



Entrapment of protein in chitosan-tripolyphosphate beads and its release in an *in vitro* digestive model



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ABSTRACT

This research sought to evaluate the entrapment and *in vitro* release behaviour of bovine serum albumin (BSA) in chitosan-tripolyphosphate (TPP) hydrogel beads. Beads were manufactured by extruding gel forming solutions containing varying concentrations of chitosan (1–2.5%w/w) and BSA (0.25–10%w/w) into TPP solutions ranging in concentration from 0.1 to 10%w/w and in ionic strength from 0.16 to 0.67 M at pH values of 4, 5 and 9.4. Beads produced at a low TPP concentration of 0.4% w/w had the highest BSA entrapment efficiency ($71.6 \pm 0.7\%$) and inhibited BSA release in simulated gastric fluid (SGF) to a greater extent. Increasing chitosan concentration resulted in a higher protein entrapment efficiency, but lowered the overall release. Increasing TPP concentration or the BSA concentration loaded, led to early release in SGF. The results indicate that the utilization of lower concentrations of TPP is a good approach to improve the protein retention ability of chitosan-TPP beads in a simulated gastric environment.

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1. Introduction

Chitosan is the deacetylated derivative of chitin found widely in nature, such as in shrimp, crab and fungi. It is a linear polysaccharide composed of N-acetyl-d-glucosamine and d-glucosamine units at different ratios (Dash, Chiellini, Ottenbrite, & Chiellini, 2011). Chitosan is a versatile polymer with health attributes, such as anti-hypertensive, anti-obesity and anti-inflammatory properties (Ngo et al., 2015). One attractive application of chitosan is as a food grade excipient for controlled release, or targeted delivery of bioactive food ingredients in the GI tract (Norton, Gonzalez Espinosa, Watson, Spyropoulos, & Norton, 2015). This is in part due to its mucoadhesive ability, penetration enhancing property, ease of chemical modification and biocompatibility (George & Abraham, 2006).

Because of the mild cationic properties of this polymer in acidic conditions, chitosan interactions with poly-anions have been extensively studied as a promising approach to the formation of various matrices for food use (Chen & Subirade, 2005; Ghanem & Skonberg, 2002; Zhang, Zhai, Zhao, Ren, & Leng, 2015). Studies have reported the use of sulfate, citrate and tripolyphosphate (TPP) as counterion salts to prepare chitosan hydrogels through ionic crosslinking, and TPP is reported to be the most popular (Shu & Zhu, 2002).

To understand the fundamental properties and gelling mechanism of chitosan-TPP matrices, Mi, Shyu, Lee, and Wong (1999) prepared beads at different pH values (from 1 to 8.6). The pH of the 10% TPP gelling solutions was found to play a key role, with a reported mechanism of gel forming, switching from phase-inversion at high pH values, to ionic crosslinking at pH values below 6. More recently, Rayment and Butler (2008) studied chitosan beads, also made using 10% TPP gelling solutions at pH 8.5 and 4. These researchers found that the pH 8.5 beads shrank upon gelling and became mechanically stronger than those made in acidic conditions, results consistent with the earlier study by Mi et al. (1999). These pH 8.5 prepared beads shrank further (–45%) when put in SGF (pH 1.2). The authors considered this shrinkage positive in terms of offering protection in a gastric environment, assuming that the shrinkage was not a result of chitosan dissolution in the simulated gastric environment. Somewhat conflictingly, Mi et al. (1999) reported that vacuum dried beads prepared at low pH showed good stability in similar acidic environments.

Chitosan-TPP beads have been used to entrap proteins, including BSA, lipase and cytochrome C (Alsarra, Neau, & Howard, 2004; Chang, Niu, Kuo, & Chen, 2007; Ghanem & Skonberg, 2002; Xu & Hanna, 2007). The concentration and pH of the TPP gelling solution, chitosan and the initial protein concentrations in the gel forming solutions were each identified as factors influencing the entrapment efficiency of proteins in these studies. However, no study examined all of these factors in a comprehensive manner.

The influence of TPP concentration on chitosan gelation has attracted some attention, but results are contradictory. Alsarra

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et al. (2004) entrapped *Candida rugose* lipase into chitosan-TPP beads using an extrusion method. It was found that the optimum TPP concentration was 0.5% (w/v), and increasing the TPP concentration to 0.8% (w/v) led to a dramatic decrease in the entrapment efficiency of the lipase. This result is interesting, as this is the only study reporting the use of such low TPP concentrations. The results were in contrast to the commonly held view that higher concentrations of TPP would lead to a higher density of crosslinking between chitosan and TPP, which would consequently result in higher entrapment efficiency of bioactives. For the most part, researchers have chosen moderate (1% to 3% w/v) or quite high (5%, 10% and 15% w/v) concentrations of TPP for protein entrapment in chitosan (Chang et al., 2007; Ghanem & Skonberg, 2002; Ma & Liu, 2010; Xu & Hanna, 2007). Moreover, in a study from Xu and Hanna (2007), BSA (with similar molecular weight to *Candida rugose* lipase) was entrapped into chitosan-TPP beads, and it was reported that increasing the TPP concentration from 5% to 10% (w/v) resulted in higher entrapment efficiency of BSA, which is contrary to the result reported for the *Candida rugose* lipase (Alsarra et al., 2004). To fully understand the role of TPP concentration on the entrapment properties of chitosan, this study was conducted with BSA as the model protein. The research sought to prepare chitosan-TPP macrobeads using a simple extrusion method to systematically study the influence of TPP concentration, pH of the gelling solution, as well as the concentrations of chitosan and BSA in the gel forming solution, on the entrapment efficiency of the protein.

In the case of bioactive proteins with health enhancing properties (e.g. antihypertensive and anti-inflammatory properties), entrapment to protect against digestion and altering of their bioactivity is essential. However, the controlled release of these proteins in the digestive system is also very important. Therefore this research also sought to establish the release behaviour of BSA from the dried chitosan beads manufactured using a range of conditions. Various *in vitro* digestion fluids have been studied to evaluate encapsulation systems. These fluids have varied in pH, salt type, ionic strength, enzyme source and digestion time (Argin, Kofinas, & Lo, 2014; Park, Kang, Haam, Park, & Kim, 2004; Simonoska Crcarevska, Glavas Dodov, & Goracinova, 2008) making the direct comparison of the data from the different systems difficult. Recently, Minekus et al. (2014) proposed a harmonised *in vitro* digestion protocol for the food research area. These recommendations were used in this study to determine the release behaviour of protein from chitosan-TPP gel. No such studies have been reported to date in the literature.

Overall, this research sought to evaluate the effect of pH and TPP concentration on the entrapment of BSA in chitosan-TPP beads and the subsequent release of BSA in a food model *in vitro* human digestion system.

2. Materials and methods

2.1. Materials

Food grade chitosan (Deacetylation degree 96% and molecular weight 210 kDa based on supplier data) was purchased from Primex ehf (Siglufjörður, Iceland). Bovine serum albumin (BSA) (lyophilized, 66 kDa), glacial acetic acid, sodium tripolyphosphate pentabasic, sodium hydroxide, hydrochloric acid and other salts for simulated digestion fluids (potassium chloride, monopotassium phosphate, sodium bicarbonate, sodium chloride, magnesium chloride hexahydrate and ammonium carbonate) were of reagent grade and purchased from Sigma-Aldrich (Arklow, Ireland). All solutions were prepared with deionized water (DI water) produced from a Millipore system (Elix type 2 pure water, Millipore, Cork, Ireland).

2.2. Preparation of BSA loaded chitosan beads

Chitosan-TPP beads with entrapped BSA were produced by an extrusion method as described by Ghanem and Skonberg (2002) with some modifications. Preliminary studies in our laboratory had identified chitosan with higher molecular weight as the best for bead formation. The concentration of acetic acid (fixed at 1% w/w) and the curing time (fixed at 20 min) used in this study were selected based on previous research in our laboratory. Beads of varying formulations were produced, and one example, in which 2% (w/w) chitosan and 0.5% (w/w) BSA gel forming solution was extruded into a 0.4% (w/w) TPP gelling solution, is described as follows. To ensure good dissolution of both polymers, chitosan and BSA were dissolved separately in 1% (w/w) acetic acid to prepare 120 g of a 2.5% (w/w) chitosan solution by overhead stirring for one hour and 30 g of a 2.5% (w/w) BSA solution by gentle agitation. The BSA solution was then mixed into the chitosan solution to obtain the final 2% (w/w) chitosan and 0.5% (w/w) BSA gel forming solution. After 30 min agitation using a magnetic stirring bar, the mixture was allowed to stand on the bench to eliminate air bubbles. An aliquot of this gel forming solution (10 g) was extruded into 20 g of a 0.4% (w/w) TPP gelling solution through 200 μ l pipette tip (inner diameter: 420 μ m; outer diameter: 1 mm) driven by a peristaltic pump (Minipuls 2, Gilson, Madison, Wisconsin, USA) with a flow rate of $\sim$ 1 ml/min. The dropping distance (from tip to surface of TPP gelling solution) was 9 cm. The curing process required 20 min after extrusion, and then the beads formed were separated and rinsed using 20 g DI water three times. The gelling solutions and rinse solutions were collected to facilitate measurement of the entrapment efficiency as described in Section 2.8. The solution preparations and the manufacture of beads were carried out at ambient temperature. The fresh beads were spread on Petri dishes and dried under laminar flow in a microbiology safety cabinet (Scanlaf Labogene Mars class 2, Lynge, Denmark) at ambient temperature for 48 h.

2.3. Effect of concentration of TPP gelling solutions on entrapment efficiency, size and weight of beads

TPP concentration in the gelling solutions was adjusted to 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.8%, 1%, 2%, 5% and 10% (w/w). The gel forming solution was kept constant at 2% (w/w) chitosan and 0.5% (w/w) BSA as described in Section 2.2.

2.4. Effect of pH of TPP gelling solutions on entrapment efficiency

The pH value of the 1% (w/w) TPP gelling solution (initial pH 9.4) was adjusted to 4 or 5 using 0.1 M HCl with negligible change in TPP concentration. The gel forming solution was maintained at 2% (w/w) chitosan and 0.5% (w/w) BSA as in Section 2.2.

2.5. Effect of ionic strength on entrapment efficiency

To increase ionic strength, NaCl was added into 0.4% (w/w) TPP solution (pH 9.4) with final concentrations of 10, 20 or 30 g/l. The gel forming solution was kept constant at 2% (w/w) chitosan and 0.5% (w/w) BSA as in Section 2.2. The ionic strength was calculated according to Eq. (1) (de Vicente, 2004).

$$I = \frac{1}{2} \sum_{i=1}^n c_i z_i^2 \quad (1)$$

where I is ionic strength (M), c_i is concentration (M) of ion i , and z_i is the charge number of ion i .

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