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Chitosan–Alginate Microcapsules for Oral Delivery of Egg Yolk Immunoglobulin (IgY): Effects of Chitosan Concentration

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Abstract In our previous study, chitosan–alginate microcapsules were developed to protect egg yolk immunoglobulin (IgY) from gastric inactivation. The present study was undertaken to determine the effect of chitosan concentration (0–0.8%; *w*/*v*) on various properties of the microcapsules in order to produce the optimum chitosan–alginate microcapsules for use in the oral delivery of IgY. The properties investigated included microcapsule morphology, loading capacity for IgY (expressed as the IgY loading percentage, *w*/*w*, of microcapsules), encapsulation efficiency (EE%), in vitro gastro-resistance, and IgY release. IgY loading percentage and EE% were both highest at 0.2% (*w*/*v*) chitosan, and, above this level, further increases were not observed. The stability of IgY in simulated gastric fluid (pH 1.2) was significantly improved by encapsulation in alginate microcapsules (IgY retained 43.5% of its activity) and was further improved by including chitosan at any of the chitosan concentrations assessed (IgY retained an average of 69.4% activity) although there was no difference in protection of gastric inactivation among concentrations of chitosan varying from 0.05% to 0.8% (*w*/*v*). Higher chitosan concentrations (i.e., ≥0.2%; *w*/*v*) prolonged the release of IgY from the microcapsules during simulated intestinal fluid incubation (pH 6.8). However, above the 0.2% (*w*/*v*) level, no significant differences were observed. We conclude that the optimum chitosan concentration for microencapsulation is 0.2% (*w*/*v*).

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Keywords Alginate · Chitosan · Chitosan concentration · Egg yolk immunoglobulin (IgY) · Gastroresistance · Microcapsules

Abbreviations

EE%	encapsulation efficiency
IgY	egg yolk immunoglobulin
IgY loading %	IgY loading percentage (*w*/*w* of microcapsules)

Introduction

Oral administration of specific egg yolk immunoglobulin (IgY) presents a promising approach for the passive immunization of animals and humans suffering from enteric diseases [1]. However, the activity of IgY may be reduced or destroyed by gastric conditions, particularly a low pH [2]. Therefore, it would be beneficial to find an effective method to preserve the therapeutic value of IgY antibodies during gastric passage.

In our previous study, the applicability of chitosan–alginate microcapsules for oral delivery of IgY was established [3]. We showed that chitosan–alginate microcapsulation enabled a larger proportion of the IgY to remain bioactive in a simulated gastrointestinal tract environment providing an effective delivery system for specific IgY. Chitosan was applied as a membrane-coating material to reduce microcapsule swelling [4], improve encapsulation efficiency (EE%) [5–9], and improve stability [10].

Several publications have reported that the concentration of chitosan in the encapsulation medium is an important factor which controls the release of various materials from the chitosan–alginate membrane [5, 8, 11]. However, the effect of chitosan concentration on the gastroresistance of the microcapsules has rarely been reported. The present study was undertaken to determine the effect of chitosan concentration on several important properties of the microcapsule in order to produce the optimum chitosan–alginate microcapsules for oral delivery of IgY. The properties investigated included microcapsule morphology, loading capacity of the microcapsules (expressed as the IgY loading percentage, *w*/*w*, of microcapsules), encapsulation efficiency, in vitro gastric stability, and IgY release.

Materials and Methods

Specific IgY against K88+ ETEC was prepared and purified as described previously [3]. IgY powder was obtained by freeze-drying and its purity was determined to be over 82% based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of IgY-Loaded Chitosan–Alginate Microcapsules

Microcapsules were prepared by a one-step procedure as previously reported [3]. Briefly, sodium alginate ($1.05–1.15\times10^3$ cps; Tianjin Guangfu Fine Chemical Research Institute, Tianjin, China; 2%; w/v) was dissolved in deionized water and IgY powder was added at a loading rate of 25% (*w*/*w*). The encapsulation media were prepared by diluting the stock chitosan (deacetylation degree ≥ 90%; viscosity ≥ 10 cps; Dalian Xindie Chitin Co., Ltd., Dalian, China) solution with 1.5% $CaCl_2$ (*w*/*v*, final concentration) to produce concentrations of 0%, 0.05%, 0.1%, 0.15%, 0.2%, 0.4%, 0.6%, and 0.8% (*w*/*v*) chitosan. The pH of the encapsulation media was adjusted to 3.5±0.03 with 4 M NaOH. Approximately 20 mL of the sodium alginate/IgY solution was extruded into 200 mL of encapsulation

medium, with stirring (200 rpm) at a rate of 4 mL/min through a 0.7-mm (outer diameter) needle. The coaxial air volume was 0.3 m^3/h, and the distance between the needle tip and the encapsulation medium was 8 cm. The microcapsules obtained were kept suspended in the encapsulation medium with stirring for 30 min and were then filtered and rinsed with 50 mL of distilled water and finally freeze-dried. Blank microcapsules were prepared under similar conditions and were used as controls. All chitosan concentrations in this study yielded microcapsules with a mean wet diameter of approximately 1,000 μm. The surface morphology of the microcapsules was examined using a scanning electron microscope (JEM-1200Ex, JEOL Ltd., Tokyo, Japan). Samples were mounted on metal stubs using double-sided adhesive tape and gold-coated under vacuum for examination.

IgY Loading Percentage and Encapsulation Efficiency

Briefly, dried IgY-loaded microcapsules (10 mg) were dissolved in 5 mL of IgY release solution (0.2 M $NaHCO_3$; 0.06 M $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 8.0) and the total protein concentration was assayed using bicinchoninic acid (BCA) protein assay reagent kit (Pierce Inc., New York, NY, USA) according to the manufacturer's suggested protocol. The IgY loading percentage was obtained using the following equation:

$$\text{IgY loading}\,\% = C_{IgY} \times V/W \times 100\% \tag{1}$$

where C_{IgY} is the concentration of IgY in the sample; V is the volume of the sample, and W is the weight of microcapsules used for determining the IgY loading percentage. Encapsulation efficiency was expressed as the percentage of the total available IgY that was actually incorporated into the microcapsules, calculated using the total initial amount of IgY and the remaining IgY content of the dried microcapsules.

In Vitro Stability of IgY to Simulated Gastric Conditions

As previously reported [3], the stability of IgY in gastric conditions was evaluated using simulated gastric fluid (3.2 mg/mL pepsin in 0.03 M NaCl, at pH 1.2). The simulated gastric fluid was added to free and microencapsulated IgY at a pepsin-to-IgY ratio of 1:20 and incubated at 37 °C with shaking. After 2 h, nonencapsulated IgY was neutralized with 2 M Tris–HCl (pH 8.0) and filtered and the IgY was released as previously described [3]. Antibody activity, defined as the ability of the anti-K88+ETEC IgY to bind to K88 fimbriae, was determined by indirect enzyme-linked immunosorbent assay and was expressed as a proportion of the activity of the untreated positive control. The intact IgY was visualized by SDS-PAGE.

In Vitro IgY Release

The release of IgY from the microcapsules was studied by incubating 50 mg of IgY-loaded microcapsules in 50-mL simulated gastric fluid without pepsin, while shaking at a temperature of 37 °C. After 2 h, the microcapsules were filtered, transferred into 50 mL of simulated intestinal fluid (0.05 M KH_2PO_4; pH 6.8 without pancreatin), and further incubated for 4 h at 37 °C. At desired intervals, aliquots (200 μL) were removed and replaced with the same amount of fresh medium. The protein concentration was assayed

using BCA method as described above. The accumulative release percentage ($Q\%$) was calculated according to the equation:

$$Q\% = \left(C_n \times V + V_i \sum_{i=0}^{n-1} C_i \right) / (W \times \text{IgY loading}\%) \times 100\% \quad (2)$$

where C_n is the sample concentration at T_n; V is the total volume of release medium; V_i is the sampling volume at T_i; C_i is the sample concentration at T_i (both V_0 and C_0 were equal to zero), and W is the weight of the microcapsules used for IgY release in vitro.

Statistical Analysis

Statistical analysis was performed using SPSS 11.5 for Windows. Analysis values were expressed as mean±standard deviation (SD). Levels of significance were evaluated using one-way analysis of variance with the Student–Neuman–Keuls test for multiple comparisons. Differences were considered significant at $p<0.05$.

Results and Discussion

When an alginate drop falls into a chitosan solution containing a suitable divalent cation like Ca^{2+} (referred to as the one-stage procedure) [12], the interphasic membrane is formed by complexation between the positively charged amine groups of chitosan and the negatively charged carboxyl groups of alginate through electrostatic interactions [13]. Calcium chloride diffuses into the alginate core more rapidly than chitosan because of its low molecular weight and subsequently forms a gel core. Thereafter, a microcapsule of a calcium–alginate gel core coated with chitosan–alginate interphasic membrane is formed. In the present study, various concentrations of chitosan (0–0.8%; *w*/*v*) were used for preparation of the IgY-loaded microcapsules. Concentrations of chitosan above those in the encapsulation media used in the present experiment were initially employed, but our preliminary tests indicated that production of microcapsules was extremely difficult above a concentration of 0.8% (*w*/*v*) chitosan, as the viscous nature of the encapsulation medium induced excessive microcapsule aggregation.

Morphological Analysis

Scanning electron microscope examination revealed that, upon freeze-drying, the spherical structure of the microcapsules was lost (data not shown). The fine surface morphologies of the IgY-loaded microcapsules are shown in Fig. 1. In the absence of chitosan, alginate microcapsules exhibited a rough surface with some micropores (Fig. 1a). Incorporation of the lowest concentration of chitosan (0.05%; *w*/*v*) modified the surface of alginate microcapsules as a result of forming a complex membrane (Fig. 1b). In contrast, the surface of alginate microcapsules prepared with higher chitosan concentrations (0.2%, 0.8%; *w*/*v*) were smoother and denser than those of the alginate microcapsules prepared with the lowest chitosan concentration which was insufficient to fully coat the surface of the alginate microcapsules (Fig. 1c, d). It appeared that the wrinkling on the surface of microcapsules increased with increasing chitosan concentration. This may be due to an increased amount of chitosan–alginate interphasic membrane formed with increasing chitosan concentration. As the thickness of the membrane is related to the amount of the interphasic membrane formed [14], increasing the chitosan concentration may result in a thicker membrane.

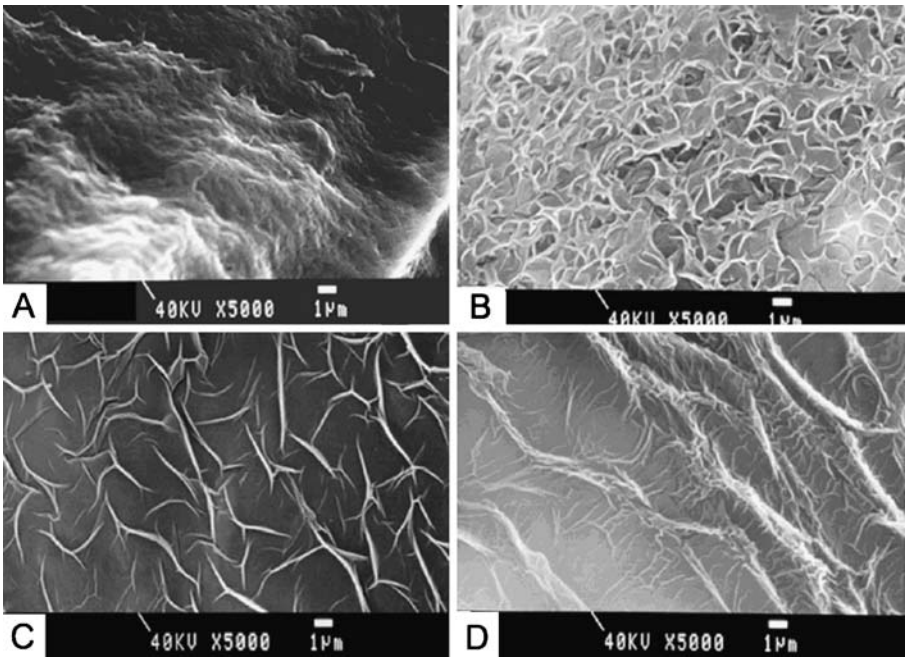

Fig. 1 Scanning electron microscope micrographs of the fine surface structures of freeze-dried IgY-loaded chitosan–alginate microcapsules prepared with various chitosan concentrations (**a** 0%; **b** 0.05%; **c** 0.2%; **d** 0.8% *w*/*v*)

IgY Loading Percentage and Encapsulation Efficiency

Figure 2a demonstrates the effect of chitosan concentration on IgY loading percentage. Including chitosan at concentrations of 0.05 to 0.1% (*w*/*v*) increased IgY loading percentage from 8.0% without chitosan to an average of 13.3% with chitosan. Chitosan concentrations of 0.2 to 0.8% (*w*/*v*) resulted in a further increase in IgY loading percentage, to an average of 19.7%. Maximal IgY loading percentage (19.6%) was achieved at 0.2% (*w*/*v*), as, above this concentration, further increases were not observed.

The study on the effect of increasing chitosan concentration on EE% revealed that the response of EE% to increasing chitosan concentration was similar to that observed for IgY loading percentage (Fig. 2b). Including chitosan during alginate microencapsulation has also been reported to improve EE% of dextran [5], ampicillin [6], bovine serum albumen [7, 8], and hemoglobin [9]. The enhanced IgY loading percentage and EE% in the presence of chitosan could be attributed to the formation of a strong complex membrane that would stabilize and strengthen the ion gel network and limit the loss of protein by diffusion during microcapsule formation.

In Vitro Stability of IgY to Simulated Gastric Conditions

The effect of chitosan concentration on the stability of the microencapsulated IgY was assessed by measuring IgY activity after 2 h of incubation in simulated gastric fluid. Nonencapsulated IgY was rapidly hydrolyzed, and antibody activity was almost completely lost during 2 h of incubation (Fig. 3). The stability of IgY was significantly improved by

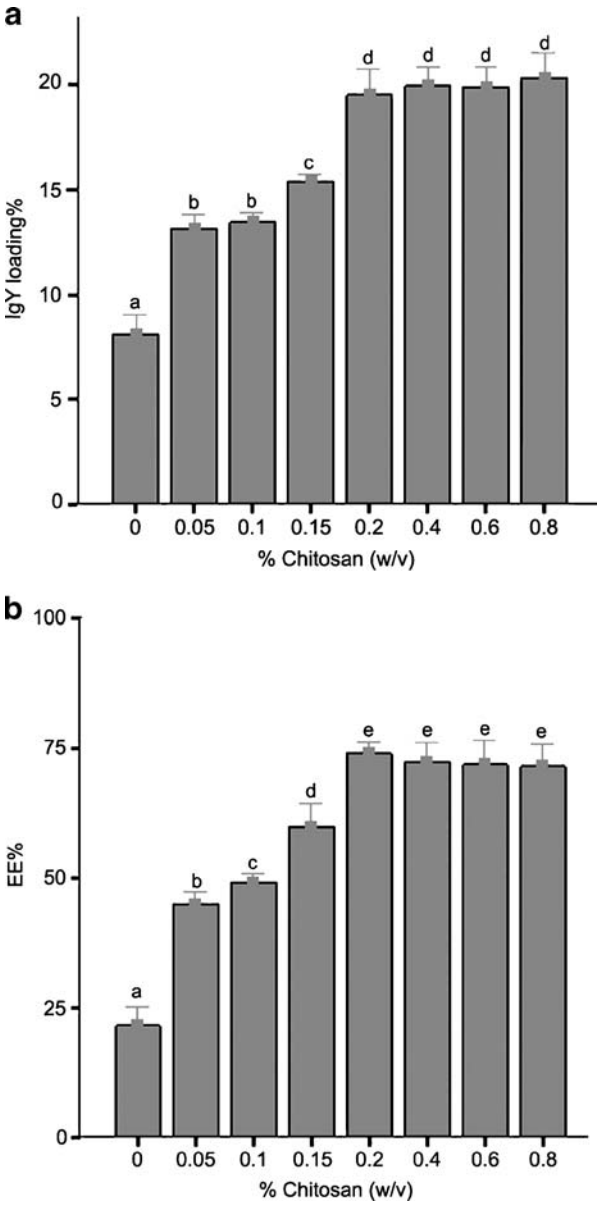

Fig. 2 Effect of chitosan concentration on IgY loading percent (**a**) and encapsulation efficiency (EE%; **b**). Data are presented as mean±SD (n=3). Means in a *column* without the *same letter* are significantly different ($p<0.05$)

encapsulation in alginate microcapsules (retaining 43.5% activity) and was further improved by including chitosan at any of the concentrations assessed (retaining an average of 69.4% activity). The addition of the lowest concentration of chitosan (0.05%; *w*/*v*) resulted in a significant increase in the amount of IgY activity retained (67.1%), but higher chitosan concentrations did not further increase protection from gastric inactivation. The

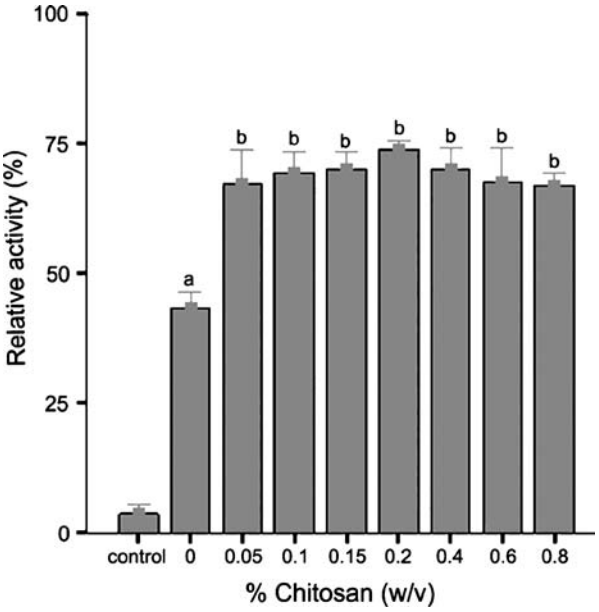

Fig. 3 Effect of chitosan concentration on microcapsules protecting IgY from gastric inactivation. Samples were incubated in simulated gastric fluid for 2 h. Data are presented as mean±SD (n=3). Means in a *column* without the *same letter* are significantly different (p<0.05). The control group was nonencapsulated IgY

retained activity of IgY was observed to be 67.4–73.9% for chitosan concentrations ranging from 0.05% to 0.8% (*w*/*v*) at the time of encapsulation.

Intact IgY was readily visualized by SDS-PAGE, as a band with an approximate molecular weight of 180 kDa in the samples from chitosan–alginate microcapsules, whereas no bands corresponding to intact IgY were observed in the microcapsules produced from alginate alone (Fig. 4). This suggests that chitosan coating significantly increased the protection of IgY from peptic hydrolysis.

Alginate microencapsulation improved the stability of IgY in simulated gastric fluid, but the majority of activity was still lost perhaps due to the gastric fluid entering the microcapsules through the micropores on the surface of the unprotected alginate core

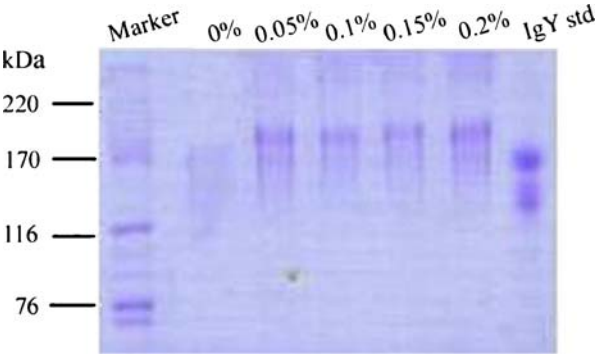

Fig. 4 Intact IgY released from IgY-loaded microcapsules formed at various concentrations after 2 h of simulated gastric fluid exposure was visualized by SDS-PAGE (nonreducing, 7.5% gel)

(Fig. 1a). Partial protection of IgY activity, even in the unprotected alginate microcapsules, may result from the inhomogeneity of the microcapsules produced by this method. When sodium alginate is extruded into a Ca^{2+} solution, a polymer gradient is produced as the alginate droplet diffusing outward meets the inward-migrating gelling zone. Thus, the alginate concentration at the surface of the microcapsule may be as much as ten times higher than that at the core [15], resulting in an outer "shell" that is relatively impermeable to hydrogen ions and peptic hydrolysis.

In Vitro IgY Release

The release characteristics of IgY-loaded microcapsules prepared with various chitosan concentrations were investigated by incubating the microencapsulated IgY in simulated gastric fluid and subsequently in simulated intestinal fluid. The accumulative release percentage of IgY is depicted in Fig. 5. During the initial incubation of microcapsules in simulated gastric fluid, release of IgY from all chitosan concentrations was negligible. In contrast, transferring to simulated intestinal fluid resulted in an initial rapid release followed by a more gradual sustained release. The initial increase was considered to be due to the effects of freeze-drying [3]. With no chitosan or lower concentrations of chitosan (i.e., <0.2%; *w*/*v*), almost 100% of the IgY release occurred within 2 h of incubation. A chitosan concentration of 0.2% (*w*/*v*) delayed the release of IgY from microcapsules and about 85% of the IgY was released within 4 h in simulated intestinal fluid, whereas, at above 0.2% (*w*/*v*) chitosan, no significant differences were observed. This is in good agreement with the results of Huguet et al. [11] who observed that the release of hemoglobin from chitosan–alginate microcapsules stored in water decreased with increasing chitosan concentrations up to a certain level and then reached a plateau where no further increase was evident, although, in that study, the plateau concentration was 0.8% (*w*/*v*).

The delayed and sustained release of IgY from chitosan–alginate microcapsules compared with alginate alone probably reflects the strengthening of the microcapsules by ionic interaction of chitosan with alginate. The thickness of the chitosan–alginate interphasic membrane is related to the chitosan concentration used [8]. The increased

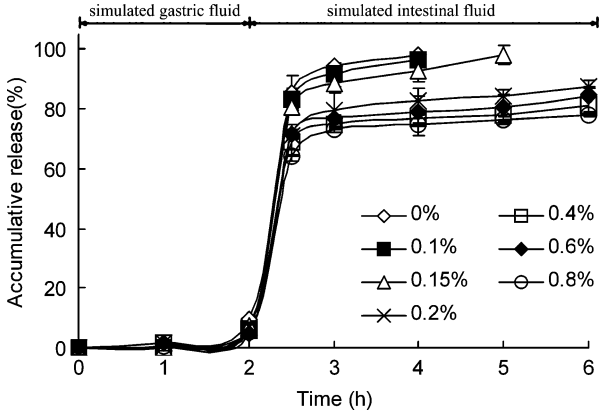

Fig. 5 Effect of chitosan concentration on IgY release from IgY-loaded microcapsules. Samples were first incubated in simulated gastric fluid and then transferred to simulated intestinal fluid. Data are presented as mean±SD (n=3)

chitosan concentration may have created a stronger (denser and thicker) membrane with reduced IgY release as a result of better cross-linking. Sezer et al. [5] reported that the release of dextran from chitosan–alginate microcapsules, incubated in a phosphate buffer, decreased with increasing chitosan concentration between 0.25% and 0.4% (*w*/*v*). Similar results were reported previously for bovine serum albumen [8].

The occurrence of a plateau concentration of chitosan may have resulted from producing the microcapsules by a one-step procedure. Gåserød et al. [12] reported that, for microcapsules made by a one-stage procedure, only low contents of chitosan are bound. Chitosan only binds to the surface, thereby creating a thin membrane with such small pores that further diffusion of chitosan into the microcapsules and subsequent binding to the gel network is restricted.

In conclusion, the present study showed that chitosan concentration had a significant effect on IgY loading percentage, EE%, and the release of IgY in simulated intestinal fluid. Therefore, chitosan–alginate microcapsules appear to be a more effective vehicle for the oral delivery of IgY compared with alginate microcapsules. The amount of chitosan coated on the alginate microcapsules increased with chitosan concentration in the initial solution but eventually reached a plateau where no further increases were observed. The optimal concentration of chitosan for microencapsulation was found to be 0.2% (*w*/*v*). This concentration resulted in maximal efficiency of production, protection of encapsulated IgY from the simulated gastric environment for at least 2 h, and effective release of IgY for delivery in the simulated intestinal fluid.

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