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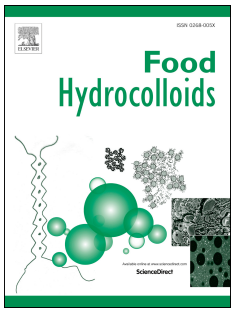

Interactions between different forms of bovine lactoferrin and sodium alginate affect the properties of their mixtures

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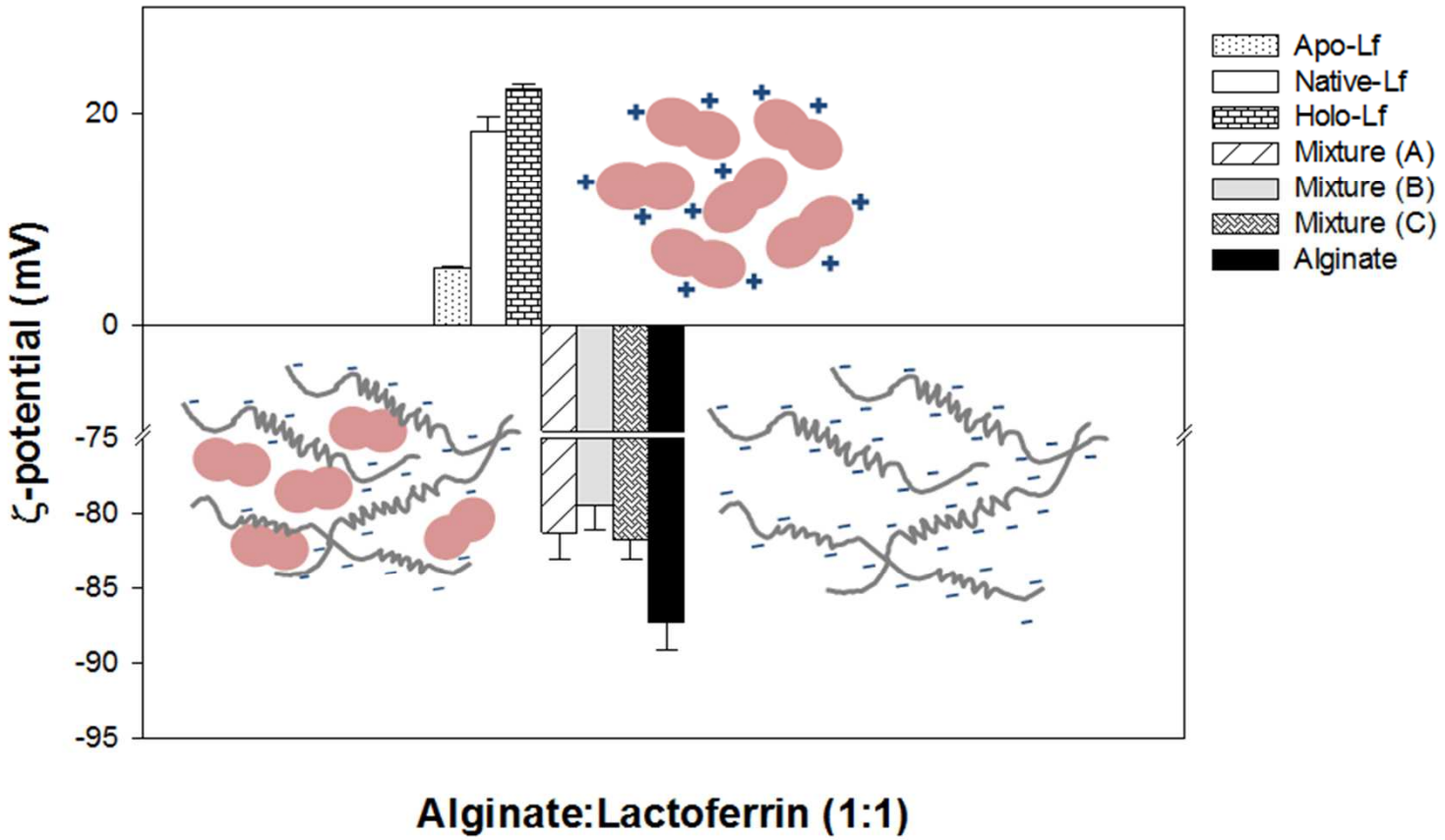
ζ-potential (mV)
20
0
-75
-80
-85
-90
-95
Alginate:Lactoferrin (1:1)
Apo-Lf
Native-Lf
Holo-Lf
Mixture (A)
Mixture (B)
Mixture (C)
Alginate

Title Page Information

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Abstract

The interactions between different forms (apo-, native- and holo-) of lactoferrin (Lf) and sodium alginate at different ratios in aqueous solution in the pH range of 4-7 were evaluated. Fourier transform infra-red (FTIR) spectra of freeze dried mixtures showed shifts only in the bands arising from the carboxylate groups of alginate relative to physical mixtures; indicating intermolecular interactions involving COO^- moieties of alginate. Circular dichroism (CD) spectroscopy showed that Lf retained its tertiary structure in the Lf-alginate mixtures. In the pH range of 4 - 7, the zeta-potential of Lf-alginate solutions was significantly less negative than that of alginate indicating charge compensation. Native-PAGE results indicated that the extent of binding of Lf by alginate was dependent of the form of Lf with apo-Lf displaying a higher binding affinity. At natural pH, the Lf-alginate mixtures generated higher viscosities than their respective sodium alginate controls indicating the existence of intermolecular interactions between the two components. A mixture of native-Lf and sodium alginate showed the highest increase in the viscosity while increasing level of iron saturation in Lf showed an inverse effect on viscosity. DSC analysis showed that the thermal denaturation temperature of native- and holo-Lf can be enhanced upon interaction with alginate in solution.

Keywords:

Lactoferrin, sodium alginate, complexation, zeta-potential, viscosity, gel electrophoresis, denaturation temperature

Introduction

Intermolecular interactions, mainly electrostatic interactions between proteins and polysaccharides, are explored in the food and pharmaceutical industries for the development

of successful protein delivery systems (Peinado, Lesmes, Andrés & McClements, 2010). These interactions are influenced by the physico-chemical properties of the proteins and polysaccharides as well as environmental factors (pH, ionic strength, protein-to-polysaccharide ratio, temperature and mixing process) and can be manipulated to fabricate protein/polysaccharide complexes with a new set of properties in comparison to the proteins and polysaccharides alone (Benichou, Aserin & Garti, 2002; Schmitt & Turgeon, 2011; Ye, 2008). As detailed by Tolstoguzov (1991), the interaction between proteins and polysaccharides may lead to either their co-solubility, incompatibility (phase separation), or complexation (soluble or insoluble). The charges on the proteins/polysaccharides, presence of oppositely charged side groups (acidic and basic) and availability of charged patches in the polyions can play decisive roles in complex formation. Soluble complexes can form when the net charges of proteins and polysaccharides are very different (Ye, 2008).

The glycoprotein Lactoferrin (Lf) possesses a broad spectrum of functional properties towards humans and animals such as cellular growth regulation and differentiation, intestinal iron homeostasis, host defense against microbial infection and inflammation, regulation of myelopoiesis, immunomodulatory and anti-oxidant activities and protection against cancer (Conneely & Ward, 2004; Guo, Pan, Rowney, & Hobman, 2007). In order to exploit these benefits, Lf is being increasingly used in health products. However, these functional properties of Lf are affected by different factors during production, storage, transport and consumption such as heat, salts, pH and enzymes (Abe et al., 1991; Naidu, 2006; Steijns, Brummer, Troost, & Saris, 2001; Eriksen et al., 2010, Onishi, 2011). Lf is a cationic protein with positively charged regions most prominently at the N-terminus, as well as in an inter-lobe region between the C- and N-lobes close to a connecting helix (Moore, Anderson, Groom, Haridas, & Baker; 1997) and as such it has a high isoelectric point (pI ~ 8.0-9.0) (Baker, 2005; Brisson, Britten, & Pouliot, 2007, Ye & Singh, 2007; Bokkhim, Bansal,

Grøndahl, & Bhandari, 2013). A substantial amount of research on Lf has been conducted by the pharmaceutical sector regarding its biochemical characterization and biological activities. In the food sector, studies have focussed on digestion and thermal stability mostly in Lf's natural form in different food systems. Lately, several researchers have explored Lf's technological properties (emulsifying and stabilizing) in oil-in-water emulsions (Bengoechea, Jones, Guerrero, & McClements, 2011; Bengoechea, Peinado, & McClements, 2011). In order to optimise stability and achieve safe delivery of Lf, its encapsulation in a high heat and acid stable matrix such as alginate is a potential avenue for new product development. The full potential of such a system can be reached only with the detailed knowledge of the intermolecular interactions between Lf and alginate and their effect on the properties of the mixture which can influence the fabrication of a delivery system and the release property of Lf.

Sodium alginate is an anionic polysaccharide extracted from the brown algae which is composed of polymeric sequences of (1-4) linked β-D-mannuronate (M) and α-L-guluronate (G) residues (Draget, 2009). Alginate molecules possess ion exchange property because of the presence of the carboxylic groups in both the M and G residues and have a high affinity for di- and tri-valent ions and cationic protein molecules molecules (Zhao, Li, Carvajal, & Harris, 2009). Electrostatic interactions between the negatively charged alginate polymer and positively charged proteins have been studied for lysozyme and chymotrypsin where gelling of the mixtures were observed (Wells & Sheardown, 2007) and for heat treated Lf particles where turbidity, dynamic light scattering and electrophoretic measurements were used to probe effect of pH and ionic strength (Peinado et al., 2010). In addition, alginate has been used to encapsulate vascular endothelial growth factor (Gu, Amsden, & Neufeld, 2004) leading to its sustained release from the alginate matrix. The interactions between the protein and alginate are mainly controlled by the charge density of the protein and the type of

alginate used (e.g. M/G ratio, molecular mass) (Turgeon, Schmitt, & Sanchez, 2007). During the process encapsulating a protein, the functional properties of the protein might be altered due to e.g. electrostatic interactions with the alginate matrix (McClements, 2006). It has been reported that with some proteins such as transforming growth factor-beta (TGF-β1), irreversible complex formation can occur between the protein and alginate molecules resulting in protein inactivation. In such cases additives that protect the protein from the alginate polymer should be added to retain protein activity (Gombotz & Wee, 1998).

This study aims to investigate the interactions between different forms of Lf (apo-, native- and holo-) and alginate in aqueous solution at the natural pH of the proteins and in the pH range of 4 to 7. As different forms of Lf demonstrate different physico-chemical properties (Bokkhim et al., 2013), it is important to investigate how those properties affect the intermolecular interactions in the Lf-alginate mixtures. For many application of proteins, it is important to evaluate if the intermolecular interactions in the protein-polyelectrolyte complex affects the secondary and/or tertiary structure and thus functional properties of the protein, and in this study, this was evaluated using Fourier transform infra-red (FTIR) and circular dichroism (CD) spectroscopy. A measure of the binding affinity of different forms of Lf to alginate was evaluated using gel electrophoresis. zeta-potential measurements were done in order to evaluate complex formation and the properties of the mixtures were investigated through the use of viscosity, as well as DSC for the thermal properties of the protein in the mixtures. The research findings will provide fundamental information regarding the potential benefits and application of Lf-alginate mixtures.

Experimental Section

Materials

Two forms of bovine lactoferrin (NatraFerrin), native- and apo- forms with iron saturation levels approximately 13 and 1% were provided by MG Nutritionals®, Burnswick, Australia. Sodium alginate (PE 12001-13.8 EN), GRINDSTED® Alginate FD 155 (M/G ratio 1.5) was donated by Danisco Australia Pty. Ltd., Sydney, Australia. The molecular mass was determined by U-tube viscometry using and the appropriate Mark-Houwink constant (Vold et al. 2006, Vold et al. 2007) and found to be 140 kDa. Bis (2-hydroxymethyl) iminotris-[hydroxymethyl] methane) (bis-tris) (purity > 98%), potassium chloride, sodium hydroxide, sodium acetate (trihydrate), Trizma® base and glycine were purchased from Sigma Aldrich Co., Castle Hill, Australia (purity > 99 %). Acetic acid (99%), hydrochloric acid (concentration ~ 31.5%) and methanol (99.8%) were from Labtek Pty. Ltd., Brendale, Australia. Sodium dodecyl sulphate (SDS) and glycerol, both of analytical grades were bought from Amresco, Solon, USA and Ajax Finechem Pty. Ltd., Taren Point, Australia respectively. The dyes, bromophenol blue and Coomassie brilliant blue G-250 were from Bio-rad, Gladesville, Australia. Millipore water was used for all experiments. All chemicals used in this study were of analytical grade. Lactoferrin having 50% iron saturation level and holo-Lf were prepared in the laboratory according to the method described by Bokkhim, Tran, Bansal, Grøndahl and Bhandari (2014).

Methods

The Lf-alginate mixtures (2 % w/w) at 1:1 mixing ratio were prepared in Millipore water, allowed to stand at room temperature (22 ± 2 ˚C) overnight and freeze dried (Christ, ALPHA 1-4 LSC, Osterode, Germany). Control samples of dry mixed Lf and alginate (1:1) were also analysed. Infra-red spectra were recorded on a FTIR 100 series Perkin-Elmer spectrometer fitted with a deuterated triglycine sulphate (DTGS) detector using the Universal attenuated total reflectance (ATR) mode. Spectra were recorded at ambient temperature (22 ± 2 ˚C) on

solid samples at a resolution of 4 cm^{-1} and a scan number of 32 over the range of 4000–650 cm^{-1} with air as background. FTIR spectra of freeze dried Lf samples were similar to that of commercial as received samples (data not shown).

The structural conformation of Lf in the Lf-alginate solutions was studied by CD spectroscopy. The spectra of Lf, alginate and Lf-alginate mixtures (1:1) in aqueous solution were recorded in aqueous solution with Millipore water as the background in the wavelength region 250–350 nm using a Jasco J-710 spectrometer with a J-700 Spectra manager software. Lf and alginate samples were diluted to 0.25 % while Lf-alginate mixtures were diluted to 0.50 % for analysis. The measurements were made at ambient temperature (22 ± 2 ˚C) and the ellipticities were expressed as Milli degrees.

Surface charge properties of the Lf, alginate and Lf-alginate mixtures were measured using NanoS Zetasizer based on electrophoretic mobility of the particles. The solutions used for the measurements were acetate buffer (pH 4 and 5) and bis-tris buffer (pH 6 and 7) all with a final ionic concentration of 1 mM potassium chloride as well as water resulting in natural pH (the pH which is achieved upon dissolution of the protein). Solutions of Lf (apo-, native- and holo-) and sodium alginate were prepared in appropriate media (1% w/w). Lf was dissolved at constant stirring for 2 h at room temperature using a magnetic stirrer. Sodium alginate was dissolved using high shear homogeniser (IKA® RW 20 digital, USA) at 600 rpm for 30 min. The alginate solution was then heated in a water bath at 40 ˚C for 90 min to remove any trapped air bubbles. The Lf and alginate solutions were mixed in a ratio of 1:1 using the high shear homogeniser (~500 rpm for 5 min). The mixture was allowed to stand at room temperature for 1 h. The zeta-potential was measured after diluting the mixtures 50 times.

Lf and alginate mixtures were examined using 12% polyacrylamide precast continuous gels (Mini-PROTEAN® TGXTM Precast Gels, BIO-RAD, Gladesville, Australia) under non-

denaturing (native) conditions in a Mini-PROTEAN tetra cell system. The samples of Lf and Lf-alginate mixtures (2 % w/w) were diluted in Millipore water to achieve 1 mg/mL of protein (Lf) content. The loading buffer contained 70 mM Tris-Cl (pH 6.8), 31 % glycerol and 0.01 % bromophenol blue as the dye. The samples were diluted with the loading buffer in the ratio of 1:2 prior to loading in the precast gel. Diluted samples (5 μL) were loaded into the wells of the precast gel and electrophoresis was carried out at 200 V. The gels were fixed for 5 min, stained with Coomassie brilliant blue R-250 solution (34 % methanol) and finally destained. The gel was scanned using Gel Densitiometer (GS-800 Calibrated Densitiometer, UMAX Technologies, Model UTA – 2100XL, Taiwan) and analysed with Quantity One® software operating in Microsoft Windows® computer system. To evaluate the effect of holding time on complexation between alginate and Lf in aqueous solution, Native-PAGE was run for Lf-alginate mixtures (1:1) on different days; day 0 (immediately after mixing), day 1 and day 7. Apart from day 0, the mixtures were kept at 5 ˚C. In addition, Native-PAGE gels with alginate and Lf-alginate mixtures in equal mixing ratio (1:1) were stained using Periodic Acid Schiff (PAS) technique used for carbohydrate staining (Dubray & Bezard, 1982). The gel was fixed in fixative solution for 5 min followed by 1% periodic acid solution dip for 10 min. It was then rinsed with Millipore water twice for an interval of 10 min each and was dipped in Schiff's reagent for 10 min. Finally the gel was allowed to remain in Millipore water overnight and scanned as described above.

The apparent viscosity of the Lf, alginate and Lf-alginate mixtures at natural pH (unaltered) was measured by a Discovery Hybrid Rheometer (DHR-1; TA Instruments, USA) using TRIOS software at 20 ˚C under the shear rate of 25 s^{-1}. A 40 mm, parallel plate, peltier plate steel geometry was used with a gap of 100 μm. Stock solutions of alginate (2 % w/w) and apo-, native- and holo-Lf as well as Lf-50 (Lf having 50% iron saturation level) (2 % w/w) were prepared in Millipore water and mixed at different ratios to achieve total Lf content of

25, 50, 60 and 75 % of the final 2 % total solid content in the mixtures. As controls, 2 % alginate stock solution was diluted with Millipore water to achieve total solid content of 0.5, 0.8, 1.0 and 1.5 % (w/w). Furthermore, Lf-alginate mixtures with similar mixing ratios but with different solid concentrations were prepared. Mixing was done using a high shear homogeniser (~500 rpm for 5 min) and the solutions were left at room temperature overnight before the measurement. In order to evaluate the contribution of addition of Lf to the viscosity of the mixture, the increase in viscosity due to Lf addition to the alginate solution was calculated as follows:

$$\text{Contribution of Lf to viscosity} = \frac{\text{Viscosity (Mixture)} - \text{Viscosity (Alginate)}}{\text{Viscosity (Mixture)}} \times 100\% \quad (1)$$

-ie. from the viscosity of 2% mixture containing x % alginate, viscosity of x % alginate was subtracted.

The thermal properties of different forms of Lf in the presence of alginate in aqueous solution were studied by Differential Scanning Calorimetry (DSC1 STAR^e System, METTLER TOLEDO, Schwerzenbach, Switzerland) according to the method described by Bokkhim, Bansal, Grøndahl and Bhandari (2013). The Lf-alginate mixtures (2 % w/w) at 1:1 mixing ratio were prepared in Millipore water, allowed to stand at room temperature (22 ± 2 ˚C) overnight and freeze dried. The freeze dried Lf-alginate samples were rehydrated in Millipore water to achieve 10 % solutions. 20 µL of rehydrated Lf-alginate mixtures were used for analysis. The temperature of maximum heat absorption (T_{max}) and the enthalpy change of denaturation (ΔH_{cal}) were determined from the transition peak using STAR^e Excellence Software (METTLER TOLEDO). It was not possible to prepare 10% Lf-alginate mixture by simply dissolving and mixing, thus freeze drying of the Lf-alginate mixture was done.

Statistical analysis

Zeta-potential values and thermal properties are presented as mean ± SD of triplicate experiments. MiniTab 16 software was used to analyse the significance of differences between the values (where applicable) using Analysis of Variance (ANOVA) with Tukey's HSD post hoc test at family error rate 5 at 95% confidence level.

Results and Discussion

This study investigated the properties of Lf-alginate mixtures prepared from different forms of Lf and at different mixing ratios with a constant final solid content of 2% at pH values of 4–7. This pH range was chosen based on the fact that Lf is unstable below pH 4.0 (Abdallah & El Hage Chahine, 2000), that the p*K*a of alginate is 3.4–3.7 and the p*I* of Lf is 7.8–9.5. Furthermore, this is the pH range most commonly encountered in food products. After combining the Lf and the alginate solutions, the mixtures were held at 4 ± 2 ˚C overnight to ensure equilibrium had been attained. Neither precipitation nor gelling was visible in the mixtures. The formation of soluble complexes is attributed to the mixing ratios studied for which the net charge of alginate far surpassed the charge of Lf. Relatively stable colloidal dispersions of a native-Lf-alginate system at pH 3–10 has previously been reported for aggregated Lf particles (Peinado et al., 2010).

Chemical Characterisation

FTIR spectra of alginate, different forms of Lf and their respective freeze dried composite Lf-alginate mixtures (1:1) were analysed in the wavelength region of 1200–1750 cm^{-1}. In addition, physical mixtures (e.g. dry-mixed powders) of alginate and Lf (1:1) were run as control samples. The wavelength region chosen includes the amide I (1600–1690 cm^{-1}), II (1480–1575 cm^{-1}) and III (1229–1301 cm^{-1}) bands of proteins (Kong & Yu, 2007) as well as the antisymmetric stretch (1595–1597 cm^{-1}) and symmetric stretch (1407–1412 cm^{-1}) of the

carboxylate groups of alginate (Lawrie et al. 2007). Figure 1 (A, B & C) show the spectral data obtained. The amide I and amide II bands observed at 1637–8 cm^{-1} and around 1520 cm^{-1}, respectively, for all three forms of Lf in the dry state are different to those reported for human-Lf in solution (1647 and 1577, respectively) (Hadden, Bloemendal, Haris, Srai & Chapman, 1994), however, fall within the expected regions for amide bands.

For apo- and native-Lf it was possible to obtain representative spectra of the physical mixture (dry mixed powders) of Lf and alginate (1:1) as control samples, however, such a sample could not be obtained for holo-Lf as the consistency of the powder prevented proper mixing in the dry state. The physical mixtures show the addition spectra of the two macromolecules in which no intermolecular interactions occur. It can be seen in Figure 1 (A & B) that the major bands and shoulders in these samples are very similar to that of the two pure macromolecules but with an apparent shift in the amide I band to lower wavenumbers of around 7–8 cm^{-1} which is attributed to the underlying antisymmetric stretching of the carboxylate group of alginate. Importantly, in the freeze-dried samples prepared from mixing Lf with alginate (1:1) in solution and subsequent drying intermolecular interactions can occur, and here the amide I band is found in a similar position to the physical mixture indicating that there is no change in the secondary structure of the protein. Likewise, the apparent shift in the amide II band to higher wavenumbers by 17–10 cm^{-1} is attributed to an additive effect as the same trend was noticed for the physical mixtures. The position of the amide III band was not affected by the addition of alginate in the mixture.

The antisymmetric stretch of alginate showed a shoulder in the physical mixture at a similar position as pure alginate (e.g. at 1595 cm^{-1}). However, in the freeze dried mixture a shift in this band towards higher wavenumber by 8–9 cm^{-1} was evident (Figure 1). Furthermore, the separation (Δ) between the antisymmetric and symmetric stretching vibrations (Lawrie et al.

240 2007) change from 188 cm^{-1} in alginate to 198–200 cm^{-1} in the freeze-dried samples and this
241 is attributed to the carboxylate group of alginate interacting with Lf, most likely with the
242 positively charged amino acid side chains. The spectral features for such amino acid side
243 chains are unfortunately not well resolved but overlap with the amide vibrational modes. In a
244 similar manner to these findings, different changes in the FTIR spectra of freeze-dried and
245 physical mixtures have also been reported by Souillac, Middaugh and Rytting (2002) in a
246 protein/carbohydrate mixture. Zhao et al. (2009) displayed in their work on bovine serum
247 albumin added to sodium alginate in solution some shifts in the FTIR bands, however, in
248 their work, the spectra were not compared to a simple addition spectrum of the two
249 macromolecules. Overall, in agreement with the general observation that proteins
250 electrostatically binds mostly with the carboxyl groups of the polysaccharides (Tolstoguzov,
251 1991) the FTIR spectra indicate the presence of intermolecular interactions between Lf and
252 alginate in the freeze-dried mixture involving the carboxylate groups of alginate, however,
253 these interactions do not cause a change the secondary structure of the protein.

254 Figures 1A, 1B and 1C

255 CD spectroscopy is a well-established tool for the assessment of changes to the tertiary
256 structure of proteins-polyelectrolyte complexes (Kayitmazer, Seeman, Minsky, Dubin, & Xu,
257 2013). The CD spectra of alginate, different forms of Lf and their respective mixtures (1:1) in
258 aqueous solution in wavelength region of 250–350 nm are shown in Figure 2. This
259 wavelength region was chosen as it was where the ellipticity values of alginate in solution lie
260 in the vicinity of zero and therefore allows evaluation of the Lf tertiary structure. All spectra
261 showed the characteristic bands of Lf in the 250–350 nm region; a negative band centered at
262 270–272 nm, a band at 291–292 nm and one at 296–298 nm (Bokkhim et al., 2013). Within
263 experimental error there was very good agreement between the spectra of each of the forms

of Lf and their respective mixtures with alginate and no significant change in the maximum wavelength or ellipticity values was observed. This demonstrates that despite the intermolecular interactions that occur between the oppositely charged Lf and alginate in aqueous solution, all forms of Lf retained their original tertiary structures. This result is in agreement with previous findings that oxidised sodium alginate does not induce a conformational change to BSA (Gao, Liu, Chen, & Chen, 2011).

Figure 2

Physicochemical Characterisation

The zeta-potential of native-Lf-alginate mixtures at different mixing ratios in the pH range 4.0– 7.0 was studied. The Native-Lf mixture displays a positive zeta-potential in this pH range in agreement with its p*I* value and it was found that the zeta-potential was significantly higher at pH 4 as seen in Figure 3A. In the absence of Lf, alginate displayed similar zeta-potential values in the pH range studied (Fig. 3A). With the addition of Lf, the negative charge decreased significantly (the zeta-potential values of the mixtures were significantly different ($P < 0.05$) to that of the alginate solution at all pH values studied) and the decrease was larger at lower pH (Fig. 3A). This correlates with the significantly more positive charge of Lf at pH 4. In addition, it was noted that the higher the amount of Lf in the mixture, the larger was the numerical decrease in the zeta-potential value (Fig. 3B). A similar trend was noted when Lf was added to negatively charged β-lactoglobulin-stabilized emulsions (Ye & Singh, 2007) and is in agreement with formation of electrostatic interactions between the two macromolecules (Ye, 2008). Since the largest decrease in zeta-potential was observed for the mixture with the alginate:Lf ratio of 1:1 (Fig. 3B) this mixing ratio was chosen for further experiments. Furthermore, the zeta-potential values of the native-Lf-alginate mixtures were not significantly different at pH 4 and 5 or at pH 6 and 7. However, the zeta-potential values

288 of the mixtures at pH 4 or 5 were significantly different ($P < 0.05$) from that at pH 6 or 7
289 (Fig. 3 A). Based on this, the three forms of Lf were studied at pH 4 and 7.

290 The zeta-potential of the three forms of Lf, alginate and Lf-alginate mixtures at pH 4 and 7
291 are presented in Figure 3C. The zeta-potential values for all three forms of Lf at both pH 4
292 and 7 were positive and the value for apo-Lf was significantly lower than that of native- and
293 holo-Lf at both pH values in agreement with that previously reported (Bokkhim et al., 2013).
294 In addition, zeta-potential values of all Lf solutions at pH 7 were much lower than at pH 4 as
295 noted above for native-Lf. It was found that the Lf-alginate mixtures generated significantly
296 different ($P < 0.05$) zeta-potential values than that of alginate at both pH values. The
297 differences in zeta-potential for the different forms of Lf in mixtures were insignificant at
298 both pH 4 and 7. It can be inferred that the negative charges of alginate were partly
299 compensated by the addition of positively charged Lf. Thus, as can be seen from Figure 3C, a
300 more positive zeta-potential of Lf at pH 4 resulted in higher charge compensation when
301 added to negatively charged alginate solution leading to a significant decrease in the zeta-
302 potential. At pH 7, which is near the p*I* of Lf, the protein had a lower positive charge thus
303 leading to lower charge compensation. The results obtained are in agreement with the
304 formation of electrostatic interaction between all forms of Lf and alginate at both pH 4 and 7.
305 The overall negative charge of the complexes similar to that of the polyelectrolyte alginate
306 indicates that the outer layer of the complex is dominated by the polyelectrolyte (Peinado et
307 al., 2010).

308 Figures 3A, 3B, 3C and 3D

309 The surface charge properties of the three forms of Lf, alginate and Lf-alginate mixtures at
310 natural (unaltered) pH are presented in Figure 3D. From previous studies it has been found
311 that pH of 1 % (w/w) natural apo-, native- and holo-Lf solutions are 5.7, 5.4 and 6.2,

312 respectively (Bokkhim, et al., 2013). It was found that the zeta-potential of apo-Lf was
313 significantly lower (5.3 ± 0.1 eV) than native- and holo-Lf (18 ± 1 eV and 22.3 ± 0.4 eV,
314 respectively) under these conditions (Fig. 3D). Furthermore, it was found that the zeta-
315 potential values of Lf-alginate mixtures at natural pH in different mixing ratios (alginate:Lf =
316 1:1 and 1:1.5) were not significantly different from each other, but were significantly
317 different from that of alginate at both mixing ratios (Fig. 3D). This lack of a discernable
318 change in the zeta-potential values with the additional Lf in the mixture could indicate that a
319 point of saturation of alginate has been reached or that the difference is too small to detect. In
320 the following sections the mixtures were studied under natural pH conditions as this is most
321 relevant to Lf encapsulation in alginate for its development as a pharmaceutical or food
322 component.

323 **Evaluation of Relative Binding Affinity of Different Forms of Lf**

324 Evaluation of protein-polyelectrolyte interactions has traditionally used capillary
325 electrophoresis and size exclusion chomatrography (Kayitmazer et al., 2013). More recently,
326 so-called gel retardation assays employing agarose gels have been used to evaluate
327 complexation between DNA and chitosan (Masotti et al., 2007) and complexation between
328 silk-polylysine block copolymers and plasmid DNA (Numata, Subramanian, Currie, &
329 Kaplan, 2009) while heparin displacement assays employing native polyacrylamide gel
330 electrophoresis has been used to evaluate complex stability for complexes between siRNA
331 and PEI polyelectrolytes (Hobel et al., 2011). Common to these techniques is the ability to
332 evaluate relative amounts of bound and unbound protein/polynucleotide. In the current study,
333 Native-PAGE gel electrophoresis was explored as a means to evaluate to which extent
334 alginate is capable of binding the different forms of Lf. The choice of native conditions for
335 the gel electrophoresis experiment was based on the protein tertiary structure being retained

under these conditions. In order to test this concept a gel was run with native-Lf at a constant amount in all lanes but in different mixing ratios with alginate and the result of a Coomassie blue stained gel (protein stain) is displayed in Figure 4A. It can be seen, that in all samples a protein band is appearing at the same position as the positive control (native-Lf in the absence of alginate, lane 1). Furthermore, in lanes containing alginate, varying amounts of Lf has remained on the top of the gel and the amount migrated (values indicated on the gel) increases with decreasing amount of alginate (from lane 2 to 10). This result is interpreted in terms of the Lf associated with alginate being prevented from migrating in the polyacrylamide gel (ie. it is being retarded) and remain at the top of the gel while un-bound Lf has migrated. This clearly demonstrates the validity in using native-PAGE to evaluate (relative) binging of Lf to alginate. It should be noted, that over the mixing ratio range studied there is a 100 fold difference in the molar ratio of alginate to Lf and this is not linear which explains the non-linear trend in the amount of migrated Lf with mixing ratio. Furthermore, this result agrees with the lack of a discernable change in the zeta-potential values for the two mixing ratios evaluated in Figure 3D.

Figures 4A and 4B

Subsequently, Native-PAGE gel electrophoresis was used to evaluate relative binding affinity of the different forms of Lf to alginate. The result shown in Figure 4B was obtained at a constant mixing ratio of 1:1 and a constant amount of Lf in all lanes and the protein stained using Coomassie blue. It can be seen that all forms of Lf appear at the same position as the respective positive control samples (lanes 1, 5, 9). Furthermore, the effect of holding time over 7 days was evaluated. Within the experimental error, there was no significant difference in the amount of Lf migrating with different loading time for either apo-Lf (lanes 2–4), native-Lf (lanes 6–8) or holo-Lf (lanes 10–12). Therefore, the amount of the different forms

of Lf bound to alginate was evaluated from the average values from the three time points and found to be 76% for apo-Lf, 60% for native-Lf and 60% for holo-Lf. Repeat experiments (data not shown) consistently showed that a larger amount of protein was retained in the apo-Lf complex compared to the other proteins. A separate gel was run and stained with PAS (polysaccharide stain, Supplementary Figure S1). This experiment showed that for all proteins a similar amount of alginate was retained for the complexes relative to the pure alginate sample. It can thus be concluded that that alginate displays higher binding capacity (larger number of protein molecules per alginate polymer) for apo-Lf than for the other forms of Lf. This result correlates with the surface charge properties of the different forms of Lf at natural pH. Thus, apo-Lf is close to its isoelectric point while the two other forms of Lf have similar positive surface charge (Fig. 3D). While one study found that proteins with higher (and opposite) charge resulted in more binding events with polyelectrolytes (Vinayahan, Williams, & Phillips, 2010), another study has shown, that not only the overall charge but also the charge distribution of proteins affect their interactions with polyelectrolytes (Xu, Mazzawi, Chen, Sun, & Dubin, 2011). In the current study it appears that the higher capacity for alginate to bind apo-Lf compared to native- and holo-Lf is related to fewer intermolecular interactions between alginate and apo-Lf. Considering that the strength of interaction between oppositely charged biopolymers is enhanced when the net charges of the biopolymers are increased (Ye, 2008), this in turn leads to the prediction that weaker interactions exist between alginate and apo-Lf than the other forms of Lf.

Properties of Mixtures Resulting from Intermolecular Interactions

The polysaccharide alginate is expected to affect the viscosity more than the protein Lf (Schmitt, Sanchez, Desorby-Banon, & Hardy, 1998). However, electrostatic interactions between Lf and alginate can affect the apparent viscosity of their mixtures. It should be noted

that the change in viscosity might not always result from an electrostatic interaction but also from other intermolecular interactions and hydration properties of the molecules involved. Mixing of polymers can influence the hydration property of a polymer in the mixture and there can be a synergistic (compatibility) or antagonistic (non-compatibility) effect (Tolstoguzov, 1991). When native-Lf (2 % w/w) having very low viscosity (~2 mPa s) was added to an alginate (2 % w/w) solution of high viscosity (~1300 mPa s) at an equal ratio (1:1) (resulting in 1% alginate), the apparent viscosity of the mixture was found to be much higher (~1000 mPa s) than that of 1 % alginate solution alone (~150 mPa s) but lower than that of a 2 % alginate solution. This clearly showed that the viscosity of the mixture was not solely due to alginate and that Lf synergistically contributed to the increase in viscosity. If the result of mixing alginate and Lf had been an additive effect, the viscosity would have decreased to a value similar to that of the 1 % alginate solution as the viscosity of the Lf solution is significantly lower than that of the alginate solution at the same concentrations. The results clearly indicated that intermolecular interactions between Lf and alginate have an effect on the viscosity of their mixtures.

The effect of iron saturation levels on the viscosity change in mixtures with constant total solid content of 2 % (w/w) was studied. From Figure 5A, it can be seen that with the increase in apo-Lf, Lf-50 and holo-Lf content in the mixture from 25 to 75 % (e.g. mixing ratios of 3:1 to 1:3), the viscosity of the mixture decreased. However, the contribution of Lf on viscosity increments as calculated by equation 1 increased for the same series of mixtures (Fig. 5B). For native-Lf, on the other hand, the viscosity of the mixture increased along with its contribution to viscosity increment. Comparing the native-Lf, Lf-50 and holo-Lf at any Lf content, it can be seen that with an increasing level of iron saturation of Lf, the viscosity increment decreased. Considering that the iron ions are buried within the interdomains of the lobes of the protein (Brisson et al., 2007) they are unlikely to affect the intermolecular

409 interactions. However, upon iron binding a change in tertiary structure of Lf is observed
410 (Bokkhim, Tran, Bansal, Grøndahl, & Bhandari, 2014; Shimazaki, Kawano, & Yoo, 1991)
411 which appears to affect the strength of intermolecular interactions with alginate and is likely
412 due to the availability of exposed functional groups on Lf changing as a consequence of the
413 change in tertiary structure. Considering that apo-Lf has the lowest iron saturation it would be
414 predicted to have the highest impact on the viscosity increment, however, this was not
415 observed. This can be related to these experiments being done at the natural pH of the Lf, and
416 the fact that apo-Lf has a significantly lower zeta-potential compared to native- and holo-Lf
417 under these conditions (Fig. 3D) thus decreasing electrostatic interaction with anionic
418 alginate as concluded above. The native-Lf showed the maximum increase in viscosity of the
419 mixture at any Lf content level. The amount of native-Lf bound to alginate for mixtures
420 containing 50 % or more native-Lf remain relatively constant (Figure 4A), yet, a large effect
421 on viscosity is seen between 50, 60 and 75 % Lf content. One explanation for this observation
422 is that as the amount of alginate decreases relative to native-Lf, there will be less alginate
423 available per Lf molecule which gives rise to network formation although not to an extent of
424 visible gelling. This would cause the viscosity to increase with increasing amount of native-
425 Lf in the mixture. The native-Lf is unique in displaying this property and this is contributed
426 to a combination of optimal tertiary structure as well as optimal surface charge properties.

427 Figures 5A and 5B

428 The changes in thermal properties of Lf in the Lf-alginate mixtures in aqueous solution at
429 natural pH were studied by DSC. The temperature of maximum heat absorption (T_{max}), also
430 known as the denaturation peak, and the enthalpy change of denaturation (ΔH_{cal}) were
431 derived from the transition peak obtained from the DSC thermograms and the values are
432 presented in Table 1. The denaturation peaks of apo-Lf and the second peak (mono- or di-

ferric saturated) of the native-Lf (Bokkhim et al., 2014) in the mixtures were not significantly different from their respective Lf samples. However, the denaturation peaks of the first (main) peak (iron free) of native-Lf and the peak of holo-Lf in the mixtures shifted significantly towards higher temperature as compared to their respective Lf samples. Though apo-Lf and the first peak (iron free) of native-Lf both are devoid of iron and similar in composition, the difference in their behaviour in the mixtures can be related to differences in the strength of intermolecular interactions with alginate as a result of their surface charge properties. The significant increase in the denaturation temperatures for native- and holo-Lf in their alginate mixtures is attributed to a larger number of electrostatic interactions as inferred from the PAGE gel electrophoresis data. The reason for the second peak of native-Lf not showing a significant increase in denaturation temperature can be attributed to the small amount of iron saturated Lf (mono- or di-ferric) present in the native-Lf (13 %). In agreement with the current study, improved heat stability of Lf in the presence of negatively charged soluble soybean polysaccharide has been reported (Uneo, Ueda, Morita, Kakehi, & Kobayashi, 2012). Tolstoguzov (1991) pointed out that in a protein-polysaccharide mixture, increasing the amount of bound protein in the mixture gives rise to an increase in the denaturation temperature of the protein as compared to free proteins. Considering that only 60 % of native- and holo-Lf is bound to alginate at the mixing ratio studied (1:1) and that this can be increased upon changing the mixing ratio (based on PAGE gel electrophoresis results), it is clear that alginate offers great opportunity for improving the thermal stability of Lf.

The enthalpy change of denaturation (ΔH_{cal}) for the main denaturation peak for all the mixtures significantly decreased as compared to their respective Lf samples. Though a higher temperature was needed to denature Lf in the mixtures attributed to intermolecular interactions, less heat energy was required to cause denaturation once the temperature of denaturation had been reached. Previous studies reported in the literature have made disparate

458 observations with regards to the effect of polyelectrolytes on protein stability. A study of
459 mixtures of polysulfoanions and clyceraldehyde-3-phosphate dehydrogenase (GADPH)
460 found that these polyelectrolytes cause a decrease in denaturation temperature and that
461 polysaccharide-based polyelectrolytes had the least of an effect (Stogov, Izumrudov &
462 Muronetz, 2010). Likewise, synthetic anionic polyelectrolytes when complexed with
463 lysozyme, chymotrypsinogen and GADPH were found to significantly reduce the thermal
464 stability of the protein (Inivova, Izumrudov, Muronetz, Galaev & Mattiasson, 2003). In
465 contrast, Burova et al. (2002) has made similar observations to the current study. They
466 reported a lowering of the denaturation enthalpy for complex of soybean tripsin (Kunitz)
467 inhibitor (STI) and dextran sulfate at pH 3 and an increase in denaturation temperature of STI
468 when complexed with pectin having low degree of esterification.

469 Overall, these and the current study highlight the importance of evaluating the thermal
470 stability of protein/polyelectrolyte complexes before proceeding for technological
471 application. Furthermore, the current study have shown that while alginate does not enhance
472 the thermal stability of Lf, it does lead to a higher denaturation temperature which is an
473 important finding for the application of Lf-alginate mixtures in food products.

474 Table 1

475 **Conclusion**

476 The results of this study have demonstrated that the anionic polysaccharide alginate has the
477 potential to be a successful carrier material for different forms of Lf since the protein is
478 conformational stable in its mixtures with alginate. A number of the experiments probed the
479 intermolecular interactions between alginate and the different forms of Lf. Specifically; the
480 zeta-potential data was consistent with charge compensation of the protein by alginate with
481 the overall complex carrying a negative charge in the pH range of 4 to 7. It was shown from

the FTIR spectra that the COO^- moieties of alginate are involved in the intermolecular interactions and the viscosity of the mixtures furthermore gave strong indications of intermolecular interactions. In addition, it was found in this study that the form of Lf has a significant effect on both the binding affinity (from PAGE gel electrophoresis) and strength of interactions (from viscosity and DSC) in these complexes. This knowledge will be of great value when fabricating alginate-based delivery systems for Lf. Further work is being carried out on the *in-vitro* release of lactoferrin from Lf-alginate encapsulate mixture.

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Caption for table supplied:

Table 1

Thermal denaturation temperatures (T_{max}) and enthalpy change of denaturation (ΔH_{cal}) of Lf samples and Lf-alginate mixtures (1:1) (10% w/w).

Table:

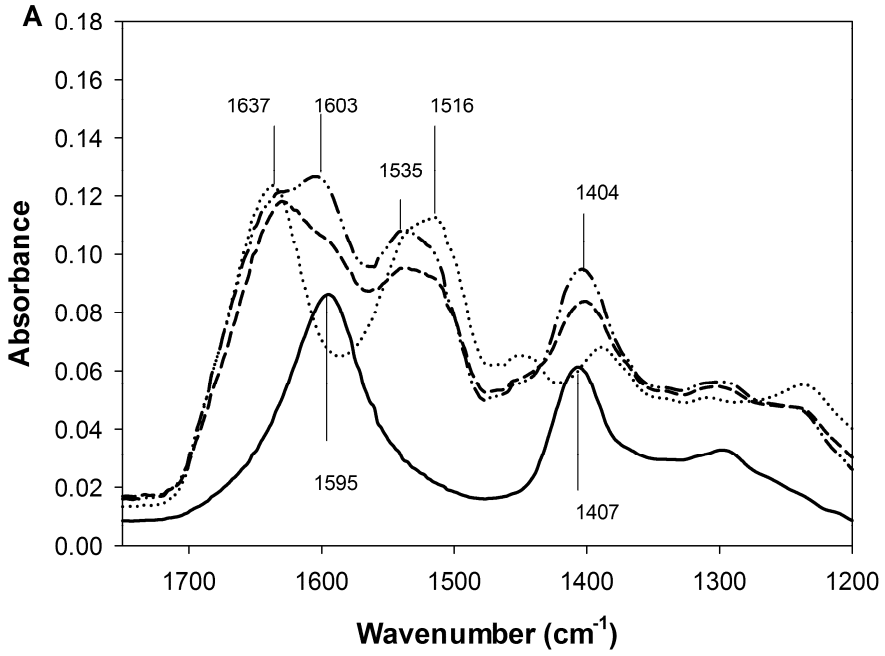
Table 1

Sample	Peak 1: T_{max1} (˚C)	ΔH_{cal} 1 (J/g)	Peak 2: T_{max2} (˚C)	ΔH_{cal} 2 (J/g)
Apo-Lf	70.7 ± 0.8^{c}	13.2 ± 0.2^{C}	-	-
Mixture (Apo-Lf)	69 ± 1^{cd}	8.1 ± 0.8^{D}	-	-
Native-Lf	60.5 ± 0.5^{e}	14 ± 1^{BC}	89.2 ± 0.5^{b}	2.0 ± 0.1^{E}
Mixture (Native-Lf)	67 ± 1^{d}	7.3 ± 0.7^{D}	88 ± 2^{b}	1.0 ± 0.1^{E}
Holo-Lf	-	-	91.3 ± 0.5^{b}	21.0 ± 0.6^{A}
Mixture (Holo-Lf)	-	-	94.2 ± 0.4^{a}	15.3 ± 0.9^{B}

Mean values of T_{max} and ΔH_{cal} (vertical column) that do not share a letter are significantly different at $P < 0.05$.

Captions for Figures supplied:

Figure	Caption	Remarks/Format
Fig. 1	FTIR spectra of alginate, different forms of Lf and their respective mixtures (Lf-alginate = 1:1); dry mixed & freeze dried mixture in the wavelength region 1750–1000 cm^{-1}. (A) Apo-Lf and its mixtures, (B) Native-Lf and its mixtures and (C) Holo-Lf and its mixtures. Alginate (—), Lf (···), dry mixed mixtures (– – –) and freeze dried mixtures (–··–). As holo-Lf was not able to form homogenous mixture when dry mixed, the spectrum has been removed.	TIFF
Fig. 2	Circular dichroism spectra of alginate (•, black), apo-Lf (o, red), native-Lf (Δ, green), holo-Lf (∇, voilet), MA (■, navy blue), MN (□, brown), MH (◊, blue) in aqueous solution. M represents mixtures of alginate & Lf (1:1) where A (apo-), N (native-) & H (holo-) Lf.	TIFF
Fig. 3	(A) The zeta-potential values of native-Lf (o), alginate (•) and Lf-alginate mixtures at 1:1 ratio (▼) in pH range 4.0 – 7.0 (1 mM KCl); (B) Zeta-potential values of native-Lf-alginate mixture at different mixing ratios at pH 4-7 (1 mM KCl). Lf (♦), Alg (•), Alg:Lf=9:1 (o), Alg:Lf=8:2 (▼), Alg:Lf=7:3 (Δ), Alg:Lf=6:4(■) & Alg:Lf=5:5 (□); (C) The zeta-potential of apo-, native- and holo-Lf and their alginate mixtures at 1:1 ratio at pH 4 and 7; (D) The zeta-potential of apo-, native- and holo-Lf and their alginate mixtures at natural pH in mixing ratios of 1:1 (ie. 5:5) and 1:1.5 (ie. 4:6).	TIFF
Fig. 4	(A) Native-PAGE of Native-Lf and its mixture with alginate (MN) in different mixing ratios (alginate: native-lf); (B) Native-PAGE of different forms of Lf (apo-, native & holo-) and their mixtures (MA, MN & MH) in equal mixing ratio (1:1) at different storage days (0, 1 & 7). The values from the densitiometer analysis (relative amount of Lf migrated from the mixture) are given at the bottom of the gels.	The figures are the original gels scanned using Gel Densitiometer (GS-800). Thus is saved in JPEG format, as converting them to TIFF led to fading of bands.
Fig. 5	(A) Comparison of apparent viscosities at shear rate 25 s^{-1} of mixtures of alginate and Lf [apo-(A), native-(N), Lf-50 & holo-(H)] at different ratios (alginate:Lf of 3:1, 1:1, 1:1.5 & 1:3) at natural pH; (B) % Contribution of Lf to viscosity increase of these mixtures as calculated from equation 1.	TIFF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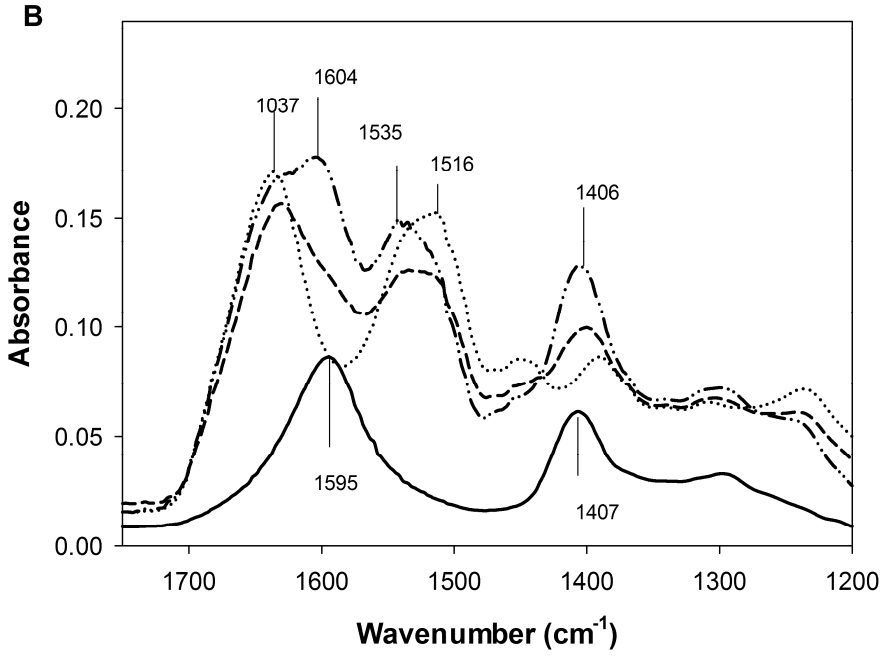
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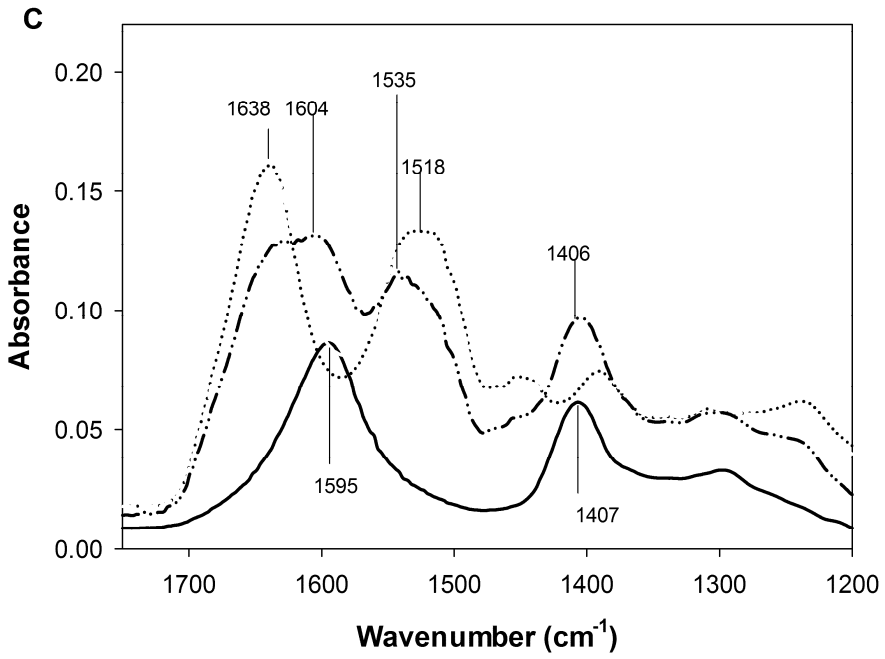
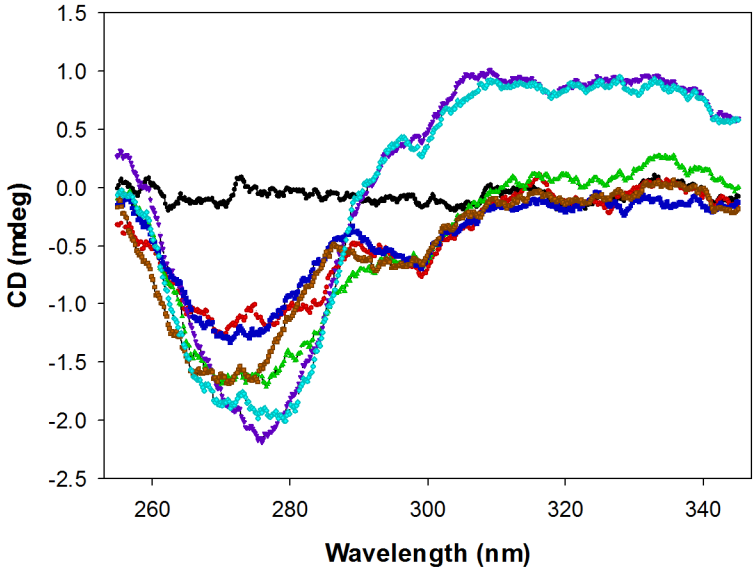
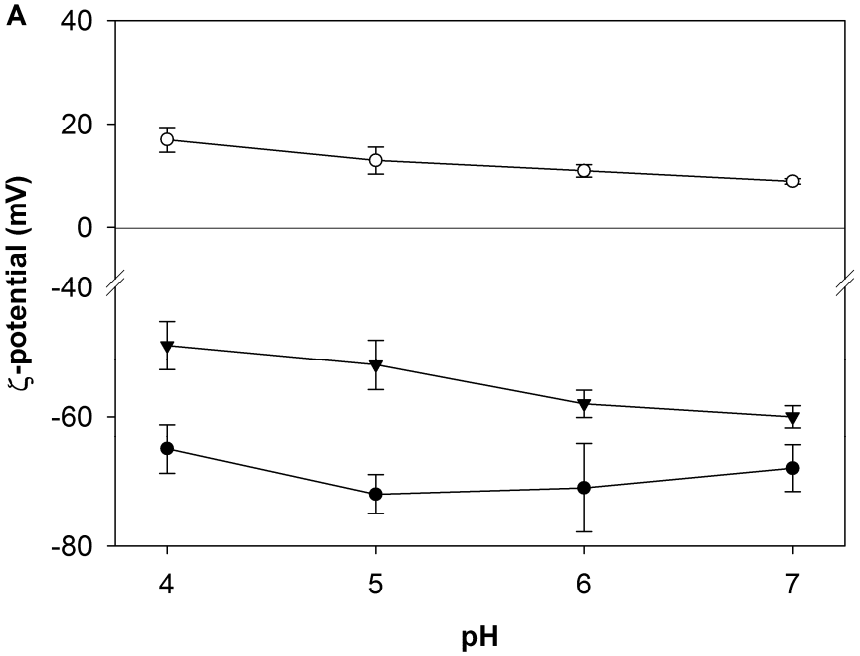
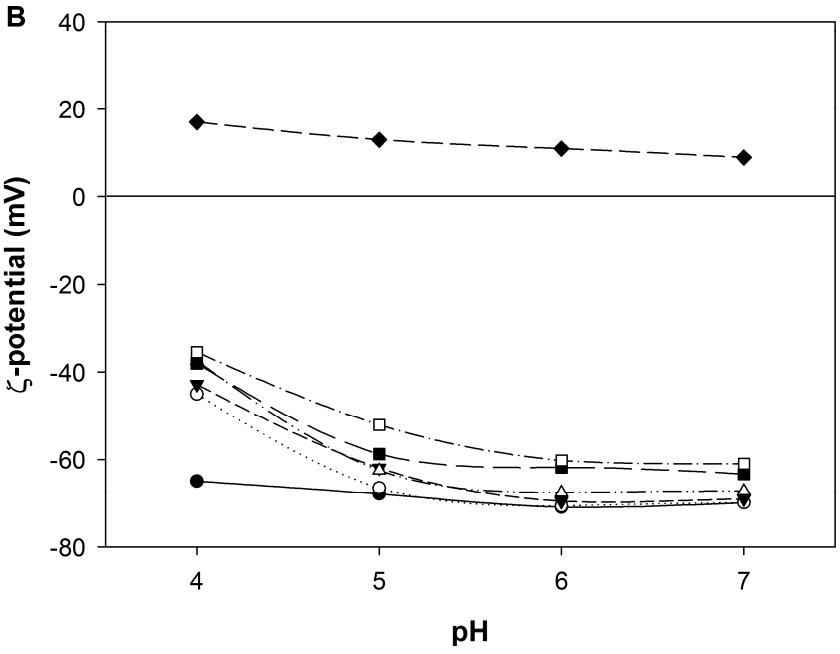
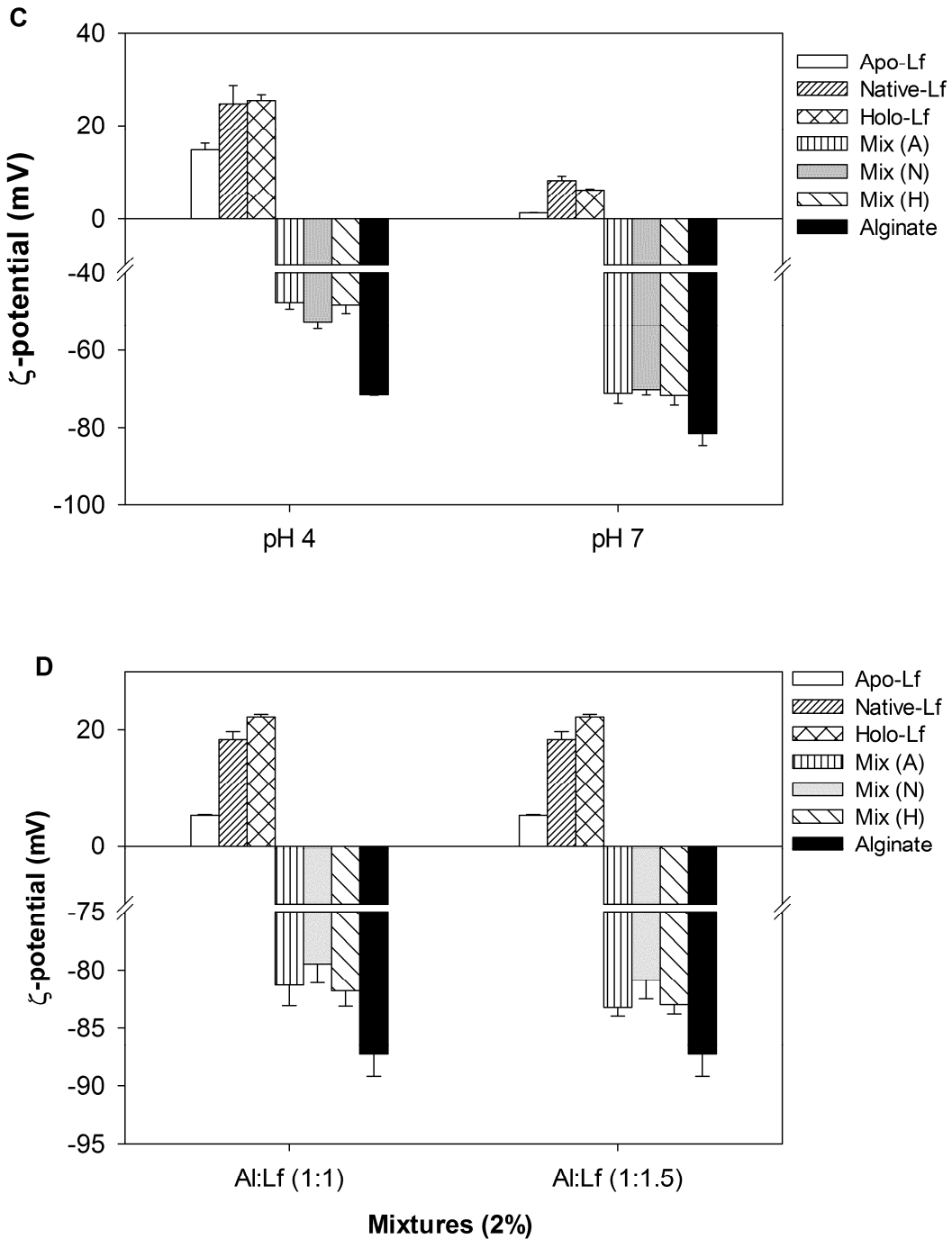
1200
Wavenumber (cm-1)

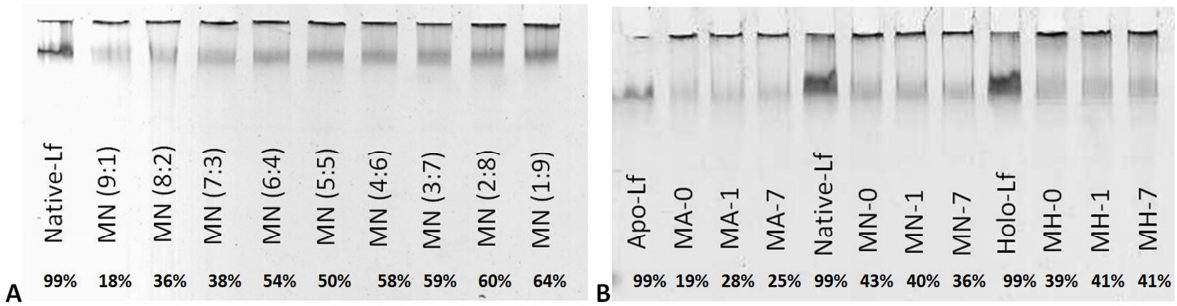

C
0.20
0.15
0.10
0.05
0.00
Absorbance
1638
1604
1535
1518
1406
1595
1407
1700
1600
1500
1400
1300
1200
Wavenumber (cm-1)

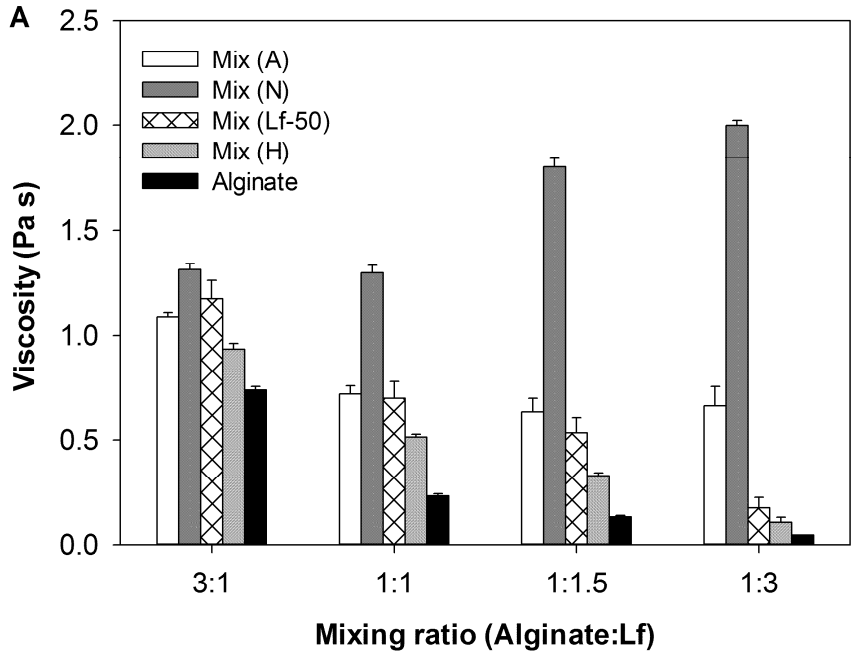

1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
CD (mdeg)
260
280
300
320
340
Wavelength (nm)

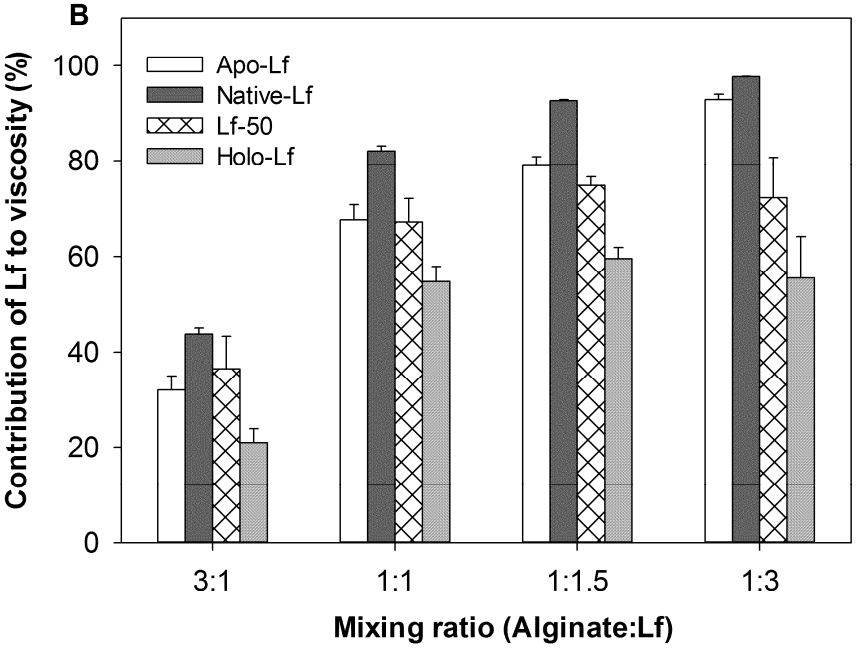

A
40
20
0
-40
-60
-80
ζ-potential (mV)
4
5
6
7
pH


B
40
20
0
-20
-40
-60
-80
ζ-potential (mV)
4
5
6
7
pH


C
40
20
0
-40
-60
-80
-100
ζ-potential (mV)
pH 4
pH 7
Apo-Lf
Native-Lf
Holo-Lf
Mix (A)
Mix (N)
Mix (H)
Alginate
D
20
0
-75
-80
-85
-90
-95
ζ-potential (mV)
Al:Lf (1:1)
Al:Lf (1:1.5)
Mixtures (2%)
Apo-Lf
Native-Lf
Holo-Lf
Mix (A)
Mix (N)
Mix (H)
Alginate




A
Mix (A)
Mix (N)
Mix (Lf-50)
Mix (H)
Alginate
Viscosity (Pa s)
2.5
2.0
1.5
1.0
0.5
0.0
3:1
1:1
1:1.5
1:3
Mixing ratio (Alginate:Lf)


B
Apo-Lf
Native-Lf
Lf-50
Holo-Lf
Contribution of Lf to viscosity (%)
100
80
60
40
20
0
3:1
1:1
1:1.5
1:3
Mixing ratio (Alginate:Lf)

Highlights:

1. All forms of Lactoferrin (Lf) retain their structural conformation in Lf-alginate mixtures.
2. Molecular interactions between Lf and alginate involve the alginate carboxylate groups.
3. Native-PAGE was used to evaluate the binding capacity of alginate towards Lf.
4. All forms of Lf contribute to an increase in viscosity of Lf-alginate mixtures.