The effect of ultrasound on casein micelle integrity

J. Chandrapala,* G. J. O. Martin,*¹ B. Zisu,† S. E. Kentish,* and M. Ashokkumar*

*School of Chemistry/Department of Chemical and Biomolecular Engineering, University of Melbourne, Victoria 3010, Australia

†Dairy Innovation Australia, Synedes Road, Werribee, Victoria 3030, Australia

ABSTRACT

Samples of fresh skim milk, reconstituted micellar casein, and casein powder were sonicated at 20 kHz to investigate the effect of ultrasonication. For fresh skim milk, the average size of the remaining fat globules was reduced by approximately 10 nm after 60 min of sonication; however, the size of the casein micelles was determined to be unchanged. A small increase in soluble whey protein and a corresponding decrease in viscosity also occurred within the first few minutes of sonication, which could be attributed to the breakup of casein-whey protein aggregates. No measurable changes in free casein content could be detected in ultracentrifuged skim milk samples sonicated for up to 60 min. A small, temporary decrease in pH resulted from sonication; however, no measurable change in soluble calcium concentration was observed. Therefore, casein micelles in fresh skim milk were stable during the exposure to ultrasonication. Similar results were obtained for reconstituted micellar casein, whereas larger viscosity changes were observed as whey protein content was increased. Controlled application of ultrasound can be usefully applied to reverse process-induced protein aggregation without affecting the native state of casein micelles.

Key words: ultrasonication, casein micelle, dynamic light scattering, viscosity

INTRODUCTION

Considerable interest has been expressed in using ultrasound for improving the functional properties of dairy products (Ashokkumar et al., 2008). Ultrasound has been shown to be a potential alternative to thermal treatment for reducing microbial activity (Piyasena et al., 2003), an effective method for homogenizing milk fat (Villamiel and de Jong, 2000), and a means of improving the heat stability of whey protein concentrates (WPC) (Ashokkumar et al., 2009a,b). Ultrasonication

can impart changes to aqueous systems such as milk by acoustic cavitation and a range of accompanying physical and chemical effects. When subjected to an acoustic field, bubbles grow in size because of both rectified diffusion and coalescence. The bubbles eventually collapse, generating extreme temperatures within the bubbles, which leads to the generation of radicals (Ashokkumar et al., 2007). Shock waves are also formed, causing localized turbulence with extreme shear forces.

Although the general mechanistic actions of ultrasound have been well studied, much is still to be learned about the effect of these actions on milk and its various components. Milk is a complex biochemical system. It is an aqueous lactose solution containing a complex mixture of mineral salts, an oil-in-water emulsion of fat globules, a variety of soluble whey proteins, and colloidal protein associations called CN micelles. Some knowledge exists regarding the effect of ultrasound on the fat and whey protein components of milk. It has been shown that ultrasound can reduce the size of milk fat globules (Villamiel and de Jong, 2000; Wu et al., 2001). This effect can be attributed to the localized shear forces induced by ultrasonication. The application of ultrasound has been shown to break apart heat-induced protein aggregates in WPC and prevent their reformation on subsequent heating (Ashokkumar et al., 2009a,b). The breakdown of the heat-induced aggregates is attributed primarily to the physical shear forces resulting from acoustic cavitation, although minor whey protein structural changes are observed (Chandrapala et al., 2011). Ultrasound has also been shown to improve the gelation properties of WPC systems (Zisu et al., 2011).

Of the work to date on CN-containing systems, considerable focus has been given to understanding the effects of ultrasound on bulk physical and functional properties. Our own work has shown that sonication of both CN-rich milk protein concentrate retentate and calcium caseinate can significantly reduce solution viscosity (Zisu et al., 2010). These effects were attributed to the disruption of CN-CN or CN-whey protein interactions, or both, but no evidence was presented to support this hypothesis. The effect of ultrasonication on milk gels has also been reported (Vercet et al., 2002;

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¹Corresponding author: gjmartin@unimelb.edu.au

Riener et al., 2009, 2010; Nguyen and Anema, 2010; Zisu et al., 2011). Acid gel firmness (G') was found to be altered when skim milk was ultrasonically treated before acidification, although the effect was attributed largely to denaturation of whey protein caused simply by temperature increases (up to about 95°C) resulting from sonication performed without temperature control (Nguyen and Anema, 2010). The simultaneous application of heat and ultrasound under moderate pressure (manothermosonication) has enabled the formation of improved yogurt gels of greater strength compared with those obtained from untreated milk (Vercet et al., 2002). The cause of this behavior was not ascertained. Although the authors suggested that a difference in the size of fat globules was not responsible, a proper comparison of the fat globules was not performed. Other researchers also found that thermosonication improved the rheological properties of yogurts (Riener et al., 2009, 2010) and presented data showing a significant decrease in the size of fat globules in the sonicated milk.

Although CN micelles are considered relatively stable particles, their composition and size respond to alterations in pH, temperature, and milk protein concentration (Martin et al., 2007; Tsioulpas et al., 2007; Dalgleish, 2011). It is possible that the localized high temperatures and shear forces created by ultrasonication could physically alter the CN micelles or their interactions with other milk components. One study found that the average size of reassembled CN micelles could be reduced by exposure to ultrasound (35 kHz frequency) for 6 h, provided the pH was above 8 (Maddalou et al., 2009). However, it is unclear how this relates to native CN micelles because the reassembled CN particles were considerably larger (275 nm) and structurally and functionally different (Mounsey et al., 2005). In another study involving true CN micelles, a decrease in particle size resulting from ultrasonication was observed. This particle size decrease was attributed to a reduction in CN micelle size; however, this was not substantiated (Nguyen and Anema, 2010).

To date, no systematic study on the effect of ultrasonication on CN micelles has been performed. It is unknown what, if any, effect ultrasound has on the structure, size, or composition of CN micelles, or their interactions with whey proteins. The purpose of this study was to investigate possible alterations to the size of CN micelles resulting from the application of high-intensity ultrasound. To investigate the effect of ultrasound on native CN micelles, experiments were performed on fresh, pasteurized skim milk, including particle size and soluble CN analysis. To isolate the effects of ultrasound on fat globules from those on CN micelles, particle size analysis was performed on skim milk after micelle dissociation and on resuspended pel-

lets of CN micelles obtained by centrifugation. To further investigate the effect of ultrasound on CN micelles and also explore possible interactions between CN micelles and whey protein, 3 CN-containing milk powder systems were also examined: reconstituted micellar CN powder (MC) containing CN micelles and very little whey protein; reconstituted CN powder (NCN) containing nonmicellar CN and even smaller quantities of whey protein; and a high-protein powder with a CN:whey protein ratio of 1:1 by mass (CNWP). The knowledge gained from this study will enable a better understanding of observed functional changes to CN-containing systems resulting from sonication.

MATERIALS AND METHODS

Materials

Fresh, pasteurized skim milk was purchased from a local supermarket and immediately stored at 4°C; all analyses were performed within 2 d. The composition of the milk was 33 g/L of protein, 1.5 g/L of fat, and 52 g/L of lactose, as determined by the manufacturer. The MC and edible NCN powders were obtained from Murray Goulburn Nutritionals (Cobram, Victoria, Australia). Scanning electron microscopy images confirmed that the CN in the reconstituted MC powder was in a micellar form (data not shown). A high-protein CNWP powder was also supplied. The composition of the powders was determined by the manufacturer using standard methods (Table 1). Ultrapure water (MilliQ; Millipore, Billerica, MA) was used at all times.

Reconstitution of the MC and NCN Powders

The MC and NCN powders were reconstituted in MilliQ water to obtain 50 and 150 g/kg solutions. To completely solubilize the powders, solutions were continuously stirred for 30 min at 50°C and a further 1 h at room temperature. The solutions were left overnight

Table 1. Composition of powders¹

Component	MC (%, wt/wt)	NCN (%, wt/wt)	CNWP (%, wt/wt)
Protein	85.5	88.7	80.1
CN	80.0	88.7	~40
Fat	1.6	0.9	3.4
Lactose	0.6	0.1	3.1
Ash	7.3	1.7	6.9
Moisture	5.3	10.2	6.5

¹MC = micellar CN powder containing CN micelles and very little whey protein; NCN = reconstituted CN powder containing nonmicellar CN and even smaller quantities of whey protein; CNWP = high-protein powder with a CN:whey protein ratio of 1:1 by mass.

at 4°C. The following day, solutions were equilibrated at 25°C for 1 h before sonication and analysis.

Ultrasonication

Milk was sonicated in 60-mL aliquots in a glass vessel equipped with a cooling jacket, using a 20-kHz, 450-W ultrasonic horn (19 mm in diameter, Sonifier 450; Branson, Danbury, CT) at amplitude of 50% for 1, 5, 10, 20, 30, or 60 min. The actual power delivered to the solution was determined to be 31 W by calorimetry. During sonication, chilled water was circulated continuously to keep the temperature of the milk to within 10°C of room temperature.

Particle Size Measurement

Photon correlation spectroscopy (PCS) was used to determine particle size with a Malvern Autosizer 4700 PCS or a ZetaSizer instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Measurements were performed at 25°C at a scattering angle of 90°C. Volume size distributions were determined using the CONTIN algorithm, and average particle size was determined on the basis of light-weighted intensity, using the method of cumulants. To determine the effects of sonication on the size of fat globules alone, particle size measurements were also conducted after dissociation of the CN micelles by diluting milk samples 10-fold in 10 g/L of EDTA solution (EDTA powder; APS Finechem, Seven Hills, New South Wales, Australia; adjusted to pH 7 by addition of sodium hydroxide).

Turbidity

The turbidity of the milk samples was measured using a UV-visible spectrophotometer (Carey 3E; Varian, Palo Alto, CA) by transmission of light ($\lambda = 860$ nm) through a 2-mm path-length quartz cuvette. The turbidity of the samples diluted in EDTA solution was multiplied by 10 to account for the dilution.

pH Measurement

The pH of the milk was measured at 25°C by using a pH electrode with an integrated temperature sensor (Mettler Toledo Ltd., Port Melbourne, Victoria, Australia) connected to a Seven Easy pH meter (Mettler Toledo Ltd.). The pH probe was calibrated before each measurement at 25°C using standard buffers at pH 4.0, 7.0, and 10.0 (Mettler Toledo Ltd.).

Ultracentrifugation

Milk samples were ultracentrifuged at $78,000 \times g$ at 25°C for 90 min by using a Beckman L8 80M ultracentrifuge with a type 50 rotor (Beckman Instruments Inc., Palo Alto, CA). All analyses were carried out on the clear supernatant.

Resuspension of Pelleted Micellar Phase

Pellets containing only CN micelles were obtained by centrifugation at $25,000 \times g$ for 60 min at room temperature by using a Beckman Avanti30 centrifuge with an F0685 rotor (Beckman Instruments Inc.). The firm pelleted phase was carefully removed to a beaker after several washes with water. The pellets were resuspended in simulated milk ultrafiltrate (i.e., 0.02 M Bis-Tris, 0.05 M sodium chloride, 0.003 M calcium chloride; pH 6.7) and stirred overnight. Before ultrasonication, the resuspended samples were centrifuged at $14,254 \times g$ for 5 min at room temperature to remove any large, undissolved CN particles.

Soluble Calcium Measurement

Soluble calcium was determined by measuring the total calcium contained in supernatants of the ultracentrifuged milk samples. Residual protein was removed by diluting an aliquot (5 g) of supernatant sample with 20 g of MilliQ water and 25 g of 24 g/100 g of TCA, followed by suction filtration through a Whatman no. 40 filter paper into a preweighed Büchner flask. The precipitated proteins were washed with 10 g of 12 g/100 g TCA solution and the total mass of the TCA filtrate was recorded. An aliquot (4.0 g) of the supernatant was mixed with a suppressant (2 g of lanthanum chloride) and diluted to a total mass of 10 g. The calcium concentration was determined by atomic absorption spectroscopy (Varian Atomic Absorption Spectrophotometer, Mulgrave Victoria, Australia) using an air acetylene flame and wavelength of 422.7 nm. Commercially available calcium standards for atomic absorption spectroscopy were used after appropriate dilution (0 to 6 mg/mL for calcium).

Total Protein Measurement

The total concentration of protein in the supernatants from ultracentrifugation was measured by Bradford assay according to the manufacturer's instructions (Sigma-Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia). Whey protein concentration was estimated by measuring the total protein remaining after precipi-

tation at pH 4.6. Casein concentration was calculated by subtracting the whey protein from the total protein.

HPLC

The supernatants of milk solutions obtained from ultracentrifugation were analyzed for CN concentration by reverse-phase HPLC after filtering through a 0.45- μ m syringe filter. Reverse-phase HPLC was performed using a Jupiter 5- μ m C18 300A column (250 $\times$ 4.6 mm i.d.; Phenomenex, Torrance, CA). Analysis was performed with a solvent gradient using 2 solutions with different ratios of acetonitrile-water-trifluoroacetic acid (100:900:1 and 900:100:0.7, by vol) as reported previously (Visser et al., 1991).

Viscosity Measurements

The viscosity of the fresh skim milk and 5 wt% powder solutions was measured using a type 531-03/0c capillary viscometer (SI Analytics GmbH, Mainz, Germany) maintained at 20°C. The dynamic viscosity was determined by comparing the relative flow times and densities of the samples with those of distilled water. The viscosity of the 15 wt% powder solutions was determined using a Rheometrics ARG2 rheometer (TA Instruments c/o Waters Australia Pty Ltd., Rydalmere, New South Wales, Australia). A steady stress sweep experiment was carried out using 40-mm parallel-plate geometry and a 1-mm gap at 25°C. The stress was varied to obtain the viscosity data between the shear rate of 50 to 200 s⁻¹.

RESULTS AND DISCUSSION

Investigation of the Stability of CN Micelles in Fresh Skim Milk

Decreases in the average particle size and turbidity were observed over the 60 min of sonication of skim milk (Figure 1A and B). A decrease in particle size upon sonication of skim milk was also observed by others (Nguyen and Anema, 2010). This was attributed to a decrease in micelle size; however, the exact mechanism was not proposed.

Although the quantity of fat in skim milk is low in comparison with the CN content, the residual fat globules have a high relative refractive index and can be of considerable size. As such, they contribute significantly to the light-scattering properties of the milk, which influence both the turbidity and dynamic light-scattering signal. Because ultrasonication has been shown to be highly effective at reducing the size of fat globules in

milk (Villamiel and de Jong, 2000; Wu et al., 2001), further investigation was required to determine whether the observed decreases in particle size and turbidity were due to alterations to the CN micelles, the fat globules, or whey or whey protein-CN aggregates formed during pasteurization. In this laboratory, it has often been observed that the fat globules in skim milk are similar in size to larger CN micelles (~200–300 nm). Although asserting an influence on the average particle size (i.e., obtained from the method of cumulants) in the presence of CN micelles, they are not detectable as a separate peak in the volume size distribution (obtained using the CONTIN algorithm). To clearly observe the size distribution of the fat globules, it is necessary to first completely dissociate the CN micelles by addition of chelating agents that remove the colloidal calcium phosphate and disrupt the micelles (Udabage et al., 2000). Skim milk samples sonicated for different times were suspended in a solution of EDTA, dissociating the CN micelles. The particle size distribution of the fat globules was then examined by PCS and the turbidity of the samples was measured (Figure 1C and D).

A reduction in size of the fat globules as a function of sonication time was clearly evident in the particle size distribution of the EDTA-treated skim milk samples (Figure 1C). The peak at larger particle sizes corresponds to the fat globules, and the other peak corresponds to the products of the dissociated CN micelles. The average size of the peak corresponding to the fat globules decreased from approximately 230 to 175 nm after 60 min of sonication (Figure 1D). The turbidity of these samples also decreased as a function of sonication, declining from approximately 1.4 to 0.5 cm⁻¹ after 60 min of sonication (Figure 1D). Further verification was provided by measuring the particle size and turbidity of sonicated skim milk samples immediately after sonication and again after 24 h. The turbidity and size of all samples remaining unchanged after 24 h, suggesting that the changes caused by sonication were not reversible. This is consistent with a breakdown of fat globules, which would be expected to remain stable after sonication. It is evident from these data and from similar results on other milk samples (data not shown) that a decrease in fat globule size causes a decrease in the average particle size and turbidity of fresh skim milk.

Although a decrease in fat globule size is responsible, at least in part, for the observed decrease in particle size, the possibility of a change in CN micelle size cannot be completely eliminated. To further investigate whether CN micelles are affected by sonication, CN micelles were isolated from the other milk components by centrifugation. Pellets obtained from centrifugation of fresh skim milk were resuspended and the particle

Figure 1. Intensity-weighted particle size distribution of fresh skim milk (A) and fresh skim milk with micelles dissociated by EDTA (C) as a function of sonication time: 0 min (solid diamonds and solid line); 1 min (shaded squares and long-dashed line); 30 min (shaded triangles and mixed-dashed line); 60 min (open circles and mixed-dashed line). Intensity-weighted average particle size (solid diamonds) and turbidity (open diamonds) of fresh skim milk (B) and fresh skim milk with micelles dissociated by EDTA (D) as a function of sonication time. Error bars are the standard deviation of triplicate measurements of the same sample.

size and turbidity were determined (data not shown). Both the turbidity and average particle size remained unchanged ($2.49 \pm 0.04 \text{ cm}^{-1}$ and $168 \pm 2 \text{ nm}$, respectively) over 60 min of ultrasonication, providing strong evidence that the size of the CN micelles remained unaltered by exposure to ultrasound.

To further examine the structural stability of the CN micelles during sonication, the protein content of

the serum phase was examined after sonication. The supernatants from ultracentrifugation were analyzed by HPLC and Bradford protein assays to determine whether sonication released any CN from the micelles or caused any interaction between whey protein and soluble CN. Consistent with previous observations (Nguyen and Anema, 2010), the results of the Bradford assay method and analysis of the individual free CN in

the serum of fresh skim milk solutions by HPLC showed no significant change in concentrations of soluble CN as a result of ultrasonication (Figure 2A and 2B). The amount of soluble whey protein was, however, observed to increase slightly in the first 1 to 5 min of sonication (Figure 2B). The observed increase in soluble whey protein could be due to the breakup of aggregated whey protein or whey protein associated with CN micelles (Ashokkumar et al., 2009b; Chandrapala et al., 2011) that may have formed during pasteurization of the fresh milk. Consistent with this explanation, the viscosity of fresh skim milk (with whey protein) was seen to decrease slightly from 1.71 to 1.65 cP over the first minute of sonication and then remain stable (1.65 ± 0.02 cP), whereas the viscosity of resuspended pellets (without soluble whey protein) was seen to remain unchanged over 60 min of sonication (1.15 ± 0.02 cP). Although these results suggest ultrasonication may break up whey protein or CN-whey protein aggregates, again no changes to the CN micelles themselves could be seen to result from ultrasonication.

Minor changes to the pH were observed as a function of increased sonication time (Figure 2C). Ultrasound can alter the pH of a solution because of the formation of small amounts of nitric acid (Supeno and Kruus, 2000). The reaction between nitrogen and oxygen within the hot zone of the cavitation bubbles generates nitric acid. For raw skim milk samples, we found that the pH change was fully reversible within a few minutes (Figure 2C), suggesting only a temporary disruption of the natural mineral equilibrium present in milk. The pH of milk is also sensitive to temperature; however, because the experiments in the present study were performed under temperature-controlled conditions (within 10°C), the observed pH decrease was more likely due primarily to nitric acid formation. Even slight changes in pH may affect the mineral equilibria of milk, which in turn could affect the composition of CN micelles. However, no measurable differences in the soluble calcium could be observed as a result of the temporary pH decrease caused by sonication (Figure 2C).

CN-Containing Powders

To further investigate the effect of ultrasonication on CN, 3 different powder systems were examined: MC, NCN, and CNWP. The turbidity and average particle size of reconstituted MC were observed to decrease rapidly in the first 10 min and then stabilize (Figure 3A). The initial decrease could presumably be due to a breakup of micelle aggregates remaining from powder solubilization, a breakup in whey protein or whey protein-CN aggregates, or simply a decrease in the size of any fat globules present. A corresponding initial de-

Figure 2. Relative concentration of soluble CN of skim milk ultra-sonicated for different times (A). Results are HPLC peak intensities of κ -CN (open and solid circles), α_{S1} -CN (open and solid diamonds), α_{S2} -CN (open and solid triangles), and β -CN (open and solid squares) in supernatants of ultracentrifuged samples normalized to that of β -CN in the unsonicated sample. Open and closed symbols represent samples ultracentrifuged immediately or 24 h after sonication, respectively. Estimated concentrations of CN (open and solid circles) and whey protein (open and solid diamonds) in the serum of fresh skim milk samples as determined by Bradford assays of total soluble protein and soluble protein remaining after CN precipitation at pH 4.6 (B). Data shown are the results of measurements of 2 different skim milk samples. The pH (open and solid diamonds) and soluble calcium concentration (open and solid circles) of fresh skim milk samples as a function of sonication time (C). Closed and open symbols represent measurements taken immediately or 24 h after sonication, respectively.

crease in turbidity was also observed (Figure 3B). As with fresh skim milk, a small and reversible decrease in pH was seen to result from sonication (Figure 3C). In this case, a slight and reversible increase in the soluble calcium could also be seen after 60 min of sonication (Figure 3C). According to the Bradford assays, ultrasonication had no measurable influence on either the whey protein or soluble CN concentration (Figure 3D).

These results are again consistent with the suggestion that ultrasonication had no effect on the CN micelles. A large reduction in both the average particle size and turbidity of reconstituted NCN powder was seen after only 10 min of sonication (Figure 4A). The extremely low turbidity is consistent with the CN being present in nonmicellar form. The particles observed by PCS were initially larger than CN micelles (280 nm) and most

Figure 3. Intensity-weighted average particle size (solid diamonds) and turbidity (open diamonds) of 5% micellar CN as a function of sonication time (A). Viscosity of 5% (open diamonds) and 15% (open squares) micellar CN as a function of sonication time (B). pH (open and solid diamonds) and soluble calcium concentration (open and solid circles) of 5% micellar CN as a function of sonication time (C). Closed and open symbols represent measurements taken immediately or 2 h after sonication, respectively. Estimated concentration of CN (open circles) and whey protein (open diamonds) in the serum of fresh skim milk samples as determined by Bradford assays of total soluble protein and soluble protein remaining after CN precipitation at pH 4.6 (D).

Figure 4. Intensity-weighted average particle size (solid diamonds) and turbidity (open diamonds) of a 5% nonmicellar CN powder (A) and a 5% high-protein powder with a CN:whey protein ratio of 1:1 (CNWP; B) as a function of sonication time. The viscosity (solid diamonds) and mass of pelleted solids (open circles) of 5% nonmicellar CN as a function of sonication time (C). The viscosity of 5% (open diamonds) and 15% (open squares) CNWP powder as a function of sonication time (D).

likely to be small fat globules (the powder contained 0.9% fat) or protein aggregates that were rapidly broken down by sonication. The size and turbidity continued to decrease, although to a much lesser extent over the 60-min period tested. The viscosity and amount of solid material pelleted by centrifugation were also observed to decrease as a function of sonication time (Figure 4C). These results in particular are consistent with sonication breaking up nonmicellar protein aggregates that remained after powder solubilization at 50°C and overnight equilibration.

It has previously been shown that ultrasonication can break aggregates of whey protein without causing denaturation of individual protein molecules (Ashokkumar et al., 2009b; Chandrapala et al., 2011). The results presented in this paper suggest analogous behavior with CN; CN aggregates held together by denatured whey proteins are disrupted without affecting the native state of the CN micelles. Further experiments were performed on the high-protein CNWP powder. Application of ultrasound decreased the viscosity by approximately 20% after 5 min and then had little fur-

ther effect (Figure 4D). The particle size was reduced from 205 to 165 nm within 10 min of sonication and to 160 nm after 60 min, and the turbidity decreased concomitantly (Figure 4B). More concentrated (15% wt/wt) solutions for both of the CNWP and MC systems showed similar trends, although the relative viscosity reduction was much greater for the CNWP that contained whey protein (cf. Figures 3B and 4D). For both systems, a much greater viscosity reduction was observed than with 5% wt/wt solutions. These results are again consistent with ultrasonication disrupting whey protein, whey protein-CN micelle aggregates, or both over a short period without appearing to modify the native whey protein or CN structure on prolonged exposure.

CONCLUSIONS

Ultrasonication did not appear to affect CN micelle size or composition or to permanently affect the mineral balance in fresh skim milk. Ultrasonication did, however, reduce the size of the already small fat globules remaining in skim milk, did appear to break up whey protein aggregates, and did assist in breaking apart CN micelle, nonmicellar CN, and whey protein aggregates present in reconstituted CN powder systems. Controlled application of ultrasonic energy can help break up large CN and whey protein aggregates, thereby influencing macroscopic properties, such as viscosity, without inducing changes to the CN micelles or mineral balance. Ultrasonication can be used as a processing tool in CN-containing milk systems with minimal damage to key milk components. In particular, it appears that ultrasound can help reverse protein aggregation caused by prior processing without affecting the native state of the milk proteins or mineral equilibrium. This is an important observation for a range of industry applications.

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