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Highlights for Preparation of alginate microgels in a simple one step process via the Leeds Jet Homogenizer by Pravinata *et al.*

- Calcium alginate microgel particles down to size 100 nm have been produced
- Their formation depends on rapid & highly turbulent mixing of separate alginate and Ca^{2+}
- Oppositely charged protein present during formation acts as a dispersant & reduces particle size

Preparation of alginate microgels in a simple one step process via the Leeds Jet Homogenizer

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Abstract

Fine calcium alginate microgel particles, down to particle sizes of size lower than 100 nm, were produced using a Jet Homogenizer previously developed in the School of Food Science and Nutrition (University of Leeds, Leeds, UK) consisting of highly turbulent mixing of two liquid streams of sodium alginate and calcium chloride solution. The final mean particle size, *d*, depended on the alginate to calcium ratio. From 0.5 to 2 wt.% alginate concentration in the presence of 1 – 10 and 20 mM [Ca^{2+}], *d* was lower than 5 μm and higher than 20 μm, respectively. However, *d* was not so significantly affected by the homogenization pressure above 150 bar at room temperature (20 ± 3 °C) or the volume ratio of the sodium alginate to calcium chloride solutions, within the limits 1:9 or 9:1. The particles initially exiting the homogenizer appeared to be slightly aggregated since sonication produced a further decrease in size. The particles were negatively charged (-31.7 mV ± 3.1 mV at pH 8) and inclusion of a suitable globular protein (lactoferrin but not lysozyme) of opposite charge led to a further reduction particle size and a slight decrease in particle ζ-potential. It suggested that some degree of protein adsorption to the particle surface occurred, akin to a surfactant, which helped to control the particle size. In addition, some lactoferrin may also be incorporated inside the microgel particles during their formation, highlighting the potential of this technique to encapsulate various materials within microgel particles formed from Ca^{2+} cross-linked biopolymers.

Introduction

Alginate is a high molecular weight polysaccharide extracted from brown seaweeds of the phylum *Phaeophyceae*. The polysaccharides are monomers of β-D-mannuronic and α-L-guluronic acids linked together by α 1-4 glycosidic bond and arranged according to different sequences with molecular weights ranging between 50 to 465 kDa, depending on the alginate sources (Stokke et al., 2000). Alginate is applied as a stabilizer and thickener in a wide range of food and pharmaceutical products, but also for the preparation of hydrogel bead or (micro)gel particles for the encapsulation of functional ingredients like vitamins and probiotics (Anal & Singh, 2007; Kailasapathy, 2006). Other applications of alginate encapsulation for controlled drugs release and wound dressing management were reviewed by Goh, Heng, & Chan (2012).

Calcium alginate beads are quite simple to prepare and the mechanism of gelation via calcium ions forming egg-box junction zones is a widely known and exhaustively studied phenomenon (Fang et al., 2007). The calcium cross-bridging is so strong that simply dripping or spraying (prilling) alginate solution into a calcium ion solution will give 'instantaneous' gelation of the alginate droplet in the calcium solution (Brun-Graeppi, Richard, Bessodes, Scherman, & Merten, 2011; Quong, Neufeld, Skjåk-Bræk, & Poncelet, 1998). At the concentrations of alginate and Ca^{2+} (0.1 to 0.5 mol dm^{-3}) generally used for the prilling method, alginate chains bind quickly to Ca^{2+} and associate into dimers then multimers of increasing size. This rapidly leads to a growing number of chain entanglements within a dense alginate gelled network (Jørgensen, Sletmoen, Draget & Stokke, 2007). Such methods have been reviewed by Shilpa, Agrawal, & Ray (2003) and more recently by Paques, van der Linden, van Rijn, & Sagis (2014). Once they have been formed, the gel particles can be extremely resilient, e.g., to boiling and shear (BeMiller & Whistler, 1996). They are therefore very attractive as encapsulating materials for oral delivery of protein or peptide drugs (George & Abraham, 2006), with the added advantage of being stable to acid, e.g., in the gastric phase of digestion. However, such simple preparation methods generally give rise to particles that are too large (typically no smaller than 25 μm) for some applications. There are disadvantages of being too large in terms of settling out of the particles, blending them into other ingredients and their organoleptic effects in foods. More advanced variations of the prilling technique have been developed with improved yield, smaller

beads and particles possessing a narrower size distribution. These variants generally involve modification of the spraying nozzles and shear fields in the receiving calcium bath, or modification of the forces between them via electric fields and/or mechanical vibration. The minimum gel particle size formed by these methods still tend to be of the order of tens of microns (Paques et al., 2013). Smaller beads have the advantage that release is more rapid if this is based on diffusion out of them or their surface erosion – the specific surface area being larger. There are other advantages of small size in terms of the ease of mixing and blending, lower tendency to settle or aggregate, plus their access to narrower capillaries and junction zones, or the relative ease to cross other biopolymer barriers, such as the mucin layers coating the gut and other epithelial surfaces (Bajka, Rigby, Cross, Macierzanka & Mackie, 2015).

It is not easy to control the spraying of alginate solution, which is rheologically complex and thus it is difficult to control and reduce the droplet size consistently before it contacts the calcium-rich phase. Various other methods have been developed to produce particles of decreasing size. One obvious route is to prepare two separate water-in-oil (W/O) emulsions or microemulsions in which alginate is already dissolved in the aqueous phase of one emulsion and calcium dissolved in the aqueous phase of the other emulsion and then mix the two (micro)emulsions (Machado et al., 2012). Microemulsion droplets spontaneously exchange but require considerable amounts of surfactant to form the droplets, whereas the W/O emulsion route requires some method to initiate the slow release and diffusion of calcium ions between the droplets to gel the droplets containing the alginate (Amici, Tetradis-Meris, Pulido de Torres, & Jousse, 2008; Poncelet et al., 1992). Recently Paques et al. (2014) described a method where calcium nanoparticles dispersed in the oil phase act as the source of cross-linking ions under relatively neutral pH (pH 6), resulting in particles of around 1 μm and even as low as 200 nm.

In the present paper our aim is to describe a relatively simple new method that can be used to produce micron or sub-micron alginate particles, or other microgel particles that rely on rapid or confined exposure of the polysaccharide to a cross-linking agent such as Ca^{2+}. The calcium alginate microgel particles were prepared using a high pressure Jet Homogenizer (University of Leeds, Leeds, UK). The instrument has typically been used to make fine (O/W or W/O) emulsions as described in numerous publications from this research group (Burgaud,

75 Dickinson, & Nelson, 1990). Here, however, the instrument has been used as a kind of high shear micro-reactor
76 (Johnson & Prud'homme, 2003), as previously performed by Casanova & Higuita (2011), to prepare $CaCO_3$
77 microparticulates.
78 Microgel particles are also just one type of novel food particle that might be exploited to stabilize Pickering
79 emulsions (de Folter & van Ruijven, Marjolein W. M.; and Velikov, 2012; Destribats, Rouvet, Gehin-Delval,
80 Schmitt, & Binks, 2014) – emulsions stabilized by an adsorbed layer of particles as opposed to molecules.
81 Traditionally, the particles in Pickering emulsions are particles of solid material that do not deform on adsorption.
82 However, as long as they maintain a size and contact angle sufficient to secure their interfacial attachment,
83 deformable particles may also stabilize via the Pickering mechanism, so that the term 'Mickering' emulsions has
84 been coined by Schmidt et al. (2011) to describe protein microgel-particle-stabilized emulsions. Improvements
85 regarding the production of truly nanoscale protein microgel particles of well-defined size or shape were reported
86 by Sağlam, Venema, van der Linden, & de Vries (2014). Many methods rely on heating globular proteins in
87 relatively dilute solution and/or at pH values far enough from their isoelectric pH, so that they are highly charged.
88 Whey protein has been particularly intensively studied (Schmitt et al., 2010; Schmitt & Ravaine, 2013) and other
89 applications of protein microgel particles in general have recently been reviewed by Dickinson (2015).

90 **2. Materials and methods**

91 *2.1. Materials*

92 Sodium alginate (Kelgin*LV) was supplied by Kelco International Ltd (London, UK). Extensive drying of the
93 alginate powder in an oven at 110 °C revealed it had a residual moisture content of 11 ± 0.5 wt.%, but solutions
94 were made up on basis of the weight of the powder as received. Calcium chloride dihydrate (99.5%, MW 147,
95 Lot 81K0252), sodium azide (99.5%), fluorescein isothiocyanate-dextran (average molecular weight 2,000,000
96 Daltons, Lot SLBB6384V) and lysozyme with protein content greater than 90% (from chicken egg white, product
97 code L6876, Lot 111H7010) were all from Sigma Chemicals, St. Louis, MO , USA. The xanthan gum (Keltrol®)
98 used was obtained from CPKelco (Surrey, UK). Bovine lactoferrin (Bioferrin 2000, bioactive whey peptide, Lot
99 #2783491) was kindly donated by Glanbia Nutritionals (Middlesbrough, UK). The lactoferrin and iron content

were specified as greater 95 % total protein and 150 mg per 100 g of protein respectively. In protein-containing samples, 0.05 mol dm^{-3} sodium bicarbonate (Fisher Scientific, Loughborough, UK) was used as buffer solution. The pH was adjusted with 0.5 mol dm^{-3} HCl solution (Convol, BDH Chemicals Poole, Dorset, UK) and 1 mol dm^{-3} NaOH (Fisher Scientific, Loughborough, UK) using Jenway pH meter (Guildford, UK) to reach pH 6, 8 and 10 after adjustment. All chemicals were used without further purification. Water purified by a MilliQ apparatus (Millipore, Bedford, UK), with a resistivity not less than 18.2 MΩ cm, was used for the preparation of all solutions.

2.2. Methods

Preparation of microgel particles

Solutions containing a range of concentrations of calcium ions [Ca^{2+}] varying from 1 to 70 mM were prepared by dissolving the $CaCl_2.2H_2O$ solid in water. Sodium alginate solutions at concentrations of 0.5, 1, and 2 wt.% alginate were prepared by dispersing the alginate powder in water while heating at 50 ºC, under magnetic stirring for at least 2 h. No further procedures were performed to purify the alginate. The alginate solutions were cooled down to room temperature (20 ± 3 ˚C) before use to prepare microgel particles. Sodium azide was also dissolved in the sodium alginate solutions at 0.01 wt.% to act as a preservative.

Figure 1 shows the essential features of the Jet Homogenizer. Two cylindrical chambers of different volumes are connected via fixed thin capillary tubing to an outlet via very small hole (0.5 mm diameter) drilled into a stainless steel disk. A compressed air driven ram pushes on pistons placed in the top of each chamber to force the liquids out of the chambers and through the hole. The fluid velocities generated through the hole can be extremely high (> 300 m s^{-1}), creating highly turbulent flow Reynolds number > 10^5 (Burgaud et al., 1990; Casanova & Higuita, 2011), depending on the pressure applied (typically 100 to 400 bar). The duration of the whole process is very short (< 1 s). When oil and appropriate surfactant solutions are placed in the separate chambers this leads to highly reproducible formation of fine (O/W or W/O) emulsions without the need for any pre-mixing of the immiscible phases (Burgaud, Dickinson, & Nelson, 1990). In the experiments described here, $CaCl_2$ solution is placed in one chamber and sodium alginate solution in the other. Under the highly turbulent conditions, cavitation, shear and impact effects are generated which result in the alginate and calcium ions

interacting to form much smaller (< 1 μm with 1 wt.% alginate and 10mM $CaCl_2$ - see below) gel particles than via prilling methods. The suspension of particles in excess calcium alginate solution exits the device at relatively low velocity and may be simply collected in a beaker. On immediately exiting the homogenizer the sample was diluted in water or buffer (depending upon the sample) to help limit any possible particle aggregation prior to particle size measurements. In some experiments protein (lysozyme or lactoferrin) was also added to the alginate solution prior to homogenization. The concentrations of lysozyme and lactoferrin were varied from 0 to 0.12 and 0.8 wt.%, respectively. These proteins were dissolved in the sodium bicarbonate buffer and stirred via a magnetic stirrer for 30 minutes with no heating required at room temperature (20 ± 3 °C). The proteins dissolved readily and clear solutions were obtained. Afterward, they were added to the 2 wt.% alginate solution at 1:1 weight ratio and stirred with magnetic stirrer for 15 minutes before placing into the Jet Homogenizer chamber. The pH of the diluted calcium alginate suspensions when buffer was not used (i.e., without proteins added) was pH 7 ± 0.2.

Microgel particle size measurement

The particle size distribution of larger gel particles produced in preliminary studies was measured via a Mastersizer Hydro 2000 (Malvern Instruments, Malvern, UK) and characterized via the Sauter mean diameter,[1] d_{32}. For microgel particles of size equal to or lower than 10 μm, dynamic light scattering (*DLS*) was used. The mean Zaverage particle diameter (μ_z) and volume-weight size distribution were measured via a Zetasizer Nano ZS (Malvern Instruments, UK) *DLS* instrument. If calcium alginate microgel particles are assumed to be spherical in shape, the apparent particle diameter (μ_z) is inversely related to the diffusion coefficient of the particles via Stokes-Einstein equation below (Nobbmann et al., 2007):

$$D = k_b T / 3\pi\eta d_H \quad (1)$$

k_b = Boltzmann constant, T = temperature, η = viscosity of buffer, *and* d_H = hydrodynamic diameter.

[1] $d_{32} = \sum_i \frac{n_i d_i^3}{n_i d_i^2}$ where n_i = the number of particles of diameter d_i

147 Prior to *DLS* measurement, 2 mL aliquots of diluted sample from the Jet Homogenizer were filtered by
148 injection through a 1 μm pore size disposable Whatman (GE Healthcare Life Sciences, Buckinghamshire, UK)
149 syringe filter. The filtrate was dripped directly into a disposable cuvette and placed in the instrument at 25 °C.
150 The scattered light was detected at 173°, the refractive index and viscosity of the buffer were assumed equal to
151 water, i.e., 1.333 and 10^{-3} Pa s, respectively. It was checked that the viscosity of these diluted suspensions was
152 approximately the same as that as pure water or buffer (i.e., 10^{-3} to 3 x 10^{-3} Pa s, data not shown). Each microgel
153 preparation was repeated at least twice and triplicate measurements were made on each preparation: mean
154 particles sizes and the standard deviation for each set of conditions are reported.
155 *Microscopy*
156 Most of the microgel particle sizes obtained were below the resolution of a typical light microscope. The
157 technique of negative fluorescent staining was employed to collect micrographs of particle samples by confocal
158 laser scanning microscopy (CLSM). Some samples were also examined after centrifugation to concentrate the
159 suspension. Samples were centrifuged at 1086 g for 1.5 h in Kendro Lab Products Gmbh (Hanau, Germany)
160 bench top centrifuge. The supernatant was discarded after centrifugation and the sediment re-dispersed in a
161 small amount of water with vigorous shaking. To the centrifuged and non-centrifuged samples 2 mg of fluorescent
162 dextran was added per ml of sample. A small quantity of xanthan solution was also added to samples to reach a
163 xanthan concentration of 0.1 ± 0.01 wt.%. The mixture was placed in a welled slide (30 mm diameter x 0.3 mm
164 depth), then imaged via a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Manheim
165 Germany) connected with a Leica Model DM RXE microscope base. Laser excitation of the samples was
166 performed with the Ar/ArKr laser source at 488 nm and fluorescence emission wavelength was at 500 nm to 550
167 nm. A 20x objective with numerical aperture 0.5 was used to obtain all images, at 1024 x 1024 pixel resolution.
168 The purpose of adding dextran was to provide a fluorescent background to highlight the non-fluorescent microgel
169 particles, assuming that molecular weight of dextran was sufficiently high to prevent its migration into the
170 particles. The xanthan was added to produce a weak gel structure that would limit Brownian motion of the
171 particles.

To prepare samples for SEM analysis, samples of the Ca-alginate suspension were freeze-dried in triplicates using a benchtop Christ Alpha 2-4 freeze-dryer (Martin Christ, Germany) at -50 ˚C and 0.04 mbar for 25 h. Before the samples were placed into the freeze-dryer, these were quickly frozen using a blast freezer at -30˚C for 3 h. By measuring the weight difference before and after drying, the solids content of the microgels was determined as 0.77 ± 0.06 wt.%. Thus the solid recovery was almost 100% since the final concentration of alginate after mixing with Ca^{2+} was 0.8 wt.%.

For SEM preparation, a small piece from the dried sample of Ca-alginate suspension was pulled apart and a cross-section of it was glued vertically to a chrome coated steel plate with carbon double-sided tape. The sample was then sputter coated with gold using a JEOL JFC-1600 Auto Fine Coater (JEOL Japan) for 200 seconds at 30 mA. The SEM images were obtained using a JEOL 6390A SEM (JEOL, Japan) at 10 to 20 kV. Within this range of conditions different samples gave consistent results but closer examination of the particles at higher magnification was not possible due to beam damage. The SEM was also connected to a JED 2300 EDS Spectrometer (JEOL, Japan), which was used to check the composition of Ca-alginate particles at various spots.

Sonication of samples

Sonication was applied to some samples to see if this affected the particle size, in view of the possibility of particle aggregation. A 2 ml aliquot of the 1 in 10 diluted microgel suspension from the Jet Homogenizer was placed in a 10 ml glass sample vial and the ultrasonic probe of a Sonics Vibra Cells Ultrasonic Processer (Sonics and Materials Inc., CT, USA) was immersed in the suspension. The sample was sonicated at 130 W power, frequency 20 kHz, in 2 second pulses for 2 to 30 min. To avoid heating, the sample tube was surrounded by an ice bath. The temperature of the sample was checked with a thermocouple and did not rise above 35 ˚C throughout the sonication.

Bulk viscosity measurements

The viscosity of some suspensions and supernatants was measured with a Kinexus Rheometer (Malvern Instruments, Worcestershire UK). The apparatus was controlled and data acquisition was performed via *rSpace* software, as supplied with the rheometer. The temperature was set at 25 °C in every experiment. The cone and

plate geometry (CP2/60:PL65) was used for each sample, i.e., a 60 mm diameter and 2° angle cone. After depositing approximately 1.5 ml of the sample between the cone and plate, the sample was then left to achieve steady state for 5 minutes. Viscosities were measured over a logarithmic ramp of shear rates ranging from 0.1 to 10 s^{-1}, the whole range taking 24 minutes in total.

3. Results and Discussion

Data collected were analyzed using IBM SPSS Statistics v.22. A paired sample t-test (for sonicated samples) and 2-way ANOVA tests (for proteins containing samples) were conducted to determine the differences in particle sizes and ζ potential with a significance level set at 0.05.

Effects of alginate and calcium concentration, phase volumes and homogenization pressure

In preliminary experiments, the volume ratio of the two chambers containing the Na alginate and $CaCl_2$ solutions and was varied between 80:20 and 90:10. In addition, placing the alginate in the larger chamber but the $CaCl_2$ in the smaller chamber, and *vice versa*, was also investigated. Following many repeat experiments, lower d_{32} was always obtained with the 80:20 alginate : Ca^{2+} configuration, irrespective of [Ca^{2+}]. The final variable tested in these preliminary experiments was the homogenization pressure. In general, as expected, increasing pressures led to particles of decreasing size. However, between 300 and 500 bar there was no significant decrease in particle size. The Ca^{2+} concentration range investigated was 1 to 70 mM: below 1 mM no particles could be detected visually or via the confocal microscope, the Mastersizer or via *DLS*. Above 20 mM significant increases in d_{32} occurred, with d_{32} values 20 to 100 μm or even larger and the results were much less reproducible. Since there are many simple ways of producing calcium alginate particles in these larger (i.e., > 20 micron) size ranges (Paques *et al.* 2014) and the objective here was to try and produce micron or sub-micron sized particles, Ca^{2+} concentration above 20 mM were not studied in detail further.

The overall outcome of the preliminary work was that the optimum conditions for small microgel particle formation appeared to be an alginate concentration = 1 wt.%, a calcium ion concentration ([Ca^{2+}]) = 10 mM, a volume ratio of 80:20 alginate : Ca^{2+} solution, plus a homogenization pressure of 350 bar. These conditions were used in all other experiments discussed below, unless otherwise stated, in order to produce small stable microgel

222 particles that could be widely utilized as a vehicle to encapsulate and deliver insoluble functional ingredients in
223 foods or similarly intractable compounds in pharmaceuticals.

224 Figure 2 shows typical confocal micrographs obtained with the fluorescently labelled dextran as a
225 negative contrast agent for non-centrifuged (a) and centrifuged (b) microgel particles obtained under these
226 conditions. Figure 2(c) shows a typical SEM image of a sample of a non-centrifuged and freeze-dried sample.
227 Figure 3 shows the detailed particle size distribution (*PSD*) determined via *DLS*. The vol.% PSD suggested that
228 there were two populations of particles – a lower volume one below 100 nm in size and a larger one with a peak
229 size at just under 1 μm. A broad distribution might be expected due to some tendency for the primary particles to
230 aggregate (Santos, Cunha, Veiga, Cordeiro-da-Silva, & Ribeiro, 2013). Sonication was therefore applied to test
231 for the presence of aggregates. Figure 3 shows that sonication caused the PSD to become narrower and
232 monomodal, with the 2 peaks at *ca.* 100 nm and 1 μm shifting to give a single peak centered on *ca.* 150 nm. This
233 suggested that the larger particles may indeed have been aggregates of the smaller microgel particles, probably
234 due to excess Ca^{2+} linking the particles together as they formed. Similar effects of sonication on alginate gel
235 particles have been observed elsewhere (Lertsutthiwong, Noomun, Jongaroonngamsang, Rojsitthisak, &
236 Nimmannit, 2008; Santos et al., 2013).

237 Due to difficulties of beam damage described above, it was difficult to obtain clear images of samples
238 above a certain magnification, but to try and compare the light scattering results of particle size with SEM
239 indications of size, detailed image analysis was performed on some images. As an example, Figure 2(c) was
240 analyzed using *ImageJ* software. After automatic thresholding and edge detection, 520 objects were counted and
241 the areas of these objects in pixels these were converted to diameters of circular objects of equivalent area, in
242 nm. This gave a mean diameter of 140 nm with standard deviation of 50 nm, reasonably close to the μ_z values
243 determined via DLS.

244 Figure 4 shows the ratio of the Z-average diameter after sonication (μ_z^s) to that before sonication (μ_z^o)
245 *versus* sonication time. Up to *ca.* 20 min sonication there was a statistically significant ($p < 0.05$) decrease in
246 particle size to a ratio value of 0.5 ±0.01. On prolonged sonication or higher power sonication (data not shown)

there appeared to be a tendency for a slight increase in particle size, e.g., to ratio = 0.53 ±0.03 after 30 min, although this was not statistically different from the 20 min value. Any slight increase in particle size might be due to microscopic local heating caused by the cavitation processes during sonication (Kardos, Luche, Esigec, & Bourget, 2001) resulting in surface melting of gel particles and enhancement of their fusion and aggregation, even though the bulk temperature of the sample was maintained below 35 ºC. All in all, it appears that sonication should be used with caution when trying to dis-aggregate such particles.

3.2. Effect of the addition of lysozyme and lactoferrin on microgel particle size

Another way of viewing the calcium alginate particle formation in the Jet Homogenizer is the emulsification of the stream of alginate solution into the stream of aqueous $CaCl_2$, whereupon local association of the Ca^{2+} and alginate occurs in microscopic regions which go on to form the subsequent microgel particles. Undoubtedly, both break-up and fusion of particles occur in the highly turbulent conditions of the homogenizer, just as with the emulsification of two immiscible phases, so that the final particle size is a result of the balance between formation and fusion. It is therefore worth speculating what type of 'emulsifier' might help to stabilize the nascent microgel particles, meaning an ingredient that might preferentially adsorb to the surface of the particles as they are being formed and limit their fusion (coalescence) on collision to form larger particles. Calcium alginate particles have a net negative ζ-potential of −30 mV in pH 8 sodium bicarbonate buffer (see below) and alginate alone is a very strongly negatively charged polymer, with a net ζ-potential −60 mV (Bokkhim, Bansal, Grøndahl, & Bhandari, 2015), as might be expected, so a cationic 'surfactant' should be appropriate to help limit particle aggregation and/or fusion, i.e., stabilize the particles. However, a cationic low molecular surfactant is clearly not suitable, since the surfaces of all microgel particles are porous (Dickinson, 2015), whereas a positively charged particle of appropriate size might be able to form an adsorbed layer on the surface of the alginate gel particles and limit their growth and/or fusion. For this reason two compact globular proteins – lysozyme and lactoferrin – with relative high positive charges at the pH of microgel particle formation were chosen to test how they might influence the particle size. Fuenzalida *et al.* (2016) have recently studied the interaction between lysozyme and alginate in detail and shown that the formation of strong electrostatic complexes depends very much on the exact chemical

structure of the alginate molecules, as well as the concentration of calcium ions. None of these factors were varied here, however.

Figure 5 shows μ_z (the Z average diameter as measured via DLS as described in the Methods section) without sonication, as a function of lysozyme concentration at pH 8 and pH 10, as well as the ζ-potential of the particles. (Filled symbols refer to ζ and open symbols to μ_z). These two different pH values were chosen to investigate any effect of the magnitude of the charge on the lysozyme, which is reported as having an isoelectric pH (pI) of 11.0 (Bayarri, Oulahal, Degraeve, & Gharsallaoui, 2014). Lysozyme should therefore have a significantly smaller positive charge at pH 10 than at pH 8. Although Figure 5 suggests trends for a slight increase in μ_z and a slight decrease in ζ (less negative) with increasing lysozyme concentration ([lysozyme]) at pH 8 or 10, taking the error bars into account, these decreases are not significant. However, ζ was significantly less negative at pH 10 than at pH 8 at all [lysozyme], including [lysozyme] = 0, when the charge on the carboxyl groups of the alginate is not expected to vary between these two pH values and a more positively charged lysozyme at pH 8 might be expected to give more charge neutralization of the alginate, i.e., a less negative ζ. On the other hand, the interaction between the two oppositely charged molecules will depend intimately on their absolute charges. In the absence of other information, we calculated the expected total charge on lysozyme at pH 8 and pH 10, based on its amino acid composition (Manwell, 1967), the mean pK_a values of the ionizable amino acid side chains as shown in Table 1 (taken from Damodaran, 1996) and the Henderson-Hasselbach equation, e.g., as expressed in the form:

$$\alpha = \left[10^{(pK_a - pH)} + 1\right]^{-1} \qquad (2)$$

: where α is the degree of dissociation of the ionizable group. This calculation confirms a significantly lower net positive charge at pH 10 (+2) than at pH 8 (+7), although the predicted pI = pH 10.4, based on these pK_a values. We note that many types of solid and liquid colloidal particles become susceptible to flocculation when the ζ-potential falls much below 30 mV in magnitude, although these microgel particles are atypical colloids. It was noticed that addition of lysozyme at and above 0.18 wt.% caused increased turbidity of the alginate solutions

before homogenization, which settled out on standing for more than 2 h. This cloudiness disappeared on filtering the solutions for *DLS* measurements but suggested that lysozyme also tended to form complexes with the alginate in solution, probably via electrostatic attraction between the oppositely charged molecules. Presumably this interaction between alginate and lysozyme before homogenization prevents the lysozyme from exerting any significant effect on the calcium alginate microgel particle size during homogenization, possibly because there is little free lysozyme available. A separate set of microgel particles was prepared where the lysozyme was added *after* microgel particle formation. The results are shown in Figure 6 but are seen to be almost the same as those in Figure 5. Thus lysozyme appeared to have little ability to decrease the tendency for microgel particle aggregation, whether it was added during or after particle formation. Lysozyme binding to pre-formed Ca-alginate particles and lysozyme binding to free alginate chains before and during the formation of egg-box junction zones will clearly not be the same, since in the former many potential lysozyme-binding sites would already be occupied by Ca^{2+} ions. This might explain why the difference in ζ between pH 8 and 10 is not so easy to explain simply in terms of lysozyme binding to the surface of alginate microgel particles.

Lactoferrin, an iron binding protein, is an interesting candidate for delivery as a functional ingredient as an antimicrobial or antiviral, immunomodulator, antioxidant or anticancer agent (Steijns & van Hooijdonk, 2007; Wakabayashi, Yamauchi, & Takase, 2006). Figure 7 shows the results of similar experiments at pH 8 to those described above but replacing lysozyme with lactoferrin up to a concentration of 0.8 wt.%. There was a significant ($p < 0.05$) decrease in μ_z (from 200 to 100 ± 8 nm) observed with increasing concentration of lactoferrin ([lactoferrin]) up to 8 wt.%. No turbidity appeared during mixing the alginate and lactoferrin solutions before homogenization up this [lactoferrin]. The ζ-potential also became significantly ($p < 0.05$) less negative, changing from ca. – 32 mV to – 22 ±2 mV as [lactoferrin] was increased from 0 to 0.8 wt.%. Both the size and ζ-potential results suggested that some lactoferrin probably adsorbed to the surface of the microgel particles and this possibly helped to limit their size during particle formation. Based on the amino acid composition of bovine lactoferrin (Pierce et al., 1991), the pI of lactoferrin was calculated (as for lysozyme, using the pKa values in Table 1) as pH = 8.6 or 8.8, depending on whether or not one considers two Fe^{3+} ions attached, respectively.

The pI of lactoferrin is lower than that of lysozyme and so experiments were also conducted at pH 6, where the ζ-potential of lactoferrin is expected to be significantly more positive. (The calculated overall net charge on lactoferrin at pH 6 was +28 or +34, depending on whether the two Fe^{3+} ions are included). Compared to lysozyme (ca. 4 nm diameter, Damodaran, 1996), lactoferrin is a larger (ca. 10 nm diameter, Nevinskii, Soboleva, Tuzikov, Buneva, & Nevinsky, 2009) and more strongly (positively) charged globular protein and both the higher molecular size and higher positive charge might explain why lactoferrin is apparently more easily trapped at the surface of the nascent microgel particles. However, the results in Figure 7 also show that there was no significant difference in the size or ζ-potential of the microgel particles at pH 6 versus pH 8, within the experimental error. This indicates that that an increase in charge of +6e on the lactoferrin is not sufficient to affect significantly the interaction between lactoferrin and alginate during or after microgel particle formation. The particle size distributions measured for lactoferrin itself at pH 6 and 8 are shown in Figure 8 and indicate a very slight shift to lower particle sizes as the pH decreased from 8 to 6, the peaks in the distributions being 10 ± 2 nm, indicating no significant change in the state of aggregation of lactoferrin in this pH range.

It is equally possible that at least some of the oppositely charged proteins become incorporated inside the gel particles as they form. It is therefore worth calculating the minimum concentration of globular protein that would be needed to coat gel particles of a certain size assuming no incorporation inside them occurs. This can be done from simple geometry if one assumes that both the gel particles and the protein molecules are hard spheres of known size. The assumption of sphericity is probably reasonable for the gel particles – the larger particles appear smooth and rounded – and is also reasonable for lactoferrin (Nevinskii, Soboleva, Tuzikov, Buneva, & Nevinsky, 2009). An additional assumption that has to be made is the maximum packing fraction of lactoferrin spheres on the surface of the gel particles. This is again unknown, but as a first assumption this was taken to be just 10% of the surface area of the gel particles, taking into account the fact that complete charge reversal was not observed, i.e., the surface must not have been 'completely' covered. Assuming a starting alginate concentration of 1 wt.% and that all this alginate is converted into microgel particles of diameter *d* of density = 1 g cm^{-3}, Figure 9 shows the theoretical mass ratio of lactoferrin to alginate (m/M) required to cover 10% of the

surface of the particles as a function of *d*, also assuming a film of hydrated globular protein of density = 1 g cm^{-3}. The trend in the graph clearly illustrates that much higher wt.% concentrations of lactoferrin than alginate would actually be required to limit the particle size below 100 nm, even if only 10% coverage was necessary to achieve stabilization. It is interesting that at the maximum concentration of lactoferrin used (0.8 wt.%, i.e., m/M = 0.8) 10% surface coverage corresponds to a microgel particle size of ca. 150 nm, which, considering the simplicity of the model, is not so very far from the minimum of *ca.* 100 nm observed experimentally (see Figure 9). However, the assumptions of: (i) a hard gel particle surface and (ii) that all the alginate forms particles (i.e., no alginate is left in solution) are questionable. How far assumption (i) holds will really depend on the pore size at the surface of the gel particles compared to the size of the lactoferrin molecules and at present we have no information on this for the particles formed under these unusual conditions, although it could be tested by measuring ingress of nanoparticles of different sizes, for example. With respect to assumption (ii), the supernatant, after separating off all the microgel particles via centrifugation, had a viscosity of 0.01 to 0.03 Pa s over the shear rate range 0.1 to 10 s^{-1} and showed negligible shear thinning (± 0.03 Pa s) over this shear rate range. Solutions of 0.01 to 0.05 % alginate + added [Ca^{2+}] up to 10 mM (but without homogenization) had viscosities that were no higher than 0.03 Pa s in the same shear rate range. The [Ca^{2+}] of the supernatant was not determined, but since the final [Ca^{2+}] in the whole system = 10 mM and the viscosity of the supernatant indicated [alginate] < 0.05 wt.%, clearly very little of the original 1 wt.% alginate added remained free in solution. Obviously, the coverage would be inversely proportional to the specific surface area so that if the actual particle size was 100 nm rather than 150 nm, then the coverage would be $(1/1.5)^2$ smaller or only 4.4%, which would certainly explain why charge reversal was not obtained under these conditions. Nevertheless, the results show that some control over the microgel particle size can be exerted by including a small globular protein of opposite net charge. Furthermore, there seems no reason why the same general method of particle formation could not be extended with the Jet Homogenizer method to other biopolymers, e.g., pectin and carageenans that also gel on cross-linking with Ca^{2+} or other bivalent cations.

4. Conclusions

370 It has been shown that a very simple technique of rapid and highly turbulent mixing of sodium alginate
371 and calcium ions in the Jet Homogenizer developed in this School can produce very fine calcium alginate
372 microgel particles. Sonication of these particles produces a further decrease in size, suggesting that the particles
373 exiting the homogenizer are slightly aggregated. The charge on the microgel particles is negative and if the
374 positively charged globular protein lactoferrin is included during particle formation this can produce a further
375 reduction in the final microgel particle size, down to a diameter of ca. 100 nm. This is accompanied by a slight
376 decrease in the negative charge of the particles, but not charge reversal, so that the gel particle surface is
377 unlikely to be completely covered in lactoferrin. Some of the lactoferrin may be incorporated inside the microgel
378 particles. Indeed, this suggests a way of encapsulating lactoferrin within such particles. Encapsulation of
379 lactoferrin (or other materials) within calcium cross-linked biopolymer microgel particles produced in this way are
380 the subject of ongoing investigations in our laboratory.

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383 and Dr. Anida Yusoff regarding the confocal microscopy of some of the samples.

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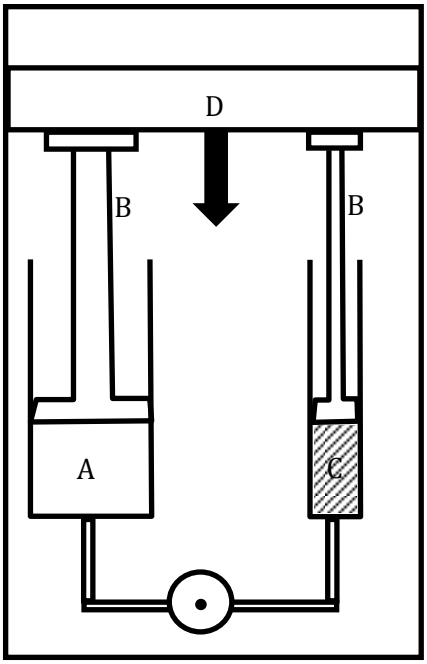
497

Table 1.

Values of pK_a of amino acid residue side chains used to calculate charge of lysozyme and lactoferrin, taken from Damodaran[29].

Amino acid residue	**pK_a**
Asp	4.6
Cys	8.8
Glu	4.6
His	7.0
Lys	10.2
Tyr	9.6
Arg	12.0

Figure 1



510 **Figure 2**

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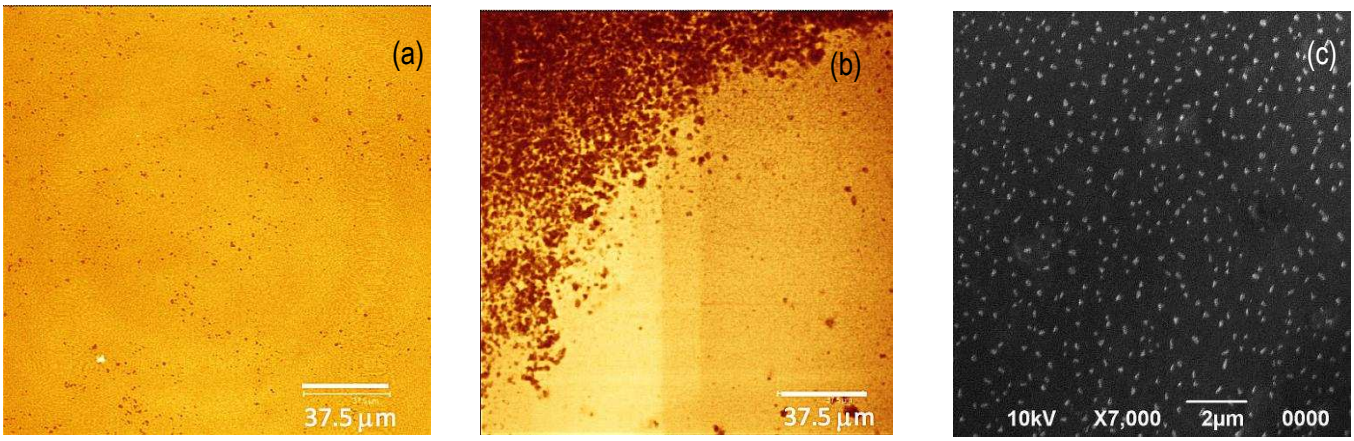

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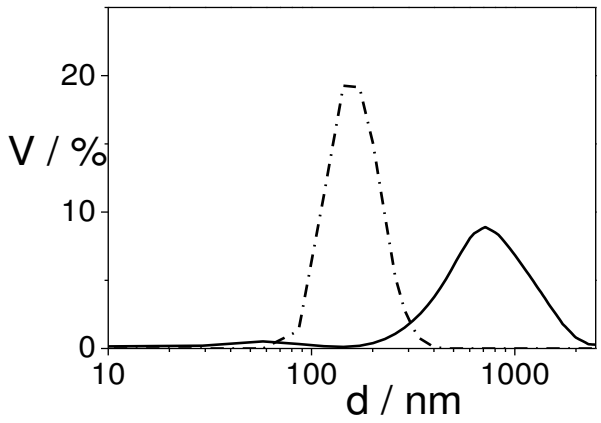

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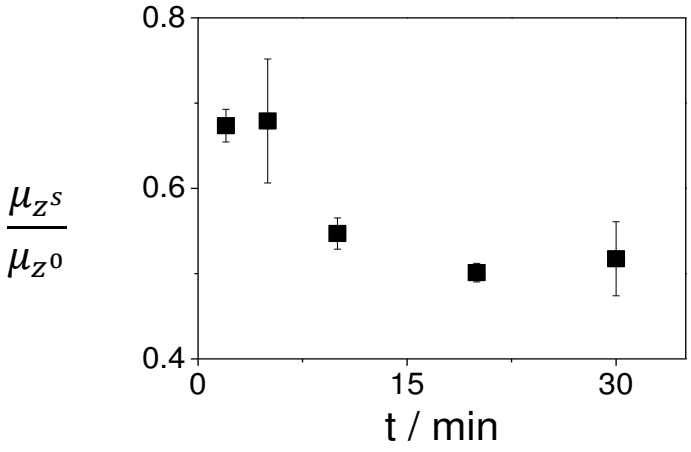
Figure 4



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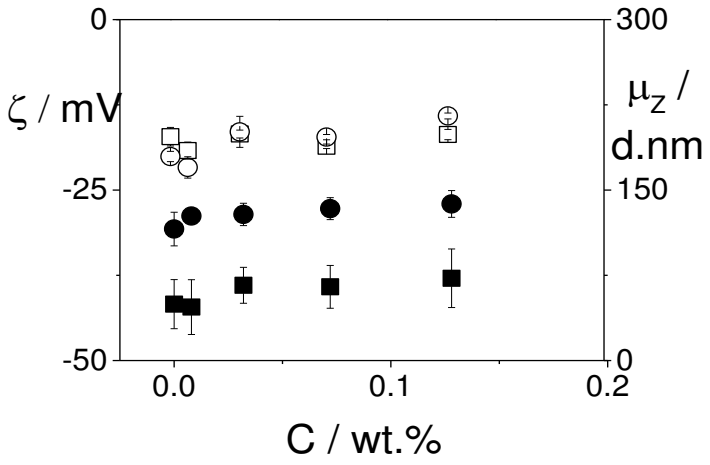

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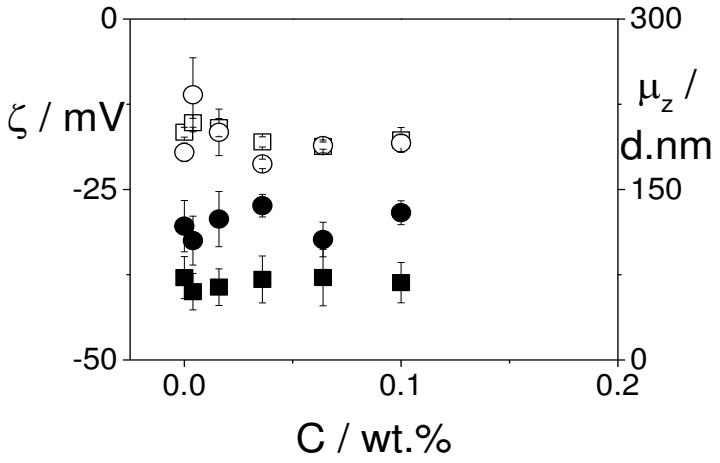
Figure 6



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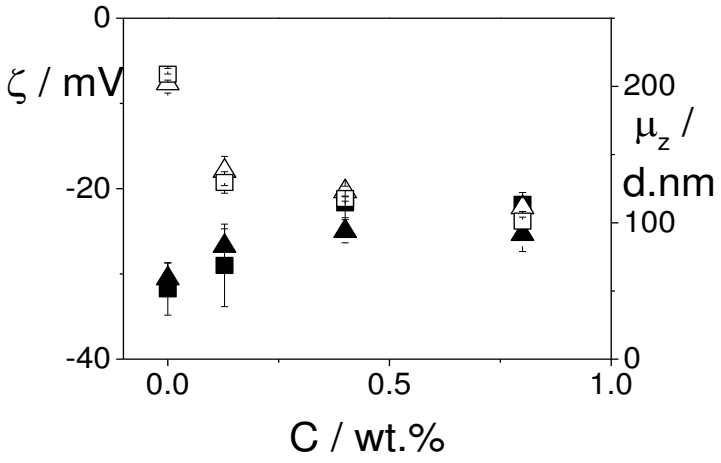

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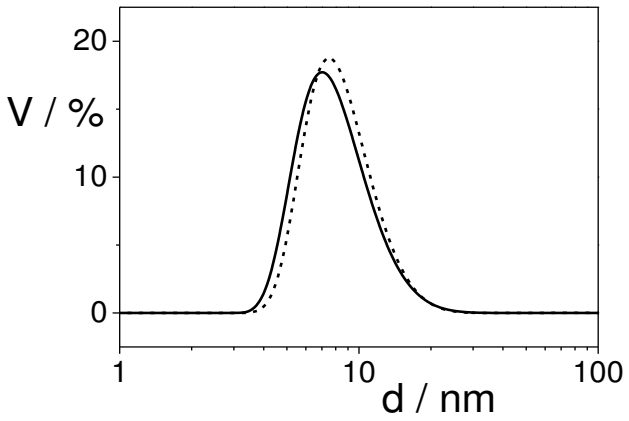
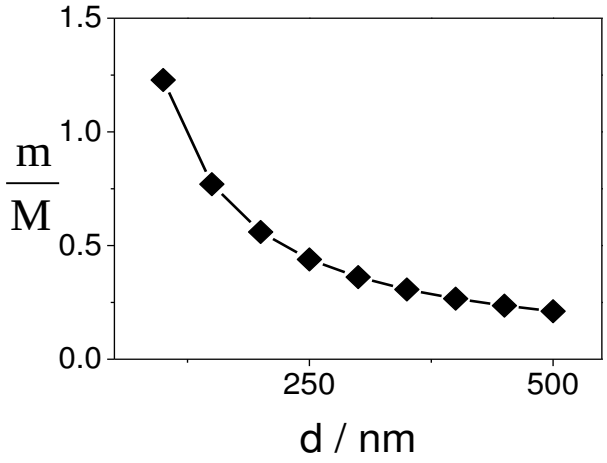


567 **Figure 9**



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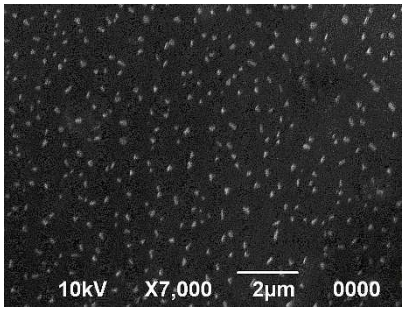
***Graphical Abstract**

TOC Graphic



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***Detailed Response to Reviewers**

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To: Brent Murray
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Title: Preparation of alginate microgels in a simple one step process via the Leeds Jet Homogenizer Food Hydrocolloids

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Thank you for your assistance and co-operation in the above matter. I look forward to receiving the revised manuscript.

Kind regards,

Peter A. Williams, DSc PhD CChem FRSC

Editor-in-Chief
Food Hydrocolloids

Note: While submitting the revised manuscript, please double check the author names provided in the submission so that authorship related changes are made in the revision stage. If your manuscript is accepted, any authorship change will involve approval from co-authors and respective editor handling the submission and this may cause a significant delay in publishing your manuscript.

Reviewers' comments:

Dear Editor, Thank you for the extra time allowed to prepare a revised manuscript, in light of he large volume of comments made by Reviewer #2 and also due to the fact that the main co-author, Linda Pravinata, had to return home to Indonesia due to serious illness of close family.

Dealing with Reviewer#2 first (since he/she requires more changes) we again draw attention to the fact of an incredibly large number of suggestions made, many of which are very minor, questions of preference of style, exact choice of words, etc., plus many of these comments seem sequential and to have been made in isolation from what is stated elsewhere or later on in the paper. This is very unusual, but life is too short and in the interests of keeping the length our response to a sensible limit, we have simply adopted all of the suggestions below in black text without question. However, some of the comments do require more lengthy response and discussion and so hopefully to separate these out more clearly we have highlighted these in red, followed by "RESPONSE:" where we elaborate on the corresponding changes made to the manuscript and why.

For consistency, we have adopted the same method of indicating our corrections to Reviewer #1, although these are much more minor. We hope this is clear.

Reviewer #2: General comment:
The manuscript deals with an innovative method to produce alginate gelled beads in the presence of calcium, of reduced size as compared to those obtained usually by prilling (alginate solution droplets that drop in a concentrated calcium bath). However, the set of data is not very convincing and more investigation/complementary experiments have to be performed, regarding especially the occurrence of protein - alginate interaction and electrostatic complex formation (in the presence/absence of calcium).
RESPONSE: Although this does not require a specific response, since we deal at length below with the issue of whether the data is convincing or not, we do take issue with the suggestions that more experiments are necessary (which we note is not really followed up on in the remainder of Reviewer #2's comments). This is because this opening sentence implies that protein –alginate interaction is the crux of this paper, when it is not. The main theme, which we think we have made clear, is to simply highlight the possibility of producing unusually small Ca alginate particles via this new method. The use of protein to try and modify and control their size is a minor issue and we think the title and Abstract of the manuscript make this clear.

Too much detail are lacking regarding beads formation (pressure applied…) and about the analytical methods used. A lot of references should be added so as to support the hypothesis displayed in the draft. Generally speaking, more rigor is required!
Also, even if I'm not native english spoken, I found a lot of grammar mistakes that hindered comprehension.
I hope that you are not discouraged, but you have to carry out wide improvements for further acceptance!
Thank you by advance to consider the comments below:

Specific comments:
Justify all the text of the manuscript!

Line 9 : Add "of size lower than". Replace "have been" by "were".
Lines 9-11 : rephrase : "… produced using a Jet Homogeneizer previously developed in… (Institute name, city, country), consisting of highly turbulent mixing of two liquid streams of sodium alginate and calcium chloride solutions."
Line 12: rephrase from "[…] relative…": the alginate-to-calcium cations ratio (specify units, i.e. wt/mM).

Line 12: add after "alginate" concentration. Change "plus" by "in the presence of 1-10 and 20 mM [Ca2+], d was lower than 5 µm and higher than 20 µm, respectively".
Line 14: talk about the effects of homogeneization procedure in another sentence for clarity. 150 bar was the pressure applied during beads formation ? What was the temperature?
Line 14: sodium alginate-to-CaCl2 volume ratio of the (sprayed) solutions.
Lines 15-16: rephrase.

Line 16: you measured net surface charge? Please specify.
RESPONSE: We do not say we measured net surface charge. We measured zeta potential and since the zeta potential was clearly negative it may be inferred that the particles we negatively charged without further justification.

What was the pH?
Line 17: "such as lactoferrin". Again, what was the pH? Specify. Replace "produced" by "resulted in" or "led to".
Lines 18-19: rephrase: "suggestive of protein adsorption to some extent, akin…"
Replace "on the other hand" by "In addition," or something similar.
Line 21: remove "a number of".
Line 26: remove "principally". Beta-D-mannuronic (M) and alpha-L-guluronic (G) are the only monomers. Add "[…] acids linked together by a 1à4 glycosidic bond". Specify that these monomers are "arranged according to different sequences". The monomer M/G molar ratio and also their sequence [dimmers MM, GG and GM (MG)] along the alginate chains affected their gelation properties.

Likewise, I strongly advise you to talk about alginate gelation via calcium cations, depending on the [Ca2+]/[G] concentration ratio: dimmers formation via the "egg-box model" and thereafter multimers formation by dimmers aggregation via the homopolymeric G junction zones.
For more information, you can refer to :
- Fang, Y. Al-Assaf, S., Philips, G. O., Nishinari, K., Funami, T., Williams, P. A. & Li, L. (2007). Multiple Steps and Critical Behavior of the Binding of Calcium to Alginate. Journal of Physics and Chemical B, 111, 2456-2562.
- Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. D., & Thom, D (1973). Biological interactions between polysaccharides and divalent cations: The egg-box model. FEBS letters, 32, 195-198.
- JØrgensen, E., Sletmoen, M., Draget, K. I. & Stokke, B. T. (2007). Influence of oligoguluronates on alginate gelation, kinetics and polymer organization. Biomacromolecules, 8, 2388-2397.

RESPONSE: This IS the journal called Food Hydrocolloids and to launch into a detailed review of the egg-box junction model, etc., would seem to be unnecessary for even the casual reader of this specialist journal. In the following paragraph we therefore made the leap to discussing the different practical methods of calcium gelation, assuming the reader is familiar with the general features of Ca alginate gels. However, to help allay any possible concerns, we have changed the first sentence at old line 31 to "Calcium alginate beads are quite simple to prepare and the mechanism of gelation through calcium ions forming egg-box junction zones is a widely known and exhaustively studied phenomenon (Fang et al., 2007)", i.e., using on the references suggested, although we note that the journal title given by Reviewer #2 was not correct.

Line 27: some values (numbers) may be more appropriate. I found in the ref. "Stokke, Draget, Smidsrød, Yuguchi, Urakawa & Kajiwara, (2000)" between 50 and 420 kDa (on average 150 kDa). Please add a ref.
Line 28: add before "products" "food and pharmaceutical…"
Line 29: add "the" after "for"..
Line 30: say: "Other applications (specify the fields) were reviewed by…"
Line 31: replace "extremely" by "quite". In the lab, you need at least a peristaltic pump!
Line 32: "gelation" may be more appropriate than solidification. Food gels are semi-hard (or soft, non-flowing) biomaterials.
Line 33: A space is lacking "solution(Brun"

Line 36: also pH.
RESPONSE: We have added BeMiller, J. E., & Whistler, R. L. (1996) Carbohydrates. In O. R. Fennema (Ed.), *Food Chemistry* (3rd ed., p.215). New York: Marcel Dekker, Inc.

Line 37: replace "agents" by "material" or "matrix". Remove "where they have". Say "with the added advantage".
Line 39: replace "microns" by "µm".

Line 41: add a ref.! You can specify that with the concentrations of alginate and CaCl2 (generally 0.1 - 0.5 M) used for the prilling method, alginate chains bind quickly Ca2+ and associated subsequently into dimmers and then multimers of increasing size; this led rapidly to a growing number of chains entanglements within a dense alginate gelled network (JØrgensen et al., 2007).
RESPONSE: This is a repeat of the comment above and we have already responded to it.

Lines 38-41: rephrase. Finish the sentence after "ingredients". Make a new sentence regarding the detrimental organoleptic properties.
Line 45: add a ref please.

Line 46: smaller than… what? release… of what? Active compounds? Please rephrase. Regardless particle size, encapsulated compounds could be released by diffusion or gelled network disruption. Size may affect kinetics of release.
RESPONSE: We mean smaller rather than larger, i.e., in general, the point being that the specific surface area is larger, so yes, the kinetics of release is affected. This is exactly what we have said, so we do not see the problem with this sentence.

Line 50: a ref. please!
RESPONSE: We have added the reference:-
Bajka, B. H., Rigby, N. M., Cross, K. L., Macierzanka, A., & Mackie, A. R. (2015). The influence of small intestinal mucus structure on particle transport ex vivo. Colloids Surf. B, 135, 73-80.

Line 51: difficulties in spraying a (concentrated) alginate solution would arise from the viscous/thickening properties. Please rephrase. Replace "contacts" by "meets" or "encounters".
Line 53: remove "to try". Replace "increasingly smaller" by "[…] produce particles of decreasing size".

Line 54: unclear. Please rephrase. Do you mean that an alginate solution is dispersed in a continuous oil phase, then calcium is added to the emulsion, resulting in the gelation of the dispersed (alginate) droplets ?
RESPONSE: We think that this is fairly obvious, but have re-worded this to the following to avoid any possible confusion:- "One obvious route is to prepare two separate water-in-oil (W/O) emulsions or microemulsions in which alginate is already dissolved in the aqueous phase of one emulsion and calcium in the aqueous phase of the other emulsion and then mix the two (micro)emulsions (Machado et al., 2012)."

Add a space between "phases(Machado…"

Line 56: "methods to initiate the slow…"
Line 60 : mild pH… a value please! Replace "micron" by "µm"
Line 60-62: repeated information, delete.
Line 62: avoid "we". More preferably, say "In the present paper, a method to produce micron or sub-micron alginate particles is displayed, simpler to carry out than that using (micro-)emulsions".

Line 65: you should replace "via the Pickering mechanism" by "for the stabilization of Pickering emulsions". Please describe a "Pickering emulsion".
Line 67: particles… rigid. What do you mean? Not solid-like (soft)? Size… of what? Droplets of the dispersed water phase?
RESPONSE: This is a good suggestion to clarify and we have changed this to read
"Microgel particles are also just one type of novel food particle that might be exploited to stabilize Pickering emulsions (de Folter & van Ruijven, Marjolein W. M.; and Velikov, 2012; Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014) – emulsions stabilized by an adsorbed layer of particles as opposed to molecules. Traditionally, the particles in Pickering emulsions are particles of solid material that do not deform on adsorption, but, as long as they maintain a size and contact angle sufficient to secure their interfacial attachment, deformable particles may also stabilize via the Pickering mechanism, so that the term 'Mickering' emulsions has been coined by Schmidt et al. (2011) to describe protein microgel-particle-stabilized emulsions."

Line 69: repharse. "In addition, some improvements regarding the production… were reported by Saglam…"

Line 72: pH values?
RESPONSE: We have changed this to:-
"Many of these methods rely on heating globular proteins in relatively dilute solution and/or at pH values far enough from their isoelectric pH, so that they are highly charged, particularly whey protein……"

Line 74: you should add a short section which describes the further investigations in your work. You should be more specific about the method you chose to consider (or to improve) to yield small alginate gelled particles.
RESPONSE: We do not understand this comment. For example "further investigations" at this point does not seem appropriate when we have not even discussed the current investigations. Perhaps the Reviewer means that we should add a short section summarizing what the rest of the paper is about. This seems to be an increasing trend but merely leads to unnecessary repetition in our opinion – because that is what the Abstract is for. Besides, in the previous paragraph, we have already said in the Introduction that "In the present paper, a method to produce micron or sub-micron alginate particles is displayed, simpler to carry out than that using (micro-)emulsion. The calcium alginate microgel particles were prepared using a high pressure jet homogenizer (University of Leeds, Leeds, UK).", which is the principal purpose of this paper – which we then go on to describe."
However, we have changed the wording in this sentence to:-
"In the present paper our aim is to describe a relatively simple new method that can be used to produce micron or sub-micron alginate particles, or other microgel particles that rely on rapid or confined exposure of the polysaccharide to a cross-linking agent such as Ca^{2+}. The calcium alginate microgel particles were prepared using a high pressure jet homogenizer (University of Leeds, Leeds, UK)……"

Line 77: you have to further characterize your alginate sample; purity, salts, water content. Chemical composition is lacking. Could you provide any information about chain size/polydispersity, or maybe viscosity at given concentration? It could be useful to compare different alginate batches in the future...

Line 79: purity of commercial protein samples and reagents?
Line 80: "used as received". Unclear. You mean these were on analytical grade/no further purification was performed?
RESPONSE: we have already added the purity of the other samples and reagents where these are known, but added the lysozyme purity (≥ 90 %). "Used as received" is the standard phrase used in many papers to indicate that no other special purification was performed, but we have changed this to "All chemicals were used without further purification."

Line 81: "Glanbia Nutritional" (specify city, country).

Line 83: pH value after adjustment?
RESPONSE: We specify what pH we adjusted to later on when we talk about methods and analysis – we are merely stating here what acid and alkali we used to make any pH adjustment.

Line 89: Are alginate concentrations corresponded to the weight of alginate powder dissolved? Or is it "true" alginate concentrations, taking into account the sodium alginate content in the powder?
We have added that "Extensive drying of the alginate powder 110 oC revealed it had a residual moisture content of 11 ± 0.5 wt.%, but solutions were made up on basis of the weight of the powder as received."

Line 90: replace "heating to 50°C" by while heating at 50°C, under magnetic stirring for at least 2 h. How did you ensure the complete solubilization of the alginate powder? Did you perform a centrifugation step to remove remaining insoluble particles?
Line 94: you should mention the use of a peculiar "high pressure jet homogenizer" in the introduction. "our school"… avoid and specify the institute name please!

Line 94-95: these specifications also may be moved in the intro. "fine… emulsions as previously described." please specify the kind of emulsion. Is it a kind of microfluidization apparatus, i. e. an homogenizer using high dynamic pressure?

Line 96: "… as previously used by Casanova & Higuita (2011), to prepare CaCO3 microparticules.
RESPONSE: This is a sensible suggestion and we agree that there is nothing lost in moving these lines to the Introduction, which we have done, but the description that follows in the Methods makes the principal of its operation clear.

Line 101: rephrase: "Compressed air from ram-air driven system pushes on pistons…"
Line 105: start another sentence "[…] < 1 s). When oil… are used… chambers, fine (O/W or W/O) emulsions can be obtained without…".

Lines 108-110: rewrite. "Under highly turbulent conditions, cavitation, shear and impact effects were generated by this technique. The components in each sprayed solution (Na-alginate and CaCl2) interacted to each other. This resulted in gel particles, which size…"
You may specify the solution volume ratios (Na-Alg)-to-CaCl2 applied and/or in terms of wtalginate/mMCaCl2 ratio, depending on the concentration ranges of each compound. What were the liquid flows and temperature/pressure applied? A lot of details are lacking.
RESPONSE: All these details are in the lines just a few lines below.

Line 110: how did you prepare the protein solutions? Concentration? How these were added to the Na-alginate solution?
Line 111-114: repeated information (line 96). Necessary?

Line 115: how do you collect the gel beads? What is the "preparation"? The beads? What is the buffer? Sodium bicarbonate?
RESPONSE: "The beads?" We do not call them beads. The whole method is about preparing what we call microgel particles, the title of this section "Preparation of microgel particles" So we think this is obvious, but we have changed these lines to
"Under the highly turbulent conditions, cavitation, shear and impact effects are generated which result in the alginate and calcium ions interacting to form much smaller (< 1 μm with 1 wt.% alginate and 10mM $CaCl_2$ - see below) gel particles than via prilling methods. The suspension of particles in any excess calcium alginate solution exits the device at relatively low velocity and may be simply collected in a beaker. On immediately exiting the homogenizer the sample was immediately diluted in water or buffer (depending upon the sample) to help limit any possible particle aggregation prior to particle size measurements."

Line 117: larger than… what? Is the "Sauter mean diameter d32" a software or peculiar measurement? Please indicate the meaning/data processing.
RESPONSE: The Sauter mean diameter and its abbreviation is an extremely common term used in colloid science and particle sizing. Nevertheless, we have added a brief description as a footnote.

Line 119: rephrase "of size equal to or lower than 10 μm, "
Line 120: rephrase "…apparent particle diameter displayed as volume-weight size distribution (as a percentage of total volume) were…".
Line 124: this is true under the hypothesis that spherical particles were analyzed. You should specify this!
Line 127: Were you in the dilute regime? What was the approximate particle "concentration" in your suspension? Was it limpid? Specify the manufacturer (Millipore, city, state).
Line 128: "scattered light" rather than "light scattering".
Line 130: rewrite: "It was checked that the viscosity of these… (3 x10-4 Pa s; Data not shown)
Line 132: specify "mean apparent diameter and SD"
Line 135: rewrite "…particle sizes obtained were…"
Line 136: rewrite "the technique… was employed to collect micrographs of particle samples by CLSM".
Line 138: value in g? not in rpm, otherwise you have to express the rotor model.
Line 142: remove "then". Write "…added to samples to reach a xanthan concentration of 0.1 ± 0.01 wt.%. The mixture was placed in a welled slide (30 mm diameter x 0.3 mm depth)".
Line 145: "… was performed with". What the fluorescence emission wavelength?
Line 146: remove "the" before "dextran".

Line 147 : "… the molecular weight of dextran…". How could you support your assumption? Was this method adapted from previously reported protocol? If possible, add a ref please.
RESPONSE: No this was not adapted from a previous method. (Our assumption is supported by the results, where a bright background appears to highlight less bright objects of a size that agrees with the other measurements).

Line 148: do you mean that xanthan was used to "immobilize" the alginate beads onto the microscope slide and coverslip? Did you suppose that your sample without xanthan may evolve during microstructure analysis?
RESPONSE: No, we are not asserting this and we do not use the word immobilize – we say "produce a weak gel structure that would limit Brownian motion of the particles", which we think is self-explanatory. The technique is widespread. The term gel refers to a 3-dimensional structure and Brownian motion is a random walk in 3-dimensional space and we are merely slowing down the translational motion of the alginate microparticles. There is no reason to suppose that the system will 'evolve' once the gel particles have been formed in the absence of xanthan, but the particles will move and this blurs the images.

Line 152: "these", not "they". "quickly", not "quick".
Line 153: remove "Triplicate samples were freeze-dried". In line 150, write "… were freeze-dried in triplicates". Delete "The end product was a sheet of fluffy white solid of dried Ca-alginate suspension." Unecessary.

Line 155: how did you measure the Na-Alginate concentration? Was it consistent across the Ca-alg bead samples? It would be more simple to display the composition (on a dry weight) or your freeze-dried samples. How could you ensure that alteration of alginate bead structure was minimized upon freeze-drying? Usually, samples containing gelled biopolymers were fixed with glutaraldehyde (covalent cross-linking agent") , then dehydrated at room temperature in a graded ethanol series up to 100%. Otherwise, if you determined the surface morphology of your beads, you can find another procedure in: Mandal, S., Kumar, S. S., Krishnamoorthy, B., Basu, S. K. (2010). Brazilian Journal of Pharmaceutical Sciences, vol. 46, 785-793. Was your own method previously reported, to support its relevance/reliability?

RESPONSE: We do not say that we measured the alginate concentration. We talk about the solids recovery and all we are saying that of the original solids added, almost all this is was recovered on drying. We used the alginate as received, as described above.

The method was not previously reported, so there is no reference. We did not want to crosslink or alter the samples in any way, to avoid artefacts as much as possible. Closer examination of the particle surface was not possible due to beam damage and all we can say is that the different samples seemed to give consistent results, which we have done at the end of the discussion of the *Microscopy* section in the Methods.
Plus we have subsequently also done some image analysis (see below) which gives particles sizes consistent with the light scattering, which gives further confidence that our method is appropriate.

Line 165: "… particle aggregation…" during beads preparation?
Line 166: "probe" may be more appropriate than "tip".
Line 167: "suspension", not "solution". How did you control temperature? With a thermostatic bath?
Line 172: rewrite "The apparatus was controlled and data acquisition was performed with the rSpace software, as supplied with the rheometer."
Lines 173-174: "geometry", not "cartridge". "Each", not "every". Sample volume deposited?
Line 175: did you use a logarithmic ramp of shear rates?
Results & Discussion

Lines 179-180: was it the (Na-Alg)-to-CaCl2 solutions volume ratio? Unclear. Also you did not provided information about the Na-Alg and CaCl2 concentration ranges investigated.
RESPONSE: Of course it was the ratio of the volume of these two solutions, which is the basis of the whole method, but we have changed this to:- "the volume ratio of the two chambers containing the Na alginate CaCl2 solutions and was varied between 80:20 and 90:10." to make this absolutely explicitly clear.
Just a few lines below we describe the range of concentrations investigated – one does not expect the manuscript to be commented on line by line like this without the reader reading further on. One can only cram so much information into one sentence before it becomes too hard to follow.

Line 181: Is this data shown somewhere in the manuscript? Again, you have to specify the meaning of d32.
RESPONSE d32 covered above. No, we have not included all this preliminary data in the manuscript, mainly because above 20 mM Ca2+ not only were the particles formed much larger as we have already said, but their sizes were not very reproducible, but we have added this extra comment now.

Line 183: "give rise to…". Rephrase "increasing pressures led to particles of decreasing size".
Line 184: rephrase: "The Ca2+ concentration range (1-70 mM), was determined since below 1 mM, …"

Line 188: replace "of producing" by "to produce". "Larger…" than what?
RESPONSE: we meant > 20 micron, as in the immediately preceding sentence, but have added "(i.e., > 20 micron)

Line 189: rephrase "Ca2+ concentrations above 20 mM were…"

Lines 190-192: rephrase. Data not shown?
RESPONSE: Yes, same point as above (line 181).

Line 193: replace "except where stated" by "Wherever not otherwise indicated". Also "in order to produce…"
In the Figure 2 caption, you have to indicate the Na-Alg and CaCl2 concentrations used to produce the beads observed. For the CLSM micrographs, what was the emission wavelength?

Lines 197-198: why don't you comment the micrographs? Why didn't you use a higher magnification for the SEM data? You should compare the average bead size by direct measurements, if possible, on the micrographs.
RESPONSE: We have already added the comment that images could not be obtained at higher magnifications or at higher resolution due to beam damage in response to Lin 155 above. However, we have since completed some image analysis on the better image in Figure 2(c). This is now described in a short section here as follows.
"Due to difficulties of beam damage described above, it was difficult to obtain clear images of samples above a certain magnification, but to try and compare the light scattering results of particle size with SEM indications of size, detailed image analysis was performed on some images. As an example, Figure 2(c) was analyzed using *ImageJ* software. After automatic thresholding and edge detection, 520 objects were counted and the areas of these objects in pixels these were converted to diameters of circular objects of equivalent area, in microns. This gave a mean diameter of 140 μm with standard deviation of 50 μm, reasonably close to the μ_z values determined via DLS."

In the Figure 3 caption, you have to add " as measured by DLS at x°C and by using fixed scattering angle of x°. For the DLS data analysis, did you use a particle size distribution following a Gaussian model for single-size particle distribution. Did you consider the possibility of a multimodal distribution (several particle sizes)? Usually, the autocorrelation function is deconvoluted by a Gaussian analysis, yielding a translational diffusion coefficient D, related to a single apparent diameter for spherical particles of uniform density. As this model makes the assumption of a normal distribution of the mean D value, it would fail for polydisperse molecules in solution. Therefore a weighting effect towards the summed Di values should be taken into account to represent the relative amount of the i particles of diameter di in suspension, so that particle size was generally may be displayed as multimodal distributions.
RESPONSE: Since we define uz as the size specifically measured by DLS in the text, we do not think it is necessary to add this to the legend, just like we do not have to describe the method of obtaining the zeta potential, since likewise only one method was also used for this. We would agree if different methods were being used to measure the same thing. We show one particle size distribution with and without sonication. Clearly, the software reveals more than one population, i.e., it is multimodal and we show this. No we did not attempt to fit to more complex models after sonication since the data fits to the Gaussian were always acceptable according to the instrument software. Quite possibly there might be minor populations, but it would be impossible to display all the results as multimodal distributions anyway as the Reviewer suggest in the space allowed. Fundamentally, we are here concerned with the major population after sonication which microscopy confirms is in the uz size range given.

Line 201: if particles aggregated, you may obtain a polydisperse suspension… In line 203, you seemed to indicate that particles were polydisperse prior to sonication. It is not clearly stated… Line 205: why your beads may further aggregate? Because of the high level of alginate chains cross-linking with the Ca2+ concentration applied? You should further investigate this "unwanted" phenomenon.

RESPONSE: We do not quite understand Reviwer#2's comments here. Nowhere are we suggesting that the microgels particles obtained are monodisperse – clearly uz is an average of a distribution. All we are saying is that some of the primary particles (which are distributed across a range of sizes) are also aggregated, broadening the distribution further, which is proven by sonication, which shifts the distribution to a slight lower (and narrower) range of sizes. We do not see why this is not clear. We thought the reason for aggregation was obvious, but we agree it would be probably best to state this, so we have changed the sentence beginning at old line 205 to "This suggested that the larger particles may indeed have been aggregates of the smaller microgel particles, probably due to excess Ca2+ linking particles together as they formed"
We agree that this phenomenon could be investigated further, though it is not necessary for this study which aims to illustrate the basic method, whilst at the same time the addition of protein as a 'emulsifier' for the systems was indeed an attempt to try and control this phenomenon, as described in the immediately following paragraph.

In Figure 4 caption, you have to indicate that mean particle diameters were assessed by DLS.
RESPONSE: As above (Figure 3 caption)

Line 208: rephrase: "… a decrease in particle size to apparent ratio plateau value of …"
Lines 209-211: delete "Sonication… achieved".

Line 211: what was the mechanism of aggregated-beads disruption?
RESPONSE: sonication, i.e., agitation of the particles with ultrasound of similar wavelengths to the particles sizes. Surely this is clear. An absolutely standard method for trying dispersing aggregated material.

Lines 211-212: could you specify the sonication times/power ranges?
Lines 212-213: rephrase.
Line 219: what were these microregions? The homopolymeric G zones of alginate chains? Again, add a ref.

Line 221: but you are not working with immiscible phases in your case. The parallel is unclear.
Line 222: a ref. please!
RESPONSE: We have changed the wording to "It is therefore worth speculating what type of 'emulsifier' might help to stabilize the nascent microgel particles, meaning an ingredient that might preferentially adsorb to the surface of the particles as they are being formed and limit their fusion (coalescence) on collision to form larger particles." There is no reference to this: the idea is novel as far as we know.

Lines 223- 225. Are these values from your own measurements or reported data? Unclear.
RESPONSE: We necessarily have to jump ahead a little to further this argument, but we have changed this to "Calcium alginate particles have a net negative ζ-potential of –30 mV in pH 8 sodium bicarbonate buffer (see below)." But again it would have been helpful if Reviewer#2 had modified their initial reaction to this line on reading subsequent lines.

Line 226: replace "to help stabilize the particles" by "to limit particles aggregation".
"…molecular weight surfactant.
RESPONSE: In colloid science these are really two aspect of the same thing, but we agree to change it to "limit particle aggregation and/or fusion, i.e., stabilize the particles".

Line 227: what kind of microgel particles? From protein and/or gelling polysaccharide?
RESPONSE: Changes to "…all microgel particles"

Line 229: particles may not overaggregate at sufficiently high level of electrostatic repulsions. What was the pH of your suspension? This information is essential!
RESPONSE: We do not understand this term "overaggregate". It is not a term we have used, nor are we aware of any such phenomenon in the literature at high electrostatic repulsion.
We specify the pH at which we tested and performed these experiments in the immediate next sections, but note that we have also added at old line 115 that "The pH of the diluted calcium alginate suspensions when buffer was not used (i.e., without proteins added) was pH 7 ± 0.2."

Line 230: delete "with a strong". Re-specify the pH. Do you aim at obtaining protein-polysaccharide electrostatic complexes? Do you suppose a competing effect between calcium cations and positively-charged lyzozyme towards carboxylate groups of alginate chains?

Molecular interactions may require further investigation… I suggest you to refer to : Juan P. Fuenzalida, Pavan K. Nareddy, Ignacio Moreno-Villoslada,Bruno M. Moerschbacher, Musti J. Swamy, Shu Pan, Marc Ostermeier, Francisco M. Goycoolea (2016). On the role of alginate structure in complexing with lysozyme and application for enzyme delivery. Food Hydrocolloids, 53, 239-248.
Line 229: was this "coating method" reported elsewhere? Likewise, I'm not sure you were peculiarly explicit when you described in the M&M section the way to incorporate lyz or lactoferrine in the alg solution. Did turbidity change subsequently to protein addition? (OK, I found the answer in lines 250-251). I strongly advise you to read carefully the section 3.2 in the Fuenzalida et al. (2016) study. Protein - alginate calcium multiple interactions were characterized. They talked about "alginate chain flexibility", a data that you did not measure and this absence -apologize me - bothers me. Anyway you it is quite hard to consider protein-polyelectrolyte interaction while omitting the role of the G sequences of the alginate chains upon calcium-induced gelation.
RESPONSE: The pH has been specified throughout, with or without protein. Lysozyme is well known as having one of the highest isoelectric pH values of most globular proteins. No we do not aim at producing "protein-polysaccharide electrostatic complexes". We have described what our aim is in the previous section - to get some positive protein adsorbed around the outside of the negatively charged microgel particles. Nor do we aim to encapsulate the protein into the particles Reviewer #2 launches into a whole set of questions around these issues which we do address when discussing the results that follow. Cloudiness being just one. We can understand how this misunderstanding might arise if the paper ended here – but it does not. However, we are grateful to Reviewer #2 to pointing out references that explain why the lysozyme did not work so well as an 'emulsifier', such as Fuenzalida et. al. (2016), which we note probably appeared after we had submitted this manuscript. We have included they key Fuenzalida et.al. (2016) one at old line 231.

Line 233-235: two "which" in the same sentence. Make two sentences.

Line 232: recall the meaning of "µz". Please rephrase: Figure 5 displays µz values and also the zeta potential of the non sonicated alginate gelled particles in the presence of lyzozyme applied at concentrations in the range… specify that you worked with 10 mM CaCl2 rather than 20 mM. What happened in this latter case?
RESPONSE: We have defined uz again here:- "the Zaverage diameter as measured via DLS as described in the Methods section."

"specify that you worked with 10 mM CaCl2 rather than 20 mM. What happened in this latter case?
RESPONSE: We are not sure what Reviewer#2 means here. We do specify 10 mM in the legend and we are not sure where the idea of 20 mM has come from. We state early in the Methods section that all this detailed work was done with 10 mM.

Line 233: A point is lacking: "particles. These".
Line 237: Rephrase: "regardless the lyzozyme concentration applied."
Lines 238-239: rephrase: "The zeta-potential was not significantly affected by the lyzozyme concentration applied, regardless pH.

In Figure 5, you should not present on the same graph distinct data (size and zeta potential). I advise you to present on a same chart zeta-potential measurements at pH 8 and 10, and in another one particle size. Why did not you show the zeta-potential of Lys. alone as a function of concentration? I'm sorry but you did not specify the attribution of the open and close symbols. It is quite embarrassing... For example, you should compare the zeta potential of each compound (Alg and lyz) in isolation and this for Alg and Lyz in admixture at same concentration as in isolation; is there an additive and/or synergistic effect regarding net charge of the two mixed compounds ?
RESPONSE: The presentation of different two sets of "y" data against one 'x' range is a very common practice and serves to highlight the trends in two data sets simultaneously rather than the reader having to scan between two separate Figures, since, in this case, we do not expect any relationship between pH 8 and pH 10. If there is any relationship, it will be a tendency for reduced particle size as the magnitude of the zeta potential (and therefore inter-particle electrostatic repulsion) increases. So there IS a very good reason for plotting them together. Furthermore, on careful reading of the legend it is seen that we do specify the meaning of the different open and closed symbols. Nevertheless, we have added to the text that "filled symbols refer to zeta and open symbols to uz" to make this even more clear. We are interested in the zeta potential of the particles, not lysozyme, which is in a size range 2 orders of magnitude lower, so that a comparison between the protein alone and the protein coating the particles would not be valid anyway.

Lines 252-253: probably true but you should further investigate these (probably) electrostatic interactions. Simply, why didn't you perform observations by CLSM of the bead size/morphology in the presence of Lyz. protein?
Lines 253-255: did you measure the level of Lyz. retention by the suggested complexation with the negatively-charged alginate chains? I advise you to centrifuge the Alg+ Lyz solution prior to bead formation, so as to remove "turbidity" - as you mentioned - attributed to the complexes? A method is given by Fuenzalida et al. (2016).
Lines 257-259: probably Lyz. and calcium did not associate to alginate chains via the same binding sites… could you develop thoroughly this point?
RESPONSE: As Reviewer#2 has already pointed out, this interaction has already been studied by others and we have included their suggested reference at old line 231 (see Response to line 229 above). In addition, we must thank the Reviewer for pointing us in the direction of an explanation of why zeta at pH 8 is more negative than at pH 10, as below. However, the visible turbidity and settling out of the material clearly indicated very large aggregates, which was not what we hoped to achieve, so for this reason we did not pursue this further, since this paper is not primarily concerned with protein-polysaccharide interactions.

Consequently, we have added a comment about material visibly settling out at old line 251, which we should have a included previously, plus we have changed the discussion of these results here to:-

"Thus lysozyme appeared to have little ability to decrease the tendency for microgel particle aggregation, whether it was added during or after particle formation. Lysozyme binding to pre-formed Ca-alginate particles and lysozyme binding to free alginate chains before and during the formation of egg-box junction zones must clearly not be the same, since in the former many potential lysozyme-binding sites would already be occupied by Ca^{2+} ions. This might explain why the difference in ζ between pH 8 and 10 is not so easy to explain simply in terms of lysozyme binding to the surface of alginate microgel particles."

Line 260: Didn't you define "+ve" parameter previously?
Lines 263-267: understandable but rephrase or cut into several sentences to improve clarity.

Lines 267-268: please rewrite. "as the lacotferrin concentration was increased from 0 to 0.8 wt%." Maybe you should find some supplementary information/data for comparison in:
Huma Bokkhim, Nidhi Bansal, Lisbeth Grøndahl, Bhesh Bhandari (2016). In-vitro digestion of different forms of bovine lactoferrin encapsulated in alginate micro-gel particles. Food Hydrocolloids, 51, 231-242.
RESPONSE: Reviwer#2's words are more elegant and we have changed this as he/she suggests. However, we have read this paper and can find no obvious complementary information since the methods and conditions are so different.

Line 270: have you measured any possible retention of lactoferrin by the alginate chains?
RESPONSE: No, because again we were mainly interested in controlling particle size, not complex formation or encapsulation, but as we suggest at the end (line 322) this method of preparation may be a new methods of encapsulating leactoferrin that could be followed up.

Lines 275-276: you should specify beforehand in the text the protein molecular weights. If you modified the pH while applying either Lyz or lacto., you should not compare the effect of the protein type as you modified another parameter (pH)… larger… more strongly charged than… Lyz? rephrase "… both these factors" by "the combination of these parameters".
RESPONSE: The protein molecular weights are not important here, but their hydrodynamic radii. We had already discussed the size of lactoferrin in the original submission, but include a value for lysozyme for comparison at old line 275. We compared both proteins at the same pH, but at more than one pH. We have rephrased as suggested "…and both these factors…" to read "…and both the higher molecular size and higher positive charge…"

Line 278: "versus" is in italics. Change. "within the exp. error" would mean no significant difference thus there is no "little change".

Figure 7 caption: same remark as that for Figure 5.
RESPONSE. Same as our response to Figure 5 caption, above.

Figure 8 caption : add: "Particle size distribution as measured by DLS at x°C of lactoferrin…"
Line 280: replace "it seen that there was only " by "indicated". Replace "was changed" by "decreased". Change in size of lacto. molecule is in fact not significant.

Line 283: "inside" is in italics. Could you support your assumption by previous study?
RESPONSE: we have referred to the other work suggested by Reviewer#2 that at least shows that this is possible?

Line 284: in the sentence above you say "incorporated", now you employ the word "coat". It is confusing…
RESPONSE: We say "incorporated inside" to distinguish it form coating, which we think is clear. We are calculating what is needed to coat ignoring any incorporation inside. We think this is already clear but to avoid any possible confusion we have changed "…that would be needed to coat gel particles…" to "…that would be needed to coat gel particles assuming no incorporation inside them occurs".

Line 285: "perfectly" is maybe exaggerated. Change "spherical hard spheres". Redundant.
RESPONSE: we used "perfectly" to emphasize that this is a model, ideal case, since spherical is sometimes used as a qualitative adjective, e.g., "it was fairly spherical", even if this is not strictly correct in a Platonic sense. Nevertheless, we have removed it.

Line 289: unclear.
Lines 289-291: how did you measure 10% of surface coverage by lacto.? What are the units?
RESPONSE: We did not measure it, this is an assumption, as we clearly state. It is a fractional coverage – it has no units, like volume fraction.

Weight (g)/cm2 Line 293: add "of diameter d and of density of around 1 g cm-3".
As I asked you for Lyz. above, have you investigated by CLSM de gel particles size/morphology in the presence of Lacto.? It could be informative.
RESPONSE. We have tried but they look no different. We would not, of course, be able to distinguish a monolayer of lactoferrin molecules around the particles with such technique which has much too low a resolution.

Line 296: by the word "stabilization", you mean that the alginate particles would not aggregate?
RESPONSE. We addressed this point above, re. lines 222 and 226.

Line 297: you have to explain how you measured (or hypothesized) 10% surf. cov.
RESPONSE: We have explained this above, re. Lines 289-291

Line 298: 150 nm is quite different from 100 nm… Line 299: "Figure 9). However".
RESPONSE: Yes it is different, but is it 'quite' different, considering the simplicity of the model? We think not. Nevertheless, we have changed "…corresponds to a microgel particle size of ca. 150 nm, which is close to the minimum of *ca.* 100 nm observed experimentally…" to "…corresponds to a microgel particle size of ca. 150 nm, which, considering the simplicity of the model, is not so very far from the minimum of *ca.* 100 nm observed experimentally"

Lines 300-303: confusing. Rephrase. I think some authors elsewhere determined porosity of alginate beads produced in similar conditions ([Alg] and [CaCl2]). However, if I understand well, you mixed Alg and lactof. in the same solution prior to the addition of CaCl2, thus beforehand bead production. Thus it may be expected that a part of lactof was entrapped in the gel matrix, another part adsorbed on the bead surface, and a third part remaining unbound (free). Lactof. retention by the particles has to be assessed.
RESPONSE: We are not quite sure why this is confusing, since the Reviewer is correct - this is exactly what we have said and what we have done, but possibly re-phrasing "…and at present we have no information on this…" to "…"…and at present we have no information on this for the particles formed under these unusual conditions…"
It would indeed be useful to measure amount of bound and unbound at a later date, but what we have shown is that to obtain very much smaller particles one would have to go up to very high ratios of protein to alginate if surface coating by protein was the controlling mechanism. If some protein was free and some trapped inside the particles, then even more protein would be required. This is the key point we have made.

Line 304-306: is the viscosity measured different from that measured for the buffer alone? As a part of Alg was removed by centrifugation owing to gelation in the presence of calcium, the

remaining concentration of soluble alginate may be very low to influence viscosity… Have you measured soluble alginate concentration in supernatant?
In line 307, you estimated "0.1 (units?)". Where does it come from?
RESPONSE: This a good point and we agree this could be more clear. We have changed these sentences to "With respect to assumption (ii), the supernatant, after separating off all the microgel particles via centrifugation, had a viscosity of 0.01 to 0.03 Pa s over the shear rate range 0.1 to 10 s^{-1} and showed negligible shear thinning (± 0.03 Pa s) over this shear rate range. Solutions of 0.01 to 0.05 % alginate + added [Ca^{2+}] up to 10 mM (but without homogenization) had viscosities that were no higher than 0.03 Pa s in the same shear rate range. The [Ca^{2+}] of the supernatant was not determined, but since the final [Ca^{2+}] in the whole system = 10 mM and the viscosity of the supernatant indicated [alginate] < 0.05 wt.%, clearly very little of the original 1 wt.% alginate added remained free in solution."
We think this answers the point above – yes the viscosity is slightly higher than buffer alone but not much, indicating very little free alginate remaining in the bulk, i.e., less than 0.1 of the overall concentration in the mix.

Lines 313: not all types of pectin and carageenans. Low-methylated Pectin and kappa-carrageenan. "bivalent cations", not all "ions".

Line 314: add a title "Conclusion".

TOC graphic: is it SEM micrograph? The scale is not viewable. I suggest you a higher magnification if possible, that may give information regarding surface structure of the gelles particles.
RESPONSE: As explained above, we could not go to higher magnification/resolution because of beam damage. We agree the automatically generated SEM scale is not easy to read and we have superimposed a scale that is clearer. Also, however, we have replaced this particular image with one Figure 2(c) that is more clear.

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Reviewer #1: This is a generally well written article which should be of interest to the readership of FH.
RESPONSE: We feel we must draw the Editor's attention to the discrpency here between thie Reviewers general view and Reviewer#2.

Perhaps it is the quality of my printer, but the graphical abstract is difficult to make out.
RESPONSE: We have covered this re. Reviewer#2

Title - is the Leeds Jet Homogenizer the standard name for this instrument?
RESPONSE: Yes it is.

Line 74 - I think the aims of the paper need to be more explicitly outlined in the introduction.
RESPONSE: We feel we have addressed this re. Reviewer#2's comments on lines 74 and Lines 94-95

Line 77 + other - the city and country of suppliers need to be added.

Line 91 C the C doesn't need to be a superscript.

Line 268 - ± 30 mV is often used as an indicator of stability perhaps you could comment on this here?
RESPONSE: This is a good idea and we have added "We note that many types of solid and liquid colloidal particles become susceptible to flocculation when the ζ-potential falls much below 30 mV in magnitude, though these microgel particles are atypical colloids." in this paragraph of discussion.

Line 314 - add a heading to make this section more clear.
Line 373 - you shouldn't really ignore the other authors of this article.
Figure 1 - In your figure the chambers look to be the same size.

Figures 4 - 7 are these values different statistically?

RESPONSE: We had already done extensive statistical analysis but hoped that the magnitude of the error bars shown would be adequate to indicate the statistically significant difference between different samples/conditions. Nevertheless,

we agree that we should say more to allay any concerns. About each Figure, where this is first introduced in the text, we have therefor added a statement about the statistical reproducibility of the results. We have also added at the start of the Results section the statement “Data collected were analyzed using IBM SPSS Statistics v.22. A paired sample t-test (for sonicated samples) and 2-way ANOVA tests (for proteins containing samples) were conducted to determine the differences in particle sizes and ζ potential with a significance level set at 0.05.