Microencapsulation of Hemoglobin in Chitosan-coated Alginate Microspheres Prepared by Emulsification/Internal Gelation

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ABSTRACT

Chitosan-coated alginate microspheres prepared by emulsification/internal gelation were chosen as carriers for a model protein, hemoglobin (Hb), owing to nontoxicity of the polymers and mild conditions of the method. The influence of process variables related to the emulsification step and microsphere recovering and formulation variables, such as alginate gelation and chitosan coating, on the size distribution and encapsulation efficiency was studied. The effect of microsphere coating as well its drying procedure on the Hb release profile was also evaluated. Chitosan coating was applied by either a continuous microencapsulation procedure or a 2-stage coating process. Microspheres with a mean diameter of less than 30 μm and an encapsulation efficiency above 90% were obtained. Calcium alginate cross-linking was optimized by using an acid/$CaCO_3$ molar ratio of 2.5, and microsphere-recovery with acetate buffer led to higher encapsulation efficiency. Hb release in gastric fluid was minimal for air-dried microspheres. Coating effect revealed a total release of 27% for 2-stage coated wet microspheres, while other formulations showed an Hb release above 50%. Lyophilized microspheres behaved similar to wet microspheres, although a higher total protein release was obtained with 2-stage coating. At pH 6.8, uncoated microspheres dissolved in less than 1 hour; however, Hb release from air-dried microspheres was incomplete. Chitosan coating decreased the release rate of Hb, but an incomplete release was obtained. The 2-stage coated microspheres showed no burst effect, whereas the 1-stage coated microspheres permitted a higher protein release.

KEYWORDS: alginate, chitosan, internal gelation, oral protein delivery, microspheres

INTRODUCTION

Oral administration of peptidic drugs is of great interest, but difficulties associated with the poor permeability across biological membranes, susceptibility to enzymatic attack, rapid postabsorptive clearance, and chemical instability have thwarted efforts to achieve an efficient formulation.[1] Several approaches have been proposed to improve oral bioavailability, among them microencapsulation, which represents a promising concept.[2] A mild encapsulation method should be adopted to minimize protein denaturation and the loss of its biological activity, avoiding exposure to high temperatures and organic solvents.

The relatively mild cross-linking conditions required for obtaining chitosan-coated alginate microparticles and the nontoxicity and biodegradability of these 2 naturally occurring biopolymers have enabled them to be used for the encapsulation of a wide variety of biologically active agents including proteins,[3-13] enzymes,[14-16] antibodies,[17] cells,[18-23] and DNA.[24-27]

Alginate is an anionic copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid residues. In the presence of divalent cations such as calcium, alginate forms a gel due to the stacking of guluronic acid (G) blocks with the formation of "egg-box" calcium-linked junctions.[28] Alginate gel microspheres have been conventionally prepared using extrusion by dropping an alginate solution through a needle into a $CaCl_2$ solution (external gelation), but this approach has several inconveniences, namely, the limitation in reducing microsphere diameter, the teardrop shape of the microparticles produced, and the difficulty in industrial scale-up.[29] The use of an emulsification/external gelation method by adding an emulsion of alginate solution in a hydrophobic phase to a $CaCl_2$ solution[30,31] could permit the reduction of microparticle diameter, but the disruption of the emulsion system equilibrium may cause deficient diameter control and a significant degree of clumping of microspheres.[32] Emulsification/internal gelation is another alternative to extrusion in which the bead diameter can be easily controlled and that has scale-up potential.[33] In this method, an alginate solution containing an insoluble calcium salt is dispersed in oil, and gelation is achieved by gentle acidification with an oil-soluble acid that causes calcium ion release.[34] The production of internally gelled alginate microspheres has also been accomplished by extruding an alginate solution mixed with $CaCO_3$ dropwise through a

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syringe needle into an oil containing an oil-soluble acid[35]; however, in this case the inconvenience related to extrusion/external gelation was also verified.

To reduce the porosity and increase stability, alginate microparticles have been coated with chitosan by electrostatic interaction.[36] The negatively charged carboxylic acid groups of alginate bind ionically with positively charged amino groups of chitosan, a cationic polymer, to form a polyelectrolyte complex on the basis of their opposite charges.[37] Moreover, chitosan has been reported to enhance absorption of various compounds across the mucosal barrier owing to its properties of mucoadhesion, which increase the contact time of the drug with the mucosa, and its ability to induce a transient opening of epithelial cell tight junctions.[38-40]

Alginate microspheres prepared by internal gelation and cross-linked with chitosan were applied in the encapsulation of DNA[24,26,27] and Sudan Orange G.[41] Encapsulation of bioactive compounds, such as proteins and DNA, by either internal gelation or external gelation revealed that internal gelation provides a better encapsulant protection from hydrolysis[24] but may permit higher losses from microspheres.[9]

In the present study, hemoglobin (Hb) was used as a model protein for evaluating the influence of some process and formulation factors on the morphology and encapsulation efficiency of calcium alginate microspheres obtained by emulsification/internal gelation. The effect of microsphere coating as well as microsphere drying procedure on the encapsulation efficiency, granulometry, and Hb release were also evaluated. Ideally, Hb should not be released in simulated gastric pH, and a slow release within 3 to 5 hours should occur in simulated intestinal fluid.

Materials and Methods

Materials

Sodium alginate SG300 was supplied by SKW Biosystems (Paris, France). This alginate exhibits an average molecular weight (MW) of 694 kd and a high guluronic content of 60%.[35] Low molecular weight (LMW) chitosan (150 kd) and Span 80 were purchased from Fluka Biochemika (Steinheim, Germany). Freeze-dried bovine hemoglobin (Hb) was obtained from Sigma (Steinheim, Germany). $CaCO_3$ was supplied by Omya (Orgon, France), and paraffin oil and Tween 80 were supplied by Vaz Pereira (Lisbon, Portugal). All other chemicals used were of analytical reagent grade.

Methods

Preparation of Microspheres

Microspheres were prepared by emulsification/internal gelation.[33] A 2% wt/vol sodium alginate solution with 1.5% wt/vol bovine Hb was prepared by dissolving alginate in a previously prepared protein solution in phosphate buffer at pH 6.8 (*United States Pharmacopeia* [*USP*] XXVI).

Microspheres were prepared by adding a suspension of 5% wt/vol ultrafine $CaCO_3$ into the alginate solution. After homogenization, the mixture was dispersed into paraffin oil containing, in some cases, a lipophilic surfactant (Span 80 1% vol/vol) by stirring at a speed rate from 200 up to 600 rpm using an Ika-Eurostar mixer (Staufen, Germany) with a marine impeller. The aqueous/oily phase ratio was varied from 20% to 40%, keeping the total volume constant. After a 15-minute emulsification, 20 mL of paraffin oil containing glacial acetic acid was added to the water/oil (w/o) emulsion, reducing the alginate pH to approximately 5.0, and stirring continued to permit $CaCO_3$ solubilization. The influence of the acetic acid/$CaCO_3$ molar ratio, a formulation parameter, was evaluated (1.25 and 2.5) by changing the volume of acetic acid added to the w/o emulsion. Oil-dispersed microspheres were recovered by using the following washing media either with or without Tween 80 (1% vol/vol): $CaCl_2$ 0.05 M, distilled water, and acetate buffer at pH 5.5 (*USP* XXVI). Microspheres were successively washed with the same washing medium used in partition, until no more oil was detected under optical microscope observation.

Chitosan coating was applied using 2 methods: a continuous microencapsulation procedure designated as 1-stage coating (C1), and a 2-stage procedure designated as 2-stage coating (C2). In the first method, coating was performed by adding an emulsion of 0.1% wt/vol chitosan in 1% (vol/vol) acetic acid, at pH 6.4, in paraffin oil (50/50 vol/vol), to the oily dispersed gelled microspheres after calcium solubilization, and stirring was performed for 30 minutes. Coated microspheres were recovered by using acetate buffer at pH 5.5, as described above. Two-stage coating method was performed by adding a 0.1% wt/vol chitosan solution in 1% vol/vol acetic acid, at pH 6.4, to the isolated microspheres under magnetic stirring for 30 minutes. Uncoated (U) microspheres were used as control.

Microspheres were kept in the washing medium to be used in the wet state (W). Otherwise, gelled microspheres were allowed to dry in air at 4ºC to 6ºC (D) until constant weight was achieved or microspheres were lyophilized (L). Lyophilization was performed at 0ºC for 48 hours (Lyph-lock 6 apparatus, Labconco, Kansas City, MS) after freezing microspheres in an ethanol bath (Benchtop shell freezer, Freezone model 79490, Labconco) at −50ºC.

Microsphere Morphology

Microsphere morphology was monitored by optical microscopic observation using an optical microscope Olympus BH2-UMA equipped with a Cue-2 image analyzer (Olympus,

Tokyo, Japan). Granulometric size distribution based on volume distribution was determined in washing media by laser diffractometry (Fraunhofer model) using a Coulter LS130 particle analyzer (Beckman Coulter Inc., Fullerton, CA), with a size range from 0.1 to 1000 μm. Measurements were made in triplicate for each batch.

Encapsulation Efficiency

The encapsulation efficiency was evaluated by an indirect method. The nonencapsulated Hb was determined by measuring its losses during microsphere preparation and recovery. Protein retention was calculated from the difference between the initial amount of protein added and the total losses.

Hb Quantification

Samples were mixed with NaOH to raise pH above 9.5 and ethanol was added (50/50 vol/vol) to the reaction mixture. The absorbance was measured at 401 nm (UV-1603, UV-visible spectrophotometer, Shimadzu, Kyoto, Japan). When using 0.05 M $CaCl_2$ as the washing medium, 0.1 M ethylenediamine tetra-acetic acid (EDTA) was added before adjusting the pH. A calibration curve was prepared for up to 0.6 g/L of hemoglobin concentration.

In Vitro Release Studies

An amount of microspheres equivalent to 10 mg of Hb was placed in glass vials containing 50 mL of artificial gastric fluid (*USP* XXVI), without enzymes, under magnetic stirring (100 rpm for 2 hours). Microspheres were then transferred into artificial intestinal fluid (*USP* XXVI), without enzymes. Samples were withdrawn at different time intervals and spectrophotometrically assayed for the Hb concentration as mentioned previously. Released Hb was calculated accordingly to the following equation:

$$\text{Hb release} = \text{Mt Min} \times 100 \qquad (1)$$

where M_t is the amount of Hb at time t and M_{in} is the amount of Hb in the microspheres at time $t = 0$. All studies were performed in triplicate.

RESULTS

Microsphere Morphology

A unimodal size distribution with a mean diameter less than 30 μm was obtained for uncoated alginate microspheres prepared using a w/o ratio of 30/70, 1% emulsifier, and mixer rotational speed of 400 rpm, and recovered with acetate buffer. The microspheres were dispersed and spherical in shape as illustrated in Figure 1.

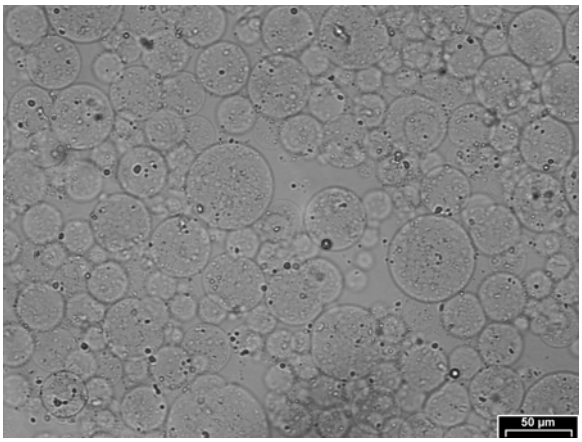

Figure 1. Optical photomicrograph of Hb-loaded alginate microspheres (magnification 200 X).

The effect of different process parameters on the mean size of microspheres was evaluated. Increasing the internal phase ratio from 20% to 40% resulted in a slight decrease in the mean size, and in the presence of Span 80 the microsphere mean diameter decreased (Table 1). The influence of the mixer rotational speed during the emulsification step on size distribution (Figure 2) was also evaluated, and it was shown that increasing the impeller rotational speed decreased the mean diameter of microspheres reaching a minimum at 500 rpm. At 600 rpm, a lower mode value was obtained, but a broader distribution increased the mean size. At 400 rpm, the standard deviation relative to the mean was 46% and at 600 rpm this value increased to 80%.

Microsphere recovery medium was determinant on microsphere size distribution (Table 2). Water-washed microspheres presented a mean size that increased up to 200 μm and a broader and bimodal granulometric distribution. An increased mean size was also verified for microspheres washed with $CaCl_2$. Detergent-containing media did not cause significant differences except for acetate buffer-washed microspheres, which presented a higher mean diameter and a broader distribution. These results, based on volume distribution, were different when a number distribution was used (Figure 3). Microspheres washed with $CaCl_2$

Table 1. Volume Mean Diameter of Microspheres in Relation to the Aqueous/Oily Phases Ratio and the Inclusion of a Surface Agent*

Water/Oil (%) (vol/vol)	Span 80 1% (vol/vol)	Diameter (μm)
20	+	26.0 ± 9.7
30	+	24.9 ± 11.6
40	+	22.7 ± 9.8
30	-	132.6 ± 36.8

*Microspheres were prepared at 400 rpm. Data are shown as mean ± SD.

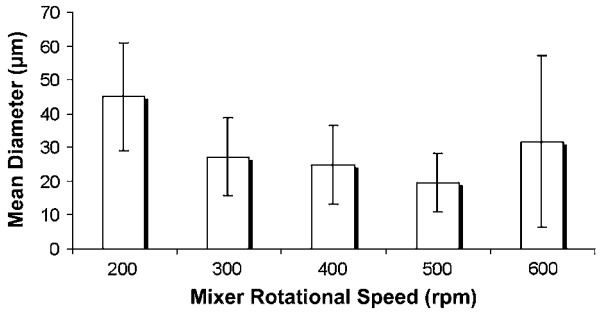

Figure 2. Volume mean diameter of microspheres formed at different mixer rotational speed. Error bars represent SD of the mean.

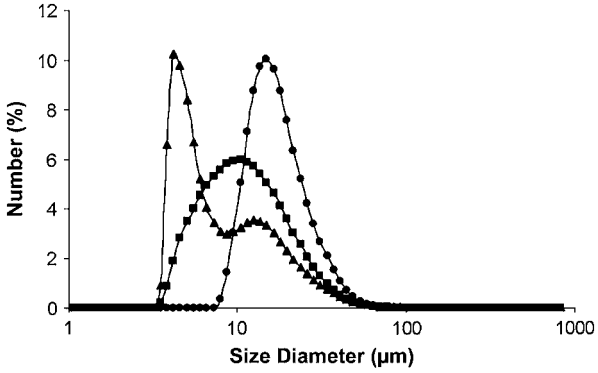

Figure 3. Number size distribution of microspheres recovered using different washing medium: $CaCl_2$ 0.05 M (▲), water (■), and acetate buffer pH 5.5 (●).

or water presented a smaller peak mode than acetate buffer-recovered microspheres.

Formulation parameters also affected the microspheres' mean diameter (Table 3). Acetic acid/$CaCO_3$ molar ratio determines the degree of alginate gelation, and the microspheres' mean size increased with the volume of acetic acid added to the emulsified droplets of alginate. Chitosan-coated microspheres presented a higher mean diameter size than uncoated microspheres due to aggregation during the coating process (Figure 4). During 2-stage coating, aggregation was more intense, leading to irregularly shaped units sizing above 1000 µm, which could not be measured by laser diffractometry.

After air drying, the macroscopic appearance of microspheres was altered. Uncoated microspheres formed a film, and clumps of microspheres were formed for coated microspheres, probably owing to cohesion of the initially wet microspheres.[31] Lyophilized microspheres presented a sponge-like structure.

Encapsulation Efficiency

Hb-loaded microspheres prepared by emulsification/internal gelation retained more than 90% of the initial Hb inside the alginate microspheres.

Table 2. Volume Mean Diameter of Microspheres Recovered in Relation to the Microsphere Washing Medium*

Washing Medium	Tween 80 1% (vol/vol)	Diameter (µm)
$CaCl_2$ 0.05 M	-	206.2 ± 108.4
	+	196.0 ± 112.9
Water	-	198.0 ± 143.7
	+	181.3 ± 135.9
Acetate buffer, pH 5.5	-	24.9 ± 11.6
	+	36.6 ± 17.8

*Microspheres were prepared with an aqueous/oily phase of 30% (vol/vol) at 400 rpm. Data are shown as mean ± SD.

Process parameters such as mixer rotational speed and concentration of emulsifier did not affect Hb retention inside microspheres (Tables 4 and 5). Varying the w/o phase ratio from 20% to 40% caused only a slight decrease in encapsulation efficiency.

Hb losses during preparation were minimal when recovery was performed either with acetate buffer or with water (Figure 5). Losses occurred preferentially during the first wash, immediately after the partition, corresponding to Hb released during alginate gelation. $CaCl_2$-recovered microspheres registered significant protein loss with an encapsulation efficiency less than 40%. Detergent-containing media significantly increased protein leakage from microspheres when water or acetate buffer were used.

Taking into consideration the reaction between acetic acid and $CaCO_3$, each mole of $CaCO_3$ reacts with 2 moles of CH_3COOH. Using an acetic acid/$CaCO_3$ molar ratio slightly higher than stoichiometric proportion (2.5/1) resulted in high encapsulation efficiencies, close to 90% (Table 6). When an insufficient volume of acetic acid was used to dissolve the $CaCO_3$ (1.25/1 ratio) higher protein losses were observed.

One-stage coating did not significantly change Hb encapsulation efficiency, in comparison to uncoated microspheres

Table 3. Effect of Acetic Acid/$CaCO_3$ Molar Ratio on Volume Mean Diameter of Uncoated and Coated Microspheres*

Acetic Acid/$CaCO_3$ Molar Ratio	Chitosan Coating	Diameter (µm)
1.25	Uncoated	18.1 ± 9.5
2.5	Uncoated	24.9 ± 11.6
2.5	1-stage	150.8 ± 85.0
2.5	2-stage	>1000†

*Microspheres were prepared with an aqueous/oily phase of 30% (vol/vol) at 400 rpm. Data are shown as mean ± SD.
†Obtained value was above the detection limit.

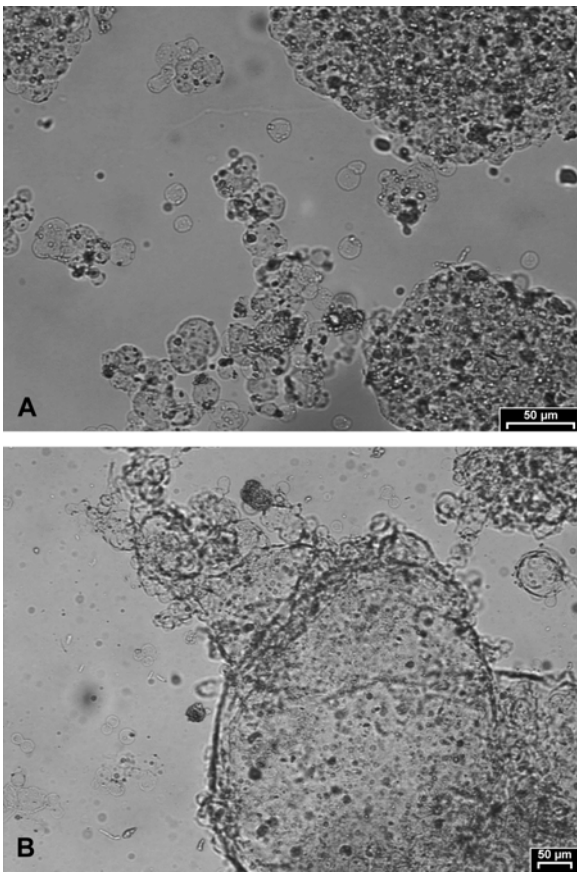

Figure 4. Optical photomicrograph of (A) 1-stage and (B) 2-stage chitosan-coated alginate microspheres (magnification 200 X and 100 X for A and B, respectively).

(Table 6). Hb losses during the 2-stage coating procedure were insignificant.

In Vitro Release Studies

The influence of chitosan coating and drying conditions of microspheres were evaluated on the release behavior of Hb in gastrointestinal simulated pH. The formulations used in the in vitro release studies are summarized in Table 7.

After 2-hour incubation at pH 1.2, a high release of Hb was obtained for W and L microspheres, while for air-dried microspheres a lower Hb release was obtained (Table 8). During assay, UD and C1D microspheres did not disperse completely and, in the case of C2D microspheres, a partial dispersion of microspheres occurred after 30 minutes.

Coating influence on Hb retention, at pH 1.2, was observed for the C2W and C1D microspheres, where protein released was lower compared with uncoated formulations. One-stage coating caused a higher retention of Hb for D and L microspheres compared with C2 microspheres, and for W microspheres the opposite occurred.

In intestinal simulated fluid (Figures 6, 7, and 8), a burst effect was observed for uncoated microspheres, although a lower effect was detected for UD microspheres. A fast Hb release (<1 hour) occurred in the UW and UL microspheres. Some microsphere fragments persisted for UD microspheres until the end of the assay, which prevented a complete Hb release.

Coating microspheres decreased the release rate of Hb, at pH 6.8, but an incomplete release was obtained. C2W microspheres released less than 10% of encapsulated Hb, while the C1W microspheres, showing a slight burst effect, released almost 20%. C2D microspheres showed a similar release profile to that obtained for C2W microspheres. C1D microspheres changed its behavior completely compared to

Table 4. Encapsulation Efficiency of Hemoglobin in Relation to the Emulsion Formulation Parameters*

Water/Oil (%) (vol/vol)	Span 80 1% (vol/vol)	Hb Losses During First Wash (%)	Encapsulation Efficiency (%)
20	+	6.0	92.9
30	+	7.6	90.9
40	+	9.1	88.9
30	-	8.2	91.2
30	+	7.6	90.9

*The rotational impeller speed was 400 rpm. Data represent the average of 3 replicates.

Table 5. Effect of the Rotational Impeller Speed on Hemoglobin Encapsulation Efficiency*

Speed Rate (rpm)	Hb Losses During First Wash (%)	Encapsulation Efficiency (%)
200	7.1	90.9
300	7.8	91.9
400	7.6	90.9
500	14.6	84.3
600	5.5	92.8

*Microspheres were prepared with an aqueous/oily phase ratio of 30% (vol/vol) at 400 rpm. Data represent the average of 3 replicates.

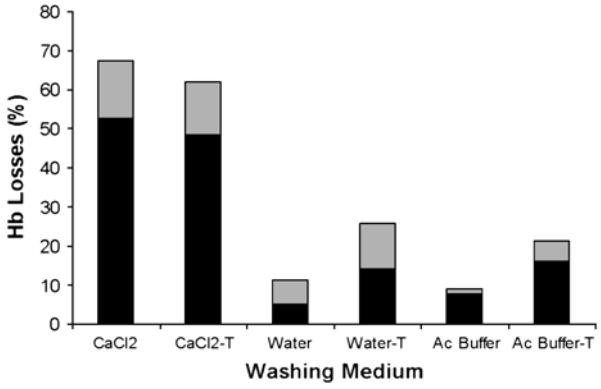

Figure 5. Hemoglobin losses from microspheres during the first wash (black) and subsequent washes (gray) with washing media either with (T) or without Tween 80 1% (vol/vol).

Table 6. Effect of Acetic Acid/$CaCO_3$ Molar Ratio on Hb Encapsulation Efficiency for Uncoated and Coated Microspheres*

Acetic Acid/ $CaCO_3$ Molar Ratio	Chitosan Coating	Hb Losses During First Wash (%)	Encapsulation Efficiency (%)
1.25	Uncoated	20.1	65.2
2.5	Uncoated	7.6	90.9
2.5	1-stage	8.7	89.3
2.5	2-stage	7.6	90.9

*Microspheres were prepared with an aqueous/oily phase ratio of 30% (vol/vol) at 400 rpm. Data represent the average of 3 replicates.

C1W microspheres, releasing less than 5% of Hb after 6 hours at pH 6.8. Hb release from C2L microspheres was reduced (less than 2%), and an incomplete total release of 65% was achieved. For C1L microspheres, a higher Hb release was observed, but the final value after 6 hours, 70%, was not significantly different from that obtained for microspheres coated in 2 stages.

DISCUSSION

Microsphere Morphology

Microsphere size was kept to a minimum to improve the surface area contact. Alginate bioadhesive[42] and chitosan mucoadhesive properties[43] as well as the polycation ability to enhance the paracellular absorption through the intestinal membrane[38] are favored by a more intimate contact between intestinal mucosa and smaller-sized microspheres.

The formation of carbon dioxide is inherent to the internal gelation method used in this work, where calcium is obtained by acid-induced solubilization of a calcium insoluble complex. One of the great concerns is the possibility of an increase in the alginate matrix porosity caused by the release of carbon dioxide, which may cause deformity and clumping of microspheres.[32] In this study, spherical and discrete microspheres without agglomeration were obtained when an appropriate recovery medium was used.

Table 7. Formulations Used in the Release Studies Prepared Using Different Drying and Coating Processes*

	Drying Process		
Coating Process	Wet	Air Dried	Lyophilized
Uncoated	UW	UD	UL
1-step coated	C1W	C1D	C1L
2-step coated	C2W	C2D	C2L

*U indicates uncoated; W, wet state; D, air dried; L, lyophilized; C1, 1-stage chitosan coated; and C2, 2-stage chitosan coated.

Table 8. Hb Release After 2 Hours in Simulated Gastric Fluid From Uncoated (U), 1-stage Chitosan Coated (C1), and 2-stage Chitosan Coated (C2) Microspheres in the Wet State (W), Air-dried (D), or Lyophilized (L)*

	Hb Released in Simulated Gastric Fluid After 2 Hours (%)		
	W	D	L
U	60.7 ± 1.7	0.2 ± 0.3	49.6 ± 6.2
C2	27.5 ± 2.0	18.7 ± 8.4	62.6 ± 5.3
C1	68.4 ± 4.0	0.00	53.4 ± 0.7

*W indicates wet state; D, air dried; L, lyophilized; U, uncoated; C2, 2-stage chitosan coated; and C1, 1-stage chitosan coated. Data are shown as mean ± SD.

Diameter distribution of microspheres obtained by emulsification/internal gelation depends on the size of the emulsion droplets that are determined by a balance between the dispersive and the surface tension forces.[44] The former tends to disperse the emulsion and the latter causes coalescence. The interfacial tension between the alginate droplets and the oil in the continuous phase is reduced in the presence of Span 80 and microsphere size is decreased. Increasing the dispersive force also decreased microsphere mean diameter, although a broader distribution was obtained, contributing to a higher heterogeneity of the batch. Mean size increased for an impeller rotational speed higher than 500 rpm, which could be explained by aggregation of small emulsion droplets during the gelation induced by acid addition. Thus, an impeller rotational speed of 400 rpm was chosen to allow the achievement of small microspheres with a narrow size distribution.

The decrease of microsphere mean size with the proportion of dispersed phase can be attributed to the higher viscosity. Preliminary results[44] showed that a higher proportion of dispersed phase causes a large increase in mean size, justified

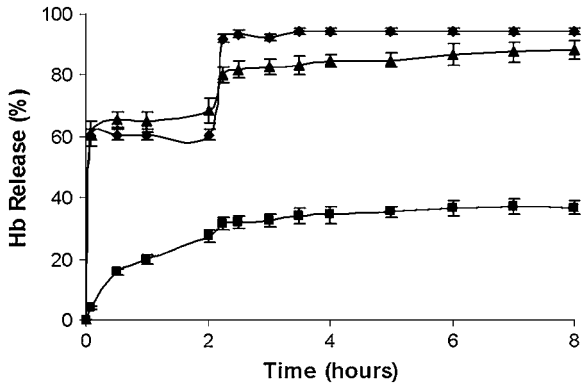

Figure 6. In vitro release profile of Hb in simulated gastric fluid for 2 hours and then in simulated intestinal fluid for (♦) uncoated, (▲) 1-stage coated, and (■) 2-stage coated wet microspheres. Error bars represent SD of the mean based on 5 replicates.

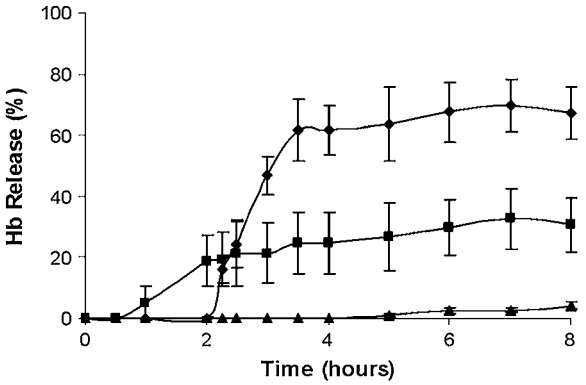

Figure 7. In vitro release profile of Hb in simulated gastric fluid for 2 hours and then in simulated intestinal fluid for (♦) uncoated, (▲) 1-stage coated, and (■) 2-stage coated air-dried microspheres. Error bars represent SD of the mean based on 5 replicates.

by an increase in the frequency of coalescence resulting in the formation of aggregates, which was not verified with the conditions used in this work.

Microspheres obtained by emulsification/internal gelation are usually oil-separated by several washing cycles with a $CaCl_2$ solution containing Tween 80 1% (vol/vol).[35,41] Among washing media tested to recover microspheres, water and $CaCl_2$ caused an increase in mean size and the appearance of a bimodal distribution attributed to aggregation of the recently formed microspheres during partition. Washing alginate microspheres with water or $CaCl_2$ probably causes water losses to the external medium. In the case of water, this effect is explained by its low osmolarity, which induces ion and, consecutively, water diffusion outside the microspheres. Recovering microspheres with $CaCl_2$ increases the degree of alginate cross-linking, which is known to cause gel shrinkage.[45] These water losses may induce adhesion between microspheres resulting in the formation of aggregates probably owing to the formation of new hydrogen bonds created between hydroxyl groups of the polymer.[46] The formation of new hydrogen bonds is apparently avoided in the case of acetate buffer, thus preventing aggregation. This hypothesis is supported by the fact that when size distribution is analyzed in a number perspective, the peak mode decreases when water and $CaCl_2$ are used as recovery media compared with acetate buffer at pH 5.5. The water losses cause a decrease in microsphere size, although the small microspheres tend to aggregate, leading to a higher mean size in volume distribution. The number of aggregates formed is very small compared with the number of discrete microspheres and, for this reason, the peak mode appears in the range of discrete microspheres when number distribution is considered.

The increase in acetic acid/$CaCO_3$ molar ratio, achieved by adding a higher volume of acid, increased the mean diameter of microspheres. Theoretically, an inverse effect would be expected, since alginate gelation induced by calcium ions causes microsphere shrinkage and a decrease in size.[45] For this reason, it is thought that microsphere granulometry could be influenced during microsphere recovery, and that acetate buffer at pH 5.5 continues the solubilization of $CaCO_3$ and therefore alginate gelation. In the absence of the oil barrier and in the case in which a lower acid/$CaCO_3$ molar ratio was used, a higher effect probably took place.

Two-stage coated microspheres underwent a visible aggregation during the coating process that occurred in lesser degree for C1 microspheres. Aggregation can be explained by strong electrostatic interaction between alginate and chitosan, 2 polyelectrolytes of opposite charge, which in the case of 1-stage coating is partially avoided by the presence of the external oil phase.

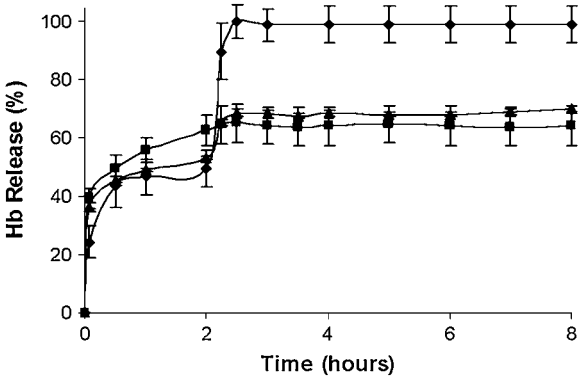

Figure 8. In vitro release profile of Hb in simulated gastric fluid for 2 hours and then in simulated intestinal fluid for (♦) uncoated, (▲) 1-stage coated, and (■) 2-stage coated lyophilized microspheres. Error bars represent SD of the mean based on 5 replicates.

Encapsulation Efficiency

Another concern related to the increase of alginate matrix porosity caused by the release of carbon dioxide during acid-induced solubilization of $CaCO_3$ consists of a possible increase in drug losses from microspheres. It was previously shown by Vandenberg et al[47] that internal gelation of alginate results in reduced encapsulation efficiency of the model protein bovine serum albumin (BSA) when compared with external gelation. In this study, high encapsulation efficiency for Hb was obtained using emulsification/internal gelation. Attempts were made to reduce protein losses during the emulsification step and also during microsphere partition and washing. Phosphate buffer at pH 6.8 was used as the alginate dissolution medium to help protein retention during the emulsification step. At this pH, Hb has a positive charge (pI 7.1), which favors the electrostatic interaction with the polyanionic alginate. This effect was previously

described for the encapsulation of transforming growth factor-β_1 (pI = 9.82)[48] and BSA[49] in alginate microspheres.

Microsphere recovery with $CaCl_2$ 0.05 M conduced to low encapsulation efficiency. The same effect was observed before with an external gelation method, where Hb-loaded alginate microspheres lost more than 90% of the protein to the $CaCl_2$ solution.[50] Microspheres did not retain the protein possibly owing to diffusion to the external medium; if ions are present in the free solution, then the protein (if electrically not neutral) always finds enough counterions for charge compensation.[51] Using the internal gelation method, protein loss from alginate microspheres was reduced because Hb does not diffuse to the external oily medium. Thus, by choosing an appropriate partition medium, it was possible to control the diffusion of protein out of microspheres and obtain high encapsulation efficiencies.

A higher encapsulation efficiency of Hb was obtained for acetate buffer–recovered microspheres because pH influences the degree of ionization of proteins and thus the interactions between proteins and polysaccharides of opposite charge,[52] which may be responsible for increasing encapsulation efficiency of proteins inside alginate microparticles.[4,48,49] Acetate buffer at pH 5.5 keeps alginate in its polyanionic state and Hb positively charged (pI = 7.1). Although Hb is instable at low pH, the dissociation only occurs at a pH below 5,[50] so acetate buffer should not interfere with the protein integrity.

Washing microspheres with water also made it possible to obtain high protein retention. In this case, the absence of ions in the free solution may have minimized Hb diffusion to the washing media; however, mean size increased up to 200 μm owing to microsphere aggregation. Therefore, acetate buffer was selected as the most appropriate medium for microsphere partition and washing.

Other process parameters, related to the emulsification step, such as mixer rotational speed, concentration of emulsifier, and w/o ratio, did not cause great changes in protein retention, although it could be expected that factors favoring the increase of mean size could increase encapsulation efficiency owing to a reduced surface area. Taking into account this fact, it can be assumed that encapsulation efficiency is mainly determined during partition after destroying the oil barrier.

The increase of encapsulation efficiency with increasing acetic acid/$CaCO_3$ molar ratio is explained by a higher extent of alginate gelation[53] that led to stronger microspheres, providing a greater resistance to solute transport.[54]

Coating microspheres with chitosan before partition did not prevent the small losses occurring after partition, which may indicate that these losses occurred during the gelation of microspheres induced by the addition of acid due to alginate shrinkage. Further losses, as those verified during external gelation process, are prevented by the dispersing-oil medium.

In Vitro Release Studies

In acidic medium, calcium alginate matrices are depleted of calcium ions and converted to insoluble alginic acid within 30 minutes without any visible changes in the morphology.[55] This may reduce the gel strength favoring drug release by diffusion. Drug solubility in the medium is another important factor. Lipophilic encapsulates such as Sudan orange G[41] and slightly soluble drugs at acidic pH values such as nitrofurantoin[56] or sodium diclofenac[57] showed a minimal release after 1 to 2 hours under gastric conditions. After 2 hours at acidic pH values, soluble drugs such as nicardipine presented a release between 30% and 70%.[58] For BSA, a protein model, with a molecular weight close to that of Hb, manufacture processes seemed very important in determining protein release after 24 hours of acid incubation.[47] For example, drying the microcapsules decreased protein release from 90% to 20%.

High Hb losses found at pH 1.2 can be attributed to an interaction existing at pH 5.5 between the negatively charged alginate and the positively charged Hb that is lost at pH 1.2 with the partial precipitation of alginate in the form of alginic acid. At pH values lower than 4, Hb can easily be dissociated into dimers (MW = 32 000), and when the pH decreases further, Hb can be dissociated into monomers (MW = 16 000), which can diffuse more easily through the alginate-chitosan membrane than the tetramer.[59]

As water is removed from the gel network during microsphere drying, alginate concentration increases and average pore size is reduced.[47,60] When a high G alginate is used, microspheres reswell only slightly upon rehydration[61] and do not regain their initial diameter. A more pronounced effect is expected for air-dried microspheres since it was verified that lyophilization of dextran-loaded alginate microspheres increased the release rate compared with heat-dried microspheres.[62] The release rate decreased as the temperature decreased, which may indicate that a slower drying rate reduces the pore size after rehydration. This finding is in accordance with the results obtained for U and C1 microspheres, where the order for total Hb release after 2 hours at pH 1.2 was W > L > D. Hb release was prevented for UD and C1D microspheres because of the aggregates formed during the drying process that did not disperse when placed in acidic medium.

In the case of chitosan-coated microspheres, Hb needs to pass through a second layer of alginate-chitosan complex membrane to be released. The influence of chitosan coating on the encapsulate behavior, in gastric conditions, is still not clear. Chitosan-coated microspheres with a chitosan solution at 0.75% (wt/vol) showed the same drug retention after

24 hours of acid incubation as uncoated BSA-loaded microparticles, but decreasing chitosan concentration to 0.25% (wt/vol) offered a much higher retention.[47] In another study, with alginate microspheres prepared by emulsification/internal gelation, less than 10% of drug was released within the first hour under gastric conditions for uncoated microspheres, and after chitosan coating only a minimal release (less than 3%) was observed.[41]

During 2-stage coating, a visible aggregation occurred that contributed to the higher retention of protein at pH 1.2 for C2W microspheres compared with UW and C1W microspheres since the surface area for diffusion was reduced. The higher protein release obtained with C2 microspheres dried by air exposition or lyophilization compared with dried U and C1 microspheres is justified by a destruction of the integrity of chitosan-alginate complex membrane by the drying process. A similar behavior was found for brilliant blue (BB) release in a saline solution.[63] For C1 microspheres, the drying step contributed to the expected reduction in Hb release; it may be assumed that this coating process leads to a more stable membrane after drying.

Complete calcium alginate microsphere dissolution in intestinal fluid is due to the reduced Ca^{2+} concentration within the microspheres after a 2-hour exposition to an acidic pH[48] and to the sequestering of Ca^{2+} ions of alginate network by the phosphate anions present in the dissolution medium.[28] Hb release from UD microspheres was maximal at 1.5 hours and was followed by a slow release from some undissolved fragments of the film formed during drying. The incomplete dissolution is due to a higher concentration of alginate obtained after microsphere drying.[3]

Chitosan coating decreases drug release rate, and an incomplete release of Hb was obtained owing to suppression of erosion of alginate gel microspheres.[36] The initial burst effect observed for C1L and C1W microspheres, followed by a sustained release, is justified by the presence of the alginate-chitosan complex membrane. C1D microspheres did not release Hb because the undispersed clumps obtained at acidic pH remained at pH 6.8, thereby decreasing the surface area for diffusion. No burst effect was observed for C2 microspheres after changing dissolution media, and only a small fraction of remaining Hb was released. One possible explanation is that the aggregation occurring between alginate microspheres during the coating process may protect more from the pH influence in the alginate network due to a decreased surface area of exposition.

Conclusion

Hb-loaded alginate microspheres with a mean diameter less than 30 μm and an encapsulation efficiency above 90% were prepared by an emulsification/internal gelation method. Acetic acid/$CaCO_3$ molar ratio was important to obtain high encapsulation efficiency, and microspheres recovered with acetate buffer made the control of protein diffusion out of microspheres easier, thus enabling the achievement of high encapsulation efficiency with a narrow size distribution.

The in vitro release of Hb at simulated gastrointestinal conditions from air-dried microspheres led to the lowest release at pH 1.2, whereas at pH 6.8 Hb release was fast for uncoated microspheres and incomplete for coated microspheres. Chitosan coating decreased the release rate at pH 6.8 and for 2-stage coating no burst effect occurred after changing dissolution media, although 1-stage coating permitted a higher total release of Hb.

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