***In situ* cross-linking of alginate during spray-drying to microencapsulate lipids in powder**

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Abstract

Microencapsulation of emulsified lipophilic bioactive compounds in dry, cross-linked alginate microcapsules (CLAMs) is a promising strategy to facilitate their incorporation into food systems, prolong shelf life, and target delivery within the gastrointestinal tract. However, current technology to produce CLAMs requires multiple time- and energy- intensive unit operations. We developed a novel technology that streamlines CLAM production into a single unit operation by accomplishing particle formation, cross-linking, and drying during spray-drying. Spray-dried CLAMs were prepared using corn oil as the cargo, and dry basis oil loadings up to 35% (w/w) were achieved. Alginate cross-linking was verified by the insolubility of CLAMs in water and ready dissolution in sodium citrate. Volume weighted mean particle size of CLAMs increased with increasing oil content: 8.1 μm , 11.8 μm and 17.9 μm for 15%, 25% and 35% oil, respectively. Spray dried CLAMs were approximately spherical, with oil droplets evenly distributed throughout each microcapsule. The size distribution of oil droplets, with average diameters ranging from approximately 200 to 300 nm, remained unchanged throughout the encapsulation process; spray drying did not induce aggregation or coalescence of oil droplets within CLAMs. CLAMs released 22-35% of oil in simulated gastric fluid (pH 1.5) and 81-93% in simulated intestinal fluid (pH 7) in 2 hours, indicating that CLAMs are an enteric system. Coupled with the scalability of this novel CLAM production method, the successful encapsulation of the model lipid suggests that spray-dried CLAMs may be of commercial use for incorporating lipophilic compounds into foods.

Keywords: Microencapsulation, spray-drying, alginate, *in situ* cross-linking

1. Introduction

Incorporating bioactive lipophilic compounds into functional foods is highly desirable, as these extra-nutritional plant and food constituents have been associated with a number of health benefits, including protection against cardiovascular disease and cancer (Kris-Etherton et al., 2002; Sanders, 1996). Microencapsulation, which produces sub-micron to millimeter scale capsules in which a coating material envelops the core material, is regarded as a means of facilitating the incorporation of lipophilic bioactive compounds into foods and beverages. In the food industry, microencapsulation offers a multitude of applications, including protecting bioactives from degradation induced by environmental factors, masking undesirable flavors and odors, improving material handling, separating otherwise incompatible components, and controlling the release of bioactives (Desai & Park, 2005; Gibbs, Kermasha, Alli, & Mulligan, 1999).

While numerous food grade coating materials are available for microencapsulation, alginate is the subject of particular interest due to its release applications (George & Abraham, 2006). A polysaccharide derived from algal cell walls, alginate is a polymer of guluronate and mannuronate. Regions of repeating guluronate units in adjacent alginate molecules selectively bind divalent cations such as calcium in egg-box structures; these cross-links induce the spontaneous formation of a gel at room temperature. The cross-linking of alginate may be reversed through the scavenging of calcium ions from the gel matrix, which allows the polymers to separate, thus breaking the gel and releasing the encapsulated material (Draget & Taylor,

2011). This property provides one **potential** mechanism for **triggering the** release of an alginate microcapsule's payload in the intestinal environment, which is high in **chelators** that scavenge calcium. **Another mechanism of physiological release of alginate gel cargo involves the protonation and displacement of calcium ions under low pH conditions in the stomach to form alginic acid gels, which readily dissolve in the intestine due to the displacement of protons by sodium ions** (Gombotz & Wee, 1998; Ostberg, Lund, & Graffner, 1994). The disintegration of alginate microcapsules under intestinal conditions makes alginate an attractive choice for nutrient delivery applications.

Dry cross-linked alginate microcapsules (CLAMs) have been used to encapsulate lipophilic bioactives such as carvacrol (Wang, Gong, Huang, Yu, & Xue, 2009), α -tocopherol (Yoo, Song, Chang, & Lee, 2006), and oils containing tocopherols, tocotrienols, carotenoids, and linolenic acid (Chan, Lim, & Heng, 2000; Durante, Lenucci, Laddomada, Mita, & Caretto, 2012). Controlled release studies of CLAMs indicate that while a relatively low fraction of core material is released in gastric conditions, the vast majority of the core material is released in intestinal conditions (Wang et al., 2009; Yoo et al., 2006). In addition to providing a mechanism of targeted and controlled release, encapsulation in CLAMs has been demonstrated to increase the shelf-stability of bioactives, compared to non-encapsulated compounds (Durante et al., 2012).

However, while alginate microcapsules offer advantages in core material protection and release, their utilization in the food industry has been limited by time- and energy- intensive manufacturing methods. A variety of production methods have been developed for preparing CLAMs, which can be broadly classified as external gelation, inverse gelation, and internal gelation methods. External gelation involves dispersing an O/W emulsion (with alginate in the

aqueous phase) into a calcium solution by extrusion or spraying. Cross-linking proceeds as calcium ions diffuse inwards, encapsulating the emulsified core material in a cross-linked hydrogel. External gelation methods have been widely used to encapsulate lipophilic compounds (Durante et al., 2012; Dongxiao Sun-Waterhouse, Wang, & Waterhouse, 2014; D. Sun-Waterhouse, Zhou, Miskelly, Wibisono, & Wadhwa, 2011; Wang et al., 2009; Yoo et al., 2006). Internal gelation methods involve forming an O/W/O multiple emulsion with alginate and an insoluble calcium salt in the aqueous phase. The core material is contained in the innermost oil phase. The addition of acid solubilizes the calcium salt to make it immediately available for cross-linking the alginate throughout the aqueous phase. As preparing stable multiple emulsions may be challenging, microfluidic techniques have been developed to produce core-shell microcapsules using this technique (Liu et al., 2013). The inverse gelation method forms core-shell microcapsules when oil droplets containing finely divided insoluble calcium chloride are dispersed in an alginate solution. Upon leaving the oil droplet, calcium chloride particles dissolve such that calcium ions are immediately available to form a cross-linked membrane around the droplet (Abang, Chan, & Poncelet, 2012). All of these CLAM preparation technologies require separation of the hydrated alginate beads, followed by a drying step.

To increase the scalability of CLAM production, spray-drying preparations have been suggested. The oldest and most prevalent microencapsulation technology used in the food industry, spray drying involves atomizing a feed solution, suspension, or emulsion in a hot gas current to obtain dry powdered particles (Desai & Park, 2005; Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). Matrix-style microcapsules are commonly produced using coating materials such as milk proteins, modified starches, maltodextrin, and gums (Gibbs et al., 1999). Alginate has been used as a wall material for spray dried microparticles containing hydrophilic

compounds, but cross-linking was induced after spray-drying by external gelation (Bagheri, Madadlou, Yarmand, & Mousavi, 2014; Schoubben, Blasi, Giovagnoli, Rossi, & Ricci, 2010). A separation step and a second drying step are still required. Others have avoided this complication by weakly cross-linking the alginate with dilute calcium chloride prior to spray-drying (Erdinc & Neufeld, 2011; Estevinho, Damas, Martins, & Rocha, 2014). However, this approach requires a vigorous mixing step prior to spray drying to produce alginate beads of small enough diameter to pass through the nozzle. Recently, we developed a single-step process for preparing cross-linked alginate microparticles by spray-drying (Jeoh, Scher, Santa-Maria, & Strobel, 2012; Santa-Maria, Scher, & Jeoh, 2012). A suspension consisting of alginate, an acid neutralized by ammonium hydroxide, and insoluble calcium salt is spray-dried. Upon atomization, the volatilization of ammonia decreases the pH in the droplet, which solubilizes the calcium salt, releasing calcium ions to cross-link the alginate. Thus, particle formation, cross-linking, and drying occur in a single, scalable unit operation. However, to our knowledge, none of the aforementioned spray-drying methods have been used to microencapsulate lipophilic compounds in cross-linked alginate.

In the present study, we investigated the potential to encapsulate lipophilic compounds in CLAMs prepared using a novel two-step process consisting of homogenization and spray-drying. CLAMs containing corn oil as a model lipophilic compound were produced at oil loadings up to 35%. We present results on the particle size, morphology, oil content, and oil droplet size of the CLAMs. Additionally, we demonstrate controlled release of oil from CLAMs in simulated digestive fluids.

2. Materials & Methods

2.1 Materials

Corn oil (Cal Western Packaging, Memphis, TN) was procured from a local market. Low viscosity sodium alginate (A1112) was purchased from Sigma-Aldrich (St. Louis, MO). Polysorbate 80 (Tween 80), dicalcium phosphate, sodium citrate dihydrate, succinic acid, ammonium hydroxide, n-hexane, isopropanol, methanol, chloroform, potassium chloride, sodium bicarbonate, sodium chloride, ammonium carbonate, potassium phosphate monobasic, magnesium chloride, and hydrochloric acid were purchased from Fisher (Fair Lawn, NJ). MilliQ water with a minimum resistivity of 18 MΩ-cm (Millipore, Billerica, MA) was used for all experiments.

2.2 Methods

2.2.1 Preparation of Cross-Linked Alginate Microcapsules (CLAMs)

Spray-dried CLAMs were prepared with target oil loadings of 0%, 15%, 25%, and 35% (w/w). All CLAMs were formulated with a 5:1 corn oil to Tween 80 ratio (w/w), and the target oil loading dictated the quantity of corn oil and Tween 80 in each formulation. An emulsion composed of corn oil, Tween 80, and 2% (w/w) succinic acid adjusted to pH 5.6 with ammonium hydroxide was dispersed at 9500 rpm for 2 min with a hand-held dispersing unit then homogenized using a high pressure homogenizer at 600 bar. The homogenized emulsion was mixed 1:1 (w/w) with a suspension of 4% alginate, 0.2% calcium phosphate dibasic dihydrate, and 0.06% sodium citrate dihydrate to form the spray dryer feed. This feed solution was promptly pumped into a Buchi B290 laboratory spray-dryer (New Castle, DE) to produce dry microcapsules. All formulations were prepared under identical operating conditions: inlet air temperature at 150°C, aspirator airflow rate at maximum (35 m³/h), feed peristaltic pump at 20% of maximum (6 ml/min), and nozzle air flow at two-thirds of maximum (40 mm on Q-flow indicator). Spray dried powders were stored in glass vials in a desiccator until analysis.

2.2.2 Oil loading and hexane extractable oil measurement

Corn oil loading of CLAMs was quantified by measuring first the oil washed from CLAMs with organic solvent and second the oil released by dissolving the solvent-washed CLAMs. In a 50 ml conical tube, 1.0 g of CLAMs was washed in 20 ml n-hexane for 2 min with gentle agitation. After centrifugation for 5 min at 4000 rpm, the organic phase was decanted, the solvent was vaporized, and the mass of extracted oil was determined using an analytical balance. To quantify the mass of oil remaining in the washed microcapsules, the cross-links were disrupted by adding 40 ml of 100 mM sodium citrate to the pellet of microcapsules, followed by end-over-end mixing until fully dissolved. After dividing the fluid equally into two 50 ml conical tubes, oil was extracted by adding 10 ml hexane with vigorous mixing followed by the addition of 10 ml of propan-2-ol. After centrifugation for 5 min at 4000 rpm, the organic phase was separated and vaporized, and the mass of encapsulated oil was determined using an analytical balance. The total quantity of oil contained within the microcapsules was represented by the sum of the hexane extractable oil and the encapsulated oil. The oil loading, which represents the percentage of CLAM powder mass comprised by oil, was calculated as follows:

$$\text{Oil Loading} = \left[\frac{\text{Extracted Oil (g)} + \text{Encapsulated Oil (g)}}{\text{Mass of Microcapsules (g)}} \right] \times 100$$

Hexane extractable oil, which indicates the percentage of oil accessible to the hexane solvent, was calculated as follows:

$$\text{Hexane Extractable Oil} = \left[\frac{\text{Extracted Oil (g)}}{\text{Extracted Oil (g)} + \text{Encapsulated Oil (g)}} \right] \times 100$$

2.2.3 Size analysis of emulsion oil droplets

Size distributions of the oil droplets before and after encapsulation process were measured at 25°C using a Zetasizer Nano ZS dynamic light scattering instrument (Malvern

Instruments, Worcestershire, UK). The settings for the analyzer were as follows — material type: oil, material refractive index = 1.47, dispersant type: water, dispersant refractive index = 1.33, temperature: 25°C. The homogenized emulsion was diluted 50X in water prior to the measurement. To measure the size of oil droplets encapsulated within CLAMs, the spray-dried powder was dissolved in 50 mM sodium citrate at half the solids content of the spray-dryer feed suspension, then diluted 50X in water prior to measurement. Each emulsion sample was measured independently in triplicate.

2.2.4 Particle size analysis of spray-dried powders

The particle size distributions of non-hydrated microcapsules were determined using a Mastersizer 2000 particle analyzer with Hydro μ P dispersion unit (Malvern Instruments, Worcestershire, UK). Spray dried powders were dispersed in propan-2-ol and added to the dispersion unit according to manufacturer guidelines. Samples were circulated in the dispersion unit for 10 min before initiating measurement. The settings for the analyzer were as follows — particle refractive index = 1.51, particle absorption index = 0.1, dispersant refractive index = 1.37, model: general purpose spherical. Each sample was measured in triplicate, with ten internal measurements per replicate. Malvern software was used to calculate size parameters for each replicate and generate an average size distribution for each sample.

2.2.5 Field emission scanning electron microscopy (SEM)

Surface topography images of uncoated powders mounted on double-sided carbon tape were obtained using a Hitachi S-4100 FE-SEM, with an electron beam acceleration voltage of 2 kV. For cross-sections, powder samples were mounted in an epoxy resin, cross-sectioned, and sputter-coated in gold. Scanning electron micrographs of sectioned CLAMs were obtained using a FEI/Philips XL30 SFEG SEM operated at 5 kV in SED mode.

2.2.6 Laser scanning confocal microscopy (LSCM)

LSCM was performed using an Olympus FluoView1000 (Center Valley, PA). Powder samples for LSCM were prepared as described but with the encapsulated oil phase containing hydrophobic fluorescent dye (30 µg/g tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride complex). Powder samples were mounted in minimal fluorescence Type DF immersion oil (Cargille, Cedar Grove, NJ), which matches the refractive index of the glass coverslip. Samples were viewed using a 60X oil objective. Samples were excited at a wavelength of 458 nm, and emission wavelengths were collected between 590 and 640 nm. Laser transmissivity was set to 10%, and vertical slices were spaced at a distance of 0.5 µm. Images dimensions were 800 x 800 pixels, with acquisition time of 10 µs/pixel. Image processing was performed using ImageJ software (Rasband, 1997-2014).

2.2.7 Release of oil in simulated digestive fluids

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared following two published recipes without enzymes or bile salts. Pharmacopeia SGF consisted of 0.2% sodium chloride at pH 1.5, and Pharmacopeia SIF consisted of 50 mM phosphate buffer at pH 7.4. SGF and SIF were also prepared using recipes modified from Minekus et al (2014). Minekus 2014 SGF (6.9 mM potassium chloride, 0.9 mM potassium phosphate monobasic, 25 mM sodium bicarbonate, 47.2 mM sodium chloride, 0.1 mM magnesium chloride, and 0.5 mM ammonium carbonate) was prepared at pH 3.0 and 1.5. Minekus 2014 SIF consisted of 6.8 mM potassium chloride, 0.8 mM potassium phosphate monobasic, 85 mM sodium bicarbonate, 38.4 mM sodium chloride, and 0.33 mM magnesium chloride, pH 7.0. Hydrochloric acid was used for all pH adjustments.

Oil release from 25% oil CLAMs was measured by adding 0.15 g CLAMs to 20 ml of each fluid in triplicate. The suspensions were incubated for 2 h at 37°C with end over end mixing at 20 rpm, then centrifuged at 5000 rpm for 2 min. The supernatant was decanted from the CLAM pellet, which was dissolved in 20 ml of 100 mM sodium citrate. Oil was extracted from the supernatant and pellet emulsions with 10 ml hexane with vortex mixing, followed by the addition of 10 ml propan-2-ol with vortex mixing. After centrifugation for 5 min at 5000 rpm, the organic phase was separated, dried, and weighed to determine the mass of oil released into the digestive fluid and the mass of oil retained in the pellet. Oil release was quantified as follows:

$$\text{Oil Released} = \left[\frac{\text{Oil Released (g)}}{\text{Oil Released (g)} + \text{Oil Retained}} \right] \times 100$$

Mass closure was verified by calculating the oil loading based on the sum of the masses of released and retained oil. In addition, a positive control was conducted with 100 mM sodium citrate as the release fluid. 100% release was achieved in 2 h, and the calculated oil loading agreed with the target and measured oil loadings.

2.2.8 Statistical analysis

Data were reported as mean $\pm$ standard deviation. One-way ANOVA and multiple comparison test were used to assess differences between factor means. Statistical analysis was performed using MATLAB R2014a (Mathworks). P-values less than 0.05 were deemed to be significant.

3. Results

3.1. Oil loadings and size distribution of spray-dried cross-linked CLAMs

Measured corn oil loadings of $17.0 \pm 1.0\%$, $26.3 \pm 1.4\%$ and $35.9 \pm 2.5\%$ were consistent with the target oil loadings of 15 %, 25 % and 35 %, respectively, indicating no loss of cargo

during the preparation of the CLAMs (Table 1). Measured oil loadings were not significantly greater than the corresponding target oil loadings.

Particle size distributions for 0%, 15%, 25%, and 35% oil CLAMs were reported on a volume basis (Figure 1). As oil loading increased, the particle size distributions broadened and a shoulder in the 50 to 200 μm range increased in magnitude. While the 10th percentile diameter ($D(0.1)$) and median diameter ($D(0.5)$) were independent of oil loadings, with average values of 1.3 μm and 6.2 μm , respectively, oil loading appeared to influence the volume-weighted mean diameter ($D_{4,3}$) and the 90th percentile diameter ($D(0.9)$) (Table 1). For oil-loaded CLAMs, both $D_{4,3}$ and $D(0.9)$ increased with oil loading. The $D_{4,3}$ of 15%, 25%, and 35% oil CLAMs were $8.1 \pm 0.2 \mu\text{m}$, $11.8 \pm 0.7 \mu\text{m}$, and $17.9 \pm 0.3 \mu\text{m}$, respectively. $D(0.9)$ also increased with increasing oil content, with values of $13.9 \pm 0.3 \mu\text{m}$, $21.4 \pm 1.9 \mu\text{m}$, and $54.9 \pm 1.3 \mu\text{m}$ for 15%, 25%, and 35% oil CLAMs, respectively. For reference, $D_{4,3}$, $D(0.1)$, $D(0.5)$, and $D(0.9)$ for 0% oil CLAMs were $9.4 \pm 0.1 \mu\text{m}$, $1.5 \pm 0.1 \mu\text{m}$, $8.7 \pm 0.1 \mu\text{m}$, and $17.2 \pm 0.3 \mu\text{m}$, respectively.

3.2. CLAM morphology

SEM images showed that 25% oil CLAMs were generally spherical with textured surfaces resembling golf balls (Figure 2a). The 25% oil CLAMs have small dark indentations on their surfaces with diameters on the order of several hundred nanometers. These indentations may indicate locations where oil droplets resided on the microcapsule surface. The oil droplets themselves vaporize in the low pressure SEM environment and cannot be visualized. Small cavities previously occupied by oil droplets were observed in SEM images of cross-sectioned 25% oil CLAMs (Figure 2b). Consistent with the size of the surface indentations, oil droplet cavities appear to be approximately several hundred nanometers to one micron in diameter, and

these dark regions appear to be distributed uniformly across the cross-sectional area of each CLAM. The two CLAMs in the field of view each appear to contain an air void several microns in diameter. Nevertheless, oil droplets were evenly distributed throughout the annular region surrounding the void. In contrast with the oil-loaded CLAMs, the 0% oil CLAMs had smooth surfaces and exhibit a collapsed appearance reminiscent of deflated beach balls (Figure 2c).

In a parallel analysis, we used LSCM to characterize 20% oil CLAMs prepared with a hydrophobic fluorescent dye in the oil phase. XY sections spaced 1 μm apart in the vertical Z-direction provide an internal view of oil droplet distribution within CLAMs (Figure 3). Fluorescence intensity was uniform across the particles in each XY section, indicating that oil droplets were distributed throughout the CLAM volume rather than being concentrated on the CLAM surface. Individual oil droplets were too small to be visualized distinctly. Regions of low fluorescence intensity within CLAMs may indicate small internal voids.

3.3. Oil droplet size is conserved during CLAM preparation

The average diameter of oil droplets was relatively consistent before and after microencapsulation. Oil droplet size distributions for the homogenized emulsion (before spray-drying) and the emulsion obtained from citrate-dissolved CLAMs (after spray-drying) overlap, with only a slight upshift in diameter after encapsulation (Figure 4). Size distributions were generally monomodal. The z-average diameters of oil droplets were 193.5 ± 2.9 nm, 190.8 ± 4.3 nm, and 161.6 ± 2.4 nm in the homogenized emulsions used for preparing 15%, 25%, and 35% oil CLAMs, respectively. The z-average diameters of oil droplets liberated from 15%, 25%, and 35% oil CLAMs were 305.5 ± 5.1 nm, 234.2 ± 2.8 nm, and 186.7 ± 9.5 nm, respectively.

Polydispersity index (PDI) of oil droplet distributions was greater after encapsulation than before encapsulation. For 15% oil CLAMs, PDI increased from 0.108 ± 0.030 in the

homogenized emulsion to 0.238 ± 0.020 when CLAMs were dissolved. For 25% oil CLAMs, the PDI increased from 0.124 ± 0.033 in the homogenized emulsion to 0.268 ± 0.012 for the dissolved CLAMs. For 35% oil CLAMs, the PDI increased from 0.130 ± 0.029 in the homogenized emulsion to 0.439 ± 0.048 in the dissolved CLAMs emulsion.

3.4. Gastric and intestinal release of oil from CLAMs

We investigated the release of corn oil from 25% oil CLAMs after a 2 h incubation in a variety of simulated gastric and simulated intestinal fluids. In Pharmacopeia SGF (pH 1.5), $21.8 \pm 0.9\%$ of the loaded oil was released, while $93.3 \pm 0.7\%$ was released in Pharmacopeia SIF (pH 7.4) (Figure 5a). In a similar experiment performed using simulated digestive fluids modified from Minekus et al (2014), $34.8 \pm 1.5\%$ of oil was released in SGF (pH 1.5) and $81.0 \pm 2.6\%$ was released in SIF (pH 7.0) (Figure 5b). The recipe composition significantly influenced the oil release. Regardless of the recipes used, CLAMs released a relatively minor fraction of oil in SGF while releasing an overwhelming fraction of oil in SIF.

While a relatively low fraction of oil was released from CLAMs in SGF fluids at pH 1.5, SGF prepared at elevated pH elicited significantly greater oil release. In Minekus 2014 SGF prepared at pH 3.0, oil release nearly doubled at pH 1.5, increasing to $64.4 \pm 3.1\%$ (Figure 5b).

4. Discussion

4.1. Cross-linked microcapsules were produced by a novel, scalable method

Cross-linked alginate microcapsules (CLAMs) were prepared using a novel two-step process consisting of emulsification and spray drying. Corn oil was encapsulated in microcapsules consisting of homogenized oil droplets embedded throughout a dry, cross-linked alginate matrix. To our knowledge, this is the first utilization of spray-drying to directly

microencapsulate lipid moieties in cross-linked alginate matrix. CLAMs immersed overnight in water with end-over-end mixing remained visibly insoluble, while alginate powder dissolved completely, demonstrating successful cross-linking in the CLAMs.

Oil loadings of 20 to 30% of total weight are common for spray-dried microcapsules (Jafari, Assadpoor, He, & Bhandari, 2008). Here, our process was used to prepare microcapsules with corn oil loadings up to 35%. These high oil loadings are promising considering the novelty of the cross-linked alginate wall material. Modifications to the wall material formulation may permit further increase in oil loading in CLAMs. For example, our previous wall material formulation detailed by Santa-Maria et al (2012) was modified to the formulation in this work, permitting increase in maximum oil loading from 5% to 35%.

This microencapsulation process is considerably more streamlined than traditional processes for encapsulating lipids in cross-linked alginate. The conventional external gelation process involves at least five steps: preparing an emulsion in an alginate solution, spraying into a calcium bath, allowing curing time for calcium diffusion into the droplets, separating the microcapsules from the solution, and drying (Figure 6). The novel process described here requires only two processing steps to produce dry CLAMs, far fewer than any of the existing external gelation, internal gelation, or inverse gelation methods. Furthermore, the homogenization and spray drying unit operations used in our process benefit from being scalable, cost-effective, continuous, and well-established in the food and pharmaceutical industries.

4.2. CLAM size & morphology

The spray-dried oil CLAMs (D_{43} of 8 to 18 μm) were considerably smaller than previously reported for CLAMs produced by external gelation. For example, CLAMs produced using an electrostatic vibrating spray nozzle had diameters ranging from 700-1200 μm when

hydrated and 500-800 μm when fully dried (Wang et al., 2009). Fully hydrated, lipid nanoparticle-loaded CLAMs with median diameter of 330-1350 μm have been prepared using an electrostatic droplet generation technique, with size dictated by needle inner diameter (Strasdat & Bunjes, 2013). The same authors reduced the median diameter of hydrated CLAMs to 45 μm by using the two-fluid nozzle of a spray-dryer to disperse their suspension in calcium solution. The small particle size achieved here for spray-dried CLAMs is desirable for minimal sensory perception in food applications.

CLAM diameters trended upward with increasing oil content. Increasing the oil content likely increased the viscosity of the spray dryer feed. Increased feed viscosity generally increases the size of the atomized droplets formed at the nozzle, and thus results in larger powder particle size (Jafari et al., 2008). While the particle size here depended on oil content, it is theoretically possible to tune particle size by adjusting the viscosity (through composition or solids content), the flow rate of the feed suspension, and the atomizing air flow. The 90th percentile diameter ($D(0.9)$) also increased with increasing oil content, which suggests aggregation of microcapsules in the powder. CLAM particle size distributions featured a secondary peak from 50-200 μm which increased in magnitude with oil content (Figure 1). This increase in volume fraction suggests increasing aggregation of CLAMs as oil loading rises.

Oil-loaded spray-dried CLAMs were generally spherical, while 0% oil CLAMs had a collapsed appearance (Figure 2). The oil droplets likely provided structure to the CLAMs as they dried, preventing the outer skin from collapsing upon itself. Dark regions in LSCM images of CLAMs may indicate small air voids (Figure 3). Similarly, the left cross-sectioned microcapsule in Figure 2b contains a central region that lacks dark oil droplet vacancies, which may indicate that the section bisects an air pocket. These small air voids were likely formed as a result of air

bubbles present in the spray-dryer feed suspension. The alginate dissolution and mixing processes introduced considerable air into the feed suspension, and excess surfactant facilitated the stabilization of micron-sized bubbles. These air voids could likely be eliminated by degassing the feed suspension prior to spray-drying. CLAMs do not exhibit any evidence of ballooning, a common undesirable phenomenon in which steam expansion creates a single large void in the centers of spray-dried microcapsules (Jafari et al., 2008). Due to the relatively low feed solids content and the lack of ballooning, CLAMs are considerably smaller in size than most spray dried microcapsules.

SEM imaging suggested that oil droplets were on the order of several hundred nanometers, and individual oil droplets were too small to be resolved by LSCM. Using dynamic light scattering, the average diameters of oil droplets in CLAMs was found to range from approximately 200 to 300 nm, with oil droplet size distributions falling almost entirely below 1 μm (Figure 4). The particle size of microencapsulated oil droplets agreed with the approximate sizes of the dimples observed on the CLAM surface (Figure 2a) and the dark indentations throughout the cross-section (Figure 2b). The sub-micron oil droplet sizes achieved by homogenization facilitated the effective encapsulation of the corn oil in spray-dried CLAMs. For most spray-drying wall materials, smaller emulsion droplet diameter in the feed results in less non-encapsulated oil on the microcapsule surface (Sootitawat, Yoshii, Furuta, Ohkawara, & Linko, 2003). Sootitawat et al (2003) suggested that coarser emulsion droplets (distributions exceeding 5 μm) break down upon atomization, leading to elevated surface oil content; meanwhile, fine emulsions (0 to 5 μm) experienced no size reduction upon atomization and dried into microcapsules with minimal surface oil. The oil droplets in our study were an order of magnitude smaller due to high-pressure homogenization. Consequently, there was minimal shift

in oil droplet size distribution before and after spray-drying (Figure 4). Atomization did not reduce the size of oil droplets; rather, size distributions increased and broadened slightly after spray-drying, which was attributable to the increased complexity of the emulsion system being measured.

4.3. Oil droplets are evenly distributed throughout CLAMs

There were no oil losses during the spray-drying process; all oil in the feed suspension was encapsulated in the CLAM powder. Although the surface indentations of 25% oil CLAMs in the SEM images suggest the presence of oil droplets at the outer surface of the particles (Figure 2a), the absence of strong fluorescence intensities at the outer surfaces of the particles in the LSCM images verified there was no preferential localization of oil at the surface. The presence of surface oil is common for matrix-type microcapsules. Successful spray drying minimizes surface oil content to improve oxidative protection and retention of volatiles, but the nature of matrix microcapsules precludes complete elimination of surface oil (Jafari et al., 2008).

An attempt at quantifying surface oil following commonly practiced hexane washing procedure (Wang et al., 2009) removed in excess of 40% of the total oil from CLAMs. Given the microscopy evidence, it is likely that our attempts to measure surface oil grossly overstate the quantity of oil on the surface of spray-dried CLAMs. By comparison, the same hexane extraction procedure removed 2% of total core material from carvacrol-loaded CLAMs prepared by external gelation (Wang et al., 2009). The matrix-type CLAMs in the Wang et al study are comparable in composition, but spray-dried CLAMs are approximately two orders of magnitude smaller in size. We attribute the high hexane extractable oil in spray-dried CLAMs to their small size ($D_{4,3}$ ranged from 8 to 18 μm). During extraction, the hexane solvent may penetrate a certain average distance below the CLAM surface and extract all oil in that penetrated shell (Figure 7).

If oil is distributed uniformly throughout the CLAM volume, the hexane extractable oil is the ratio of the penetrated shell volume to the CLAM volume. For a CLAM with a diameter of 10 μm and hexane extractable oil of 41%, the corresponding hexane penetration depth was 0.8 μm . Assuming this penetration depth is constant and independent of radius, Figure 7 shows how the hexane extractable oil increases dramatically as the CLAM radius decreases. For CLAMs with a diameter of 600 μm as produced by Wang et al (2009), the predicted hexane extractable oil is 0.8%, which is in reasonable agreement with their measured value of 2%. Thus, while solvent extraction procedures are common for determining non-encapsulated oil in spray-drying literature, this procedure breaks down for small particles of diameters $\leq 10\text{ }\mu\text{m}$.

4.4. Spray-dried CLAMs are an enteric system

Spray-dried CLAMs demonstrated enteric behavior, retaining the majority of their cargo under gastric conditions while experiencing nearly complete release under intestinal conditions. To facilitate comparisons with previous studies involving CLAMs prepared by external gelation, oil release was assessed in simulated gastric and simulated intestinal buffers prepared based on U.S. Pharmacopeia recipes. Despite spray-dried CLAMs being two orders of magnitude smaller in diameter, the release of oil in gastrointestinal conditions was generally in agreement with the release of oil from CLAMs prepared by external gelation. In one study which included digestive enzymes in their U.S. Pharmacopeia-based digestion buffers, CLAMs released 20% of their cargo after 2 h in SGF and 70% once transferred to SIF for 2 h (Wang et al., 2009). In a similar study which did not include digestive enzymes, α -tocopherol-loaded CLAMs released approximately 20% of their cargo after 2 h in SGF and 55% after 2 h in SIF (Yoo et al., 2006). Despite their smaller particle size, spray-dried CLAMs retained a comparable fraction of their cargo in Pharmacopeia gastric buffer. Intestinal release at 2 h was greater for spray-dried

CLAMs than for externally-gelled CLAMs due to their considerably smaller particle size, which favors more rapid dissolution. Similar gastric retention and enhanced intestinal release suggest that spray-dried CLAMs provide an improved enteric system compared to larger CLAMs prepared by external gelation.

Oil release in Minekus 2014 SGF mimicking human gastric electrolyte composition and concentrations was greater than oil release in Pharmacopeia SGF containing only NaCl at the same pH of 1.5. At this pH, the carboxyl groups on the alginates will be predominantly protonated in both SGFs and the CLAMs would most likely remain insoluble as acid gels. Proximity of the alginates facilitate hydrogen bonding between alginates, further shrinking the particles in the solution (Doumeche et al., 2004). The difference in electrolyte concentration and composition in the two SGFs appear to play a role in oil release from the particles, most likely by affecting particle properties (e.g. by exerting secondary hydration forces or by charge screening (Israelachvili, 2006)). The precise mechanism by which such interactions influence oil release from the CLAMs, however, cannot be fathomed from these studies.

The release of oil in Minekus 2014 SIF was consistent with the release in Pharmacopeia SIF, with over 80% released after 2 h. When immersing CLAMs in SIF, the mechanism of release is the sequestration of calcium ions from the alginate matrix by carbonate and phosphate ions, resulting in the dissolution of the alginate matrix (Draget & Taylor, 2011). It is worth noting that the release mechanism is different when performing simulated gastric and intestinal digestion sequentially, although the same end result is achieved. In low pH gastric fluid, protons displace calcium ions in the alginate matrix, forming an alginic acid gel. When emptied into the intestine, the gel is exposed to high relative concentrations of monovalent cations such as sodium, which displace protons, causing the alginic acid gel to disintegrate and release its cargo

(Ostberg et al., 1994). Here, dissolution of CLAMs was visibly observed after 2h in both Pharmacopeia and Minekus 2014 SIF. The near complete release of microcapsule payload in intestinal conditions is highly desirable for intestinal delivery applications.

Prior to intestinal release, it is desirable for CLAMs to release minimal oil in the gastric environment. In the low pH gastric environment, alginate becomes highly protonated, displacing calcium from the matrix but leaving the alginate matrix morphology and swelling largely unchanged (Ostberg et al., 1994). In this study, oil release in SGF was strongly dependent on pH. At pH 1.5, which corresponds to an empty stomach, the release in Pharmacopeia and Minekus 2014 SGF was significantly lower than the release at pH 3.0, which corresponds to a full stomach. At pH 1.5, which is well below the pKa of the alginate carboxylic acid groups, extensive protonation of the alginate to insoluble alginic acid inhibits dissolution and swelling of the polymer. However, at pH 3.0, only slightly below the pKa of the carboxylic acid groups, the alginate is less protonated. Calcium in the CLAMs may be displaced by monovalent cations, inducing some swelling and gradual dissolution of alginate.

This study demonstrates that spray-dried CLAMs are a promising enteric system, minimizing gastric release while releasing the vast majority of payload in the intestinal environment. The oil release results are in agreement with previous studies of CLAMs. However, it is important to note that the simulated gastrointestinal conditions are characteristic of an empty stomach, featuring relatively low solids and high rate of mixing. In the full stomach condition, which may be relevant if CLAMs were incorporated into a food product or tableted and consumed with food, the powder would experience a relatively high solids and low mixing environment. The use of a digestion model encompassing a food bolus and pH gradients in the

stomach would shed more light on release behavior of CLAMs in the human gastrointestinal system.

5. Conclusion

Corn oil was effectively microencapsulated in a cross-linked alginate matrix in two scalable steps: homogenization and spray-drying. This novel process is commercially scalable and involves considerably fewer unit operations than existing methods for CLAM preparation. While corn oil was used as a model lipid in this study, this research suggests that our novel process could be applied to encapsulate lipophilic bioactive compounds for food and pharmaceutical applications. Oil loadings up to 35% were achieved, and modifications to the wall material formulation may facilitate the preparation of CLAMs with oil loading beyond 35%. Spray dried CLAMs were micron scale and contained emulsified oil droplets on the order of hundreds of nanometers. The emulsion maintained stability throughout the encapsulation process. Oil droplets were evenly distributed throughout the volume of the microcapsules, not concentrated near the surface. Oil release in simulated gastric fluid was highly pH dependent, with lower release at pH 1.5. The high oil release in simulated intestinal fluid and comparatively low release in simulated gastric fluid suggest that spray dried CLAMs may be a suitable vehicle for delivering bioactive lipophiles to the intestine. Overall, this study demonstrated a novel CLAM preparation method that can be readily scaled up in the food industry. The use of this technique to microencapsulate bioactive lipophiles may facilitate their inclusion in foods and delivery in the human gastrointestinal tract.

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Figure Captions: *In situ* cross-linking of alginate during spray-drying to microencapsulate lipids in powder

Figure 1. Powder size distributions for spray-dried CLAMs.

Figure 2. SEM images of CLAMs. 25% oil CLAMs appear approximately spherical with dimpled surfaces (A). Cross-section of 25% oil CLAMs shows oil droplet distribution within these CLAMs (B). 0% oil CLAMs have a collapsed appearance with smooth surfaces (C). Scale bars represent 5 μm .

Figure 3. Confocal cross-sections of 20% oil CLAMs. XY sections with vertical separation of 1 μm show regions of diffuse fluorescence across CLAMs at all depths, indicating the dyed oil droplets were evenly distributed throughout the CLAM volume. Depth indicates displacement into the sample in the direction away from the cover glass.

Figure 4. Size distributions of oil droplets. Size distributions are shown before (—) and after (---) encapsulation for 15% (A), 25% (B), and 35% (C) oil CLAMs.

Figure 5. Release of oil from 25% oil CLAMs in simulated gastric and intestinal fluids based on the U.S. Pharmacopeia (A) and Minekus et al (2014) (B).

Figure 6. Schematic of novel method of CLAM production contrasted with conventional external gelation process.

Figure 7. Theoretical solvent penetration during hexane extraction. Hexane solvent penetrates a distance (a) below the CLAM surface, removing oil from the penetrated shell. If oil is evenly distributed throughout the CLAM volume, hexane extractable oil becomes the ratio of the penetrated shell volume to the CLAM volume. Assuming solvent penetration depth (a) is

constant ($a = 0.8 \mu\text{m}$ in the chart) and independent of CLAM radius (b), hexane extractable oil is expected to increase dramatically with decreasing CLAM radius.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Table 1. Oil loading and powder size of CLAMs

Sample	Measured Oil Loading (%)	Powder Size Measurement (μm)			
		D _{4,3}	D(0.1)	D(0.5)	D(0.9)
15% Oil CLAMs	17.0 ± 1.0	8.1 ± 0.2	1.3 ± 0.01	6.0 ± 0.1	13.9 ± 0.3
25% Oil CLAMs	26.3 ± 1.4	11.8 ± 0.7	1.3 ± 0.05	6.2 ± 0.5	21.4 ± 1.9
35% Oil CLAMs	35.9 ± 2.5	17.9 ± 0.3	1.3 ± 0.01	6.4 ± 0.1	54.9 ± 1.3
0% Oil CLAMs	--	9.4 ± 0.1	1.5 ± 0.09	8.7 ± 0.1	17.2 ± 0.3