**Inhibition of lipid oxidation in nanoemulsions and filled microgels
fortified with omega-3 fatty acids using casein as a natural
antioxidant**

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Abstract

The susceptibility of omega-3 fatty acids to oxidation in foods can often be retarded using well-designed colloidal delivery systems. In this study, the ability of a natural antioxidant (casein) to inhibit the oxidation of flaxseed oil in oil-in-water nanoemulsions and filled hydrogel beads (microgels) was investigated. Nanoemulsions ($D_{43} = 0.31 \mu\text{m}$) were fabricated by high-pressure homogenization using a quillaja saponin extract (Q-Naturale) as an emulsifier, whereas filled microgels ($D_{43} = 970 \mu\text{m}$) were fabricated by injecting a 0.8% alginate solution containing nanoemulsions into a calcium solution using an extrusion device. The size of the particles in these colloidal delivery systems exhibited no major changes during 14-days storage at 55 °C. The oxidative stability of flaxseed oil droplets was better when they were encapsulated in microgels than when they were free, which was attributed to the greater surface area of exposed lipid in the latter case. The oxidative stability of both nanoemulsions and filled microgels was improved by adding caseinate as an antioxidant. Simultaneous incorporation of both caseinate and lipid droplets within microgels proved to be the most effective means of inhibiting lipid oxidation. Overall, these results provide valuable information for the development of colloidal delivery systems to encapsulate and protect omega-3 fatty acids for functional food applications.

Keywords: omega-3 fatty acid; casein; alginate; hydrogel beads; oxidation; nanoemulsions.

1. Introduction

Consumption of sufficiently high levels of omega-3 fatty acids may have numerous health benefits, including promoting brain development and function, decreasing the risk of cardiovascular diseases, and reducing the risk of certain types of cancer (Adkins & Kelley, 2010; Lorente-Cebrián, et al., 2013; Papanikolaou, Brooks, Reider, & Fulgoni, 2014; Riediger, Othman, Suh, & Moghadasian, 2009; Zhao, et al., 2004). Western-style diets do not currently meet the levels of omega-3 fatty acids required to demonstrate these potential health benefits (Papanikolaou, et al., 2014; Waraho, McClements, & Decker, 2011). The food industry is therefore taking measures to help consumers increase their omega-3 fatty acid intake by incorporating these beneficial lipids into various functional food and beverage products. However, the supplementation of these products with omega-3 fatty acids is often challenging due to their extremely low water solubility, susceptibility to oxidation, and variable bioavailability (Jacobsen, Let, Nielsen, & Meyer, 2008; McClements, Decker, Park, & Weiss, 2008; Sanders, 2000). Consequently, there is growing interest in the development of appropriate delivery systems to incorporate omega-3 fatty acids into the human diet (Kennedy, Luo, & Ausman, 2012). Emulsion-based delivery systems, such as emulsions, nanoemulsions, and filled microgels, are particularly useful for this purpose since they can easily be incorporated into aqueous-based food and beverage products (McClements & Rao, 2011).

Oil-in-water nanoemulsions consist of small lipid droplets dispersed within an aqueous phase, with the mean droplet diameter typically being defined as < 200 nm (McClements, Decker, & Weiss, 2007; McClements, et al., 2011). Nevertheless, this definition is somewhat arbitrary as there is no distinct change in physicochemical

properties or physiological response at this specific particle size. In addition, the type of mean diameter used to express the particle size (*e.g.*, number, surface or volume weighted mean) may also determine whether a system is an emulsion or nanoemulsion. The term nanoemulsion was used in the current study because the surface weighted mean diameter (d_{32}) was below 200 nm. Nanoemulsions can be designed to overcome some of the challenges associated with incorporating omega-3 oils into foods, including improving their water-dispersibility and oral bioavailability (Walker, Decker, & McClements, 2015). However, conventional nanoemulsions are not particularly effective at inhibiting lipid oxidation due to their high interfacial area, thin interfacial thickness, and high light penetration (Jacobsen, et al., 2008; Langevin, 2000). Consequently, it is necessary to take additional steps to protect omega-3 oil nanoemulsions against lipid oxidation.

In recent years, there has been considerable interest in the utilization of filled hydrogel beads (microgels) as food-grade colloidal delivery systems to improve the chemical stability, water dispersibility, and bioavailability characteristics of various lipophilic bioactives (Das & Ng, 2010; Kosaraju, Weerakkody, & Augustin, 2009; Liang, Line, Remondetto, & Subirade, 2010; Silva, Favaro - Trindade, Rocha, & Thomazini, 2012). One of the potential advantages of using hydrogel beads for protecting omega-3 oils from oxidation is that their interiors can be designed to inhibit undesirable chemical reactions (Griffith, 2000) *e.g.*, by incorporating antioxidants or by excluding the entry of pro-oxidants. Indeed, previous studies in our laboratory have shown that polyunsaturated lipid droplets trapped within filled hydrogel beads were more stable to lipid oxidation than free lipid droplets (Matalanis, Decker, & McClements, 2012; Salcedo-Sandoval, et al., 2015; Zhang, Decker, & McClements, 2014). One of the potential advantages of these systems is that antioxidants can be

incorporated into the interior of the hydrogel beads so that they are in close proximity to the oxidizable lipid droplets.

Food-grade hydrogel beads are typically fabricated from natural polymers (proteins and polysaccharides) because of their low cost, desirable functional attributes, label friendliness, and low toxicity (Chen, Remondetto, & Subirade, 2006; Shewan & Stokes, 2013). Alginate, a linear anionic biopolymer, is commonly used to fabricate filled hydrogel beads to encapsulate lipophilic bioactives (Hoad, et al., 2011; Rayment, et al., 2009). Alginate forms ionotropic gels at ambient temperature due to electrostatic cross-linking of anionic carboxyl groups (-COO-) on alginate molecules by multivalent cations (typically Ca^{2+}) in the surrounding aqueous solution (Lertsutthiwong, Noomun, Jongaroonngamsang, Rojsitthisak, & Nimmannit, 2008). Alginate hydrogel beads can therefore simply be formed by injecting a solution of alginate into a solution of calcium ions. Similarly, filled hydrogel beads, which consist of lipid droplets trapped inside microgels, can be formed by injecting a mixture of alginate and nanoemulsion into a calcium ion solution. Previous studies have shown that alginate hydrogel beads are particularly effective at improving the chemical/biochemical stability of certain lipophilic bioactive agents, such as olive oil (Sun-Waterhouse, Zhou, Miskelly, Wibisono, & Wadhwa, 2011), curcumin (Zhang, et al., 2016b), β -carotene (Zhang, Zhang, & McClements, 2016a) and astaxanthin (Lin, et al., 2016). Therefore, alginate beads may also be useful to provide a local environment that protects omega-3 oils from oxidation.

The oxidation of omega-3 oil nanoemulsions can also be inhibited by adding appropriate antioxidants to the oil and/or aqueous phase (Qian, Decker, Xiao, & McClements, 2012; Salvia-Trujillo, Decker, & McClements, 2016; Yi, Li, Zhong, &

Yokoyama, 2014). Ideally, the food industry would like to utilize natural antioxidants (rather than synthetic ones) due to consumer demand for “cleaner” food labels. Many proteins can inhibit lipid oxidation by inactivating reactive oxygen species (ROS), scavenging free radicals, or chelating transition metals (Donnelly, Decker, & McClements, 1998; Elias, Kellerby, & Decker, 2008; McClements & Decker, 2000). The addition of caseinate to emulsions has previously been shown to inhibit lipid oxidation due to these antioxidant mechanisms (Diaz & Decker, 2004; Hu, McClements, & Decker, 2003; Kargar, Spyropoulos, & Norton, 2011; Ma, et al., 2012). The main objective of the current study was therefore to determine if sodium caseinate could also be used as a natural antioxidant in nanoemulsions and filled hydrogel beads (microgels) fortified with omega-3 fatty acids. In addition, a natural emulsifier (quillaja saponin) was used to stabilize the nanoemulsions, so as to keep the delivery systems all-natural.

2. Materials and methods

2.1 Materials

Flaxseed oil was obtained from a supermarket and used without further purification (AAK Ltd., England, UK). As stated by the manufacturer, the flaxseed oil contained approximately 7.1, 14.3, and 71.4 wt.% of saturated, monounsaturated, and polyunsaturated fats, respectively. Quillaja saponin (Q-Naturale 200) was provided by National Starch LLC (Bridgewater, N.J.). The molecular weight of quillaja saponin has been reported to be around 1650 g/mol (Mitra & Dungan, 1997). The manufacturer reported that this ingredient contained approximately 78-80% water and 65-75% saponin (dry basis), with the majority of other components in the dried material being carbohydrates. Powdered sodium caseinate (92-96% protein, NaC) was

obtained from Biomedicals LLC (Solon, OH). Sodium alginate (Lot# 180947, viscosity of 1% dissolved in water = 15-20 mPa s) was purchased from Sigma-Aldrich (St. Louis, MO). The manufacturer reported that the molecular weight of this product was around 120 to 190 kDa. All other chemicals used in this study, including technical grade Nile red dye (CAS #7385-67-3) and fluorescein thiocyanate isomer I (FITC), were purchased from Sigma-Aldrich (St. Louis, MO). Double distilled water from a water purification system (Nanopure Infinity, Barnstead International, Dubuque, IA) was used to prepare all solutions and nanoemulsions. A TBA reagent was prepared for the lipid oxidation experiments by dissolving 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, and 0.25 M HCl with 2% BHT in ethanol solution. All concentrations are expressed on a percentage weight-by-weight (%w/w) basis unless otherwise stated.

2.2 Emulsion preparation

A coarse emulsion was prepared by homogenizing 10% (w/w) flaxseed oil and 90% (w/w) aqueous emulsifier solution (1.12% (w/w) quillaja saponin, 5 mM phosphate buffer, pH 7.0) together with a high-shear mixer for 2 min (M133/1281-0, Biospec Product, Inc., ESGC, Switzerland). The droplet size was then reduced by passing the coarse emulsion through a high-pressure homogenizer (Microfluidics Microfluidizer M-110Y, Newton, MA USA) three times at an operating pressure of 12,000 psi. The resulting system was referred to as the stock nanoemulsion.

2.3 Preparation of unfilled hydrogel beads

Preliminary experiments were performed to identify suitable preparation conditions for producing the hydrogel beads. These experiments indicated that small spherical beads (600~1000 μm) could be formed by injecting 0.8% (w/w) alginate into a 100 mM calcium chloride solution using an encapsulation unit with a vibrating nozzle

diameter of 300 μm , electrode potential of 1000 V, and operating frequency of 800 Hz (Encapsulator B-390, BUCHI, Switzerland). Consequently, these conditions were used to prepare the unfilled and filled hydrogel beads in the remainder of the experiments.

For the unfilled hydrogel beads, a stock solution of alginate (1.2%, w/w) was prepared by dissolving alginate powder into phosphate buffer (pH 7) and stirring overnight. A mixed alginate (1.2%, w/w) and sodium caseinate (6%, w/w) stock solution was then prepared by dispersing alginate and NaC powders into phosphate buffer solution (pH 7.0) together with continuous stirring. The solutions were then adjusted back to pH 7.0 (if necessary) using 2 M sodium hydroxide. Unfilled hydrogel beads were formed using the encapsulation unit to inject 0.8% (w/w) alginate solution containing either 0 or 4% (w/w) caseinate into a 0.1 M calcium chloride solution with continuous stirring. The beads were allowed to crosslink with Ca^{2+} for 30 min at ambient temperature and then collected by filtration and washed with distilled water to remove excess Ca^{2+} from their surfaces. The unfilled hydrogel beads produced were then stored at 4 °C.

2.4 Encapsulation of filled hydrogel beads

Alginate solution (1.2%, w/w) or a mixed alginate-casein solution (1.2% and 6%, w/w, respectively) were mixed with stock nanoemulsion by stirring for 1 h to obtain a system containing 2% (w/w) fat, 0.8% alginate, and either 0 or 4% (w/w) casein. The resulting mixtures were then left to stand for 1 h to remove any air bubbles. Filled hydrogel beads were prepared using the encapsulation unit by injecting the droplet-biopolymer mixture into a 0.1 M calcium chloride solution with continuous stirring. The beads were allowed to crosslink with Ca^{2+} for 30 min at ambient temperature and then collected by filtration and washed with distilled water. The filled hydrogel beads

were then stored at 4 °C. The oil content of the filled hydrogel beads was calculated as follows:

$$\text{Oil content (g oil per g beads)} = (W_{\text{final}} - W_{\text{initial}}) * 2\% / W_{\text{beads}}$$

Here, W_{initial} and W_{final} are the weights of the beaker containing calcium chloride solution before and after injecting the droplet-biopolymer mixture (g), and W_{beads} is the wet weight of the filled hydrogel beads after filtration (g).

2.5 Particle size measurements

The mean particle diameter and particle size distribution of nanoemulsions and beads were determined using static light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). This instrument infers the size of the particles from measurements of their angular scattering pattern. Refractive indexes of the dispersed and continuous phases used in the calculations were 1.474 and 1.330, respectively. Emulsions and hydrogel beads were diluted in 5 mM phosphate buffer (pH 7.0) prior to analysis to avoid multiple scattering. Mean particle sizes are reported as the surface-weighted (d_{32}) or volume-weighted (d_{43}) mean diameters. All samples were measured at least in triplicate.

2.6 Peroxide value (PV)

Emulsions and filled hydrogel beads were incubated in the dark at 55 °C for 14 days, with measurements made everyday. The concentration of lipid hydroperoxides (primary lipid oxidation reaction products) was measured according to a method reported previously (Shantha & Decker, 1994). For the nanoemulsions, lipids were extracted by adding a small volume of nanoemulsion (0.3 mL) to a mixture of isooctane/2-propanol (1.5 mL, 3:1 v/v) and then vigorously vortexing 3 times for 10 s each, followed by centrifugation for 2 min at 1000 g. For the filled hydrogel beads, a saturated solution of disodium EDTA was first mixed with the system to degrade the

hydrogel matrix through calcium ion sequestration. 0.3 mL of the resulting sample was then used to extract the lipids as described for the nanoemulsions. After mixing with the organic solvent, 0.2 mL of the isooctane/2-propanol extract (top organic layer) was mixed with 2.8 mL of methanol/1-butanol (2:1 v/v), followed by 30 μ L of 1:1 v/v 3.94 M ammonium thiocyanate/ferrous iron solution (solution prepared by mixing equal amounts of 0.132 M barium chloride and 0.144 M ferrous sulfate). After reacting for 20 min at room temperature in the dark, the absorbance was measured at 510 nm using a spectrophotometer (Ultraspec 3000 pro, Biochrom Ltd., Cambridge, UK). The concentration of hydroperoxides was calculated using a standard curve of cumene hydroperoxide at concentrations of 0-5 μ M. All samples were measured in triplicate.

2.7 Thiobarbituric acid-reactive substances (TBARS)

The level of lipid TBARS (secondary lipid reaction products) was measured according to a method described previously (McDonald & Hultin, 1987). One microliter of nanoemulsions or EDTA-dissociated filled hydrogel beads was mixed with TBA reagent (2.0 mL) and then vortexed in glass screw cap test tubes. These tubes were then placed in a boiling water bath for 15 min, and then cooled down to room temperature in a water bath for 10 min. The tubes were centrifuged at 1000 g for 15 min. After standing for 10 min, the absorbance of the supernatant was measured at 532 nm. TBARS concentrations were calculated as μ M using a standard curve of 1,1,3,3-tetraethoxypropane (TEP) at concentrations of 0-5 μ M. All samples were measured in triplicate.

2.8. Microstructure analysis

A confocal scanning laser microscope with a 20 $\times$ objective lens and 10 $\times$ eyepiece (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, US.) was used to determine the

microstructure of the hydrogel beads. The oil phase in the filled hydrogel beads was dyed prior to nanoemulsion formation by adding 0.1 mL Nile red solution (1 mg Nile red /mL ethanol) to 2 mL of oil, covering, and stirring overnight. The excitation and emission spectra for Nile red were 543 nm and 605 nm, respectively. The caseinate was dyed prior to hydrogel bead formation by adding 0.1 mL FITC dye solution (1 mg FITC/mL dimethyl sulfoxide) to 2 mL of caseinate solution, and storing at 4 °C overnight. The excitation and emission spectrum for FITC were 488 nm and 515 nm, respectively. A small aliquot of hydrogel beads was placed on a microscope slide and covered with a cover slip prior to visualization. All microstructure images for confocal microscopy were taken and processed using the image analysis software (NIS-Elements, Nikon, Melville, NY).

2.9. Statistical analysis

All experiments were carried out in triplicate. The results are reported as mean and standard deviations, and the differences among samples were calculated from these values based on an analysis of variance (ANOVA) using the statistical analysis software (SPSS, IBM Corporation, Armonk, NY, USA).

3. Results and discussion

3.1 Preparation of nanoemulsions and filled hydrogel beads

Initially, we characterized the properties of the flaxseed-oil fortified nanoemulsions and filled hydrogel beads. In both the absence and presence of caseinate, the nanoemulsions formed optically opaque homogenous systems, which were stable to gravitational separation over the tested incubation period (**Figure 1**). This effect can be attributed to the fact that they initially contained relatively small particles (**Table 1**): $D_{32} = 0.198$ and $0.213 \mu\text{m}$ in the absence and presence of

caseinate, respectively. These results suggest that the addition of caseinate did not promote destabilization of the saponin-coated lipid droplets in the nanoemulsions. This is presumably because both the caseinate and saponins are negatively charged at pH 7, and so there will be an electrostatic repulsion between them. The fact that the mean surface weighted droplet diameter (D_{32}) produced after homogenization was below 200 nm means that the initial systems could be referred to as nanoemulsions (rather than emulsions). Nevertheless, this is somewhat arbitrary because the mean volume weighted droplet diameter (D_{43}) was above 200 nm (**Table 1**).

In the absence of caseinate, the unfilled hydrogel beads had a fairly transparent appearance and sedimented to the bottom of the test tubes due to their relatively high density and large particle size (**Figure 1, Table 1**). The filled hydrogel beads also sedimented to the bottom of the test tubes, but they had a whitish appearance. This suggests that the density of the filled hydrogel beads was still greater than that of water, which is probably because there were insufficient lipid droplets present to reach density matching. The fact that the filled beads had a whitish appearance can be attributed to light scattering by the nanoemulsion droplets inside them.

In the presence of caseinate, both the unfilled and filled hydrogel beads sedimented to the bottom of the test tubes, and had a whitish appearance. The whiteness of the unfilled hydrogel beads can be attributed to the relatively high protein concentration inside them, which increases their refractive index and leads to stronger light scattering. The sedimentation of the hydrogel beads can again be attributed to their high density and large size (**Table 1**).

The size of the hydrogel beads produced by the injection method depended on their composition (**Table 1**). The mean particle diameter of the filled hydrogel beads was higher than that of the unfilled ones, which suggested that the presence of the

lipid droplets increased bead dimensions. In addition, the size of the hydrogel beads containing caseinate was larger than those containing no caseinate, which suggests that the protein also increased bead dimensions. The particle size distributions of the different colloidal delivery systems were monomodal (**Figures 2a and 2b**), which suggests that the fabrication methods used were effective. Previous studies have reported that the crosslinking of alginate by Ca^{2+} occurs rapidly (Li, Hu, Du, Xiao, & McClements, 2011), which would account for the fact that spherical hydrogel beads with smooth surfaces were formed in our study (see later).

3.2 Physical stability of nanoemulsions and filled hydrogel beads

The size of the droplets in the nanoemulsions remained constant throughout 14-days storage at 55 °C, with the mean droplet diameter being 206 ± 11 nm and 217 ± 5 nm in the absence and presence of caseinate, respectively. This result is in agreement with a previous study on nanoemulsions prepared using quillaja saponins, which also reported that the lipid droplets were stable to aggregation at neutral pH when incubated at temperatures ranging from 30 to 90 °C (Uluata, McClements, & Decker, 2015). The origin of this effect was attributed to a strong electrostatic repulsion between the negatively charged saponin-coated droplets. There appeared to be a slight increase in the mean diameter of the hydrogel beads after storage at 55 °C in both the absence and presence of caseinate (**Figure 3**), but they remained as spherical particles. Alginate beads are known to be resistant to dissociation because of the strong electrostatic cross-linking of the anionic alginate molecules by the cationic Ca^{2+} ions (Chan, 2011). The appearance of all the delivery systems changed during storage, suggesting that there were some changes in their physicochemical properties over time (**Figure 1**). First, all of the systems containing lipid droplets and/or proteins changed from bright white initially to slightly brownish after storage, which

suggested that some form of chemical change had occurred, *e.g.*, oxidation of lipids and/or proteins. Second, in the systems containing hydrogel beads, the thickness of the sediment layer increased after storage, which suggests that some swelling of the beads had occurred, which is consistent with the light scattering measurements (**Figure 3**). This increase in bead size suggests that the biopolymer molecules in the hydrogel matrix may have undergone some form of rearrangement, presumably driven by the fact that there is a higher osmotic pressure inside the beads than outside them. Consequently, water may flow into the beads and cause them to swell.

3.3 Oxidation stability of nanoemulsions and filled hydrogel beads

The high susceptibility to polyunsaturated lipids to oxidation is one of the major challenges associated with incorporating omega-3 fatty acids into food (McClements, et al., 2000). We therefore examined the effectiveness of the different colloidal delivery systems at inhibiting lipid oxidation under accelerated storage conditions (55 °C for 14 days). The concentration of primary (lipid hydroperoxides) and secondary (TBARS) reaction oxidation products in nanoemulsions and filled hydrogel beads was measured (**Figure 4**). At the early stages of storage, the peroxide and TBARS values of all the delivery systems (except the nanoemulsions without caseinate) were fairly similar ($P>0.05$), but at longer storage times there were appreciable differences depending on the nature of the system:

Nanoemulsions (Without caseinate): In the absence of caseinate, the peroxide and TBARS values of the nanoemulsions increased moderately until day 4 and then increased sharply between days 4 and 8. The peroxides then continued to increase moderately, while the TBARS decreased. The concentration of lipid hydroperoxides and TBARS formed at the end of storage was much higher for these systems than for any of the other systems studied ($P<0.05$).

Nanoemulsions (With caseinate): In the presence of caseinate, the peroxide and TBARS values of the nanoemulsions increased moderately throughout storage, but at a much slower rate than for the nanoemulsions without caseinate. The relatively low level of lipid oxidation observed in the nanoemulsions containing caseinate may be due to its inherent antioxidant activity, since this protein is known to inactivate reactive oxygen species (ROS), scavenge free radicals, and chelate transition metals (Gallaher, Hollender, Peterson, Roberts, & Coupland, 2005).

Filled hydrogel beads (Without caseinate): In the absence of caseinate, the peroxide values of the filled hydrogel beads only increased slightly during storage, while there was no change in the TBARS values during storage. Consequently, encapsulation of the nanoemulsion droplets inside the alginate beads clearly improved their stability to oxidation. A possible explanation of this effect is that the hydrogel beads contained alginate, which can chelate transition metals and therefore protect against lipid oxidation (Salvia-Trujillo, et al., 2016).

Filled hydrogel beads (With caseinate): In the presence of caseinate, there was little change in the peroxide or TBARS values of the filled hydrogel beads throughout storage, indicating that this system was the most stable to lipid oxidation. These results indicate that encapsulation of the nanoemulsion droplets inside alginate beads that also contain casein is the most effective means of improving the oxidative stability of the omega-3 oils. Presumably, this is because the antioxidant caseinate molecules are located in close proximity to the omega-3 oil droplets, and are therefore more effective at inhibiting oxidation because they are at a higher local concentration.

In summary, the oxidative stability of the colloidal delivery systems decreased in the following order: filled hydrogel beads containing casein > filled hydrogel beads without casein > nanoemulsions mixed with casein > nanoemulsions without casein.

These results differ from those of another study where it was reported that filled casein-rich microspheres and casein-stabilized emulsions had fairly similar oxidation rates (Matalanis, Lesmes, Decker, & McClements, 2010). This difference could be due to differences in the total amount of caseinate incorporated within the hydrogel beads (4% *versus* 2.7%) and/or due to differences in the nature of the polysaccharides in the hydrogel beads (alginate/caseinate *versus* pectin/caseinate).

Overall, these results indicate that encapsulating flaxseed oil droplets within antioxidant-rich hydrogel beads is an effective means of protecting the omega-3 fatty acids from oxidation.

3.4 Effect of location of casein and nanoemulsions on lipid droplet oxidation

Further insight into the importance of the relative location of the caseinate molecules and nanoemulsion droplets on lipid oxidation was obtained by preparing four delivery systems containing hydrogel beads (HB), nanoemulsion droplets (NE) and caseinate molecules (C) in different arrangements (**Figure 5**):

- *HB+NE+C*: This system consisted of unfilled hydrogel beads, free nanoemulsion droplets, and free caseinate molecules all dispersed separately in water.
- *[HB+NE]+C*: This system consisted of nanoemulsion droplets trapped inside hydrogel beads, as well as free caseinate molecules dispersed in water.
- *[HB+NE+C]*: This system consisted of nanoemulsion droplets and caseinate molecules trapped inside hydrogel beads that were dispersed in water.
- *[HB+C]+NE*: This system consisted of caseinate molecules trapped inside hydrogel beads (HB+NE) and free caseinate molecules (C) dispersed in water.

The microstructure and visual appearance of the various delivery systems was measured before and after storage (**Figure 5**). The microstructure was determined by

confocal fluorescence imaging using a green color probe for the proteins and a red color probe for the lipids. The initial hydrogel beads fabricated in Sections 2.3 and 2.4 were too large to measure their microstructures using microscopy. We therefore prepared smaller hydrogel beads loaded with 2% flaxseed oil or/and 1.5% casein using a vibrating nozzle diameter of 120 μm and the same conditions as described in Sections 2.3 and 2.4. There were appreciable differences in the microstructure and properties of the samples depending on the relative location of the different components.

HB+NE+C: The confocal microscopy images of these systems exhibited a bright yellow fluorescence signal in the continuous phase and no fluorescence within the hydrogel beads (**Figure 5a**). This confirmed that both the protein and lipids were present in the aqueous phase outside of the beads. Unexpectedly, the unfilled hydrogel beads moved to the top of the test tubes after storage (**Figure 5b**). This may have been because the caseinate increased the density of the surrounding aqueous phase so that it was greater than that of the hydrogel beads.

[HB+NE]+C: The confocal microscopy images of these systems exhibited a bright green fluorescence signal in the continuous phase and a bright red fluorescence signal in the hydrogel beads (**Figure 5a**). This confirmed that the protein was in the aqueous phase, whereas the lipid droplets were inside the hydrogel beads. Again the hydrogel beads in this system creamed, which suggests that they were less dense than the aqueous phase containing caseinate (**Figure 5b**).

[HB+C]+NE: The confocal microscopy images of these systems exhibited a bright green fluorescence signal in the hydrogel beads and a bright red fluorescence signal in the continuous phase (**Figure 5a**). This confirmed that the proteins were trapped inside the hydrogel beads, whereas the lipid droplets were present in the

surrounding aqueous phase. In this case, the mixed systems had a fairly homogeneous appearance, which can be attributed to the presence of the nanoemulsion droplets in the continuous phase (**Figure 5b**). Nevertheless, close visual inspection of the samples indicated that the hydrogel beads did form a sediment layer at the bottom of the tubes, which can be attributed to the fact that the caseinate-loaded beads were heavier than the surrounding aqueous phase.

[*HB+NE+C*]: The confocal microscopy images of these systems exhibited a bright yellow fluorescence signal in the hydrogel beads and no fluorescence signal in the continuous phase (**Figure 5a**). This confirmed that both the proteins and lipid droplets were trapped inside the hydrogel beads. In this case, the hydrogel beads sedimented (**Figure 5b**), which suggested that they were denser than the surrounding aqueous phase, probably because they contained high levels of caseinate.

The effect of lipid droplet and caseinate location on the oxidative stability of the mixed systems was determined by measuring PV and TBARS values during 14-days storage at 55 °C (**Figures 5c and 5d**). Interestingly, the [*HB+C*]+*NE* systems were the most unstable to oxidation, and behaved fairly similarly to the systems of *NE* without caseinate (**Figures 6a and 6b**). This effect can be attributed to the fact that the antioxidant caseinate molecules were trapped inside the alginate beads and therefore they were not effective at inhibiting oxidation of the lipid droplets in the continuous phase. Conversely, the [*HB+NE+C*] systems exhibited the highest oxidative stability, which can be attributed to the fact that the lipid droplets were trapped inside alginate beads and were in close proximity to high levels of antioxidant caseinate. The other two systems exhibited intermediate oxidative stability. In the case of the *HB+NE+C* systems, both the free lipid droplets and free caseinate molecules were in the aqueous phase, and so the protein could exhibit some

antioxidant effects. In the case of the $[HB+NE]+C$ systems, the lipid droplets were partly protected from oxidation due to the fact they were encapsulated within the alginate beads. The oxidative stability of the $[HB+NE+C]$ systems was higher than the $[HB+NE]+C$ systems (**Figures 6a and 6b**), which was in agreement with a recent study that reported antioxidants (quercetin) were more effective when they were close to the lipid substrate (Waterhouse, Wang, & Sun-Waterhouse, 2014).

Typically, the PV values increase and then decrease as oxidation proceeds, because they are initially formed and are then decomposed (McClements, et al., 2000). The maximum in the PV values depends on the rate of peroxide formation compared to peroxide degradation, which is highly system dependent. Consequently, it is possible for TBARS (secondary reaction products) to increase, while PV values still increase or reach a maximum value.

Overall, these results indicate that the physical location of both the lipid droplets and the antioxidant proteins in hydrogel systems impacts the stability of the lipids to oxidation.

4. Conclusions

Filled hydrogel beads were fabricated by injecting a mixture of flaxseed oil nanoemulsions and alginate molecules into a calcium solution. Storage studies indicated that the nanoemulsions and hydrogel beads were physically stable when stored at 55 °C for 14 days, although there was some swelling of the beads. In addition, this study demonstrated that the oxidation stability of the flaxseed oil droplets could be improved by adding caseinate molecules as a natural antioxidant, or by encapsulating them within hydrogel beads. The best oxidative stability was obtained by encapsulating both the lipid droplets and caseinate molecules within the

hydrogel beads. This effect was attributed to the fact that there was a high level of antioxidant protein in close proximity to the lipid droplets within the beads. Overall, this study showed that encapsulating flaxseed oil droplets within an antioxidant protein-rich hydrogel bead is highly effective at protecting them against oxidation. This information may be useful for formulating functional foods and beverages with improved nutritional profiles.

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Figure 1

Figure 2a

Figure 2b

Figure 3a

Figure 3b

Figure 4a

Figure 4b

Figure 5a

Figure 5b

Day 0

- (I) HB + NE + C
- (II) [HB+NE] + C
- (III) [HB +C] + NE
- (IV) [HB +NE +C]

(i) (II) (III) (IV)

Day 14

Figure 5c

Figure 5d

Figure 6a

Figure 6b

Table 1. The mean particle diameter of different colloidal delivery systems. ^a

Systems	Particle size	
	D ₃₂ (μm)	D ₄₃ (μm)
NE	0.198±0.011	0.307±0.023
NE + casein	0.213±0.019	0.322±0.025
U-HB	527±23	889±27
U-HB + casein	966±41	1047±61
F-HB	902±37	974±42
F-HB + casein	1069±25	1137±21

^a Values are expressed as mean ± standard deviation of triplicate analysis.

Graphical Abstract

“Influence of casein on the oxidation stability of omega-3 nanoemulsions and hydrogel beads: Antioxidation effects of casein”