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RESEARCH PAPER

Chitosan-Alginate Microparticles as a Protein Carrier

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ABSTRACT

The oral administration of peptidic drugs requires their protection from degradation in the gastric environment and the improvement of their absorption in the intestinal tract. For these requirements, a microsystem based on cross-linked alginate as the carrier of bovine serum albumin (BSA), used as a model protein, was proposed. A spray-drying technique was applied to BSA/sodium alginate solutions to obtain spherical particles having a mean diameter less than 10 μm. The microparticles were hardened using first a solution of calcium chloride and then a solution of chitosan (CS) to obtain stable microsystems. The cross-linking process was carried out at different CS concentrations and pH values of the cross-linking medium. The CS concentration affected the BSA loading in the microparticles prepared at a pH value less than the protein isoelectric point (pI). Moreover, the BSA loading at a pH value less than the pI was higher than that at a pH similar to the pI regardless of the CS concentration. This finding could be attributable to the formation of a BSA/alginate complex. The evaluation of the interaction between BSA and alginate at different pH values by means rheological measurements confirmed this hypothesis. This approach may represent a promising way to devise a microcarrier system with appropriate size for targeting the Peyer's patches, with appropriate immobilization capacity, and suitable for the oral administration of peptidic drugs.

Key Words: *Alginate; Chitosan; Microparticles; Protein complexation; Spray-drying.*

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INTRODUCTION

Active compounds such as peptide and protein drugs are increasingly becoming a very important class of therapeutic agents as a result of the rapid advances in biotechnology and genetic research. Peptides and proteins are normally administered by the parenteral route. However, complications such as thrombophlebitis or tissue necrosis and poor patient compliance have stimulated the investigation of alternative nonparenteral routes (1). Nevertheless, several extremely efficient barriers, such as proteolytic enzymes present in various epithelia at different locations, may restrict the absorption of peptide and protein drugs administered by nonparenteral routes (2). In addition, most peptides pass through biological barriers poorly due to low diffusivity and a partition coefficient that is unfavorable for uptake by lipid membranes. Therefore, these macromolecules are absorbed preferentially through the paracellular transport and the intercellular junction (3).

To overcome these problems, several approaches have been proposed, such as the inhibition of the enzymatic degradation (4), the enhancement of membrane permeability or the widening of the tight junctions (5), the chemical modification of the protein (6), and the formulation of carrier systems (7). Among nonparenteral routes, oral administration is usually preferred because it is the most acceptable and convenient for the patient. To overcome the problems due to the acidic environment and enzymatic degradation in the gastrointestinal tract, the formulation of drug delivery systems as liposomes, nanoparticles, and microspheres has shown very interesting results (8). Furthermore, it was shown that microcapsules were able to be taken up by Peyer's patches; the uptake of particles less than 10 μm was much greater than that of the larger microspheres (9,10).

To minimize protein denaturation and the loss of its biological activity, a mild microencapsulation method, avoiding exposure to elevated heating and to organic solvents, should be adopted (11). Among many other microencapsulation procedures, the spray-drying technique could be considered proper for protein encapsulation (12,13).

In protein carrier selection, natural polymer hydrogels may be preferable candidates as protein release matrices because they are biosafe, highly inert toward protein drugs, and do not need organic solvents (11). In this regard, the use of alginate gelled by the addition of calcium ions has been proposed to prepare beads (14) or microcapsules (15,16) applied in the controlled release of macromolecules and cells. Alginates are known to be nontoxic when taken orally and to sustain drug release due to their cation-induced gelation (14). Moreover, alginates possess a bioadhesive property (17) and may be useful in increasing drug residence time at the site of resorption, thereby improving overall drug effectiveness and bioavailability. However, the cation cross-linked alginate network can degrade by removal of the calcium ions by chelating agents such as lactate, citrate, and phosphate. These ions are virtually nonexistent in human intestinal fluid (18), but they can be introduced with the diet. As calcium ions are removed, the cross-linking in the gel decreases, and the gels are destabilized, leading to fast drug delivery rates (19).

Alginates form strong complexes with polycations that are more resistant in the presence of calcium chelators and can be used to both stabilize the gel and reduce its porosity (17). Among these polycations, chitosan (CS) has received considerable attention for its safety and its mucoadhesive properties (20). Also, CS enhances the penetration of macromolecules across the intestinal barrier (21). Due to its hydrophilic and cationic characteristics, CS has the ability to gel on contact with counteranions. It has been reported that the amino groups of CS are capable of interacting with an anionic polymer that has carboxylic groups, such as carboxymethylcellulose (22) or alginate (15,23), by ionic binding. Moreover, the CS-alginate complex was noted to be stable to pH values ranging from 3.7 to 4.7 (23). Therefore, it is expected that the interaction of alginate with CS could result in a stronger material that could be more stable in both acidic media and in media containing calcium chelators.

Thus, the aim of this work was to investigate the feasibility of the spray-drying technique to produce less than 10-μm microparticles of alginate cross-linked by calcium ions and CS for the transport of bovine serum albumin (BSA) as a model protein. In particular, microparticle dimensions, morphology, and protein loading were evaluated as a function of the preparation procedure.

MATERIALS

Sodium alginate (Na-A) (molecular weight about 147,000, containing 62% mannuronic acid and 38% guluronic acid) was donated by Kelko International (Bagnolex Cedex, France). BSA was purchased from Sigma Chemical Company (St. Louis, MO). CS (low molecular weight, about 70,000) and calcium chloride dihydrate were obtained from Fluka Chemie (Buchs, Switzerland). The protein reagent for the Lowry assay was supplied by Bio-Rad Laboratories (Milan, Italy).

METHODS

Microparticle Preparation

Water solutions containing a 2% w/v of (1/1) BSA/Na-A mixture were spray-dried by a Buechi 190 Mini-Spray Dryer (Buechi Laboratoriums, Technik AG, Flawil, Switzerland) in the following operating conditions: inlet temperature 140°C, outlet temperature 60°C–65°C, pump setting 5 ml min^{-1}, spray flow 600 Nlh^{-1}, aspirator setting 10. A 0.5-mm nozzle cap was used, and the nozzle body was cooled with water.

The resultant microparticles (termed *temporary microparticles* throughout this article) were dispersed at room temperature under vigorous stirring at 3000 rpm (Ultraturrax, IKA, Labortechnik, Staufen, Germany) in a 2.5% w/v calcium chloride water solution (corresponding to a $CaCl_2$/Na-A ratio of 5/1 w/w) at pH 5.5 for acetic acid and cured by an interfacial gelation process for 5 min (24). Then, an equal volume of a CS water solution at different concentrations (ratios of CS/Na-A ranged from 0/1 to 4/1 w/w) at pH 5.5 for acetic acid was added. The system was maintained under stirring (3000 rpm) for 10 min at room temperature. The resultant cross-linked microparticles (termed *microparticles* throughout this article) were recovered by centrifugation at 3000 rpm, rinsed with water, and freeze-dried (Lyovac GT2, Leybold-Heraues GMBH, Köln, Germany). The same procedure was adopted for the microparticle preparation at pH 3.5.

Morphological and Particle Size Analysis

The morphological structure of both the temporary microparticles and the microparticles was examined with a scanning electron microscope (SEM) (XL-40, Philips, Eindhoven, The Netherlands) after coating under an argon atmosphere with a 10-nm gold-palladium thickness (Emitech K550 Sputter Coated, Emitech Limited, Ashford, KY). The particle size was determined by image analysis (Image Proplus, Media Cybernetics, USA) of each sample on SEM micrographs. The mean diameter of at least 100 particles was calculated from the average diameter measured at 2° intervals and passing through the centroid of the particle perimeter.

Bovine Serum Albumin Content

BSA content was determined by dissolving an exactly weighed amount (10 mg) of both temporary microparticles and microparticles in 3% w/v sodium citrate water solution (10 ml) for 48 h with magnetic stirring at room temperature, with the citration breaking down the structure entrapping the protein (15,25). After centrifugation at 10,000 rpm (Sorvall RC-28S, Du Pont Co., Wilmington, DE) for 10 min, BSA concentrations in the clear supernatant solutions (1 ml) were assayed spectrophotometrically (750 nm) (Lambda 3B, Perkin-Elmer, Norwalk, CT) by the well-documented Lowry assay (26). The reported data were averaged from three determinations from three different batches.

Evaluation of Bovine Serum Albumin/Sodium Alginate Interaction

A Na-A solution (1% w/v in acetic acid solution at pH 3.5 and 5.5) was mixed with an equal volume of a BSA solution (0%–12% w/v in acetic acid solution at pH 3.5 and 5.5) to prepare samples having a BSA/Na-A ratio ranging from 0 to 6. After 24 h, the rheological behavior of the supernatant solutions, after centrifugation at 20,000 rpm (Sorvall RC-28S) for 15 min, was determined at 25°C by placing 9 ml of the sample in a coaxial cylinder (radii ratio 1.02) rheometer (Rotovisco RV12, Haake, Karlsruhe, Germany) and measuring the shear stress as a function of the shear rate. In the same conditions, solutions containing pure Na-A and pure BSA, as well as the media, were examined. The rheological experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Spray-drying is a viable commercial method of forming microparticles. Such a technique was considered in the present study to evaluate its usefulness in producing protein-loaded microparticles with a size less than 10 μm. Moreover, since the polymer material selected, sodium alginate, needs cross-linking reactions, the effect of two formulative variables (i.e., the ratio between chitosan and alginate and the pH value of the reaction medium) on the loading capacity of the microparticles was investigated.

It is known that charged proteins interact with polyelectrolytes, leading to complexes that are useful in several fields of application (22). In our case, as the BSA has an isoelectric point (pI) of 5.0 (27), at a pH value less than its pI, it is positively charged and could be complexed with a negatively charged polymer such as alginate. Therefore, to investigate the possible effect of an interaction between BSA and alginate on the entrapment capacity of the microparticles, two different pH values (3.5 and 5.5) were selected for the crosslinking reaction.

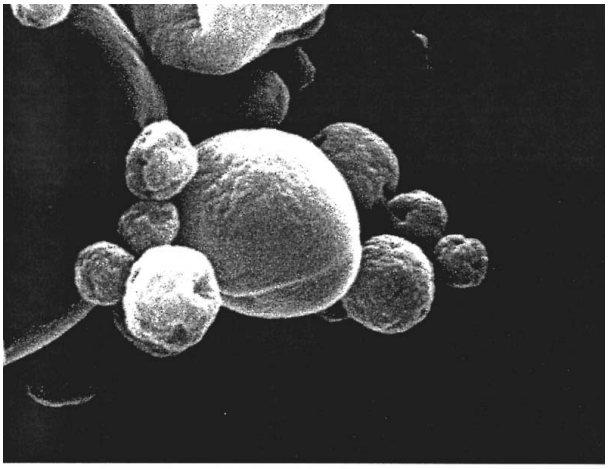

Figure 1. SEM micrograph of temporary microparticles (CS/Na-A = 0.4/1).

The spray-drying technique adopted in the present study produced temporary microparticles that were spherical and smooth (Fig. 1), whereas the microparticles (obtained by the cross-linking reaction) were spheroidal and quite rough regardless of the formulative variables (Fig. 2).

The size and the dimensional distribution varied as a consequence of the cross-linking procedure (Table 1). Although the one-way analysis of variance (ANOVA) method showed a statistical difference ($P < .01$) between the diameter values of the temporary microparticles (mean value 1.72 μm) and the microparticles (mean value 2.81 μm), the average size of all the samples ranged within 10 μm.

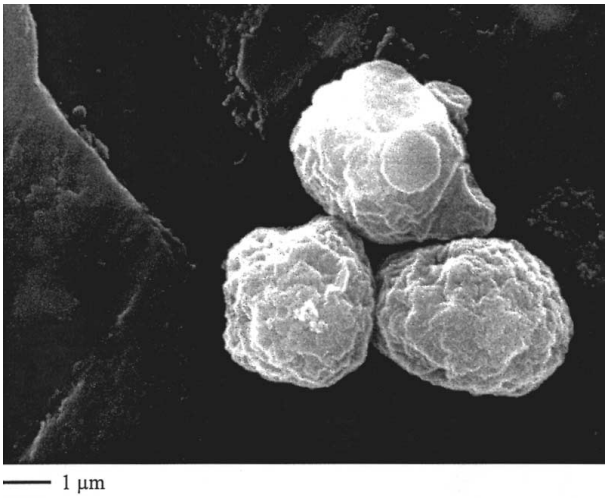

Figure 2. SEM micrograph of microparticles (CS/Na-A = 0.4/1).

Table 1

Size and Dimensional Distribution of Temporary Microparticles and Microparticles

	Temporary Microparticles	Microparticles
Minimum diameter (μm)	0.19	1.06
Mean diameter (μm)	1.72 ± 1.00	2.81 ± 1.33
Maximum diameter (μm)	5.97	7.26
<1 μm	18%	—
1–2 μm	60%	35%
2–3 μm	7%	28%
3–4 μm	13%	20%
4–5 μm	—	6%
5–10 μm	2%	11%

Moreover, the formulative variables adopted during the cross-linking process affected the BSA loading level. In fact, the microparticles prepared at pH 3.5 showed a satisfactory BSA content compared with the theoretical value, and it resulted in about twice as much as that obtained at pH 5.5 for all the CS/Na-A ratios used (Table 2). Since it was previously established that the composition of the complex between chitosan and alginate is not a function of the pH of the reaction mixture (23), this result could be reasonably attributable to the polycationic state of the protein at pH 3.5. Therefore, an electrostatic interaction between the charged protein and the polyanionic alginate would determine a higher capacity of protein immobilization inside the microparticles.

Table 2

Bovine Serum Albumin (BSA) Content in the Microparticles Obtained at Different CS/Na-A Ratios and Different pH

CS/Na-A (w/w)	BSA Content (%), pH 5.5 (Theoretical Content = 50%)	BSA Content (%), pH 3.5 (Theoretical Content = 50%)
0.0/1	11.2 ± 1.0	21.3 ± 1.2
0.2/1	9.1 ± 1.2	26.3 ± 1.5
0.4/1	12.1 ± 1.5	23.6 ± 1.5
0.8/1	8.3 ± 1.1	16.0 ± 1.1
2.0/1	8.5 ± 1.1	15.7 ± 1.3
4.0/1	9.1 ± 1.2	16.5 ± 1.2

CS/Na-A, chitosan/sodium alginate.

To evaluate the interaction occurring between BSA and alginate, rheological measurements were carried out on BSA/Na-A water solutions at pH 3.5 and 5.5. Rheological characterization of polyelectrolytes, such as alginates, provided relevant information about the electrostatic interaction between a polysaccharide and an oppositely charged compound (28).

In this regard, two different approaches could be adopted. The first involved the evaluation of the reduced viscosity owing to an ''electroviscous effect'' by dilute solutions of polyelectrolytes (29); the second involved the evaluation of the reduced viscosity in the supernatant solutions after the removal of the polysaccharide involved in a solid complex formation (23). In the present work, the second approach was considered since it was impossible to obtain true solutions at a pH value of 3.5. Therefore, the analysis was performed on the supernatant solutions after centrifugation.

The flow behaviors of the solutions at pH 3.5 and 5.5 are depicted in Figs. 3 and 4. Since the rheological flow behavior of BSA corresponded to that of the medium, the rheograms of BSA/Na-A supernatant solutions represent only the rheological properties of the alginate, which did not interact with BSA. Consequently, the viscosity of the supernatant solution will be a function of the alginate concentration, and a decreased viscosity would indicate a decreased concentration of alginate owing to its involvement in the complex formation.

All the flow curves showed a pseudoplastic behavior without hysteresis loops, indicating that no change in the structure of the molecules under shear stress occurred. However, a deviation from the ideal pseudoplastic behavior was observed at low shear rates. The presence of BSA led to reduced viscosity only at pH 3.5, whereas no significant changes in the viscosity compared with that of the initial alginate solution were observed at pH 5.5. Therefore, the decreased viscosity observed at pH 3.5 suggests that an electrostatic interaction between the polycationic BSA and alginate occurred. Also, with the increase of BSA concentration, the viscosity of the supernatant solution decreased progressively, reaching values comparable to those of the medium at a BSA/Na-A ratio of 4/1, denoting the complete precipitation of alginate. This behavior is clearly indicated by the plot of the relative apparent viscosity, calculated for a shear rate of 1039 s^{-1}, versus the BSA/Na-A ratio, for which 100% is the value of the pure alginate solution (Fig. 5).

The occurrence of an interaction between the protein and alginate at a pH value of less than the pI of the protein could account for the statistically significant ($P < .01$) decrease of the BSA content in the microparticles ob-

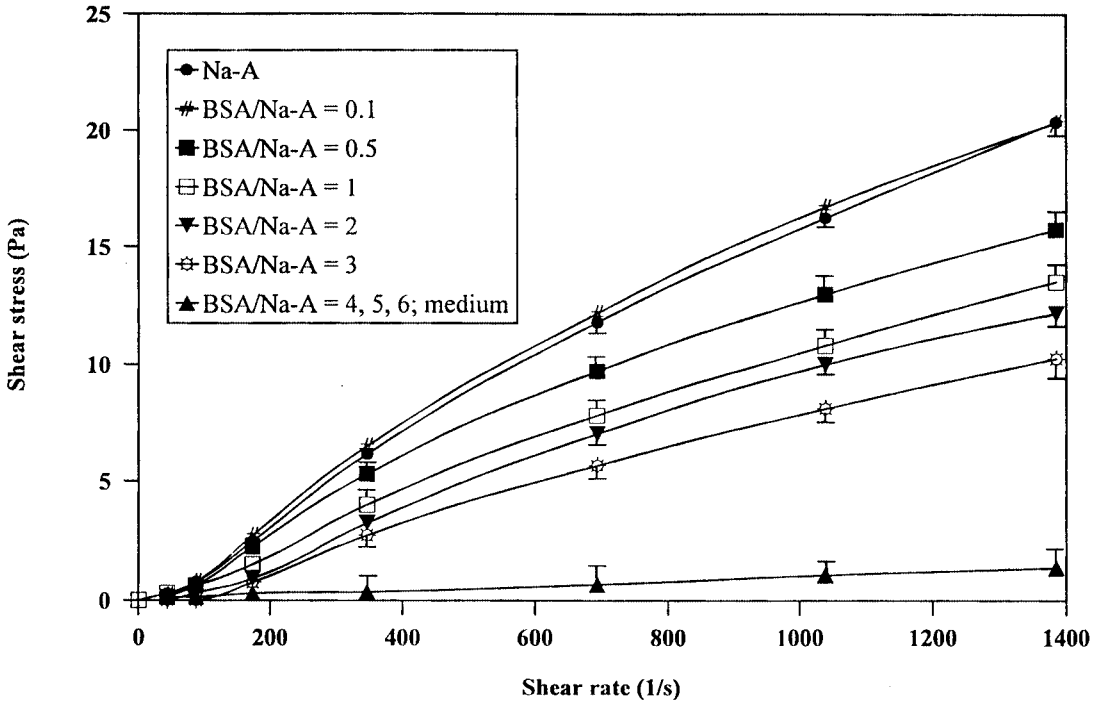

Figure 3. Flow behavior of pH 3.5 supernatant solutions at different BSA/Na-A ratios.

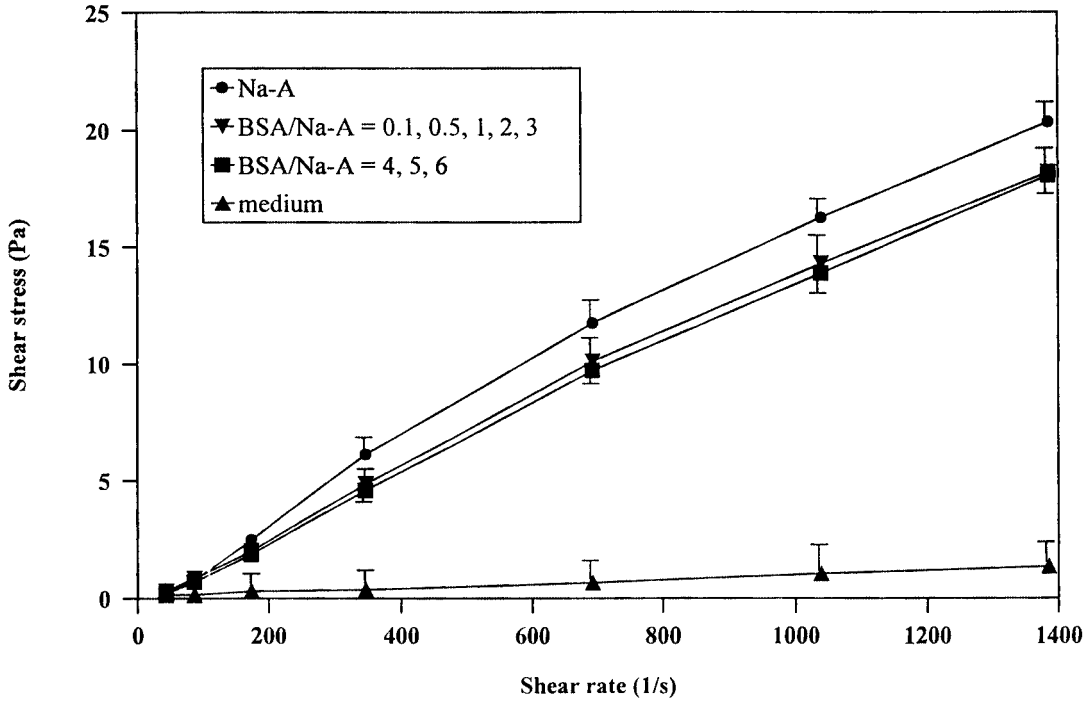

Figure 4. Flow behavior of pH 5.5 supernatant solutions at different BSA/Na-A ratios.

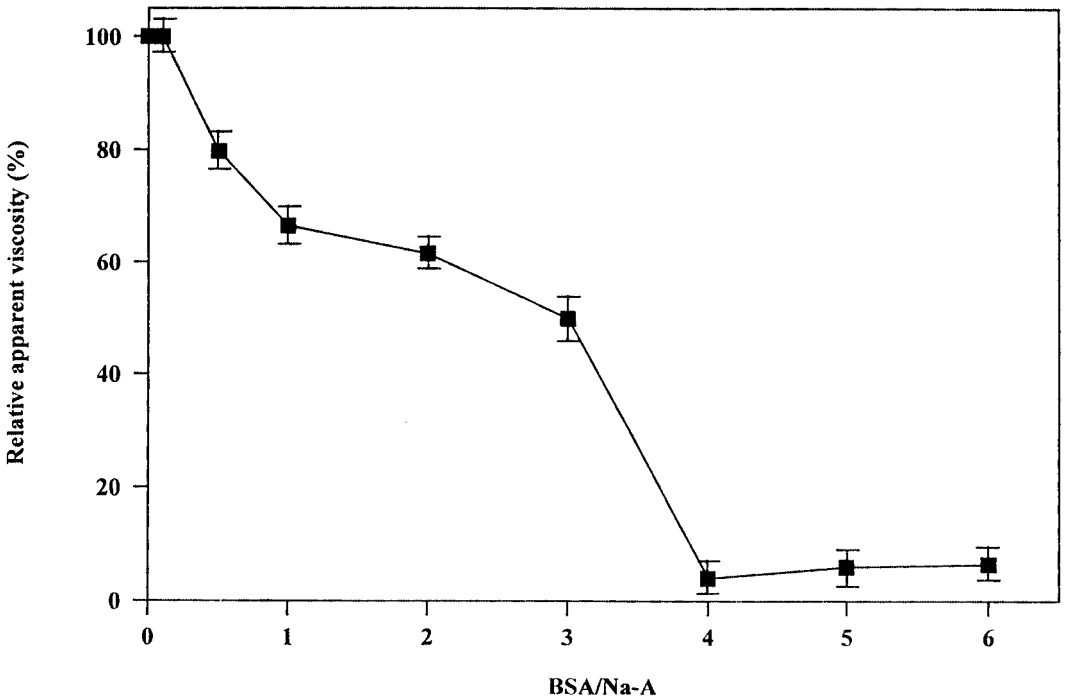

Figure 5. Relative apparent viscosity of pH 3.5 supernatant solutions at different BSA/Na-A ratios.

tained using the highest CS/Na-A ratio at pH 3.5 (Table 2). In fact, a competition between CS and positively charged BSA for available acid sites on the alginate chain could occur. This has been observed previously in several investigations with small drugs (30,31).

In contrast, the BSA content in the microparticles cross-linked at pH 5.5 was not statistically ($P > .05$) affected by the CS/Na-A ratio. Since this pH condition prevents the occurrence of an electrostatic interaction between BSA and alginate, drug loading was merely related to the capacity of the polymer backbone structure to entrap the protein.

CONCLUSIONS

The spray-drying method here reported provided small-size, protein-loaded chitosan-alginate microparticles with appropriate protein immobilization capacity. A complex between BSA and alginate was formed at a pH value less than the isoelectric point of the protein, leading to a significant increase in protein loading. Therefore, the possibility of immobilizing a protein in a biosafe hydrogel matrix on the basis of polyion complexation by means of complex formation between the protein and the polymer was demonstrated.

In such a condition, the polycationic chitosan would compete with BSA, producing a slight decrease in protein loading. Therefore, further investigations are necessary to evaluate the role of chitosan in improving protein release and microparticle mucoadhesive properties, as well as to indicate that this approach is effective in modifying stability and targeting of an orally administered protein and then in enhancing its therapeutic efficacy.

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