delivery.[2]

To date, various methods are available to produce NPs including polymerisation-based methods and those employing preformed polymers such as the emulsion diffusion method, solvent evaporation, nanoprecipitation etc.[3] Most of these methods involve the use of organic solvents, toxic surfactants, high energy processing procedures (vigorous stirring, sonication, heat, etc.), which are potentially harmful to sensitive biomolecules. In recent years, low energy production methods and the use of water-soluble polymers that are able to self-assemble into complexes under gentle stirring, have attracted increasing attention. The method employs natural or synthetic polyelectrolytes that spontaneously interact with the protein due to the electrostatic forces leading to coacervation complexes.[3,4]

The use of polysaccharides and especially natural biopolymers, has attracted particular interest due to their desirable biocompatible, biodegradable, hydrophilic and protective properties.[5,6] The interaction between cationic and anionic biopolymers leads to the formation of the polyelectrolyte complex, which has demonstrated favourable characteristics for drug entrapment and delivery. In this study, two polysaccharides, alginate and chitosan were used as polyionic polymers to associate with the model protein drug ovalbumin.

Alginate is found in numerous pharmaceutical and biomedical applications such as drug delivery and cell encapsulation. It consists of an anionic chain of (1–4)-linked

β-D-mannuronic acid (M) and α-L-guluronic acid (G) in different arrangements and proportions. In the presence of calcium ions (Ca), interaction between the bivalent ions and guluronic acid residues causes the alginate to form a gel. The gelling properties depend on the composition and extent of the G and M sequence, molecular weight of the polymer and concentration of the counter ions during gelation. The solubility characteristics of the alginate network are also affected by the pH of the medium. The pKa values of mannuronic acid and guluronic acids are 3.4 and 3.6, respectively.[5,6]

Chitosan is a natural cationic polysaccharide obtained by the N-deacetylation of chitin, a product found in the shell of crustaceans. It consists of repeating units of glucosamine and N-acetyl-glucosamine. Due to its specific properties, chitosan has found a number of applications including drug delivery and as an absorption enhancer.[7] With the pKa of approximately 6.5 of the amine groups, chitosan is insoluble at a neutral pH but is soluble and positively charged in an acidic pH. [5,6]

The purpose of this study was to develop polyelectrolyte complex NPs to deliver a model protein drug ovalbumin using the method of self-assembly of opposite charged polysaccharides, alginate and chitosan. The influence of various experimental parameters on the complex formation and their stability were evaluated, including the ratio and concentration of polymers, the solution's pH and ionic strength, the addition of calcium ions to the alginate, and the stirring time. Polyelectrolyte complexes were characterized in terms of particle size, polydispersity index, zeta potential, efficacy of their association (concentration of nanocomplexes formed), and ovalbumin loading. Interactions between polyelectrolytes and protein were verified by differential scanning calorimetry (DSC), and the morphology of the complexes was studied using scanning electron microscopy (SEM).

2. Experimental

2. 1. Materials

Albumin from a chicken egg white (ovalbumin, grade VI) and low molecular weight chitosan (50–190 kDa, degree of deacetylation 75–85% , and viscosity of 1% w/v acetic solution 20–200 mPas) were obtained from Sigma – Aldrich, USA. Sodium alginate with 35–45% guluronic acid content (Protanal® LF 10/60LS), viscosity 20–70 mPas (1% solution) and molecular weigh of 253,000 g/mol[8] was purchased from FMC BioPolymer, Norway. All other chemicals used in this study were of analytical grade.

2. 2. Selection of Parameters for Optimal Polyelectrolyte Complex (PEC) Formation

To optimize the composition and complex formation of alginate, ovalbumin and chitosan, continuous titration of one polymer with another was performed monitoring the particle size, zeta potential and average count rate throughout the experiments. A Zetasizer Nano ZS ZEN 3600 and an MPT-2 autotitrator unit, both from Malvern Instruments, UK, were used for these studies.

2. 2. 1. Influence of the Polymer Concentration

The alginate and chitosan were dissolved in 0.25% acetic acid, pH 4.0, and the buffer solution of ovalbumin was diluted with 0.25% acetic acid in a volume ratio 1:2. The starting concentrations of the alginate, ovalbumin and chitosan were 2.0, 2.0 and 0.1 mg/ml, respectively. All solutions were adjusted to pH 4.0, and filtered through a 5μm filter (cellulose acetate syringe filters, ALBET® LabScience, Hahnemühle, Germany). Briefly, the alginate solution was titrated with ovalbumin solution to reach the minimum size of the complexes formed. This dispersion of nanocomplexes was further titrated with a chitosan solution. The composition yielding particles with the lowest size and sufficiently high zeta potential and average count rate was selected for further experiments.

2. 2. 2. Influence of Calcium Chloride on the Alginate Pregelation

To check whether pregelation of the alginate molecules with calcium ions is necessary for their transformation into NPs, the alginate (1 mg/ml in 0.25% acetic acid) was titrated with an aqueous solution of calcium chloride (1mg/ml) and the size and zeta potential of the alginate was monitored throughout the experiment.

2. 2. 3. Influence of pH on the Nanocomplex Formation

The influence of a higher pH on the complexation of alginate with ovalbumin was investigated in the same way as described in the section *"Influence of the polymer concentration"* except that both solutions were adjusted to pH 5.0.

2. 2. 4. Influence of Additives (Electrolytes and Non-electrolytes) on the Nanocomplex Formation

The influence of the electrolyte and non-electrolyte on the complex formation between the alginate and ovalbumin was studied in the same way as presented in the section *"Influence of the polymer concentration"* except that the buffer salts – PBS (NaCl 0.14 M, KCl 2.7 mM, Na_2HPO_4 0.01M and KH_2PO_4 1.7 mM) or mannitol (2% w/v) were dissolved in the starting alginate and ovalbumin solutions before the titration experiments.

2. 3. Polyelectrolyte Complex Preparation

After the optimisation of the polyelectrolyte complex formation, the optimal formulation was found as follows: 2 ml of ovalbumin in a buffer solution (5 mg/ml, pH 7.0) was added drop by drop into the beaker containing a 16 ml of alginate solution (0.625 mg/ml in 0.25% acetic acid, pH 3.9) and stirred for 30 min on the magnetic stirrer. Then, 2 ml of the chitosan solution (0.5 mg/ml in 0.25% acetic acid, pH 3.9) was added in drops to the dispersion and stirred for 45 min, giving a final alginate, ovalbumin and chitosan concentration of 0.5, 0.5 and 0.05 mg/ml, respectively. The colloidal dispersion of the PEC (pH 4.0) showed a characteristic Tyndall effect, and the mean particle size and zeta potential were 284 nm and –40 mV, respectively.

2. 4. Characterisation of the Polyelectrolyte Complexes

The size and zeta potential of the complexes were measured with a Zetasizer Nano ZS ZEN 3600 (4mW He–Ne laser, 633nm) from Malvern instruments, UK. Scattering light was detected at 173° by the automatically adjusted laser attenuation filters and the measurement position within the cell at 25 °C. For data analysis, a viscosity (0.8863 mPa s) and a refractive index (1.330 at 633 nm) of distilled water at 25 °C were used.

The average count rate, which is the measure of a nanocomplex concentration in the sample, was also monitored during the experiments. This parameter represents the scattering intensity of the sample in the absence of laser attenuation filters (adjusters of the laser power), therefore it can be used for comparison in the scattering intensity (kcps) between samples and indicates the particle concentration in the sample. The average count rate is a calculated value obtained from the measured count rate divided by the attenuation factor, as shown in the expression below.

$$\text{Average count rate} = \frac{\text{(measured count rate)}}{\text{(attenuation factor)}}$$

Using the Zetasizer Nano software, the quality reports, which are displayed as a warning message ("result meets/does not meet quality criteria"), were also checked after each measurement. This report reflects the suitability of the sample for DLS measurement, and the reliability of the data obtained, since it is based on a collection of measurement parameters that need to be examined to obtain an adequate degree of confidence regarding the measurement result.

The instrument was routinely checked and calibrated using the standard reference latex dispersion (Malvern Zeta potential transfer standards, Malvern, UK) and polystyrene particles (Nanosphere size standards, Duke Scientific Corporation, USA). The particle size and zeta potential of the polyelectrolyte complexes were given as a mean ± SD (n = 3–5).

2. 5. Morphology of the Polyelectrolyte Complexes

Scanning electron microscopy (SEM) was used for the morphological evaluation of the polyelectrolyte complexes using a JSM-7001F Jeol (Japan) instrument with an acceleration voltage of 1.5 kV and a secondary electron detector. Samples of the complexes were deposited on a double-sided carbon tape (diameter 12 mm, Oxon, Oxford instruments, UK) and then analysed.

2. 6. Differential Scanning Calorimetry (DSC) Analysis

DSC was used to analyse the interactions between the polymers and protein. The DSC scans were obtained using a DSC I Star System (Mettler Tolledo, Switzerland). Powdered samples (2–4 mg) were sealed in a standard aluminium pan and heated from 20 to 350 °C at a heating rate of 10 °C/min in normal atmospheric conditions. DSC scans of untreated ingredients (alginate, chitosan, ovalbumin) and their physical mixtures as well as of the treated ingredients and their mixtures were taken. Untreated ingredients were powders as received by the manufacturers, and were scanned individually and in physical mixtures (alginate:chitosan (1:0.1), and alginate:ovalbumin:chitosan (1:1:0.1)). Treated alginate, chitosan, and ovalbumin were obtained through separate dissolution in diluted acetic acid (the same as the final medium of the PEC dispersion) and further lyophilisation. A bi- and ternary mixture of all treated ingredients (alginate:chitosan (1:0.1), and alginate:ovalbumin:chitosan (1:1:0.1)) were also prepared and analysed. The alginate:chitosan complex (1:0.1) was prepared following the same procedure as in the PEC production without ovalbumin, and then lyophilized and analysed. All scans were compared to that of the freeze-dried PEC.

2. 7. Influence of Mixing Time on the Stability of the Complexes in the Presence of the Electrolyte and Non-electrolyte

The stability of the nanocomplexes was evaluated in the presence of NaCl (0.9%) and mannitol (2%), by adding both additives to the dispersion immediately after their preparation and on the next day after stirring for over 24 hours. The samples were characterised by dynamic light scattering (DLS).

2. 8. Ovalbumin Association Efficiency and Loading Capacity

The association efficiency (AE) of the ovalbumin in the PEC was determined indirectly after separating NPs

from the aqueous medium containing non-associated ovalbumin. The amount of ovalbumin associated with the nanocomplexes was calculated by the difference between the total amount of ovalbumin used to prepare the complexes and the amount of ovalbumin present in the aqueous phase after centrifugation.

$$AE = \frac{\text{Total amount of ovalbumin} - \text{Free ovalbumin in the supernatant}}{\text{Total amount of ovalbumin}} \times 100$$

The amount of ovalbumin associated with the nanocomplexes is referred to as the loading capacity (LC). It represents the difference between the total ovalbumin amount initially used to prepare the complex and the amount of non-associated ovalbumin after the complexes separation as a percentage of the total NP mass.

$$LC = \frac{\text{Total amount of ovalbumin} - \text{Free ovalbumin in the supernatant}}{\text{Total weight of nanocomplexes}} \times 100$$

2. 9. Ovalbumin Determination

Ovalbumin was determined by HPLC running with the Agilent 1100 system (Hewlett Packard, Germany) on the column for gel filtration with the surface-stabilised, hydrophilic stationary phase (4–4.5 μm particle size, 250 × 9.4 mm; Zorbax GF-250, Aligent technology, USA). To protect the column from possible aggregates in the sample, a guard column (4.6 × 12.5 mm) was inserted between the injector and the column. A mobile phase composed of a phosphate buffer solution (0.13 M NaCl, 20 mM Na_2HPO_4, pH 7.0) operated at a flow rate of 1.0 ml/min and 23 °C for 30 min. Protein identification was by UV detection at 210 nm. The method was validated and showed linearity in concentration range of 0.006–1.20 mg/ml ($R^2 = 0.9993$).

3. Results and Discussion

The self-assembly of polyelectrolytes is an attractive low-energy method for encapsulating sensitive biomolecules in NPs. The method has the advantages of not using organic solvents and harsh processing conditions, which are usually used in the formation of NPs using other methods, therefore; the possible damage of the biomolecule is minimized. Moreover, in the light of an industrial scale up, this method is superior to high-energy processes since it does not face technical and economical hurdles. But the specificity of this method is the spontaneous association of polymers in NPs, which is governed mostly by the intrinsic physicochemical behaviour of the polymers. This is not the case for conventional high-energy methods, where the formation of NPs is directly controllable by formulation parameters (quantity of energy, amount of surfactants etc.).[9] Several factors must be investigated that may influence the interaction between the charged polymers including the ionic strength of the polymer, its conformation, stirring intensity, pH of the medium and ionic strength, temperature, hydrophobicity of the complex formed and others.[3,4,10]

There are three main types of aqueous polyelectrolyte systems, which may be produced: soluble PEC, turbid colloidal PEC and a two-phase system consisting of precipitated PEC and supernatant liquid.[4] To deliver pharmaceutical proteins a turbid colloidal system should be developed to enable the incorporation of the protein into the nanoparticulate carrier. A soluble PEC system does not seem to be suitable since it comprises small PEC aggregates where the interactions are weak, and the complex formation would most likely form a system with poor protein association. A two-phase system is also not suitable as a delivery system since the precipitation of the PEC yields large sedimenting aggregates, not representing a desirable stable nanosized system.

In our study we were aiming to produce turbid polyelectrolyte complexes consisting of an alginate core entrapping the protein ovalbumin, and the outer coating of the chitosan for its contribution to better mucosal delivery.[7] Such polyelectrolyte complexes may be formed using different methods. In this study, we used the procedure where the first polymer solution is slowly added in a drop by drop manner into the second solution. Alternatively, an additional step may be included in this process, a pregelation of alginate with calcium ions, which yields beads to further coat with chitosan. The PEC are formed in a dilute solutions of polymers (0.1–1 mg/ml) in a narrow concentration range of each ingredient.[4,6] Beside this, other factors also influence the complex formation and characteristics of the NPs, and they need to be studied systematically.

3. 1. Influence of Formulation Parameters on Polyelectrolyte Complexation

Typically, the charge of polyelectrolytes is the most important for their self-assembly in NPs. Protein and polymers must be oppositely charged to form turbid colloidal complexes, which are strongly dependant on the pH of the medium. For a polyanion such as alginate (having a pKa of guluronic and mannuronic acid 3.6 and 3.4, respectively) the protein must be positively charged to electrostatically interact with the alginate, meaning that the formation will require a pH lower than the isoelectric point (pI) of the protein. The ovalbumin pI was determined to be 4.8 (results not shown), therefore the complexation was performed at pH 4.0.

To determine the interaction between the alginate and ovalbumin during the polyelectrolyte complexation, titration experiments were performed (Figure 1). During the stepwise addition of the ovalbumin solution (2 mg/ml) to the alginate (2 mg/ml) a decrease in particle size was observed. The assembly mechanism probably involves strong electrostatic

forces of attraction between the polyanion alginate and the positively charged ovalbumin since a significant decrease in particle size was already observed after the first addition of ovalbumin. A decrease in particle size of alginate can be explained by the shrinkage of the alginate molecules into an assembly with ovalbumin yielding a smaller particle size. Also the quality report message displayed good measurement data after the first and all further additions of ovalbumin, including the low polydispersity index, from 0.252 to 0.087. Before the addition of ovalbumin, the size of the alginate with a low scattering intensity (1355 kcps) did not reflect a reliable size measurement due to the high polydispersity in the sample, which is inappropriate for cumulant size analysis. To point out, the DLS measures the hydrodynamic diameter of the moving particles and represents the diameter of a spherical particle. Factors that affect the diffusion speed will therefore affect its hydrodynamic size. The diffusion speed depends not only on the size of the particle "core" but also on its surface structure. A freely flowing polymer chain, without a globular structure, would therefore probably variably affect the hydrodynamic size. Figure 1 also shows a significant increase in the average count rate, giving evidence that the addition of ovalbumin to the alginate increased the formation of the turbid polyelectrolyte complexes. After several additions of ovalbumin the average count rate curve reached a plateau, which denoted the end of the titration, revealing that the capacity of alginate to complex ovalbumin is fulfilled.

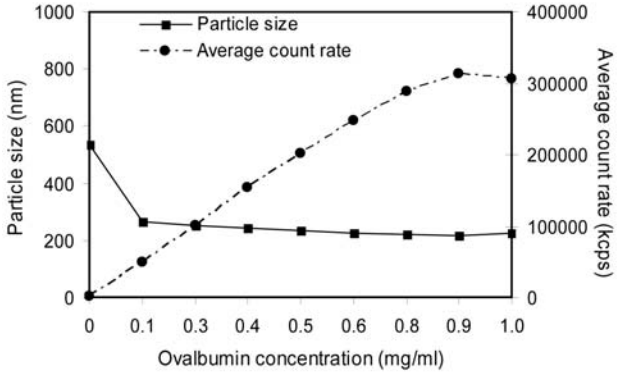

Figure 1. Particle size and average count rate of the complexes prepared by the step-wise addition of ovalbumin (2 mg/ml) to alginate solution (2 mg/ml).

The dispersion of the polyelectrolyte complexes of alginate and ovalbumin, both having a final concentration of 1 mg/ml, was further titrated with a chitosan solution. The starting concentration of chitosan had to be reduced significantly, since high concentrations yielded large aggregates (results not shown). Figure 2 shows a slight increase in the particle size after the addition of chitosan to the preformed alginate/ovalbumin complexes up to a concentration of 0.06 mg/ml. This can be attributed to the chitosan coating the complexes. However, after a further addition of chitosan, an extraordinary increase in particle size was observed, which is probably because of particle aggregation. Such aggregates are inappropriate as a delivery system because of possible particle flocculation and sedimentation leading to the two-phase systems, and loss in the unique nanosized system properties. The aggregation of particles can also be seen from the count rate curve (Figure 2). At the beginning, a slight decrease of complex concentration was noticed which was due to the dilution of the dispersion (addition of diluted chitosan solution). However, after the last addition of chitosan, a sharp decrease in kcps was observed, which can be attributed to the particles' aggregation and flocculation (Figure 2 and 3). Also, the poly-

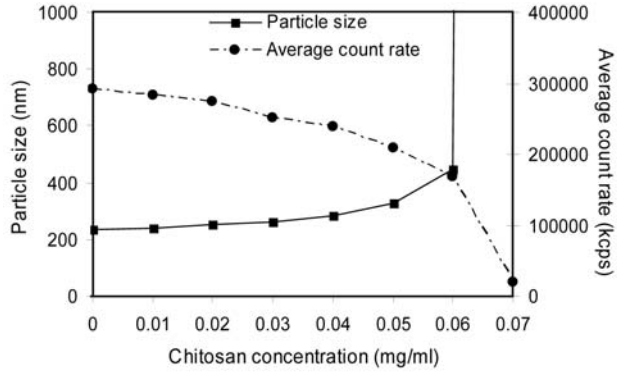

Figure 2. Particle size and average count rate of complexes after a step-wise addition of chitosan solution (0.1 mg/ml) to the alginate/ovalbumin complexes.

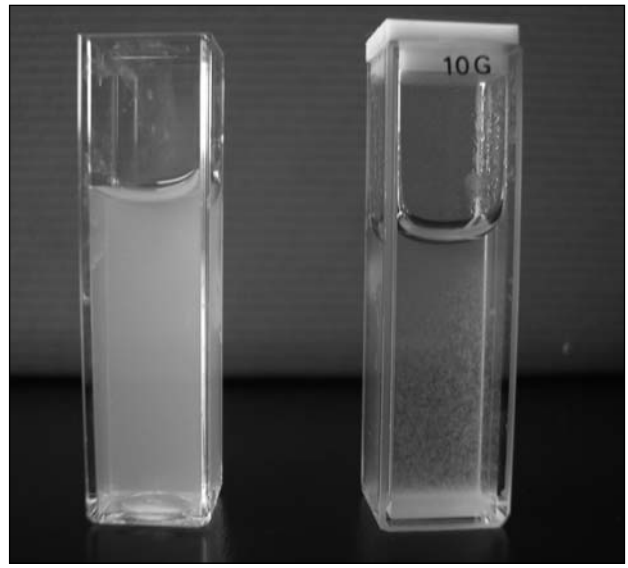

Figure 3. A photograph image of the polyelectrolyte complexes before (left) and after (right) the last addition of chitosan. The excess of chitosan changes the turbid dispersion of the PEC into a two-phase system consisting of a clear supernatant and large flocculates, slowly sedimenting (right).

dispersity index increased in parallel with the size, indicating that the uniformity of the particle size is becoming more heterogeneous with the excess addition of chitosan. We assume that aggregate formation may occur due to the interaction between the particles during chitosan coating, or due to the loss of negative zeta potential above –35 mV (Figure 4).

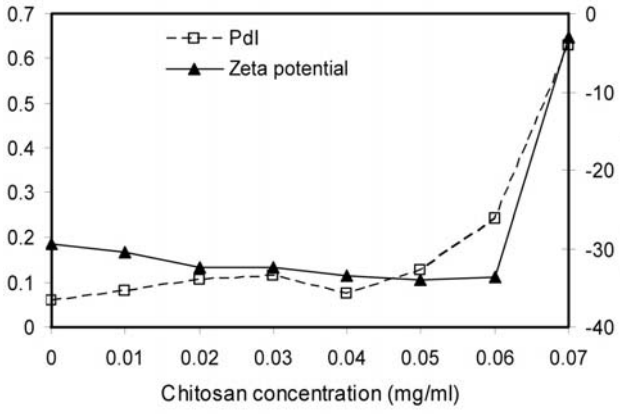

Figure 4. The polydispersity index and zeta potential of the complexes after a step-wise addition of chitosan solution (0.1 mg/ml) to the alginate/ovalbumin complexes.

Based on the particle size, particle concentration (count rate), polydispersity and sample quality reports, the most favourable mass ratio for the polyelectrolyte nanocomplexes of alginate:ovalbumin:chitosan was 1:1:0.1, having a final concentration 0.5, 0.5, and 0.05 mg/ml, respectively. De and Robinson also studied the interaction between alginate and chitosan.[11] They observed that alginate and chitosan show the best interaction with the mass ratio of 0.1, however, they used calcium ions instead of ovalbumin to pre-gel the alginate prior to its complexation with the chitosan. They postulated that the pre-gel state of the alginate molecules is essential for the formation of NPs, providing an alginate beads as a starting nucleus for nanoparticle growth, as previously proposed by Rajonarivony.[12] On the contrary, Dauglas and Tabrizian opposed this theory, showing that the complete omission of calcium ions does not prevent the formation of polyelectrolyte nanocomplexes.[13] To elucidate the influence of the calcium ion on the gelation of alginate we performed additional experiments. Alginate was titrated with calcium chloride and the size and zeta potential was measured throughout the experiment.

Table 1 shows the size, polydispersity index and zeta potential of the alginate molecules in the pre-gel state prepared in different ratios with calcium ions. It can be observed that the influence of calcium ions on the formation of a uniform size of alginate nucleus is minimal. At the beginning (at a charge ratio close to 1), calcium ions slightly reduce the particle size and polydispersity index. At a higher calcium content (above the $CaCl_2$: alginate mass ratio 0.57), larger particles were observed with high polydispersity. The excess of calcium ions likely encounters a higher degree of ionic interaction, causing an alginate gelation. Other authors have also observed this effect of alginate particle size going through a minimum by the addition of calcium ions. They used the alginate with Mw between 100–291 kDa and the guluronic acid content F_G 0.4, similar to our ingredient. De and Robinson observed the smallest particle size for calcium chloride to sodium alginate ratios between 0.08–0.35.[11] Sarmento et al. triggered pre-gelation of alginate with calcium chloride in the mass ratio 0.2,[14] similar to that of Rajaonarivory.[12] They all explained the reduction in polymer size due to the shrinkage of the individual alginate chain into a more compact coiled structure, where the guluronic region in the same alginate chain is linked by calcium ions. The composition of alginate, more specific guluronic and mannuronic acids contents, was manifested as an important role in the gelation process. A low G content was selected to be the most suitable to stimulate the nucleus formation omitting the gelation of the alginate. A higher G-content alginate probably encounters a higher degree of ionic interaction in the same alginate chain and between

Table 1. Charge ratio (P/N), particle size, polydispersity index (PdI) and zeta potential of alginate molecules for various calcium chloride:sodium alginate mass ratios.

$CaCl_2$ (mg/ml)	$CaCl_2$: Na-alginate (m/m)	Charge ratio (P/N)*	Particle size (nm)	PdI	Zeta potential (mV)
0	0		573	0.63	–44.1
0.07	0.14	0.5	399	0.70	–37.0
0.14	0.28	0.9	268	0.80	–27.1
0.21	0.41	1.4	284	0.52	–25.5
0.29	0.57	2.0	251	0.48	–22.8
0.36	0.71	2.5	327	0.45	–20.3
0.43	0.86	3.0	420	0.78	–21.3
0.50	1	3.5	627	0.70	–12.2

* The charge ratio (P/N) was calculated based on the number of positive charges (calcium) over that of negative charges (alginate) for different mass ratios of calcium chloride/sodium alginate.

the chains within the forming beads, resulting in larger alginate aggregates. Additionally, gelation can be triggered by a higher concentration of calcium ions and/or alginate, resulting in intermolecular crosslinking, alginate aggregation and gelation.[5,12,14]

Table 1 also shows that the zeta potential of the alginate is markedly reduced by calcium ions. The loss in zeta potential is not a good indicator regarding the stability of colloidal systems, and can potentially lead to particles aggregation and phase separation. Moreover, we observed that pre-gelation with calcium ions influenced the capacity of the alginate to complex polycations, ovalbumin and chitosan (not shown), meaning that protein loading or chitosan coating should in this case be reduced. Since high loading and coating were our targets, the pre-gelation with calcium ions was omitted and we focused only on the polyelectrolyte complexation. Figure 5 shows the size distribution of each component obtained separately and after their complexation according to the optimized method of production. The peak of ovalbumin is found around 6 nm with a low polydispersity index (< 0.2). On the contrary, alginate and chitosan have both polymodal and polydispersal distributions, but after the complexation of alginate with ovalbumin, alginate's wide distribution narrows into one peak yielding good measurement data with an average particle size around 280 nm and a low polydispersity index (PdI < 0.3). During further coating with chitosan only a small increase in particle size was observed.

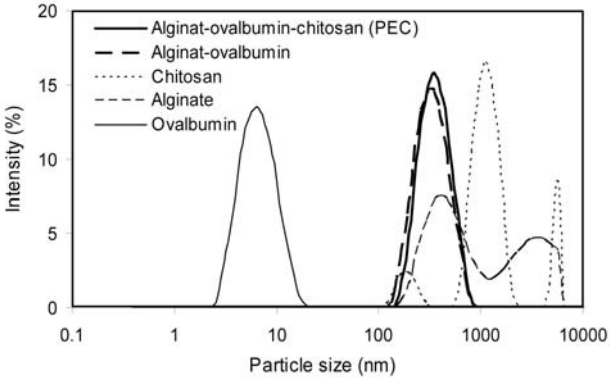

Figure 5. Size distribution of ovalbumin (6 nm), alginate and chitosan obtained separately, and after their complexation: alginate-ovalbumin (bold dashed line) and alginate-ovalbumin-chitosan (bold continuous line).

The SEM image of the PEC is presented in Figure 6. In the dry state, the PEC show an irregular and spherical to polyhedral shape. They are not as spherical as hydrophobic NPs usually are, which is most likely due to the loss of water from the hydrophilic polymers on drying before imaging. In general, the estimated size from microscopy is smaller than that obtained using DLS, which is due to the swelling capacity of the hydrophilic particles in water when measuring their hydrodynamic radius using DLS.

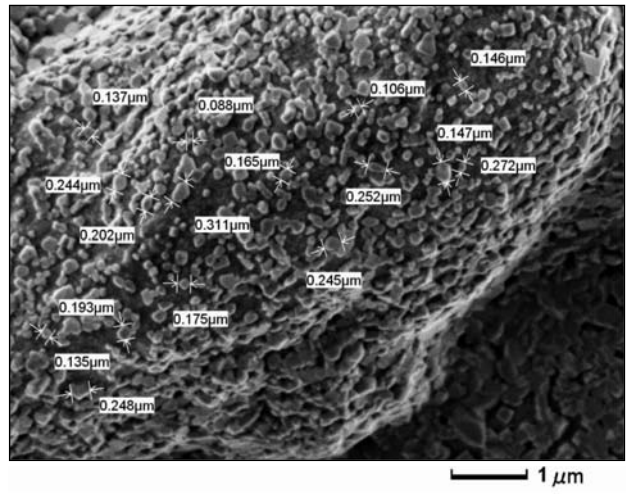

Figure 6. The SEM image of the PEC prepared from alginate, ovalbumin and chitosan in a concentration 0.5, 0.5 and 0.05 mg /ml, respectively.

The most predominant molecular forces for the polyelectrolyte complex assembly are strong electrostatic interactions.[15] Hydrogen bonding, hydrophobic interaction and van der Waals forces also complement the PEC formation. Electrostatically driven complexation between the polyelectrolytes with pH ionisable groups (such as carboxylic groups) is strongly dependent on the pH of the medium, which determines the charge of polyelectrolytes and the net charge of protein. To disclose the driving force of the interaction between the polymers and protein, we investigated the influence of the pH on the complex formation by selecting a pH that was either above or below the pI of the protein. Titration experiments were performed at pH 4.0 and pH 5.0 by the stepwise addition of ovalbumin to the alginate solution and the count rate (kcps) was measured during titration to assess the amount of nanocomplexes in the sample (Figure 7). When the solution pH was below the pI of the protein, a gradual increase in the kcps was observed, which reflects the complex formation. On the contrary, when the pH was 5.0, the titrated sample retained the same kcps value as prior to titration. Furthermore, this sample showed no turbidity as in the dispersion prepared at pH 4.0 that had a milky look (Figure 4, left). These results gave the evidence that the pH and the charge of polymers play a crucial role determining polyelectrolyte complexation. Alginate being negatively charged at both pH values cannot interact with the ovalbumin at pH 5.0, where its net charge is negative. However, at pH 4.0, ovalbumin is positively charged thus is able to electrostatically interact with the alginate.

It is also known that electrolytes and non-electrolytes affect the polymer complexation between opposite charged polymers since they may interfere in their electrostatic interactions. In order to investigate the effect of additives on the polyelectrolyte complexation between alginate and ovalbumin, either buffer salts (NaCl 0.14 M, KCl

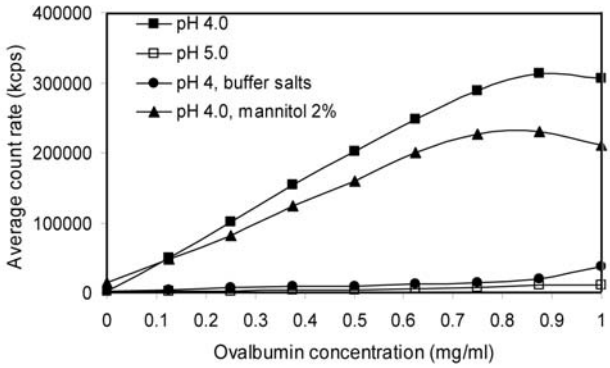

Figure 7. The average count rate as a measure of the alginate-ovalbumin complex concentration in the sample prepared at pH 4.0 and pH 5.0, and in the presence of mannitol (2% w/v) or buffer salts (154.4 mM), both at pH 4.0.

2.7 mM, Na_2HPO_4 0.01M and KH_2PO_4 1.7 mM) or mannitol (2%, w/v) were added to both solutions (pH 4.0) prior to titration. Figure 7 shows that alginate and ovalbumin cannot associate in the presence of buffer salts although the pH of the medium was 4.0. The count rate was low throughout the titration, which gave the evidence that electrolytes form ion pairs with the charged ionic site on both polymers, occupying them, thus reducing their capacity to assemble in polymer complexes. Strong electrolytes seem to have a higher charge screening effect than any of the used polymers.

Mannitol as a non-electrolyte also slightly affected the complexation between the charged polymers although not being a charged molecule. The efficiency of the complex formation was reduced in the presence of mannitol (Figure 7), however not to such a large extent as electrolytes. This gave the evidence that mannitol mitigated electrostatic interaction between polymers, but did not completely prevent their association.

3. 2. DSC Analysis

DSC was used to investigate the interactions between the polyelectrolytes. Endo and exothermic peaks can be obtained due to the energy change associated with the loss of water in the polymers, with depolymerisation of the polymers and denaturation of the protein at higher temperatures. Profile changes and shifts of peaks are usually associated with the interaction between the drug and the polymers.[16–18]

As shown in Figure 8, DSC scans of the untreated alginate and chitosan exhibit initial endothermic peaks close to 80 and 70 °C, respectively, which have been attributed to the evaporation of water associated with hydrophilic polymers. A higher exothermic peak of alginate at around 250 °C, and an exothermic baseline deviation of chitosan beginning around 250 °C with a peak at 310 °C indicate the degradation of both polymers, as reported.[17–20] The DSC scan of the ovalbumin shows an endothermic peak of around 60–80 °C, which can be attributed to the water loss and/or denaturation process, and an exothermic baseline deviation beginning around 230 °C (with its peak at 290 °C and above), which can be related to the degradation process of the ovalbumin.[21]

In the physical mixture of alginate and chitosan with a mass ratio of 1 : 0.1 (as in the PEC), alginate dominated the shape of the curve, showing the exothermic peak at 250 °C with a further exothermic deviation (Figure 9a). The physical mixture of alginate, ovalbumin and chitosan with a mass ratio similar to the PEC shows a thermogram with an endothermic peak of around 80 °C and an exothermic baseline deviation starting at 230 °C with an additional exothermic peak at 250 °C (Figure 9b). The latter peak is characteristic for alginate, and the baseline devia-

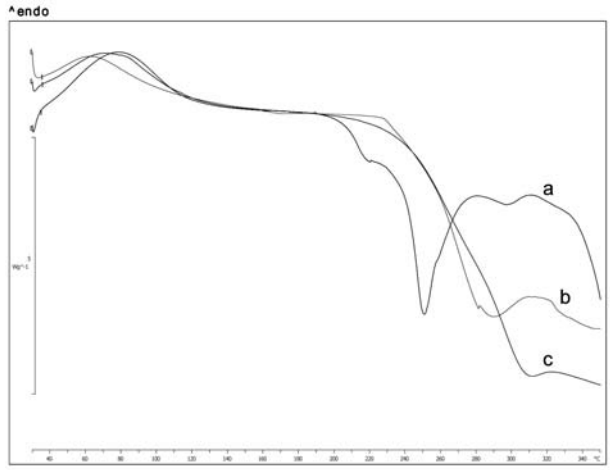

Figure 8. DSC scans of untreated samples of alginate (a), ovalbumin (b) and chitosan (c).

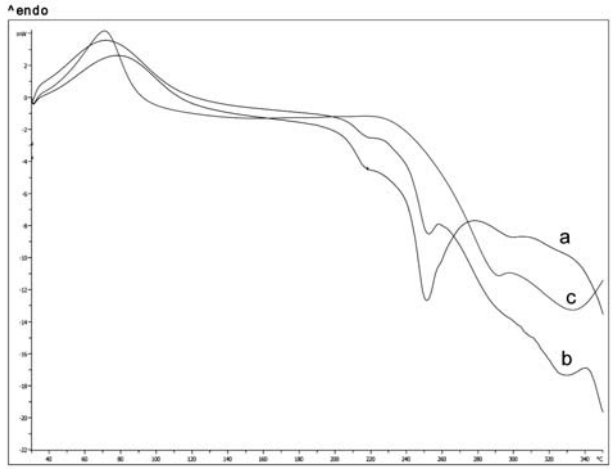

Figure 9. DSC scans of untreated materials in the physical mixtures of alginate and chitosan with a mass ratio of 1:0.1 (a), alginate, ovalbumin and chitosan with a mass ratio of 1 : 1 : 0.1 (b), and polyelectrolyte complexes of alginate, ovalbumin and chitosan produced with a mass ratio of 1 : 1 : 0.1 (c).

tion was observed for ovalbumin and also chitosan, although there was only a small amount of chitosan in the physical mixture. The shape of this curve thus combines the individual contribution of each material. For comparison, the DSC scan of the PEC has also been added to the image (Figure 9c) showing a different behaviour, but the control samples being treated before scanning in the same way as the PEC production are more decisive and are presented in Figure 10.

The DSC scans of the treated alginate, chitosan, ovalbumin, and alginate/chitosan complex were also obtained. After treatment of the materials the shape of the curves of all three ingredients changed indicating that the processing conditions influenced the properties of the polymers (not shown). The endothermic peaks of all three ingredients showed the same profile as the untreated ingredients. However, the exothermic peak of the alginate shifted to a higher temperature of 310 °C, and the curve of the ovalbumin showed the two exothermic peaks more intensively at 290 and 340 °C (Figure 10c). The contribution of chitosan in the mixture as well as in the complex with alginate was minor due to its smaller amount in the sample (Figure 10a, 10b). The DSC curve of the mixture of all the treated components showed some similarity with the DSC scan of the treated ovalbumin but with a smaller deviation of the exothermic peaks at temperatures 290 °C and 345 °C (Figure 10d). On the other hand, the PEC showed a different behaviour in the DSC scans (Figure 10e). The curve flattened, the characteristic exothermic peaks of either the ovalbumin or alginate almost disappeared, and a gradual exothermic baseline deviation was observed. The obvious difference between the thermal behaviour of the mixtures and PEC is probably due to the complexation of polyelectrolytes indicating interactions between the ingredients.

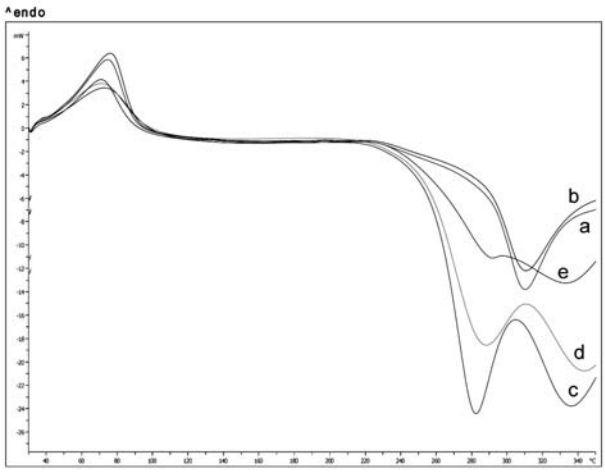

Figure 10. DSC scans of treated materials (after dissolving in an acetic acid solution and freeze-drying): the binary mixture of alginate and chitosan with a mass ratio of 1:0.1 (a), the alginate/chitosan complex with a mass ratio of 1:0.1 (b), ovalbumin (c), the ternary mixture of alginate, ovalbumin and chitosan with a mass ratio of 1:1:0.1 (d), and polyelectrolyte complexes of alginate, ovalbumin and chitosan with a mass ratio of 1:1:0.1 (e).

3. 3. Stability of the Complexes and the Effect of Stirring Time

During the stability studies, NaCl as an electrolyte, and mannitol as a non-electrolyte similarly affected the PEC integrity as observed in the titration experiments. The electrolyte replaced the electrostatic interaction between the polymers causing the PEC to dissociate, which is evident in the significant reduction in the average count rate (Table 2). The non-electrolyte also influenced the dissociation of the polymer assembly, but this effect was minor compared to the electrolytes. After stirring both dispersions with either NaCl or mannitol for an additional 24 hours, we observed very interesting results. In the presence of mannitol the polymers were able to reassemble into complexes reaching the same concentration as before the addition of mannitol (evident from the average count rate). We suspected that at the beginning, the non-electrolyte – mannitol probably temporarily interfered in the polymer interactions reducing their affinity to associate, but after prolonged stirring of the dispersion, the polymers were able to reinstate their original state, forming the complexes irrespective of the presence of mannitol. This effect was not observed with the electrolytes, where NaCl induced a permanent dissociation of the complexes, which could not recover even after prolonged stirring. Only an insignificant increase in kcps was noticed and the dispersion also remained optically transparent.

In an additional experiment we evaluated how the stirring time after the PEC formation influences the recovery and integrity of the complex formation, and how they are affected by the electrolyte and the non-electrolyte after their prolonged stirring (24 hours) prior to their addition. The concentration of the complexes as well as their size remained quite similar after prolonged stirring of the dispersion (Table 2). Moreover, mannitol showed no inf-

Table 2. Particle size, polydispersity index (PdI) and an average count rate of PEC dispersion in the presence of mannitol (2%, w/v) or NaCl (0.9%, w/v), and the effect of stirring over 24 hours before and after the addition of mannitol or NaCl (n = 3).

Sample / additive / process	Particle size (nm)	PdI	Count rate (kcps)
PEC	284	0.227	216 701
PEC + mannitol	256	0.202	141 284
PEC + mannitol + 24h	287	0.222	215 156
PEC + 24h	284	0.207	217 277
PEC + 24h + mannitol	300	0.244	218 155
PEC	284	0.227	216 701
PEC + NaCl	381	0.268	26 124
PEC + NaCl + 24h	607	0.399	67 888
PEC + 24h	259	0.215	216 333
PEC + 24h + NaCl	387	0.277	30 118

luence on the complexes when the dispersion was stirred for 24 hours prior to its addition, evident as the same value in kcps. On the contrary, the addition of NaCl caused the disintegration of the complexes irrespective of the overnight stirring prior to its addition. The reduction in the average count rate and the increase in dispersion transparency provided the evidence that the complexes had dissociated. Prolonged stirring was beneficial for the polymers to associate efficiently into complexes having a more recovered structure. Such complexes are therefore not affected by a non-electrolyte such as mannitol, but are still sensitive to a strong electrolyte such as NaCl, causing their disintegration.

In view of the formulation and the applicability of nanocomplexes for drug delivery, these data have important messages. Polymeric nanocomplexes must dissociate under conditions *in vivo* in order to release the incorporated drug. Since the strong electrolytes are present in the physiological solutions they can trigger the disintegration of the complexes and thus release the associated drug. Mannitol, on the other hand, can be used for example as a cryo/lyoprotectant during the freeze-drying process. This process is necessary regarding the stability of the PEC complexes which cannot be stored as an aqueous dispersion. It is very important that the cryo/lyoprotectants do not influence the integrity and thus the turbidity of the PEC (kcps). Additionally, they must provide properties to provide the freeze-dried product with suitable characteristics (retention of homogenous properties and an inner structure of the material, a quick and easy reconstitution in water etc.), these investigations are under further evaluation.

3. 4. Ovalbumin Loading

The efficiency of the ovalbumin association in the PEC was evaluated for different initial loadings of ovalbumin in the PEC. The concentrations of ovalbumin used to produce the PEC were within the range 0.05–0.5 mg/ml. The size of the PEC slightly increased by decreasing the initial loading of ovalbumin, however not significantly, and the zeta potential was the same. Table 3 shows that the loading of ovalbumin in the PEC was increased by increasing the initial concentration of ovalbumin used to produce nanocomplexes. This provided the evidence that polymer carriers have enough capacity to complex ovalbumin up to a concentration of 0.5 mg/ml. The association efficiencies of ovalbumin in the PEC were high and comparable for all loadings of ovalbumin in the PEC, reaching a value of 80–85%.

Table 3. Influence of initial loading of ovalbumin on the PEC properties: particle size, polydispersity index (PdI), zeta potential, ovalbumin loading capacity (LC) and ovalbumin association efficiency (AE).

Ovalbumin (mg/ml)	Particle size (nm)	PdI	Zeta potential (mV)	LC (%)	AE (%)
0.5	284	0.227	–44.0	38.2	80.2
0.25	286	0.274	–43.4	26.1	83.6
0.1	331	0.348	–44.5	12.6	82.3
0.05	332	0.296	–44.1	7.1	84.6

4. Conclusion

The PEC produced by the self-assembly of oppositely charged polymers can provide a stable protein delivery system, since it is formulated under mild processing conditions without the need of organic solvents, and results in a high protein association efficiency. Results showed that the complexation between polymers and protein is based on their electrostatic interaction, and very much depends upon their properties (pI of protein and pKa of polymer units) and the conditions used to prepare the NPs. The DSC confirmed the interaction of the complexation of polyelectrolytes and protein into complexes. Electrolytes such as NaCl or buffer salts markedly influence the interaction between protein and polymers, disabling their association in complexes or causing the dissociation of complexes. In contrast, non-electrolytes such as mannitol, have a minor effect on the polyelectrolyte assembly; this effect also vanished after prolonged stirring of the PEC dispersion. The association efficiency of ovalbumin in the PEC was high, i.e. 80–85%, for all tested concentrations of ovalbumin (0.05–0.5 mg/ml). The loading capacity of ovalbumin in the PEC ranged between 7 and 38% relative to the initial amount of ovalbumin used to produced the PEC.

These studies contribute significantly to the understanding of PEC formation and the complexation of polymers with the model protein ovalbumin. The approach can also be applied and suitably adjusted to other protein drugs. This work will be furthered by investigating protein release from the PEC, lyophilisation of the PEC, and the surface characteristic of the protein after complexation with polymers.

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Povzetek

Polielektrolitne komplekse za dostavo modelnega proteina ovalbumina smo izdelali iz dveh polisaharidnih polimerov, alginata in hitosana. Parametre, ki vplivajo na tvorbo polielektrolitnih kompleksov smo preučevali s koloidnim titriranjem raztopin v kombinaciji z dinamičnim sipanjem laserske svetlobe. Interakcije med polielektroliti smo preverjali z diferenčno dinamično kalorimetrijo, morfologijo nastalih kompleksov pa z vrstično elektronsko mikroskopijo. Ugotovili smo, da je tvorba kompleksov odvisna od pH raztopine in koncentracije polimerov. Ovalbumin tvori komplekse z negativno nabitim alginatom le v raztopini, katere pH je pod izoelektrično točko ovalbumina. Nastale komplekse iz alginata in ovalbumina smo nadalje obložili še s hitosanom. Najustreznejša sestava polielektrolitnih kompleksov z velikostjo 280 nm in nabojem –40 mV, je vsebovala alginat, ovalbumin in hitosan v razmerju 1 : 1 : 0,1 oz. njihovih koncentracijah 0,5 : 0,5 : 0,05 mg/mL. Vgradnja ovalbumina v polielektrolitne komplekse je odvisna od njegove začetne količine pri izdelavi kompleksov in je za različne formulacije znašala od 7–38%, pri čemer je bila učinkovitost asociiranja visoka za vse formulacije, t.j. 80–85 %. Blagi pogoji izdelave, nanometrska velikost delcev in visoka učinkovitost asociiranja proteina v komplekse so obetavne lastnosti za razvoj ustreznega dostavnega sistema za proteinske učinkovine.