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Novel alginate gel microspheres produced by impinging aerosols for oral delivery of proteins

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Abstract

Lysozyme and insulin were encapsulated in alginate gel microspheres using impinging aerosols method. High loadings of around 50% weight/dry microspheres weight were obtained with encapsulation efficiencies of at least 48%. Environmental scanning electron microscopy revealed smooth spherical hydrated microspheres (30–60 μm) in diameter. No lysozyme or insulin release was measured in simulated gastric fluid (HCl, pH 1.2, 37°C). Total insulin release occurred in simulated intestinal fluid (SIF; phosphate buffer saline, pH 7.4, 37°C) in 8 h following 2 h incubation in SGF and was found to retain 75% activity using the ARCHITECT® assay. Lysozyme was released completely in SIF in 10 h following 2 h incubation in SGF and was found to exhibit at least 80% bioactivity using the *Micrococcus lysodeikticus* assay. The absence of protein release in HCl and the retention of high levels of biological activity demonstrate the potential of alginate gel microspheres, for improving oral delivery of biopharmaceuticals.

Keywords: alginate gel microspheres, aerosols, lysozyme, insulin, protein release, bioactivity, ARCHITECT assay

Introduction

Successful oral delivery of therapeutic proteins such as insulin, calcitonin and growth factors continues to define one of the leading challenges in drug development and presents a major obstacle to their wider clinical use. The low oral bioavailability of proteins stems in part from their large molecular size and generally high hydrophilicity, which restricts transcellular transport by partition and passive diffusion across the intestinal epithelium. Oral bioavailability is poor for molecules of mass greater than several hundred daltons. In addition, proteins are susceptible to hydrolytic degradation by low gastric pH and proteolytic breakdown by digestive enzymes such as trypsin and alpha-chymotrypsin. The mucus layer or glycocalyx of the small intestine and lysosomal proteases within epithelial cells present further barriers. These factors have prompted a variety of strategies aimed at improving bioavailability including the co-administration of protease inhibitors (e.g. aprotinin, trypsin inhibitor) and absorption enhancers (e.g. bile salts, medium chain glycerides; Lee, 1992; Lueßen et al., 1996; Aungst, 2000; Babu et al., 2008). The latter approach is problematical, however, because of the potential for transport of potentially toxic metabolites from the gastrointestinal (GI) tract that are produced by several species of intestinal bacteria. Proteins have been conjugated with functional groups such as vitamin B12 (Russell-Jones, 1998) to exploit endogenous transport mechanisms across the intestinal epithelium and by the covalent addition of groups that improve lipophilicity (Clement et al., 2002; Kipnes et al., 2003). The latter approach has shown efficacy by influencing glucose levels in type I and type II diabetic patients, administered oral insulin formulations. Small organic molecules

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(200–400 Da) such as amino acids and dipeptide derivatives have also been exploited as transport mediators or carriers resulting in improved oral absorption of insulin, heparin and calcitonin in human clinical trials (Wu and Robinson, 1999; Abbas et al., 2002). Since protein modifications do not prevent exposure of the macromolecule to hydrolytic and proteolytic environments in the intestinal lumen, microencapsulation has been widely investigated to protect proteins and present high concentrations of active compounds at absorption surfaces, thereby enhancing the prospects for transport across the epithelium. Encapsulated oral protein formulations have encompassed a wide range of polymers and carrier systems including liposomes, pH responsive hydrogels and microemulsions (Damge et al., 1988; Masuda et al., 2002; Toorisaka et al., 2003; Vauthier et al., 2003; Morishita et al., 2006). Alginate polysaccharides in particular have featured strongly in such studies because of the polymer's processability by completely water-based methods, a mild cross-linking reaction based on divalent metal ions, favourable biocompatibility and generally regarded as safe status as a food additive (Tonnesen and Karlsen, 2002). However, conventional extrusion methods in which alginate droplets are sprayed or fall into cross-linking solution, can give rise to microspheres of irregular shape and porosity (Onal and Zihnioglu, 2002; Martins et al., 2007) due to impact of the droplets with the solution. Emulsification methods involving organic solvents, heat and shear forces may denature proteins and raise toxicity issues (You et al., 2001; Silva et al., 2006a; Reis et al., 2007a; Schoubben et al., 2009).

We previously encapsulated small molecule hydrophilic (gentamicin sulphate, MW 477.6) and hydrophobic drugs (ibuprofen, MW 206.2) in alginate gel microspheres using a new impinging aerosols technique and maintained drug activity during microspheres incubation in simulated intestinal fluids (Bhandari, 2009; Hariyadi et al., 2010). This study describes the encapsulation of insulin and lysozyme in sub-60 μm alginate microspheres using the impinging aerosols method. Currently, insulin (MW 5.73 kDa) is delivered subcutaneously to control glucose levels in diabetic patients but attempts to develop oral formulations to eliminate the discomfort of injection have stretched over several decades. Lysozyme (MW 14.3 kDa) possesses antibacterial and antiviral properties (Zorzin et al., 2006) and has been used extensively as a model therapeutic since its activity may be measured simply and reliably. The influence of formulation conditions on microsphere morphology, protein loading, release behaviour in SIFs and bioactivity is described to assess the utility of alginate microspheres, prepared using the impinging aerosols technique, for oral delivery of protein therapeutics.

Materials and methods

Materials

Sodium alginate (Protanal SF 120 L, MW 204 kDa, guluronate/mannuronate ratio (G/M = 2:1), viscosity of a 1% aqueous solution = 400–600 mPas) was provided by Swift and Company Limited, Mulgrave, Victoria, Australia. Calcium chloride (UNILAB Taren Point, NSW, Australia), hydrochloric acid (UNIVAR) and sodium hydroxide were obtained from Ajax Finechem Pty Ltd (NSW, Australia). Phosphate buffer saline (PBS) was obtained from Amresco Inc. (Solon, OH). Lysozyme (from chicken egg white), insulin (from bovine pancreas), Bicinchoninic acid (BCA) solution, Copper (II) sulphate solution and *Micrococcus lysodeikticus* cell suspension were purchased from Sigma–Aldrich, Castle Hill, NSW, Australia.

Production of protein-loaded alginate gel microspheres using the impinging aerosol technique

Lysozyme solution in distilled water (20 mL, 0.5%w/v) was added to sodium alginate solution in distilled water (200 mL, 1.5%w/v). Insulin (50 mg) was first dissolved in 0.1 M HCl (10 mL), adjusted to pH 7 with 0.1 M NaOH, then added to sodium alginate solution in distilled water (100 mL, 1.5%w/v). The co-solutions of protein and alginate were subsequently sprayed from an upper nozzle (flow rate 12 mL/min, pressure 75 Pa) into an aerosol mist of $CaCl_2$ solution produced by a lower nozzle (flow rate 9 mL/min, pressure 50 Pa) (Figure 1). The resulting alginate gel microspheres which formed in the spray chamber were collected and characterised in terms of particle size (Malvern Mastersizer), morphology (light microscopy and ESEM, environmental scanning electron microscopy), protein loading, *in vitro* release behaviour in SGF and SIF and protein activity.

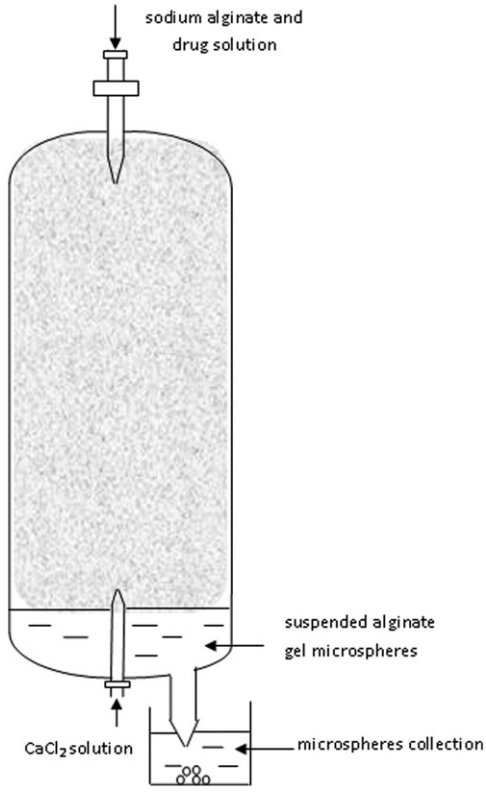

Figure 1. Diagram of impinging aerosol technique for production of alginate gel microspheres.

Measurement of protein loading of alginate gel microspheres

Insulin or lysozyme loading of alginate gel microspheres was analysed using the BCA total protein assay, which is based on the principle that Cu^{2+} reduction to Cu^{+} by protein in an alkaline environment is proportional to the concentration of protein. BCA forms a purple complex with Cu^{+}, which strongly absorbs light at a wavelength of 562 nm, and provides a colorimetric assay of protein concentration. The insulin or lysozyme loading of alginate gel microspheres was determined following breakdown of 1 mL aliquots of suspension over 24 h at room temperature in 0.5 M sodium citrate solution (2 mL, pH 8.5). The content of insulin or lysozyme was determined using the BCA total protein assay (Cary 50 Bio UV spectrophotometer, Varian Australia) and calculated by comparison with a calibration curve constructed using a series dilution of each protein in sodium citrate (concentrations of 5, 10, 15, 20, 25 μg/mL for insulin and 125, 200, 250, 400, 800 μg/mL for lysozyme), Protein loading was expressed as %w/w of the dried alginate microspheres.

In vitro *studies of protein release in SIF*

Suspensions (1 mL) of insulin or lysozyme-loaded alginate gel microspheres in triplicate were incubated in 1 mL PBS (pH 7.4) in 10 mL polypropylene tubes at 37°C for 10–16 h. The release medium was replaced completely by fresh PBS every 20 min in the first 2 h then every hour until the conclusion of the test. The concentration of insulin or lysozyme in the release medium was determined using the BCA total protein assay as described above (''Measurement of protein loading of alginate gel microspheres'' section). The amount of lysozyme or insulin released from the microspheres was calculated by comparison with a calibration curve constructed using a series dilution of each protein in PBS and expressed as cumulative release (%) versus time (h).

In vitro *studies of protein release in simulated gastric fluid and SIF*

Release studies were carried out in simulated gastric fluid (SGF) followed by SIF. Suspensions (1 mL) of insulin or lysozyme-loaded alginate gel microspheres in triplicate were incubated in 1 mL HCl (0.05 M, pH 1.2) at 37°C for 2 h. The HCl release medium was replaced with 1 mL of PBS and the PBS medium was subsequently replaced with fresh PBS every 1 h in the first four hours then every 2 h until the conclusion of the test. The concentration of protein in SGF and SIF was determined using the BCA assay as described above. The amount of insulin or lysozyme released was calculated by comparison with a calibration curve constructed using a series dilution of each compound in HCl or PBS and expressed as cumulative release (%) versus time (h).

Measurement of particle size

Alginate gel microspheres suspensions were analysed to provide data on mean particle size and size range using low angle laser light scattering (Mastersizer 2000, Malvern Instruments, UK).

Morphology of protein-loaded alginate microspheres

Alginate gel microspheres were examined using optical microscopy (Olympus BH-2, Japan), with camera attachment (C-5050 Olympus) and ESEM (FEI Quanta 200, USA) in environmental mode using an accelerating voltage of 20 kV and 8 mm working distance. The sample chamber pressure was set to 5.0 Torr and the chamber gas was water vapour. Samples were examined over a range of hydration conditions by varying the pressure and temperature within the sample chamber and the output from a gaseous secondary electron detector was used for image construction.

Activity of lysozyme released from alginate gel microspheres

The activity of lysozyme released from alginate gel microspheres in PBS was evaluated using the fluorescent *M. lysodeikticus* assay (Sigma Aldrich, St. Louis, MO, USA). Lysozyme activity results in cell wall lysis, which reduces the turbidity of the cell suspension and yields a dramatic increase in the fluorescence caused by labelled *M. lysodeikticus* cells, in proportion to lysozyme activity (Salton, 1952). A fluorescence labelled *M. lysodeikticus* cell suspension in PBS (0.01%w/v) was used as the substrate working suspension. Lysozyme standard solutions in PBS were used to prepare a series dilution for construction of a calibration curve of activity (400–900 U/mL) versus enzyme concentration. Release media containing lysozyme were collected and stored at −20°C prior to determination of the enzyme concentration by BCA assay and subsequently activity testing. Lysozyme standard solutions, calibration and test samples (100 microL) were added to *Micrococcus* suspension (2 mL) for spectrophotometric analysis at 450 nm (Cary 50 Bio, Varian, Australia). Readings taken over a 5 min time period were converted into activity values using Varian® Enzyme Kinetics software. The relative activity of lysozyme released from the gel microspheres was determined by comparison with the activity of fresh lysozyme solution in PBS of the same concentration. Measurements were performed in triplicate for each sample of release medium and the mean was calculated.

Insulin immunoassay

The bioactivity of insulin released from alginate gel microspheres was measured using the ARCHITECT® one-step chemiluminescent immunoassay (Abbott Laboratories Inc., Abbott Park, IL, USA). The release sample, paramagnetic microbeads (coated with anti-insulin monoclonal antibody) and acridinium-labelled anti-insulin monoclonal antibody conjugate are combined to form a microbead-insulin-conjugate sandwich. Unbound materials are removed by washing. The addition of pretrigger reagent (containing hydrogen peroxide) and trigger reagent (containing sodium hydroxide) leads to acridinium-produced chemiluminescence, measured as relative light units, which is proportional to the insulin concentration in the sample. Calibration samples (0–300 μU/mL concentration) are measured in duplicate to generate a calibration curve according to a four-parameter logistic curve (Y

Table 1. Protein loading, microspheres yield and encapsulation efficiency of alginate gel microspheres.

Concentration of $CaCl_2$ (M)	Protein loading (%w/w)		Yield of microspheres (%)		Encapsulation efficiency (%)	
	Lysozyme	Insulin	Lysozyme	Insulin	Lysozyme	Insulin
0.1	38.4–48.2	34.5–54.4	32.9–37.4	45.3–46.5	50.1 ± 9.0	48.4 ± 1.2
0.25	32.9–37.0	46.1–52.7	35.5–41.9	51.1–52.3	53.8 ± 4.7	48.0 ± 4.7
0.5	35.6–41.8	56.1–62.1	38.7–48.4	51.3–55.0	48.0 ± 6.5	50.0 ± 4.8

Note: Protein loading measured relative to dry microspheres.

weighted) method. The concentration of active insulin in standard solutions in PBS and HCl and the concentration of active insulin released into PBS media (1, 4 and 8 h) and into PBS following 2 h incubation in HCl was determined. Samples were diluted with non-fat, dry milk powder solution (60 μg/mL) prior to assay to eliminate non-specific binding to the anti-insulin antibody coated beads. Each sample was measured twice in a 1000-fold serial dilution and active insulin concentration was calculated in units of μU/mL. The relative activity of insulin released from the alginate gel microspheres was calculated by comparison with the activity of insulin standard solution of the same concentration.

Results and discussion

Characteristics of protein-loaded alginate gel microspheres

The impinging aerosols method brings droplets of alginate solution in contact with a fine mist of $CaCl_2$ cross-linking solution to induce gelation. Impact of droplets with the surface of cross-linking solution is avoided, thereby resulting in microspheres of uniformly spherical form. High protein loadings of around 50% (w/w; dry particles) were measured for both lysozyme and insulin (Table 1) and no significant differences were apparent on changing the concentration of the $CaCl_2$ cross-linking solution. The yield of microspheres calculated by comparison of the weight of alginate plus protein in the aerosol solution and the dry weight of collected particles, was typically 33–48% for lysozyme-loaded microspheres and 45–55% for insulin-loaded microspheres. Loss of material is explained by adhesion to the walls of the spray chamber. The 20% lower yield of lysozyme-loaded microspheres may be due to changes in spray geometry caused by the positively charged lysozyme molecules (p*I* 11.0) interacting with the negatively charged carboxyl groups along the alginate chain but requires further investigation. Insulin (p*I* 5.3) is negatively charged at the pH (7.4) of the aerosol solution (pH 7.4). Encapsulation efficiencies were obtained by comparing the weight of encapsulated protein with the starting weight in the alginate aerosol solution and were at least 48% for both proteins.

Optical microscopy revealed the smooth, roughly spherical form of both hydrated unloaded and protein-loaded alginate gel microspheres produced using the impinging aerosols method (Figure 2). The microspheres were generally in the range of 30–60 microns. The average size of unloaded and lysozyme-loaded microspheres, measured using low angle laser light scattering, was consistently around 36 microns and independent of the concentration of $CaCl_2$ cross-linking solution (Table 2). The d_{90} values however indicate aggregation of a fraction of the microspheres within the sample. Use of a low concentration (0.1 M) $CaCl_2$ solution resulted in an increase in size of insulin-loaded alginate microspheres. This behaviour may be indicative of electrostatic repulsion effects between insulin and alginate macromolecules (having the same charge) which cause expansion of the lower cross-link density gel.

Lysozyme-loaded microspheres exhibited minor swelling after incubation in PBS for 1 h but major expansion occurred on incubation in PBS at 37°C for 6 h. Expansion characteristics were independent of the concentration of $CaCl_2$ solution used to cross-link the microspheres (Table 3). Insulin-loaded microspheres exhibited a more variable swelling behaviour in PBS. Microspheres cross-linked using 0.5 M $CaCl_2$ solution were more resistant to swelling (53% in 6 h) than lysozyme-loaded particles (150% in 6 h) whereas microspheres cross-linked using lower concentration $CaCl_2$ solutions (0.1 and 0.25 M) swelled rapidly in the first hour by around 80–90%. The swelling behaviour of alginate gel microspheres and beads on exposure to simulated body fluids such as PBS has been widely documented and explained by a process of exchange/displacement of Ca^{2+} ions by monovalent Na^{+} cations, which reduces the cross-link density of the alginate gel and subsequently the gel strength (Martinsen et al., 1989; Tonnesen and Karlsen, 2002; Shilpa et al., 2003). The increased swelling tendency of insulin-loaded microspheres in the first hour in PBS suggests that the presence of negatively charged insulin macromolecules decreases the alginate gel strength and density by electrostatic repulsion effects involving the negatively charged alginate chains and augments the reduction in cross-link density caused by ion exchange. The lower swelling tendency of insulin-loaded microspheres cross-linked using 0.5 M $CaCl_2$ cannot be explained by this mechanism, but instead infers that Ca^{2+} ions displacement from the more highly cross-linked gel network by ion exchange is impeded by the presence of insulin.

ESEM was employed to provide additional information on the size and morphology of hydrated alginate gel microspheres. The decided advantage of ESEM compared to conventional SEM is that samples can be analysed in the hydrated state without coating and investigations of material behaviour can be made dynamically under controlled

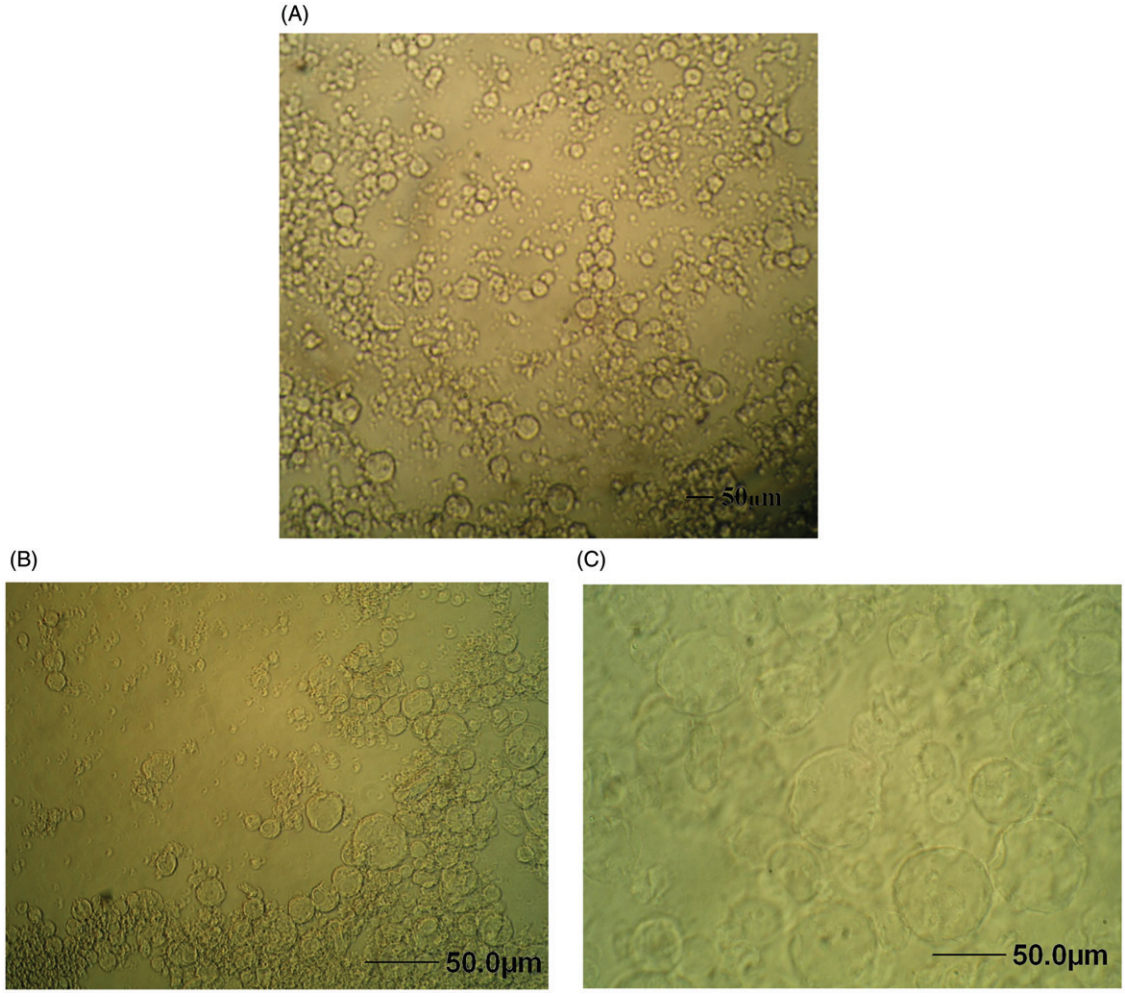

Figure 2. Optical micrographs of hydrated: (A) blank, (B) lysozyme-loaded and (C) insulin-loaded alginate gel microspheres.

Table 2. Particle size of hydrated lysozyme and insulin-loaded alginate gel microspheres.

Protein	d_{10}			d_{90}			Average size (μm)		
	Concentration of $CaCl_2$ (M)								
	0.1	0.25	0.5	0.1	0.25	0.5	0.1	0.25	0.5
Lysozyme	19.9	16.9	17.6	193.3	251.1	147.6	37.2	36.2	36.1
Insulin	20.6	15.1	14.9	300.2	329.8	286.5	45.3	34.2	32.9

Notes: d_{10}: 10% and d_{90}: 90% of particles smaller than this value.
Average size is the surface weighted mean $D[3,2]$.

Table 3. Effect of incubation period on the size of hydrated protein-loaded alginate gel microspheres.

Timescale	Lysozyme-loaded (μm)			Insulin-loaded (μm)		
	Concentration of $CaCl_2$ (M)					
	0.1	0.25	0.5	0.1	0.25	0.5
After 1 h incubation in PBS at 37°C	43.4	37.2	36.2	82.9	67.2	37.4
After 6 h incubation in PBS at 37°C	88.3	85.4	90.4	92.6	84	50.4
After 1 h incubation in HCl at 37°C	37.3	37.3	36.2	55.8	35.1	33.4

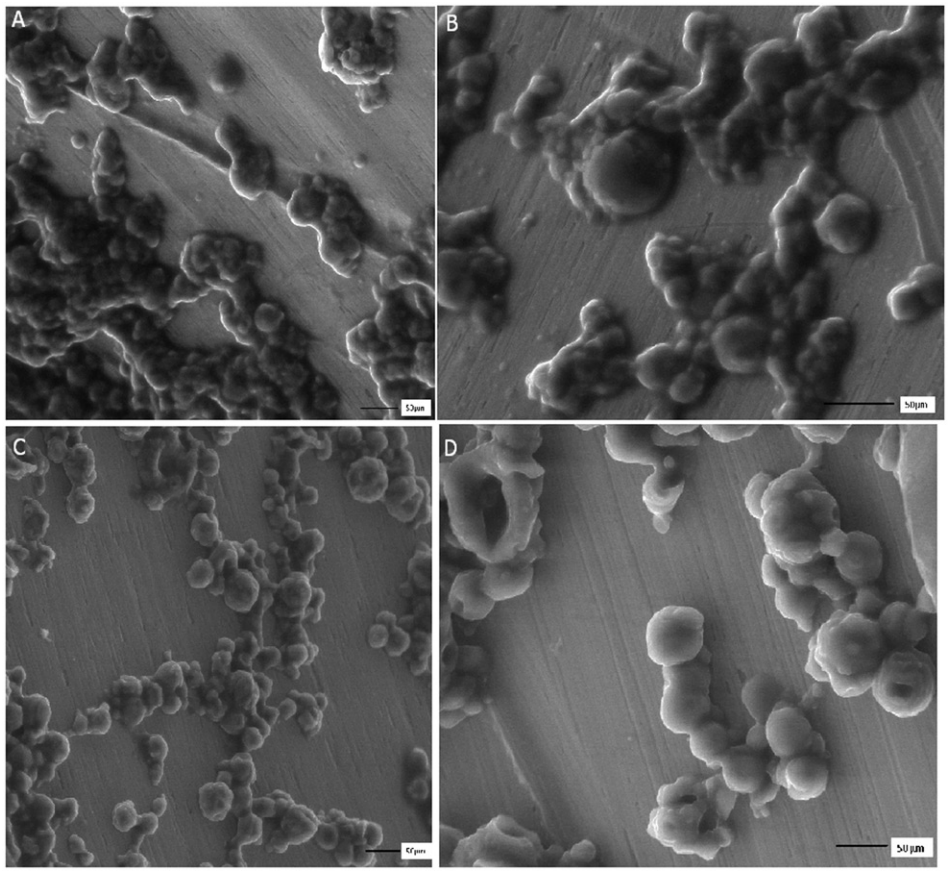

Figure 3. ESEM images of (A, B) hydrated insulin-loaded alginate gel microspheres cross-linked using 0.5 M $CaCl_2$ (C, D) hydrated lysozyme-loaded alginate gel microspheres cross-linked using 0.5 M $CaCl_2$.

humidity atmospheres. The sequence of ESEM images of hydrated insulin-loaded and lysozyme-loaded alginate gel microspheres in Figure 3 captures the dehydration behaviour. Lowering the vapour pressure in the sample chamber results in gradual removal of the water phase, allowing visualisation of the spherical shape and smooth surface of the microspheres, which are in the 10–40 μm size range. Lysozyme-loaded microspheres appeared more resistant to dehydration than insulin-loaded particles as indicated by retention of the spherical form for longer dehydration times. This behaviour suggests that lysozyme may stabilise and strengthen the alginate gel structure by electrostatic attraction compared with insulin-loaded microspheres.

Protein release from alginate gel microspheres

Lysozyme-loaded alginate gel microspheres prepared using 0.1 and 0.25 M $CaCl_2$ cross-linking solution, exhibited fairly rapid release of 60–70% of the enzyme load in 2 h in PBS (pH 7.4, 37°C) and complete release occurred in 6 h (Figure 4). More gradual loss of enzyme occurred from alginate gel microspheres cross-linked using a higher concentration of $CaCl_2$ (0.5 M) and was sustained for 8 h. Protein release in this case was confined to approximately 40% of the initial load at 2 h. This behaviour may be explained by a lower initial cross-link density for alginate gels formed using lower $CaCl_2$ concentrations, which facilitates expansion of the gel network and subsequent diffusion of lysozyme into the external medium. The study by Aslani and Kennedy (1996) clearly illustrated the significant influence of cross-link density on drug release from hydrated alginate films. A five-fold decrease in the permeability to acetaminophen ensued when the concentration of divalent metal cations was increased from 0.1–0.7 M.

When lysozyme-loaded microspheres were retained in SGF (pH 1.2, 37°C) for 2 h, no enzyme release was detected from alginate microspheres prepared using 0.5 M $CaCl_2$ solution (Figure 5) and less than 8% release was measured for microspheres cross-linked using lower $CaCl_2$ concentrations (0.1 and 0.25 M). Gradual but complete release of lysozyme subsequently occurred in PBS (pH 7.4) in 10 h for alginate microspheres cross-linked using 0.5 M $CaCl_2$ solution and in 8 h for microspheres cross-linked using 0.1 and 0.25 M $CaCl_2$ solution. This finding indicates that therapeutic macromolecules may be protected from breakdown and loss of activity in gastric fluid following oral administration and transported into the small and large intestine for absorption across the epithelium or to provide local therapeutic effect.

The release profiles of lysozyme are similar for alginate microspheres either incubated in PBS (Figure 4) or in HCl followed by PBS (Figure 5), but the release process is extended for around 2 h under the latter conditions.

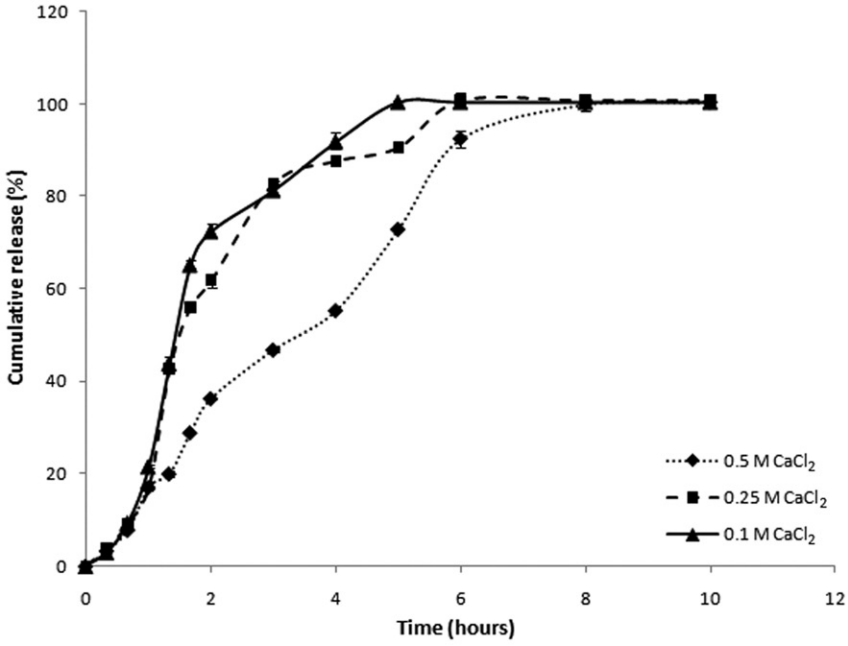

Figure 4. Release of lysozyme from alginate gel microspheres in PBS at 37°C.

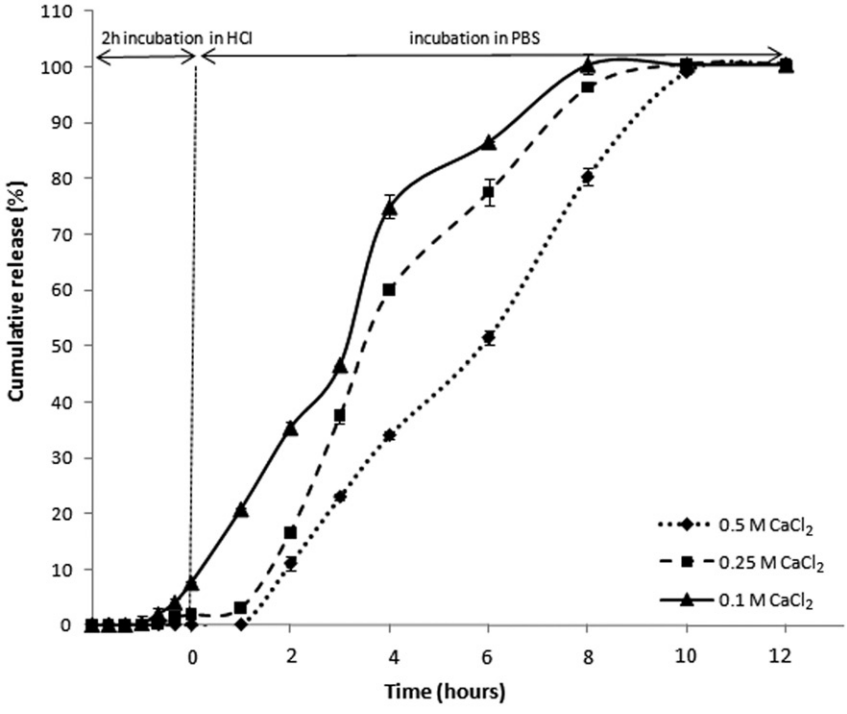

Figure 5. Release of lysozyme from alginate gel microspheres during 2 h incubation in HCl followed by incubation in PBS at 37°C.

A possible mechanism giving rise to this behaviour involves conversion of carboxyl groups along the alginate chain to the unionised form at low pH, accompanied by displacement of Ca^{2+} ions from junction points in the gel network. On transfer to PBS, competitive binding between Ca^{2+}, Na^{+} and positively charged lysozyme macromolecules (p*I* 11.0) with negatively charged carboxyl groups along the alginate chain may increase protein interactions with the gel network and impede lysozyme diffusion from the microspheres. The lag phase of around 1 h observed prior to lysozyme release, from microspheres cross-linked using 0.25 and 0.5 M $CaCl_2$ solution may result from the extra time required for exchange of Ca^{2+} by Na^{+} ions in the more highly cross-linked gels, to allow expansion of the network structure.

Zorzin et al. (2006) produced chitosan-coated alginate microspheres containing positively charged lysozyme (p*I* 11.0) using an emulsification method. Spherical particles

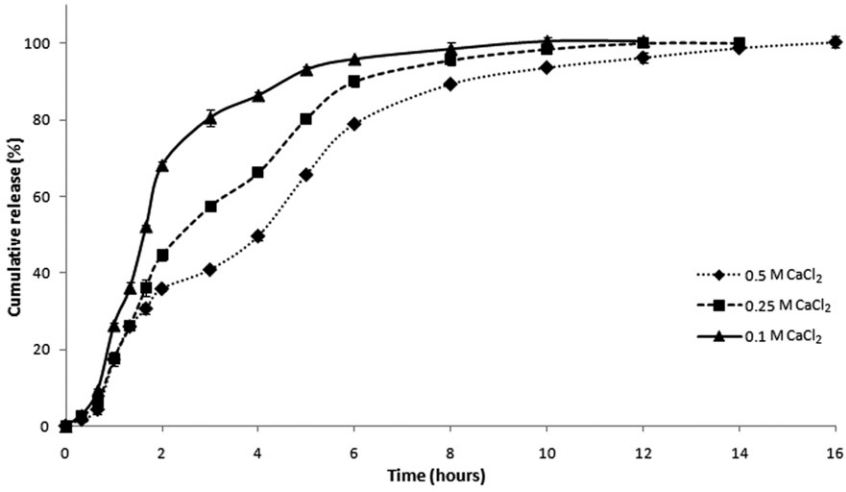

Figure 6. Release of insulin from alginate gel microspheres in PBS at 37°C.

less than 5 μm were produced but release of a major fraction of the enzyme load (45%) occurred at pH 3, indicating poor protective capacity in the gastric environment (Zorzin et al., 2006). Wells and Sheardown (2007) encapsulated lysozyme by immersing blank alginate microspheres in an alternative approach a solution of the enzyme in 0.15% sodium chloride solution. Lysozyme release in SGF was not reported but rapid release of lysozyme occurred in PBS in 3 h, amounting to 90% of the initial loading. Mumper et al. (1994) also used alginate to encapsulate a positively charged protein (TGF-β, pI 9.8) for oral delivery and found that gradual release of around 80% of the protein load occurred from the microspheres over 24 h in PBS but not in HCl. Complete release occurred in less than 2 h in PBS following incubation of microspheres in HCl but little protein activity was measured using an ELISA assay of antibody/protein binding. This behaviour may be explained by the extended microspheres residence time of 24 h in HCl prior to PBS, which is expected to result in swelling of alginate and consequently denaturation of protein in the low pH environment.

Insulin-loaded alginate microspheres exhibited a similar release pattern to lysozyme in PBS but the duration of release tends to be extended for insulin (Figures 4 and 6). Around 90% of the protein load is delivered in 8 h from microspheres cross-linked using 0.5 M $CaCl_2$ and release is sustained for a further 6 h. As expected, the rate of insulin delivery increases with decreasing concentration of $CaCl_2$ used in microspheres production, corresponding with the lower density of the alginate gel network, which facilitates protein diffusion.

Insulin release was generally inhibited in SGF. No insulin was detected by BCA assay in the release medium when insulin-loaded alginate gel microspheres prepared using 0.5 M $CaCl_2$ were incubated for 2 h in SGF (Figure 7). Minor release of insulin (less than 6%) was found for microspheres prepared using 0.1 and 0.25 M $CaCl_2$ corresponding with the swelling behaviour recorded in Table 3. Restricted drug release from alginate gel beads and microspheres at low pH has been widely reported (Martinsen et al., 1989; Tonnesen and Karlsen, 2002; Shilpa et al., 2003) and results from the production of high viscosity alginic acid gels, which display minor expansion under such conditions. Protonation of carboxylic acid groups effectively reduces electrostatic repulsion between alginate molecules, resulting in a more compact network which retards drug diffusion (You et al., 2001). On transfer to PBS, complete release of insulin was recorded in 6–8 h. This findings provide further indications that therapeutic polypeptides such as insulin may be protected from breakdown and loss of activity in gastric fluid following oral administration and transported at high concentrations into the small and large intestine for absorption or to provide therapeutic effect.

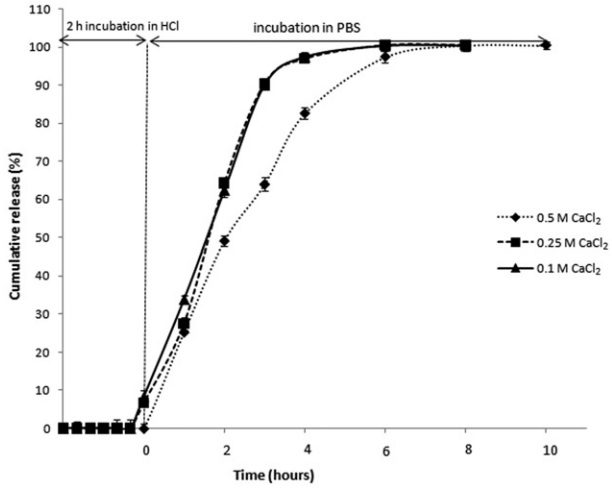

Figure 7. Release of insulin from alginate gel microspheres during 2 h incubation in HCl followed by incubation in PBS.

When samples were incubated in PBS following 2 h incubation in HCl (simulating transfer from gastric to intestinal fluid), the rate of insulin release increased significantly compared with microspheres incubated only in PBS (Figures 6 and 7). For example, approximately 80%

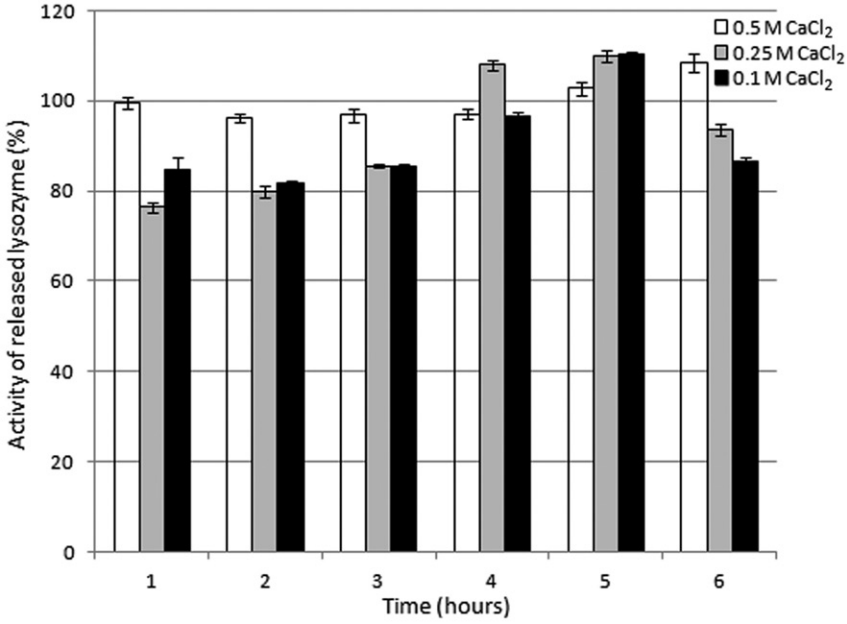

Figure 8. Activity of lysozyme released from alginate microspheres in PBS.

and over 90% of the insulin load, respectively, was released in 4 h from microspheres cross-linked using 0.5 and 0.25 M $CaCl_2$, which were incubated in PBS following 2 h in HCl. This compares with cumulative release amounts of 50% and 70% for microspheres incubated only in PBS. Complete insulin release was achieved in around 6 h when microspheres were retained in HCl followed by PBS, compared with 12 h for microspheres incubated in PBS alone. The proposed mechanism involves exclusion of Ca^{2+} ions from binding sites with alginate macromolecules at low pH and competitive binding between Ca^{2+} and Na^{+} ions at higher pH in PBS, resulting in reduced gel cross-link density. This condition facilitates insulin diffusion through the gel network.

This data clearly demonstrate the marked effect of exposing alginate gel microspheres to low pH conditions in accelerating release of certain proteins in a more neutral environment (PBS). *In vitro* release studies involving alginate microspheres generally expose separate samples to SGF and SIF rather than exposing the same sample in sequence to these media to mimic (in a basic fashion) movement of the dosage form along the GI tract. Potentially, important changes in release characteristics will not be detected by the former approach and predictions of *in vivo* behaviour may be rendered invalid. These findings hold particular significance for formulations designed to deliver proteins which show optimum absorption in specific regions of the intestinal tract.

Several studies have sought to protect insulin from degradation in the acidic environment of the GI tract by encapsulation in alginate microspheres. Builders et al. (2008) prepared large (260–860 μm) enteric-coated alginate/mucin microspheres loaded with human insulin by diffusion of the drug into empty microspheres. Microspheres were prepared by a complicated coacervation approach which involved dispersing sodium alginate/mucin solutions in liquid paraffin followed by addition to acetone at −30°C and finally enteric coating using solutions of cellulose acetate phthalate in acetone. Despite loading the microspheres in hard gelatin capsules, almost 40% of the insulin content was released in SGF (pH 2.2) in 1 h indicating poor protection efficiency and complete insulin release occurred in 2 h in SIF (pH 6.5). The emulsification/internal gelation method investigated by Silva et al. (2006a, b) and Reis et al. (2007b) has also proved highly inadequate for protecting insulin against the low pH conditions of the GI tract. This method involves homogenisation of a suspension of $CaCO_3$ in alginate solution in paraffin oil containing Span 80 as a surfactant. Addition of glacial acetic acid solubilises the $CaCO_3$ producing calcium ions for gelation of the alginate solution droplets within the oil phase. Microspheres are harvested using acetate buffer (pH 4.5). Although microspheres may be produced in the size range 20–70 μm or less than 10 μm depending on process conditions, major loss of 75–100% of the insulin content was reported in SGF in 5 min, indicating association of the protein with the microspheres surface. Application of the conventional ionotropic gelation method which involves extrusion of droplets of alginate solution into a $CaCl_2$ cross-linking solution has proved more successful for limiting insulin release in SGF and allowing gradual release in SIF. Less than 6% of the insulin load was released from the large insulin-loaded alginate microspheres prepared by Martins et al. (2007) in SGF in 2 h and almost 90% was released in SIF (pH 6.8) in 2 h. Similarly, Onal and Zihnioglu (2002) limited insulin release to 30% in SGF in 2 h and obtained gradual release in SIF over 6 h by including chitosan to the $CaCl_2$ cross-linking solution. Formation of a polyelectrolyte complex between the negatively charged alginate and positively charged chitosan molecules at the microspheres surface was considered to stabilise the gel and reduce its porosity. The insulin-loaded

microspheres investigated in the above studies were around 1800 and 1000 μm in size, respectively, which does not favour efficient interaction with absorption sites in the intestinal lumen due to the low surface area/volume ratio. In contrast, the impinging aerosols method, which results in microspheres less than 60 μm in size that almost completely limit protein release in SGF, presents distinct advantages in this respect.

Bioactivity of lysozyme and insulin proteins released from alginate gel microspheres

Lysozyme released from alginate gel microspheres into PBS over 6 h retained at least 80% activity relative to control lysozyme solutions in PBS and released enzyme activity was similar for microspheres prepared using increasing concentrations of $CaCl_2$ solution (0.1., 0.25. and 0.5 M) (Figure 8). Similar percentage activity levels of 80–100% were measured for lysozyme released from alginate microspheres in PBS following 2 h incubation in HCl (Figure 9).

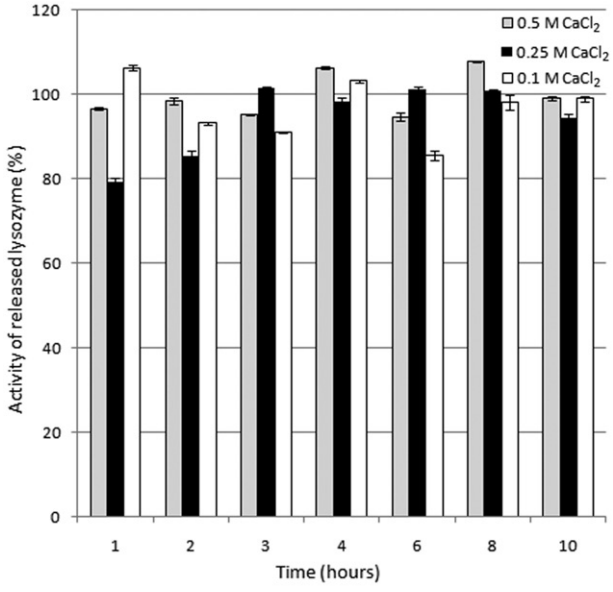

Figure 9. Activity of lysozyme released from alginate gel microspheres in PBS following 2 h incubation of the microspheres in HCl.

The ARCHITECT® chemiluminescent immunoassay is used routinely in the clinical laboratory setting to quantify insulin levels in blood samples to provide diagnostic information for diabetes mellitus and pharmacokinetic evaluations. Cross reactivity also enables measurement of the concentration of recombinant insulin analogues (Moriyama et al., 2006). The assay determines the concentration of therapeutically active insulin indirectly through measurements of the binding activity with anti-insulin monoclonal antibodies and was applied in this study as a convenient, clinically relevant method for assessing the bioactivity of insulin released from alginate gel microspheres.

The amino acid sequences of human and bovine insulin are identical other than at position A8, A10 and B30 (Reeves and Kelly, 1982; Marks et al., 1985). The terminus of the B-chain and the A-chain loop define the two antigenic determinants in human insulin (Marks et al., 1985), thus, the reactivity of bovine insulin released from the alginate microspheres in the ARCHITECT assay demonstrates conservation of antigenic regions between the two variants. The relative activity of insulin released from alginate microspheres (calculated by comparison with the activity of insulin standard solution of the same concentration) was found to be dependent on microspheres incubation time in PBS and microspheres exposure to SGF. The relative activity of insulin released from alginate gel microspheres increased gradually from 75% to over 90% with microspheres incubation time of 1–8 h in PBS and from 65% to over 75% in PBS following 2 h incubation in HCl (Figure 10).

The reduction of activity of released insulin in PBS following microspheres incubation in HCl may be explained by diffusion of the acidic medium into gel matrix. Our investigations revealed that the activity of insulin in PBS

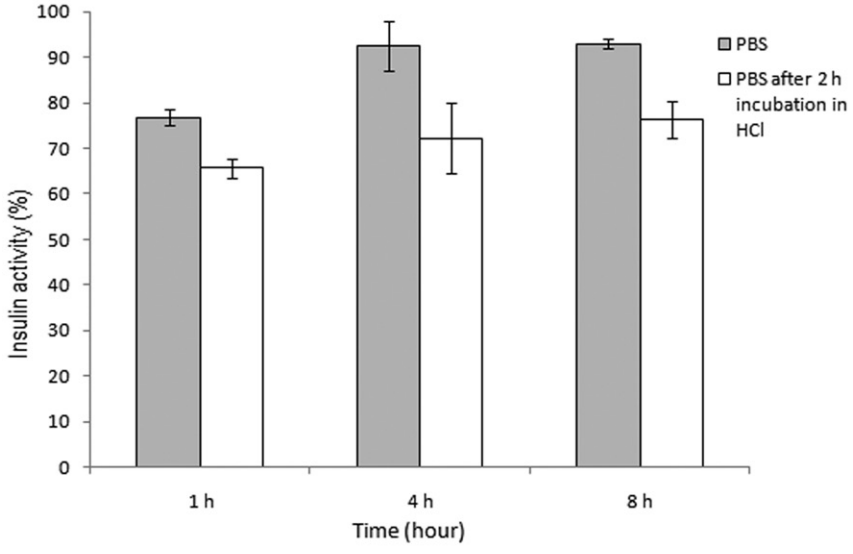

Figure 10. Relative activity of insulin released from alginate gel microspheres in PBS at 37°C and in PBS following 2 h incubation of the microspheres in HCl at 37°C.

decreased significantly when the pH is adjusted between 2 and 3, but it is maintained at high levels at pH 7.0–7.2. Sarmento et al. (2007) reported insulin denaturation at low pH due to alteration of the secondary structure involving changes in α-helix, β-sheet and β-turn components. García-Fuentes et al. (2003) observed higher aggregation of insulin at low pH compared to a neutral pH. These conformational changes may shield epitopes from binding with insulin antibodies. The reduction in activity of insulin released into PBS at early time points (1 h) is more difficult to explain but may involve the increased Ca^{2+} concentration at the microsphere surface associated with higher cross-linking density (Reis et al., 2007a; Wan et al., 2008) and the sensitivity of insulin molecules to aggregation in the presence of divalent metal ions (Silva et al., 2006b).

Conclusion

Lysozyme and insulin can be encapsulated in alginate gel microspheres at high loadings using impinging aerosols of alginate solution and $CaCl_2$ cross-linking solution. The ability to obtain an absence of lysozyme and insulin release in SGF but complete release in SIF with retention of high levels of activity for both proteins demonstrate the major potential of alginate gel microspheres, produced using the impinging aerosols technique, for improving oral delivery of protein therapeutics.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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