Hemoglobin Encapsulation in Chitosan/Calcium Alginate Beads

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SYNOPSIS

A mild chitosan/calcium alginate encapsulation process, as applied to encapsulation of hemoglobin, was investigated. The first procedure consisted of adding dropwise a hemoglobin-containing sodium alginate mixture in a chitosan solution, then hardening the interior of capsules thus formed, in the presence of $CaCl_2$. In the second method, the droplets were directly pulled off in a chitosan–$CaCl_2$ mixture. Both procedures led to beads containing a high concentration in entrapped hemoglobin as more than 90% of the initial concentration (150 g/L) were retained inside the beads provided that the chitosan concentration was great enough. The molecular weight of chitosan ($\overline{M}_v$ 245,000 or 390,000) and the pH of its solution (2, 4, or 5.4) had only a slight effect, the best retention being obtained with beads prepared at pH 5.4. The hemoglobin release during the bead storage in water was found to depend on the conditions of their formation and especially on the chitosan molecular weight. The best retention during storage in water was obtained with beads prepared with the high $\overline{M}_v$ chitosan solution at pH 2. Considering the total loss in hemoglobin during the bead formation and after 1 month of storage in water, the best results were obtained by preparing the beads in an 8 g/L solution of a 390,000 chitosan at pH 4 (less than 7% of loss with regard to the 150 mg/L initial concentration). © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Immobilization of biological molecules, especially of proteins and enzymes, is of particular interest for protecting, separating, stabilizing, and/or controlling the release of immobilized material. Among the various immobilization methods proposed so far, microencapsulation in calcium alginate beads presents the advantage of being very mild and of maintaining the activity of biological macromolecules or even cells.[1] To limit the loss of encapsulated material, the microcapsules are sometimes coated with a polycationic polymer that forms a membrane at the bead surface. Thus, the poly(L-lysine)/alginate system has been used for the encapsulation of various molecules such as drugs,[2] proteins,[3] and viable cells.[4–6] Chitosan is a cationic polysaccharide derived from the natural polymer, chitin, and it is known to form polyelectrolyte complexes with polyanionic polymers.[7–9] Some of its derivatives were proposed in the preparation of calcium alginate beads coated with a polyelectrolyte complex membrane.[10] Encapsulating processes based on the electrostatic interaction between sodium alginate and chitosan resulting in the formation of a complex membrane were also described.[11–13]

In the present study, different procedures aimed to the production of chitosan/calcium alginate beads containing hemoglobin (Hb) were tested and the influence of various parameters on the bead permeability toward the immobilized protein was investigated. As material to be encapsulated, Hb is a good model for other globular proteins. Moreover, such a mild encapsulating procedure could lead to Hb-containing vesicles with a potential interest as a red blood cell substitute, provided that their sizes could be very small. Various methods of Hb encapsulation have already been reported including the encapsulation in membranes constituted of crosslinked Hb,[14–16] of organic polymers,[17,18] and of phos-

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pholipids,[19-21] but so far all the described capsules present different kinds of drawbacks. Among them, a low concentration or a loss of activity of encapsulated Hb and a lack of *in vivo* stability are often mentioned and our results could bring some improvement in this field.

EXPERIMENTAL

Materials

S550 sodium alginate was a gift from Sanofi (France). It has a low content in guluronic groups and, as mentioned by the supplier, its solution (1.2% w/v) exhibits at 20°C a viscosity of 550 cps, which corresponds to a molecular weight lying between 80,000 and 120,000.[22] Chitosan (Seacure 123) was purchased from Protan Inc. (Redmond, WA). Two different samples whose solution viscosities (2% w/v in 0.1% HCl) were, respectively, 45 and 12 cps as determined at 20°C with a Brookfield digital rotary viscosimeter at 30 rpm were used. The values of $\overline{M_v}$ were calculated from the viscosities of the diluted solutions (< 1% w/v) with a capillary viscometer (Ubbelhode type) from the Mark–Houwink relationship:

$$[\eta] = K(\overline{M_v})^a$$

with $[\eta]$ the intrinsic viscosity in mL/g, $K = 3.04 \times 10^{-5}$, and $a = 1.26$ (0.1M acetic acid, 0.02M NaCl).[23] They were found to be, respectively, 390,000 for the high viscosity sample and 245,000 for the other one. Freeze-dried bovine Hb was purchased from Sigma (USA). Its concentration was calculated by direct measurement of the optical density at 570 nm.

Methods

Bead Formation

Sodium alginate was dissolved in the Hb solution (40 or 150 g/L) to obtain a final concentration of 1.8% (w/v). Twenty milliliters of this viscous solution were introduced in a 50 mL syringe and extruded through a 0.15 mm-diameter needle using an EFD 1000XL dispenser (East Providence, Rhode Island). The droplets were pulled off in 150 mL of a 0.05M $CaCl_2$ stirred solution, then allowed to harden for 30 min. The beads were rinsed with distilled water, then transferred into distilled water for storage at 4°C.

Another procedure using chitosan was also investigated. A first method consisted in extruding the Hb-containing alginate droplets in a 0.1% HCl chitosan solution (4 or 8 g/L) containing 0.05M $CaCl_2$ and whose pH was adjusted at a given value (2, 4, or 5.4) with 1M NaOH (treatment I). In the second method, the alginate droplets were pulled off in a chitosan solution (in 0.1% HCl) whose pH was adjusted as described above and the capsules thus obtained were isolated, then treated with 0.05M $CaCl_2$ (treatment II). In both cases, the beads were treated and stored as those prepared in the absence of chitosan. The beads (about 1 mm diameter) were examined with a Wild M3 stereomicroscope (Leica, Switzerland).

Hb Release

Hb release from beads was monitored by direct measurement of the optical density of the solutions at 570 nm. First, the release during the bead formation was determined by measuring at the end of the procedure (i.e., 30 mn after all the alginate solution was introduced into the gelling solution) the Hb concentration of the solutions used for the preparation. After 1 week or 1 month of bead storage in water, the release of Hb was determined by the measurement of the optical density of the supernatant after a brief stirring in the presence of beads.

RESULTS

Alginate Beads with a Low Hb Content

These beads were prepared from an initial alginate solution containing 40 g/L of Hb, following the various procedures described above. The results of the Hb release are given in Table I. This table shows that, when formed in pure 0.05M $CaCl_2$, the Hb-loaded beads do not retain the protein, as over 90% were lost to the solution at the end of the preparation. On the other hand, when the alginate–Hb mixture was added dropwise to an 8 g/L chitosan solution, this resulted in the formation of capsules with no loss of Hb during this step. No Hb loss was observed when the capsules were allowed to gel and harden for 30 min in the presence of 0.05M $CaCl_2$, or during the 1-week storage of the beads in water. When the beads were formed directly in a 8 g/L chitosan–0.05M $CaCl_2$ mixture, similar results were obtained with only a 1% Hb loss during the preparation and no further release during the storage. In contrast, the use of a 2 g/L chitosan solution led to

Table I Hb Release from Alginate Beads Prepared from a Low Hb Concentration Solution (40 g/L)

	Solution of Bead Preparation			
		8 g/L Chitosan 0.05*M* $CaCl_2$		2 g/L Chitosan 0.05*M* $CaCl_2$
	0.05*M* $CaCl_2$	I[a]	II[a]	I[a]
Released Hb %[b]				
A	92	1.2	0	91
B	100	1.2	0	91
Hb concentration in the beads g/L	0	39.5	40	3.6

[a] I: Treatment I ($CaCl_2$ added in the chitosan solution at the beginning of the bead formation). II: Treatment II ($CaCl_2$ added after the capsule formation in chitosan). The pH of chitosan solutions was 5.4

[b] With regard to the total Hb amount of the initial solution: A, release in the solutions of bead preparation; B, total release after an 1 week storage in water at 4°C.

a very large Hb loss during the capsule preparation and, consequently, no significant release during the storage in water.

Alginate Beads with a High Hb Content

These beads were prepared from an initial alginate solution containing 150 g/L of Hb, using the chitosan/calcium alginate system, under the conditions of both treatments I and II described in the Experimental part. The Hb release from these beads was studied both during the bead formation and after a 30 day storage in water at 4°C as a function of the characteristics of the chitosan solutions (pH and viscosity). Moreover, the influence of the time of the $CaCl_2$ addition in the chitosan solution (treatment I or II) on the Hb release was also studied.

First, considering the Hb release during the bead formation and the total one after 30 days of storage, Figure 1 shows that the capsule formation in the chitosan solution followed by the total gelation with $CaCl_2$ (treatment II) resulted in a better Hb retention. Second, whatever the time of the $CaCl_2$ addition, the Hb loss during the bead formation decreased when the pH of the chitosan solution increased and became very low when the capsules were formed in a chitosan solution at pH 5.4. In contrast, during the storage in water, the lower the pH of the chitosan solution used for the bead formation, the lower the Hb release.

Figure 2 shows the influence of the viscosity of the chitosan solution (pH 5.4), i.e., of the polysac-

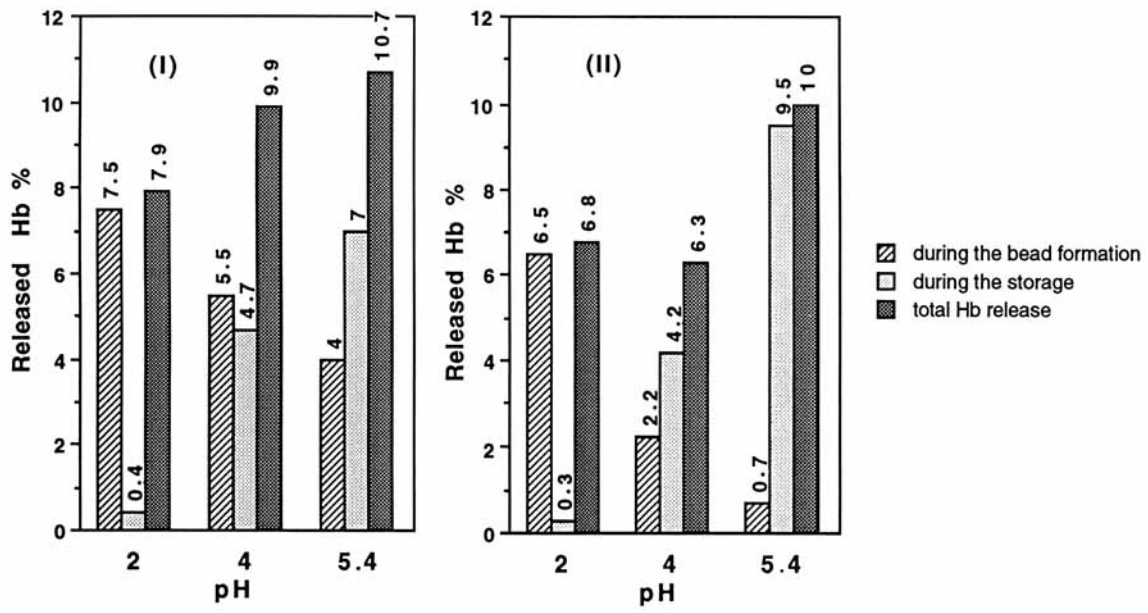

Figure 1 Hb release as a function of the pH of the chitosan solution ($\overline{M_v}$ = 390,000, [chitosan] = 8 g/L). (I) $CaCl_2$ was added at the beginning of the bead formation; (II) $CaCl_2$ was added after the chitosan–alginate membrane formation. The percentage of Hb released after the 30 day storage (in water) is expressed with respect to the amount of Hb remaining in the beads after their preparation. The other percentages are related to the initial Hb amount.

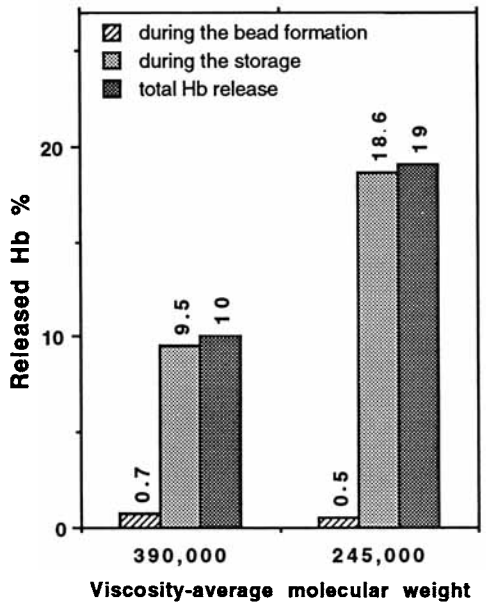

Figure 2 Hb release as a function of the molecular weight of chitosan. pH of the bead preparation: 5.4. [Chitosan] = 8 g/L. $CaCl_2$ added after the formation of alginate–chitosan capsules (treatment II). Other conditions as in Figure 1.

charide molecular weight, on the Hb release during the bead formation and after a 30 day storage in water at 4°C. It appears that the chitosan viscosity did not affect significantly the Hb loss during the bead formation but that a lower viscosity resulted in an increase in the Hb release during the storage in water.

Figure 3 shows the photographs of capsules prepared in the presence of chitosan ($\overline{M_v}$ = 390,000) at pH 5.4 with $CaCl_2$ present at the beginning of bead formation (I) or added after the sodium alginate–chitosan capsule formation (II). The beads are spherical in both cases. However, those shown in (I) are quite smooth, whereas those shown in (II) present some irregularities such as cracks, blisters, or small cavities.

DISCUSSION

The use of a calcium alginate/polycation system for Hb encapsulation leads to a suitable immobilization of the protein, whereas the classical system of protein entrapment by calcium alginate gelation gives rise, as already mentioned,[1] to an almost total Hb release during the first hour, i.e., during the time necessary to the bead preparation (Table I). Such calcium alginate/polycation systems have already been used as a mild method for encapsulation of drugs,[2] proteins,[3] and viable cells,[4-6] and the polycation that has been the most used, is poly(L-lysine).

We conducted a series of investigations using chitosan as the polycation and identified some parameters that influence the Hb permeability of the coated beads thus obtained. Since early in our study we encountered some problems with the loss of Hb payload during the bead preparation, we used a modification of the classical procedure in which, generally, the calcium alginate beads are first formed, then coated with a polycation.[3] In fact, our procedure consisted either in preforming Hb-containing capsules by adding dropwise the sodium alginate–Hb mixture into a chitosan solution, then gelling the interior by addition and diffusion of $CaCl_2$, or directly in adding dropwise the sodium alginate–Hb mixture into a chitosan–$CaCl_2$ solution.

In this procedure, whatever the time of the $CaCl_2$ addition (in the initial chitosan solution or after the capsule formation in pure chitosan), we found that

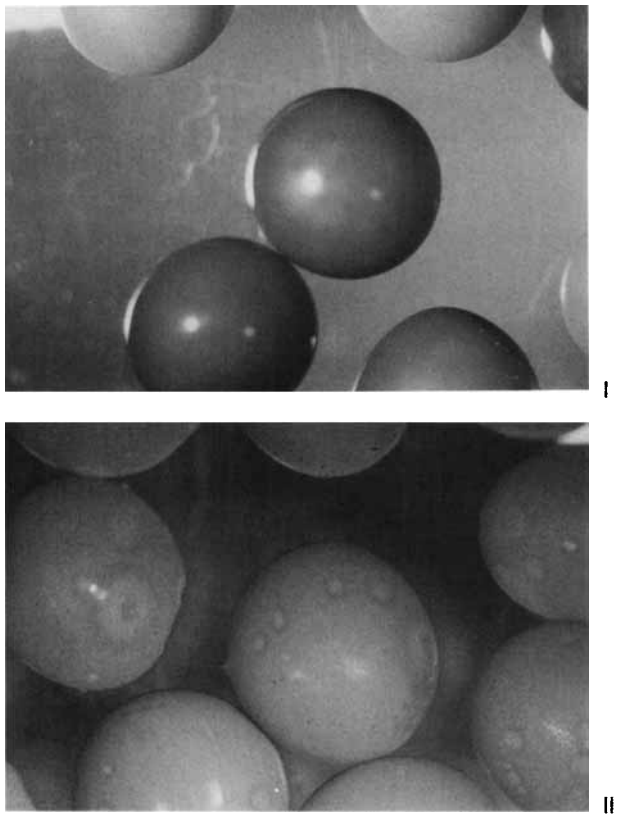

Figure 3 Photographs of Hb-containing beads (magnification 30×). (I) $CaCl_2$ was added at the beginning of the bead formation; (II) $CaCl_2$ was added after the chitosan–alginate membrane formation.

the characteristics of the chitosan solution influenced the Hb permeability of the beads thus obtained. Among these, the chitosan concentration, the solution viscosity, and the pH were investigated mainly. Thus, when the concentration was increased from 2 to 8 g/L, there was no more Hb loss during the bead formation and during the storage in water (Table I). Similarly, an increase in the viscosity of the chitosan solution, i.e., in its molecular weight, from 245,000 to 390,000, resulted in a decrease of the Hb release during the storage in water (Fig. 2). Concerning the effect of the chitosan concentration, it has been already observed that an increasing concentration of the polycation solution resulted in a lower release of proteins.[2] On the other hand, the results on the relationship between chitosan molecular weight and permeability toward globular proteins are also in agreement with those obtained with poly(L-lysine)-coated beads.[3] In fact, in this case, it was proved by transmission electron microscopy that there was a direct relationship between the molecular weight of the poly(L-lysine) and the density of the outer region of the capsule. The denser this region, the lower the protein diffusion through the membrane.

The effect of the pH of the chitosan solution is more complex. In fact, the Hb loss during the bead formation decreased with the pH increase but the results were opposite for the Hb release during the storage in water (Fig. 1). The Hb release during the bead formation at low pH may be caused by the dissociation of the tetrameric protein into dimers. In fact, it is known that Hb easily dissociates at pH below 5; the dissociation process goes beyond the ($\alpha\beta$) dimer and, at lower pH, it even involves single chains (M_r 16,000)[24] that can more easily diffuse through the chitosan–alginate membrane than can the tetrameric species. The same effect of the molecular weight of macromolecules on their diffusion through poly(L-lysine)-coated alginate beads has been already observed.[2,3] However, it is more difficult to understand why the porosity of the polycation–polyanion membrane was lower when it was obtained at pH 2 rather than at pH 5.4. The alginate chains are constituted of mannuronic acid and guluronic acid units whose pK's are 3.38 and 3.65, respectively.[25] Consequently, when a droplet of the alginate solution arrives in the chitosan solution at pH 2, a great concentration of COOH functions is formed at the interface. In contrast, when the procedure is carried out at pH 5.4, the alginate chains keep the greater part of their COO^- functions. Since, between these two pH values, the ionization rate of chitosan does not change a lot (pK = 6.3),[26] one should obtain a denser membrane at pH 5.4 than at pH 2, because of a greater number of alginate–chitosan ionic linkages. On the other hand, Fukuda and Kikuchi[7] showed that the yield of the solid polyelectrolyte complex obtained from sodium carboxymethyl cellulose and chitosan is higher when prepared at pH 2.5 than at pH 5. In our case, this would correspond to a thicker membrane but less dense, due to the formation of some kinds of loops as schematically shown in Figure 4. The lower density would also explain the greater Hb loss during the bead formation at pH 2. The better Hb retention during the storage in water, for low pH-formed beads, would be the result of the strengthening of the membrane due to the ionization of the chitosan-associated alginate chains in the presence of water and to the subsequent association of calcium ions with the resulting alginate anions.

In conclusion, we found that formation of Hb-containing calcium alginate beads in the presence of chitosan gave rise to a high load in protein, since in treatment II, pH 5.4, and with an initial Hb concentration of 150 g/L, the Hb loss during the bead formation is only 0.7%. After storage in water for 30 days, the higher total release is 10%. Modifying some parameters such as the chitosan molecular weight and the pH of the chitosan solution optimized the total loss after 30 days of storage at about 6.3%. However, if addition of $CaCl_2$ after the formation of the polyelectrolyte complex membrane led to a slightly better retention of the encapsulated protein, the beads thus obtained were not as regular as those prepared directly in a chitosan–$CaCl_2$ mixture (Fig. 3). Such a procedure can be applied to other globular proteins and also to cells. Experiments aimed to apply this kind of coating to the preparation of smaller diameter beads (microspheres) by emulsification/internal gelation[27] are presently under investigation.

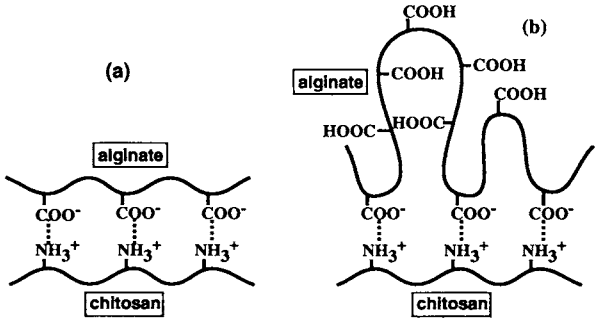

Figure 4 Schematic representation of the ionic interactions between alginate and chitosan: (a) pH 5.4; (b) pH 2.

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