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Freeze-Drying and Release Characteristics of Polyelectrolyte Nanocarriers for the Mucosal Delivery of Ovalbumin

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Abstract

Polyelectrolyte complexes (PEC) consisting of an alginate core entrapping the protein ovalbumin and the chitosan coating were prepared by the self-assembly of oppositely charged polyelectrolytes. The PEC were prepared at pH 4.0 and consisted of alginate, ovalbumin and chitosan in a concentration of 0.5, 0.5 and 0.05 mg/ml, respectively, having a particle size of around 300nm, a zeta potential of –44 mV and a protein association efficiency of 80%. The release of ovalbumin from PEC was mostly dependant on the pH of release medium and the presence of strong electrolytes contributed to higher release. Approximately 90% of the ovalbumin was released in a phosphate buffer media, pH 7.4. The release was lower in media with pH 4.0, reaching the value of app. 40% and 60% of ovalbumin released in water (pH 4.0) and NaCl solution (0.9% w/v, pH 4.0), respectively. In an acidic saline solution, pH 3.0, there was only 5% of ovalbumin release, however, increasing the pH to 6.8, approximately 70% of ovalbumin immediately released from the PEC. The PEC were freeze-dried aided by various excipients. Their efficiency on the redispersibility of the freeze-dried product was evaluated according to the mean particle diameter, polydispersity, average scattering intensity (particle concentration) and visual appearance of the PEC (Tyndal effect). In the presence of trehalose and mannitol, the aggregation and integrity of the PEC were prevented, yielding properties similar to the PEC dispersion before lyophilisation. The surface hydrophobicity of the ovalbumin either free or formulated in the nanocomplexes was determined by the bis-ANS fluorescence intensity, indicating a higher surface hydrophobicity for the PEC. The mild formulation conditions, nanometre-sized particles, high protein association efficiency, pH-dependant release, and modified surface properties are promising factors towards the development of an oral delivery system for protein made by the self-assembly of oppositely charged polyelectrolytes.

Keywords: Oral protein delivery, nanoparticles, polyelectrolyte complexes, self-assembly, alginate, chitosan, ovalbumin, freeze-drying, bis-ANS, surface hydrophobicity

1. Introduction

Peptides and proteins have become the drug of choice for the treatment of numerous diseases due to their selectivity and ability to provide effective and potent action. Various peptides and proteins are now produced on a large scale as a result of advances in biotechnology. Since it became clear that the clinical development and administration of these types of drugs is not possible without some sort of sophisticated formulation approaches, an increased interest in formulating and delivering biological drugs has arisen. Oral drug delivery is by far the most preferred route of drug administration; however, for proteins it may not be feasible because of their low oral bioavailability. Therefore, the administration of protein drugs in clinical practice is restricted to more invasive parenteral routes. Obstacles leading to poor protein oral bioavailability are the highly acidic pH in the stomach, the presence of secreted and brush-border enzymes, and physical barriers of the intestinal cells. The latter is especially problematic for macromolecular drugs being too large to pass between the cells through the paracellular pathway, and too hydrophilic to be absorbed passively through the cell membranes (transcellular pathway).[1,2] The use of NPs for the mucosal

delivery of macromolecules is one of the most promising strategies to overcome these limitations.

Nanoparticles (NPs) are particularly advantageous because they physically entrap the protein into the carrier material, which partly gives protection from degradation in the gastrointestinal tract. Recent studies have shown that NPs can also be transported through the gastrointestinal tract.[3,4] However, it should be noted that under normal conditions the absorption of particles is extremely low.[5,6] Improved particles uptake has been obtained by using NPs with mucoadhesive properties, which are mainly intended to enhance the absorption by prolonging the residence time of the particles on the intestinal epithelia. In fact, NPs have the intrinsic capacity to interact with mucosal epithelial membranes due to their nanometric size and large surface area. Several studies have shown that optimal interaction is achieved with NPs in the smallest size range, 50–500 nm.[3,4,6,7] Apart from the size, the carrier material is also important in promoting NP bioadhesion.[4,7] Furthermore, it has been found that the mucoadhesive properties of the carrier can induce transcytosis and the transient dilation of the intercellular spaces, giving evidence that the enhancement of the particles uptake could be higher than anticipated.[3,6] The absorption of NPs was not only shown to occur through the Peyer's patch M cells in the gut-associated lymphoid tissue but also through the enterocytes, which are the majority of the cells of the gut epithelium. Although there is no question that the NPs are being absorbed, the complete mechanism of this absorption remains to be clarified. The parameters favouring the NPs absorption are: the small particle diameter, colloidal stability, suitable hydrophilic/hydrophobic surface properties and others.[4,6,7] It has been found that nanoparticles with more hydrophobic surface are more efficiently taken up by the epithelial cells.[2,7] Besides this, the physical barrier of the mucus also plays an important role in the absorption of particles by entrapping the particles, causing their aggregation and preventing their further penetration.[8] Therefore, the colloidal stability as well as the mucus permeability that can be provided by certain hydrophilic polymers, has also been found to influence the absorption of nanoparticle-delivered molecules, suggesting very important impact of the hydrophilic/hydrophobic balance of the nanoparticle's surface.[4,8–10]

Nanoparticles made by polyelectrolyte complexation have shown a great potential in formulating biological drugs since this method avoids the need for severe technological procedures that are usually harmful to sensitive biomolecules.[11,12] Polyelectrolyte complexes (PEC) are formed in dilute aqueous solutions of oppositely charged polyelectrolytes that have the ability to spontaneously associate in complexes on mixing under gentle stirring.[12–14] In this work, alginate and chitosan were used to form PEC for the delivery of the model protein ovalbumin.[15] Alginate was used as the core material to incorporate the protein, and chitosan was used as the coating due to its favourable mucoadhesive and permeation enhancing properties.[16]

Previously we investigated various parameters influencing the formation of the PEC.[15] In this study we evaluated nanocomplexes according to the protein release in different media that simulates the conditions in GIT, and the possibility of freeze-drying to gain long-term stability of the PEC. There have been several studies on the lyophilisation of NPs, evaluating various additives against the adverse effect of freeze-drying. But this complex process of drying is mostly governed by the particular type of nanoparticles; therefore, it needs to be investigated for each formulation. Various excipients in different concentrations and combinations were used to freeze-dry the PEC, and their efficiency after the reconstitution of the freeze-dried product in water was evaluated with regard to the mean particle diameter, polydispersity, integrity (determined by the average counts detected per second) and the visual appearance of the PEC (Tyndal effect). In addition, the surface properties of the PEC were also investigated, since this characteristic was reported to be a very important factor influencing the uptake by biological membranes.[4,7–10] For this evaluation, a new approach using the extrinsic fluorescent dye has been set up for the first time to identify the extent of the hydrophobicity of the formulated nanosystem.

2. Materials and Methods

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 a 35–45% guluronic acid content (Protanal® LF 10/60LS), viscosity 20–70 mPas (1% solution) and a molecular weight of 250 kDa was from FMC BioPolymer, Norway. Excipients: trehalose, mannitol and glycine were from Sigma – Aldrich, USA; poloxamer 188 (Lutrol F68, polyethylene-polypropylene glycol) was from BASF, Germany; and polysorbate 80 (Tween 80, polyoxyethylene-20-sorbitan monooleate) was from Croda, USA. Noncovalent extrinsic fluorescent dye, 4,4'-bis-1-anilinonaphthalene-8-sulfonate (bis-ANS) was from Molecular Probes, USA. All other chemicals used in this study were of an analytical grade.

2. 2. Polyelectrolyte Complex Preparation

The PEC were prepared as follows: 2 ml of ovalbumin in a buffer solution (5 mg/ml, pH 7.0) was added drop by drop into a beaker containing 16ml of the 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 into the dispersion and stirred for

45 min, giving a final colloidal dispersion of alginate, ovalbumin and chitosan in concentrations of 0.5, 0.5 and 0.05 mg/ml, respectively.

2. 3. Characterization of the Polyelectrolyte Complex

The PEC were characterized according to the mean particle diameter, polydispersity, average scattering intensity (particle concentration) and zeta potential by using a Zetasizer Nano ZS ZEN 3600 (4mW He-Ne laser, 633 nm) 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. A detailed description is published elsewhere.[15]

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. 4. Ovalbumin Loading and Release from the Nanocomplex

The loading of ovalbumin in the PEC was determined indirectly after separation of the nanoparticles from the aqueous media by centrifugation at 40,000 rpm for 30 min (Beckman Coulter Centrifuge L7-55, USA). The content of the entrapped ovalbumin in the PEC was calculated from the difference of the total ovalbumin used for the PEC formation and the unassociated ovalbumin determined in the supernatant.

The release profiles of the ovalbumin from the PEC were obtained from freeze-dried PEC (containing 5% mannitol and trehalose as cryo/lyoprotectants), which were redispersed in a release medium [phosphate buffered saline (PBS, pH 7.4), water (PEC dispersion had pH 4.0), 0.9% NaCl solution (pH 4.0), and an acidic saline solution (0.1M HCl, 0.9% NaCl, adjusted to pH 3.0) raised to pH 6.8 after 2h incubation (with 0.02M Na_3PO_4)], and incubated at 37 °C under slow magnetic stirring. At determined times, 1.0 ml samples were taken and centrifuged at 15,000 rpm for 15 min (Centrifuge Sigma 2–15, Germany). The supernatants were analyzed for protein content, and the sediment was returned to the incubating medium using the fresh release medium.

2. 5. Ovalbumin Determination

Ovalbumin was determined by the HPLC running with an Agilent 1100 system (Hewlett Packard, Germany) on the column for gel filtration with a surface-stabilized, 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 × 12.5mm) 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 at 23 °C for 30 min. Protein identification was by UV detection at 210 nm. The method was validated and showed linearity in a concentration range of 0.006–1.20 mg/ml (R^2 = 0.9993).

2. 6. Freeze-drying the Polyelectrolyte Complexes

Trehalose, mannitol, glycine, poloxamer 188 and polysorbate 80 were used as the freeze-drying excipients for the formulation to be freeze-dried. They were evaluated alone and in the binary and ternary mixtures, which were prepared with an equal amount of each excipient. Briefly, the PEC dispersion was purified by centrifugation at 15,000 rpm and 4 °C for 30 min (Sigma 3–30 K, Sartorius, Germany). The sediment was redispersed in one half of the initial volume of distilled water. Aliquots of the PEC dispersion were added to the same volume of excipients solutions to achieve final PEC concentration 1.05 mg/ml and final excipient concentrations of 0.2%, 0.5%, 1%, 2% and 5% w/v. A control sample was prepared in the same way adding distilled water instead of the excipient solution. All samples were first characterized, and then poured into a 25 ml glass vials (Nuova Ompi, Piombino Dese, Italia), which were partially closed using lyo rubber stoppers (Helvoet Pharma, Alken, Belgium). The samples were frozen in liquid nitrogen, placed on pre-cooled shelves (–100 °C) and then lyophilized at –100 °C and 0.002 mbar for over 12 hours using a freeze-dryer (Lio-5P LT, Kambič, Slovenia).

Samples having crystalline excipients (mannitol and glycine) were also annealed to enable the total crystallization of the crystalline components. This was done by holding a frozen sample at –20 °C for 10 hours.

The freeze-dried products were redispersed in distilled water of the same volume as the original dispersion before freeze-drying. Redispersion was achieved by manual shaking and then stirring for a few minutes on the magnetic stirrer. All samples were characterized for their mean particle diameter, polydispersity, average count rate (particle concentration) and visual appearance of the PEC (Tyndal effect).

2. 7. Morphology of the Polyelectrolyte Complexes

Scanning electron microscopy (SEM) was used for the morphological evaluation of the polyelectrolyte com-

plexes using a JSM-7001F Jeol (Japan) instrument with an acceleration voltage of 1.5kV and a secondary electron detector. The SEM images were taken of the freshly prepared PEC that were dried in a vacuum dryer at 25 °C for 24 h, and of the freeze-dried PEC without any excipients, which were redispersed in water and vacuum dried as well. Samples were deposited on a double-sided carbon tape (diameter 12 mm, Oxford instruments, Oxon, UK) and then analysed.

2. 8. Assessment of the Surface Characteristics of the Nanocomplex Using the Extrinsic Fluorescence Dye – Bis-ANS

A noncovalent extrinsic fluorescent dye was used for the qualitative assessment of the surface hydrophobicity of the protein either alone or in a complex with polymers. Fluorescence emission spectra were recorded in the presence of bis-ANS (5 μM) on the ovalbumin (0.5 mg/ml), alginate (0.5 mg/ml), chitosan (0.05 mg/ml), alginate/chitosan complexes (0.5 : 0.05 mg/ml) without ovalbumin and the PEC (alginate/ovalbumin/chitosan, 0.5 : 0.5 : 0.05 mg/ml). All solutions/dispersions had the same composition media and pH (see 2.2 Polyelectrolyte complex preparation). The excitation wavelength was 385nm. The emission scans were recorded from 430 nm to 600 nm.

3. Results and Discussion

3. 1. Characterization of the PEC

The control of the particle size and the retention of the protein biological activity are the major challenges in formulating nanosized systems. Recently, great attention has been focused on the nearly spontaneous formation of nanoparticles that avoids the use of harsh processing conditions, i.e. the self-assembly of polyelectrolyte and protein in an aqueous media. The self-assembly is achieved through the weak and specific intermolecular interactions, and is governed by particular protein and carrier materials.

Ovalbumin was associated with alginate to form the core of the nanoparticles, and chitosan was used as the coating material. Previously we have found that this macromolecular association mostly depends on the net charge of the protein at the working pH, and that the electrostatic interaction between positively and negatively charged macromolecules were predominant.[15] The PEC were prepared following the complexation of the ovalbumin with the alginate at pH 4.0, which is below the isoelectric point of ovalbumin (pI 4.8), where its net charge is positive, thus providing a strong interaction with the negatively charged alginate. Alginate consists of repeated units of mannuronic and guluronic acid with pKa 3.4 and 3.6, respectively, therefore it is negatively charged at pH 4.0. Chitosan was further deposited onto the negatively charged alginate-ovalbumin complexes for a better mucoadhesiveness with the mucin.[3,16] The PEC prepared showed a characteristic Tyndall effect (milky look, average count rate 125,000 ± 10,000 kcps), and the mean particle size and zeta potential were 290 ± 20 nm and –44 ± 5 mV, respectively.

3. 2. Ovalbumin Loading and Release from the PEC

The loading of ovalbumin in the PEC prepared with alginate/ovalbumin/chitosan in a concentration of 0.5 : 0.5 : 0.05 mg/ml, respectively, was 38.2%, reaching a high ovalbumin association efficiency of 80.2%.[15]

The release profile of ovalbumin from freeze-dried polyelectrolyte complexes in PBS (pH 7.4), water (pH 4.0), 0.9% NaCl solution (pH 4.0) and acidic saline (pH 3.0) followed by simulated intestinal fluid (pH 6.8) is presented in Figure 1. In the PBS (pH 7.4), up to 75% of the ovalbumin was released from the PEC within the first 15 min, and increased to 85% after 0.5 hours of incubation. The release of ovalbumin from PEC in water and 0.9% NaCl solution (both dispersions having pH 4.0) reached the value of app. 40% and 60%, respectively. In acidic saline solution (pH 3.0) the release of ovalbumin was low, only 5%, and showed no progress within 2 hours of incubation. However, when increasing the pH to 6.8, ovalbumin immediately released from the PEC, reaching the value of app. 70%.

The differences in the release profiles suggest that the release of ovalbumin from PEC is mostly dependent on the pH of the medium. The presence of electrolytes also contributes to higher release. The pH of the medium primarily affects the charges of polyelectrolytes and hence their attraction or repulsion forces. In PBS with pH 7.4, the net charge of ovalbumin became negative, what led to the repulsive interaction with negative alginate and dissociation of PEC. Chitosan showed no significant influence on the release profiles, probably due to its low content in PEC composition (results not shown). The release in water and 0.9% NaCl solution (both media having pH 4.0) was much lower compared to PBS. Incomplete release in these media was probably because of the positive charges on ovalbumin (pI of ovalbumin is 4.8), still enabling interaction with alginate. In addition to that, the release was substantially higher in NaCl solution with higher ionic strength compared to water, what indicated that strong electrolytes also promoted the dissociation of the complexes and protein release from the PEC. This was probably due to the higher charge shielding effect of the salts that weakened the interaction between ovalbumin and alginate.[17,18] These profiles are characterized by an initial burst release, which is common in the nanometre size PEC system, since the rate of protein diffusion is proportional to the particle's surface area.[19] The initial stage of ovalbumin

release can therefore be attributed to the dissociation of protein located closer at the surface of nanoparticles and the remaining of the unreleased ovalbumin is most probably still entrapped with polymers.

In acidic saline solution pH 3.0, the release of ovalbumin was retained in spite of high ionic strength of the medium. It is possible that at this pH even more intense attractive interactions between ovalbumin and alginate took place and became predominant. It is also possible that at this pH some alginate molecules partly precipitated (pKa of mannuronic and guluronic acids of alginate are 3.4 and 3.6, respectively) forming a network that prevented ovalbumin release.[20] Solubility of ovalbumin was not impaired at this pH (not shown). When the pH was increased, the net charge of protein changed, which led to repulsion with alginate. At higher pH alginate was also converted into ionic and more soluble state, which contributed to higher ovalbumin release.

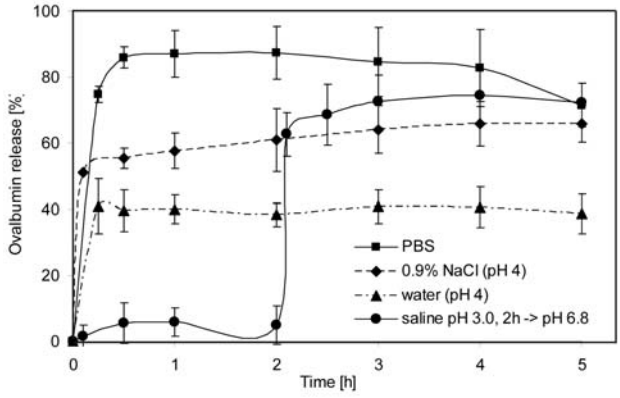

Figure 1. Ovalbumin release from the PEC (alginate:ovalbumin:chitosan 0.5:0.5:0.05 mg/ml, respectively) in the PBS (pH 7.4), 0.9% NaCl solution (pH 4.0), water (pH 4.0), and acidic saline solution (pH 3.0) raised to pH 6.8 after 2 h of incubation (n = 3).

In this study, only the influence of pH and ionic strength on the protein release from PEC was examined, however, it should be stress that the release from the PEC is affected by the properties of the associating macromolecules (weak or strong polyelectrolytes, their charge density and degree of hydrophobicity, their molecular weight or number of monomeric unit), the size of the particles, and the environmental parameters such as the pH, T, concentration, diffusion gradient etc.[12,18–20] From the HPLC chromatograms based on the gel filtration, we can speculate that ovalbumin might have retained its conformational integrity, since neither an alteration in size nor degradation products of ovalbumin were detected by the HPLC analysis, only a well-defined peak appeared on each chromatogram at the retention time similar to that of the native ovalbumin. However, to verify the biological activity of the protein drug, other methods for protein characterization should additionally be performed.

3. 3. Freeze-drying the PEC

Polyelectrolyte nanocomplexes in an aqueous dispersion may face physical and/or chemical instability, such as the aggregation or fusion of particles, the dissociation of polyelectrolyte complexes, hydrolysis of polymers, loss of drug activity etc.[21] In order to improve the physical and chemical stability of the nanocomplexes, water has to be removed. Freeze-drying is the most suitable process for drying biologically active substances such as protein drugs, and provides the product with desirable properties, which are the system's homogeneity as found in the solution, preserved inner structure, porous structure, quick and easy reconstitution in water etc.[21–23] Nevertheless, several physicochemical phenomena may occur during this complex process of drying, which may lead to the protein denaturation, loss of protein from the vials during drying, an unpleasant appearance of the freeze-dried cake, difficulties in redispersion of the product after reconstitution in an aqueous media and others.[23,24]

The freeze-drying process is often approached with a "trial and error" experimental plan. There are some simple rules based on well-accepted scientific principles that should be followed when freeze-drying biopharmaceutical drugs.[24,25] Drying the colloidal system presents an additional challenge. An inadequate reconstitution of the freeze-dried nanometre-size particles in the aqueous media represents a major obstacle leading to the formation of large particle aggregates and the loss of the beneficial colloidal properties of the nanosystem. There are some studies on freeze-drying nanoparticles in the literature, but optimal conditions depend mainly on the nature of the particle systems thus have to be customized for each formulation.[21,23,26] Moreover, no systematic studies have been performed for freeze-drying PEC, which consist of water-soluble polyelectrolytes on the basis of weak and reversible interaction, that are greatly influenced by the medium parameters such as pH, ionic strength, temperature, etc.[15,18]

In order to choose the appropriate excipients, which effectively retain the size and integrity of the PEC after the reconstitution of the lyophilisate in water, a screening of various excipients at variable concentrations and combinations was performed. Excipients used for freeze-drying can be distinguished according to their function as bulking agents, stabilizers or surfactants.[25,26] Bulking agents are additives that usually crystallize during the freezing stage and provide support to the amorphous nanoparticle-rich phase forming a mechanically strong and elegant cake structure. Stabilizers are used to replace the water that is lost during freeze-drying. They should remain in an amorphous state to provide adequate bonding with the drying product and stability during dehydration. In the literature this mechanism is called "water replacement".[21] Nonionic surfactants are used to prevent particle aggregation, which is mostly seen in the final stage of rehydration of the freeze-dried product.[25]

Trehalose was selected as an amorphous stabilizing excipient, mannitol and glycine as crystalline bulking excipients, and polysorbate and poloxamer as nonionic surfactants. The prepared PEC were first purified by centrifugation to remove unassociated polymers. After purification, the size of the PEC slightly increased compared to freshly prepared PEC, and were in the range 290–330nm. Liquid nitrogen was used to freeze the samples to obtain high supercooling of the sample. This resulted in the formation of smaller ice crystals, which produced less mechanical stress on nanoparticles compared to slow freezing with larger ice crystals.[21,24] Table 1 shows the effect of various excipients on the properties of freeze-dried PEC after their reconstitution in water. Without any excipients, the size of the freeze-dried PEC after rehydration increased remarkably, by a factor of 3.3, which represents the ratio between the final (after lyophilisation) and initial mean diameter (before lyophilisation) of the particles (S_f/S_i). Trehalose was found to be the most efficient excipient relative to other excipients tested individually. The factor of particle size increase of the reconstituted sample was only 1.4 for all trehalose solutions. Mannitol as a bulking agent also showed a protecting effect on the PEC during freeze-drying, yielding a factor of particles size increase of 1.6 for 2 and 5% mannitol solutions. The efficiency of mannitol in the reconstitution of the freeze-dried PEC increased with an increasing concentration of mannitol (up to 5%), whereas for trehalose the influence of concentration was minimal, indicating good stabilization properties. On the other hand, glycine did not provide the product with good redispersion properties. Large aggregates were observed after reconstitution in water, and the samples were therefore not suitable for DLS measurement.

Frozen samples with the crystalline bulking agents, e.g. mannitol and glycine, were also annealed for 10 hours at –20 °C, which is higher than the freezing temperature, to obtain their complete crystallization before lyophilisation. If the bulking agent does not crystallize completely, the liberation of the water associated with the crystallization of the bulking agent during drying or storage may lead to stability problems.[27] Our results showed that the redispersion properties of the freeze-dried PEC with either mannitol or glycine at various concentrations were not improved by annealing. What is more, much larger particle sizes with a high PdI (> 0.5) were obtained, which exceeded size of 800 and 1000 nm for mannitol and glycine, respectively (results not shown). The crystallization of mannitol or glycine probably caused a phase separation in the cryo-concentrated nanoparticle dispersion with no opportunity for the stabilization interaction. Growing crystals of water and mannitol or glycine could exert mechanical forces on the nanoparticles leading to their fusion. Also, nanoparticles in the nanoparticle-rich phase might interact and form aggregates.[21] Therefore, it is necessary that some of the excipient remains molecularly dispersed in the amorphous nanoparticle phase to stabilize the NPs and prevent their aggregation, enabling their reconstitution. This explanation is also supported by the results when trehalose was used, which is an amorphous excipients and yielded the most suitable protective properties, replacing water during freeze-drying, providing hydrogen bonding with drying material. Annealing was omitted from the lyophilisation process in further experiments.

Nonionic surfactants (polysorbate and poloxamer) influenced the particle size on their addition to the PEC dispersion before lyophilisation (not shown). For both polymers, large particles were observed with increasing concentration above 1%, i.e. 600 nm and 800 nm for poloxamer (5%, w/v) and polysorbate (5%, w/v), respectively. Since polysorbate and poloxamer are polymers they might interfere in different ways increasing the size of the nanosystem. The redispersion properties of the freeze-dried product having these excipients were poor, also at concentrations below 1%, the S_f/S_i index was from 2–2.8 (Table 1).

In order to investigate whether excipients have synergistic effects on nanoparticle stabilization, binary and ternary mixtures of different excipients in the same ratio were prepared. Glycine was omitted from further experiments due to its poor protective properties. Table 1 shows that the combination of trehalose and mannitol was the most promising, with a S_f/S_i index of around 1.6, whereas other binary and ternary mixtures of excipients did not promote the stability and redispersion of the freeze-dried PEC.

It is also important that the integrity and the turbidity of the PEC are preserved upon the addition of excipients and after the reconstitution of the freeze-dried product in water, indicating that PEC did not disintegrate.[21,23] In our previous study we also observed that strong electrolytes (NaCl) influence the integrity of the PEC, causing their dissociation (turbid dispersion became almost transparent).[15] The visual appearance (Tyndall effect) as well the average count rate that reflects the amount of particles in the sample were therefore examined after the addition of the excipients before freeze-drying and after the reconstitution of the freeze-dried product in water (the same volume as the dispersion prior to lyophilisation). After the addition of the excipients, all dispersions showed a similar turbidity (average count rate) as before their addition. But after freeze-drying, only trehalose and mannitol either alone or in combination were able to retain the turbidity after reconstitution similar to the dispersion prior to lyophilisation (measured by the average count rate) (Table 1). This indicated that the PEC were not affected by the addition of those excipients and that the PEC did not disintegrate. All other samples were characterized by a more or less reduction in the average count rate, probably due to an inadequate redispersion of the freeze-dried product in water yielding larger – aggregated or flocculated particles that slowly sedimented.

Table 1. Particle size, polydispersity index, S_f/S_i index (the ratio between the final and initial mean diameter) and the Tyndal effect (estimated on the visual appearance and average count rate) for PEC dispersion before and after freeze-drying using various excipients.

		Particle size (nm)	PdI	S_f/S_i index	Tyndal effect*
Before freeze drying					
No excipients		310 ± 20	0.147		3
After freeze drying					
No excipients		1029 ± 20	0.277	3.3	1
Excipients	Concentration (%)				
Trehalose	0.5	432 ± 60	0.226	1.4	3
	1	421 ± 30	0.279	1.4	3
	2	452 ± 10	0.276	1.5	3
	5	421± 30	0.276	1.4	3
Mannitol	0.5	662 ± 37	0.400	2.1	3
	1	591 ± 29	0.283	1.9	3
	2	482 ± 10	0.252	1.6	3
	5	495 ± 30	0.211	1.6	3
Glycine	1	Nd	Nd	Nd	1
	2	Nd	Nd	Nd	1
	5	Nd	Nd	Nd	1
Polysorbate 80V	0.2	857 ± 120	0.322	2.8	2
	0.5	720 ± 60	0.339	2.3	2
	1	651 ± 60	0.672	2.1	2
	2	Nd	Nd	Nd	1
	5	Nd	Nd	Nd	1
Poloxamer 188	0.2	946 ± 16	0.287	3.0	2
	0.5	923 ± 10	0.255	3.0	2
	1	899 ± 40	0.378	2.9	2
	2	1030 ± 15	0.337	3.3	2
	5	1413 ± 60	0.341	4.6	2
Trehalose/mannitol	0.1/0.1	490 ± 20	0.528	1.6	3
	0.25/0.25	482 ± 30	0.243	1.6	3
	0.5/0.5	519 ± 19	0.280	1.7	3
	1/1	483 ± 10	0.248	1.6	3
	2.5/2.5	501 ± 13	0.247	1.6	3
Trehalose/poloxamer	0.1/0.1	845 ± 20	0.376	2.7	2
	0.25/0.25	823 ± 30	0.311	2.7	2
	0.5/0.5	740 ± 29	0.270	2.4	2
	1/1	811 ± 36	0.359	2.6	2
	2.5/2.5	1030 ± 11	0.293	3.3	2
Trehalose/polysorbate	0.1/0.1	735 ± 33	0.295	2.4	2
	0.25/0.25	758 ± 25	0.409	2.4	2
	0.5/0.5	752 ± 7	0.327	2.4	2
	1/1	635 ± 40	0.577	2.0	2
	2.5/2.5	Nd	Nd	Nd	1
Trehalose/mannitol/polysorbate	0.067/0.067/0.067	702 ± 20	0.305	2.3	2
	0.16/0.16/0.16	760 ± 42	0.291	2.5	2
	0.33/0.33/0.33	729 ± 19	0.281	2.4	2
	0.66/0.66/0.66	700 ± 30	0.337	2.3	2
	1.66/1.66/1.66	Nd	Nd	Nd	1
Trehalose/mannitol/poloxamer	0.067/0.067/0.067	774 ± 20	0.296	2.5	2
	0.16/0.16/0.16	752 ± 15	0.304	2.4	2
	0.33/0.33/0.33	796 ± 25	0.263	2.6	2
	0.66/0.66/0.66	755 ± 34	0.337	2.4	2
	1.66/1.66/1.66	Nd	Nd	Nd	1

* The Tyndal effect estimated visually and scaled from 1 to 3 on the basis of the average count rate: 1: absent, 0–40,000 kcps; 2: scarce, 40,000–90,000 kcps; 3: significant, 90,000–130,000 kcps). The average count rate of freshly prepared PEC was 125,000 ± 10,000 kcps.

The analysis of the freeze-dried product also includes the visual observation of the final volume and the appearance of the cake. One of the desired characteristics of the freeze-dried pharmaceutical form includes an intact cake occupying the same volume as the initial frozen mass. Using visual inspection, the final volume and the appearance of the cake were markedly improved with an increasing amount of excipients added in comparison to the sample without any excipients.

The SEM images of the freshly prepared PEC as well as of the freeze-dried PEC after redispersion in water were taken (Figure 2). The freshly prepared PEC show an irregular and polyhedral shape with a size comparable to the DLS measurements (Fig 2A). The PEC, that were processed through the freeze-drying, showed some changes in their shape and size as observed in Figure 2B. Their surface is not as smooth as the vacuum dried PEC and some roughness of their shape can be observed. Areas of large clusters are also found with no clear boundaries between individual particles, indicating that aggregation and fusion of particles occurred during freeze-drying.

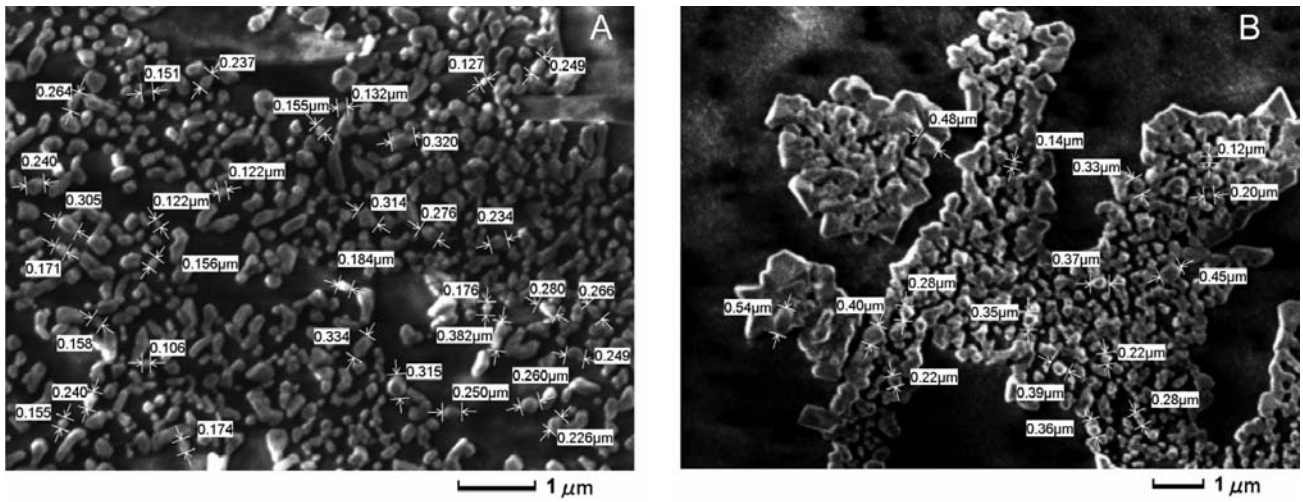

Figure 2. The SEM images of freshly prepared PEC (A) and freeze-dried PEC without excipients after redispersion in water (B).

3. 4. The Surface Characteristic of Ovalbumin after Complexation with Polymer Carriers

Non-covalent extrinsic fluorescent dye – bis-ANS was used to evaluate the surface characteristic of ovalbumin after its complexation with polymers. Bis-ANS is applied in various fields of protein analysis, but mostly to detect the surface hydrophobicity of the protein.[28] The fluorescent properties of the dye are governed by the solvent relaxation process and intramolecular charge transfer reactions, which are affected by the environment and by the interaction of the dye with the protein. Molecules such as 1-anilinonaphtalene-8-sulfonate (ANS) are hardly fluorescent in an aqueous environment, but become highly fluorescent in an apolar, organic solvent (low dielectric constants) or upon contact with hydrophobic surfaces. Besides the hydrophobic interaction, the electrostatic interaction was also discussed as a possible binding mechanism for various ANS dyes. Those may instate through ion pairing between the negatively charged sulfonate groups of the ANS with positively charged groups of solutes (e.g. histidine, lysine or arginine in the protein). However, for bis-ANS, it was found that hydrophobic interaction dominates the electrostatic interaction, and that its fluorescence intensity readily increases with the increased hydrophobic area in the sample.[29]

Results showed that the fluorescence intensity of the PEC containing ovalbumin is significantly higher compared to the free ovalbumin recorded under the same conditions (the same composition of the solvent and pH) (Figure 3). Alginate and chitosan, recorded solely and in combination, negligibly influenced the bis-ANS fluorescence intensity. Moreover, the fluorescence intensity of chitosan excluded the contribution of the electrostatic interaction to the increase in the bis-ANS fluorescence, which could eventually occur between the negatively charged bis-ANS and the positively charged chitosan. The zeta potential of the ovalbumin, alginate, chitosan, alginate/chitosan complex and the PEC was determined to be +3.9, –35.9, +20.0, –45.3, and –44.0 mV, respectively. The PEC also have a negative zeta potential and thus cannot electrostatically interact with the bis-ANS dye. The increase in the bis-ANS fluorescence intensity for nanocomplexes is most likely due to the changes in the surface properties of the ovalbumin when it is complexed with polymer carriers. Additionally, it was also reported that the dielectric environment of the protein is reduced when interacting with polymer carriers, which may be also reflected in the

increase of the bis-ANS fluorescence intensity.[30] The complexation of ovalbumin with polymer carriers might therefore result in a greater susceptibility of the PEC for hydrophobic interaction. This might contribute to greater affinity for the lipidic biological membrane, which is necessary for the protein to cross the cell barrier (epithelium) and to be absorbed.

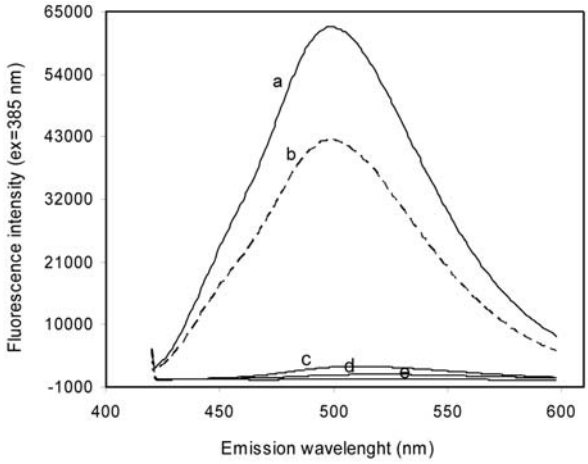

Figure 3. The bis-ANS fluorescence intensity in ovalbumin/alginate/chitosan dispersions; a – PEC; b – ovalbumin; c – chitosan; d – alginate/chitosan complex; e – alginate. All samples were recorded in the same dispersion media, having a pH of 4.0.

However, for nanoparticles to be absorbed, they must pass two barriers, the mucus gel layer and the mucosa, the cellular/epithelial layer. Mucus, which consists of high molecular weight glycoprotein, is the first layer that hinders the diffusion of particles to the intestinal epithelium. A number of investigators have studied the effect of surface properties of nanoparticles on their transport through the mucus barrier, albeit with discordant results. Norris and Sinko reported that small size particles (cut-off 500nm) with a more hydrophobic surface can penetrate easier through the mucus where hydrophobic interactions are involved.[7] But several recent publication gave the evidence that hydrophilic and near neutrally-charged nanoparticles readily penetrate through the mucus because such surface characteristics minimize mucoadhesion by reducing hydrophobic or electrostatic interaction.[10,31] Other factors such as colloidal stability, which is also provided by certain hydrophilic polymers (such as PEG coating), play also an important role preventing nanoparticle aggregation into larger particles.[32] Particle size is however one of the key factor in the transport of nanoparticles. Indeed it is agreed that nanoparticle transport increases when the particle diameter decreases.[4,7,9,33] To conclude, nanoparticles should provide certain hydrophilic character to ensure colloidal stability and mucus permeability.[10,32] In addition, their hydrophobicity plays also very important role since it significantly affects the interaction with the lipid biomembranes, enabling better nanoparticle/protein adsorption or inclusion into the bilayer of epithelial cells.[2,34,35]

4. Conclusion

Polyelectrolyte complexes (PEC) consisting of an alginate core entrapping the protein ovalbumin and the chitosan coating were prepared by the self-assembly of oppositely charged polymers. Positively charged ovalbumin at pH 4.0 (below the isoelectric point of ovalbumin, pI 4.8) successfully associated with the negatively charged alginate, achieving high association efficiency, e.g. 80%. Further coating with chitosan minimally influenced the PEC properties. Release studies showed that almost 90% of the incorporated ovalbumin was released in the buffer solution with pH 7.4 after 1h of incubation, whereas the release in media with pH 4.0 was substantially lower, reaching 40% in water (pH 4.0) and 60% in 0.9% NaCl solution (pH 4.0). These results showed that the dissociation of the PEC can be triggered by pH of the medium and that electrolytes (salts) might also interfere with the electrostatic interaction between oppositely charges polyelectrolytes. The prepared PEC showed a pH-dependent release: a great retention of ovalbumin release in acidic saline solution (simulated gastric fluid), and a significant release at pH 6.8.

PEC were successfully freeze-dried aided by the excipients. In particular, trehalose was found to be the most efficient in all concentrations tested enabling the successful redispersion of PEC with almost completely recovered properties compared to those before lyophilisation. Mannitol also showed protective properties against the adverse effects of freeze-drying, but its efficiency was observed at higher concentrations. Additionally, higher concentrations of both excipients improved the appearance of the lyophilisate – cake, having a mechanically strong and elegant structure.

The surface hydrophobicity of ovalbumin either alone or complexed with polymer carriers was evaluated using the fluorescence emission spectra in the presence of the non-covalent extrinsic fluorescent dye bis-ANS. Results showed that the PEC exhibited higher surface hydrophobicity in comparison to free ovalbumin, which indicates a potentially more favourable interaction with the biological membranes. However, others factors such as colloidal stability, mucus permeability, proteolytic resistance etc. should also be considered when formulating nanoparticles for oral protein delivery.

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Povzetek

Polielektrolitne komplekse (PEK) smo pripravili s postopkom samoasociiranja nasprotno nabitih polimerov, pri katerem smo protein vključili v alginatno jedro, nastale komplekse pa obložili s hitosanom. PEK z velikostjo okoli 300 nm, zeta potencialom –44 mV in 80% učinkovitostjo associiranja proteina v PEK smo pri pH 4,0 pripravili pri koncentracijah alginata, ovalbumina in hitosana 0,5:0,5:0,05 mg/ml. Ugotovili smo, da pH medija močno vpliva na sproščanje ovalbumina iz PEK ter da prisotnost elektrolitov sproščanje poveča. Največje sproščanje, blizu 90 %, smo dosegli v fosfatnem pufru pH 7,0, medtem ko je bilo pri pogojih s pH 4,0 sproščanje opazno nižje: 40 % v vodi (pH 4,0) in 60 % v 0,9 % raztopini NaCl (pH 4,0). Disperzija PEK je pokazala pH-odvisno sproščanje: v kisli raztopini soli pH 3,0 se je sprostilo le 5 % vgrajenega proteina, sproščanje pa se je bistveno izboljšalo in doseglo 70 %, ko smo pH dvignili na 6,8. PEK smo liofilizirali z dodatkom pomožnih snovi. Njihovo učinkovitost na redispergiranje liofilizata smo ocenili z merjenjem velikosti redispergiranih delcev, polidisperznega indeksa in povprečnega sipanja svetlobe (koncentracija delcev) ter po vizualnem izgledu. Agregacijo delcev smo preprečili z dodatkom trehaloze in manitola, ki sta po redispergiranju liofilizata dala disperzijo s podobnimi lastnosti kot pred liofilizacijo. Površinsko hidrofobnost ovalbumina, bodisi prostega ali vezanega v PEK, smo določali z merjenjem intenzitete fluorescentnega barvila bis-ANS. Dokazali smo, da se po vezavi ovalbumina v PEK površinska hidrofobnost poveča. Blagi pogoji izdelave, nanometrska velikost delcev, visoka učinkovitost associiranja proteina v komplekse, pH-odvisno sproščanje in spremenjene površinske lastnosti sistema so obetavne karakteristike za razvoj ustreznega dostavnega sistema za peroralni vnos proteinske učinkovine.