Effect of solvent and temperature on the size distribution of casein micelles measured by dynamic light scattering

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ABSTRACT

The objectives of this study were to investigate the effect of the solvent on the accuracy of casein micelle particle size determination by dynamic light scattering (DLS) at different temperatures and to establish a clear protocol for these measurements. Dynamic light scattering analyses were performed at 6, 20, and 50°C using a 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments, Holtsville, NY). Raw and pasteurized skim milk were used as sources of casein micelles. Simulated milk ultrafiltrate, ultrafiltered water, and permeate obtained by ultrafiltration of skim milk using a 10-kDa cutoff membrane were used as solvents. The pH, ionic concentration, refractive index, and viscosity of all solvents were determined. The solvents were evaluated by DLS to ensure that they did not have a significant influence on the results of the particle size measurements. Experimental protocols were developed for accurate measurement of particle sizes in all solvents and experimental conditions. All measurements had good reproducibility, with coefficients of variation below 5%. Both the solvent and the temperature had a significant effect on the measured effective diameter of the casein micelles. When ultrafiltered permeate was used as a solvent, the particle size and polydispersity of casein micelles decreased as temperature increased. The effective diameter of casein micelles from raw skim milk diluted with ultrafiltered permeate was 176.4 ± 5.3 nm at 6°C, 177.4 ± 1.9 nm at 20°C, and 137.3 ± 2.7 nm at 50°C. This trend was justified by the increased strength of hydrophobic bonds with increasing temperature. Overall, the results of this study suggest that the most suitable solvent for the DLS analyses of casein micelles was casein-depleted ultrafiltered permeate. Dilution with water led to micelle dissociation, which significantly affected the DLS measurements, especially at 6 and 20°C. Simulated milk ultrafiltrate seemed to give accurate results only at 20°C. Results obtained in simu-

lated milk ultrafiltrate at 6°C could not be explained based on the known effects of temperature on the casein micelle, whereas at 50°C, precipitation of amorphous calcium phosphate affected the DLS measurement.

Key words: casein micelle, dynamic light scattering, particle size, particle size distribution

INTRODUCTION

Particle size and particle size distribution (PSD) of dispersed systems affect key properties such as surface area, reactivity, opacity, packing density, and rheological properties. Quantifiable changes in particle size or PSD give valuable indications of aggregation or dissociation phenomena, which can help predict the stability and a range of macroscopic properties of colloidal systems.

The particle size and structure of CN micelles are of great interest and have been the focus of numerous investigations. Holt (1985) critically compared different methods used for measuring the average size and size distribution of CN micelles, including electron microscopy, light scattering, and controlled pore glass chromatography. He concluded that natural variations in average micelle size, deficiencies in electron microscopy methods, and overestimation of micelle effective diameter by light scattering methods have led to discrepancies in reported CN micelle size and particle distribution data. In recent years, dynamic light scattering (DLS), which is both fast and noninvasive, has become the method of choice for evaluating particle size and PSD in a range of colloidal systems. Because CN micelles scatter light very well, the development of DLS holds great promise for the accurate determination of CN micelle size, as well as the investigation of its interactions with other molecules or its response to environmental factors. The main advantages of light scattering are that it avoids drastic changes in the environment of micelles and that the measurements are performed on a large number of particles (Holt, 1975).

Dagleish et al. (1987) studied the effect of high heat treatment of milk on CN micelle size, and were able to show effectively that micelle size increased because of aggregation of κ -CN and serum proteins. Walstra et al. (1981) used DLS to show that the hydrodynamic

Received June 19, 2008.

Accepted December 23, 2008.

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diameter of CN micelles decreased by approximately 10 nm when rennet was used to cleave the κ -CN and remove the stabilizing macropeptide. Dalglish et al. (1989) confirmed that the surface of the CN micelle is covered predominantly by κ -CN by analyzing the protein composition of micellar fractions whose sizes had been established by DLS. In another study, Griffin et al. (1988) used DLS to investigate the effect of the partial removal of micellar calcium phosphate on the structure and size of the CN micelle. Later, de Kruif (1997) successfully used DLS to study and characterize the kinetics of CN micelle aggregation resulting from acidification.

The DLS method correlates the fluctuations of the average intensity of scattered light over time with the size of particles in suspension. The main quantity measured is the translational diffusion coefficient D , which can be used to determine the apparent particle diameter d by using the Stokes-Einstein equation:

$$D = \frac{k_B T}{3\pi\eta(t)d}, \quad [1]$$

where k_B is the Boltzmann constant ($1.38 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1}$), T is temperature (K), $\eta(t)$ is viscosity (Pa s), and t = time (s).

In biological systems, in which a wide distribution of particle sizes is typically present, the measured effective diameter ($\overline{d_{eff}}$) is an average diameter weighted by the intensity of light scattered by each particle.

One of the main challenges associated with the particle size measurement by DLS of food colloids, including CN micelles, is the fact that measurements need to be performed in clear solutions (Alexander and Dalglish, 2006). Moreover, because the autocorrelation function of the DLS software compares the signal with a time-delayed version of itself, the accuracy of the measurement is susceptible to secondary scattering. To avoid this, protein systems must be diluted to very low concentrations for the incident photons to be scattered only once by the sample, typically in the range of $10^{-3} \text{ }\mu\text{g/mL}$. Because most food systems, including milk, are both opaque and concentrated, considerable dilution is required (Dalglish and Hallett, 1995). When doing this, much consideration must be given to ensuring that the analyzed particles maintain their native structure during the DLS analyses. This is rather challenging, because the use of the wrong diluting agent can lead to changes in the native pH and ionic equilibrium, which in turn can result in dissociation or aggregation of the studied colloidal particles, thus affecting the results of the particle size measurement.

Although DLS has been used for some time for measuring the size of CN micelles, the protocols used for performing these measurements have not been consistent from one study to another. Different solvents have been used for diluting the milk. Some studies have used lactose-free simulated milk ultrafiltrate (SMUF; Walstra et al., 1981; Roesch et al., 2004). Simulated milk ultrafiltrate was originally designed by Jenness and Koops (1962) as a lactose-free buffer for the dispersion of CN or serum proteins in studies of physicochemical properties such as heat stability, electrophoresis, or ultracentrifugation. Simulated milk ultrafiltrate has since become a very popular solvent or model system for various investigations, including for studying the effect of milk salt concentration on the structure of acidified micellar CN systems (Auty et al., 2005), or for studying the influence of whey protein heat denaturation on the acid-induced gelation of CN (Schorch et al., 2001). de Kruif (1997) used UF water as a solvent in measurements of CN micelle sizes, whereas Ono et al. (1983, 1990) used both lactose-free SMUF and permeate from UF skim milk. In the latter case, the size of CN micelles was calculated based on a wavelength vs. turbidity relationship. Tuinier et al. (1999) and Maroziane and de Kruif (2000) used milk UF permeate as a solvent in their investigations of CN micelle interactions with exocellular polysaccharides and pectin. Karlsson et al. (2005) also used UF permeate as a solvent in their DLS investigation, whereas Anema and Li (2003) used a calcium-imidazole buffer. Strawbridge et al. (1995) and Spagnuolo et al. (2005) also used a buffer containing 5 mM calcium chloride and 20 mM imidazole as a solvent for DLS analysis.

The choice of solvent and the different protocols used for DLS analyses could be the reasons why the micelle sizes reported in the literature are not always consistent from one study to another. Therefore, the objectives of this study were to investigate the effect of the diluting medium on the CN micelle particle size measurement by using DLS and to establish a clear methodology for these measurements. The effect of temperature on the CN micelle particle size was also investigated.

MATERIALS AND METHODS

Materials

Raw skim milk and HTST pasteurized skim milk (Cornell Dairy, Ithaca, NY) with a residual fat content of approximately 0.1% were used as sources of CN micelles in this study. Three different solvents were used as diluting media for particle size measurements: UF water (water), lactose-free SMUF (SMUF), and CN-depleted fresh permeate from UF milk (UF permeate).

Table 1. Composition of the lactose-free simulated milk ultrafiltrate¹

Ingredient	Amount, g/L
KH ₂ PO ₄	1.58
K ₃ citrate·H ₂ O	1.20
Na ₃ citrate·2H ₂ O	1.79
K ₂ SO ₄	0.18
CaCl ₂ ·2H ₂ O	1.32
MgCl ₂ ·6H ₂ O	0.65
K ₂ CO ₃	0.30
KCl	0.60
KOH	Add to pH 6.6

¹Adapted from Jenness and Koops (1962).

The SMUF was prepared using analytical grade mineral salts according to the method reported by Jenness and Koops (1962). The composition of SMUF is shown in Table 1. The UF permeate was obtained in the pilot plant at Cornell University (Ithaca, NY) from pasteurized skim milk, using a polyethersulfone spiral-wound membrane with a nominal cutoff of 10 kDa (GEA Niro Inc., Hudson, WI). The total residual protein concentration in the UF permeate was 0.06% (wt/wt). To remove any impurities, all solvents were filtered immediately before use in the particle size analyses by using a 0.2- μ m nylon syringe filter (Fisher Scientific, Pittsburgh, PA).

Particle Size Analyses

The DLS analyses were performed using a 90Plus Nanoparticle Size Analyzer equipped with a Peltier temperature control system (Brookhaven Instruments Corp., Holtsville, NY). The particle size measurements were conducted at a fixed 90° angle and a wavelength of 658 nm.

To maximize the accuracy of the measurement, skim milk was diluted before the DLS analysis by using the 3 solvents mentioned above. As a measure of an acceptable sample concentration, we followed the recommendation of the DLS equipment manufacturer that the signal intensity measured by the instrument be between 700 and 900 kilocounts per second (**kcps**). The milk:solvent ratio required to achieve the recommended signal intensity was 150 μ L of milk/1 L of solvent.

The particle size measurements were performed at constant temperatures of 6, 20, and 50°C, respectively. The lower temperature was chosen to represent refrigeration conditions, 20°C was chosen because this is a temperature commonly used for DLS analysis, and the higher temperature was selected to be just below the denaturation temperature of serum proteins. The skim milk samples were equilibrated either in the refrigerator (at 6°C) or in a water bath (for 20 and 50°C) for at least 1 h before the measurements. The solvents were also tempered at the measurement temperature and

were filtered immediately before dilution. The exception was the SMUF used in the 50°C measurements. In this particular case, SMUF was kept under moderate stirring at room temperature, then filtered using the 0.2- μ m syringe filter and heated on a water bath immediately before the dilution step. For all solvents and temperatures, the disposable cuvettes used for the measurements were equilibrated in the temperature-controlled chamber of the particle size analyzer for 5 min. After that, the diluted test samples were pipetted in the cuvette and allowed a 2.5-min temperature equilibration, after which the DLS measurement was promptly started.

Data collection and analysis were performed using BIC software (Brookhaven Instruments Corp.), which converted the experimental data into size distributions. The software contains a Dust Filter algorithm that improves the quality of the measurements by rejecting data that are corrupted by scattering from random particles such as air bubbles or dust. The Dust Filter cutoff parameter was set at 30, which is the optimal value suggested by the manufacturer when the expected particle size is in the range of hundreds of nanometers. Seven replicate measurements were performed for each experimental condition. Each measurement consisted of 7 subsequent individual runs of 30-s duration. For each measurement, the relative particle size distribution, the intensity weighted effective diameter ($\overline{d_{eff}}$), and the polydispersity index (p) were determined.

Identification of Milk Protein Classes and Residual Fat Globules

To achieve a positive identification of each class of milk proteins by DLS, a native solution of serum proteins was obtained by precipitating CN out of skim milk at isoelectric pH (4.6) according to the IDF sample preparation procedure for quantifying the non-CN nitrogen in milk (International Dairy Federation, 1964). Ten milliliters of milk was diluted with 75 mL of 40°C distilled water in a volumetric flask that was then placed in a water bath at 40°C. One milliliter of 10% acetic acid was added to the flask, which was gently mixed for 10 min before the addition of 1 mL of 1 N sodium acetate solution. The flask was cooled in an ice bath to 20°C, diluted to 100 mL with 20°C distilled water, and filtered through dry Whatman no. 42 filter paper (Whatman Int. Ltd., Maidstone, UK). The serum protein solution obtained using this procedure was very clear and had a protein concentration that did not require further dilution before particle size analyses.

The residual fat globules in the skim milk were isolated and their size distribution was evaluated by

Table 2. Viscosity and refractive index of the solvents studied at the 3 test temperatures

Solvent ¹	Temperature, °C	Average viscosity $\pm$ SD, cP	Refractive index
UF permeate	6	1.809 $\pm$ 0.022	1.341
	20	1.154 $\pm$ 0.019	1.342
	50	0.804 $\pm$ 0.041	1.341
SMUF	6	1.756 $\pm$ 0.037	1.335
	20	0.996 $\pm$ 0.021	1.334
	50	0.521 $\pm$ 0.008	1.334
Water	6	1.652 $\pm$ 0.077	1.333
	20	1.023 $\pm$ 0.015	1.333
	50	0.467 $\pm$ 0.029	1.333

¹UF permeate = CN-depleted fresh permeate from UF milk; SMUF = simulated milk ultrafiltrate; water = UF water.

DLS using the method of Michalski et al. (2001). Skim milk (0.1% residual fat) was diluted (1:1 vol/vol) with 35 mM EDTA/NaOH, pH 7.0 buffer to dissociate CN micelles and aggregates. This mixture was dispersed in a 0.1% SDS solution and analyzed by DLS at 20°C. Immediately before the dilution step, both the 35 mM/L EDTA:NaOH buffer, pH 7.0, and the 0.1% SDS solution were filtered using a 0.2- μ m nylon syringe filter. In this experiment, the dilution ratio was adjusted so that the skim milk:solvent ratio was also 150 μ L of skim milk/1 L of solvent.

Viscosity and Refractive Index Measurements

To account for the solvent properties in the calculation of particle sizes, the viscosity and refractive index for each solvent and experimental temperature were determined and their respective values were introduced manually in the BIC software. The viscosities of each solvent at the 3 experimental temperatures were determined using an Advanced Rheometric Expansion System strain-controlled rheometer, in conjunction with Orchestrator data collection and analysis software (TA Instruments, New Castle, DE). Measurements were performed using double-wall Couette geometry. Temperature control was achieved using a Julabo FS18-MW heating and refrigerated circulator (Julabo USA Inc., Allentown, PA). The viscosity was measured using a steady-mode, single-point test setup at a shear rate of 1 s⁻¹.

The refractive index for each solvent was determined using a digital fiber-optic refractometer after the filtration step (Misco Products Division, Cleveland, OH). The viscosity and refractive index values of the solvents under the test conditions are shown in Table 2.

Mineral Composition Analysis

The ionic concentration in the solvents was tested at the Dairy One Forage Analysis Laboratory (Ithaca,

NY). The following ions were quantified: calcium, phosphorous, magnesium, potassium, sodium, and chloride. All ion, with the exception of chloride, were determined using a Thermo Jarrell Ash IRIS Advantage HX Inductively Coupled Plasma Radial Spectrophotometer (Thermo Scientific, Madison, WI). Samples were acidified to match standards (5 mL of sample + 545 μ L of 1.5 N HNO₃ + 250 μ L of 0.5 N HCl), then aspirated by inductively coupled plasma spectrophotometry. Results from inductively coupled plasma spectrophotometry were multiplied by a correction factor of 1.159 to account for the dilution during acidification. The chloride content was determined by acidifying 5 mL of sample with 45 mL of 0.2 N HNO₃, followed by potentiometric titration with AgNO₃ using a Brinkman Metrohm 716 Titrino Titration Unit (Brinkmann Instruments Inc., Westbury, NY) equipped with a silver electrode. All ionic concentrations were reported in milligrams per liter (Table 3).

Statistical Analysis of Data

One-way ANOVA and 2-way ANOVA with an interaction effect were performed to determine significant differences between the values of effective diameter and polydispersity ($P < 0.05$). Differences between means were compared using the Tukey-Kramer honestly significant difference test and the statistical software package JMP, version 7.0 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Before the results of the study are presented, a brief discussion of the representation of DLS results is necessary. The results of PSD measurements can be displayed either as a lognormal distribution or as a multimodal size distribution (Figure 1). The lognormal distribution was widely used before software packages associated with the PSD instrumentation had the capacity to

Figure 1. Different representations of particle size distributions: a) multimodal size distribution; b) lognormal distribution. Sample: raw skim milk in UF permeate at 50°C.

create multimodal size distributions. This distribution offers a simplified representation of PSD, which allows relatively easy comparisons between different samples or replicate measurements of the same sample, as well as the calculation of a single effective diameter for the analyzed sample. The multimodal size distribution, on the other hand, offers more information regarding the presence of groups of particles or molecules of different sizes, but the accuracy of such representations depends greatly on the algorithms used by the specific software. When determining PSD in systems known to have multiple classes of particles, it is very important that both the lognormal and multimodal representations be used when analyzing the data, which was the approach used in the current study.

Evaluation of Solvents

Before determining the PSD of CN micelles, PSD measurements were performed for all 3 solvents at 6, 20, and 50°C, and the results of these measurements were

defined as the solvent baselines. This was an important step in ensuring that the solvents themselves did not bring any significant contributions to the particle size distribution of the samples.

As expected, water was virtually free of particles, as indicated by the negligible signal intensity (1.5 to 2 kcps). For SMUF, the baselines at 6 and 20°C were also free of particles and showed a very low signal intensity (1.5 to 2.5 kcps). When determining the baseline of fresh SMUF at 50°C, a pronounced and rapid increase was observed in particle count and size during the measurement (Figure 2). This phenomenon was noted by Jenness and Koops (1962) when heating SMUF to 100°C, and was attributed to the dissolution and precipitation of amorphous calcium phosphate (Andritsos et al., 2002; Spanos et al. 2007). Although Jenness and Koops (1962) reported that the process was reversible on cooling to 3°C, in the current study the presence of particles was noticed even after cooling, which indicated that the precipitation process was not fully reversible. It is also worth noting that precipitation was observed when

Table 3. Ionic concentrations of the solvents used to dilute the milk samples (measured values) and milk serum (values from literature)

Ion	Ion concentration ¹			
	UF permeate, mg/L	SMUF, mg/L	Water, mg/L	Milk serum, ² mg/kg
1. Calcium	253	351	2.10	390
2. Phosphorus	407	352	0.05	360 ³
3. Magnesium	69	78	4.10	70
4. Potassium	1,423	1,429	<0.01	1,500
5. Sodium	268	368	0.70	450
6. Chloride	1,026	1,140	1.10	1,100
Total 1 through 6	3,446	3,718	<8.06	3,870

¹UF permeate = CN-depleted fresh permeate from UF milk; SMUF = simulated milk ultrafiltrate; water = UF water.

²Average values. Source: Walstra et al. (1999, p. 8).

³Estimated from total phosphate

Figure 2. Dynamics of signal intensity (count rate) and effective diameter of unfiltered simulated milk ultrafiltrate at 50°C. kcps = kilocounts per second.

a sample of SMUF was kept under stirring overnight at room temperature ($T \sim 23^\circ\text{C}$) which indicated that, given sufficient time, precipitation could take place at lower temperatures than reported previously. To avoid this problem, the SMUF was filtered using a 0.2- μm syringe filter immediately before using it to dilute the skim milk samples. Baselines of filtered SMUF at 50°C had signal intensities that never increased above 9 kcps because precipitation occurred at a much slower rate when the seed particles were removed.

The UF permeate was the solvent closest to milk serum, the natural environment of CN micelles. Baseline determinations performed at 6, 20, and 50°C were characterized by a low signal intensity (<23 kcps). The DLS analyses indicated the presence of 2 classes of particles: one in the nanometer to tens of nanometers range, and a second one in the hundreds of nanometers range. These particles were presumed to represent serum proteins and CN micelles, respectively. This hypothesis was verified by performing DLS measurements on a solution of serum proteins obtained from skim milk by using a quantitative CN precipitation procedure. According to the multimodal size distributions in Figure 3a and 3b, which show the results obtained at 20°C, the particles in the smaller range represent serum proteins, whereas the larger particle size group represents CN micelles. The presence of CN micelles in permeate obtained by UF with a 10-kDa cutoff was due to the membrane having a minor leak. However, because the classes of particles present in permeate were the same as in skim milk, it is reasonable to assume that they would not affect the trends observed in this study.

Size distribution data from multiple runs was shown to define the particle size ranges clearly, because distribution curves varied slightly among different replicate runs. When evaluating the multimodal particle size

distributions in Figure 3, it is important to keep in mind that they offer information about the size range of particles, not quantitative information about the size of individual particles. The calculated effective diameter of the serum proteins at 6, 20, and 50°C is shown in Table 4. A one-way ANOVA test indicated that these values were not statistically different. The important conclusion was that, because of the low signal intensity (7 to 23 kcps), the UF permeate was not considered to influence the results of the CN micelle size measurement significantly.

Effect of Fat Globules and Serum Proteins on the Particle Size Distribution in Skim Milk

Particle size measurements were performed both in raw skim milk and HTST pasteurized skim milk. For all solvents and temperatures, the particles observed by DLS ranged between several tens of nanometers to several hundreds of nanometers.

In addition to the influence of the solvent, which was discussed above, we considered that the presence of residual fat globules in the skim milk might also affect the accurate evaluation of CN particle size distribu-

Figure 3. Multimodal size distributions: a) UF permeate, and b) serum protein solution at 20°C.

Figure 4. Comparative particle size distributions: a) skim milk in UF permeate, and b) skim milk after dissociation of CN micelles in EDTA:SDS. Measurements were performed at 20°C.

tion. Although the fat content of the skim milk samples was very low ($\sim 0.1\%$), the slightly larger size of the fat globules, as compared with the CN micelles, may have affected the accuracy of PSD analyses, as was reported previously (Holt, 1985). To assess this influence, CN micelles were dissociated by treating the skim milk with a mixture of EDTA and SDS, as reported previously by several researchers (Holt, 1985; Michalski et al., 2001; Ye et al., 2004a,b). Particle size measurements were then performed at 20°C. The particles observed after this treatment ranged in diameter from 50 to 1,400 nm (Figure 4b), and the average effective diameter was 299.1 ± 21.4 nm. Two relatively distinct populations of particles were observed: one in the 100- to 180-nm range and a second one in the 400- to 1,100-nm range. It is possible that the smaller particles represented CN micelles that were not dissociated as a result of the EDTA:SDS treatment, whereas the second class was most likely predominantly composed of fat globules. It is interesting to note that the size of the latter class of particles was not observed during the DLS analyses of skim milk, in which the largest particles were in the 300- to 400-nm range (Figure 4a). The signal intensity

obtained in the measurement of the residual fat globules was very weak (36.7 ± 9.8 kcps) as compared with the skim milk analyses (700 to 900 kcps). However, because the fat globules are larger than the CN micelles, it is possible that even if they are present in trace amounts, they slightly distort the size distribution of the CN micelles, shifting it toward larger sizes. This effect of the fat globules needs to be acknowledged, but it is very difficult to account for it accurately and quantitatively when determining the size of the CN micelles. As discussed later in this manuscript, the effect of fat globules can become significant if an unsuitable solvent is used for dilution before DLS analyses.

Serum proteins were also present in the skim milk samples. However, because of the small amount and their small size (see Figure 3), their effect on the effective diameter, which was an intensity-weighted value, could be considered insignificant.

Particle Size Measurements in Skim Milk: Temperature and Solvent Effects

Based on the discussion above, the quantitative results of the particle size analyses performed on skim milk were considered to be due predominantly to the CN micelles, and from this point on, the discussion focuses on the CN micelles alone.

Table 5 contains the values of the effective diameters calculated after the PSD analyses of raw skim milk and pasteurized skim milk. All measurements had good reproducibility, with standard deviations in the range of 1.9 to 8.8 nm, corresponding to coefficients of variation of 1.1 to 4.9%.

Two-way ANOVA analysis indicated that the nature of the solvent had a significant effect on the measured effective diameter of the CN micelles, both in raw skim milk [$F(2,54) = 128.4$, $P < 0.0001$] and in pasteurized skim milk [$F(2,54) = 132.52$, $P < 0.0001$]. Temperature also had a significant influence on particle size [$F(2,54) = 20.5$, $P < 0.0001$ for raw skim milk, and $F(2,54) = 11.23$, $P < 0.0001$ for pasteurized skim milk], and a very strong interaction between solvent and temperature was also observed [$F(4,54) = 187.8$, $P < 0.0001$ for raw skim milk, and $F(4,54) = 125.6$, $P < 0.0001$ for pasteurized

Table 4. Effective diameters $\pm$ standard deviations of particles in a serum protein solution separated using the International Dairy Federation CN precipitation procedure

Temperature, °C	Average effective diameter, nm
6	8.1 ± 1.0^a
20	8.2 ± 0.7^a
50	8.4 ± 0.7^a

^aValues were not statistically different ($P < 0.05$).

Table 5. Calculated average effective diameter $\pm$ standard deviations for CN micelles in skim milk as measured by dynamic light scattering under different experimental conditions

Sample or solvent ¹	Average effective diameter, nm		
	6°C	20°C	50°C
Raw skim milk			
UF permeate	176.4 $\pm$ 5.3 ^a	177.4 $\pm$ 1.9 ^a	137.3 $\pm$ 2.7 ^d
SMUF	153.3 $\pm$ 2.7 ^b	176.3 $\pm$ 2.5 ^a	194.9 $\pm$ 6.0 ^c
Water	180.5 $\pm$ 8.8 ^a	180.0 $\pm$ 2.7 ^a	200.6 $\pm$ 2.5 ^c
HTST pasteurized skim milk			
UF permeate	190.5 $\pm$ 4.3 ^{bc}	176.9 $\pm$ 4.1 ^a	141.0 $\pm$ 2.1 ^e
SMUF	176.6 $\pm$ 4.8 ^a	198.8 $\pm$ 4.1 ^{bd}	205.9 $\pm$ 7.6 ^d
Water	178.8 $\pm$ 5.5 ^a	189.6 $\pm$ 5.3 ^c	198.7 $\pm$ 6.3 ^{bd}

^{a-e}Different letters indicate statistically significant differences ($P < 0.05$) for each source of casein micelles (raw skim milk or HTST pasteurized skim milk).

¹UF permeate = CN-depleted fresh permeate from UF milk; SMUF = simulated milk ultrafiltrate; water = UF water.

milk]. A Tukey-Kramer honestly significant difference analysis was then performed to identify statistically significant differences between the means.

When UF permeate was used as a solvent, the effective diameter of CN micelles decreased as temperature increased, with the effective diameter being significantly lower at 50°C as compared with 6 and 20°C (Table 5). The decrease in micelle size at 50°C, as compared with the other 2 temperatures, was in agreement with previous reports (Davies and Law, 1983; Ono et al., 1990; Gaucheron, 2005), and it can be explained by the increased strength of hydrophobic interactions at that temperature. At low temperatures, the hydrophobic bonds become weaker, which causes the micelle structure to become looser and more porous and the micelle diameter to become larger. Additionally, the solubility of calcium phosphate increases, leading to a partial dissolution of micellar calcium phosphate, which also causes the voluminosity of the CN micelle to increase

(Gaucheron, 2005). Whereas particle sizes were larger at the lower temperatures for both types of milk, a significantly higher particle size at 6°C, as compared with 20°C, was observed only in the pasteurized skim milk samples. The shrinking of CN micelles at 50°C and their loosening at 6°C could also be observed when analyzing the polydispersity (p) data. The interpretation of p-values is as follows: $0 \leq p \leq 0.02$, which indicates monodisperse or nearly monodisperse systems; $0.02 < p \leq 0.08$, which indicates narrow particle size distributions; and $p > 0.08$, which is characteristic of broader size distributions (Brookhaven Instruments, 1995). According to the polydispersity data in Table 6, micelles in UF permeate and SMUF displayed narrow distributions at 50°C ($p \leq 0.08$) and broader size distributions at the lower temperatures.

When SMUF and water were used as solvents, the effect of temperature on particle size was different from what was observed in UF permeate, with particle sizes

Table 6. Polydispersity values $\pm$ standard deviations for CN micelles in skim milk measured by dynamic light scattering under different experimental conditions

Sample or solvent ¹	Average polydispersity		
	6°C	20°C	50°C
Raw skim milk			
UF permeate	0.16 $\pm$ 0.02 ^a	0.09 $\pm$ 0.02 ^b	0.05 $\pm$ 0.01 ^c
SMUF	0.15 $\pm$ 0.01 ^a	0.09 $\pm$ 0.01 ^b	0.05 $\pm$ 0.01 ^c
Water	0.14 $\pm$ 0.03 ^a	0.10 $\pm$ 0.01 ^b	0.09 $\pm$ 0.03 ^b
HTST pasteurized skim milk			
UF permeate	0.21 $\pm$ 0.01 ^b	0.12 $\pm$ 0.01 ^{ac}	0.07 $\pm$ 0.01 ^d
SMUF	0.20 $\pm$ 0.03 ^b	0.12 $\pm$ 0.01 ^{ac}	0.08 $\pm$ 0.02 ^{cd}
Water	0.13 $\pm$ 0.02 ^a	0.07 $\pm$ 0.02 ^d	0.12 $\pm$ 0.05 ^{ac}

^{a-d}Different letters indicate statistically significant differences ($P < 0.05$) for each source of casein micelles (raw skim milk or HTST pasteurized skim milk).

¹UF permeate = CN-depleted fresh permeate from UF milk; SMUF = simulated milk ultrafiltrate; water = UF water.

Figure 5. Dynamics of the effective diameter of CN micelles in raw skim milk diluted with a) UF permeate, b) simulated milk ultrafiltrate, or c) water measured at 6, 20, and 50°C.

becoming larger as the temperature increased. To understand the reason for this behavior, a more detailed analysis of the PSD data was performed. Specifically, the evolution of the effective diameter and of the signal intensity during the PSD measurement was examined. As mentioned in the Materials and Methods section, each measurement consisted of 7 subsequent individual runs of 30-s duration, whose results were combined by the software into one final effective diameter calculation. The data for the individual runs was manually deconvoluted and analyzed, and the dynamics of the

Figure 6. Changes in signal intensity (particle counts) during the dynamic light scattering analyses of raw skim milk diluted with a) UF permeate, b) simulated milk ultrafiltrate, or c) water measured at 6, 20, and 50°C.

effective diameter over the duration of the measurement (3.5 min) were represented for each solvent and measurement condition. The data for the effective diameter measured in raw skim milk is shown in Figure 5 and that for signal intensity is shown in Figure 6. The trends observed for pasteurized skim milk were virtually identical to those for raw skim milk (data not shown).

When UF permeate was used as a solvent, the effective diameter was virtually constant throughout the

measurement, indicating that the micelles maintained their structure and size over the duration of the experiment (Figure 5a). A slight decreasing trend was observed at 50°C, but the increase was not statistically significant. Signal intensity was also constant throughout the measurements at 6 and 20°C, but it did increase slightly over time at 50°C, possibly because of precipitation of amorphous calcium phosphate at that temperature (Figure 6a). This increase in signal intensity reflected the small decrease in effective diameter discussed above, but was too small to affect in any significant way the outcome of the measurement.

In SMUF, the solvent with the greatest ionic strength (see Table 3), the effective diameter of the CN micelles was virtually constant at 6 and 20°C, but showed a statistically significant decreasing trend at 50°C ($P < 0.01$), with a 9.5% decrease taking place over 3.5 min (Figure 5b). This decrease in particle size was mirrored by an increase in signal intensity throughout the measurement. This is similar to the phenomenon observed for measurements performed in UF permeate, and it was possibly caused by the precipitation of amorphous calcium phosphate. In SMUF, however, these changes had a statistically significant effect on the results of the DLS analyses at 50°C, which changed throughout the duration of the measurement. It is important to note that at 20°C, the results of the DLS measurements performed in SMUF were very similar to those obtained when using UF permeate. Differences, however, were large at the other 2 temperatures (6 and 50°C). The increase in the effective diameter with temperature when SMUF was used as a solvent was surprising, and was not consistent with the known effects of temperature on the CN micelles that were discussed above. The reasons for this effect of temperature are still unclear. Although precipitation of calcium phosphate and formation of particles of approximately 200-nm size (see Figure 2) might explain in part the values obtained at 50°C, it is not clear what led to the decrease in effective diameter by approximately 20 nm between 20 and 6°C (Table 5).

Measurements performed in water, the solvent with the lowest ionic strength, also resulted in values that were not consistent with those obtained in UF permeate. At 6 and 20°C, a statistically significant ($P < 0.05$) increase in the effective diameter was observed from the beginning to the end of the measurement (Figure 5c). When testing a sample of skim milk in water at 6°C over a longer period of time (45 min), a 42% increase in size was observed, with the effective diameter increasing from 179.2 nm at 30 s to 254.7 nm at 45 min (data not shown). At 50°C, no significant changes in size were observed throughout the measurement. The linear, significant decrease in signal intensity observed

for all measurements performed in water (Figure 6c) suggests dissociation of the CN micelles in water. As a result, it is likely that the contribution of the residual fat globules to the measured effective diameter became more significant, which would explain the increase in size for the measurements performed at 6 and 20°C. For water, the influence of temperature on the measured effective diameter was similar to what was observed for SMUF, and thus was inconsistent with the expected trend.

When evaluating the influence of the solvents on the particle size of CN micelles, it is worth noting that the influence of their viscosity, although accounted for in the calculations, has not been investigated systematically. This issue deserves further consideration.

CONCLUSIONS

The findings of this study illustrate the unsuitability of water as a solvent and the limitations of SMUF as a solvent at 6 and 50°C. When water was used as a solvent, significant dissociation of CN micelles occurred, making the results of PSD analyses inaccurate. In SMUF at 50°C, precipitation of amorphous calcium phosphate affected the results of the DLS analyses, whereas at 6°C, the measured effective diameter of the CN micelles was lower than at the higher temperatures, for reasons that are still unclear.

Overall, it can be concluded that to obtain accurate measurements of CN micelle sizes, it is critically important to use a solvent with a chemical composition as close as possible to that of the milk serum. In this study, that solvent was identified to be UF permeate, whose use as a solvent resulted in CN micelle size values at the 3 different temperatures consistent with those reported in the literature. Although relatively minor, the slight overestimation of the effective diameter of CN micelles caused by the presence of residual fat globules in skim milk, in all solvents, must be acknowledged.

Another conclusion of this work was the importance of using a clear and consistent protocol for the measurement of particle sizes of CN micelles or of any other colloidal particles. Because in the literature, these important aspects of DLS measurement of CN micelles are, in many cases, mentioned briefly and somewhat inconsistently, this work provides a guide for conducting particle size analyses in milk.

ACKNOWLEDGMENTS

The authors thank the New York State Milk Promotion Board (Albany, NY) for funding this work and David Barbano's research group from Cornell University for kindly providing the milk UF permeate and its composition.

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