Characterization of alginate-lactoferrin beads prepared by extrusion gelation method

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Characterization of beads

Alginate-lactoferrin mixture

CaCl$_2$ solution

Calcium content (mg Ca$^{2+}$/g Alg)
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Abstract

The potential use of lactoferrin (Lf) as a food ingredient is attracting increasing attention. Encapsulation of Lf in alginate gel beads can potentially enhance its stability and safe delivery. It is therefore important to understand the properties of alginate-Lf beads in order to develop a successful encapsulation method. Three different forms (apo-, native- and holo-) of bovine Lactoferrin (Lf) were encapsulated in alginate beads by the gel entrapment method using calcium as the cross-linking ion. A minimum of 40% alginate was required in the beading mixture to retain the form of a bead. Higher proportion of alginate reduced the water holding capacity and calcium uptake by the beads and these effects were similar for all forms of Lf. Longer cross-linking time led a greater amount of Lf and iron leaching from the beads and this was most pronounced for holo-Lf compared to apo- and native-Lf. The elastic modulus was affected by the composition (mixing ratio) of beads and not by the forms of Lf with decreasing elasticity of the beads with increasing alginate content which was attributed to the decreasing crosslinking density. The stability of the encapsulated Lf was evaluated based on the amount leached into pH adjusted Millipore water and was affected by pH (4 and 7) for holo-Lf but not for apo- and native-Lf. The relative rate of Lf leaching at pH 4 for the different forms of Lf was found not to directly correlate with the pI of the different forms of Lf.

Keywords: Gel entrapment, bovine lactoferrin, alginate, electrostatic interaction, stability

1. Introduction

Lactoferrin (Lf) is an iron-binding glycoprotein (MW = 80 kDa) found in various biological fluids of mammals (Marnila & Korhonen, 2009) and it has many health benefits to humans and animals (Wakabayashi, Yamauchi, & Takase, 2006). Lf can bind iron with high affinity ($K_D \sim 10^{-20}$ M) and can exist in either iron depleted (apo-) or in iron saturated (holo-) forms. In
nature (native-), it exists as a mixture of apo-, holo- (Steijns & van Hooijdonk, 2000) and also monoferric Lf species saturated at either their N or C lobe (Brisson, Britten & Pouliot, 2007). The iron binding ability along with its cationic nature is responsible for a diverse range of physiological functions such as regulation of cellular growth and differentiation, intestinal iron homeostasis, host defence against microbial infection and inflammation, regulation of myelopoiesis, immunomodulatory and protection against cancer (Conneely & Ward, 2004; Guo, Pan, Rowney & Hobman, 2007). These benefits enable Lf to be a potential supplement in commercial food products (i.e. infant milk, supplemental tablet, yoghurt, skim milk, and drinks). However, Lf can easily be denatured by heat treatment (Abe et al., 1991) and processing condition including storage, freezing/thawing, and spray drying (Naidu, 2006) thereby limiting its application. In addition, conditions such as pH, temperature and conductivity can affect the functional properties of Lf and furthermore, Lf is susceptible to degradation by proteolytic enzymes in the gastrointestinal tract of the human body (Onishi, 2011). Thus, a delivery system that protects Lf is required to be developed to deliver Lf as a food component for maximum health benefit.

Alginate is a natural polysaccharide produced by brown algae (Phaeophyceae) and bacteria (Azobacter vinelandii). It is composed of unbranched binary copolymers of (1→4) linked β- D-mannuronic acid (M) and α-L-guluronic acid (G) residues of widely varying composition and sequence (Draget, 2009). It has the ability to retain water and form viscous solutions, stabilize aqueous mixtures and form gels in the presence of divalent cations. Based on these biophysical properties, alginate has been classified as a food additive and has been used in food preparations as thickeners, emulsifiers and gelling and stabilizing agents (Brownlee, Seal, Wilcox, Dettmar, & Pearson, 2009). Because of its unique gelling properties under mild and non-toxic conditions, the use of alginate has been extended to biotechnological and
biomedical applications such as encapsulation and immobilization of enzymes and live cells (Donati & Paoletti, 2009; Martinsen, Storrø, & Skjåk-Bræk, 1992).

Alginates possess ion exchange property due to the presence of the carboxylic groups in both the M and G residues which have been shown to interact with cationic protein molecules electrostatically. Examples of reported electrostatic interactions between the anionic alginate and cationic proteins includes the proteins lysozyme and chymotrypsin (Wells & Sheardown, 2007) and Lf (Peinado, Lesmes, Andrés, & McClements, 2010). Such electrostatic interactions will affect the release of proteins from the alginate gel system. Furthermore, increased charge neutralization by positively charged components will cause a reduction in intramolecular repulsion between individual alginate chains such that the system will be able to adapt a tighter, more compact configuration and thereby affect diffusional pathways (Stockwell, Davis, & Walker, 1986).

An alginate gel can be prepared by a simple diffusion method where a crosslinking ion is allowed to diffuse from a large outer reservoir into an alginate solution forming a single gel bead entrapping the active agent. Diffusion setting produces inhomogeneous alginate gels due to the formation of a sharp gelling zone moving from the surface towards the centre of the gel (Draget, 2009; Draget, Smidsrød, & Skjåk-Bræk, 2005; Draget & Taylor, 2011). An extrusion technique is used for the formation of gel particles by the diffusion method (Desai & Park, 2005) and is useful for encapsulation of heat labile active agents as they are completely surrounded by wall material (Pegg & Shahidi, 2007). The gelling process is influenced by several factors such as alginate concentration, molecular mass and the M/G sequence of the alginate, the ratio between gelling and non-gelling ions and the presence of complexing agents (eg. phosphates and citrates). In addition, gelation of alginate in a mixed system, where charged polymers such as proteins interact electrostatically under favourable conditions may lead to alterations in mechanical properties of the gel beads (Draget, 2009).
Pore size and pore size distribution in alginate gels are of importance as they determine the diffusion properties. Anderson, Skipnes and Smidsrød (1977) reported that calcium-treated alginate forms networks characterized by a pore size between 5 and 150 nm. Diffusion of small molecules is not strongly affected by the alginate gel pore size, but the diffusion of larger molecules, such as proteins, is somewhat restricted, although proteins with molecular weight as high as 300 kDa are able to diffuse through the gel beads with a rate that depends on their molecular size (Tanaka, Matsumura, & Veliky, 1984; Donati & Paoletti, 2009). The M/G ratio of the alginate also affects the diffusivity of proteins in the gel matrix (Amsden & Turner, 1999). Diffusion within the gel depends on porosity, however, since the gel matrix is negatively charged, the influence of electrostatic forces between the matrix and the ionic substrates must also be considered (Martinsen et al., 1992).

In this study, the gel encapsulation behaviour of different (apo-, native- and holo-) forms of bovine Lf in calcium alginate beads was studied. The physico-chemical properties of Lf are affected by its form (Bokkhim, Bansal, Grøndahl & Bhandari, 2013) and the technological applications of Lf therefore, are likely to also be affected by the form of Lf used. This warrants for a detailed study of the different forms of Lf. In addition, the effect of alginate-Lf mixing ratio and crosslinking time on the efficiency of encapsulation and physical and mechanical properties as well as stability of the beads were investigated.

2. Materials and Methods

2.1. Materials

Native- and apo-bovine lactoferrin (NatraFerrin) with 13 and 0.9% iron saturation respectively were provided by MG Nutritionals®, Australia. Sodium alginate (PE 12001-13.8 EN, GRINDSTED® Alginate FD 155, M/G ratio 1.5) was donated by Danisco Australia Pty. Ltd., Australia. The molecular mass of this alginate as determined by U-tube viscometry
using the appropriate Mark-Houwink constant was found to be 140 kDa (Vold, Kristiansen & Christensen, 2006; Vold, Kristiansen & Christensen, 2007). Calcium chloride dihydrate (99%) and sodium azide (99.5%) were purchased from Chem-supply Pty. Ltd. and Sigma Aldrich Co., Australia, respectively. Millipore water was used in the preparation of all samples. Holo-Lf was prepared in the laboratory according to the method of Bokkhim, Bansal, Grøndahl and Bhandari (2013).

2.2. Fabrication of beads

Stock solutions (2, 3, 4 & 5%) by weight of alginate (Alg) and of Lf (apo-, native- and holo-) were prepared in Millipore water. Alginate was dissolved in Millipore water using a high shear homogeniser (IKA ® RW 20 digital, USA) at 600 rpm for 30 minutes. The alginate solution was then heated at 40 °C in a water bath for 90 minutes to remove any trapped air bubbles and then allowed to cool to room temperature. The Lf samples were dissolved in Millipore water under constant stirring using a magnetic stirrer for 2 hours at room temperature.

The first series of beads were extruded by mixing alginate and Lf solutions of equal concentration at equal mixing ratio (1:1) to achieve the final solid contents of 2%, 3%, 4% and 5% in the mixture. Mixing was done at 600 rpm for 20 minutes. No attempts were made to adjust the pH or the ionic concentration of the mixtures. 3 mL of the mixtures were extruded into18 mL of 0.1 M calcium chloride (CaCl₂) solution using 25G ⁵/₈” (internal diameter ~ 0.26 mm) stainless steel PrecisionGlide® needles (Becton Dickinson and Co., USA) under constant stirring with a magnetic stirrer. The beads were allowed to crosslink in the CaCl₂ solution for 30 minutes, removed from the crosslinking solution and subsequently washed three times with Millipore water and finally drained on a sieve.
The second series of beads were extruded as above but using 30G 1/2” (internal diameter ~ 0.16 mm) stainless steel PrecisionGlide® needles.

The third series of beads were extruded by mixing alginate (2%) and Lf (2%) solutions in different ratios of Alg:Lf, specifically in the ratios 1:3, 1:1.5, 1:1, 1.5:1 and 3:1 using 30G 1/2” PrecisionGlide® needles.

The term mixture should be understood as an alginate-Lf mixture and will be denoted as Mix in the captions for figures. In addition, alginate-Lf mixtures having apo-, native- and holo-Lf will be denoted by MixA, MixN and MixH, respectively. All the samples were prepared on weight percentage.

2.3. Characterization of beads

2.3.1. Bead size

The extruded beads were measured for their diameter (mm). 20 beads of same composition were lined in contact in a straight row and the total length measured using a calibrated scale. The total length was divided by the number of beads to achieve the diameter of a single bead.

2.3.2. Water holding capacity

The water content (expressed as g water/g alginate) was determined as a measure of the water holding capacity of the beads. The weight of alginate or the alginate-Lf mixture used for extrusion as well as the weight of the extruded beads after draining was measured. The water content was calculated from equation 1.

\[
\text{Water content} = \frac{\text{Wt. of wet beads} - \text{Wt. of total dry matter}}{\text{Wt. of alginate}} \quad (1)
\]

Where Wt. of total dry matter = Lactoferrin and alginate in the beads.

2.3.3. Bead composition
The Lf content (%) of the beads was determined from the initial amount of Lf present in the alginate-Lf mixture and the amount of Lf which had leached out into the cross-linking CaCl$_2$ solution during bead formation. The absorbance at a wavelength of 280 nm was measured using UV-Visible Spectrometry (UV-Visible Spectrophotometer, Pharmacia, Ultrospec III, LKB, England) and the concentration of Lf determined based on standard curves which were plotted for the different forms of bovine Lf. This was measured at two time-points: at 30 and 60 minutes of cross-linking to determine the effect of cross-linking time on the amount of Lf leaching from the beads.

The iron content (%) of the beads was determined based on the iron content measured in the crosslinking CaCl$_2$ solution by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Varian Vista Pro Radial ICP-OES system, Melbourne, Australia).

The calcium content (%) of the beads was quantified by ICP-OES measurement and used as a measure of the calcium uptake by the beads during their formation. The beads were freeze dried (Christ, ALPHA 1-4 LSC, Osterode, Germany) for 72 hours, acid digested and analysed by ICP-OES.

2.4. Properties of beads

2.4.1. Mechanical property (Young’s Modulus)

To investigate the mechanical property in terms of elastic (Young’s) modulus of the extruded beads, the beads (2 %) with mixing ratios (Alg:Lf) of 1:1.5, 1:1 and 1.5:1 were used. This range of mixing ratios was selected as a mixing ratio of 3:1 produced soft beads which disintegrated on handling. After extrusion, the beads were stored submerged in Millipore water for 24 hours prior to compression testing. A TA.XT Texture Analyser (Stable Micro Systems, UK) with TA10 Cylinder (D = 12.7 mm, L = 35 mm) was used for compression analysis. A single bead was compressed with a test speed of 0.1 mm/sec with a trigger force
of 5 g. The bead was compressed to 20% of its diameter. The Young’s modulus was obtained from the gradient of the stress-strain curve. The gradient was calculated from the linear part of the slope (15 – 40% of total strain).

2.4.2. Stability

The amount of Lf leaching from the beads (Alg:Lf = 1:1) into Millipore water at pH 4 and 7 (22 ± 2 °C) for 6 weeks were determined as a measure of bead stability. 2 grams of beads were placed in 20 mL of pH-adjusted water. 0.02% of sodium azide was used as preservative in the beading mixture as well as in pH-adjusted Millipore water. The stability test of the beads was conducted under constant shaking at 240 rpm (IKA® KS 130 basic, GMBH & Co. KG, Germany). The amount of Lf leaching into the pH-adjusted water was determined at an interval of 1 week, using UV-visible spectrometry at 280 nm (Aitken & Learmonth, 1996) and corresponding Lf standard curves.

2.5. Statistical analysis

For experiments with three or more replicates, significance of differences between the values was analysed by Analysis of Variance (ANOVA) with Tukey’s HSD post hoc test, family error rate 5 at 95% confidence level using MiniTab 16. The number of experiments is indicated by ‘n’ in the figures.

3. Results and discussion

3.1. Characterisation of beads from alginate-Lf mixtures with equal mixing ratios

Beads were extruded using two different sized needles (25G⁵/₈” and 30G¹/₂”) from alginate-Lf beading mixtures, containing different forms of Lf, at equal mixing ratios. The concentration of CaCl₂ in the cross-linking bath and time for cross-linking were kept constant at 0.1 M and 30 min, respectively.
With a bigger diameter needle (25G 5/8"), an effect of solid matter content of the beading mixture on the size (bead diameter) was noticed only at and beyond 4% total solids (Fig. 1 A & B). In addition, total solids content above 3% in alginate and 4% in alginate-Lf mixtures led to bead deformation (tailing), which is a very common phenomenon with viscous alginate solutions (Fundueanu, Nastruzzi, Carpov, Desbrieres, & Rinaudo, 1999). This shows that alginate beads were more prone to tailing than the alginate-Lf beads when compared beads of same total solid content. The diameter of the beads were 2.5 ± 0.2 mm, 2.6 ± 0.3 mm and 3.1 ± 0.3 mm for 2%, 3% and 4% solid contents, respectively. A value for 5% solid content beads could not be obtained due to the irregular shape of the beads (Fig. 1 A & B).

Figure 1(A & B).

When a smaller diameter needle (30G 1/2") was used for extrusion of the beads having similar composition, the effect of total solids content on bead diameter (2.3 ± 0.3 mm) was negligible and no tailing was observed (Fig. 2A). When different forms of Lf (native- and holo-) were added to alginate, a change in bead colour was evident. While alginate beads are transparent those containing Lf were translucent and represented the colour of the alginate and Lf solutions from which they were produced. The colours of different forms of Lf powders were different; apo-Lf appeared white while native-Lf appeared light pink and holo-Lf reddish brown. They imparted similar colour to their respective beads. The beads with apo-Lf were opaque (Fig. 2B). The colour intensity increased with the total Lf concentration in the mixture (Fig. 2A). These visual pictures showed that Lf is retained by the beads. Because of the uniformity in size, PrecisionGlide® needle (30G 1/2") was used for extrusion of beads for all further experiments.

Figure 2 (A & B).
From Figure 3, it can be seen that alginate is the responsible component in the mixture for the water holding capacity of beads as adding Lf to the mixture did not significantly change the water content, e.g., the water content of alginate-Lf beads was not significantly different \( (P > 0.05) \) compared to that of alginate control beads. With lower alginate content (in alginate control as well as in alginate-Lf beads), a higher water holding capacity of the beads was observed. Such a trend has been widely reported (Yotsuyanagi, Ohkubo, Ohhashi, & Ikeda, 1986) and is related to a higher polymer density leading to partial collapse of the network and a reduction in pore size (Donati & Paoletti, 2009). The water holding capacity of the beads from mixtures having different forms of Lf was not significantly different \( (P > 0.05) \) at all concentration levels. This indicates that all alginate-Lf beads would have similar pore size.

3.2. Characterisation of beads extruded from alginate-Lf mixtures with different mixing ratios

3.2.1. Bead composition

Beads extruded through 30G\(1/2\)" needles from alginate-Lf beading mixtures (2%), containing different forms of Lf, at different mixing ratios (Alg:Lf of 1:3, 1:1.5, 1:1 and 3:1) were used in this section of the study. Evaluation of the amount Lf and iron leaching into the cross-linking solution was done for all four mixing ratios. The reason for considering 2% total solids in the mixtures was due to practical limitations because of viscosity increment.

3.2.1.1. Effect of cross-linking time

The amount of Lf leaching into the cross-linking solution during bead formation is characterised by the Lf content of the beads (Supplementary Fig. S1). Depending on the bead composition, the Lf content varied from 92–97 %. The Lf detected in the solution can be
attributed to Lf leaching from the gel beads into the bath solution during cross-linking (George & Abraham, 2006) or rapid escape of Lf into the solution at the initial stage of bead formation. When the cross-linking time was increased from 30 to 60 minutes, a larger amount of Lf had leached (about 2%) into the curing bath (Supplementary Fig. S1). The additional leaching of Lf after cross-linking is attributed to Lf (80 kDa) diffusing into the bath from the cross-linked beads as research has shown that molecules with molecular weight as high as 300 kDa can diffuse from alginate gel beads into the water system (Tanaka et al., 1984; Martinsen, Skjåk-Bræk, & Smidsrød, 1989; Donati & Paoletti, 2009). In addition, a trend was noticed that with increasing amount of alginate in the Lf-alginate mixture (eg. from Alg:Lf of 1:3 to 3:1), the leaching of Lf from the beads was lower (eg. resulted in higher Lf content, Supplementary Fig. S1). This trend is attributed to a higher concentration of alginate in the bead leading to a denser matrix (eg. beads were all of the same diameter). For Lf-alginate beads, a crosslinking time of 30 minutes was subsequently adapted for preparation of the beads for further investigation.

3.2.1.2. Effect of different forms of Lf

The amount of different forms of Lf and iron that leaches into the cross-linking solution after a cross-linking time of 30 min are presented in Figures 4 A and B, respectively. A clear trend was seen for the effect of form of Lf in the alginate-Lf mixture; the Lf content of holo-Lf being the lowest (Fig. 4 A). For all mixing ratios, the differences in Lf content of apo-Lf and native-Lf were non-significant ($P > 0.05$) but were significantly higher ($P < 0.05$) than that of holo-Lf. The iron content of the beads displayed the same trend. Iron retained by beads with apo-Lf was significantly higher ($P < 0.05$) than that of holo-Lf, whereas beads with native-Lf did not follow the trend (Fig. 4 B). It should, however, be noted that the determination of the Lf content is considered more accurate due to the higher absorbance values measured. This data indicate that the iron detected in the cross-linking solution is bound to the Lf. These
values were verified from iron analysis by ICP-OES and protein analysis by Dumas method conducted on the freeze dried beads (data not shown). The differences in the molecular structures of the different forms of Lf, in particular the structure of holo-Lf being more compact compared to the structure of apo- and native-Lf (Sánchez et al., 1992; Brisson, Britten, & Pouliot, 2007) and the differences in their physico-chemical properties (Bokkhim et al., 2013) may be responsible for the differences observed.

Figure 4 (A & B).

### 3.2.2. Water holding capacity

The beads from section 3.2.1.2 were characterized for their water holding capacity except beads produced from the mixing ratio (Alg:Lf) of 1:3 which were excluded as they disintegrated during handling due to the low alginate content. Inclusion of any form of Lf from 25 % (3:1) to 60 % (1:1.5) in the alginate-Lf mixture (total solids content of 2 %) did not significantly affect the water holding capacity of the beads when compared to the alginate control beads of similar alginate content (Fig. 5). However, the alginate content in the mixture, affected the water held by the beads. Lower water content was observed for higher alginate content, which followed a trend similar to beads extruded with same mixing ratio but different solid contents (Fig. 3). This indicates that beads containing higher alginate content have a denser matrix. Higher water holding capacity correlated with softer beads, and as mentioned above the beads with 75 % Lf (1:3) disintegrated during bead handling. From this experiment, it can be concluded that a minimum of 40 % alginate is required in the Lf-alginate mixture (2 %) to form firm beads.

Figure 5.
3.2.3. Calcium uptake by the beads

The beads from section 3.2.1.2 (except for the mixing ration (Alg:Lf) of 1:3) were characterized with regards to the amount of calcium uptake during bead formation and the data for different compositions is shown in Figure 6. The calcium uptake by the beads having different forms of Lf, calculated on the basis of unit mass of alginate, was not significantly affected by the forms of Lf used in beading mixture but was significantly different from that of control alginate beads at mixing ratios 1:1.5 and 1:1. However, the difference diminished as the alginate content increased to 75% (3:1). This showed that addition of Lf to alginate, thereby decreasing the alginate content in the mixture, increases the calcium uptake by the alginate component during bead formation at lower alginate content (below 75%). On the other hand, the calcium uptake by the alginate control beads having different final concentration was not significantly different ($P > 0.05$). An explanation for these observations is that the presence of protein in the alginate-Lf mixture delays the formation of a sharp gelling zone during gelation allowing calcium to diffuse relatively fast towards the centre of the bead, ultimately leading to a higher amount of calcium participation in crosslinking. The amount of calcium (mg) per unit mass of alginate (g) ranged from $109 \pm 2 - 117 \pm 4$ for the controls alginate beads to $137 \pm 5 - 147 \pm 5$ mg/g for mixtures (1:1.5). These results are very similar to the values of $160 \pm 30$ mg/g for beads prepared from a 2 % sodium alginate (M/G ratio 1.5) in 0.27 M CaCl$_2$ reported by Tan et al. (2008).

Figure 6.

3.3. Properties of beads

3.3.1. Mechanical property of extruded beads

Beads containing native-Lf described in sections 3.2.2 and 3.2.3 formed from solutions with mixing ratios (Alg:Lf) of 1:1.5 and 1:1 as well as with a mixing ratio of 1.5:1 were
characterised for their compressibility. As it was found that the water holding capacity and the calcium content of the beads were not significantly affected by the forms of Lf used, only native-Lf encapsulated in alginate was included in this experiment. The mechanical property, specifically the elastic modulus of the extruded beads is presented in Figure 7. It is evident that alginate beads show no significant difference in the elasticity of the gel beads when the concentration of alginate is increased from 0.8 to 1.0 % but the gel elasticity increases significantly when the alginate content is increased to 1.2 %. This agrees with the previous results by Martinsen, Skjåk-Bræk, & Smidsrød (1989) and considering that the beads showed the same level of calcium uptake (Fig. 6), it is attributed to the difference in their water holding capacity (Fig. 5). On the other hand, among the beads having native-Lf in their compositions, the elasticity of beads with 60% Lf (Alg:Lf = 1:1.5) were not significantly different from that of beads with 50% Lf (1:1) but significantly different ($P < 0.05$) from that of beads with 40% Lf (1.5:1). The form of Lf showed no significant difference in the elasticity of the beads (results not shown). This trend of decreasing elasticity of the beads with increasing alginate content for the alginate-Lf beads correlates with the higher crosslinking density (eg. higher calcium uptake) seen for the 1:1.5 Alg:Lf beads (Fig. 6) and is consistent with previous reports (Martinsen et al., 1989). Furthermore, it can be seen that the alginate-Lf beads (1:1) are not significantly different than the control beads (1% alginate) yet by increasing or decreasing either Lf or alginate in the mixing ratio by more than 20%, the elasticity of the beads was significantly altered.

Figure 7.

3.3.2. Stability of extruded beads

Beads described in sections 3.2.2 and 3.2.3 formed from solutions with a single mixing ratio (Alg:Lf) of 1:1 were characterised for their stability with respect to the amount of Lf leaching
into pH-adjusted water at two different pH values. The pH values were chosen so as to ensure that only diffusion process would take place. At lower pH (< 4.0), alginate gels become unstable due to proton catalysed hydrolysis (Draget, 2009) and breaks down into lower molecular components (Gombotz & Wee, 1998). This will ultimately affect the crosslinking and stability of the beads and thereby the mechanism of release of Lf. The choice of a single mixing ratio was based on the Lf content following a similar trend for beads with different ratios (see Fig. 4). The cumulative leaching of Lf from the alginate-Lf beads against time (days) at pH 4 and 7 is shown in Figure 8 A and B, respectively, and is expressed in percentage based on the initial Lf content of the beads at day 0. No significant effect of pH (4 and 7) was observed on the leaching of apo- and native-Lf from their respective beads, but the leaching of holo-Lf was significantly ($P < 0.05$) affected by pH and was higher at pH 7 than at pH 4. Leaching of a higher amount of holo-Lf at pH 7 as compared to pH 4 might be due to higher swelling ratio of alginate beads at neutral pH (Shi, Alves & Mano, 2006). At the same time, it has been reported that holo-Lf has a more compact molecular conformation as compared to apo- and native-Lf (Sánchez et al., 1992; Brisson et al., 2007). Thus, the combination of the gel network size and the protein size might have contributed to leaching of higher amounts of holo-Lf at pH 7. Similar trends with respect to pH have been reported by Shi, Alves and Mano (2006) for the drug indomethacin and by Huguet and Dellacherie (1996) for bovine serum albumin. On the other hand, the amount of apo-Lf leaching from the beads was significantly higher than that of native- and holo-Lf at pH 4. It has been reported that a protein with a low $pI$ may be released more rapidly from the alginate matrix (Gombotz & Wee, 1998). In our past research (Bokkhim et al., 2013), it was found that apo-Lf demonstrates a net charge of zero in pH range of 5.5-6.5, which is lower than the actual $pI$ (8.5-9.5) of Lf. This means that while native- and holo-Lf have an overall positive charge at both pH values used in the current stability study, apo-Lf will have a positive charge only at
pH 4. It thus appears that the relative rates of leaching at pH 4 are not solely related to the
charge of the different forms of Lf. However, our previous study also concluded that apo-Lf
has different physicochemical properties as well as iron binding capacity compared to native-
and holo-Lf and it thus appears that the differences in Lf leaching for the different forms of Lf
is related to more subtle differences between the proteins. The maximum amount of apo-Lf
which had leached from the beads in 6 weeks did not exceed 9% of the initial Lf content in
the beads and lower amounts were observed for the other forms of Lf. This indicates a high
overall stability of Lf within the alginate gel matrix.

Figure 8 (A & B).

4. Conclusion

Lf (apo-, native- and holo-) can be encapsulated in alginate beads by the extrusion gel
entrainment method. Alginate in the alginate-Lf mixture was found to be the major
contributing factor in determining the water holding capacity of the beads, and it was
inversely proportional to the alginate content in the mixture. It was found that a minimum of
40% alginate was necessary in the alginate-Lf mixture to form firm beads. At low alginate
content, the presence of Lf in the mixture led to an increase in calcium uptake by the beads.
The leaching of holo-Lf from alginate-Lf mixture during gelation was found to be higher than
apo- and native-Lf and correlate with the leaching of iron. The gel strength of the beads could
be significantly altered by increasing either alginate or Lf content by 20% in the mixture. The
cumulative leaching of apo-Lf from the beads into pH-adjusted water was less than 9% in 42
days and was higher than that of native- and holo-Lf at both pH values. This indicates a high
overall stability of Lf within the alginate gel matrix. Apart from leaching of Lf during
gelation and upon immersing the beads in pH-adjusted water, the form of Lf in the mixture
did not generate significant differences in the properties of the beads. The findings of this
study clearly showed that Lf can be encapsulated efficiently in alginate beads using gel
entrapment method to produce beads with desired properties and can be used as the basis for commercial scale up.
References


using size-exclusion chromatography combined with light scattering and viscosity detectors. Biomacromolecules, 8, 2627.


Captions for figures supplied:

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<tr>
<td>Fig. 1</td>
<td>Photographs of beads extruded through PrecisionGlide® 25G 1/8&quot; needles. (A) Alg and (B) MixN (1:1) with different total solid contents.</td>
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<td>Fig. 2</td>
<td>Photographs of beads extruded through PrecisionGlide® 30G 1/2&quot; needles. (A) Beads extruded from MixH (1:1) with different total solids content in the mixture. (B) Beads extruded from alginate-Lf mixtures (total solids content, 3%; 1:1) having different forms of Lf. Alginate beads (1.5%) were extruded as control.</td>
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<td>Fig. 3</td>
<td>Water holding capacity of beads extruded from alginate-Lf mixtures (1:1). Alginate beads having total alginate content of 1, 1.5, 2 &amp; 2.5% were extruded as controls. The collective bars for each group that do not share a letter is significantly different at P &lt; 0.05 (n=3).</td>
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<td>Fig. 4</td>
<td>(A) Amount of Lf remaining and (B) Iron remaining in beads prepared from mixtures (2%) having different forms of Lf (apo-, native- &amp; holo-) and alginate in different ratios. The bars within the same column that did not share a letter are significantly different (P &lt; 0.05) (n=3).</td>
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<td>Fig. 5</td>
<td>Water holding capacity of beads prepared from mixtures (2%) with different composition. Alginate beads (0.8, 1.0 &amp; 1.5%) were extruded as control (n=3).</td>
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<td>Fig. 6</td>
<td>Calcium uptake of beads extruded from different composition mixtures (2%). Alginate beads (0.8, 1.0 &amp; 1.5%) were extruded as control. The collective bars for each group that do not share a letter are significantly different at (P &lt; 0.05) (n=3).</td>
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<td>Fig. 7</td>
<td>Mechanical property (Young’s Modulus) of alginate-Lf beads (2%) having different mixing ratios. Alginate control beads (0.8, 1.0 &amp; 1.2%) were extruded accordingly (n=6).</td>
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<td>Fig. 8</td>
<td>Cumulative leaching of Lf (%) from Lf-alginate beads (1:1) in Millipore water at different pH. (A) pH 4 and (B) pH 7. MixA (− – ), MixN (+○+), MixH (− – − − ).</td>
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<td>Fig. S1</td>
<td>Amount of Lf remaining in the alginate-Lf beads (MixN; total solids 2%) at different mixing ratios, cross-linked for 30 and 60 minutes based on the amount of Lf leached into the cross-linking solution (n=1).</td>
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