**Casein-maltodextrin conjugate as an emulsifier for fabrication of structured calcium
carbonate particles as dispersible fat globule mimetics**

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Abstract

Dispersible fat globule mimetics (FGMs) are demanded in manufacturing reduced fat products. The objective of the present study was to fabricate a group of FGMs with calcium carbonate particles (CCPs) as the core, coated with two sequential layers of anhydrous milk fat (AMF) and milk protein. The fabrication involved two steps: emulsifying an oil phase with CCPs suspended in AMF into an aqueous phase with dissolved emulsifiers to create an S/O/W suspension and spray drying the suspension. Experimentally, CCPs were suspended at 20-33.3% w/w in melted AMF at 80 °C that was then emulsified at 5% w/v into a neutral aqueous phase with 5% w/v sodium caseinate (NaCas), 10% w/v casein–maltodextrin (MD) conjugates, or 5% w/v NaCas and 5% w/v MD. Particle structures, encapsulation performance, and properties of skim milk hydrated with spray-dried powder were studied. The casein-MD conjugate treatment had the smallest particles (3.25 μm), the best encapsulation performance, and the best stability in skim milk (an increase of particle size by 30.5% after 10-day storage at 5 °C). Overall, casein-MD conjugates are better emulsifiers than NaCas in preparation of the studied FGMs that may be used to simultaneously improve sensory properties and increase the calcium content of reduced-fat products.

Keywords: Casein-maltodextrin conjugates; calcium carbonate particle; S/O/W suspension; fat globule mimetics; reduced fat dairy products.

1. Introduction

Skim milk produced after removing fat globules in full fat milk is associated with quality defects such as the altered appearance, i.e., translucency and bluish color (Drake, 2007), losses of flavors, richness, and creaminess native to dairy products, and formation of undesirable flavors due to processing (Ohmes, Marshall, & Heymann, 1998). Particulates such as those of modified starch, polysaccharide, and/or protein have been studied to mimic the characteristic physicochemical properties of fat globules (Liu, Xu, & Guo, 2007; Ma, Cai, Wang, & Sun, 2006; McClements & Demetriades, 1998). Most mimetics, however, can only replace partial properties of fat globules.

On the other hand, improving nutritional quality of reduced fat dairy products, such as increasing calcium content, is demanded. Calcium is an essential mineral for human nutrition, with the currently recommended dietary allowance being 1000 mg daily for adults (Ross, et al., 2011). Dairy products are important sources of calcium, but one 8-ounce (~240 mL) cup of skim milk has only 250-300 mg calcium (Wang & Li, 2008). Calcium carbonate, citrate, gluconate, and lactate are forms of calcium used in dietary supplements (Straub, 2007). Due to the highest percentage of elemental calcium (40% w/w) and chemical stability, calcium carbonate is the most widely used form of calcium (Kopic & Geibel, 2013). However, low solubility and sensory defects are two major concerns when incorporating calcium carbonate particles (CCPs) in liquid products. Conversely, direct addition of ionic calcium may induce the aggregation of food proteins during processing and storage. Therefore, effective methods of fortifying calcium in liquid food products are to be studied.

Properly designed colloidal particles may solve many challenges of incorporating calcium in liquid foods. Fabricating these particles requires ingredients such as milk proteins and lipids to

obtain physicochemical properties needed for their functionalities. Sodium caseinate (NaCas) is commonly used as an emulsifier in fabricating delivery systems. Conjugating proteins with reducing saccharides such as maltodextrins (MD) can improve emulsifying and stabilizing functions important to delivery systems (Akhtar & Dickinson, 2007; O'Regan & Mulvihill, 2010; Shah, Ikeda, Michael Davidson, & Zhong, 2012). Conjugation is commonly done by heating mixtures of proteins and reducing saccharides in solutions or their powder via the Maillard reaction (Oliver, Melton, & Stanley, 2006). Currently, very little has been done to produce structured particles to disperse CCPs using protein–saccharide conjugates in the form of solid/oil/water (S/O/W) emulsions.

In the present study, the first objective was to study a novel group of dispersible fat globule mimetics (FGMs) by coating CCPs with a layer of anhydrous milk fat (AMF) emulsified by dairy proteins. AMF was used to compensate density of CCPs and partially improve flavor and texture properties, while reducing lipid content. To produce FGMs, CCPs were suspended in the melted AMF dissolved with Span[®] 80 that was used to reduce S/O and W/O interfacial tensions. The S/O suspension was emulsified in the aqueous phase with NaCas, NaCas-MD mixture, or casein-MD conjugates, followed by spray drying. In addition to encapsulation performance, FGMs were studied for particle size distribution and structures using confocal laser scanning microscopy (CLSM). The second objective was to study impacts of addition of spray-dried FGMs on turbidity, fat content, viscosity, and color of skim milk. The FGMs studied may improve quality properties of reduced fat dairy products within the regulated overall fat level.

2. Materials and methods

2.1. Materials

AMF was obtained from Land-O-Lakes, Inc. (Arden Hill, MN, USA). NaCas was purchased from American Casein Co. (Burlington, NJ, USA). MD, with average dextrose equivalent of 18, was supplied by Grain Processing Corp. (Muscatine, IA, USA). Food grade CCPs under trade name of ViCALity[®] Albafil[®] were received as a gift from Minerals Technologies, Inc. (Bronx, NY, USA). Pasteurized skim milk and full-fat milk (The Simple Truth[™] brand) were products from Kroger Co. (Cincinnati, OH, USA). Supplies in protein electrophoresis were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA)

2.2. Preparation of casein-MD conjugates

NaCas and MD were hydrated at 5.0% w/v each in deionized water overnight at room temperature (~21 °C). The mixture solution was adjusted to pH 7.0 followed by spray drying at an inlet temperature of 170 °C, a feed rate of 12%, and an airflow rate of 35 m³/h using a B-290 mini spray-dryer (BÜCHI Labortechnik AG, Flawil, Switzerland). The outlet temperature was recorded to be 90-100 °C. The spray-dried powder was incubated at 80 °C and 79% relative humidity for 2.5 h to induce conjugation in a humidity-controlled incubator (model IG420U Environmental chamber, Yamato Scientific America Inc., Santa Clara, CA, USA). The powder was then collected and stored at -18 °C before further use.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS-PAGE was performed according to a literature method (Wang & Zhong, 2014) with some modifications. The protein sample was prepared by mixing 0.5 mg/mL powder in a SDS-PAGE loading buffer, followed by heating at 95 °C for 5 min in a water bath. A 15% precast linear gradient Tris-HCl polyacrylamide gel was used in electrophoresis at a constant voltage of

200 V for 40 min using a Mini Protean Tetra Cell. The gel was stained with Coomassie Blue.

2.4. Encapsulation of CCPs

AMF was melted by heating at 80 °C, followed by adding varying amounts of CCPs and Span[®] 80. The CCP: AMF: Span[®] 80 mass ratios were studied for 1:2:0.5, 1:2:1, 1:2:1.5, and 1:2:2. The mixture was blended at 15,000 rpm for 5 min using a high-speed homogenizer (model T 25 digital ULTRA-TURRAX[®], IKA[®] Works, Inc., Wilmington, NC, USA) to prepare an S/O suspension. The aqueous phase was prepared by hydrating 5% w/v NaCas, 5% w/v NaCas and 5% w/v MD mixture, or 10% w/v casein-MD conjugate in deionized water at room temperature (21 °C) overnight and adjusting pH to 7.0 using 1.0 M HCl or NaOH. The S/O suspension was emulsified at 5% w/v into the aqueous phase using the above high-speed homogenizer at 15,000 rpm for 5 min. The S/O/W suspension was spray-dried as in Section 2.2, with the exception of the feed rate being 10%.

2.5. Particle size analysis

Particle size distributions of suspensions were determined using a laser (wavelength being 750 nm) diffraction particle size analyzer (LS 13 320, Beckman Coulter Inc., Brea, CA, USA). Samples were stirred at 300 rpm for 20 min before measurement to fully suspend particles. A sample was pipetted into the measurement chamber containing water until an optimum obscuration rate between 8 and 12% was obtained. The particle size distribution was calculated using the light scattering pattern based on the Mie theory. A refractive index of 1.33 and 1.472 was set for the continuous and dispersed phase, respectively (Saveyn, Le Thu, Govoreanu, Van der Meeren, & Vanrolleghem, 2006). The volume-weighted mean diameter ($d_{4,3}$, Eq. 1) was compared because it is more sensitive than area-based mean diameter ($d_{3,2}$) for systems with large particles (McClements, 2004).

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad (1)$$

where n_i is the number of particles with diameter d_i .

2.6. Microstructure of particles

CLSM was conducted using a Leica TCS SP2 confocal microscope (Leica Microsystems, Heidelberg GmbH, Germany) in the fluorescence mode. Nile red was dissolved at 0.2 mg/mL in the lipid phase before preparing the S/O suspension, and the S/O/W suspension prepared as above was added with 0.4-0.6 mg/mL fluorescein isothiocyanate (FITC) to stain proteins. Confocal images were acquired using a 63× oil objective with a numerical aperture of 0.7 at an excitation wavelength of 488 and 543 nm for FITC and Nile red, respectively. The emission spectrum was recorded between 512 and 542 nm for FITC and between 557 and 625 nm for Nile red. The acquired images were analyzed using the Leica Confocal software. Pictures acquired for protein and fat in the same field of observation were also merged to present an overview of structures.

2.7. Encapsulation properties

2.7.1. Mass yield of spray dried samples

Mass yield was defined as the percentage of the collected mass of spray-dried powder with reference to the non-solvent mass in the corresponding suspension (feed) before spray drying:

$$\text{Mass yield\%} = (\text{Mass of collected product} / \text{Non-solvent mass in the feed}) \times 100 \quad (2)$$

2.7.2. CCP loading

One gram of the spray dried powder was added into 15 mL of 0.1 N HCl to dissolve unencapsulated CCPs. After vortexing for 5 min, the suspension was centrifuged at 4194 g and 5 °C for 30 min (Sorvall RC 5B Plus centrifuge, DuPont, Norwalk, CT, USA). The precipitate was collected and resuspended in 15 mL of 0.1 N HCl, followed by centrifugation as above. The

process was repeated for a third time to remove free CCPs. The final precipitate was washed using deionized water, followed by drying at 58 °C overnight in an oven (Precision Economy Oven, Thermo Scientific, Barrington, IL, USA). The ash content of the sample was then measured based on the AOAC Official Method 950.49 with some modification (AOAC, 2006). Briefly, the dried precipitate was contained in a pre-ashed ceramic crucible and heated in a model 1400 Thermolyne furnace (Barnstead, Dubuque, IA, USA) at 550 °C overnight (12-18 h). After cooling in a desiccator, the sample was weighed to determine the amount of ash. The same amounts of Span[®] 80, AMF, NaCas, NaCas-MD mixture, and casein-MD conjugates as in fresh S/O/W suspension were also measured similarly. The net mass of ashed CCPs (CaO) was converted to the mass of calcium carbonate by dividing 56% (mass percentage of CaO with respect to CaCO₃). The actual loading of CCP was then determined according to Eq. (3).

$$\text{Loading\%} = \frac{\text{Mass of encapsulated CCP in spray-dried powder}}{\text{Total mass of spray-dried powder}} \times 100 \quad (3)$$

2.7.3. Encapsulation efficiency (EE)

The EE was defined as the percentage of encapsulated CCP with respect to the CCP mass before spray-drying (Eq. 4).

$$\text{EE\%} = \frac{\text{Mass of encapsulated CCP in spray-dried powder}}{\text{Mass of CCP before spray drying}} \times 100 \quad (4)$$

Because factors such as sample collection errors from the lab-scale spray dryer and a small amount of solids being processed can cause inaccurate assessment of encapsulation (Eq. 4), another parameter, loading efficiency%, was calculated to reflect how well different components in the feed were retained according to their proportion after spray drying (Eq. 5). This was based on comparing the maximal loading (Eq. 6), assuming the removal of solvent (water) only and 100% encapsulation after spray drying, and the actual loading determined in Eq. 3.

$$\text{Loading efficiency\%} = (\text{Actual loading/Maximal loading}) \times 100 \quad (5)$$

Maximal loading% = (Mass of CCPs in the feed/Non-solvent mass in the feed) ×100 (6)

2.8. Scanning electron microscopy (SEM)

The morphology of CCPs and spray-dried powder was observed using SEM (LEO 1525 SEM, LEO Electron Microscopy, Oberkochen, Germany). A small amount of powder was spread onto an adhesive tape fixed on a stainless steel stub and coated with a gold layer of about ~5 nm thickness before imaging.

2.9. Properties of skim milk hydrated with spray-dried powder

Spray-dried FGMs prepared with casein-MD conjugates were hydrated at 1, 2, 5, and 10% w/v in skim milk at room temperature (~21 °C) for 2 h while being mildly stirred on a magnetic stirring plate. Sodium azide was added at 0.02% w/v as an antimicrobial to prevent microbial spoilage. Three independent replicates were prepared for each sample. Full-fat milk of the same brand was compared to skim milk.

2.9.1. Turbidity

The turbidity of milk samples with and without FGMs was measured for absorbance at 600 nm using UV–vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA). Each sample was diluted 200 times in deionized water. Triplicate samples were measured.

2.9.2. Fat content

Total fat content of full-fat milk and skim milk with and without FGMs was measured using the Mojonnier method, based on the AOAC Official Method 989.05 (AOAC, 2006). Two gram of milk sample was weighed into a Mojonnier fat extraction flask. Then 0.3 mL of ammonium hydroxide was added and mixed with milk, followed by adding 2 mL of ethanol and mixing. Subsequently, 5 mL of diethyl ether was added in the mixture and was vortexed vigorously for 90 s, followed by adding 5 mL of petroleum ether and shaking for another 90 s. The final

mixture was centrifuged (model Sorvall RC 5B Plus, DuPont, Norwalk, CT, USA) at 4194 g for 1 min, and the top organic phase was transferred into a pre-dried clean dish. The remaining aqueous phase was extracted two more times using the above procedures. The organic phase from three extractions was combined and the organic solvents were evaporated in a model N7595-1 vacuum oven (Baxter Healthcare Corp. Deerfield, IL, USA) at 100 °C for 1 h. After cooling to room temperature in a desiccator, the mass of dish with lipids was determined. A blank sample was performed using 2 g of water to replace milk. The total fat content was then calculated using Eq. (7):

$$\text{Fat\%} = [(\text{Fat mass} - \text{blank mass}) / \text{Total sample mass}] \times 100 \quad (7)$$

2.9.3. Viscosity

The shear viscosity of samples was measured using a cone-plate set of an AR2000 rheometer (TA Instruments, New Castle, DE, USA). The aluminum cone has an angle of 1° and a diameter of 40 mm. The shear rate ramps were conducted from 0.1 to 100 s⁻¹ at 21 °C for milk samples and 80 °C for the mixtures of Span[®] 80 and AMF. Samples were measured in triplicate.

2.9.4. Color measurement

The influence of FGMs on the color of skim milk was determined using a MiniScan XE Plus Hunter Colorimeter (Hunter Associates Laboratory, Inc., Reston, VA, USA). The instrument was calibrated with a standard white tile ($L = 88.2$, $a = 0.309$ and $b = 0.316$) before measurement. The Hunter chromaticity coordinates (L^* , a^* , b^*) of each sample were measured three times. Based on Eq. 8, the total color difference of skim milk (ΔE_1) and whole milk (ΔE_2) was additionally calculated using the differences between L^* , a^* and b^* values of a sample with respect to those of the white tile (Chung, Rojanasasithara, Mutilangi, & McClements, 2015).

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (8)$$

2.10. Statistical analysis

All experiments were carried out at least in duplicate, and the mean and standard error of replications were reported. Duncan analysis was used to compare significant differences of encapsulation properties, particle size of FGMs, and physicochemical properties of skim milk hydrated with spray-dried FGMs. SPSS 16.0 statistical analysis system (SPSS Inc., Chicago, IL) was used. The significance level (P) was set at 0.05.

3. Results and Discussion

3.1. SDS-PAGE analysis of conjugates

SDS-PAGE was performed to confirm conjugation of casein and MD (Fig. 1). The unprocessed NaCas and NaCas-MD mixture showed a weak band of κ -casein (MW = 19 kDa), a major band that did not allow the clear separation of α - (MW = 23.5 kDa) and β -caseins (MW = 24 kDa) at the studied conditions, and some fragments (Anema & De Kruif, 2013). A distinct increase in MW by ~4 kDa of the major band was observed for the casein-MD conjugates sample, verifying successful conjugation.

3.2. Properties of fresh S/O/W suspensions

3.2.1. Droplet size

S/O/W suspensions prepared with various amounts of Span[®] 80 were measured for particle dimension. These suspensions were prepared with an aqueous phase with 5% w/v NaCas, NaCas-MD mixture with 5% w/v each, and 10% w/v casein-MD conjugates. The results (Fig. 2) showed that increasing Span[®] 80 content led to a decrease in $d_{4,3}$. The findings agreed with earlier studies indicating that the addition of Span[®] 80 as a surfactant decreased the particle size of emulsions primarily emulsified by Tween[®] 80 (Leong, Wooster, Kentish, & Ashokkumar,

2009). The viscosity of dispersed phase and the O/W interfacial tension are two important parameters determining the disruption of the dispersed phase during high shear homogenization (Jafari, Assadpoor, He, & Bhandari, 2008; Vladisavljević, Shimizu, & Nakashima, 2006). A higher ratio of dispersed: continuous phase viscosity requires a higher critical shear stress to break oil droplets, which in our study would result in bigger droplets because of the same shear homogenization conditions. When the AMF and Span[®] 80 mixtures were measured, the viscosity was higher at a higher percentage of Span[®] 80 (Fig. 3). Therefore, smaller $d_{4,3}$ at a higher concentration of Span[®] 80 can be contributed by a greater reduction of O/W interfacial tension during homogenization.

When treatments with different surfactants in the aqueous phase were compared, casein-MD conjugates resulted in the smallest $d_{4,3}$, and treatments with the NaCas-MD mixture had smaller $d_{4,3}$ than those with NaCas only (Fig. 2). The better emulsifying properties of conjugates than unconjugated mixture of protein and oligosaccharides are well-known (Dickinson, 2011). As for the NaCas treatments with and without MD, the aqueous phase with MD has a higher viscosity and therefore a lower ratio of dispersed: continuous phase viscosity, which requires a smaller critical shear stress to disrupt droplets (Dickinson, 2008; O'Regan & Mulvihill, 2009), resulting in smaller droplets. Physically, the increased continuous phase viscosity also reduces the Brownian motion of droplets and thus droplet coalescence during homogenization (O'Regan, et al., 2010). The treatment with CCP:AMF:Span[®] 80 mass ratio of 1:2:2 in the S/O suspension was chosen for further studies.

3.2.2. *Structure of particles*

The CLSM micrographs are presented in Fig. 4. FGMs prepared with the NaCas-MD mixture had bigger particles (up to ~10 μm) that showed some flocculation (Fig. 4A). In contrast,

discrete particles were observed in the dispersion prepared with casein-MD conjugates (Fig. 4B) and were smaller ($< 7 \mu\text{m}$). The CLSM results were in good agreement with the above particle size analysis that showed the $d_{4,3}$ of 7.41 ± 0.11 and $5.35 \pm 0.77 \mu\text{m}$ for the suspension prepared with the NaCas-MD mixture and conjugates, respectively (Fig. 2).

3.3. Properties of spray-dried capsules

3.3.1. Morphology of spray-dried capsules

SEM images of spray-dried capsules are presented in Fig. 5. CCPs had heterogeneous structures with varying dimensions that were mostly below $1 \mu\text{m}$ (Fig. 5A). Spray-dried samples had mostly spherical particles with a size from 5 to $10 \mu\text{m}$ (Fig. 5B-D). These dimensions were similar to fresh suspensions (Fig. 4), suggesting the presence of one capsule per spray-dried particle. This indicates the particles in S/O/W suspensions are structurally robust enough to prevent coalescence during spray drying (Rodea-González, et al., 2012). The sample prepared with NaCas only had wrinkled surface (Fig. 5B), while those of NaCas-MD mixture or conjugates had smoother surfaces. The difference likely was caused by the smaller amount of solids in the aqueous phase containing NaCas only that caused wrinkling when the solvent in the atomized droplets is rapidly evaporated during drying (Wang, Jiang, & Zhou, 2013).

Additionally, it has been reported that MD can impact glass transition of proteins during solidification and result in spray-dried particles with a smooth surface (Danviriyakul, McClements, Decker, Nawar, & Chinachoti, 2002; Franceschinis, Salvatori, Sosa, & Schebor, 2014). Also, MD fills in defects on the particle surface (Ferrari, Germer, & de Aguirre, 2012). Several particles prepared from the NaCas-MD mixture showed minor defects such as holes and cracks (Fig. 5C), caused by water evaporation, while those of conjugated NaCas did not (Fig. 5D). The difference can be explained by the fact that casein-MD conjugates are more surface

active than NaCas and have better properties in forming solid films during drying atomized droplets.

3.3.2. Encapsulation performance

The encapsulation performance is summarized in Table 1. The treatments with MD, free or conjugated, had similar mass yields that were much lower than that with NaCas only ($P < 0.05$). It is known that MD is hygroscopic, lowers the drying rate, and increases the stickiness of powder (Goula & Adamopoulos, 2008; Maury, Murphy, Kumar, Shi, & Lee, 2005). Due to this property, it is difficult to collect powder from the drying chamber, which lowers the mass yield. The low mass yield resulted in low EE (Table 1) as estimated according to Eq. 4. This is typical in lab-scale spray drying experiments using a small sample volume and a low concentration of solids (Prinn, Costantino, & Tracy, 2002).

The loading of CCPs in spray-dried powders was evaluated (Table 1), showing the increasing order of NaCas < NaCas-MD mixture < casein-MD conjugate treatments. This enables the additional evaluation of encapsulation performance by comparing to the maximal loading (Eq. 6), using the term of loading efficiency% (Eq. 5). The casein-MD conjugate treatment had a much higher loading efficiency% (92.1%) than those of NaCas-MD mixture (67.3%) and NaCas only (33.7%). This suggests that CCPs were encapsulated efficiently in droplets by casein-MD conjugates, droplets maintained their structures and most components in the S/O/W suspension precipitated according to their proportions in atomized droplets during spray drying. This can again be explained by the better surface activity of casein-MD conjugates than caseins enabling better emulsification of S/O suspensions and better film forming properties during spray drying (Rodea-González, et al., 2012). The exact interfacial and transport phenomena however are to be studied.

3.4. Physicochemical properties of skim milk hydrated with spray-dried FGMs

The stability of FGMs prepared from NaCas, NaCas-MD mixture, and casein-MD conjugates in skim milk was first compared by visual inspection of precipitation and quantification of particle size changes during storage at 5 °C for 10 days. Spray-dried FGMs prepared from casein-MD conjugates were then hydrated at 1, 2, 5 and 10% w/v in skim milk, and several quality parameters were quantified and compared to those of full-fat milk.

3.4.1. Particle size distribution and stability

Particle size distributions of skim milk hydrated with 10% w/v of three types of FGMs before and after storage at 5 °C for 10 days are shown in Fig. 6. Skim milk had a mono-modal distribution with $d_{4,3}$ of 0.46 µm. After hydrating spray-dried FGMs prepared with NaCas, there was a peak overlapping that of skim milk and a second peak centered on around 6 µm (Fig. 6A). The distribution of the smaller group of particles did not change after storage, while the other group grew to be over 10 µm. Two overlapping peaks were observed for the treatment of NaCas-MD mixture in skim milk, which also showed the growth of the bigger group of particles after storage (Fig. 6B). The treatment with casein-MD conjugates had a broad peak with smaller particles than the other two treatments, and the increase in particle size after storage was also the least significant among three treatments (Fig. 6C). It was also observed that particle dimensions of skim milk with hydrated FGMs (Fig. 6) were smaller than those of fresh suspensions (Fig. 2). This indicates spray-dried powder was hydrated well in skim milk. The smaller particles of FGMs hydrated in skim milk than those in fresh dispersions may result from the increased viscosity in milk and additional surface-active proteins in milk improving the stability of dispersed droplets against aggregation and coalescence (Rouimi, Schorsch, Valentini, & Vaslin, 2005).

The properties of FGMs in skim milk were additionally studied using CLSM (Fig. 7). Similar to fresh dispersions (Fig. 4), the dimension of FGMs hydrated in skim milk followed the decreasing order of NaCas, NaCas-MD mixture, and casein-MD conjugate, with respective average particle size of 6.06, 5.01, and 3.25 μm . Flocculated particles were observed for those prepared with NaCas and its mixture with MD, while those of casein-MD conjugates were discrete. The dimension ranges of FGMs hydrated in skim milk observed in CLSM (Fig. 7) were similar to particle size distributions in Fig. 6.

The stability of FGMs in skim milk was compared during refrigerated storage for 10 days. It was observed that the treatments prepared using NaCas with and without MD showed precipitation, while no obvious precipitation was observed in the treatment prepared with casein-MD conjugates (photographs not shown). Changes in $d_{4,3}$ of treatments are listed in Table 2. The treatment prepared using NaCas, with and without MD, showed a three-fold increase in $d_{4,3}$ after 10-day storage, while the increase was only 30.5% for that prepared with casein-MD conjugates. The particle size distributions of skim milk with FGMs are shown in Fig. 6. A distinct peak of bigger particles was observed for all treatments indicating the aggregation or coalescence of FGMs during storage. Overall, FGMs prepared with casein-MD conjugates had the best stability. This is well established that, during emulsification, the protein moiety of conjugates is anchored on oil droplets whereas the conjugated MD protrudes in the continuous aqueous phase, which provides a thick steric stabilizing layer inhibiting the aggregation and coalescence of oil droplets (O'Regan, et al., 2009; Oliver, et al., 2006).

3.4.2. Turbidity and fat content

The turbidity of skim milk with various amounts of spray dried FGMs prepared with casein-MD conjugates was compared with full-fat milk, presented in Table 3 for absorbance at 600 nm

after 200-fold dilution. The increase in turbidity was observed for a larger amount of FGMs as micrometer-sized particles (Table 2) scattered light more extensively. The treatment with 10% w/v FGMs showed similar turbidity as full-fat milk. Fat content is another critical concern in studying FGMs, since AMF was used in encapsulating CCPs. Based on the U.S. Code of Federal Regulations (CFR, 2016), milk containing less than 0.5% fat can be labeled as “skim”. It was observed that increases in fat content were insignificant when 1% and 2% FGMs were added to skim milk (Table 3). The total fat content of skim milk with 5% and 10% FGMs had a respective total fat content of 1% and 2% that may be used in reduced fat milk and other dairy products to fortify calcium.

3.4.3. Viscosity analysis

Increasing the viscosity of skim milk is an important goal in studying FGMs. Viscosity of skim milk with various amounts of spray-dried FGMs prepared with casein-MD conjugates was compared with full-fat milk at the shear rate range of 1–100 s⁻¹ (Fig. 8). Both skim and whole milk showed the Newtonian behavior (shear-rate independent viscosity). The treatments with 2, 5 and 10% w/v FGMs in skim milk showed the shear thinning behavior. The shear-thinning behavior of colloidal dispersions can result from the breakup of aggregated particles and/or deformation of particles with fluid lipids (Dickinson & McClements, 1995). In the present case, the breakup of FGM particles is more likely because AMF is mostly solid-like at ambient conditions and CCPs are enclosed in FGMs. Furthermore, the shear rate during oral processing of liquid foods is around 50 s⁻¹ (Stokes, Boehm, & Baier, 2013). Skim milk with 2, 5, and 10% w/v FGMs had an apparent viscosity at 50 s⁻¹ being 1.3, 1.7, and 3.6 times of that of full-fat milk, which may increase creaminess (Frøst & Janhøj, 2007).

3.4.4. Color measurements

The influence of adding FGMs prepared with casein-MD conjugates on the color parameters of skim milk was characterized and compared to whole milk (Table 4). The lightness (L^*) decreased after addition of FGMs in skim milk. In addition, greenness (negative a^*) also decreased, which means an increase in redness (positive a^*). On the other hand, yellowness (positive b^*) increased as the FGMs powder concentration increased. Increases in redness and yellowness are expected, due to pigments in conjugates produced during the Maillard reaction (Akhtar, et al., 2007). When compared with skim milk, total color difference (ΔE_1) increased from 1.77 to 6.36 when 1-10% FGMs were added. When compared to whole milk, total color difference (ΔE_2) after adding 1-10% FGMs increased from 5.31 to 7.53. Both these two ΔE results indicated that the difference in color could be visually perceived as the FGM concentration increased (O'Regan, et al., 2009). After storage for 10 days at 5°C, there were no significant changes in color parameters. This indicates the systems were stable because color parameters of emulsions are sensitive to changes in particle size growth (David Julian McClements, 2002). In our recent study, we showed that the color of conjugates can be much reduced if the Maillard reaction is conducted for powder at a high temperature and a short time (130 °C for 15-30 min for whey protein isolate and MD mixture at 79% humidity) (Liu & Zhong, 2015). This strategy can be adopted in real applications if color becomes a concern for specific food products.

4. Conclusions

The present study showed that casein-MD conjugates were more feasible than NaCas in encapsulating CCPs as dispersible FGMs because of the reduced particle size and improved stability against aggregation and coalescence. The reduction of interfacial tension by Span[®] 80

was also critical to reduce the dimension of FGMs. Spray-dried FGMs were hydrated well in skim milk, with those prepared from casein-MD conjugates showing no precipitation during 10-day storage at 5 °C. The addition of spray-dried FGMs prepared from conjugates in skim milk increased the desirable turbidity and viscosity but induced the undesirable redness and yellowness. The addition of 10% FGMs in skim milk resulted in a total fat content of about 2% but the turbidity similar to and viscosity higher than full-fat milk. Therefore, dispersible FGMs prepared from casein-MD conjugates can be used to simultaneously reduce fat and increase calcium contents of food products.

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Figure captions

Fig. 1. SDS-PAGE analysis of NaCas (Lane 2), MD (Lane 3), mixture of NaCas and MD (Lane 4), and casein-MD conjugate (Lane 5). Lane 1 shows protein markers.

Fig. 2. Volume fraction-length mean diameter ($d_{4,3}$) of S/O/W suspensions prepared by emulsifying 5% w/v of a S/O suspension containing CCPs into an aqueous phase with 5% w/v NaCas, 5% w/v NaCas and 5% w/v MD, or 10% w/v casein-MD conjugates. The lipid phase was a binary mixture of Span[®] 80 and milk fat, with the amount of Span[®] 80 in the overall S/O suspension mass plotted on the X-axis. Error bars are standard errors (n = 3).

Fig. 3. Rheograms of mixtures with different mass ratios of anhydrous milk fat (AMF) and Span[®] 80 at 80 °C. Data are averages from 3 replicates.

Fig. 4. CLSM images of S/O/W suspensions prepared by emulsifying 5% w/v of a S/O suspension containing 20% w/w CCPs into an aqueous phase with 5% w/v NaCas and 5% w/v MD (A) or 10% w/v casein-MD conjugates (B). The lipid phase with equal masses of Span[®] 80 and milk fat was stained with Nile Red before preparation of S/O suspensions. The S/O/W suspensions were later stained by green FITC probe. Suspensions were diluted by 10 folds before imaging for FITC (left) and Nile Red (middle) fluorescence, with co-localized images shown on the right.

Fig. 5. SEM images of CCPs as received (A), and spray-dried FGM powders prepared from suspensions emulsified by NaCas (B), NaCas-MD mixture (C), and casein-MD conjugates (D), with S/O suspension conditions detailed in Fig. 4.

Fig. 6. Size distributions of skim milk with 10% w/v spray-dried FGM powder prepared from S/O/W suspensions emulsified by (A) NaCas, (B) NaCas-MD mixture, and (C) casein-MD conjugates before and after storage at 5 °C for 10 days. S/O suspension compositions are detailed

541 in Fig. 4. Data are averages from 3 replicates.

542 Fig. 7. CLSM images of skim milk with 10% w/v spray-dried FGM powder prepared from

543 S/O/W suspensions emulsified by NaCas (A), NaCas-MD mixture (B) or casein-MD conjugates

544 (C). For each sample, images were acquired for FTIC staining proteins in the continuous phase

545 (left) and Nile Red staining lipids (middle), which were also co-localized (right). S/O suspension

546 compositions are detailed in Fig. 4.

547 Fig. 8. Rheograms of skim milk hydrated with 0-10% w/v spray-dried FGM powder prepared

548 with casein-MD conjugates at 21 °C, in comparison to whole milk. Data are averages from 3

549 replicates.

Table 1. Encapsulation properties after spray-drying S/O/W suspensions prepared with various aqueous phase emulsifiers.*

Emulsifier	Maximal loading (% w/w)	Mass yield (%)	Actual loading (% w/w)	Loading efficiency (%)	Encapsulation efficiency (%)
5% w/v NaCas	6.7	54.8±5.8 ^a	2.2±0.5 ^c	33.7±8.9 ^c	18.1±2.7 ^c
5% w/v NaCas + 5% w/v MD	6.7	37.7±1.8 ^b	4.5±0.6 ^b	67.3±9.1 ^b	25.3±2.5 ^b
10% w/v casein-MD conjugates	6.7	37.6±3.8 ^b	6.1±0.2 ^a	92.1±0.4 ^a	34.7±4.6 ^a

*Suspensions were prepared by emulsifying a 5% w/v S/O suspension containing CCP/AMF/Span 80 at a mass ratio of 1:2:2 into the aqueous phase. Values are means ± standard errors of means from three replicates. Different superscript letters represent a significant difference in the mean of the same parameter ($P < 0.05$).

Table 2. Volume fraction-length mean diameters ($d_{4,3}$, μm) of skim milk hydrated with spray-dried FGMs before and after 10-day storage at 5 °C.*

Storage duration (d)	Skim milk (control)	Emulsifier in the aqueous phase used to prepare a suspension for spray drying		
		5% w/v NaCas	5% w/v NaCas + 5% w/v MD	10% casein-MD conjugates
0	0.46±0.01 ^g	6.06±0.38 ^c	5.01±0.29 ^d	3.25±0.35 ^f
10	0.99±0.09 ^g	17.48±0.69 ^a	15.07±0.76 ^b	4.24±0.37 ^e

*Suspensions were prepared by emulsifying a 5% w/v S/O suspension containing CCP/AMF/Span 80 at a mass ratio of 1:2:2 into the aqueous phase. Values are mean $\pm$ standard error of mean from three replicates. Different superscript letters represent a significant difference in the mean ($P < 0.05$).

Table 3. Turbidity and fat content of skim milk with various amounts of spray-dried FGMs, in comparison to whole milk.[#]

Powder% w/v added	Absorbance at 600 nm	Fat content%
0 (skim milk control)	0.24±0.02 ^d	0.16±0.05 ^d
1	0.61±0.08 ^{cd}	0.23±0.14 ^d
2	0.91±0.12 ^c	0.51±0.12 ^d
5	1.46±0.44 ^b	1.17±0.23 ^c
10	1.99±0.26 ^a	2.11±0.10 ^b
Whole milk	2.01±0.15 ^a	3.81±0.58 ^a

[#]Values are mean ± standard error of mean from three replicates. Absorbance was measured after 200-fold dilution. Different superscript letters represent a significant difference in the mean of the same parameter (P < 0.05).

Table 4. Color parameters of skim milk with various amounts of FGMs, in comparison to whole milk.[#]

Fresh samples						After 10-day storage at 5 °C				
Powder%										
w/v	L^*	a^*	b^*	ΔE_1	ΔE_2	L^*	a^*	b^*	ΔE_1	ΔE_2
added										
0	86.00±0.83 ^c	-4.43±0.35 ^a	6.42±0.59 ^{ab}	0 ^a	4.74±1.01 ^b	84.94±0.72 ^{abc}	-4.27±0.36 ^a	5.58±0.80 ^a	0 ^a	5.23±1.60 ^b
1	84.98±1.32 ^{abc}	-3.47±0.09 ^b	6.64±0.30 ^{abc}	1.77±0.59 ^b	5.31±1.54 ^b	84.31±1.38 ^{abc}	-4.08±0.68 ^a	6.32±0.20 ^{ab}	1.14±0.40 ^{ab}	5.62±2.52 ^b
2	85.43±0.66 ^{bc}	-3.12±0.12 ^b	7.99±0.29 ^d	2.25±0.18 ^b	4.85±0.90 ^b	84.29±1.39 ^{abc}	-3.07±0.13 ^b	7.69±0.43 ^{cd}	2.38±0.09 ^b	5.40±2.23 ^b
5	84.11±0.84 ^{abc}	-1.91±0.18 ^c	10.14±0.48 ^e	4.91±0.51 ^{cd}	6.74±1.16 ^b	83.19±1.53 ^a	-1.99±0.28 ^c	9.98±0.75 ^e	5.32±1.36 ^{cde}	7.20±0.95 ^b
10	83.90±0.91 ^{ab}	-0.63±0.30 ^d	11.01±0.78 ^e	6.36±0.63 ^e	7.53±1.27 ^b	83.66±1.04 ^{ab}	-0.80±0.34 ^d	10.85±1.21 ^e	6.28±0.70 ^{de}	7.40±1.72 ^b
Whole milk	90.12±0.27 ^d	-2.29±0.09 ^c	7.13±0.09 ^{bcd}	4.74±1.01 ^c	0 ^a	89.59±1.08 ^d	-2.26±0.09 ^c	6.78±0.38 ^{abc}	5.23±1.60 ^{cde}	0 ^a

[#] ΔE_1 and ΔE_2 (Eq. 7) are based on the comparison of skim milk and whole milk to a white tile, respectively. Values are mean ± standard error of mean from three replicates. Different superscript letters represent a significant difference in the mean of the same parameter ($P < 0.05$).

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

(A)

(B)

Fig. 5.

Fig. 6.

Fig. 7.

(A)

(B)

(C)

Fig. 8.