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DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY®
Vol. 29, No. 6, pp. 713–724, 2003

Chitosan-Alginate Multilayer Beads for Gastric Passage and Controlled Intestinal Release of Protein

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ABSTRACT

Chitosan-alginate beads loaded with a model protein, bovine serum albumin (BSA) were investigated to explore the temporary protection of protein against acidic and enzymatic degradation during gastric passage. Optimum conditions were established for preparation of homogenous, spherical, and smooth chitosan-alginate beads loaded with BSA. Multilayer beads were prepared by additional treatment with either chitosan or alginate or both. The presence of chitosan in the coagulation bath during bead preparation resulted in increased entrapment of BSA. During incubation in simulated gastric fluid (SGF pH 1.2), the beads showed swelling and started to float but did not show any sign of erosion. Inclusion of pepsin in the gastric fluid did not show a further effect on the properties of the beads. Release studies were done in simulated gastric fluid (SGF pH 1.2) and subsequently in simulated intestinal fluid (SIF pH 7.5) to mimic the physiological gastrointestinal conditions. After transfer to intestinal fluid, the beads were found to erode, burst, and release the protein. Microscopic and macroscopic observations confirmed that the release of protein was brought about by the burst of beads. Chitosan-reinforced calcium-alginate beads showed delay in the release of BSA. The multilayer beads disintegrated very slowly. The enzymes pepsin and pancreatin did not change the characteristics of BSA-loaded chitosan-alginate beads. Single layer chitosan-alginate beads released 80–90% of the model protein within 12 h while multilayer beads released only 40–50% in the same period of time. The release from chitosan-alginate beads and multilayer beads in SIF was further delayed without prior incubation in SGF. It is concluded that alginate beads reinforced with chitosan offer an excellent perspective for controlled gastrointestinal passage of protein drugs.

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DOI: 10.1081/DDC-120021320
0363-9045 (Print); 1520-5762 (Online)

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Key Words: Chitosan; Alginate; Multilayer beads; Sustained release; Protein delivery; Gastrointestinal passage.

INTRODUCTION

The conventional and most convenient route of drug application, the oral route, is problematic for protein and peptide drugs. The susceptibility of protein drugs to enzymatic attack, acidic hydrolysis, extension of their short half-life, and limiting transit time in the gastrointestinal region remain major challenges to formulate protein drugs with maximum bioavailability.[1–4] Various approaches have been used in an attempt to overcome these barriers and increase the oral bioavailability of proteins, including the use of polymeric microencapsulation. Microencapsulation is used to modify and retard the drug release.[5–8] A microcapsule containing protein drugs that will be consumed by gastrointestinal fluids must not be fractured until it passes through the stomach. A coating can therefore be used that is able to withstand stomach acids and enzymes and allow the protein drugs to pass through as such.[9]

Recently, natural polysaccharides have shown to be very useful for drug entrapment and sustained release of drug. The natural polymers used as carrier materials in the encapsulation technology have the great advantage of being nontoxic, biocompatible, and biodegradable.[10–13] Among various encapsulating methods proposed so far, microencapsulation in calcium-alginate beads is attractive due to the mild encapsulation conditions that allow preservation of the activity of biological macromolecules and even that of cells.[14,15] However, early bead erosion is an important problem of alginate beads prepared by coagulation with Ca^{2+} ions resulting in the early release of drug. To limit the loss of encapsulated materials, polyanionic calcium-alginate microcapsules can be coagulated or coated with a polycationic polymer like chitosan that tightens and stabilizes the bead surface. The chitosan can form a polyelectrolyte complex membrane with the alginate in the presence of calcium chloride.[10,16,17]

Chitosan, the N-deacetylated form of chitin, can be prepared by treatment of chitin in aqueous solution of 40–50% sodium hydroxide. When solubilized in dilute acid, chitosan becomes a cationic polymer, linear in structure, with a highly positive charge density.[18–20] Chitosan, being biocompatible and biodegradable, has potential in the design of dosage forms for peptides and proteins that can avoid inactivation due to direct contact of the protein drugs with the gastric environment.[10,11,21] Chitosan has the ability to swell and to form a hydrogel in the acidic gastric juice. This makes the substance suitable as an excipient in the preparation of floating dosage forms that do not adhere to the gastric mucosa.[22]

In this study, it was intended to prepare microcapsules containing protein that will resist the conditions that prevail during passage through the stomach but will release the protein under conditions that prevail in the upper intestinal region. In most of the recent studies, posttreatment of alginate and chitosan beads with glutaraldehyde is necessary for sustained release.[23] However, glutaraldehyde should be avoided due to its toxicity. This study focuses on the effect of chitosan on the entrapment of bovine serum albumin (BSA), a model representative for protein drugs, in calcium-alginate gel beads and the subsequent controlled and sustained release of the protein. Alginate gel beads have been formulated in the presence of various ratios of calcium chloride and chitosan. Entrapment, physical characteristics, and release behavior of these beads have been investigated. In this study, we have prepared multilayer chitosan-alginate beads by giving additional treatment either with chitosan or alginate or both. In vitro release of protein from chitosan-reinforced alginate beads has been investigated in normal saline and phosphate buffer at pH 7.4.[10–12,24] In our study, we focused on in vitro release of BSA by incubating the beads first in simulated gastric fluid (SGF) (pH 1.2) and then consecutively in simulated intestinal fluid (SIF) (pH 7.5) to mimic the gastrointestinal conditions.

EXPERIMENTAL SECTION

Reagents and Materials

Alginic acid (sodium salt) extracted from brown algae (Mw 2×10^5) and calcium chloride dihydrate were obtained from Fluka Chemika, and bovine serum albumin Fraction V from Sigma. Chitosan (85% degree of deacetylation, Mw 1×10^6) was prepared from shrimp chitin.[13] Pepsin and pancreatin powder were supplied by Acros Organics and Carlo Erba Reagenti, respectively. Simulated gastric fluid (SGF pH 1.2) containing 7 mL HCl, 2 g NaCl

with and without 3.2 g pepsin, diluted to 1 L and simulated intestinal fluid (SIF pH 7.5), containing 6.8 g of K_2HPO_4 and 190 mL of 0.2 N NaOH with and without 10 g pancreatin, diluted to 1 L were prepared as prescribed in US Pharmacopoeia (USP 1995). All other reagents were of analytical grade and used without further purification.

Methods

1. Preparation of Chitosan-Alginate Beads

Chitosan-alginate beads were produced by the gelation method as follows:

The homogenous mixture of 2% (w/v) sodium alginate and 1% (w/w) bovine serum albumin (BSA) in distilled water was used as dope. The pH was adjusted to 5.5 ± 0.1. The homogenous mixtures of calcium chloride ($CaCl_2$) and chitosan (CTS) at various ratios were used as coagulation fluid. Chitosan (1% w/v) was dissolved in 1% (v/v) acetic acid at room temperature and filtered through a nylon cloth to remove any remaining insoluble.

The actual coagulation solutions were prepared by diluting chitosan solution with distilled water containing 0.5–3% $CaCl_2$. The solutions were mixed for 2 h before use. The pH of the coagulation bath was adjusted to 4.5 ± 0.1. Dope (20 mL) was dropped through a 27 gauge blunt ended needle into 200 mL of coagulation fluid under mechanical stirring at 200 rpm. The flow rate of the dope was maintained at 10 mL/h by using compressed nitrogen. The smooth, spherical, and homogenous beads obtained were kept in the coagulation fluid with stirring for 2 h. Beads were collected, washed with distilled water, and air-dried. Their properties are listed in Table 1. Three batches of beads were prepared for further study.

2. Preparation of Multilayer Chitosan-Alginate Beads

Table 2 illustrates the various codes for formulations and characterizations of multilayer

Table 1. Size, entrapment efficiency, swelling index, and disintegration time of various chitosan (CTS)-calcium-alginate beads.

Formulations	CTS (%)	$CaCl_2$ (%)	(a)	(b)	(c)	(d)	(e)
A0	0.0	0.5	460 ± 60	15.7 ± 2	30 ± 6	1–2	1–2
A1	0.0	1.0	464 ± 85	25.8 ± 4	59 ± 4	2–3	2–3
A2	0.0	2.0	477 ± 44	31.5 ± 3	67 ± 9	3–4	3–4
A3	0.0	3.0	480 ± 27	34.8 ± 2	89 ± 9	3–4	3–4
B0	0.2	0.5	544 ± 52	61.2 ± 4	98 ± 7	4–5	4–5
B1	0.2	1.0	548 ± 65	67.9 ± 2	105 ± 8	4–5	4–5
B2	0.2	2.0	555 ± 21	71.1 ± 2	139 ± 4	7–8	7–9
B3	0.2	3.0	559 ± 42	74.2 ± 2	182 ± 5	7–9	7–9
C0	0.4	0.5	591 ± 33	64.7 ± 2	95 ± 12	2–3	2–4
C1	0.4	1.0	600 ± 16	67.6 ± 3	172 ± 12	3–5	3–5
C2	0.4	2.0	606 ± 19	73.9 ± 3	186 ± 10	9–11	9–12
C3	0.4	3.0	619 ± 24	78.9 ± 2	198 ± 7	10–12	10–12
D0	0.6	0.5	626 ± 28	64.7 ± 2	105 ± 8	3–5	3–5
D1	0.6	1.0	632 ± 42	69.3 ± 1	182 ± 10	3–5	3–5
D2	0.6	2.0	645 ± 31	77.0 ± 3	187 ± 6	8–10	8–11
D3	0.6	3.0	651 ± 17	84.4 ± 3	204 ± 7	10–12	10–13
E0	0.8	0.5	655 ± 24	65.6 ± 4	99 ± 10	3–4	3–4
E1	0.8	1.0	644 ± 22	73.2 ± 3	186 ± 5	4–6	4–6
E2	0.8	2.0	651 ± 19	76.7 ± 1	188 ± 6	10–12	10–14
E3	0.8	3.0	654 ± 25	88.5 ± 4	212 ± 8	12–14	12–14

(a) Mean size (μm) (± SD).
(b) Entrapment efficiency (%) (± SD).
(c) Swelling index (%) (± SD).
(d) Disintegration time (h) in SIF without pancreatin, prior incubated in SGF for 4 h.
(e) Disintegration time (h) in SIF with pancreatin, prior incubated in SGF for 4 h.

Table 2. Size, entrapment efficiency, swelling index, and disintegration time of various chitosan (CTS)-calcium-alginate multilayer beads.

Formulations	Coagulation fluid		Postcoagulation fluid			Characteristic				
	CTS (%)	$CaCl_2$ (%)	CTS (%)	Alginate (%)	$CaCl_2$ (%)	(a)	(b)	(c)	(d)	(e)
F1	0.0	3	0.02	0.00	0.0	563 ± 19	21.9 ± 4	80 ± 10	10–12	10–12
F2	0.0	3	0.04	0.00	0.0	592 ± 23	24.7 ± 5	88 ± 8	10–12	10–12
F3	0.0	3	0.08	0.00	0.0	650 ± 35	29.3 ± 7	99 ± 7	12–14	12–14
G1	0.4	3	0.02	0.00	0.0	830 ± 39	62.4 ± 3	186 ± 7	20–22	20–24
G2	0.4	3	0.04	0.00	0.0	887 ± 42	64.2 ± 4	191 ± 8	20–22	20–24
G3	0.4	3	0.08	0.00	0.0	920 ± 22	68.8 ± 7	196 ± 4	> 24	> 24
H1	0.4	3	0.08	0.02	0.5	830 ± 39	69.8 ± 7	67 ± 8	> 24	> 24
H2	0.4	3	0.08	0.04	0.5	850 ± 32	64.7 ± 7	72 ± 10	> 24	> 24
H3	0.4	3	0.08	0.08	0.5	910 ± 21	67.3 ± 9	74 ± 10	> 24	> 24

(a) Mean size (μm) (± SD).
(b) Entrapment efficiency (%) (± SD).
(c) Swelling index (%) (± SD) in SGF.
(d) Disintegration time (h) in SIF without pancreatin, prior incubated in SGF for 4 h.
(e) Disintegration time (h) in SIF with pancreatin, prior incubated in SGF for 4 h.

chitosan-alginate beads. In case of formulations (F1, F2, and F3), beads were prepared as described above but 3% $CaCl_2$ solution without chitosan was used as coagulation fluid. The resultant beads were washed once with distilled water and transferred into 100 mL of 0.02% (F1), 0.04% (F2), or 0.08% (F3) chitosan solution for 30 min. In case of G, the beads were first obtained by extruding sodium alginate and BSA solution in $CaCl_2$ and 0.4% CTS solution, followed by transfer to 0.02% (G1), 0.04% (G2), or 0.08% (G3) CTS solution for 30 min. In formulation H, the beads obtained as G3 were further incubated in 0.02% (H1), 0.04% (H2), or 0.08% (H3) sodium alginate solution for 30 min. The formulations H1, H2, and H3 were further incubated in 0.5% $CaCl_2$ aqueous solution for 10 min. The chitosan-alginate multilayer beads were rinsed with distilled water and subsequently air-dried. Preliminary experiments were done to optimize the suitable postcoagulation fluid. Three batches of each type of multilayer beads were prepared for further analysis.

3. Particle Size Determination

The particle size of 100 beads was measured with a micrometer (Mittotuyo micrometer, NSK Co., Japan) and the average values are presented.

4. Determination of the Encapsulation Efficiency

The BSA content in beads was determined by a digestion method. The BSA-loaded beads (10 mg) were pulverized and incubated in 10 mL phosphate buffer (pH = 7.4) at room temperature for 24 h. The suspensions were then centrifuged at 6000 rpm for 30 min. The supernatant was assayed for BSA content at the wavelength of 280 nm[10,25] and at 595 nm by BioRad Bradford method.[26] Supernatant from the blank beads (without BSA) was taken as reference. All samples were analyzed in triplicate.

5. Swelling Studies

The swelling properties of chitosan-alginate beads were determined in SGF (pH 1.2) with and without pepsin. Beads of known weight (10 mg) were placed in a glass vial containing 10 mL of swelling solution and allowed to swell at 37°C. The swollen beads were periodically removed and weighed. The wet weight of the swollen beads was determined by blotting them with filter paper to remove moisture adhering to the surface, immediately followed by weighing on an electronic balance. All experiments were done in triplicate.

The percentage of swelling of the beads was calculated from the formula:

$$[\text{Final weight of microbeads } (W_t) - \text{initial weight of microbeads } (W_0) / \text{initial weight of microbeads } (W_0)] \times 100$$

6. Disintegration Behavior of Beads

Disintegration of beads in SIF (pH 7.5) was studied at different intervals of time. Beads (10 mg) were preincubated with 10 mL of SGF with and without pepsin for 4 h at 37°C in an incubator with 100 rpm shaking. After filtering, the swollen beads were replaced in another vial, containing 10 mL of SIF with and without pancreatin. The samples were incubated at 37°C at 100 rpm in a shaking incubator. The physical appearance of beads and their fragments during incubation was observed under the microscope. The time of complete disintegration of different beads was registered. All the experiments were done in quadruplet and the time range was recorded.

7. In Vitro Drug Release Studies

The BSA release from beads was studied by incubating 50 mg of beads in 50 mL of SGF without pepsin (pH 1.2) in 125 mL conical flask kept in a shaking water bath at 37°C at 100 strokes per min. After 4 h, the beads were filtered and transferred to 50 mL of SIF without pancreatin (pH 7.5) and incubated at 37°C in a water bath at 100 strokes/min. At desired intervals of time, a 1.5-mL sample was withdrawn and replaced with same amount of fresh medium. Protein (BSA) in the release medium was measured directly by the optical density at 280 nm (10.27) after removing the debris by centrifugation at 6000 rpm for 30 min. Supernatants of few samples were analyzed by BioRad Bradford method at 595 nm.[28] The release studies were also carried out in intestinal fluid only, without prior incubation in gastric fluid. The respective blank chitosan-alginate beads (without BSA) were taken as reference. Each experiment was repeated at least three times.

8. Statistical Analysis

Results were analyzed and expressed as mean ± SD. Statistical analysis was done by using factorial design (Randomized Complete Block Design) for characterization of chitosan-alginate beads. Effects of various posttreatments on multilayer chitosan-alginate beads were statistically analyzed by one-way ANOVA. The differences were considered significant at the level of $p < 0.05$. SPSS 7.5 for Windows was used for all statistical analysis.

RESULTS AND DISCUSSION

The polyanionic polymer sodium alginate in aqueous solution dropped into an aqueous solution containing a suitable divalent counter cation like Ca^{2+} has the tendency to form beads. Smooth, spherical, and homogenous beads were formed. Addition of chitosan to the coagulation fluid improved the mechanical properties of the beads. When the alginate solution was dropped into chitosan solution only, in absence of Ca^{2+}, droplets were formed at the surface of the coagulation fluid, like oil on water. Later, these droplets coalesced together and formed clumps. Beads formed without $CaCl_2$ were very fragile. These observations confirm earlier findings.[10] Various concentrations of $CaCl_2$ (0.5–3%) and chitosan (0–0.8%) have been used for preparation and in vitro characterization of single layer beads. The formulations were coded as shown in Table 1.

Beads were also obtained by postcoagulation treatment of calcium-alginate beads with chitosan (Table 2, formulation F). The multilayered beads (Table 2, formulations G and H) were obtained by treating the beads more times in a coagulation fluid. The beads after additional treatment with chitosan were intact and compact. But the beads, additionally treated with sodium alginate, became like clumps after washing. To avoid clumps and to obtain smooth and spherical beads, these beads were given additional treatment with 0.5% $CaCl_2$ solution.

1. Particle Size

The shape of calcium alginate and chitosan--treated calcium-alginate beads was spherical. Weight of the beads was found to increase with the increase in concentration of chitosan in coagulation fluid, suggesting formation of a thicker chitosan layer. Mean particle size of different formulations was between 456 ± 60 μm and 649 ± 55 μm

[Table 1, (a)]. For a given chitosan concentration, increase in the concentration of $CaCl_2$ led to a slight increase in the size of the beads. Earlier reports also suggested that size of the beads increases with the use of chitosan along with $CaCl_2$ in coagulation fluid.[10,15,16] The multilayered beads showed an increase in particle size, probably due to extra coating [Table 2, (a)].

2. Entrapment Efficiency of BSA in Beads

Various concentrations of chitosan and $CaCl_2$ had significant effect on the entrapment of BSA in the beads. Entrapment is expressed as the percentage of total available BSA in the dope that actually is entrapped in the beads. At low $CaCl_2$ concentration and in the absence of chitosan, entrapment of BSA was low (15%). This may be due to insufficient crosslinking and large pore size permitting the protein to diffuse out during and after gelation. Addition of 0.2% of chitosan to the coagulation fluid resulted in a large increase of the entrapment. A further rise in chitosan concentration has only a limited additional effect. The entrapment efficiency of beads was 16–35% and 60–89% for calcium-alginate and calcium-chitosan-alginate beads, respectively [Table 1, (b)]. Statistical analysis further shows that chitosan and $CaCl_2$ separately, and in combination significantly affect the entrapment of BSA ($p < 0.05$). The enhanced loading of BSA in presence of chitosan may be due to reduction in the release rate of protein from the alginate droplet during gelation by ionic interaction between the carboxylate groups in the alginate and the protonated amine groups in the chitosan. A denser membrane can be formed because of the greater number of alginate-chitosan ionic linkages. The pH of coagulation fluid (pH 4.5) may also strengthen the beads' membranes by enhancing the interaction between negatively charged alginate and positively charged chitosan. An increased load ($\geq 70\%$) of Dextran[15] and BSA[16] has been reported in the chitosan-alginate beads compared to 40% by the classical method with the calcium-alginate system. Huguet et al.[11] obtained increased payload of hemoglobin to calcium-alginate beads in the presence of chitosan. The entrapment efficiency of BSA in multilayer beads shows the same trends as in the single layer beads [Table 2, (b)]. There was no leakage of BSA from beads in the postcoagulation fluid during the extra treatment.

3. Swelling Index of Dried Chitosan-Alginate Beads

In order to obtain data on the behavior of chitosan-alginate beads during gastrointestinal passage, the swelling, stability, and release of BSA have been studied by incubating BSA-loaded beads in SGF and SIF in presence and absence of pepsin and pancreatin, respectively. The swelling behavior of beads has been studied in SGF. The beads showed swelling and floating, but without any sign of disintegration during 4 h. The single layer calcium-alginate dried beads prepared with 3% $CaCl_2$ expanded three times more than the beads prepared in 0.5% $CaCl_2$. This might be due to osmotic value differences between the beads and the swelling fluid. Coating with chitosan might reduce the permeability for calcium even further, resulting in a higher swelling index. The swelling index increased up to 200% with the addition of chitosan in the coagulation fluid [Table 1, (c)]. Swelling index was found similar in SGF with or without pepsin. Statistical analysis shows that concentrations of chitosan and $CaCl_2$ and in combination significantly affect the swelling pattern of chitosan-alginate beads ($p < 0.05$).

The beads again showed maximal swelling (185–190%) in the case where chitosan was present [Table 2, (c)]. Beads with an extra treatment with sodium alginate showed low swelling. Each of the postcoagulation treatments has significant effect ($p < 0.05$) on the swelling index of multilayer beads, when analyzed among types of formulation. However, the analysis shows no significant difference ($p > 0.05$) within one type of multilayer formulations.

4. Behavior of Beads in Simulated Gastrointestinal Fluids

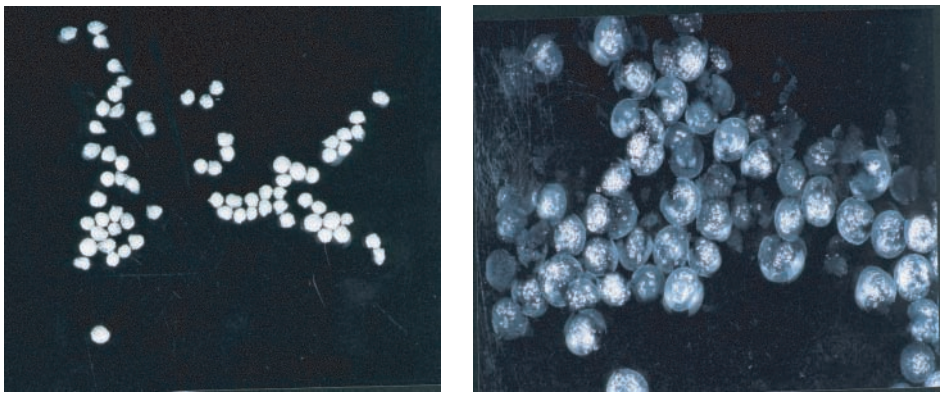
In the next series of experiments, the swollen beads in gastric fluid were incubated in intestinal fluid with and without pancreatin. The calcium-alginate beads swollen in SGF appeared not to be stable for longer periods in the intestinal fluid. Complete disintegration by bursting of the calcium-alginate beads was observed within 3–4 h. Chitosan in the coagulation fluid resulted in the chitosan-alginate beads with delayed disintegration [Table 1, (d,e)]. Multilayer beads showed slower disintegration and almost all beads except F formulations remained intact for more than 24 h. Extra coating of the alginate complex with chitosan and extra crosslinking of chitosan-alginate slowed the erosion process.

Swelling, erosion, and disintegration in simulated gastrointestinal fluids had been investigated by light microscopy. Figure 1 illustrates the swelling, erosion, and disintegration mechanisms of various beads. Pancreatin did not affect the erosion and disintegration of beads. The times taken to complete disintegration of beads in both media, with and without pancreatin were similar.

5. In Vitro Release

Beads were found swollen and floating in gastric medium but remained intact without any release of BSA. Upon transfer to intestinal fluid, the beads started to disintegrate. Based on many observations, release of BSA might occur only through the burst of the eroded beads. The disintegration of the beads was pH-dependent. At low pH, the ionic bonds in the beads persist, so that bead matrix material remained intact. After transfer to neutral pH, the anionic alginate in the Ca-alginate-chitosan complex could be displaced by hydroxyl ions. Even more important, the chitosan would lose its positive charge. As a result, the complex dissociates, the matrix erodes, and the protein is released in the surrounding fluid.

Figures 2 and 3 illustrate the release behavior of BSA-loaded beads. Various calcium-alginate and chitosan-alginate beads were incubated in SGF (pH 1.2) during the initial 4 h and then in SIF (pH 7.5) for the next 8 h. In gastric fluid, release of BSA from all different formulations was negligible. In intestinal fluid, the release was faster from the calcium-alginate beads than from the chitosan-reinforced beads. The $CaCl_2$ concentration during coagulation affected the release of BSA from the beads. At a low concentration of $CaCl_2$ (0.5%) and no chitosan reinforcement, almost 100% BSA release occurred within 2 h in SIF. The coagulation by $CaCl_2$ might have occurred only at the surface of the beads, which during exposure to intestinal fluid, started to dissociate easily. This may



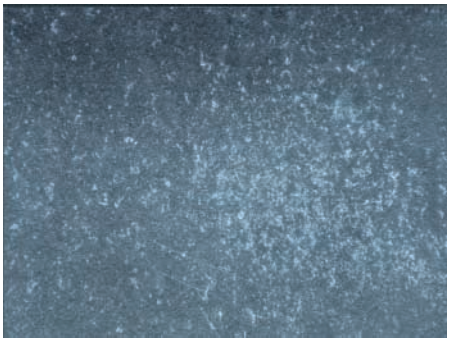
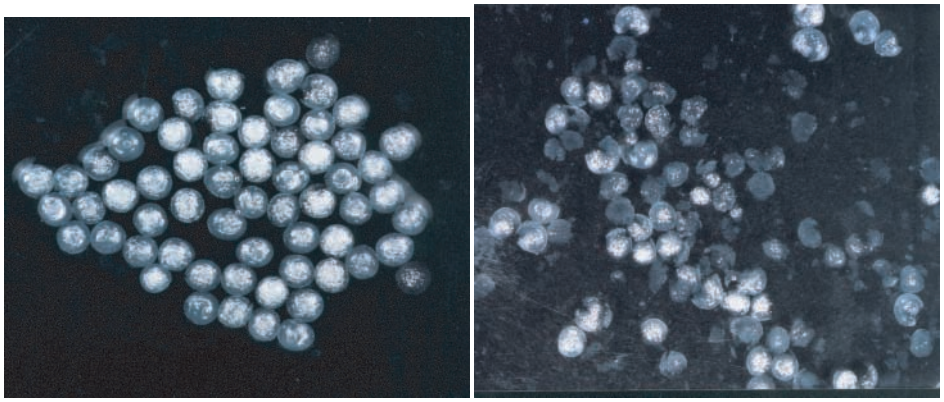


Figure 1. Photographs showing the disintegration mechanism (swelling and burst effect) of chitosan-alginate beads incubated in SGF and in SIF: (a) swollen beads (E3) in SGF; (b) beads (A3) prepared with 3% $CaCl_2$ but not reinforced by chitosan incubated in SIF for 2 h; (c) for 12 h; (d) beads (E3) prepared with 3% $CaCl_2$ and 0.8% chitosan incubated in SIF for 2 h; (e) for 12 h; (f) multilayer beads (G3) incubated in SIF for 2 h.

(*continued*)


(d) (e)

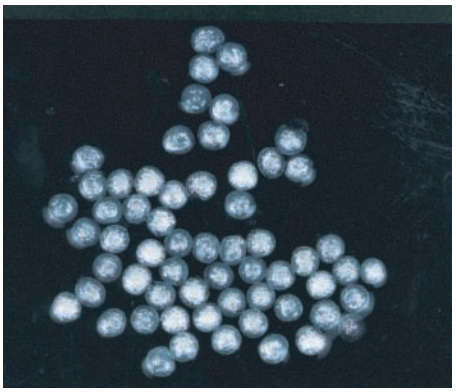

(f)

Figure 1. Continued.

also be due to the presence of phosphate ions in intestinal fluid, which have a high affinity for Ca^{2+} ions. Dainty et al.[27] reported that the disruption of calcium-alginate gel matrix occurs faster in phosphate buffer above a pH 5.5 by the chelating action of the phosphate ions. At these higher pH values, the affinity of phosphate for calcium is higher than that of alginate, and the solubility of calcium-phosphate complex is high.

The delay in the release by chitosan-containing beads (Figs. 2 and 3) coincides with the delay in the erosion of beads. Statistical analysis of BSA release was done at T-12 (release of BSA at 12 h). Analysis shows that there is significant difference ($p < 0.05$) among each formulation except between formulations C and D ($p > 0.05$). The delayed erosion and concomitantly more sustained release of BSA from chitosan-reinforced alginate beads probably reflect the strengthening of the beads by ionic interaction of chitosan (NH_3^+) with alginate (COO^-) ions.

Figure 4 depicts the release of BSA from chitosan-alginate multilayer beads incubated in SGF for 4 h and then in SIF for 8 h. The formulations F1, F2, and F3 showed erosion and burst leading to the release of 85% of BSA in 12 h. The postcoagulation treatment with low concentration of chitosan gave similar patterns, as with chitosan used simultaneously with $CaCl_2$. The use of chitosan delayed the release of BSA from multilayer beads. Only 50% of entrapped BSA was released during 12 h from the other G and H multilayer beads.

Chitosan may have both a cross-linking and a coating effect on the resultant beads. The thickness of chitosan coating layer is related to the chitosan concentration used (Table 1). Beads additionally treated with alginate and cross-linked with $CaCl_2$ showed similar release pattern. Statistical analysis by ANOVA shows the significant effect ($p < 0.05$) of various posttreatment fluids on release of BSA in intestinal fluid at 12 h after the start of experiment. There was no significant effect ($p < 0.05$) within the groups of formulations of multilayer beads except H1 and H3, which show significant difference ($p < 0.05$) from each other.

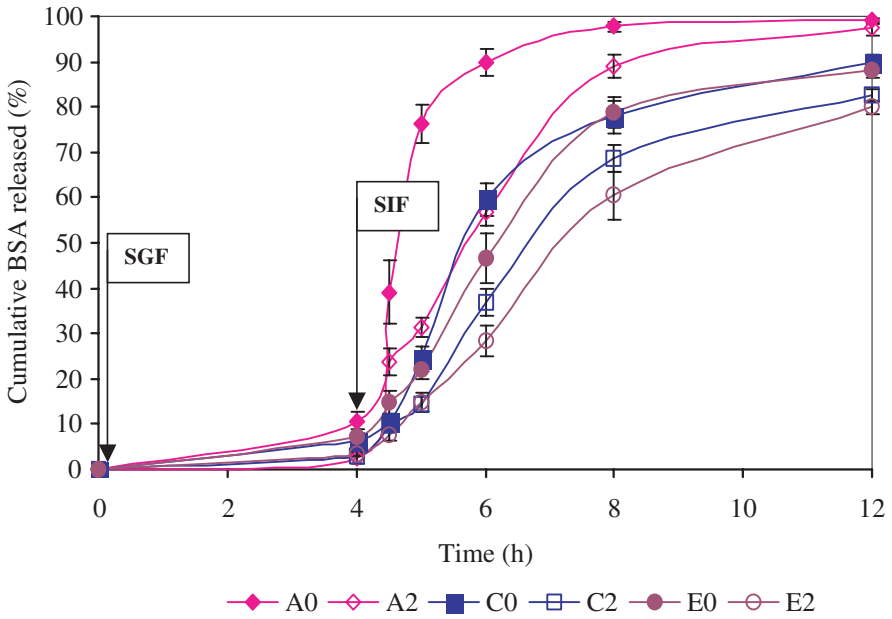


Figure 2. Effect of $CaCl_2$ and interaction of $CaCl_2$-chitosan concentration on release of BSA from chitosan-alginate beads in SGF/SIF. The notation of the samples is explained in Table 1.

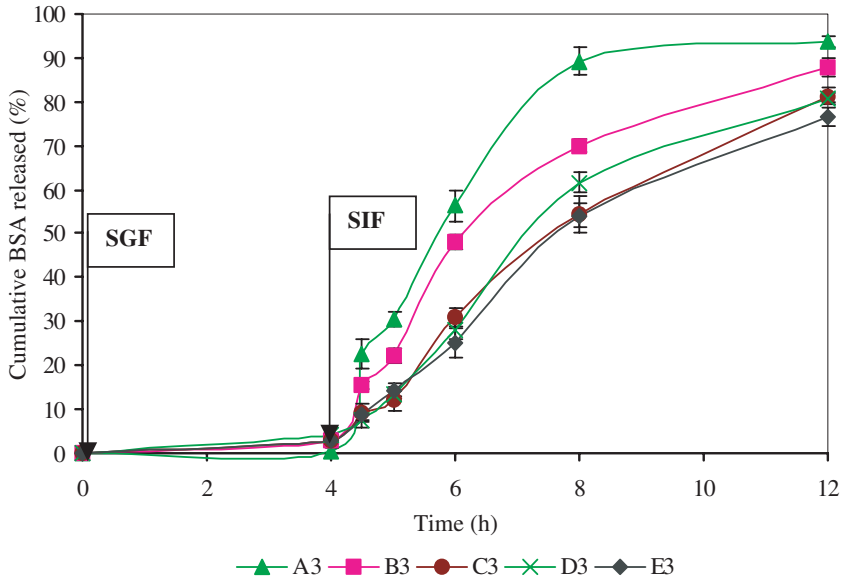


Figure 3. Effect of chitosan concentration (0–0.8%) on BSA release in SGF/ SIF. The notation of the samples is explained in Table 1.

The release of BSA from beads has been investigated during incubation in SIF only. Without prior incubation in gastric fluid (Fig. 5), single layered beads were more stable showing a delayed release of protein. The calcium alginate beads (A3, containing no chitosan) started to disintegrate only after 4 h and dissociated completely in 20 h.

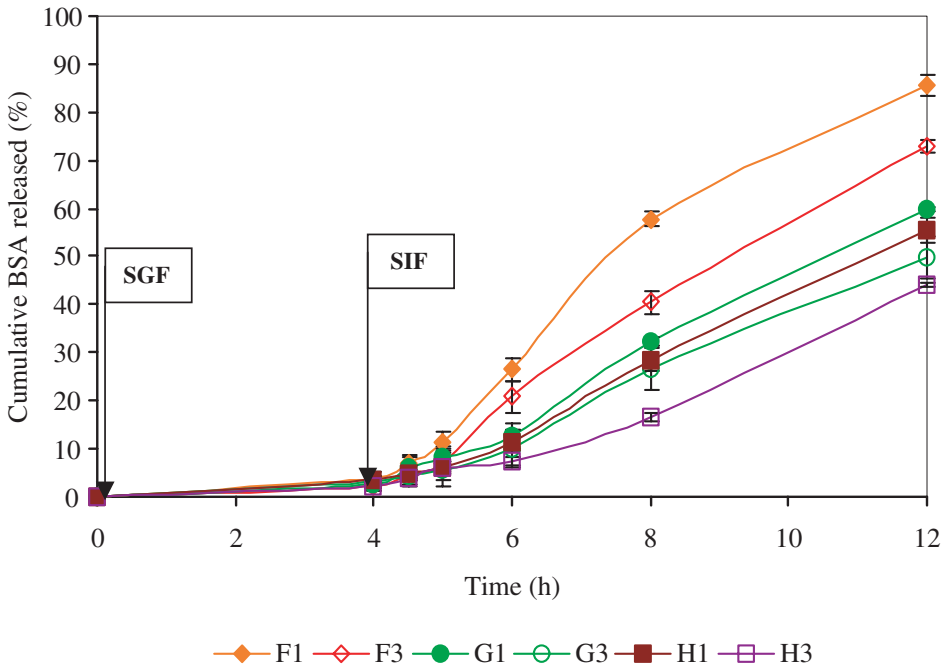


Figure 4. Release of BSA from different chitosan-alginate multilayer beads. The symbols and notations are explained in Table 2.

Beads prepared with chitosan were swollen after 2 h but without any substantial release of protein. The beads reinforced by 0.2% chitosan still showed some structure till 24 h. Higher chitosan concentration ($\geq 0.4\%$) delayed the release of BSA. There was no early erosion and only a small initial ($\leq 15\%$) release of protein. These chitosan beads remained intact till 24 h and gave no more release than 50%. The effect of the chitosan concentration on the release of BSA was significant ($p < 0.05$).

Also, the multilayer beads were found to be much more stable in intestinal fluid, without preincubation in gastric fluid. BSA release was extended over a longer period of time, especially for beads treated after coagulation with chitosan and alginate (Formulations H1, H2, and H3) (Fig. 6). The multilayer beads showed only 40–50% release of the entrapped BSA during 24 h in SIF. The effect of postcoagulation treatment on release of BSA is significant ($p < 0.05$).

CONCLUSIONS

Experiments were done to establish the optimum conditions for preparation of homogenous and spherical chitosan-alginate single and multilayer beads with a smooth surface. The swelling index increased with the increase in concentration of calcium chloride and chitosan. The multilayer beads prepared by using chitosan during and after coagulation were found to swell more in gastric fluid. The beads after postcoagulation treatment with sodium alginate showed less swelling. Addition of chitosan in the coagulation fluid resulted in a significant increase in the entrapment of BSA. The beads were intact, swollen, and floating in SGF. The use of chitosan during and after coagulation delayed the disintegration of beads and the release of BSA. The release of BSA seems to occur only concomitantly with the erosion of the beads. The disintegration of the beads was pH-dependent. The multilayer beads showed more delay in the release of BSA, more than chitosan-alginate beads prepared in a single step. The release was more sustained in intestinal fluid without prior incubating in gastric fluid. The classical calcium-alginate beads eroded completely within 20 h while the beads reinforced by chitosan remained intact. Based on findings, the multilayer beads seemed to be more effective oral formulations for sustained release of protein drugs. These bead systems can help to bypass the acidity of gastric fluid without liberating substantial amounts of loaded compound. such beads may be effective as

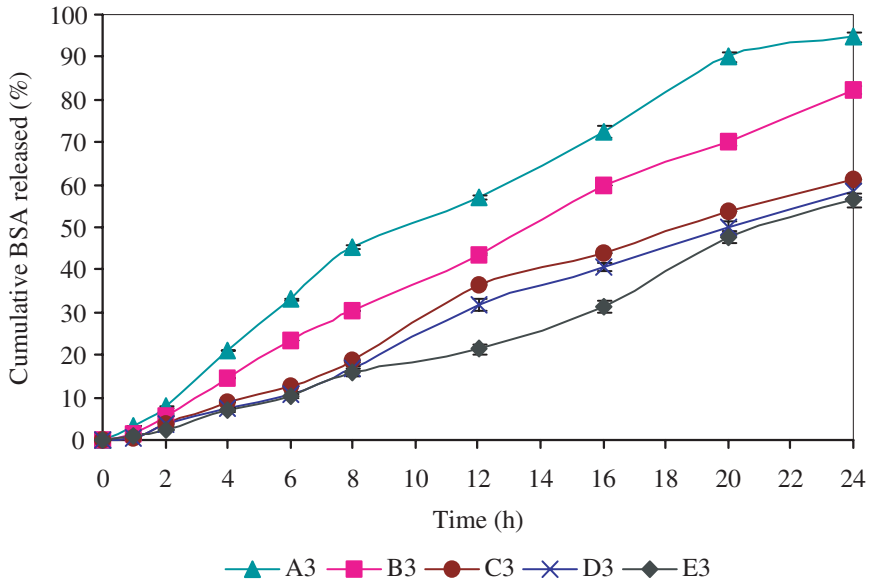


Figure 5. Effect of chitosan concentration (0–0.8%) on release of BSA incubated in SIF only. The symbols and notations of conditions are listed in Table 1.

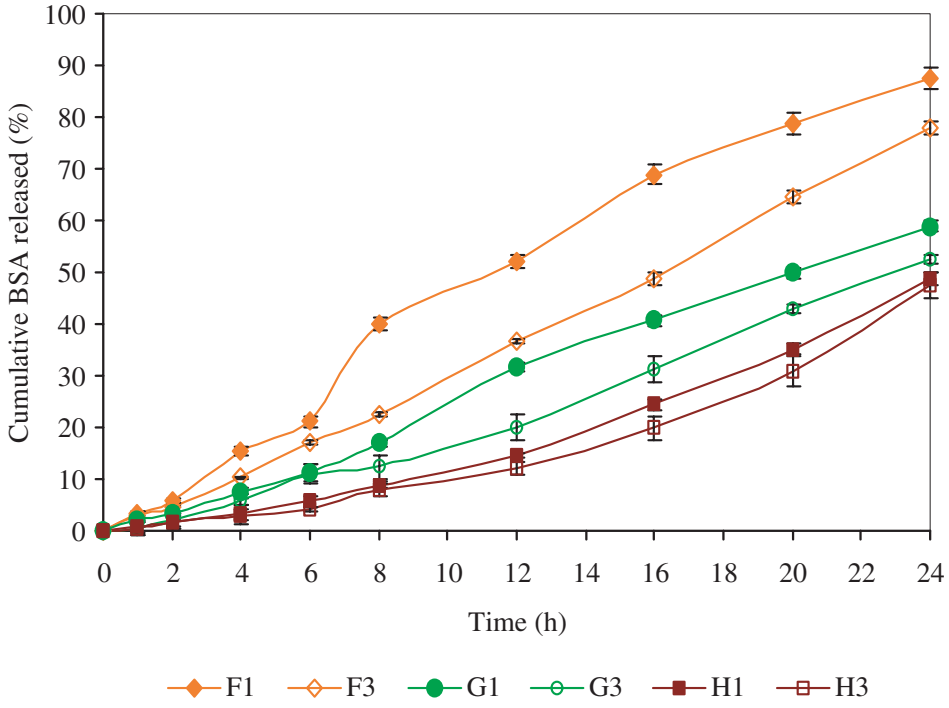


Figure 6. Release of BSA from chitosan-alginate multilayer beads incubated in SIF only. The symbols and notations refer to the conditions listed in Table 2.

site-specific peptide and protein drug delivery system for the intestine.

ACKNOWLEDGMENTS

The authors are grateful to the late Prince Leo de Lignac for his financial support and Dr. Carmen Remuñán-López, University of Santiago de Compostela, Spain, for her technical advice. Dr. Korbtham Sathirakul, Prof. Suwalee Chandrkrachang and Dr. M. Shrinivas Rao are acknowledged for their valuable suggestions.

REFERENCES

1. Zhou, X.H.; Po, L.W. Int. J. Pharm. **1991**, *75*, 117–130.
2. Fix, J.A. Pharm. Res. **1996**, *13* (12), 1760–1763.
3. Breimer, D.D. J. Control. Rel. **1999**, *62*, 3–6.
4. Ramadas, M.; Paul, W.; Dileep, K.J.; Anitha, Y.; Sharma, C.P. J. Microencap. **2000**, *17* (4), 405–411.
5. Niwa, T.; Takeuchi, H.; Hino, T.; Nohara, M.; Kawashima, Y. Int. J. Pharm. **1995**, *121*, 45–54.
6. Kim, H.K.; Park, T.G. Biotech. Bioeng. **1999**, *65* (6), 659–667.
7. Heller, H.; Barr, J.; Ng, S.; Shen, H.; Gurny, R.; Schwach-Abdelaoui, K.; Rothen-Seinhold, A.; Weert, M.V. J. Control. Rel. **2002**, *78*, 133–141.
8. Bouillot, P.; Ubrich, N.; Sommer, F.; Duc, T.M.; Loeffler, J.P.; Dellacherie, E. Int. J. Pharm. **1999**, *181*, 159–172.
9. Whateley, T.L. *Microencapsulation of Drugs*; Harwood Academic Publishers: Switzerland, 1992.
10. Polk, A.; Amsden, B.; Yao, K.D.; Peng, T.; Goosen, M.F.A. J. Pharm. Sci. **1994**, *83* (2), 178–185.
11. Huguet, M.L.; Groboillot, A.; Neufeld, R.J.; Poncelet, D.; Dellacherie, E. J. Appl. Polym. Sci. **1994**, (51), 1427–1432.
12. Aiedeh, K.; Gianasi, E.; Orienti, I.; Zecchi, V. J. Microencap. **1997**, *14* (5), 567–576.
13. Murano, E. J. Appl. Ichthyol. **1998**, (14), 245–249.
14. Aral, C.; Akbuga, J. Int. J. Pharm. **1998**, (168), 9–15.
15. Sezer, A.D.; Akbuga, J. J. Microencap. **1999**, *16* (2), 195–203.
16. Hari, P.R.; Chandy, T.; Sharma, C.P. J. Appl. Polym. Sci. **1996**, *59* (11), 1795–1801.
17. Tokura, S.; Tamura, H. Mat. Sci. Eng. C., *in press*.
18. Tokura, S.; Nishi, N.; Itoyama, K.; Shirai, A.; Nishimura, S.I.; Saiki, I.; Azuma, I. Chitin World: Proceedings from the Sixth International Conference on Chitin and Chitosan held in Gdynia, Poland. Karnicki, Z.S., Brezeski, M.M., Bykowski, P.J., Wojtasz-Pajak, A., Eds.; Germany, 1994, 287–300.
19. Stevens, W.F. Chitin and Chitosan in Life Science, Proceedings of 8th Int. Conf. on Chitin and Chitosan and 4th Asia Pacific Chitin and Chitosan Symp, ISBN4-906464-13-0, Yamaguchi, 2000, 293–300.
20. Bodmeier, R.; Oh, K.H.; Pramar, Y. Drug Dev. Ind. Pharm. **1989**, *15* (9), 1475–1494.
21. Murata, Y.; Maeda, T.; Miyamoto, E.; Kawashima, S. Int. J. Pharm. **1993**, *96*, 139–145.
22. Dorozynski, P.; Jachowicz, R.; Bystrowska, B.; Brandys, J. *Chitosan per os: From Dietary Supplement to Drug Carrier*; Muzzarelli, R.A.A., Ed.; Atec, Grottammare, Ancona: Italy, 2000; 221–237.
23. Yamamoto, A.; Tozaki, H.; Okada, N.; Fujita. S.T.P. Pharma Sci. **2000**, *10* (1), 23–34.
24. Ramadas, M.; Dileep, K.J.; Anitha, Y.; Paul, W.; Sharma, C.P. J. Biomater. Apll. **1999**, *13* (4), 290–296.
25. Huguet, M.L.; Dellacherie, E. Proc. Biochem. **1996**, *31* (8), 745–751.
26. Bradford, M.M. Anal. Biochem. **1976**, (72), 248–254.
27. Dainty, A.L.; Goulding, K.H.; Robinson, P.K.; Sinpkins, I.; Trevan, M.D. Biotechnol. Bioeng. **1986**, *28*, 210–216.