Preparation and Characterization of BSA-Loaded Alginate Microspheres

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**INTRODUCTION**

There are a variety of both natural and synthetic polymeric systems that have been investigated for the controlled release of proteins. Many of the procedures employed to incorporate proteins into a polymeric matrix can be harsh and often cause denaturation of the active agent. Alginate, a naturally occurring biopolymer extracted from brown algae (kelp), has several unique properties that have enabled it to be used as a matrix for the entrapment and/or delivery of a variety of biological agents (1). Alginate is a non-toxic, biodegradable, naturally occurring polysaccharide obtained from marine brown algae (2). Alginites can be ionically cross-linked by the addition of divalent cations in aqueous solution. Alginate microspheres have been used for the encapsulation of a wide variety of biologically active agents (proteins (3-5), enzymes (6), DNA (7)), and the relatively mild gelation process has enabled not only proteins, but cells and DNA to be incorporated into alginate matrices with retention of full biological activity (1). These delivery systems have unique challenges associated with their development that are related to both protein stability and protein release kinetics (8). This high degree of flexibility can result in delivery of active agents over time periods ranging from minutes to months (9).
By selection of the alginate type and the formulation conditions, it is possible to control, to some extent, the pore size distribution, the network density and the swelling ratio (10-13). Neutral molecules or biomolecules with a low isoelectric point (pI) are mostly released according to a diffusion mechanism, with diffusion coefficients depending on the ratio of their molecular weight to the network mesh size. In general, biomolecules that do not interact ionically with the alginate negative charges are rapidly released (within a few hours), and the release profiles are often characterized by a more or less pronounced burst effect (11).

The objective of this work was to prepare and characterize alginate microspheres as protein delivery systems. The method of microsphere preparation was adapted from the method described by Wan et al. (15), which allowed the preparation of alginate microspheres of about 12 µm. BSA was chosen as a water-soluble model protein. The BSA loading and entrapment efficiency were determined. Finally, in vitro BSA release studies were carried out.

MATERIALS and METHODS

Materials

BSA, sodium alginate, Span 85, Tween 85 and hydroxypropyl methylcellulose (HPMC) were purchased from Sigma (USA). BCA Protein Assay Reagent Kit was provided by Pierce Biotechnology, Inc. (USA). Bio-Rad Mini Protean II Electrophoresis System was supplied by Bio-Rad (USA) by Biological Industries (Israel), and calcium chloride was purchased from Merck (Germany). All other chemicals and reagents were of analytical grade.

Methods

Preparation of BSA-Loaded Alginate Microspheres

The preparation method of alginate microspheres was adapted from an emulsification method previously described (15,16). The resulting data obtained from preformulation studies was used for preparing the alginate microspheres. Briefly, an aqueous solution containing 5.0% w/w sodium alginate: HPMC (9:1) and 50 mg BSA (2% w:w BSA:polymer) was dispersed in soybean oil containing a lipophilic surfactant (Span 85; 2.0% w/w) with Silverson L4R (Silverson Machines, Ltd, UK) at 7800 rpm for 15 min, and then the aqueous solution containing a hydrophilic surfactant (Tween 85; 20% w/w) and calcium chloride solution (8.0% w/v) were added and stirred for another 15 min. Stirring was continued for 10 min after addition of isopropl alcohol to harden the microspheres. The microspheres were then washed with hexane and dried for 2 h at 37°C.

Characterization of Microspheres

Morphological and particle size analysis

The shape of the alginate microspheres was characterized by optical microscopy (Leica DMR, Germany) (17). The volume mean diameters of the alginate microspheres were determined in water by the HELOS system (Sympatec GmbH, Germany) and measured in triplicate for each batch (18). Particle size is expressed as volume mean diameter (µm) ± standard deviation (SD).

Encapsulation efficiency

The encapsulation efficiency of BSA was determined by hydrolyzing triplicate samples of BSA-alginate microspheres (10 mg each) in 250 ml of 2 M NaOH solution overnight. The samples were then neutralized with 250 ml of 2 M HCl and centrifuged at 1000 rpm for 10 min. The supernatants were collected and used for protein estimation by the BCA protein assay (19).

In vitro release study

The release of BSA from the alginate microspheres was determined in phosphate buffered saline solution (PBS, pH 7.4). The alginate microspheres (15 mg) were suspended in 1 mL of PBS. The samples were incubated at 37±0.5° C with continuous shaking (50 rpm). At predetermined time intervals, the sample was withdrawn and centrifuged at 2000 rpm for 5 min. The supernatant was collected and used for protein estimation by the BCA protein assay

SDS-PAGE Studies

The integrity of BSA was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method described by Laemmli (20). The released
samples from the alginate microspheres were also lyophilized and reconstituted in PBS. The BSA samples, native BSA, and a molecular weight reference marker (wide range, Sigma, USA) were mixed with sample dilution buffer [1.5 M Tris/HCl pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol and 0.01% bromophenol blue], boiled for 2 min prior to electrophoresis, and then separated by SDS-PAGE in a 1.5 mm thick 4% stacking gel and 12% resolving gel. The electrophoresis was performed in a Protean II vertical electrophoresis cell (Bio-Rad) at 200 V. The bands were visualized by Coomassie blue staining.

RESULTS and DISCUSSION

Alginate microspheres were prepared by methods described previously (15,16). Wan et al. (15) and Chun et al. (21) previously reported that the alginate microspheres containing HPMC were found to be generally spherical, discrete and had smoother surfaces when compared to those without HPMC, and the incorporated HPMC enhanced the formation of alginate microspheres and prevented aggregation. Therefore, we prepared the alginate microspheres using a mixture of sodium alginate and HPMC and assessed the morphology of the alginate microspheres via optical microscopy. As observed in Figure 1, microspheres appear spherical in shape with a relatively monodispersed size, similar to the other studies. Since the present method of microsphere preparation involves emulsification, the surfactants could play an important role in the microsphere production. Tween 85 produced less heterogeneous and smaller microspheres, similar to the results of a previous study (16). Protein levels were determined by the BCA assay. The encapsulation efficiency, BSA loading and particle size of alginate microspheres were 37.0%, 0.73% and 11.96±0.043 µm, respectively (Table 1). The release profile of BSA from the alginate microspheres is presented in Figure 2. There was a rapid release of 70% in 5 h and then a gradual release to 90% at 48 h. Two release mechanisms can explain the release of BSA from the alginate microspheres: 1. BSA could diffuse out of the alginate microspheres, and 2. BSA could be released from the alginate microspheres through the erosion of the matrix. The latter phenomenon could be attributed to the removal of the cross-linker bivalent cation, calcium, from the alginate microspheres by the monovalent cations (sodium, potassium etc.) under physiological conditions (15,16,22). This ion...
exchange would increase the swelling potential and at the same time reduce the stabilizing forces and cause the erosion of the alginate microspheres. Even if the microspheres did not burst, large swelling occurred and thus increased matrix porosity (22). The disruption of the calcium alginate matrix occurred faster in a phosphate buffer above pH 5.5 because of the chelating action of phosphate ions and at pH 7.4, the affinity of calcium to phosphate was higher than that to alginate, and consequently, BSA was released from alginate microspheres through the continuous erosion of the microspheres (22,23). The erosion of the microspheres could cause the fast release of BSA from the microspheres. The time required for 50% and 90% release from the microspheres was 3 h and 48 h, respectively, similar to previous results (15,16).

In this study, the structural integrity of BSA released from alginate microspheres under PBS medium (pH 7.4) was analyzed by SDS-PAGE analysis followed by Coomassie brilliant blue staining. SDS-PAGE is the most widely used principal method of separating proteins based only on differences in their molecular weight (24). According to Figure 3, the molecular weight marker and BSA standard shown in Lanes 1 and 2, respectively, exhibited a clear band at about 66 KD. The released samples of BSA (3rd hour and 2nd day) also showed similar clear banding patterns to the molecular weight marker (Fig. 3). The single lines in the gels provided evidence that the entrapped proteins in the release samples tested did not suffer a significant covalent aggregation or fragmentation during the protein release from the alginate microspheres (Fig. 3). As expected, the conformational change of BSA released from the microspheres did not occur when compared to that of BSA standard, and our results showed that the structural integrity of BSA was not affected by the entrapment procedure or release conditions, similar to previous studies (20,25).

**CONCLUSION**

This study demonstrated that spherical and non-aggregated alginate microspheres with a mean diameter of 11.96 ± 0.043 µm can be prepared by an emulsification method. The integrity of encapsulated BSA was not affected by the entrapment procedure. The release of BSA from alginate microspheres was quite fast. These results showed that the emulsification method can be used for the preparation of protein-loaded microspheres. Consequently, alginate microspheres may be an adequate system for protein delivery.

**REFERENCES**


**Table 1.** Encapsulation efficiency, BSA-loading and particle size of BSA-loaded alginate microspheres (mean±SD, n=3)

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Theoretical protein loading (%)</th>
<th>Mean diameter (µm)</th>
<th>Practical protein loading (%)</th>
<th>Encapsulation efficiency (%)</th>
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</thead>
<tbody>
<tr>
<td>BSA-Alginate*</td>
<td>1.96</td>
<td>11.96 ± 0.043</td>
<td>0.725 ± 0.0015</td>
<td>37.01 ± 0.02</td>
</tr>
</tbody>
</table>

* The formulation contained 8% w/w calcium chloride, 2% w/w Span 85 and 20% w/w Tween 85.


