Drug Development and Industrial Pharmacy, 2011; 37(6): 619–627

ISSN 0363-9045 print/ISSN 1520-5762 online
DOI: 10.3109/03639045.2010.533681

informa
healthcare

ORIGINAL ARTICLE

Protein micro and nanoencapsulation within glycol-chitosan/Ca^{2+}/alginate matrix by spray drying

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Abstract

Encapsulation of therapeutic peptides and proteins into polymeric micro and nanoparticulates has been proposed as a strategy to overcome limitations to oral protein administration. Particles having diameter less than 5 μm are able to be taken up by the M cells of Peyer's patches found in intestinal mucosa. Current formulation methodologies involve organic solvents and several time consuming steps. In this study, spray drying was investigated to produce protein loaded micro/nanoparticles, as it offers the potential for single step operation, producing dry active-loaded particles within the micro to nano-range. Spherical, smooth surfaced particles were produced from alginate/protein feed solutions. The effect of operational parameters on particle properties such as recovery, residual activity and particle size was studied using subtilisin as model protein. Particle recovery depended on the inlet temperature of the drying air, and mean particle size ranged from 2.2 to 4.5 μm, affected by the feed rate and the alginate concentration in the feed solution. Increase in alginate:protein ratio increased protein stability. Presence of 0.2 g trehalose/g particle increased the residual activity up to 90%. Glycol-chitosan-Ca^{2+}alginate particles were produced in a single step operation, with resulting mean diameter of 3.5 μm. Particles showed fluorescein isothiocyanate labeled bovine serum albumin (BSA)-protein entrapment with increasing concentration toward the particle surface. Similar, limited release profiles of BSA, subtilisin and lysozyme were observed in gastric simulation, with ultimate full release of the proteins in gastrointestinal simulation.

Key words: Micro/nano encapsulation, spray drying, protein stability, protein distribution

Introduction

Therapeutic proteins and peptides are typically delivered subcutaneously, as they are readily denatured in the acidic, protease rich environment of the stomach or gastrointestinal (GI) track and low bioavailability results from poor intestinal absorption through the paracellular route. Encapsulation into polymeric micro and nanoparticle systems has been proposed as a strategy to overcome limitations to oral protein administration (Pinto et al., 2006). Nanoparticles having diameters less than 5 μm are able to be taken up by the M cells of Peyer's patches found in intestinal mucosa (Hussain et al., 2001).

Several formulation techniques have been investigated previously to produce micron and sub-micron sized particles (<5 μm), including nanoemulsion dispersion (Reis et al., 2004), ionotropic pre-gelation (Sarmento et al., 2006) and spray drying (Coppi et al., 2001). Although, the desired particle sizes were achieved through nanoemulsion dispersion and ionotropic pre-gelation techniques, these methods have the drawback of requiring organic solvents and multiple, time consuming steps.

Spray drying is a single step process, which can be operated continuously. Heat from a hot gas stream evaporates atomized droplets of a continuous polymer/protein feed, encapsulating the drug. Formation of a particle from droplets proceeds in three stages. After atomization, the drying rate increases until the surface temperature of the droplet reaches the wet bulb temperature of the drying air, which corresponds to 100% relative humidity. In the constant rate period, the surface temperature of the droplet remains constant due to the continuous evaporation of the water. In the last stage, the drying rate decreases and crust formation occurs encapsulating the active (Masters, 1991). During spray drying, proteins can unfold

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(Received 20 July 2010; revised 06 October 2010; accepted 17 October 2010)

due to dehydration stress (Carpenter and Manning, 2002) although the droplets only reach the relatively low wet bulb temperature due to latent heat effects associated with high rates of evaporation (Broadhead et al., 1992). Additives such as sucrose, trehalose, dextran, or maltodextrin remain in the amorphous phase with the protein, and/or hydrogen bond to the protein in the place of water during drying, such as is the case with sucrose or trehalose (DePaz et al., 2002). Denaturation and protein adsorption at the water/air interface (Adler and Lee, 1999) are additional stresses involved, however, shearing stress occurring during pumping, flow and nozzle atomization, do not appear to cause major damage to proteins (Maa and Hsu, 1997).

Spray drying has been previously studied to produce polymer particles containing proteins, with diameters ranging from nanometers to several microns. Several polymeric matrices have been studied such as poly (D-lactide), poly(lactide-co-glycolide) (Blanco et al., 2005, Wang and Wang, 2003, Pavanetto et al., 1993, Wagenaar and Müller, 1994), and poly (ε-caprolactone) (Blanco et al., 2003). However, these polymers require organic solvents such as dichloromethane in the feed solution. Hydrogels such as alginate as encapsulation matrix are a good alternative, since the polymers are water soluble, biocompatible, inert toward proteins, and bioadhesive, increasing drug residence time at the site of intestinal absorption (Wee and Gombotz, 1998, Tønnesen and Karlsen, 2002). Alginates undergo gelation with di or multivalent cations such as Ca^{++}, producing gels that provide sustained release characteristics (Kim and Lee, 1992). The alginate gel network can solubilize in presence of Ca^{++} chelators such as, citrate, lactate, and phosphate, however, these ions generally do not appear in human intestinal fluid (Bhagat et al., 1994). Alginates form strong complexes with polycations such as chitosan (Wee and Gombotz, 1998) and glycol-chitosan (Sakai et al., 2000) and these complexes do not dissolve in the presence of Ca^{++} chelators and can be used to stabilize the gel and reduce its porosity.

Previously, spray drying was investigated to produce alginate based particles. Takeuchi et al., (1998, 2000) investigated the properties of lactose-chitosan-alginate composite particles produced by rotary atomizer for direct tabletting purposes. Coppi et al., (2001, 2002, 2004) studied production of alginate microparticles for oral drug delivery, where bovine serum albumin (BSA), L-lactate hydrogenase and a peptide antibiotic, polymyxin, were used as model systems. In addition, several researchers studied spray-dried alginate particle systems focusing on production of particles with specific applications, such as encapsulation of volatile materials (Rosenberg et al., 1990), and immobilization of cells (Begin et al., 1991).

In the present study, alginate and alginate-trehalose formulations were investigated using the protease subtilisin as a model protein. Since it is desired to demonstrate residual activity of encapsulated proteins, the use of an enzyme as model, allows for activity determination post-encapsulation through kinetic assay. The effect of inlet temperature, feed rate, and protein:polymer and protein:polymer:disaccharide ratios, on the properties of the resulting particles, including mean diameter, residual activity, storage stability, moisture content, and product recovery was studied. A single encapsulation step procedure was proposed for preparation of micron and submicron particles for oral administration of proteins. Ca^{++} ions were added to dilute alginate feed, along with the protein and glycol-chitosan, where physical crosslinking of the alginate and polyion complex formation takes place resulting in sprayable low viscosity gel. Glycol-chitosan was used as an additive, to further reinforce the alginate network and improve absorption of the particles, as it is water soluble at pH 7.4 and forms polyion complexes with negatively charged polymers, such as alginate. The resulting particles were investigated for size, protein content and protein release kinetics with lysozyme, albumin and subtilisin as model proteins with different molecular weights.

Materials and methods

Low viscosity sodium alginate (Na-A) (Sigma–Aldrich, Oakville, Canada) with specifications: 250 cP for 2% solution at 25°C; molecular weight about 147 000; 61% mannuronic acid and 39% guluronic acid; batch number 112K0931. Subtilisin enzyme concentrate was provided as gift from commercial supplier. BSA, lysozyme, glycol-chitosan, maltose, sucrose, trehalose, peptide substrate (*N*-succinyl-L-Ala-L-Ala-Pro-L-Phe-*p*-nitroanaline) and other excipients were purchased from Sigma–Aldrich.

Spray dryer operation

Sodium alginate at noted concentrations was dissolved in deionized water, then deaerated for 30 min. Proteins were dissolved in distilled water and added to the alginate solution. $CaCl_2$ or glycol-chitosan solutions, when added were mixed 5 min with a three-blade upward directing marine-type impeller rotating at 250 rpm. Feed solutions were continuously mixed and spray-dried with a Büchi B-290 lab scale spray dryer. The dryer nozzle and walls of the particle collection vessel were cooled with chilled water, yet a thermocouple placed into the collection vessel showed a gradual increase in vessel temperature over the run to about 45°C. From the results it was determined that elevated temperatures of the collection vessel did not have an important effect on protein activity, likely due to the protective effect of the matrix polymer. Activity, yield, particle size, and distribution and residual moisture content were determined on formulated particles. Spray drying parameters, such as liquid feed rate (Q_{lf}, L/h), drying air flow rate (Q_{da}, m^3/h), atomizing air flow rate (Q_{aa}, L/h), and pressure of atomizing air (P, atm) were held constant unless otherwise stated. Inlet and corresponding outlet temperatures remained constant for each run, at the values noted. Particle recovery was defined as amount of particles collected in the collection vessel compared

to total solids introduced to the feed solution. In spray drying operations, C (%, w/v) was defined as total solids concentration in the feed solution. Encapsulation yield (%) is the ratio of total protein present in final particles divided by the total protein present in the feed solution.

Determination of subtilisin concentration and activity

Subtilisin concentration was determined spectrophotomerically at 280 nm against BSA standards in *tris*-HCl buffer. The catalytic activity of subtilisin was determined by measuring release of *p*-nitroanalide at 410 nm from 1 mg/mL *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanalide substrate in 100 mM Tris, 0.005% Tween 80, pH 8.6. Activity was expressed in international units defined as µmol substrate hydrolyzed per min at 25°C and pH 8.6. Subtilisin loaded particles were dissolved in tris buffer, and solution assayed for released activity. The residual activity was determined by dividing the activity after rehydration of the particles, by the activity measured in the stock solution prior to spray drying.

Protein release from particles in GI simulated environment

Protein release was carried out under simulated GI conditions by suspending 10 mg particles into 20 mL 0.1 M HCl at pH 1.2, 37°C for 2 h, followed by transfer to 0.05 M phosphate buffer at pH 6.8 for 3 h. Experiments were performed in triplicate with mixing. At sample points, 1.2 mL aliquots were centrifuged and supernatant assayed for protein content spectrophotometrically (Cary 1, Varian, Australia) at 595 nm using the Bradford modified method (Coomassie plus kit, Pierce, Fisher, Canada). Reaction volume was held constant by addition of 1.2 mL buffer solution following sampling. Released protein and encapsulation efficiency were calculated assuming that all protein was released after 4 h from the point when the particles were initially added to pH 1.2 solution. Encapsulation efficiency was determined by ratio of the initial protein load to the particle formulation to the determined amount after rehydration. Particle yield was determined by dividing the amount of recovered spray-dried particles by the initial mass of solids introduced to the spray dryer.

Particle characterization

Residual moisture content was determined by ratio of the weight difference before and after particle lyophilization. The particles were assumed to be moisture free after lyophilizing for 48 h.

Spray dryed particles were sized using a laser diffraction particle sizer (Malvern Mastersizer 2000 with dry particle sizing accessory, Sirocco 2000, Malvern, UK). For each batch, the mean diameter was determined in triplicate. The size distribution was estimated by a SPAN factor;

$$\text{SPAN} = \frac{(D_{90} - D_{10})}{D_{50}},$$

where D_{90}, D_{50}, and D_{10}, are the mean diameters at which cumulative volume percent of 90, 50, and 10% of the particles are determined. A high SPAN indicates a wide size distribution, whereas a low value indicates a narrow size distribution.

Morphology was examined by scanning electron microscopy (JEOL, JSM-840) with gold coated particles. Protein distribution within the particle matrix was determined by confocal laser scanning microscope (Leica TCS SP2, Germany). A 3 mL amount of BSA-FITC solution (10 mg/mL) was added to a sodium alginate, Ca^{++}, glycol-chitosan solution (alginate/protein ratio of 9:1, for 0.3% Na-A solution). Imaging was performed with dry particles to prevent swelling.

Results and discussion

Alginate solutions containing the proteins BSA, lysozyme or subtilisin, and additives $CaCl_2$ or glycol-chitosan, were spray-dried to produce micron and sub-micron sized particles in a single step operation, and effect of operational parameters investigated. Total solid concentration (2%) and alginate:subtilisin ratio (9:1) in the feed solution was held constant. The effect of inlet air temperature on residual activity of resulting particles consisting of alginate/subtilisin, or subtilisin alone as control is presented in Table 1.

Increasing T_{inlet} from 125 to 175°C, and consequently T_{outlet} from 63 to 81°C, did not significantly affect the final residual activity yield of the subtilisin within particles, as for all temperatures, the activity yield ranged from 76 to 81%. In contrast, when subtilisin was spray-dried alone, the activity yield was 65%, showing that alginate polymer is stabilizing the enzyme. Subtilisin stability can be affected by thermal stress due to the drying air, shear forces in the nozzle during atomization, adsorption between air and liquid interface during generation of new air/water interfaces and dehydration stress caused by the rapid evaporation of the solvent (Lee et al., 2002). Globular proteins such as subtilisin are rigid and resist changes in conformation upon adsorption at air and liquid interface during generation of droplets (Tripp et al., 1995) and show pressure stability up to 1975 atm (Webb et al., 2000). Therefore, activity loss due to conformational changes as a result of adsorption and shear stresses might

Table 1. Residual activity of subtilisin within alginate microparticles produced by spray drying at different T_{inlet}.

T_{inlet} (°C)	T_{outlet} (°C)	Residual activity (%)	Particle recovery (%)	Moisture content (%)
125	63	76 ± 2	21 ± 2	7.1 ± 2
150	69	81 ± 2	33 ± 2	5.6 ± 2
175	81	77 ± 3	37 ± 3	5.5 ± 2
150*	75	34 ± 4	—	—

Protein loading = 0.1 g subtilisin/ g particle.
*Free soluble subtilisin C = 0.2%, w/v.
Q_{lf} = 0.3 L/h, Q_{da} = 38 m³/h, Q_{aa} = 600 L/h, P = 5.44 atm.

be minor compared to dehydration and thermal stresses. Immediately after atomization, the droplet surface temperature approximates the wet-bulb temperature of the inlet air. For the free soluble subtilisin solution which has a low solids concentration, the enthalpy of pure water can be used. For inlet temperature of 150°C, the surface temperature of the droplet value can be estimated to be 41°C, thus subtilisin solution droplets experience much lower temperature than the hottest region of the dryer. However, the maximum temperature of the resulting microparticles can attain the outlet temperature of the drying air where $T_{outlet} = 75$°C, and the time period of exposure of the drying droplets to the elevated temperature ranges from 5 to 30 s. (Broadhead et al., 1992). Polysaccharides such as dextran form an amorphous phase with proteins which limits the conformational changes due to the stresses involved in spray drying (DePaz et al., 2002). Alginate which is a polysaccharide, may also form an amorphous matrix structure around subtilisin, and hydrogen bonding which limits conformational changes caused by dehydration and thermal stresses. Moisture content of the alginate/subtilisin particles was not significantly affected when inlet temperature of the drying air increased, and particle recovery increased as the inlet temperature increased from 125 to 175°C. At 125°C, deposition of particles on the cyclone wall was observed, which might be due to insufficient droplet/particle drying within the drying chamber, resulting in adhesion to the cyclone wall, leading to a lower product yield. At inlet temperatures 150 and 175°C, higher moisture removal and product yield was observed. In both cases, the final moisture content of the particles was around 6%, and about 35% of the particles were recovered in the collection vessel.

Effect of protein loading was examined by varying subtilisin from 0.1 to 0.33 g subtilisin/g particle, keeping the total solid concentration constant. The formulation was spray-dried at inlet temperatures of 150 and 175°C, and the results are presented in Figure 1. The formulations containing 0.1 g subtilisin/g particle showed around 80% residual activity, and as the subtilisin concentration increased to 0.33 g subtilisin/g particle, a decrease in the residual activity was observed. The residual activities for 0.25 and 0.33 g subtilisin/g particle were 75% and 65%. Figure 1A shows that the effect of inlet temperature on the residual activity of subtilisin is minor compared to the ratio of alginate:subtilisin. As the subtilisin in the formulation increases, the alginate concentration decreases, as does the residual activity.

Several mono and disaccharides, and several polyols and amino acids are known stabilizers against water removal stress, including lactose, trehalose, sucrose, mannitol, sorbitol, lysine, histidine, and arginine (Arakawa et al., 1993). Among these examples, trehalose is known to be the best sugar for stabilizing proteins during spray drying (Adler and Lee, 1999). Effect of trehalose loading on residual activity of subtilisin is shown in Figure 1B. The total solids of the feed was kept constant and the trehalose amount changed from 0 to 0.33 g/g particle, with the subtilisin loading at 0.1 g/g particle. The presence of 20% trehalose increases residual activity of subtilisin up to 90% from 80%. Further increase in trehalose did not affect the residual activity. It might be concluded that the mixture of alginate and trehalose appear to promote the stabilization of subtilisin during spray drying. This might be due to the ability of trehalose to hydrogen bond with the proteins in the place of water during dehydration.

The experiment was repeated with a higher subtilisin content (0.33 g subtilisin/g particle). Residual activity increased from 64 ± 3 to 71 ± 4%, when going from 0 to 0.17 g trehalose/g particle, representing a 7% increase in subtilisin activity. More then 60% of the residual activity can be recovered for particles having high protein concentration (0.33 g/g particle). Similar trends were reported using sugar excipients with polysachharides. Barre et al. (1999) spray-dried cold adapted subtilisin in the presence of gum arabic and lactose mixture, observing about 55% absolute increase in residual activity of subtilisin. DePaz et al.

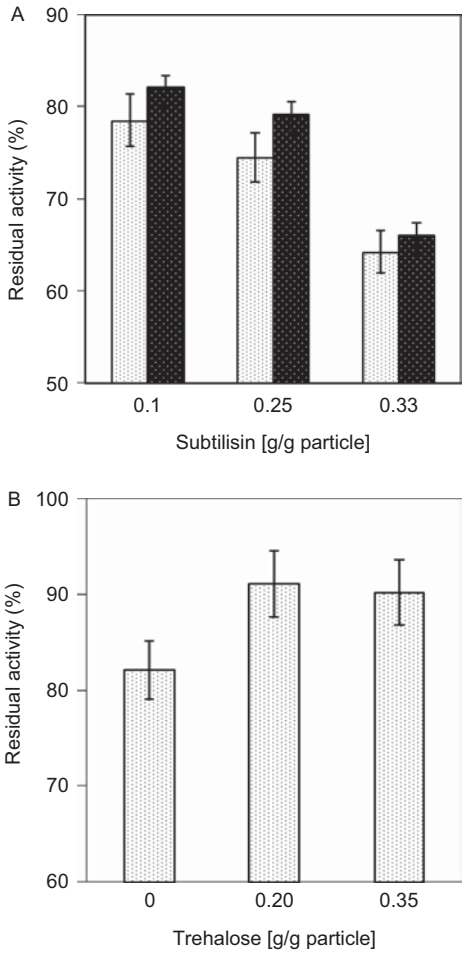

Figure 1. (A) Effect of protein loading at inlet temperature of 175°C (light bars) and 150°C (dark bars) on residual activity of subtilisin. Operating conditions; $Q_{lf} = 300$ mL/h, $Q_{da} = 38$ m^3/h, $Q_{aa} = 600$ L/h, $P = 5.44$ atm. (B) Effect of trehalose loading on residual activity of subtilisin. Operating conditions were the same except total solids concentration in the feed solution kept constant at 2% w/v, and protein loading = 0.1 g subtilisin/g particle.

(2002) reported that dextran-trehalose mixture showed full recovery of subtilisin activity during freeze drying.

The total solid content in the feed solution may affect particle size since it shortens the constant rate period. The change in total solids from 0.2 to 2% in the feed, increased the mean particle size (D[0.5]) from 2.2 to 4.9 μm, still within the appropriate size for intestinal absorption. The SPAN also increased with higher alginate concentration, and thus viscosity, and vice versa. Residual activity and particle recovery decreased around 15%, as the alginate concentration decreased from 2 to 0.2% in the feed. When the particle size is less than 2 μm, the centrifugal force may not be sufficient for recovery in the cyclone separator, allowing some particles less than 2 μm to exit with exhaust air (Prinn et al., 2002). Therefore for particles prepared with low alginate concentration, lower particle recovery resulted. Size distribution of the particles can also be affected, due to insufficient trapping of particles with lower size. If necessary, recovery of the sub-micron particles could be increased with electrostatic or high-efficiency particulate air filters. The outlet temperature increased to 85°C as the alginate in the feed decreased to 0.2% leading to a lower residual activity of encapsulated subtilisin.

The volumetric size distribution of the particles is presented in Table 3. It can be seen that less than 5% of the particles recovered in the cyclone were within the sub-micron range. This doesn't account for sub-micron particles lost to the exhaust. For all concentrations of alginate in the feed, around 90% of the particles are within the range of 1–5 μm, which is the desired size for oral absorption within the intestinal mucosa.

At lower alginate concentration in the feed, the resulting particle size distribution will have a narrower range, which can be seen from the SPAN values in Table 2. However, at lower concentrations, particles will have a smaller size as seen from the mean diameters in Table 3, making particle separation more difficult affecting recovery yields. Thus both mean particle size and volumetric population depend on the alginate feed solution concentration, with particles prepared at lower alginate concentration having narrower size distribution.

Spray-dried particles showed mainly spherical morphology as seen in Figure 2. Particles appeared smooth, although dimpling was evident and more prominent in the 0.2% versus 2% alginate particles. When trehalose was added, less dimpling was observed in larger particles, while smaller particles showed a heavily dimpled, rough surface. Scanning electron microscope (SEM) images also showed that particle diameters ranged between 0.5 and 10 μm which confirms the results of particle size distribution obtained by laser diffraction, as presented in Table 3. Moreover, the particles produced with 0.2% alginate had relatively smaller diameter and showed much smaller variation in size compared to 2% alginate feed solution, again consistent with the differences in mean and SPAN values in Table 3. The dimpled structure of the particles may indicate a hollow center, where the shell thickness is not sufficient to support the pressure difference between the inside and outside of the particle during drying.

Alginate micron and sub-micron particles have been previously produced through nanoemulsion dispersion (Reis et al., 2004), through ionotropic pre-gelation/polyelectrolyte complex coating (Sarmento et al., 2006) and by spray drying followed by coating in liquid suspension (Coppi et al., 2001, 2002, 2004), methodologies involving several complex steps. The method proposed by Coppi et al. (2001) involved spray drying of an alginate/protein solution forming particles which were subsequently subjected to several additional steps to enhance the properties, such as ionic crosslinking of the particles in $CaCl_2$ aqueous solution and treatment of the particles in chitosan solution to reinforce the alginate network and to improve absorption across the intestinal epithelia. Freeze drying was used as a second drying step, to remove water and to recover the particles. These additional steps can alter some of the properties of the particles. For example, the entrapped drug can be released to the aqueous medium during the gelation step, and during surface treatment with chitosan. pH sensitive proteins can be affected due to the solubility requirements of chitosan (pH < 5.5) and lyophylization (freeze drying) causes additional stress on proteins, by changing particle morphology and denaturation through dehydration (Wang, 2000).

Protein loaded particles were formulated in a single step procedure involved introduction of dilute Ca^{++} to an alginate feed solution, along with the active protein and glycol-chitosan, where physical crosslinking of the alginate by dilute calcium solution takes place forming sprayable low viscosity gel. Glycol-chitosan was used as an additive, to further reinforce the alginate network and improve absorption of the particles, as it is water soluble at pH 7.4 and forms polyion complexes with negatively charged polymers, including alginate (Sakai et al., 2000). Using this formulation, alginate particles were loaded

Table 2. Effect of alginate concentration on particle size and residual activity of subtilisin.

Alginate (%)	Viscosity (cp)	T_{out}	D[0.1] (μm)	D[0.5] (μm)	D[0.9] (μm)	SPAN	Residual activity (%)	Particle recovery (g particle/g solids)	Moisture content (%)
0.2	43	85	1.3	2.2	3.6	1.1	68 ± 4	21 ± 2	5.2 ± 2.4
0.5	93	78	1.6	3.7	7.3	1.6	73 ± 3	19 ± 3	5.3 ± 2.1
1	127	73	1.8	4.1	11.8	2.4	74 ± 2	37 ± 3	5.3 ± 2.2
2	189	69	1.9	4.9	13.5	2.3	81 ± 3	33 ± 2	5.6 ± 2.2

Protein loading 0.2 g protein/ g particle.
Operating conditions; Q_{lf} = 0.3 L/h, Q_{da} = 38 m³/h, Q_{aa} = 600 L/h, P = 5.44 atm.

with proteins of molecular sizes less than BSA, namely subtilisin (25–35 kDa) or lysozyme (11.4 kDa).

Particle size, protein loading and morphology were examined in the glycol-chitosan-Ca^{++}-alginate particles, as presented in Table 4. The mean particle sizes (D[0.5]) were similar for all protein formulations, at around 3.5 μm. The particles had smooth surface and spherical morphology, when examined under SEM, similar to those presented in Figure 2.

Protein release profiles were determined for the various formulated particulate proteins, under simulated gastric, followed by GI conditions as shown in Figure 3. All particles released more than 35% of the protein load within 30 min in the gastric simulation, but following that initial release, the remaining protein was fully retained. Alginate collapses, forming an insoluble complex in acid pH, thus the early release is likely associated with the particles forming a compact, and increasingly impermeable structure, at which point the protein is retained. Since burst release is likely due to release of protein from the particle surface, differences in release profiles between proteins with different molecular weights was not expected. After 120 min, the particles were transferred to a simulated intestinal environment at pH 6.8, where swelling of the particles resulted in protein release. Although the positively charged lysozyme (pI = 11.4) and subtilisin (pI = 9.4) had different molecular weights, similar release profiles were observed, possibly due to the large pore size of the resulting particles compared to the molecular size of the proteins. However, BSA showed a slightly slower release profile, which might be due to the larger molecular size of the protein compared to the pore size of the particles. As phosphate buffer saline is destabilizing to alginate due to competition for Ca^{++}, it is expected that the release profile *in vivo* will be considerably dampened compared to that observed in the simulation.

The distribution of the FITC-BSA within the polymer matrix was examined by confocal laser scanning microscopy as shown in Figure 4. The particles show a spherical structure with hollow core, and protein deposition toward the inner and outer particle surface. Deposition of protein toward the particle surface is likely due to the water extraction and evaporation carrying solutes from the particle core to the surface during formation. In addition, surface active substances like proteins tend to move to the surface during the drying process. This might explain, the high load of protein on the particle outer surface and on the inner surface surrounding the vacuole within the particle.

The time to dry a single atomized droplet is expressed in milliseconds for small scale spray dryers (Adler and Lee, 1999). During that extremely short period, solutes can be assumed to remain stationary during particle formation, however, this may not be the case when total solid concentration is low, and when the encapsulation matrix can form porous networks, such as the alginate polymer system.

During spray drying, two characteristic times are important, the first being the time required for a solute to diffuse from the edge of the droplet to its center, defined

Table 3. Size and dimensional distribution of alginate microparticles.

Total solid concentration	2%	1%	0.5%	0.2%
Mean diameter (μm)	4.91	4.12	3.67	2.15
<1 μm(%)	3	3	4	5
1–2 μm (%)	25	31	35	43
2–3 μm (%)	26	24	29	31
3–4 μm (%)	18	17	16	15
4–5 μm (%)	24	17	10	4
5–10 μm (%)	10	8	5	2

Protein loading is 0.1 g protein/g particle.
Operating conditions; $Q_{lf} = 0.3$ L/h, $Q_{da} = 38$ m^3/h, $Q_{aa} = 600$ L/h, $P = 5.44$ atm.

Table 4. Comparison of mean and size distribution of glycol-chitosan-alginate particles carrying different proteins.

Protein	D[0.1]	D[0.5]	D[0.9]	SPAN	Encapsulation (%)
BSA	2.2	3.2	12.8	3.4	96 ± 2
Subtilisin	2.1	3.5	12.1	2.8	94 ± 1
Lysozyme	2.0	3.2	13.1	3.5	95 ± 2

The glycol-chitosan:protein:alginate ratio was 4:2:14. The feed solution contained 0.3% alginate and 10 mM Ca^{++}.
Spray drying conditions; $Q_{lf} = 0.30$ L/min, $Q_{da} = 38$ m^3/h, $Q_{aa} = 600$ L/h, $P = 5.44$ atm.
BSA, bovine serum albumin.

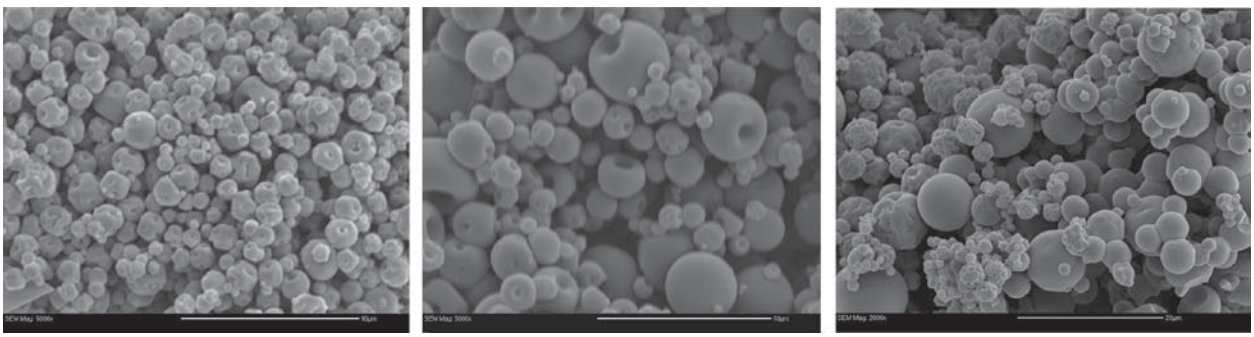

Figure 2. Scanning electron microscope images of spray-dried alginate particles carrying subtilisin. Left, centre and right image show particles prepared with 0.2 and 2% alginate, and trehalose/alginate, respectively. The scale bar for left and centre images is 10 microns and for right image is 20 microns.

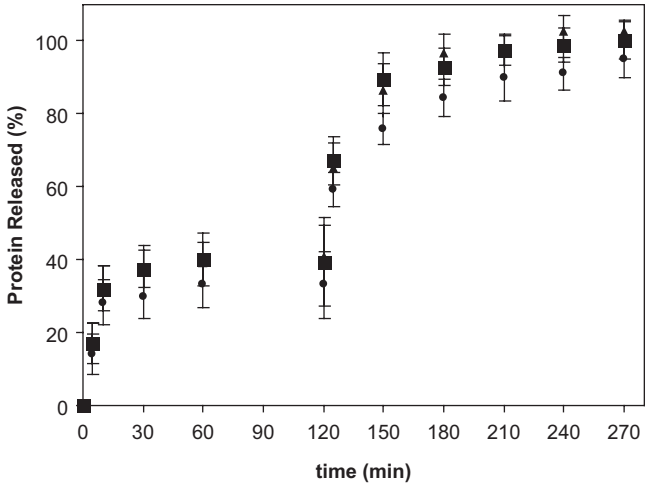

Figure 3. Release profile of bovine serum albumin (•), subtilisin (▲) and lysozyme (■) from glycol-chitosan-Ca^{++}-alginate particles in hydrochloric acid buffer at pH 1.2 for 120 min, followed by phosphate buffer at pH 6.8, simulating gastric, followed by gastrointestinal conditions. Particles were formulated with glycol-chitosan:protein:alginate ratio of 4:2:14 and from 0.3% alginate and 10 mM Ca^{++}. Data shown are the standard deviation of the mean values of a minimum of three repeated experiments.

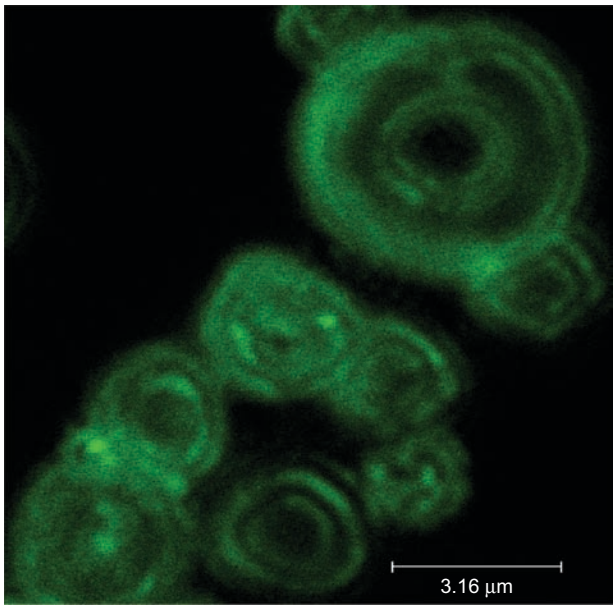

Figure 4. Confocal laser scanning microscope image of glycol-chitosan alginate particles carrying fluorescein isothiocyanate labeled bovine serum albumin.

by R^2/D, where R is the radius of the droplet and D is the solute diffusion coefficient; the second being the total drying time, t_d, for a droplet. The ratio of these two characteristic times defines the Peclet number, $Pe = R^2/ t_d D$ which characterizes the relative importance of diffusion to convection (Vehring, 2008). If the drying time is large, Pe < 1, and solutes have time to diffuse throughout the droplet, yielding relatively dense particles. When Pe > 1, drying time is small and solutes have insufficient time to diffuse from the surface to the center of the droplet (Tsapis et al., 2002). Also when Pe > 1, water molecules must diffuse from the center to the surface, since evaporation only occurs from the surface, carrying solutes from within the droplet toward the surface. In this case, diffusion of solutes from the center to the surface of the particle might be more favorable than the reverse, due to the water serving as mobile phase, diffusing from the particle core to the surface. This would be a stronger possibility, if the solids concentration was low. In addition, the encapsulation matrix which tends to form a porous polymeric network structure, can play a role in the final distribution of solutes within the matrix. For example, if the proteins have a smaller size than the network pores, the protein can migrate along with the water toward the particle surface, during evaporation.

Total drying time of a droplet was calculated by Adler and Lee (1999), using heat and mass transfer equations presented in Masters (1991). The same set of equations was used in the present study to estimate total drying time and calculated as 8.5 ms, and the diffusion coefficient of BSA calculated to be 99 $\mu m^2/s$, using Stokes–Einstein equation (calculation provided on request). Pe was calculated as 240, which indicates that for proteins, the driving force is dominated by convention, instead of diffusion. It might be concluded that the proteins which were at the surface of the droplet during formation of the particle, did not have sufficient time to diffuse from the surface to the center. Moreover, the aqueous mobile phase, continuously diffuses from the center to the surface of the droplet, carrying solutes, such as polymer chains and proteins. Fluorescent label in Figure 4 indicates presence of FITC-BSA. The hollow dark region at the centre of the particles shows that the other solutes, such as polymer chains, were being drawn outwards during evaporation, forming a hollow core. From the confocal image, shell thickness of the particles can also be seen, sufficient to withstand the stresses involved during particle formation, such as pressure differences between inside and outside the particle. In addition particles had a mean size lower than the critical diameter necessary to be orally absorbed by M cell's of the Peyer's patches in

the GI tract making them promising technology for oral protein delivery.

Conclusion

Protein loaded micron sized alginate particles were produced by spray drying. Particles were formed with mean diameter less than 3.5 μm, which is a desired range for oral protein drug delivery and can be produced in a single step process. Residual activity of model protein subtilisin was up to 80% of the initial activity. Formulations containing 0.2 g trehalose/g showed residual activity of 90%. Increase in the amount of trehalose (0.33 g trehalose/g particle) did not significantly affect residual activity. Alginate concentration in the feed solution affected the particle size. Particles prepared with less alginate had smaller mean diameter and narrower size distribution.

Glycol-chitosan-Ca^{++}-alginate particles were produced in a single step process by spray drying. The particles showed 35% protein release in a simulated gastric environment. *In vitro* release profiles of the resulting particles did not show significant variation for different molecular weight proteins. Total drying time for particles was calculated as 8.5 ms and the diffusion coefficient of the BSA molecule was estimated to be 99 $\mu m^2/s$. Pe was calculated as 240, which indicated that aqueous mobile phase, continuously diffused from the center to the surface of the droplet, carrying polymer chains and proteins. Confocal laser scanning microscope images confirmed FITC-protein concentrating toward the outer particle surface with a hollow core. Particles had a mean size lower than the critical diameter necessary to be orally absorbed by M cell's of the Peyer's patches in the GI tract and thus can be considered as a promising technology for oral peptide and protein delivery.

Declaration of interest

Financial support of the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

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