Solubilization of rehydrated frozen highly concentrated micellar casein for use in liquid food applications

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ABSTRACT

Highly concentrated micellar casein concentrate (HC-MCC), a potential ingredient of protein-fortified food, is a gel at cold temperature. It contains ~17 to 21% casein, with most serum proteins and lactose removed by microfiltration and diafiltration, and it is then further concentrated using vacuum evaporation. The HC-MCC can be stored frozen, and our objective was to determine the conditions needed to obtain complete solubility of thawed HC-MCC in water and to understand its gelation upon cooling. Dispersibility (ability to pass through a 250-μm mesh sieve), suspendability (percentage of protein not sedimented at 80 × g within 5 min), and solubility (percentage of protein not sedimented at 20,000 × g within 5 min) were measured at 4, 12, or 20°C after various mixing conditions. Gelation upon cooling from 50 to 5°C was monitored based on storage (G′) and loss (G″) moduli. The gelled HC-MCC was also examined by transmission electron microscopy. Thawed HC-MCC was added to water to reach a protein concentration of 3% and mixed using high shear (7,500 rpm) for 1 min or low shear (800 rpm) for 30 min at 4, 12, 20, or 50°C and at pH 6.4 to 7.2. The HC-MCC completely dispersed at 50°C, or at ≤20°C followed by overnight storage at 4°C. Suspendability at 50°C was ~90% whereas mixing at ≤20°C followed by overnight storage resulted in only ~57% suspendability. Solubility followed a similar trend with ~83% at 50°C and only ~29% at ≤20°C. Mixing HC-MCC with 60 mM trisodium citrate increased dispersibility to 99% and suspendability and solubility to 81% at 20°C. Cold-gelling temperature, defined as the temperature at which G′ = G″ when cooling from 50 to 5°C, was positively correlated with protein level in HC-MCC. Gelation occurred at 38, 28, and 7°C with 23, 20, and 17% of protein, respectively. Gelation was reversible upon heating, although after a second cooling cycle the HC-MCC gel had lower G′. In micrographs of gelled HC-MCC, the casein micelles were observed to be within the normal size range but packed very closely together, with only ~20 to 50 nm of space between them. We proposed that cold-gelation of HC-MCC occurs when the kinetic energy of the casein micelles is sufficiently reduced to inhibit their mobility in relation to adjacent casein micelles. Understanding solubilization of rehydrated frozen HC-MCC and its rheological properties can help in designing process systems for using HC-MCC as a potential ingredient in liquid food. Key words: solubility, micellar casein, microfiltration

INTRODUCTION

Casein is a food ingredient that is widely used in the dairy, bakery, meat, beverage, and nutraceutical industry based on its diverse functions in emulsifying, foaming, whipping, water-binding, and cheesemaking. Texture properties and nutritional value of casein further support its application as a food additive (Fox, 2001; Fox and Kelly, 2004; Séverin and Wenshui, 2005). Traditionally, casein or caseinate was manufactured in industry by either isoelectric precipitation or by chymosin coagulation (Fox, 2001). Through these processes, casein micelles have irreversibly changed their native colloidal structure into spherical or linear aggregates (Farrell et al., 1988; Oommen, 2004; McMahon and Oommen, 2013).

Since the 1990s, microfiltration (MF) of skim milk has been applied to produce micellar casein concentrate (MCC), with casein levels ranging from 7 to 20% and concomitant serum protein removal ranging from 46 to 79% based on the composition of MCC, or 60 to 95% based on the serum protein level in MF permeate (Pierre et al., 1992; Garem et al., 2000; Brandsma and Rizvi, 2001; Schuck et al., 2002; Fox and Kelly, 2004; Nelson and Barbano, 2005; Hurt et al., 2010; Marella et al., 2013). Typically, MCC manufactured using MF
contains only 7 to 10% casein, which translates into a 3- to 4-fold concentration (St-Gelais et al., 1995; Jost et al., 1999; Nelson and Barbano, 2005; Beckman et al., 2010; Hurt et al., 2010; Amelia et al., 2013; Beckman and Barbano, 2013; Hurt et al., 2015). Higher concentrations of 7- to 8-fold are achievable when milk is acidified (Brandsma and Rizvi, 1999; Brandsma and Rizvi, 2001) or when concentrated further using ultrafiltration or vacuum evaporation (unpublished data, L. E. Metzger; Amelia and Barbano, 2013).

Such highly concentrated MCC (HC-MCC) has many prospective applications in food industry, as it offers several potential advantages over caseinate or milk protein concentrate (MPC) made using ultrafiltration. (1) Compared with caseinate, casein micelles in HC-MCC still exhibit their native structure (Saboyainsta and Maubois, 2000); (2) HC-MCC has lower levels of serum protein compared with MPC, which in turn may lead to improved heat stability or storage stability by reducing binding of denatured serum protein to casein; and (3) HC-MCC is still hydrated with water. Hence, it may show improved functionality compared with milk protein powders that can lose solubility because of heat exposure during drying as well as during storage (Baldwin and Truong, 2007; Mimouni et al., 2010; Sikand et al., 2011). (4) Additionally, HC-MCC can be stored under refrigeration or frozen (Schokker et al., 2011; Sauer et al., 2012). This is beneficial because bacterial growth is repressed during refrigerated storage at 4°C. For example, Amelia and Barbano (2013) reported that the bacterial count of refrigerated HC-MCC stayed below 20,000 cfu/mL for 16 wk.

It is very difficult to resolubilize MCC that has been spray-dried (Schuck et al., 1999, 2002). Solubility index has been measured by volume of sediment after dispersing a specified amount of powder and centrifugation under well-defined conditions. Thus, solubility is inversely correlated with solubility index. Schuck et al. (1999) reported a solubility index of 15 mL of MCC powder at 24°C, indicating an extremely low solubility compared with low-heat NDM powder with a solubility index of <0.5 mL. Having a nondried form of HC-MCC would be advantageous for use in liquid food systems where high solubility is needed. However, HC-MCC forms into a gel when it is cooled and it is then crucial to disrupt the gel structure to fully disperse the casein micelles.

To test the extent of disruption of the HC-MCC gel, it was necessary to adapt tests used for measuring solubility of milk powders. Many reports have been published on the solubility of dairy protein powders, such as caseinate, MPC, or MCC powder (Schuck et al., 2002; Gaiani et al., 2005, 2007; Fang et al., 2007; Schokker et al., 2011; Hussain et al., 2012; Richard et al., 2013; Chandrapala et al., 2014; Crowley et al., 2015). The International Dairy Federation standard dispersibility test involves pouring reconstituted milk powder through a sieve with a mesh size of 250 μm (Westergaard, 2004). This dispersibility test is used to determine if any of the HC-MCC gel remains in relatively large pieces when dispersed in water. Such macrogel pieces (≥250 μm) would be too big to remain dispersed and would rapidly sediment. Smaller microgel pieces (containing aggregates of casein micelles) could be dispersed but probably not visibly observable. However, these smaller microgel pieces would sediment at centrifugation speeds used by researchers who have studied dispersibility of milk powders, such as 700 × g for 10 min (Moughal et al., 2000; Havea, 2006), 750 × g for 15 min (Schokker et al., 2011), or 36 × g for 10 min followed by 168 × g for 10 min (Crowley et al., 2015). In preliminary studies, we observed some sedimentation from pasteurized skim milk when centrifuging conditions were greater than 80 × g for 10 min.

The final step in fully solubilizing a dried or a cold-gelled HC-MCC includes disruption of any remaining aggregates into individual casein micelles. This can be measured by centrifuging at a speed at which any particles larger than individual casein micelles would sediment, such as centrifuging at 20,000 × g for 5 min. Our objective was to determine the best way to disperse and solubilize cold-gelled HC-MCC for its use in liquid food applications as a function of shear speed and time combinations, mixing temperatures, pH, and extended time. We further investigated the effect of citrate addition on dispersibility, suspendability, and solubility of HC-MCC. To better understand the factors affecting solvation of HC-MCC, we also studied rheological properties and microstructure of HC-MCC gel.

**MATERIALS AND METHODS**

**HC-MCC Manufacture**

**MF.** Pasteurized skim milk (72°C for 20 s) was processed into MCC in a 4-vessel, continuous MF unit (Filtration Engineering Inc., Champlin, MN; Figure 1) at the Institute for Dairy Ingredient Processing, South Dakota State University, Brookings. The 4 vessels were 161 mm in diameter and 965 mm in length and were fitted with polyvinylidene fluoride membranes in spiral wound configuration. The 4 membranes used were FH6438-OS03S, FH6430-OS03S, FH6430-OS03S, and FH6430-OS03S (Parker Process Advanced Filtration Division, Oxnard, CA), respectively, for vessels 1, 2, 3, and 4. The total surface area of the 4 membranes was 57.4 m². Immediately before processing skim milk, membranes were subjected to a short clean and sanitization. The short clean consisted of a water rinse to
neutral pH, followed by a 30-min 50°C alkaline wash [1.46% (vol/vol) Ultrasil 110 and 0.11% (vol/vol) Ultrasil 01, Ecolab Inc., St. Paul, MN]. Alkaline solution was flushed out with water to neutral pH, and a sanitizer solution [0.42% (vol/vol) Oxonia Active, Ecolab Inc.] was circulated through the membranes for 10 min at 75°C. Sanitizer was flushed out with water to neutral pH, and the system was ready to begin milk processing. The process used a feed temperature of 18 to 20°C, base line pressure of 35 kPa, differential pressure of 103 kPa, volume reduction of 4.0, and diafiltration level of 100% (based on volume of skim milk) with 20, 30, 30, and 20% of the diafiltration water added at vessel 1, 2, 3, and 4, respectively. During start-up, the feed and circulating boost pumps were sequentially started and the combined MF concentrate obtained from all 4 vessels was recycled back to the balance tank until the desired concentration factor was achieved. After about 10 min, diafiltration was started and after about 30 min, when the required concentration was reached, the MF concentrate flow was diverted to continuous forward mode and the concentrate was collected in a 2,000-L double-jacketed tank. Two separate batches of 5,500 L of skim milk were processed to obtain 1,324 L of MCC. The total processing time for each batch was 17 h and the average overall flux was 9.3 L/m² per hour.

Membranes were subjected to a long clean after skim milk processing. The long clean consisted of a water flush until retentate exited clear from the system followed by a 30-min 50°C alkaline wash [1.46% (vol/vol) Ultrasil 110, 0.11% (vol/vol) Ultrasil 01, and 0.11% (vol/vol) XY-12, Ecolab Inc.], a 45-min 46°C enzyme wash [0.26% (vol/vol) Ultrasil 110, and 0.84% (vol/vol) Ultrasil 63, Ecolab, Inc.], a 30-min 43°C acid wash [0.53% (vol/vol) Ultrasil 76, Ecolab Inc.], a second 30-min 50°C alkaline wash, and a 15-min 32°C soak wash [0.53% (vol/vol) Ultrasil MP, Ecolab Inc.]. Water flushes to neutral pH were performed between each wash step, except after the final soak wash, which was allowed to stay on the membrane during storage for preservation. Pressures for all cleaning cycles were maintained at 69, 83, and 152 kPa, for baseline, stage boost, and membrane inlet pressure, respectively.

Based on the mass of serum protein collected in the permeate relative to the mass of serum protein in the pasteurized skim milk, 70 to 75% of the serum protein present in the skim milk was removed during the MF process. The composition of the skim milk and MF retentate for the 2 batches are shown in Table 1.

### Table 1. Composition of pasteurized skim milk and microfiltration (MF) retentate

<table>
<thead>
<tr>
<th>Component</th>
<th>Batch I</th>
<th>Batch II</th>
<th>Batch I</th>
<th>Batch II</th>
</tr>
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<tbody>
<tr>
<td>TS (%)</td>
<td>8.93</td>
<td>8.99</td>
<td>12.52</td>
<td>11.26</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>0.13</td>
<td>0.12</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>Total N (%)</td>
<td>2.96</td>
<td>3.24</td>
<td>9.60</td>
<td>8.60</td>
</tr>
<tr>
<td>Noncasein N (%)</td>
<td>0.66</td>
<td>0.72</td>
<td>0.96</td>
<td>0.79</td>
</tr>
<tr>
<td>NPN (%)</td>
<td>0.19</td>
<td>0.21</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td>Casein N/total N</td>
<td>0.78</td>
<td>0.78</td>
<td>0.90</td>
<td>0.91</td>
</tr>
</tbody>
</table>

**Vacuum Evaporation.** The 1,324 L of MCC obtained from MF of each batch of skim milk were further processed to produce HC-MCC in a multipass falling
film vacuum evaporator (Dahmes Stainless Inc., New London, MN). The 2-stage falling film evaporator had a primary stage with 3 passes and a finisher stage with 2 passes. The MCC was vacuum-evaporated at a condensing temperature of 63°C and a pressure of −680 mbar (−20 in. Hg). The evaporator was started using water as the feed and, after achieving the required operating conditions, MCC was fed to the balance tank. The concentrate from the final pass was recycled back to the feed tank until the desired concentration was achieved. The target concentration was 30% solids for batch I and 25% solids for batch II. The solids target was reduced from batch I to II because excessive fouling of the evaporator was observed with batch I. After reaching the required concentration, the evaporator was diverted to continuous forward mode, and HC-MCC was collected in a 1,800-L jacketed tank. The HC-MCC was transferred to 1.89-L containers, frozen at −20°C, shipped from South Dakota State University to Utah State University, and stored at −20°C until further analysis. The composition of the HC-MCC from batch I and II is shown in Table 2.

**Composition Analysis.** The pasteurized skim milk, MF retentate, and HC-MCC from each batch was analyzed for TS, total fat, and ash using standard chemical analysis procedures as described by Hooi et al. (2004). Kjeldahl analysis was used to determine total N, noncasein N, and NPN (Hooi et al., 2004), with casein and serum protein content calculated by the difference. Lactose and organic acids were determined using an HPLC-based method as described by Upreti et al. (2006). The HPLC system (Beckman Coulter Inc., Fullerton, CA) included a solvent-delivery module (System Gold 125), a 20-μL sample injection loop (Rheodyne, Rohnert Park, CA), a model 631 column heater (Alltech, Deerfield, IL), a multichannel wavelength scanning detector (190–600 nm; System Gold 168 detector), and a refractive index detector (RI-2031, Jasco Corporation, Hachioji, Japan). A 300 × 7.80-mm ion exclusion column (ROAOrganic Acid, Phenomenex Inc., Torrance, CA) maintained at 65°C with sulfuric acid (0.013 N) mobile phase at a flow rate of 0.6 mL/min was used. The mobile phase was prepared by dissolving 360 μL of HPLC-grade sulfuric acid (Sigma-Aldrich, St. Louis, MO) in 1 L of HPLC-grade water (Thermo Fisher Scientific, Pittsburgh, PA). Calcium was determined by atomic absorption spectroscopy (Analyst 200, PerkinElmer Instruments LLC, Waltham, MA) at a wavelength of 423 nm (Metzger et al., 2000).

**Sample Preparation.** One hundred-gram portions of frozen HC-MCC were partially thawed at room temperature (~22°C), and then sufficient HC-MCC (30.02 ± 0.01 g for batch I and 37.50 ± 0.04 g for batch II) was added to 200 mL of deionized water to achieve 3% (wt/wt) protein. Water temperatures were adjusted to produce mixtures at 4, 12, 20, and 50°C. After combining HC-MCC and water, ~0.5 mL of antifoam B emulsion (J. T. Baker, Avantor Performance Materials Inc., Center Valley, PA) were added and pH was adjusted to 6.4, 6.8, 7.0, or 7.2 with 1 N HCl or 1 N NaOH as needed. Mixing was performed (a) at high shear (HS) using a high-speed mixer (model GLH; OMNI International, Kennesaw, GA) at 7,500 rpm, (b) at low shear (LS) using a magnetic stirrer (~800 rpm), or with a combination of LS and HS mixing. High-shear mixing was for 1 min whereas LS mixing was for 10 or 30 min. In addition, after mixing, some samples were stored overnight at 4°C with LS stirring for 18 h. Each sample was tested once for dispersibility, suspendability, and solubility. Each preparation treatment was performed in at least triplicate.

**Dispersibility.** The dispersibility test was adapted from International Dairy Federation standard method for MPC (Fang et al., 2007). After mixing, the dispersed samples were poured through a sieve (U.S.A. Standard Testing Sieve, VWR International LLC, Radnor, PA) with mesh size of 250 μm. The beaker was rinsed 3 times using the filtrate. Material retained on the sieve was washed with deionized water, and the filter paper was placed on a weighted filter paper (Bunn-o-Matic Corp., Springfield, IL) using ~800 mL of water. After draining, the filter paper was placed on a weighing scale and weighed. The dried material was stored at −20°C until analysis. Dispersibility was calculated as the percentage of dry weight of retained particles compared with weight of DM in the HC-MCC mixture.

**Suspendability and Solubility.** Once dispersibility reached ~90%, the filtrate through the sieve from the dispersibility test was centrifuged at 80 or 20,000 × g for 5 min to measure suspendability and solubility, respectively. Samples prepared at 4, 12, 20, and 50°C would be centrifuged under these conditions.
were centrifuged at 4, 12, 20, and 20°C, respectively. The rationale being that at 80 x g, particles of the HC-MCC gel that were small enough to pass through the 250-μm sieve would be easily sedimented and the remaining material could be considered as being suspended, whereas at 20,000 x g only individual casein micelles would remain in the supernatant and these could be considered as being soluble or fully solvated. The supernatant liquid was obtained and measured for protein using an infrared milk analyzer (B2000, Bentley Instruments, Inc., Chaska, MN) that had been calibrated with milk standards before the measurements. Suspendability and solubility were calculated as the percentage of protein concentration of the supernatant compared with 3% protein in the initial HC-MCC mixture. As a reference for the extent of sedimentation that occurs at 80 and 20,000 x g, suspendability and solubility were measured for homogenized pasteurized skim milk.

Adding Trisodium Citrate. Dispersibility, suspendability, and solubility were measured on samples of HC-MCC mixed with 60 mM trisodium citrate (TSC; Thermo Fisher Scientific, Fair Lawn, NJ) instead of water using HS for 1 min at 4 and 20°C with pH adjusted to 7.0 using 1 M HCl. An overnight storage at 4°C was optionally performed after mixing at 4°C. Another mixing was performed with 120 mM TSC at 4°C. Calibration curves were made for correcting protein levels measured by infrared milk analyzer of diluted HC-MCC gel that were small enough to pass through the 250-μm sieve would be easily sedimented and the remaining material could be considered as being suspended, whereas at 20,000 x g only individual casein micelles would remain in the supernatant and these could be considered as being soluble or fully solvated. The supernatant liquid was obtained and measured for protein using an infrared milk analyzer (B2000, Bentley Instruments, Inc., Chaska, MN) that had been calibrated with milk standards before the measurements. Suspendability and solubility were calculated as the percentage of protein concentration of the supernatant compared with 3% protein in the initial HC-MCC mixture. As a reference for the extent of sedimentation that occurs at 80 and 20,000 x g, suspendability and solubility were measured for homogenized pasteurized skim milk.

Rheological Properties

Sample Preparation. Frozen HC-MCC containing 23% protein was partially thawed at room temperature, then ~25 g was heated to 50°C for 10 min to liquefy the gel, poured into a petri dish to about 1-mm thickness, and cold-gelled at room temperature for 20 min. The gel sample was slowly flooded with 2% glutaraldehyde (Electron Microscopy Services, Hatfield, PA) in distilled water and fixed in situ for 1 h at room temperature. The fixed gel was cut into small pieces with a razor blade and pieces were carefully transferred into a vial filled with 2% glutaraldehyde. Samples were rinsed in 2 changes of sodium cacodylate buffer (0.1 M with 16 mM calcium chloride and 4.8% sucrose at pH 7.4) for 10 min each. Samples were postfixed in 2% osmium tetroxide in cacodylate buffer for 1 h at room temperature followed by a 5-min rinse with nanopure water. Samples were treated with saturated aqueous uranyl acetate for 1 h, then dehydrated through a graded series of ethanol (50% for 10 min, 70% for 10 min, 2× 95% for 10 min, 4× 100% for 10 min) and transitioned into Epon plastic with 4 changes of 100% acetone, 10 min each. Samples were infiltrated in plastic:acetone, 1:1, for 1 h, followed by a change to 3:1 overnight. The next day, samples were infiltrated with pure plastic with 3 changes, each first for 1 h on a rotator followed by 1 h under vacuum. The specimens were then individually embedded in a flat mold and polymerized overnight at 65°C. Sections (~70 to 100 nm) were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL) using a diamond knife (Diatome, Hatfield, PA). Sections were double-stained for 20 min with saturated aqueous uranyl acetate followed by 10 min with Reynold’s lead citrate. Sections were analyzed using a transmission electron microscope (TEM; JEOL 1400 Plus, Jeol USA Inc., Peabody, MA) operated at 120 kV, and digital images were captured with a Gatan camera (Gatan Inc., Pleasanton, CA).

Experimental Design

Effects of mixing method (HS for 1 min, LS for 30 min, with optional overnight storage) and mixing tem-
perature (4, 12, 20, and 50°C) on dispersibility were analyzed using a 4 × 4 factorial design. Effects of mixing with overnight storage and mixing temperature on suspendability and solubility were studied using a 2 × 4 factorial design. Effects of mixing method at 50°C on suspendability and solubility, different mixing pH and addition of citrate on dispersibility, suspendability, and solubility were studied using a completely randomized design. A t-test was used to compare dispersibility of HC-MCC in water and 60 mM TSC. Linear regression was performed to investigate the effect of protein levels on CGT. Data were analyzed for statistical significance at 95% confidence level using PROC GLM function in statistical analysis software (SAS version 9.3, SAS Institute Inc., Cary, NC). Significance was declared at P < 0.05 and trend at 0.05 ≤ P < 0.1. Post-hoc means comparisons were made based on P-values (α = 0.05) using Tukey-Kramer adjustment to obtain differences of least mean squares.

**RESULTS**

**Dispersibility, Suspensibility, and Solubility**

Two batches of HC-MCC were manufactured and their compositions are listed in Table 2. Batch I was used in all experiments whereas batch II was only used in dispersibility, suspendability, and solubility tests. Thawed HC-MCC completely dispersed when mixed using HS for 1 min or LS for 30 min at 50°C. At lower temperatures, there was incomplete dispersion without a significant difference between 4, 12, and 20°C (P > 0.05, Table 3). Using HS for 1 min increased dispersion at lower temperatures (~65%) compared with LS for 30 min (~38%). Oommen (2004) reported a similar effect of shear rate on the dispersion of powders. When HC-MCC samples, which dispersed initially at 4, 12, and 20°C, were subsequently stored overnight at 4°C, dispersibility increased to 100% in all cases.

Suspendability (measured as lack of sedimented protein at 80 × g) of HC-MCC dispersed at 50°C was 89% with HS and 91% with LS (Table 4); solubility (measured as lack of sedimented protein at 20,000 × g) was 85 and 82%, respectively (Table 5). As suspendability and solubility were only determined in samples with dispersibility ≥90%, these values were not determined for HC-MCC mixed at 4, 12, and 20°C without any overnight storage. When the additional overnight storage at 4°C was included, mean suspendability and solubility were 57 and 29%, respectively, for HC-MCC mixed at ≤20°C (Tables 4 and 5). However, solvation at 50°C still provided significantly higher (P < 0.001) solubility than solvation at 4, 12, or 20°C whether or not overnight storage was included (Tables 4 and 5). No difference in suspendability was observed upon overnight storage at 4°C after mixing at 50°C, and a slight difference in solubility was noted but solubility was still in the range of 80 to 85%. Using overnight storage as a way to achieve improved solvation has commonly been used in reconstituting nonfat dry milk (Berridge, 1952). In our experiment, however, overnight storage did not produce complete solubilization of HC-MCC without prior mixing at 50°C.

While investigating the effect of time on solvation of HC-MCC, we observed that providing 10 min of LS mixing in water at 20°C followed by 1 min of HS mixing increased dispersibility. Using this mixing method, dispersibility of 94 to 98% was achieved (Table 6); compared with only 67% when using HS alone at 20°C. However, both suspendability and solubility remained low with values of ~32 and ~15%, respectively. Modifying pH within the range from 6.4 to 7.2 had only a slight effect with a trend (P < 0.05) for increased solubility.

### Table 3. Mean dispersibility of rehydrated highly concentrated micellar casein concentrate (HC-MCC), with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50°C, followed by optional overnight hydration (−O) at 4°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Method</th>
<th>4</th>
<th>12</th>
<th>20</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HS</td>
<td>60.2b</td>
<td>67.7b</td>
<td>66.6b</td>
<td>98.8c</td>
</tr>
<tr>
<td></td>
<td>HS-O</td>
<td>98.7</td>
<td>99.3</td>
<td>99.8</td>
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<tr>
<td></td>
<td>LS</td>
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<td>—</td>
<td>37.9a</td>
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<tr>
<td></td>
<td>LS-O</td>
<td>99.5c</td>
<td>100.0c</td>
<td>100.7</td>
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</tr>
</tbody>
</table>

Means with the same superscript letter were not significantly different, α = 0.05.

**Table 4. Mean suspendability of rehydrated highly concentrated micellar casein concentrate (HC-MCC), with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50°C, followed by optional overnight storage (−O) at 4°C.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>89.1A</td>
</tr>
<tr>
<td>HS-O</td>
<td>87.4b</td>
</tr>
<tr>
<td>LS</td>
<td>91.9g</td>
</tr>
<tr>
<td>LS-O</td>
<td>92.8g</td>
</tr>
</tbody>
</table>

Means with the same letter were not significantly different, α = 0.05.

*Dispersibility (%) = 100 × (DM in HC-MCC − dry weight of particles with size ≥250 μm)/(DM in HC-MCC).

*Not tested.

*Not tested.
Addition of 60 mM TSC significantly \((P < 0.001)\) increased dispersibility of HC-MCC at 4 and 20°C to 97 and 99%, respectively (Table 7). Suspendability also increased to 74 and 81%, respectively, with solubility values being similar (Table 7). Including overnight storage at 4°C after mixing at 4°C had a tendency \((P < 0.10)\) to increase suspendability and solubility to 86%. Increasing the citrate concentration to 120 mM citrate was less effective in improving solvation of HC-MCC at 4°C than adding overnight storage.

### Rheological Properties

Cold-gelling temperature was linearly correlated \((P < 0.001)\) with protein concentration (Figure 2). Nondiluted HC-MCC (~23% protein) and diluted HC-MCC (20 and 17% protein) gelled at a mean temperature of 38, 28, and 7°C, respectively. For every percent unit decrease of protein, mean CGT decreased about 5°C. When HC-MCC protein concentration had been reduced to 16%, gelation did not occur until the diluted HC-MCC was cooled to 5°C.

At 50°C, nondiluted HC-MCC was fluid and had \(G'\) of 10 Pa (Figure 3). Upon gelation and cooling to 5°C, \(G'\) was 3.3 kPa and further increased to 5.1 kPa after holding at 5°C for 30 min. Upon reheating to 50°C, the HC-MCC reliquefied with \(G'\) decreasing to <10 Pa. When HC-MCC was held at 50°C for 30 min, and then recooled, the same pattern in \(G'\) of the cold HC-MCC occurring after the second cooling cycle.

### TEM

In transmission electron micrographs of cold-gelled HC-MCC, casein micelles were observed as being close together and evenly distributed with approximately 20- to 50-nm spaces separating them (Figure 4). Most of casein micelles were intact, more or less spherical, and fit the typical size range of 20 to 600 nm (McMahon and Oommen, 2013). Some casein micelles were collapsed and others were aggregated (Figure 4, asterisks). Several casein micelles were nonspherical in shape, which was also observed in skim milk treated with high pressure (~150 MPa; Knudsen and Skibsted, 2010). In contrast, casein micelles with more spherical structure have been detected in skim milk or ultrafiltrated skim milk at 20°C (Karlsson et al., 2007; McMahon et

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**Table 5.** Mean solubility\(^1\) of rehydrated highly concentrated micellar casein concentrate (HC-MCC), with pH adjusted to 7.0, using high shear (HS) for 1 min, or low shear (LS) for 30 min at 4, 12, 20, or 50°C, followed by optional overnight storage (-O) at 4°C

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature (°C)</th>
<th>HS</th>
<th>HS-O</th>
<th>LS</th>
<th>LS-O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>12</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>HS</td>
<td>4</td>
<td>29.3(^{ab})</td>
<td>2.7(^{ab})</td>
<td>32.1(^{b})</td>
<td>81.7(^{ab})</td>
</tr>
<tr>
<td>HS-O</td>
<td>4</td>
<td>29.3(^{ab})</td>
<td>2.7(^{ab})</td>
<td>32.1(^{b})</td>
<td>81.7(^{ab})</td>
</tr>
<tr>
<td>LS</td>
<td>4</td>
<td>26.9(^{a})</td>
<td>30.2(^{ab})</td>
<td>30.2(^{ab})</td>
<td>85.0(^{a})</td>
</tr>
<tr>
<td>LS-O</td>
<td>4</td>
<td>26.9(^{a})</td>
<td>30.2(^{ab})</td>
<td>30.2(^{ab})</td>
<td>85.0(^{a})</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Means with the same letter were not significantly different, \(\alpha = 0.05\).

**Table 6.** Mean dispersibility, suspendability, and solubility of rehydrated highly concentrated micellar casein concentrate, with pH adjusted to 7.2, 6.8, or 6.4, mixing using low shearing for 10 min, followed by high shearing for 1 min at 20°C

<table>
<thead>
<tr>
<th>pH</th>
<th>Dispersibility (%)</th>
<th>Suspendability (%)</th>
<th>Solubility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>98.3(^{a})</td>
<td>32.8(^{a})</td>
<td>16.0(^{b})</td>
</tr>
<tr>
<td>6.8</td>
<td>97.1(^{a})</td>
<td>30.8(^{b})</td>
<td>14.3(^{b})</td>
</tr>
<tr>
<td>6.4</td>
<td>94.0(^{a})</td>
<td>32.8(^{a})</td>
<td>13.4(^{a})</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Means within a column with the same letter were not significantly different, \(\alpha = 0.05\).

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\(^{1}\)Solubility (%) = 100 × (protein in HC-MCC − protein in supernatant of 20,000 × g centrifugation for 5 min)/(protein in HC-MCC).

\(^{2}\)Not tested as incomplete dispersibility; <90% as shown in Table 2.

**Table 7.** Mean dispersibility, suspendability, and solubility of rehydrated highly concentrated micellar casein concentrate in 60 or 120 mM trisodium citrate, with pH adjusted to 7.0, mixing using high shearing (HS) for 1 min at 4 or 20°C, followed by optional overnight storage (-O) at 4°C

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature (°C)</th>
<th>Citrate (mM)</th>
<th>Dispersibility (%)</th>
<th>Suspendability (%)</th>
<th>Solubility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>4</td>
<td>60</td>
<td>96.9(^{a})</td>
<td>73.8(^{ab})</td>
<td>73.9(^{ab})</td>
</tr>
<tr>
<td>HS-O</td>
<td>4</td>
<td>60</td>
<td>99.9(^{b})</td>
<td>85.7(^{b})</td>
<td>85.7(^{b})</td>
</tr>
<tr>
<td>LS</td>
<td>20</td>
<td>60</td>
<td>98.5(^{a})</td>
<td>81.2(^{b})</td>
<td>79.8(^{b})</td>
</tr>
<tr>
<td>HS</td>
<td>4</td>
<td>120</td>
<td>99.3(^{b})</td>
<td>66.6(^{b})</td>
<td>65.6(^{a})</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Means within a column with the same letter were not significantly different, \(\alpha = 0.05\).
al., 2009). Some low electron-dense protuberances of 20 to 30 nm were observed extending from the body of the casein micelles (Figure 4, black arrows), which is in accordance with previous observations made by other researchers (Dalgleish et al., 2004; McMahon and Oommen, 2008). Occasionally, small fat droplets in size ranging from 50 to 100 nm were observed between the casein micelles (Figure 4, white arrows).

**DISCUSSION**

**Dispersibility, Suspendability, and Solubility**

Similar to studies of milk powders (Westergaard, 2004), we used the term dispersibility to refer to the ability of the HC-MCC gel to be sufficiently reduced in size to pass through a 250-μm mesh sieve. Commonly, the assessment of solubility of milk powders is based on lack of sedimentation upon centrifuging at 170 to 750 × g for 10 to 15 min at 20°C (Moughal et al., 2000; Havea, 2006; Schokker et al., 2011; Crowley et al., 2015). However, this does not mean that the powder particles have been hydrated completely into individual casein micelles, only that any remaining particles are small enough that they do not sediment under the test conditions used. In our view, complete solvation is achieved when only individual, nonaggregated colloidal casein micelle particles are dispersed in the medium. This should apply to powders and was the requirement we set for the HC-MCC gel to be considered to have 100% solubility.

Milk powders need to go through a hydration process to release individual casein micelles contained within the dry powder particles. In contrast, the cold HC-MCC already contains hydrated casein micelles but the casein micelles are locked together as a gel. Release, or dispersion, of all the individual casein micelles from HC-MCC gel particles is necessary for 100% solubility to be obtained. To better understand the reduction in particle size that occurs during shearing and dispersion of the HC-MCC gel into water, we designated the term suspendability to refer to particles that were small enough to pass through the 250-μm mesh sieve but were large enough to sediment at a low centrifugal force (i.e., 80 × g for 5 min). These were the conditions under which no sediment was observed from pasteurized skim milk.

The 3 tests used in our study provide an indication of particle size reduction of the HC-MCC gel as it was dispersed, suspended, and solubilized in water. The dispersibility test effectively quantified visually observable macrogel particles (>250 μm). The suspendability and solubility centrifugation tests at 80 or 20,000 × g for 5 min separated microgel particles (~1 to 250 μm) and casein micelle aggregates (0.5 to 1 μm), respectively, from the fraction of HC-MCC that was solubilized into individual casein micelles (Marchin et al., 2007).

To find optimum conditions of solubilizing HC-MCC, we chose 5 parameters including shearing, overnight storage, temperature, pH, and the addition of TSC. Combinations of 10-min LS and 1-min HS reached full dispersibility (~98%) of HC-MCC, but only 33%
of suspendability and 16% of solubility at 20°C and pH 7.2 (Table 6). These results indicate that by solely increasing shearing rate, 65% of the HC-MCC gel was in particles smaller than 250 μm. Still, complete solubilization was not achieved, as only 16% particles were found separated into individual casein micelles (Figure 5). Increasing shearing rate has been associated with reduced rehydration time or smaller particle sizes of dairy powder dispersions, presumably by physically breaking particles apart, increasing turbulence, accelerating mass transfer, and releasing of casein micelles into solution (Hixson and Crowell, 1931b; Oommen, 2004; Bock et al., 2008; Jeantet et al., 2010; Richard et al., 2013; Chandrapala et al., 2014). High shear is usually more effective in reducing particle size and rehydration time during solubilization of dairy powder than LS (Hixson and Crowell, 1931b; Oommen, 2004; Bock et al., 2008; Jeantet et al., 2010; Richard et al., 2013; Chandrapala et al., 2014). More aggregates with larger sizes were observed in sodium caseinate dispersions hydrated using LS for 10 h than using HS for 10 min followed by hydration for 1 h (Oommen, 2004).

In our study, increasing mixing temperature seemed to be more effective in solubilizing HC-MCC than overnight storage. The next most effective treatment included mixing under increased shear rate. When fol-

Figure 4. Transmission electron micrographs of thin sections of cold-gelled highly concentrated micellar casein concentrate showing casein micelles (gray-black) of various sizes, including chains of protein extending out from casein micelles (black arrows), some casein micelles that appear aggregated (asterisks), as well as some very small milkfat globules (white arrows).

Figure 5. Approximate size distribution of protein particles in highly concentrated micellar casein concentrate and water mixture containing 3% protein. Black bars: mixing with low shear for 10 min and subsequent high shear for 1 min at 20°C. Gray bars: mixing with high shear for 1 min and subsequent overnight storage at 4°C. Bars with diagonal stripes: mixing with high shear for 1 min at 4°C with addition of 60 mM of trisodium citrate. White bars: mixing with high shear for 1 min at 50°C. Size distribution was calculated as >250 μm = 1 – dispersibility; 1–250 μm = suspendability – dispersibility; 0.5–1 μm = solubility – suspendability; <0.5 μm = solubility. Error bars represent SE; n = 3.
lowed by overnight storage, all HC-MCC gel particles were reduced in size to <250 μm with ~30% of the gel dissolved into individual casein micelles. Both heating to 50°C and adding 60 mM TSC melted the HC-MCC gel with ~90 and 80% of casein micelles reaching sizes of individual casein micelles, respectively. Increasing hydration temperature, time and shear rate are commonly used parameters to increase solubility of dairy proteins in industry. Increasing mixing temperature and time has been associated with a decrease in the amount of sediment or rehydration time of dairy powders (Hixson and Crowell, 1931a; Mimouni et al., 2009; Jeantet et al., 2010; Schokker et al., 2011; Richard et al., 2013; Crowley et al., 2015). Increased solvation after overnight storage at 4°C may partially be attributed to the dissociation of β-CN from casein micelles due to decreased hydrophobic interactions at low temperature. However, other interactions are likely to play more important roles, as hydrophobic interaction could not explain higher solubility at 50°C when hydrophobic interactions would be stronger.

Increasing mixing temperature accelerates the release of material from powder particles into solution, which appears to be the rate-limiting step in solubilization of MPC (Mimouni et al., 2009). Moreover, the rehydration process is more sensitive to mixing temperature than to shearing speed (Jeantet et al., 2010; Richard et al., 2013). For example, increasing mixing temperature by 4°C was as effective as doubling stirring rate (from 400 to 800 rpm) in rehydration of MCC powder (Jeantet et al., 2010).

At pH of milk (~6.6), phosphate salts exist mostly in the form of HPO$_4^{2-}$, which has a buffering capacity from pH of 5.8 to 7.8 (Lucey and Horne, 2009). Therefore, pH change from 6.4 to 7.2 has minimal effect on HC-MCC solvation.

**Calcium-Mediated Protein Linkage**

Adding 60 mM TSC increased dispersibility of the HC-MCC gel at ≤20°C to 100% and solubility to 80% with the remaining 20% being microgel pieces (Figure 5). This disruption of HC-MCC into smaller particles with use of TSC suggests that calcium is possibly involved as part of the linkages between casein in cold-gelled HC-MCC. However, it could also be argued that disruption of the casein micelles by chelating calcium leads to a breakup of the HC-MCC gel particles. In addition to the interlocking of casein within the casein micelles by nanoclusters of calcium phosphate (McMahon and Oommen, 2008), calcium can bind directly to the casein, as has been shown for metal cations in general (Reddy and Mahoney, 1992). Dispersion of HC-MCC (3% protein) contains about 23 mM of total calcium, and 60 mM of citrate is sufficient to complex all available calcium. It was shown that by adding a calcium-chelating agent (i.e., TSC), free calcium ions favorably attached to TSC and formed a soluble complex (Lucey and Horne, 2009). We hypothesized this to be the reason for the disruption of calcium-mediated protein-protein linkages with the result of increased solvation of the HC-MCC gel.

Existence of noncovalent bonding was suggested in MPC powder (Anema et al., 2006; Havea, 2006). Swelling of casein micelles has been observed upon addition of citrate, urea, or EDTA to casein micelle dispersions, as evidenced by the increased volume of casein micelles after addition of these substances (Sood et al., 1979; Huppertz et al., 2007; de Kort et al., 2011). Similarly, increased particle sizes and decreased turbidity were observed in casein micelles after addition of citrate or urea (Huppertz et al., 2007). These results are in agreement with our observations that addition of calcium chelators increased the solvation of casein micelles. However, as chelating calcium by adding TSC did not fully solubilize HC-MCC (~23% of the protein remained as microgel pieces), additional factors must be involved in linking casein micelles together in the HC-MCC gels.

It is noted that addition of TSC also causes reduction of casein-bound calcium, which may lead to dissociation of casein micelle in MPC and reduction of casein-bound phosphate (Kaliappan and Lucey, 2011). Dissociated casein molecules can be present as small aggregates, which are smaller and lighter, and therefore harder to sediment according to Stoke’s Law. Small clusters of particles measuring less than 50 nm were observed in calcium-depleted casein micelles using TEM (Oommen, 2004; McMahon and Oommen, 2013). Thus, the role of calcium chelation in increasing solubilization of HC-MCC gels may involve decreasing calcium-induced interactions between casein micelles and dissociating casein micelles into smaller particles.

**Microstructure of HC-MCC**

The observed close packing of casein micelles in transmission electron micrographs of the HC-MCC gel (separation distances of 20 to 50 nm) can be related to a casein concentration factor of ~4.5 in HC-MCC. In comparison, the average distance between casein micelles in skim milk is ~120 nm (Walstra et al., 1984). The observation of casein micelles in the HC-MCC gel with nonspherical shape may indicate deformation of the periphery of the casein micelles because of shear during manufacture.

It has been calculated that casein micelles contain from 1 to 8 g of water/g of protein depending on how
the hygroscopicity is measured (Kumosinski et al., 1988). Theoretically, fat globules in cream are closely packed (i.e., touching each other without compression) when fat level reaches 72% (Bylund and Pak, 2003). Similarly, if casein micelles contain 4 g of water/g of casein, then MCC would become closely packed when a casein concentration of 14% is approached. Therefore, in HC-MCC draining of water from the outer portion of the casein micelles needs to occur to reach ~20% casein during the manufacture. This results in close packing of the casein micelles in which there is overlap of their hydration spheres (Figure 6).

Given the closeness of neighboring casein micelles, the gap between casein micelles in HC-MCC is likely to be filled with surface protuberances radiating from the same casein micelles (Figure 7). Protuberances of tubular shape with a diameter of 10 to 20 nm extending from the bulk of casein micelles have been reported (Dalgleish et al., 2004; McMahon and Oommen, 2008). Being able to view fine-stranded protein protuberances or tendrils at high magnifications is problematic for at least 2 reasons. First, the protuberance structure can easily be changed during sample preparation such as coating with heavy metal. Second, fine detailed structure with low electron density may be lost if contrast is set too high during image capture (McMahon and McMamus, 1998).

If the observed gaps between casein micelles were devoid of protein mass associated with the casein micelles, it would be expected that the casein micelles would be randomly distributed throughout the sample volume. In contrast, these gaps are uniformly, and consistently, of approximately the same size, suggesting that the casein micelles have been pushed together as closely as possible and are held separate through steric hindrance of their outer peripheral protein protuberances and tendrils that in the past have been referred to as a hairy layer. In conclusion, based on moisture content, results from TEM, and the effect of calcium chelation on solubilization of HC-MCC, it would appear that the outer portions of the casein micelles sterically overlap with each other and some linking between casein micelles occurs via calcium bridging between adjacent peripheral proteins in the cold HC-MCC gel.

Small fat droplets were found interspersed between casein micelles. The presence of some fat droplets in HC-MCC was expected as HC-MCC contains a small amount of fat (0.5 to 1%, Table 2). Those fat droplets remaining would be the smallest fat droplets that have too slow a sedimentation rate to be present in the cream factor, leaving the cream separator. The shearing and temperature changes (i.e., freezing and thawing) during preparation and handling of HC-MCC may have caused some collapse and aggregation of casein micelles (Gebhardt, 2014), leading to these unusually large aggregates.

**Cold-Gellation of HC-MCC**

The observation that HC-MCC with 23% protein content forms a gel at temperatures of ≤38°C corresponds well with its low dispersibility, suspendability, and solubility at temperatures of ≤20°C and its high solubility at 50°C when the gel has melted. Such gelling and melting behaviors are mainly temperature-dependent, and much less affected by storage time or mixing speed. Similar G’ patterns upon reheating or recooling cycles indicate that the cold-gellation of HC-MCC is thermally reversible (Figure 3). The slight decrease (~1.3 kPa) in G’ suggests slightly weaker gel strength after recooling; although gel strength may be restored if held for longer than 30 min during recooling.

We proposed that cold-gellation of HC-MCC was caused by steric interference between the closely packed casein micelles through overlapping protuberances on their periphery (Figure 7), and that these interactions were strengthened through calcium bridging. That is, the casein micelles in HC-MCC are packed so closely together that their outer tendrils overlap and interpenetrate into the hydration sphere of each other. The spatial closeness of casein micelles and their tendrils permits calcium bridging to form between negatively charged locations of the proteins. Thus, cold-gelling of HC-MCC occurs when the kinetic energy of the casein micelles is sufficiently reduced to inhibit mobility and ability to move in relation to adjacent casein micelles. At high temperature, kinetic energy of casein micelles is sufficient to enhance mobility of surface protuberances and the casein micelles themselves, resulting in less drag in moving past each other and the gel melts. When the temperature is again decreased, the reduced kinetic energy leads to restricted movement and gelation of HC-MCC gel. The higher the casein concentration of HC-MCC, the shorter the distance between casein micelles, the sooner steric interference slows down casein micelle mobility and CGT occurs at higher temperature. This casein concentration is reached during the last stages of HC-MCC concentration by MF with further dewatering of the casein micelles occurring during evaporation.

**CONCLUSIONS**

Understanding solubilization of rehydrated frozen HC-MCC and its rheological properties can help in designing process systems for using HC-MCC as an
ingredient in liquid food systems. Either mixing at high temperature (~50°C) or addition of TSC can achieve complete dispersion and more than 80% solubility of HC-MCC in water (3% protein). Overnight storage helps to fully disperse HC-MCC, but only reaches ~30% of solubility at ≤20°C. High shearing is more effective than low shearing in increasing dispersibility, although it provides no advantage in increasing suspendability or solubility. Cold-gelation of HC-MCC is thermally reversible, and reducing protein levels in HC-MCC can decrease its CGT. The HC-MCC with less than 16% of protein does not gel at 5°C. We propose that cold-gelation of HC-MCC occurs when the kinetic energy of the casein micelles is sufficiently reduced to inhibit their mobility in relation to adjacent casein micelles. Based on these results, using high shear rates followed by moderate heating is suggested to maximize solubility of HC-MCC in liquid foods.

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REFERENCES


