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Carbohydrate Polymers 69 (2007) 725–731
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Insulin-loaded alginate microspheres for oral delivery – Effect of polysaccharide reinforcement on physicochemical properties and release profile

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Received 27 April 2006; received in revised form 11 January 2007; accepted 12 February 2007
Available online 20 February 2007

Abstract

Oral administration of insulin requires protein protection from degradation in the gastric environment and its absorption improvement in the intestinal tract. To achieve this objective several types of microspheres composed of alginate, chitosan and dextran sulphate have been prepared by ionotropic gelation. Parameters such as the mean particle size, swelling behaviour, insulin encapsulation efficiency, loading capacity and release profiles in simulated gastric and intestinal fluids have been compared for the systems developed. In this study, attempts have been made to increase insulin protection and to improve its release from microspheres by reinforcing the alginate matrix with chitosan and/or dextran sulphate. Dextran sulphate was able to avoid insulin release at pH 1.2, protecting the protein from the acidic environment and reducing the total insulin released at pH 6.8. This effect was explained by an interaction between the permanent negatively charged groups of dextran sulphate and insulin molecules.

Keywords: Alginate; Chitosan; Dextran sulphate; Insulin; Microspheres; Gastric retention

1. Introduction

The development of improved delivery systems for oral administration of insulin is a field of great interest in the treatment of diabetes mellitus to overcome the problem of daily subcutaneous injections. Oral administration is a more convenient approach considering the pain and possible infections caused by injections, thus leading to a higher patient compliance. This administration route prevents the occasional hyperinsulinaemia observed by subcutaneous administration, since the principal organ in glucose homeostasis is the liver and this should obviously be the prime target for intervention (Arbit, 2004). A successful oral drug delivery system needs to be developed on the basis of increased resistance of the drug carrier against gastrointestinal enzymes and pH gradients (i.e. from 1to 3 in the stomach to 6 to7 in the intestine). In addition, time-controlled release is also a pre-requisite for such oral drug delivery systems (Janes & Alonso, 2003).

It is known that microencapsulation of labile proteins improves their protection against gastric pH and enzymatic attack, providing a controlled release profile of the entrapped molecules (Onal & Zihnioglu, 2002), and further enhancement of their intestinal absorption. Microencapsulation of proteins can be performed using alginate, chitosan and dextran sulphate. Being biodegradable, biocompatible, non-toxic and mucoadhesive macromolecules these natural polysaccharides have been widely used in the formulation of several drug delivery systems (Lin, Yu, & Chien, 2005; Onal & Zihnioglu, 2002; Yu et al., 2004).

Alginate is an anionic polysaccharide consisting of various ratios of guluronic and mannuronic acid units linked by glycosidic bonds. Gelation can be induced by cross-linking the guluronic acid units with di- or polyvalent cations such as calcium. Major disadvantages of alginate beads

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0144-8617/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2007.02.012

are low drug encapsulation efficiency (EE) and rapid release of the loaded molecules (Halder, Maiti, & Sa, 2005). Low EE and loading capacity (LC) of alginate beads are attributed to the gel porosity, which is large enough to cause leakage of the loaded drugs and further drug diffusion from the gel network to the $CaCl_2$ solution (Setty, Sahoo, & Sa, 2005). In order to modify the encapsulation parameters (EE and LC) and protein release profiles from alginate beads, several polymers such as chitosan (Onal & Zihnioglu, 2002; Ramadas, Paul, Dileep, Anitha, & Sharma, 2000), dextran sulphate (Silva, Ribeiro, Veiga, & Sousa, 2006), pectin (Bajpai, Bajpai, & Mishra, 2006; El-Kamel, Al-Gohary, & Hosny, 2003) and methylcellulose (El-Kamel et al., 2003) have been used in combination with sodium alginate. Chitosan, a positively charged polymer, interacts with negatively charged alginate to form a polyelectrolyte complex membrane, which is able to control the drug release profile (Shu & Zhu, 2002). Dextran sulphate is a negatively charged polymer that shows a high affinity for proteins (Fuentes et al., 2004).

This paper assesses the physicochemical properties of microspheres composed solely of alginate, in comparison to those that have been reinforced with chitosan or dextran sulphate, or a combination of both polysaccharides, on the gastrointestinal retention of insulin.

2. Materials and methods

2.1. Materials

Sodium alginate of low-G content ($F_G = 0.39$), low viscosity, chitosan of low molecular weight ($M_W \approx 50$ kDa), and calcium chloride were purchased from Sigma (Portugal). Dextran sulphate ($M_W \approx 500$ kDa) was obtained from PKC® (Denmark). Acetic acid was acquired from Pronalab (Lisboa). Human zinc–insulin (lot RS0325, 7.0 mg lyophilized human biosynthetic insulin per vial) was a generous gift from Lilly Portugal.

2.2. Microspheres preparation

Microspheres were prepared by ionotropic gelation of alginate with Ca^{2+} ions. The powders (alginate, dextran sulphate, $CaCl_2$ and chitosan) were weighed separately and further dissolved in water under magnetic stirring to prepare stock solutions of alginate 2% (w/v), $CaCl_2$ 500 mM, dextran sulphate 1% (w/v) and chitosan 1% in 1% of acetic acid (w/v), respectively. To prepare alginate/insulin and alginate/insulin/dextran sulphate solutions, freshly prepared stock solutions of each compound were mixed gently to obtain a homogeneous final solution. To avoid insulin fibrillation (one of the degradation mechanisms of insulin (Brange, Anderson, Laursen, Meyn, & Rasmussen, 1997)), stirring of the final solution has been shortened. This assumption was made because insulin fibrillation was not observed to any significant extent in different pH aqueous solutions at moderate temperature in the absence of agitation (Sluzky, Tamada, Klibanov, & Langer, 1991). Then, alginate/insulin or alginate/insulin/dextran sulphate solutions were extruded dropwise through a needle with an internal diameter of 0.2 mm into a $CaCl_2$ or $CaCl_2$–chitosan solution under magnetic stirring. Microspheres were additionally stirred for 15 min to allow the alginate gel to stabilize and then washed with deionized water. Particles were stored in a desiccator under vacuum and dried until constant weight.

2.3. Particle size analysis

The size of at least 100 fresh and dried microspheres was routinely measured by light microscopy using the magnification of 40×. The average diameter of 100 microspheres was considered as the mean particle size.

2.4. Morphology

The morphology of microspheres has been studied by scanning electron microscopy (SEM) using the JEOL JSM-840 microscope (Japan). The samples were mounted on metal stubs, gold coated under vacuum and then examined one day after production. Microsphere matrices were also studied using transmission electron microscope (TEM). Microspheres were appropriately cut in transversal section using a micrometer, treated with uranil acetate and observed in a Zeiss EM 902A TEM microscope (Germany).

2.5. Swelling behaviour

The water-sorption behaviour was determined by swelling in deionized water an amount of 75 mg of accurately weighted dried microspheres at room temperature for 3 and 24 h. The wet weight of the swollen particles was determined by first blotting the dried systems with filter paper to remove surface water and then weighted immediately. The percentage of swelling was then calculated using the following equation:

$$S = \frac{W_e - W_o}{W_o} \times 100\%$$

where W_e stands for the weight of the gel microspheres at swelling equilibrium and W_o for the initial weight of the microspheres. Each swelling experiment was repeated three times and the average value was recorded.

2.6. Insulin encapsulation efficiency and loading capacity

The encapsulation efficiency (EE) was determined indirectly. The amount of insulin entrapped in the microspheres was calculated by the difference between the total amount used to prepare the systems and the amount of insulin that remained in the aqueous phase after microspheres isolation.

$$EE = \frac{\text{total amount of insulin} - \text{free insulin in supernatant}}{\text{total amount of insulin}} \times 100$$

The difference between the total amount of insulin initially used to prepare the microspheres and the amount of residual non-entrapped insulin after microspheres isolation as a percentage of total microsphere dry mass is referred to as the loading capacity (LC).

$$LC = \frac{\text{total amount of insulin} - \text{free insulin in supernatant}}{\text{total weight of microspheres}} \times 100$$

Insulin concentration was determined spectrophotometrically using the Coomassie Plus TM (Pierce, Rockford, USA) modified Bradford assay (Bradford, 1976). Briefly, insulin samples and Bradford reagent were admixed at 1:1 (v/v) ratio in a 96-well plate and incubated at room temperature for 15 min. The absorbance was measured at 595 nm on a thermomax plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA) against appropriate calibration curves.

2.7. Insulin release

Dried microspheres were placed on two different release media, i.e. 20 ml of HCl pH 1.2 USP XXVI buffer (24 h/100 rpm), or on 20 ml of phosphate pH 6.8 USP XXVI buffer (24 h/100 rpm). Aliquots of both release media were collected at predetermined time intervals, being the same volume replaced immediately with freshly prepared media solutions. The amount of insulin released from the microspheres was evaluated by Bradford assay.

3. Results and discussion

The use of natural polysaccharides as multiparticulate matrices for delivery of peptides and proteins has been widely documented in the scientific literature (Dumitriu & Chornet, 1998; Gombotz & Wee, 1998; Silva, Ribeiro, Figueiredo, Goncalves, & Veiga, 2006). These carriers have in general the potential to be used for targeting and controlled release of drugs due to their small size. In addition, the enhancement of drug absorption and bioavailability can also be achieved due to this high surface to volume ratio, which is responsible for a much more intimate contact with the mucus layer and specific targeting of drugs to the absorption site.

In the present work, different alginate microspheres were produced by the typical external gelation (i.e. ionotropic gelation) method extruding alginate or alginate/dextran sulphate solution into a calcium chloride bath. Table 1 summarises the properties of different microspheres according to their polysaccharide concentrations.

When the matrix of alginate microspheres was reinforced with dextran sulphate a slight increase of size from 1.78 to 1.83 mm was detected (Table 1). Since the initial alginate concentration remained the same (2%), the size increase (approx. 50 μm) was most likely a result of the increase in viscosity due to the incorporation of 0.5% dextran sulphate which resulted in larger drops extruded from the needle before reaching the calcium gelation bath. Chitosan-coated microspheres also showed a size increase due the contribution of the chitosan outside the layer.

Table 1
Characterization of insulin-loaded microspheres prepared in a $CaCl_2$ 100 mM solution dropwise from a needle of 0.20 mm intern diameter

Formulation	Polysaccharide concentrations (%)	Diameter (fresh microspheres) (±SD, mm)	EE (±SD, %)	LC (±SD, %)
A	Alginate 2	1.78 ± 0.09	80.0 ± 0.8	2.48 ± 0.01
B	Alginate 2 Dextran sulphate 0.5	1.83 ± 0.09	97.6 ± 0.1	2.88 ± 0.01
C	Alginate 2 Chitosan 0.5	1.85 ± 0.12	90.9 ± 1.7	2.81 ± 0.01
D	Alginate 2 Dextran sulphate 0.5 Chitosan 0.5	2.13 ± 0.09	100.0 ± 1.8	3.00 ± 0.01

A final alginate concentration of 2% (Table 1) was chosen on the basis that at lower initial alginate concentrations e.g. 0.5%–1.5% (w/v) were responsible for lower EE values for insulin (data not shown), probably due to the creation of a less compact gel matrix, which is not able to retain high amounts of protein (Quong, Yeo, & Neufeld, 1999). Encapsulation parameters were higher in alginate/chitosan microspheres (90.0%) than in uncoated alginate microspheres (80.0%) most probably due the decrease of surface porosity of the final microspheres because positively charged amino groups of chitosan formed strong membranes through ionic interactions with carboxylic residues of the alginate. Consequently, the insulin loss during gelation was lower than without chitosan coating.

The introduction of dextran sulphate into uncoated alginate microspheres and those coated with chitosan provided a higher retention of insulin than pure alginate matrices (Table 1A). The increase of EE promoted by dextran sulphate could be explained by a higher network density causing physical retention of the insulin inside the microspheres. The electrostatic interaction between the protein and the polyanion might also favour the retention of insulin within the microsphere matrix.

Surface morphology and cross section information for dried microspheres have been obtained by SEM analysis and are shown in Figs. 1–3. All the carriers showed irregular shape and had a relatively smooth surface with some wrinkles. The surface morphology and the internal structures of dextran sulphate microspheres showed a typical porous structure. On the contrary, alginate/dextran sulphate matrix seemed to be less porous. As expected, the spherical shape of microspheres was lost after drying. These morphological changes are common, especially for microspheres prepared with low alginate concentration (Halder et al., 2005).

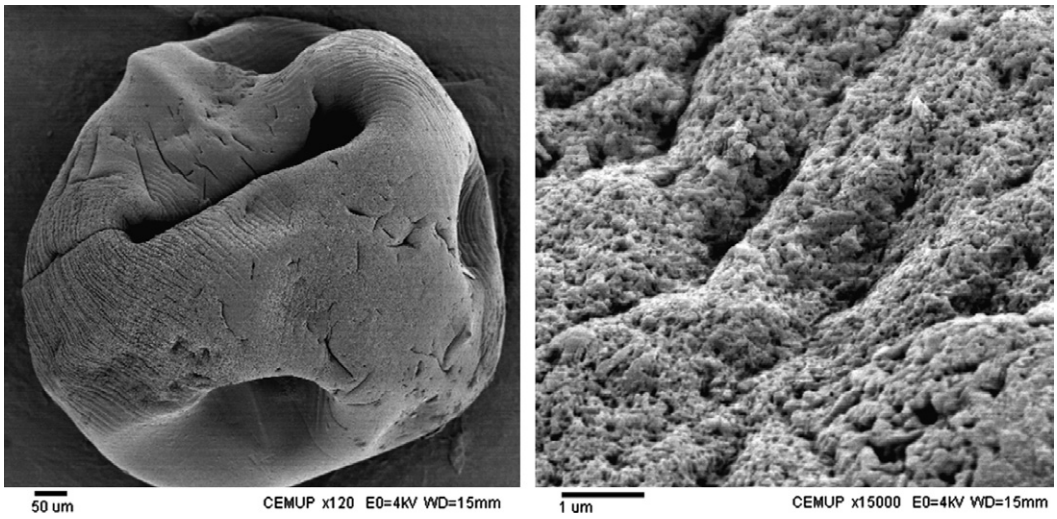

Fig. 1. SEM micrographs of insulin-loaded alginate microspheres (left) and detail of their porous surface (right).

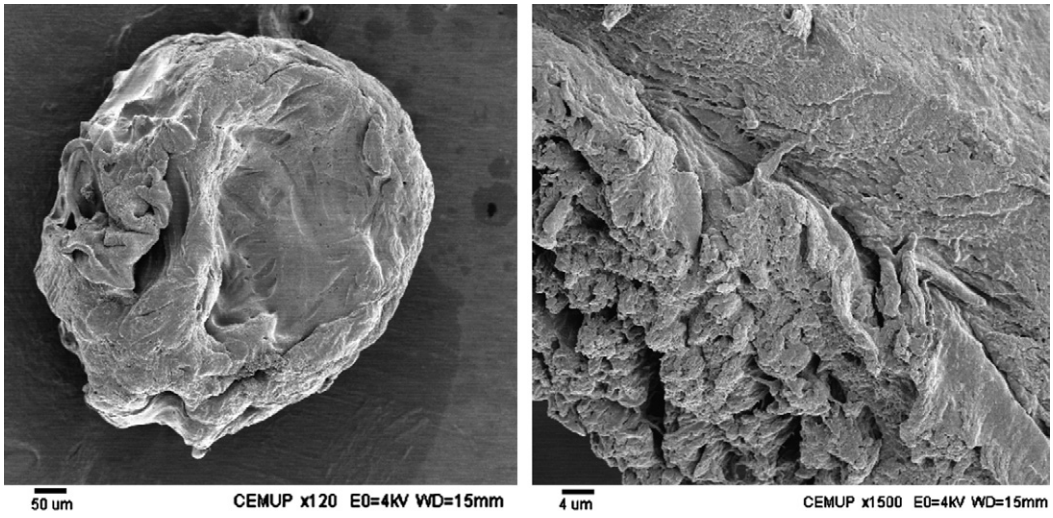

Fig. 2. SEM micrographs of insulin-loaded alginate/chitosan microspheres (left) and cross section showing chitosan (up) and alginate (down) portions of the same structure (right).

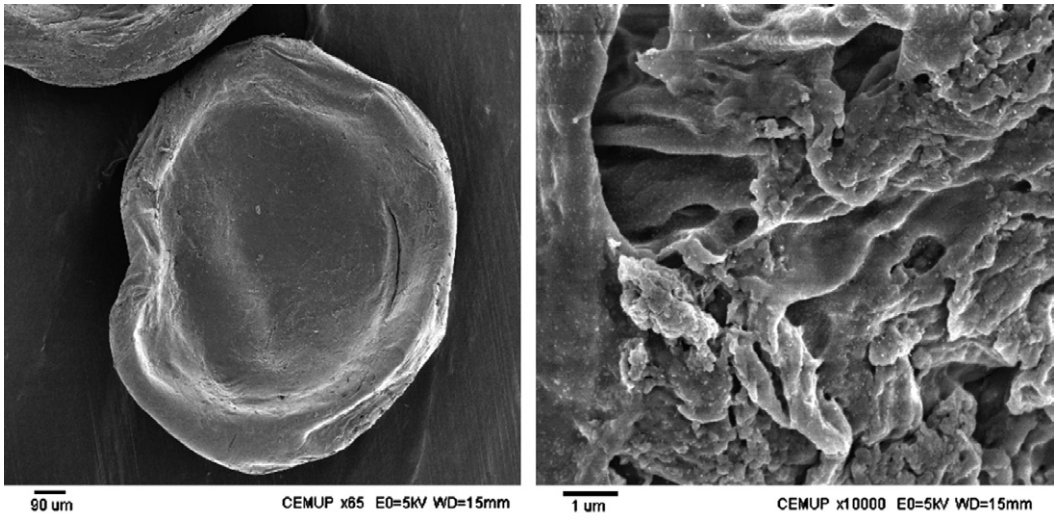

Fig. 3. SEM micrographs of insulin-loaded alginate/dextran sulphate microspheres (left) and cross section showing a dense matrix (right).

In order to analyse the degree of homogeneity, the inner matrix of the different microspheres was assessed by TEM. Fig. 4 shows the cross section of each system. Alginate microspheres (A) are characterized by a homogeneous, organized and porous matrix, however after reinforcing the structure with dextran sulphate (B) the microspheres revealed a random alginate distribution and the presence of dextran sulphate aggregates (white dots). A similar well-ordered alginate matrix could also be observed for chitosan-coated alginate microspheres (C). In this case, it is clear the presence of the dense chitosan layer, as observed in SEM cross section (Fig. 2, right). Chitosan-coated alginate/dextran sulphate microspheres (D) show an irregular and less porous inner matrix, most likely due the indiscriminate distribution of sulphate dextran between the alginate gel. The presence of denser and less porous matrices of alginate/dextran sulphate-based microspheres explains the higher values of insulin entrapment obtained comparing the alginate-based microspheres (Table 1).

Table 2 shows the change in microsphere weight by water uptake after 3 and 24 h. Alginate (Table 2A) and chitosan-coated alginate (Table 2C) microspheres showed a maximum swelling rate within the first 3 h (36% or 37%), but after the swelling rate the water uptake did not significantly increase (46% or 59%). Alginate was mainly responsible for the water uptake by microspheres since at pH 7 this polymer has the property of swelling and thus increasing its weight. At pH lower than its pK_a value (3.65) (Haug, 1964), alginate precipitates. Coating microspheres with chitosan was not strong enough to prevent the swelling and water uptake by the alginate matrix.

Table 2
Swelling behaviour of the developed insulin-loaded microspheres after 3 and 24 h ($n = 3$)

Formulation	% swelling after 3 h (mean ± SD)	% swelling after 24 h (mean ± SD)
A	36.2 ± 1.4	46.4 ± 10.9
B	49.8 ± 1.1	113.5 ± 5.4
C	37.0 ± 1.0	58.5 ± 6.6
D	50.1 ± 0.7	138.2 ± 22.4

Although alginate/dextran sulphate matrix (Table 2B) revealed a less porous surface than alginate matrix (Fig. 3), the swelling behaviour of the former was significantly different. It was observed a continuous swelling rate 24 h onwards and the percentage of water absorption was much larger than for microspheres free of dextran sulphate. After 24 h the microspheres reached the double of their initial weight. This behaviour may be due to the higher number of water-binding sites of dextran sulphate comparing with alginate on the alginate/dextran sulphate network. Thus, the water diffusion into

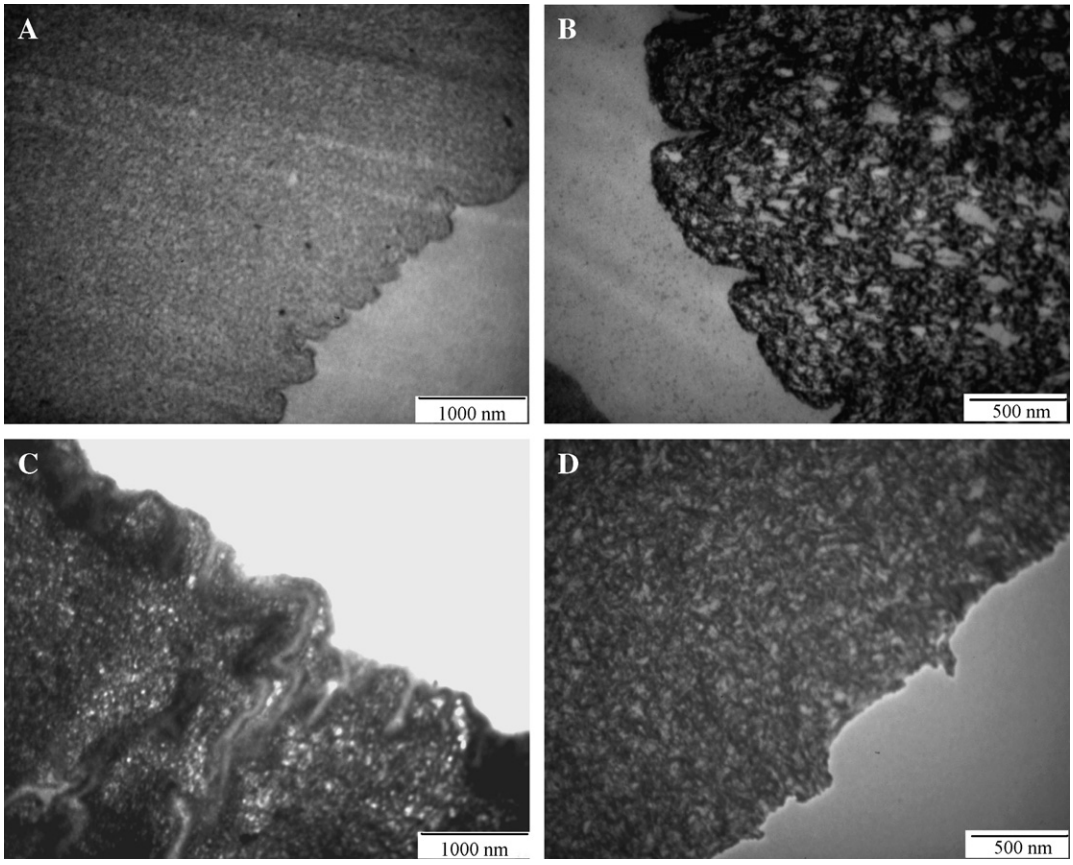

Fig. 4. TEM micrographs of alginate microspheres (A), alginate/dextran sulphate microspheres (B), chitosan-coated alginate microspheres (C) and chitosan-coated alginate/dextran sulphate microspheres (D). Insulin is present in all microspheres.

dextran sulphate containing matrix occurred in a faster and more extended degree, as also observed for swelling properties of chitosan/dextran sulphate microspheres (Lin et al., 2005) (Table 2D).

In order to overcome the hostile environment of the gastric media, i.e. acid and enzymatic effects, it is desirable to avoid insulin release from the carriers which cross the stomach, but the protein should be released under intestinal media to promote insulin uptake. The insulin release from microspheres was investigated at pH 1.2 and 6.8. Figs. 4 and 5 show the obtained cumulative insulin release patterns. The insulin was released very slowly at pH 1.2. Up to 24 h at this pH a value of less than 8% of protein was released from alginate and chitosan-coated alginate microspheres, and less than 5% from alginate/dextran sulphate, and chitosan-coated alginate/dextran sulphate microspheres. The burst release observed within 2 h is attributed to insulin bound onto the surface of the microspheres.

The effect observed on insulin release at pH 1.2 due the presence of dextran sulphate may be primarily attributed to electrostatic interactions. Since the p*I* of insulin is approx. 5.3 (Brange, 1987) the protein shows a positive charge at pH 1.2. The physical mixture of alginate and dextran sulphate provides both pH-sensitive (carboxylic) and permanently charged (sulphate) groups. The presence of sulphate groups, permanent negatively charged may be responsible for a stronger interaction with insulin at acidic pH, preventing its release Fig. 6.

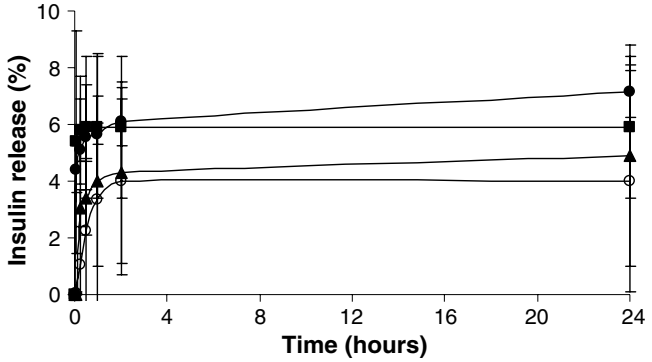

Fig. 5. Insulin release profile at simulated gastric conditions (pH 1.2 buffer) of formulations A (●), B (■), C (▲) and D (○). ($n = 3$).

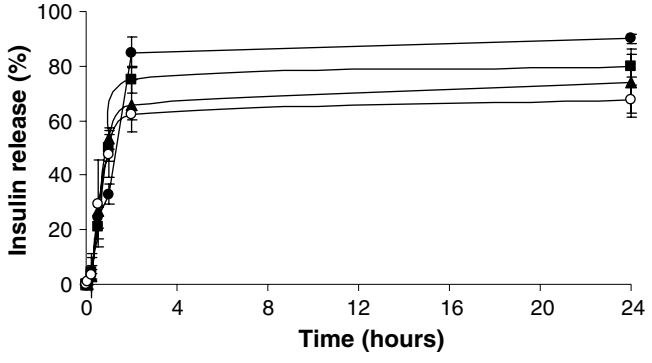

Fig. 6. Insulin release profile at simulated intestinal conditions (pH 6.8 buffer) of formulations A (●), B (■), C (▲) and D (○). ($n = 3$).

At higher pH values, a higher diffusion rate of insulin from the microspheres occurred. By increasing the pH value to 6.8, the alginic acid formed during acid incubation was converted to a soluble salt of sodium alginate. This caused the matrix to swell and disintegrate, enabling the more extended release of insulin. In fact, the increase of pH to a value higher than insulin pH causes a charge alteration of the insulin molecule and probably the reversion of the interaction with the negatively charged groups of dextran sulphate, consequently insulin is released.

For all developed microspheres an insulin burst release was observed up to 2 h, which was followed by a decrease of the release rate. Alginate microspheres released nearly 90% of insulin after 24 h and both chitosan and dextran sulphate were responsible for an improvement of insulin retention in microspheres. Chitosan-coated alginate, alginate/dextran sulphate and chitosan-coated alginate/dextran sulphate microspheres released 80%, 74% and 67% of insulin, respectively, after 24 h of assay.

Because of its amphiphile properties due to the 6 amino acid residues capable of attaining a positive charge and 10 amino acid residues capable of attaching a negative charge (Brange, 1987), at pH 6.8 insulin probably interacts with the permanently charged (sulphate) groups of dextran sulphate explaining the higher affinity of insulin for alginate/dextran sulphate microspheres. Coating with chitosan can also contribute for the sustained release of insulin due its physical barrier against insulin diffusion. Increasing the dextran sulphate concentration did not compromise the retention of insulin at gastric pH conditions, however it did not contribute to increase insulin retention at intestinal pH conditions.

4. Conclusions

The aim of this study was to develop microspheres protecting insulin from gastric passage by reinforcement of the calcium alginate matrix with another polyanion, such as chitosan or dextran sulphate. In order to reinforce the structure of microspheres it is considered essential to maintain the optimal characteristics of size distribution and encapsulation efficiency. Dextran sulphate displayed higher protective properties of alginate microspheres, enhancing protein retention under gastric conditions in comparison to chitosan coating. The addition of the polyanionic dextran also increased the encapsulation parameter values. These results indicate that dextran sulphate reinforced microspheres are good candidates for the oral delivery of insulin. The obtained *in vitro* release profile reveal that these systems could not only control the protein release but also were able to decrease the burst release. The formulation can further be optimised for oral delivery of several pharmaceutical peptides and proteins.

Acknowledgements

The authors wish to thank Lilly Portugal for insulin supply and Rui Fernandes from the Institute for Molecular and Cell Biology (IBMC, Porto, Portugal) for TEM analyses.

References

Arbit, E. (2004). The physiological rationale for oral insulin administration. *Diabetes Technology and Therapeutics, 6*(4), 510–517.

Bajpai, J., Bajpai, A., & Mishra, S. (2006). Dynamics of controlled release of potassium nitrate from a highly swelling binary biopolymeric blend of alginate and pectin. *Journal of Macromolecular Science, Part A: Pure and Applied Chemistry, 43*(1), 165–186.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry, 72*, 248–254.

Brange, J. (1987). *Galenics of insulin: The physico-chemical and pharmaceutical aspects of insulin and insulin preparations*. Springer-Verlag.

Brange, J., Anderson, L., Laursen, E. D., Meyn, G., & Rasmussen, E. (1997). Toward understanding insulin fibrilation. *Journal of Pharmaceutical Sciences, 86*, 517–525.

Dumitriu, S., & Chornet, E. (1998). Inclusion and release of proteins from polysaccharide-based polyion complexes. *Advanced Drug Delivery Reviews, 31*, 223–246.

El-Kamel, A. H., Al-Gohary, O. M. N., & Hosny, E. A. (2003). Alginate-diltiazem hydrochloride beads: optimization of formulation factors, in vitro and in vivo availability. *Journal of Microencapsulation, 20*(2), 211–225.

Fuentes, M., Pessela, B. C. C., Maquiese, J. V., Ortiz, C., Segura, R. L., Palomo, J. M., et al. (2004). Reversible and strong immobilization of proteins by ionic exchange on supports coated with sulfate–dextran. *Biotechnology Progress, 20*(4), 1134–1139.

Gombotz, W. R., & Wee, S. F. (1998). Protein release from alginate matrices. *Advanced Drug Delivery Reviews, 31*, 267–285.

Halder, A., Maiti, S., & Sa, B. (2005). Entrapment efficiency and release characteristics of polyethyleneimine-treated or -untreated calcium alginate beads loaded with propranolol-resin complex. *International Journal of Pharmaceutics, 302*(1–2), 84–94.

Haug, A. (1964). *Composition and properties of alginates*. Thesis Trondheim Norwegian Institute of Technology.

Janes, K. A., & Alonso, M. J. (2003). Depolymerized chitosan nanoparticles for protein delivery: preparation and characterization. *Journal of Applied Polymer Science, 88*, 2769–2776.

Lin, W. C., Yu, D. G., & Chien, M. (2005). pH-sensitive polyelectrolyte complex gel microspheres composed of chitosan/sodium tripolyphosphate/dextran sulfate: swelling kinetics and drug delivery properties. *Colloids and Surfaces B: Biointerfaces, 44*(2–3), 143–151.

Onal, S., & Zihnioglu, F. (2002). Encapsulation of insulin in chitosan-coated alginate beads: oral therapeutic peptide delivery. *Artificial Cells, Blood Substitutes, and Immobilization. Biotechnology, 30*(3), 229–237.

Quong, D., Yeo, J. N., & Neufeld, R. J. (1999). Stability of chitosan and poly-L-lysine membranes coating DNA-alginate beads when exposed to hydrolytic enzymes. *Journal of Microencapsulation, 16*(1), 73–82.

Ramadas, M., Paul, W., Dileep, K. J., Anitha, Y., & Sharma, C. P. (2000). Lipoinsulin encapsulated alginate–chitosan capsules: intestinal delivery in diabetic rats. *Journal of Microencapsulation, 17*(4), 405–411.

Setty, C. M., Sahoo, S. S., & Sa, B. (2005). alginate-coated alginate–polyethyleneimine beads for prolonged release of furosemide in simulated intestinal fluid. *Drug Development and Industrial Pharmacy, 31*, 435–446.

Shu, X. Z., & Zhu, K. J. (2002). The release behavior of brilliant blue from calcium–alginate gel beads coated by chitosan: the preparation method effect. *European Journal of Pharmaceutical Sciences, 53*(2), 193–201.

Silva, C. M., Ribeiro, A. J., Figueiredo, I. V., Goncalves, A. R., & Veiga, F. (2006). Alginate microspheres prepared by internal gelation: development and effect on insulin stability. *International Journal of Pharmaceutics, 311*(1-2), 1–10.

Silva, C., Ribeiro, A., Veiga, F., & Sousa, A. (2006). Insulin release from alginate microspheres reinforced with dextran sulfate. *Chemical Industry and Chemical Engineering Quarterly, 12*, 40–46.

Sluzky, V., Tamada, J. A., Klibanov, A. M., & Langer, R. (1991). Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. *Proceedings of the National Academy of Sciences of the United States of America, 88*(21), 9377–9381.

Yu, S., Zhao, Y., Wu, F., Zhang, X., Lu, W., Zhang, H., et al. (2004). Nasal insulin delivery in the chitosan solution: in vitro and in vivo studies. *International Journal of Pharmaceutics, 281*, 11–23.