Journal of Pharmaceutical Research Vol. 8, No. 2, April 2009 : 76-80.

PROTEIN RETENTION IN CHITOSAN-ALGINATE MICROCAPSULES MODIFIED FOR POSSIBLE ORAL PROTEIN DELIVERY WITH SELECTED EXCIPIENTS

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Received on : 21.05.2008 Revised : 18.03.09 Accepted : 21.03.09

ABSTRACT
The protein retention characteristics of chitosan-alginate microcapsules modified with selected excipients were evaluated *in vitro* for oral protein delivery using Bovine serum albumin (BSA) as the 'model' protein. The microcapsules were prepared by extruding alginate solution containing BSA and either talc, Eudragit L100, Eudragit RSPM, microcrystalline cellulose (MCC) or HPMCAS, into chitosan/calcium chloride solution. Protein retention in microcapsules at different pH of the elution medium was determined spectrophotometrically at $\ddot{e}_{max}$ of 280 nm. Microcapsules containing MCC and talc had the highest protein retention capacity, with 68% and 60%, respectively, of protein still available in the core of the microcapsules after 9 h at pH 1.2, while HPMCAS failed to retain any protein after 9 h at the same pH. Protein retention in microcapsules modified with other additives was intermediate. At higher pH values, microcapsules containing talc still exerted the highest protein retention of 77% (pH 3) and 85% (pH 6), followed by microcapsules containing Eudragit RS PM, 65% (pH 3) and 80% (pH 6), after 9 h. This work has demonstrated that blending the core with suitable excipient enhanced protein retention capacity of chitosan-alginate microcapsules. This technology can be employed in oral delivery of protein/vaccines to human and aquaculture.

Keywords: *Chitosan-alginate; excipients; microcapsules; protein retention; oral delivery.*

INTRODUCTION

An area that has continued to attract attention in the field of drug delivery is the delivery of bioactive/ therapeutic proteins and peptides into the body via the oral route. Most protein/peptide drugs are still administered parenterally, i.e., by injection rather than orally due to their susceptibility to acid and enzyme degradation in the stomach as well as their poor gastrointestinal absorption. The disadvantages of parenteral administration are obvious and they include poor patient acceptance, invasiveness and cumbersomeness since the drug is often administered repeatedly over a long period of time. Furthermore, injections are costly, and peptides generally display a short half-life [1] thus requiring frequent administration to maintain adequate blood levels. If protein/peptide drugs can be protected effectively from enzyme and acid assault in the GIT, then their delivery by the oral route would be feasible. Thus, any realistic attempt to deliver proteins and peptides through the oral route must take into account probable losses in the gut as a result of degradation by acids and enzymes as well as the physicochemical properties of the drug itself. [2] Bovine serum albumin (BSA), used as a model drug in this work is a large globular protein that has been well characterized with a molecular weight of about 66,000 Dalton. It has been used extensively as model protein in protein diffusion studies and it is widely recommended for use as a primary standard for protein assays partly because it is inexpensive, easily available in pure form, and easy to analyses.[3]

On the other hand, the colon is thought to have lower enzymatic activity, a close to neutral pH and lacks vigorous stirring than other regions of the GIT, leading to an ability to create local conditions favourable for stabilization and absorption enhancement. [4] Thus if therapeutic proteins can be delivered intact to this region, a greater efficiency of absorption could be achieved. [4] A suitable system for the oral delivery of peptide drugs should be able to retain the drug in the dosage form while it passes through the stomach, duodenum and small intestine until it gets to the site of optimal absorption in the colon where it should be released.[5] Various systems for achieving site-specific delivery of orally administered protein/peptide drugs have been investigated in recent years including coating systems based on pH changes and enzymatic activity of intestinal microflora, [4-6] nanoparticles, [7] matrix devices [8] and conjugate (degradable prodrug) formation.[9] These often produced very variable release profiles, partly because the transit time through the colon can vary substantially from as low as 6 h to as high as 30 h. Furthermore, several of these approaches are somewhat complex and if they were to be translated

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into actual manufacture of oral delivery systems, the products would be expensive and, therefore, unaffordable in most developing countries.[10]

This work, therefore, sought to suitably modulate the release characteristics of low-cost chitosan-alginate microcapsules by incorporating well-known pharmaceutical excipients – talc, microcrystalline cellulose and polymethacrylates – in the alginate core in order to achieve optimum protein retention within the microcapsules in simulated pH conditions of the gut.

MATERIALS AND METHOD

Materials

Sodium alginate (Kelco. Chicago, U.S.A.) and chitosan (medium viscosity grade, Vansom Chem. Co., Redmond, U.S.A.) were the polymers used for microcapsule production. Bovine serum albumin (used as a 'model' protein) was obtained from Fluka AG Chem, Fabrik, Germany. Hydroxypropyl methylcellulose acetate succinate (HPMCAS) (a pH-sensitive polymer) was manufactured by Shin-Etsu Chemical Co. Ltd. Tokyo but supplied by Biddle Sawyer Corp, New York. Calcium chloride dihydrate and talc were obtained from BDH Chemicals, Toronto, Canada and Riedel de Haen AG, Seetze, Hanover, Germany, respectively. Avicel PH 102 (microcrystalline cellulose) (MCC) was produced by FMC Corporation, Philadelphia, while Eudragit RS PM and Eudragit L100 were manufactured by Rohm Pharma GMBH, Weiterstadt, Germany. All other chemicals used were of reagent grade.

Method

Preparation of encapsulation solutions

Chitosan solution (0.1 % $^{w}/_{v}$) was prepared by dissolving 1 g of chitosan in 900 ml of distilled water containing 10 ml of 1 % $^{w}/_{v}$ tartaric acid with the aid of a magnetic stirrer. The molecular weight of the polymer and hence the viscosity of its solution were reduced by digesting overnight with 2.14 ml of 1% w/v sodium nitrite ($NaNO_2$) solution based on a ratio of 0.05 mole sodium nitrite to 1.0 mole chitosan.[11] Following the dissolution of 19.80 g of calcium chloride dihydrate and two drops of polysorbate 80 (Tween 80) in the solution, its pH was adjusted to 2.78, using sodium hydroxide pellets and solution. The chitosan solution was then filtered and the volume made up to 1000 ml.

To prepare a 2.0 % w/v sodium alginate solution, 2.0 g of the polymer was dissolved in distilled water using a magnetic stirrer. The volume was then adjusted to 100 ml.

Preparation of release media

Hydrochloric acid solution, pH 1.2

This was prepared by adding 8.5 ml of concentrated hydrochloric acid to distilled water and the volume made up to 1000 ml with more distilled water.

Glycine Buffer, pH 3

2 g of NaCl was dissolved in 1000 ml of 0.15M aqueous glycine solution and adjusted to pH 3 with 1M hydrochloric acid solution.

Acetate Buffer, pH 6

100 g of ammonium acetate was dissolved in 300 ml of water and 4.1 ml of glacial acetic acid was added. The pH was adjusted to 6 using ammonia or 5M acetic acid (as necessary) and then diluted to 500 ml with water.

Microencapsulation of BSA

10 ml of the sodium alginate solution containing 2.5% bovine serum albumin (BSA) alone or in combination with 1% of either HPMCAS, Eudragit L100, Eudragit RS-PM, MCC or talc was extruded into 70 ml of the chitosan solution in a glass petri dish from a 50 ml burette with a 1 mm diameter orifice. The extrusion distance (i.e., the distance between the burette tip and the surface of the chitosan solution) was fixed at 10 cm while the extrusion rate was found to vary with the viscosity of the alginate fluid. On reaction with calcium ions (Ca^{2+}) in the chitosan solution, the polyanionic sodium alginate droplets gel into calcium alginate beads, which then rapidly react with the polycationic chitosan. The resulting microcapsules were allowed an additional reaction time of 2 min following the termination of alginate extrusion, and then examined for size and shape consistency. The chitosan solution was removed from the petri dish by filtration. The microcapsules obtained were washed twice with distilled water and then rinsed rapidly with isopropyl alcohol to remove as much water as possible before air-drying at ambient temperature.

Protein retention studies

About 200 mg of dried microcapsules, accurately weighed, was placed in each of 300 ml wide-mouthed glass bottles in a thermostatted shaker bath (Gallenkamp) set at 30 + 0.5°C. This temperature was chosen because the microcapsules were also being studied for delivering vaccines in aquaculture to fish (which has a roughly equivalent pH profile with man). A temperature, which was considered midway between normal human body temperature and that of fish[12]. 200 ml of the elution fluid, i.e., 0.1M HCl (pH 1.2), glycine buffer (pH 3) containing NaCl (0.2% w/v) and acetate buffer (pH 6) was put in each of the bottles and capped. The shaker bath was agitated at a speed of 80 r.p.m. and elution of BSA from the microcapsules was followed spectrophotometrically using Cecil CE UV/Visible spectrophotometer 202, Series 2 at 280 nm. The experiment was carried out in quadruplicate. Assay of the microcapsules for the initial BSA content (load) was also carried out spectrophotometrically at 280 nm thus: 50 mg of the microcapsules was 'citrated' by dispersing in 20 ml of 0.5M sodium citrate in a scintillation vial and kept overnight. (Citration is used to break down

the gel structure containing the entrapped protein[11]). The microcapsules, together with the citrate solution, were then transferred to a mortar and crushed with a pestle to effect maximum BSA release into solution. Protein retention was taken as the difference of the BSA content prior to elution and the protein released during elution.

RESULTS AND DISCUSSION

Results

Figs. 1-3 show the effect of the pharmaceutical excipients on protein retention in microcapsules at different pH of the elution medium. The microcapsules were modified with either talc (T), Eudragit RS PM (RS), Microcrystalline cellulose (M), HPMCAS (H) or Eudragit L100 (L). The control (C) is the unmodified microcapsules, i.e., without any of the additives in the alginate core. At pH 1.2, which simulates gastric pH, mean protein retention in microcapsules modified with talc and MCC were 60 and 68%, respectively, after 9 h (see Fig. 1). These were significantly higher ($P < 0.05$) than the control, which had 17% protein retained after the same period. Protein retention at pH 1.2 for microcapsules modified with Eudragit RS PM and Eudragit L were 25 and 36%, respectively, after 9 h. These values were not significantly different ($P > 0.05$) from the control while microcapsules containing HPMCAS had no protein retained after 9 h. When the

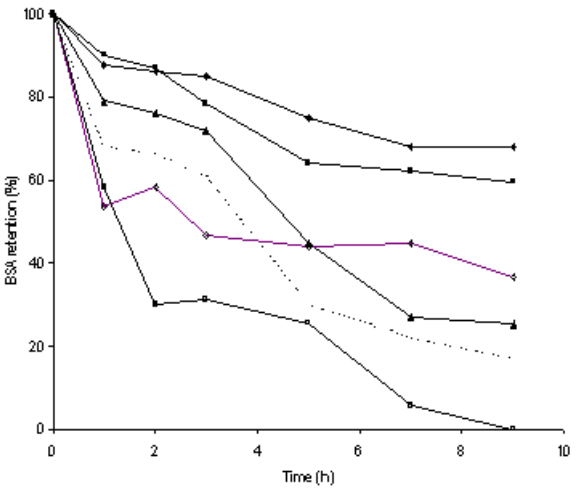

Fig. 1: *Mean Protein retention at pH 1.2 in microcapsules modified with Microcrystalline cellulose (M) (◆), Talc (T) (■), Eudragit RS PM (RS) (▲), Eudragit L100 (L) ( ), HPMCAS (H) ( ), Control (C) (…).*

pH of the release medium was increased to 3 (upper end of gastric pH) (Fig. 2), talc enhanced protein retention to 77%, Eudragit RS PM, 65%, MCC, 70%, HPMCAS, 66% and Eudragit L100, 71% after 9 h. Thus there was a general increase in the protein retention capacity of all the modified microcapsules. These values were significantly higher ($p< 0.05$) than for the control (44%). When the pH of the release medium

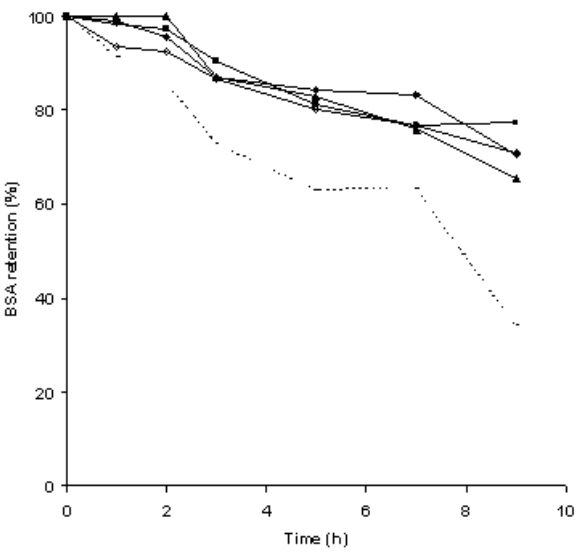

Fig. 2: *Mean Protein retention at pH 3 in microcapsules modified with Microcrystalline cellulose (M) (◆), Talc (T) (■), Eudragit RS PM (RS) (▲), Eudragit L100 (L) ( ), HPMCAS (H) ( ), Control (C) (…).*

was further increased to 6 (upper end of intestinal pH) (Fig. 3), 100% protein retention was attained in the first 3 h in microcapsules containing talc, Eudragit RS PM and Eudragit L100 and 2 h in microcapsules containing HPMCAS followed by varying levels of decline in protein retention. For example after 9 h, 85% protein was still retained in the core of the microcapsules containing talc, compared to microcapsules loaded with HPMCAS which had 64% of protein retained; Eudragit RS PM, 80%; and Eudragit L100, 59%. These values are significantly higher ($p< 0.05$) than for the control, which

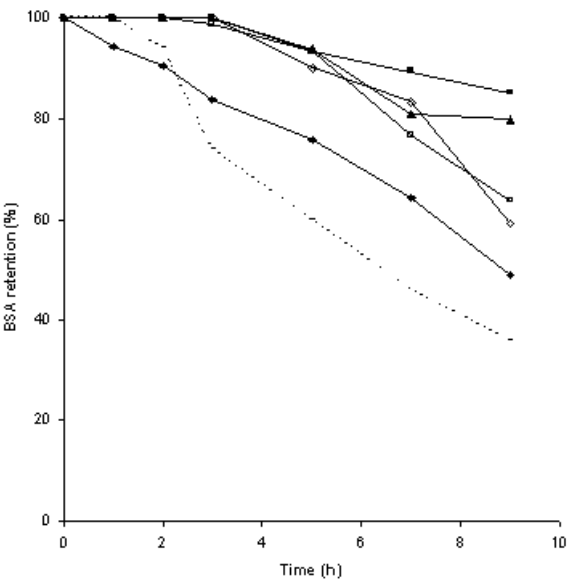

Fig. 3: *Mean Protein retention at pH 6 in microcapsules modified with microcrystalline cellulose (M) (◆), Talc (T) (■), Eudragit RS PM (RS) (▲), Eudragit L100 (L) ( ), HPMCAS (H) ( ), Control (C) (…).*

showed 36% protein retention after the same period. Protein retention in microcapsules containing MCC (48%) was not significantly different ($p > 0.05$) from the control. The order of protein retention in relation to the pH of the release medium is given in Table 1. As the pH of the release medium increased, protein retention also increased except for the microcapsules loaded with MCC.

Table 1. *Order of protein retention in microcapsules modified with core additives.*

Excipients	Order of protein retention in pH of release medium
MCC	3 > 1.2 > 6
Talc	6 > 3 > 1.2
Eudragit L100	6 > 3 > 1.2
Eudragit RS PM	6 > 3 > 1.2
HPMCAS	6 > 3 > 1.2
Control	6 > 3 > 1.2

Discussion

At pH 1.2, 'burst' release was particularly manifest in microcapsules modified with Eudragit L100, HPMCAS and the control as over 30 % of the protein was released within the first 1 h. On the other hand at pH 3 and 6, less than 20 % protein was released after 3 h from all the microcapsules except the control. These tallies with an earlier work, [13] which showed that protein release from unmodified chitosan-alginate microcapsules was markedly dependent on the pH of the release medium. In pH 1.2 medium, the chitosan membrane appears to slowly erode as the polymer dissolves. This probably accounts for the burst phenomenon. Chitosan membrane however retains its integrity in elution media of pH 3 and higher in which the polymer is insoluble.[14] Thus protein diffusion across the membrane would occur via the membrane pores.

However, the fact that protein retention in pH 1.2 medium was enhanced by incorporating some excipients (talc, MCC and Eudragit RS PM) in the microcapsule alginate core suggests that other factors besides membrane barrier also influenced BSA retention in the microcapsules. One of such factors is the nature and/or type of polymer-polymer or polymer-pigment interaction in the alginate core. The effect of fillers (solid particles) on polymeric film properties have been categorized into two main types - hydrodynamic and reinforcing.[15, 16] Hydrodynamic effect is due largely to the size, shape, volume, concentration and orientation of the filler particles and usually results in the obstruction of the diffusion pathways of the polymer matrix by the impervious particles thus creating a more tortuous diffusion channels for molecules. On the other hand, reinforcing effect occurs as a result of strong physical and/or chemical bonding of the filler to the polymer, thus stiffening the molecular chains of the polymer matrix at the filler-polymer interface. This also reduces segmental mobility leading to narrower diffusion channels. These two effects are believed to be responsible, in part, for the effects of the excipients on protein retention.

Previous studies indicate that the chitosan membrane remains intact at pH 3 and higher with the membrane manifesting pores. The protein retention data suggest that the presence of talc which has a greater axial ratio (particle length: thickness ratio) than the other additives would likely create a greater physical barrier to protein diffusion out of the alginate core by lengthening the diffusion pathway through increased tortuosity.[16] This is a hydrodynamic effect. Again, talc is basic while the other additives and alginate are acidic. Thus talc would be expected, more than the other additives, to interact more effectively with the alginate core, based on the acid-base concept. [16]

The impact on protein retention at pH 3 and 6 did not significantly differ ($p<0.05$) among the additives, except for microcrystalline cellulose (MCC) at pH 6. It is not clear why MCC failed to enhance the protein retention as effectively as other additives at this pH. However, it is known that MCC is insoluble in acid but exhibits a tendency to gel in alkaline conditions. Thus, increased MCC hydrophilicity at pH 6 may actually have promoted the diffusion of fluid in and out of the alginate core while also hindering protein diffusivity by exerting a hydrodynamic effect.

Nonetheless, the results indicate that suitable blending of alginate with water insoluble additives in the core of chitosan-alginate microcapsules affords a simple, safe and effective approach to protecting encapsulated proteins as they transit through the gastric and upper intestinal regions. As stated earlier, delivery of oral proteins and peptides can only be feasible if they are adequately protected from the hostile acid and enzyme environment to enable them reach that part of the intestine where they should be maximally released and absorbed. While the issue of adequate protein release at the target region of the gut for optimal absorption was not addressed in this work, it seems, however, that by incorporating additives in the chitosan-alginate microcapsules, approximately 61% protein retention could be attainable after 9 h transit from pH 1.2 through pH 3 to pH 6. This figure is based on the correlation and extrapolation of the data in Figs.1-3. A subsequent work will attempt to test the validity of this statement.

CONCLUSION

This work has shown that using well-known medically safe materials and a simple approach, it is feasible to produce microcapsules that are capable of retaining proteins for an extended time under conditions that span gut pH. Translated *in vivo,* it means that this system could facilitate passage of a significant amount of protein into the colon, which is the region for optimal absorption of proteins when administered per orally. Thus the findings from this study may have implications for the continuing efforts to develop suitable oral delivery systems for proteins and peptides.

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