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Improving Clarity and Stability of Skim Milk Powder Dispersions by Dissociation of Casein Micelles at pH 11.0 and Acidification with Citric Acid

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Supporting Information

ABSTRACT: Casein micelles in milk cause turbidity and have poor stability at acidic conditions. In this study, skim milk powder dispersions were alkalized to pH 10.0 or 11.0, corresponding to reduced particle mass. In the following acidification with hydrochloric or citric acid, the re-formation of casein particles was observed. The combination of treatment at pH 11.0 and acidification with citric acid resulted in dispersions with the lowest turbidity and smallest particles, which enabled translucent dispersions at pH 5.5−7.0, corresponding to discrete nanoparticles. The concentration of ionic calcium was lower when acidified with citric acid than hydrochloric acid, corresponding to smaller particles with less negative zeta potential. The pH 11.0 treatment followed by acidification with citric acid also resulted in smaller particles than the simple chelating effects (directly implementing sodium citrate). The produced casein nanoparticles with reduced dimensions can be used for beverage and other novel applications.

KEYWORDS: *skim milk powder, dispersibility, alkaline dissociation, casein micelles, citric acid, nanoscale structure*

INTRODUCTION

Bovine milk is an important source of proteins, vitamins, minerals, and energy. Caseins consist of about 80% of the bovine milk proteins, with the rest being whey proteins.[1] There are four types of caseins (α_{s1}, α_{s2}, β, and κ) that exist in bovine milk as mostly spherical casein micelles with a diameter of 50−250 nm.[2] Although the internal structure of casein micelle is still being debated, it is generally agreed that hydrophobic interactions and bridging by calcium phosphate are important to the micelle structure.[3,4] The κ-casein is located mostly on the outer surface of micelles, with the calcium-insensitive segment (glycomacropeptide) not being associated with other caseins.[5] The glycomacropeptide protrudes from the micelle surface by about 10 nm and acts as a "hairy layer" providing repulsive steric interactions that stabilize casein micelles against aggregation in bovine milk with acidity of about pH 6.8.[6−8]

Fabrication of casein structures is important for several applications. Casein micelles are sufficiently big to scatter visible light, and disruption of casein micelles is required to incorporate milk protein ingredients in beverages that are transparent or translucent. There also has been great interest in applying dairy proteins as encapsulants to deliver bioactive food components in food matrices.[9−12] Particularly, because nanoscale delivery systems have unique properties such as dispersion stability and low turbidity, dissociation of casein micelles to smaller structures may be important for developing relevant applications.

There are several methods that have been studied to control casein structures. High pressure treatment is a well-studied physical method[13−15] that changes the arrangement of water molecules around proteins[16] and thus hydrophobic interactions between caseins. However, the process is energy consuming because a pressure of 150 MPa is needed to trigger the dissociation of casein micelles.[15] When heated to above 65 °C in aqueous ethanol with more than 35% ethanol, dissociation of casein micelles was observed because of the improved solvent quality and the shifting of pK_a values of phosphoserine.[17,18]

Dissociation of casein micelles at acidic or alkaline pH has also been studied at ambient conditions without applying high energy and ethanol. Dissociation of casein micelles was observed during acidification from neutral pH to about pH 5.4, followed by reassociation upon further acidification.[19] The dissociation during acidification is favored at a lower temperature.[20−22] The alkaline dissociation of casein micelles has been studied by Vaia et al.,[23] with a higher dissociation extent at a higher temperature and a faster dissociation rate at a higher pH. The authors also reported that the alkaline disintegration of casein micelles was largely reversible and the reassociated caseins could closely resemble the properties of native casein micelles if controlled properly. The reversibility of casein micelle structure after alkaline disruption is probably caused by the incomplete dissociation at the studied pH (up to pH 10.0) conditions and the increased solubility and activity of calcium during re-formation.

Additionally, several calcium-chelating agents such as ethylenediaminetetraacetate (EDTA), phosphate, and citrate have been reported to be effective in dissociating casein micelles.[4,24,25] These compounds are competing with calcium in casein micelles that is present as colloidal calcium phosphate.[3,26] Colloidal calcium phosphate plays an important role in the association of calcium-sensitive caseins and, together

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with hydrophobic interactions, is critical to the structure of casein micelles. The loss of calcium in micelles causes the dissociation of β- and κ-caseins from casein micelles.[24]

The existing literature suggests the possibility of dissociating casein micelles at alkaline conditions and controlling the re-formation of casein micelles during the subsequent acidification process, which can be further controlled by chelating agents such as citrate. In this work, our overall objective was to study the structural re-formation after acidification of alkaline-treated dispersions constituted from skim milk powder, with and without citrate. Skim milk powder was chosen because of the need to improve stability, especially at acid pH. Particularly, the controlled re-formation of casein micelles to smaller dimensions is desired because nanostructures reduce turbidity and improve the stability against gravitational sedimentation due to the significance of Brownian motion. To understand structure re-formation, dispersions were characterized for macroscopic turbidity, hydrodynamic diameter using dynamic light scattering (DLS), morphology using atomic force microscopy (AFM), and ionic calcium concentrations. The engineered nanostructures enable the expanded use of milk protein in the beverage, cosmetic, and pharmaceutical industries where transparent appearance is desired. The improved understanding of nanoscale structure formation during acidification may also be significant to develop relevant nanoscale delivery systems discussed previously.

MATERIALS AND METHODS

Materials. Skim milk powder (Carnation nonfat dry milk) was a product from Nestlé Baking Inc. (Solon, OH). According to the label, the product had 34.8% protein, dry-basis, and was a pasteurized product. Other chemical grade reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Bio-Rad Laboratories, Inc. (Hercules, CA).

Sample Preparation. Sample preparations were conducted at room temperature (21 °C). Skim milk powder was hydrated at 9% w/w in deionized water for 6 h. The control samples were acidified directly using 4.0 M HCl. To dissociate casein micelles, samples were adjusted to pH 10.0 or 11.0 with 4.0 M NaOH and were incubated for 1 h.[23] The re-formation of casein micelles was studied by acidification to pH 7.0, 6.5, 6.0, and 5.5 with 2.0 M citric acid or 4.0 M HCl under vigorous agitation using a stirring plate. To simplify description, the pH cycle treatments in Table 1 are referred to as different routes hereafter. Approximately 300 μL of 4.0 M NaOH was used to adjust 20 mL of 9% w/w skim milk dispersion to pH 11.0, and 150 μL of 2.0 M citric acid was needed to subsequently acidify the dispersion to pH 7.0. Another set of samples were prepared by dissolving 0−60 mM dihydrate sodium citrate in the skim milk dispersion that was adjusted to pH 7.0 using 1.0 M NaOH or HCl after vigorous stirring for 2 h. To prevent microbial spoilage, 0.05% w/w sodium azide was added to each sample. Three independent replicates were prepared for each sample.

Table 1. Codes Representing Different Routes Used To Dissociate Casein Micelles at Alkaline pH and Re-Form Casein Micelles by Acidification

route code	dissociation pH	acid used to re-form casein micelles
A (control)	none (control)	4.0 M HCl
B (pH 10/HCl)	10.0	4.0 M HCl
C (pH 10/citric)	10.0	2.0 M citric acid
D (pH 11/HCl)	11.0	4.0 M HCl
E (pH 11/citric)	11.0	2.0 M citric acid

Turbidity Measurement. To determine the normalized turbidity (eq 1) using the literature method,[23] a milk serum sample was prepared by centrifugal ultrafiltration. 6 mL of the 9% skim milk dispersion at pH 7.0 was placed in a Vivaspin 6 centrifuge tube (Vivaproducts, Inc., Littleton, MA) with the bottom mounted with an ultrafiltration membrane with a molecular weight cutoff of 10,000 kDa, and the centrifugation was carried out at 10000g for 20 min (model 4540, Eppendoff, Hamburg, Germany). The absorbance of milk serum (Abs_{serum}) and skim milk dispersions (Abs_{sample}) was measured at 600 nm using a UV−vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA).

$$\text{normalized turbidity} = \frac{Abs_{sample} - Abs_{serum}}{Abs_{untreated} - Abs_{serum}} \quad (1)$$

where $Abs_{untreated}$ is the absorbance of skim milk dispersion without pH adjustment.

Particle Size and Zeta-Potential. Zeta-potential and particle size distribution were measured for samples adjusted to pH 7.0 using a Delsa Nano analyzer (Beckman Coulter, Inc., Atlanta, GA). Particle size of samples directly adjusted to pH 9.0−11.0 and subsequently acidified from pH 11.0 to pH 7.0−10.0 using 4.0 M HCl or 2.0 M citric acid was also measured. Samples were diluted 100 times using deionized water adjusted to the corresponding pH using 0.05 or 1.0 M NaOH. Particle size distributions were used to calculate the volume-length mean particle diameter ($D_{4,3}$) using eq 2.

$$D_{4,3} = \frac{\sum_{i=1} n_i D_i^4}{\sum_{i=1} n_i D_i^3} \quad (2)$$

where n_i is the number of particles corresponding to diameter D_i.

Morphological Studies. The skim milk dispersions at pH 5.5 and 7.0 were diluted to an overall solids content of 10 ppm in deionized water adjusted to the same pH using 0.05 M NaOH or HCl. 2 μL of each diluted sample was spread evenly onto freshly cleaved mica sheets that were mounted on sample disks (Bruker Corp., Santa Barbara, CA) for AFM. A rectangular cantilever having an aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m (FESPA, Bruker Corp., Santa Barbara, CA) and a Multimode microscope (Bruker AXS, Billerica, MA) were used to scan the sample. Images were generated at the tapping mode with a preset scan area of 2.0 × 2.0 μm at a scanning speed of 1 Hz. The dimension of particles was analyzed using the particle analyzer function of the NanoScope Analysis software (Version 1.4, Bruker Corp., Santa Barbara, CA).

Determination of Ionic Calcium Concentration. The concentration of ionic calcium in skim milk dispersions was determined with a calcium-selective electrode (Denver Instrument Company, Bohemia, NY). The standard curve was established using a series of solutions with 10−1000 ppm $CaCl_2$ and 0.08 M KCl in distilled water. Results were normalized by the concentration of ionic calcium in skim milk dispersion directly adjusted to pH 7.0 using 1.0 M NaOH.

Analytical Ultracentrifugation (AUC). A Beckman XL-I analytical ultracentrifuge (Beckman Coulter, Inc., Palo Alto, CA) was used in AUC experiments according to the procedures in our earlier work.[27] Briefly, samples were diluted to a protein concentration of 1.2 mg/mL in deionized water and readjusted to the target pH. The centrifugation was performed at 50000 rpm and 25 °C. The SEDFIT software of the instrument was used to analyze data using the continuous $c(s)$ distribution model, with the anhydrous frictional ratio (f/f_0) set at 1.2. Because molecular weights (MW) of individual caseins are higher than 19 kDa,[28] signals corresponding to a MW higher than 19 kDa were compared and further used to calculate the number average MW of the mixture using the following equation.

$$MW = \frac{\sum_1^n MW_i p_i}{\sum_1^n p_i} \quad (3)$$

where MW_i and p_i represent the MW and mass percentage of the ith fitted peak.

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Occurrence of Maillard Reaction. The possible Maillard reaction between dairy protein and lactose at the studied conditions can be qualitatively compared for absorbance at 420 nm (Abs_{420}).[29] Dispersions were adjusted to pH 4.6 with 4.0 M HCl to precipitate caseins. After centrifugation at 10000*g* for 20 min (model 4540, Eppendoff, Hamburg, Germany), the supernatant was transferred and was measured for Abs_{420} using a UV−vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA).

Statistical Analysis. Statistical analyses were performed using the JMP Statistical Software (SAS Institute, Cary, NC). One-way analysis of variance was carried out. Differences between pairs of means were compared using the Tukey test. The significance level P was set at 0.05.

RESULTS AND DISCUSSION

Although skim milk powder contains whey proteins, lactose and small quantities of lipids, it can be expected that caseins are mostly responsible for physicochemical changes observed in the present study. This can be ascribed to the fact that whey proteins do not usually precipitate in the pH range (5.5−11.0) presented below. The discussion hereafter is thus focused on casein only. Furthermore, it is expected that casein structures in skim milk powder dispersions are different from native casein micelles in bovine milk because of the processes used to prepare powder and reconstitution. We use "casein micelles (CM)" nevertheless hereafter for simplicity. Likewise, casein structures re-formed from dissociated CM may not resemble CM because of the possible changes in composition (types of caseins and amounts of colloidal calcium phosphate), but they are called "re-formed CM (rCM)" for simplicity and consistency with the literature.[30,31] The structures of CM and rCM are sensitive to the environment and could be changed when samples were diluted for characterization using a specific technique. Additionally, the skim milk powder used in this study is expected to be different from that directly spray-dried from skim milk, because surfactants such as lecithin can be used to improve product properties.[32] Heat treatment conditions used to produce skim milk powder can also significantly impact the properties of milk powder dispersions,[32] although it was shown that preheating at 90 °C for 10 min did not impact the alkaline disruption of casein micelles.[23] These variables were not investigated in the present study.

Structure Characteristics at Alkaline pH. The dispersions showed reduced turbidity when samples were gradually adjusted to pH 11.0 (Figure 1a), which agrees with Huppertz et al.,[30] who studied up to pH 10.0. The turbidity increased only slightly when acidified with HCl to pH 7.0 and was practically unchanged when acidified with citric acid (Figure 1a). This contrasts with the almost complete recovery of sample turbidity after the pH cycle (from 7.0 to 10.0, then to 7.0) found by Huppertz et al.[30] When hydrodynamic diameters were measured, the reduction of $D_{4,3}$ was observed when pH was increased from 7.0 to 10.0, followed by an increase at pH 11.0 (Figure 1b). When samples were acidified from pH 11.0, $D_{4,3}$ gradually decreased for both acid treatments (Figure 1b). The data in Figure 1 showed that alkaline treatments at pH 10.0, as in the work of Huppertz et al.,[30] and 11.0, in the present study, had different effects on casein structure evolution.

Because turbidity is a function of both particle dimension and density,[33] AUC was used to study samples alkalized to pH 7.0−11.0 and acidified from pH 11.0 to 7.0 using HCl, with the structures bigger than 19 kDa summarized in Table 2. A monotonic decrease in particle mass was observed with an

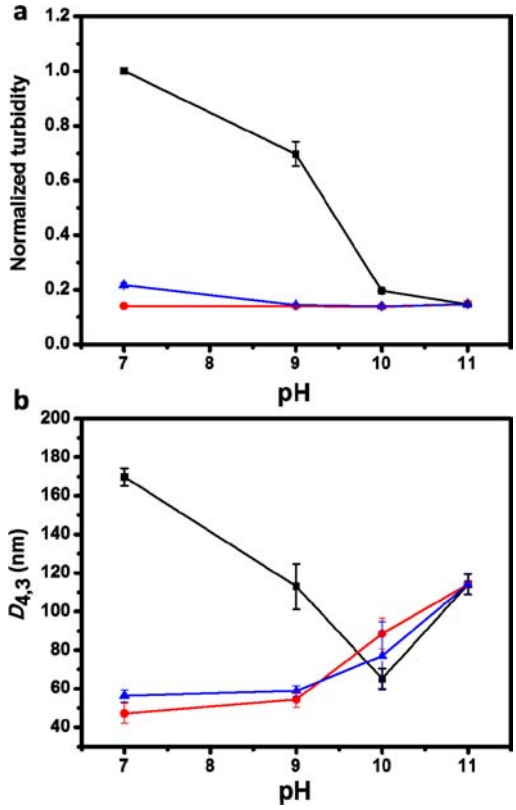

Figure 1. Turbidity (a) and $D_{4,3}$ (b) of samples when increasing pH from 7.0 to 9.0−11.0 (black squares) and subsequent acidification with citric acid (red circles) or HCl (blue triangles). Error bars are standard deviations from triplicate samples.

increase in pH during alkalization, which suggests a greater extent of CM dissociation at a higher pH. At pH 11.0, there were on average about 26 caseins per particle, based on an average MW of 25 kDa for caseins, which is about one-third of particle mass at pH 10.0. The bigger $D_{4,3}$ at pH 11.0 (Figure 1b) than that at pH 10.0 can then be caused by fewer loosely associated caseins that take a larger volume. Physically, the dimension difference between pH 10.0 and pH 11.0 treatments can be contributed by basic amino acid residues such as lysine and tyrosine whose side chain pK_a is 10.5 and 10.1, respectively.[34] At pH 11.0, caseins are (negatively) ionized to a greater extent than at pH 10.0, which increases the hydrodynamic diameter of polyelectrolytes and the intraparticle repulsion, corresponding to the lowered turbidity (Figure 1a) and increased dimension (Figure 1b).

When samples were acidified from pH 11.0 to pH 7.0, the particle mass increased gradually (Table 2), which indicates the reassociation of caseins, as reported by Huppertz et al.[30] The reduced dimension during acidification (Figure 1b) can be interpreted by the increased particle density as the weakened electrostatic repulsion between basic amino acid residues decreases the excluded volume of associated caseins. The increased particle mass (Table 2) and the reduced dimension (Figure 1b) contribute to similar or slightly increased turbidity during acidification (Figure 1a). The drastic difference in particle mass (~3 times) after alkalization to pH 10.0 and pH 11.0 (Table 2) may have contributed to the differences between our findings and Huppertz et al.[30] Physically, CM are dissociated to a greater extent at pH 11.0, which, analogous to the nucleation theory, provides more sites for attracting the

Table 2. AUC Molecular Weight (MW) Characteristics of Skim Milk Dispersions When Increasing pH from 7.0 to 11.0 (↑) Using 4 M NaOH and Subsequent Acidification from pH 11.0 to 7.0 (↓) Using 4 M HCl

sample pH	MW ($\times 10^6$ Da) and percentage (in parentheses) of each fitted peak						number av MW ($\times 10^6$ Da)
7↑		0.926 (8.66%)	1.20 (8.26%)	1.68 (13.0%)	2.94 (22.8%)	6.87 (47.4%)	4.32
9↑	0.162 (13.4%)	0.348 (22.0%)	1.07 (9.42%)	1.50 (11.4%)	2.36 (17.5%)	4.74 (26.3%)	2.15
10↑	0.162 (13.8%)	0.418 (29.3%)		1.50 (11.7%)	2.41 (19.0%)	4.83 (26.2%)	2.01
11		0.340 (79.7%)	1.13 (6.06%)	1.58 (6.21%)	2.66 (8.03%)		0.65
10↓	0.170 (17.1%)	0.481 (60.5%)			2.50 (13.5%)	4.83 (8.85%)	1.08
9↓		0.347 (46.4%)		1.34 (17.6%)	2.26 (17.9%)	4.90 (18.1%)	1.69
7↓	0.263 (18.9%)	0.790 (21.0%)	1.32 (17.2%)	2.02 (17.2%)	3.53 (13.1%)	7.63 (12.6%)	2.21

dissociated caseins during acidification. This can increase the population and reduce the dimension of rCM and therefore turbidity for treatments at pH 11.0 when compared to those at pH 10.0. The following part focuses on samples at acidic and neutral conditions after processing using different routes in Table 1.

Occurrence of Covalent Bonding during Treatments. Alkaline treatment of milk dispersions can potentially cause covalent bonding and Maillard-type reactions between protein and protein, and between protein and lactose.[35] The impacts of different routes in Table 1 were studied for SDS−PAGE and Abs_{420}, with the former examining MW changes of proteins and the latter the occurrence of Maillard reaction. SDS−PAGE data in Figure S1 in the Supporting Information did not show apparent difference in MW of proteins. For Abs_{420}, samples treated with routes C (pH 10/citric), D (pH 11/HCl), and E (pH 11/citric) had a significantly lower magnitude than those of A and B (Figure 2A in the Supporting Information). As presented below, treatments with routes A and B had much higher turbidity and bigger particle dimension, which corresponded to greater turbidity of supernatants after centrifugation of samples adjusted to pH 4.6 (Figure 2B in the Supporting Information). Therefore, there is no indication of chemical reactions at the studied conditions.

Properties of Alkaline-Treated Dispersions Acidified to pH 5.5−7.0. *Sample Appearance and Turbidity.* Visual appearance and normalized turbidity of samples acidified to pH 5.5−7.0 are shown in Figure 2 and Figure 3, respectively. At pH 3.0−5.0, precipitation was observed for all samples and the precipitated proteins separated into a bottom phase below the transparent serum (not shown). At pH 5.5−7.0, sample turbidity varied significantly with methods of acidification and pH. Overall, samples were more turbid at a lower pH, which is expected because the reduced number of net negative charges and thus weakened electrostatic repulsion between caseins favor protein aggregation as pH approaches the isoelectric point (p*I*) of ∼4.6.[34] Samples acidified with routes B (pH 10/HCl) through E (pH 11/citric) generally had lower turbidity than those directly acidified using HCl (route A, control). Route B (pH 10/HCl) was the least effective in reducing sample turbidity, while route E (pH 11/citric) was the most effective. Routes C (pH 10/citric) and D (pH 11/HCl) appeared to have had similar impacts on sample turbidity when pH was 7.0. At pH 5.5−6.5, citric acid was more effective than HCl in reducing turbidity. Results in Figures 2 and 3 indicate that the dissociated CM re-formed to different structures upon acidification using the studied routes.

Route B (pH 10/HCl) was used by Huppertz et al.,[30] and our results verified the properties of rCM upon acidification from pH 10.0. By substituting HCl (route B, pH 10/HCl) with citric acid during acidification (route C, pH 10/citric), calcium

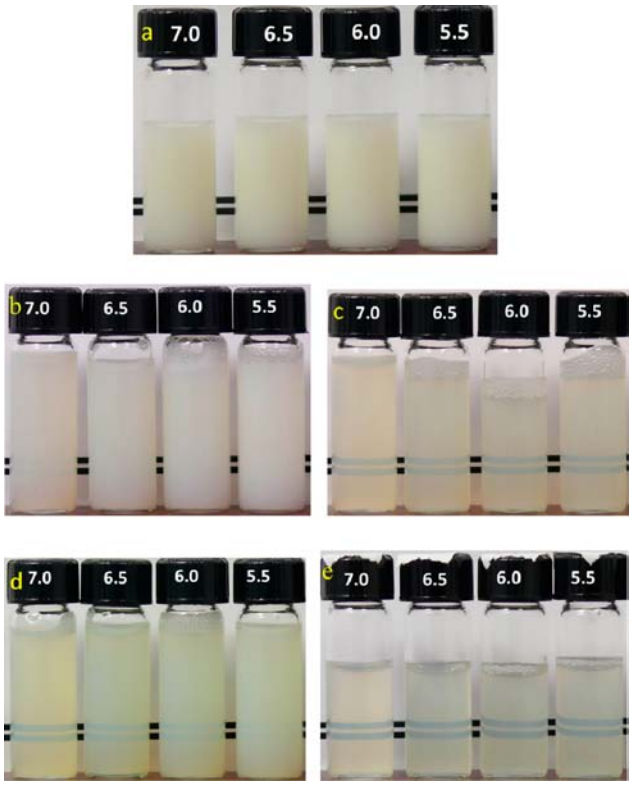

Figure 2. Appearance of dispersions constituted with 9% w/w skim milk powder after acidification to pH 5.5−7.0, as labeled on vials, using (a) route A (control), (b) route B (pH 10/HCl), (c) route C (pH 10/citric), (d) route D (pH 11/HCl), and (e) route E (pH 11/citric), as detailed in Table 1

ions are partially chelated by titrating citrate[25,36] and have reduced availability for bridging calcium-sensitive caseins,[37] corresponding to theoretically smaller rCM and reduced turbidity. For samples acidified from pH 11.0 using HCl (route D, pH 11/HCl), the effectiveness in lowering turbidity was similar to route C (pH 10/citric) (but without using citrate) at pH 7.0 but was less significant than route C (pH 10/citric) at pH 5.5−6.5 (Figures 2 and 3). The combined chelating effects of citric acid and extensive dissociation of CM at pH 11.0 enabled the greatest reduction in turbidity for route E (pH 11/citric).

Concentration of Ionic Calcium during Acidification. To study the role of calcium in formation of rCM during acidification, concentrations of ionic calcium in samples prepared from routes A (control), D (pH 11/HCl), and E (pH 11/citric) were determined (Figure 4). Direct acidification of dispersions from pH 7.0 using HCl (route A, control) liberated calcium from CM, corresponding to a higher ionic

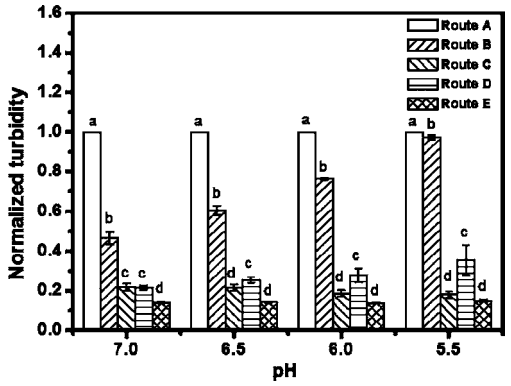

Figure 3. Normalized turbidity of dispersions constituted with 9% w/w skim milk powder after acidification to pH 5.5−7.0 using routes A−E as described in Table 1. Error bars are standard deviations from 3 replicates. Different letters above bars indicate statistical difference among samples at the same pH ($p < 0.05$).

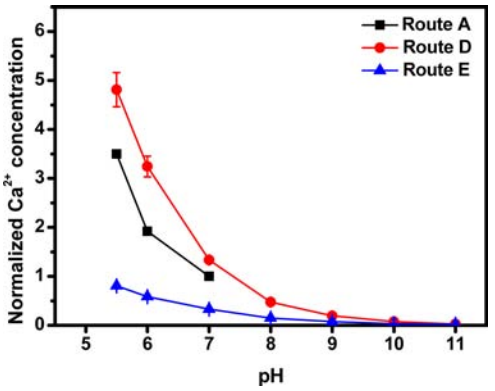

Figure 4. Concentrations of ionic calcium in skim milk dispersions after acidification using routes A (control), D (pH 11/HCl), and E (pH 11/citric), normalized by that at pH 7.0 adjusted using route A (100.5 ppm). Error bars are standard deviations from 3 replicates.

calcium concentration at a lower pH. When dispersion pH was increased to 11.0, ionic calcium concentration was only 2.2 ppm. This can be caused by two effects: precipitation of calcium phosphate at alkaline pH and binding of calcium to highly negatively charged dairy proteins. Upon acidification from pH 11.0 using HCl, samples from route D (pH 11/HCl) had higher concentrations of ionic calcium than those from route A (control), possibly because calcium phosphate initially liberated during the extensive dissociation of CM at pH 11.0 was gradually dissolved as ionic calcium during acidification. As for samples from route E (pH 11/citric), citrate ions effectively chelated the liberated calcium ions during acidification, corresponding to a concentration not higher than the dispersion at pH 7.0 without derivitization.

Additionally, there was no difference in ionic calcium concentration between routes D (pH 11/HCl) and E (pH 11/citric) at pH 9 and above (Figure 4), which agrees with no difference in turbidity and $D_{4,3}$ of the two treatments (Figure 1). At pH 7.0 and below, the higher ionic strength (ionic calcium) of treatments in route D (pH 11/HCl) than route E (pH 11/citric) weakens electrostatic repulsion due to the shortened Debye length and increases the possibility of particle aggregation, corresponding to higher turbidity and bigger $D_{4,3}$ for route D (pH 11/HCl) (Figures 1, 2 and 3).

Particle Size and Morphology of Dispersions. The $D_{4,3}$ of dispersions adjusted to pH 7.0 is shown in Figure 5. Samples

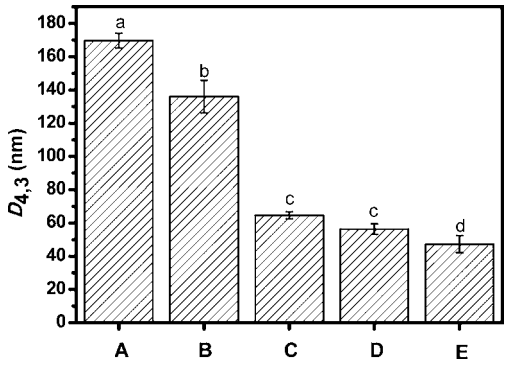

Figure 5. Volume-length mean particle diameter ($D_{4,3}$) (a), and the protein concentration measured using the Bradford method (b) of the dispersions constituted with 9% w/w skim milk powder after adjusting to pH 7.0 using routes A−E (as labeled on *x*-axis) as detailed in Table 1. Error bars are standard deviations from 3 replicates. Different letters above bars indicate statistical difference ($p < 0.05$).

from routes C (pH 10/citric), D (pH 11/HCl), and E (pH 11/citric) have much smaller $D_{4,3}$ than those from routes A (control) and B (pH 10/HCl), with that from route E (pH 11/citric) being the smallest. The DLS results are in agreement with turbidity data in Figure 3. The correlation between turbidity and particle size of a colloidal dispersion suggests that, unlike at alkaline pH (Figure 1), particle size is the major contributor of turbidity at neutral pH when intraparticle repulsion is not as strong.

AFM was conducted to study the morphology of structures after acidification to pH 7.0 and 5.5 using routes A (control) and E (pH 11/citric) (Figure 6). At pH 7.0, spherical particles were observed for the sample from route A (without derivitization, control), and the mean diameter of particles marked in azure was 84.4 nm. Although the particle dimension from AFM is smaller than the DLS result (~170 nm) because of the drying effect during AFM sample preparation, the particle mean diameter is in the 50−250 nm range of CM diameters reported in the literature.[2] When the dispersion was acidified to pH 5.5 using HCl directly (route A, control), irregularly shaped aggregates were observed, with a much bigger mean diameter of 172 nm (Figure 6a vs Figure 6b), and could have been caused by localized low pH when 4 M HCl was used in acidification. The coarse aggregate was evident as visible precipitates in Figure 2.

For samples prepared using route E, discrete particles were observed at both pHs (Figure 6c and 6d). At pH 7.0, mean particle diameter was about one-half of the sample prepared from route A (control) (Figure 6c vs Figure 6a). This indicates fewer casein molecules in rCM when processed using route E (pH 11/citric) than route A (control). The dissociation and re-formation of CM also agree with the less spherical structures in Figure 6C than those in Figure 6A. At pH 5.5, the sample from route E (pH 11/citric) showed individual particles with mean particle diameters similar to those in the sample at pH 7.0 (Figure 6d vs Figure 6c). AFM results support the turbidity data in Figures 2 and 3, and different stability against aggregation is due to structure differences of rCM processed with various routes.

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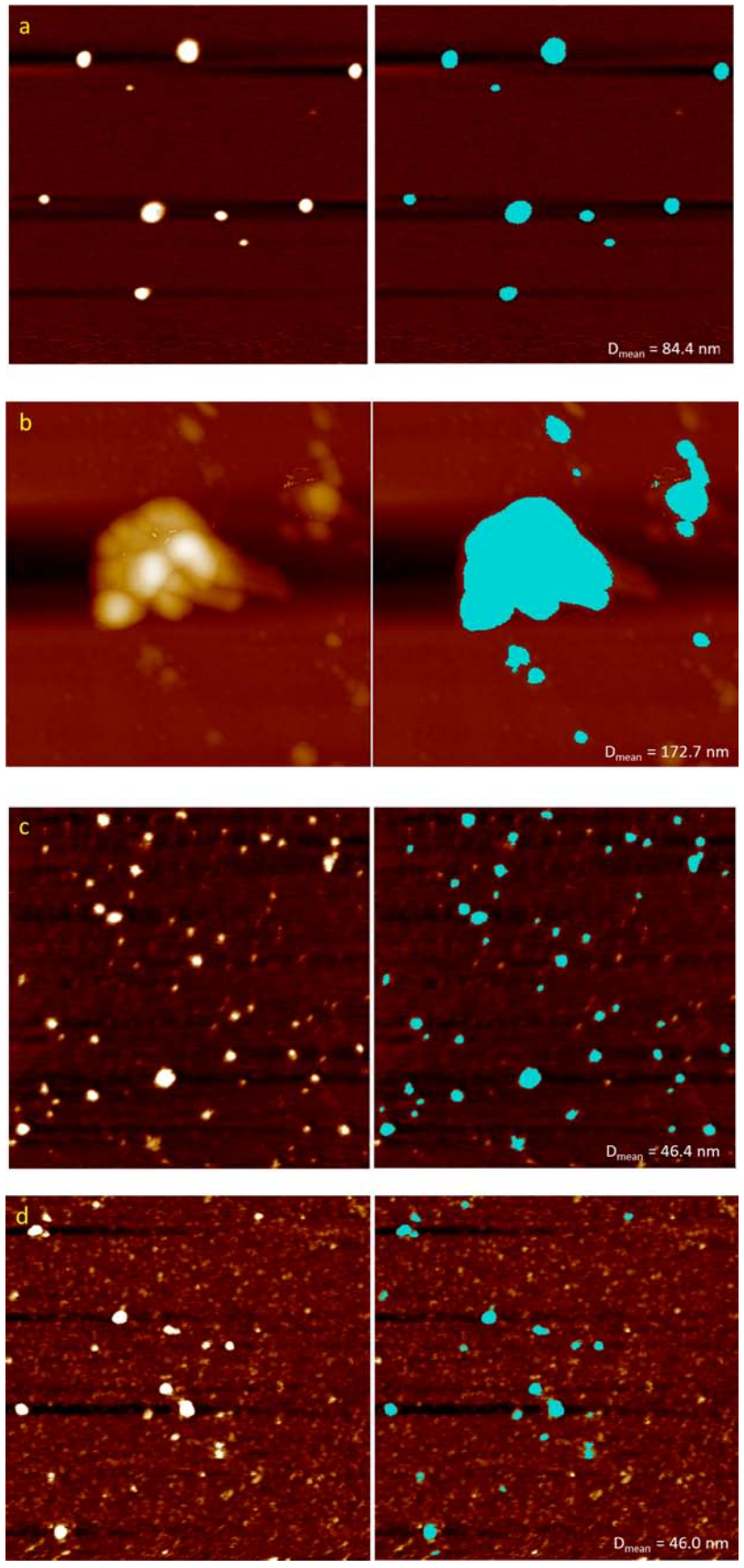

Figure 6. Atomic force microscopy topography images (left) of skim milk dispersions after adjusting pH to (a) 7.0 and (b) 5.5 using route A (control), in comparison to pH adjustment to (c) 7.0 and (d) 5.5 using route E (pH 11/citric). Particles picked by the software and the analyzed mean particle diameter are shown on the right. The scanned area is 2.0 × 2.0 μm in each image.

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Zeta-Potential of Dispersions at pH 7.0. The zeta-potentials of dispersions adjusted to pH 7.0 using different routes are shown in Figure 7. The data followed the opposite trend from

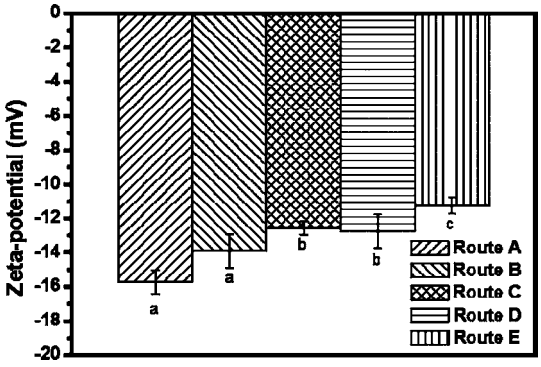

Figure 7. Zeta-potential of dispersions constituted with 9% w/w skim milk powder after adjusting to pH 7.0 using routes A–E as detailed in Table 1. Different letters below bars indicate statistical difference ($p < 0.05$).

the particle size (Figure 5). The p*I* of α_{s1}-, α_{s2}-, β-, and κ-casein is 4.94, 5.23, 5.14, and 5.90, respectively, with net charges of −21.9, −17.1, −13.3, and −2.0 at pH 6.6, respectively.[38] Theoretically, smaller rCM correspond to a smaller quantity of κ-casein per particle, and therefore the highest zeta-potential magnitude is expected for the sample processed with route E (pH 11/citric), which is opposite to results in Figure 7. Conversely, zeta-potentials in Figure 7 are in line with the increase of electrolytes associated with pH treatments. As a greater amount of NaOH is needed to increase pH to 11.0 than 10.0 and a greater amount of citric acid (weak acid, with trivalent citrate) is used in acidification, the treatment processed with route E (pH 11/citric) to pH 7.0 has the highest ionic strength, which reduces the Debye length, electrophoretic mobility, and thus the measured magnitude of zeta potential.[39] The treatments of routes A (control) and B (pH 10/HCl) had similar zeta-potentials, which agrees with the results of Huppertz et al.[30] It is likely that the above phenomena due to the alkalization to pH 10.0 and acidification to pH 7.0 with HCl are unable to cause statistical significance under the studied conditions.

Dephosphorylation of caseins by alkaline phosphase[40] or alkaline pH[41] can change p*I* and thereby surface charge and solubility characteristics of caseins. However, Freimuth and Krause[41] observed no more than 0.5% dephosphorylation after 24 h treatment at a temperature below 30 °C and a pH no higher than 11. Because samples were treated at 21 °C and pH 11 for 1 h in the present study, dephosphorylation was expected to be minor and was therefore not quantified.

Differences between Chelating Effects and Structural Re-Formation. Because CM can be dissociated by simply adding citrate,[4,25,36] the last set of experiments was used to study skim milk dispersions supplemented with 0–60 mM sodium citrate and adjusted to pH 7.0 using 1 M HCl, where 15 mM citrate was equivalent to the treatment using route E (pH 11/citric). The turbidity and $D_{4,3}$ of this group of treatments are shown in Figure 8. There was a negative correlation between the turbidity and the citrate concentration (Figure 8a), verifying that chelating of calcium by citrate directly leads to the dissociation of CM.[25,36] Smaller $D_{4,3}$ at a higher citrate concentration (Figure 8b) further verified the dissociation

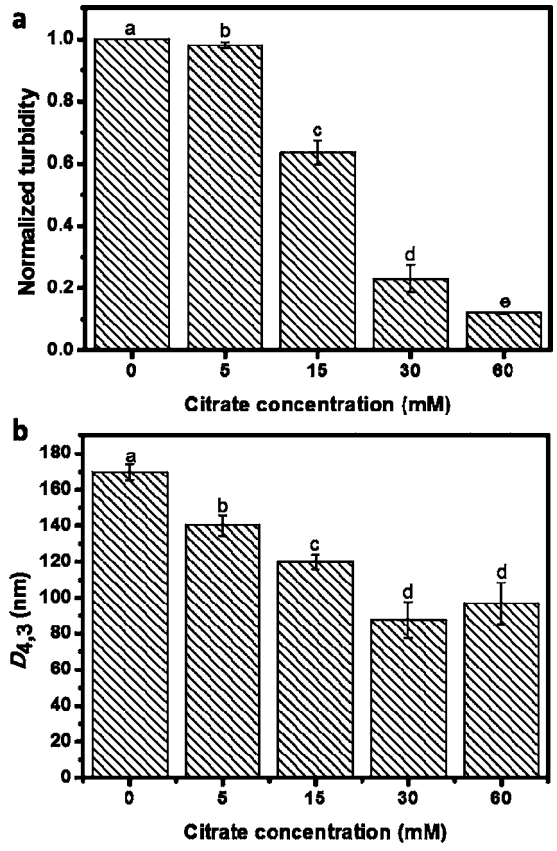

Figure 8. Normalized turbidity (a) and volume-length mean particle diameter ($D_{4,3}$) (b) of skim milk dispersions supplemented with different concentrations of sodium citrate after direct adjustment of pH to 7.0 using 1 M NaOH or HCl. Error bars are standard deviations from 3 replicates. Different letters above bars indicate statistical difference ($p < 0.05$).

properties of the chelating agent. The treatment with 15 mM citrate had a normalized turbidity of 0.64 and $D_{4,3}$ of 119.9 nm, bigger than the normalized turbidity of 0.14 and $D_{4,3}$ of 47.3 nm for the comparable treatment from route E (pH 11/citric). Therefore, route E (pH 11/citric) used in this study is more effective in improving dispersibility of skim milk powder than simply supplementing citrate, resulting from the controlled formation of rCM as discussed above.

In conclusion, CM in skim milk dispersions dissociated to a greater extent at more alkaline conditions, which allowed the re-formation to smaller particles that were further assisted by the calcium-chelating citrate during acidification. The combination of alkaline treatment at pH 11.0 and acidification with citric acid corresponded to the smallest rCM, the lowest dispersion turbidity, and the best stability against acid coagulation, especially at acidic conditions down to pH 5.5. The smaller rCM in route E (pH 11/citric) resulted from a lower concentration of ionic calcium due to the chelating effect by citrate. The structural re-formation in route E (pH 11/citric) was also observed to be different from the simple chelating function of citrate. This work provides a novel approach to reduce the dimension of casein particles, which can be directly used in aqueous dispersions such as transparent beverages requiring stability and clarity and further explored for other applications such as encapsulation.

■ ASSOCIATED CONTENT

Supporting Information

SDS−PAGE and Abs_{420} verifying no covalent bonding during treatments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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