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Physicochemical Variables Affecting the Rheology and Microstructure of Rennet Casein Gels

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The rheology and microstructure of a rennet casein system were studied in the pH range from 5.8 to 12.0 during cooling from 80 to 5 °C at four cooling rates: 0.5, 0.1, 0.05, and 0.025 °C/min. A dramatic increase in storage modulus with pH was observed during cooling at a fixed cooling rate. Continuous networks were formed for gels at pH 7.2 and above, while a discontinuous network was observed for gels below pH 6.5. The monotonic increase in storage modulus with pH could be correlated to the number of net (negative) charges and the strength of the hydrophobic interactions. At a higher pH, the protein micelles were larger due to weaker hydrophobic interactions and stronger repulsive electrostatic interactions resulting from more charges. When these protein micelles aggregated into flocs during cooling, the flocs had similar sizes at different pH values but a smaller fractal dimension at a higher pH. Consequently, for systems of the same protein and salt concentrations, more flocs were present in the gels at a higher pH, which subsequently generated more cross-links and a higher storage modulus. The pH also determined how the cooling rate affected the gel properties. At pH 5.8 and 6.5, the gels were firmest at the fastest cooling schedule, and the cooling rate did not show a trend in affecting the gel strength at the other three rates. On the other hand, a slower cooling rate generated a firmer gel at pH 7.2 and 12.0. The analysis of casein interactions suggests that the cooling rate affected the casein floc size only when repulsive interactions enabled a slow flocculation (at higher pH values) comparable with temperature change rates during cooling. For rennet casein gels of pH within the range of processed cheese products (pH 5.8 and 6.5), particle or cluster rearrangements created more uniform networks for gels cooled at slower schedules and weakened the structure.

KEYWORDS: Processed cheese; rennet casein; gelation; pH; cooling rate

INTRODUCTION

Caseins are the primary class of milk proteins and are the network formers in dairy products such as yogurt and cheese (*1*). In milk, caseins exist as micelles, consisting of four types (α_{s1}, α_{s2}, β, and κ) (*2*). The casein micelle structure is not yet established, and many models have been proposed. One of the well-known models proposed that casein micelles are composed of submicelles linked together by calcium phosphate bridges and hydrophobic interactions (*2*). The "hairy" κ-casein layer on the micelle surface provides strong repulsive steric interactions that prevent casein aggregation (*3*). Lowering the pH to the isoelectric point (pI) of casein (pH 4.6) diminishes the net electrostatic charge and repulsive steric interactions, enabling the aggregation of casein micelles in the production of yogurt. The enzymatic cleavage of κ-casein (renneting) is another approach enabling the aggregation of casein micelles, which is used in the production of natural cheeses. The portion of cleaved κ-casein, called glycomacropeptide, is streamed into whey during cheese production, and the remainder of the casein micelles, i.e., paracasein aggregates, are used to form a protein network in cheeses. A small change in pH significantly alters the quality and functionality of casein gelation products (*4*).

Complex physicochemical changes occur when the milk pH is adjusted. Chemically, the acidification process is accompanied by the dissociation of salts, including micellar calcium, magnesium, inorganic phosphate, and citrate (*5*, *6*). Physically, the volume of casein micelles increases when the pH is decreased from the milk pH of ca. 6.7, reaching a maximum at pH 5.3 (*5*). Visser et al. (*7*) also argued that, despite losing minerals, the micellar skeleton remained intact even once all micellar calcium phosphate had been released at pH 5.1 and casein micelles no longer had the submicellar structure. Despite the lack of details on an exact mechanism, dissolving calcium

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phosphate from casein micelles is clearly more significant at a lower pH (*5*−*7*), promoting the weakening and swelling of internal casein micelles (*8*, *9*).

Casein gels, produced by renneting or acidification, have been extensively studied due to the relevance and applications in a large variety of dairy products. The casein gel rheology and microstructure are directly impacted by pH (*10*): an important factor in protein interactions. The pH directly determines the number and balance of the charge distribution of amino acids on casein molecules (*11*). Further, the hydrophobic interactions between caseins become more significant as the pH gets closer to the pI (*8*). The pH also influences the rate of renneting: an initial increase in renneting rate when the pH is lowered from 7.5 to 6.0 is followed by a slight decrease in rate at a pH lower than 6.0 (*12*). The rate of renneting profoundly changes the casein gel rheology and microstructure.

For rennet-induced gels, no monotonic relationship between gel storage modulus and pH has been reported. Swelling of casein micelles seemed to result in a weaker gel, because the minimum storage modulus within a pH range from 4.6 to 7.5 coincided with the maximum volume per gram of protein at pH 5.3 (*5*). However, Zoon et al. (*13*) observed that for the pH range between 5.77 and 6.75 the storage modulus reached a maximum at pH 6.15. The trends at lower pH may be explained by the swelling of the casein micelles due to dissolution of calcium phosphate. However, the interpretation at the higher pH regime was not convincing, because proteins are more negatively charged at a pH above the pI and the electrostatic force should be more repulsive at a higher pH.

Roefs et al. (*14*) studied the combined renneting and acidification effects on casein gel systems in the pH range between 4.4 and 5.8. When the pH was lowered from 5.8, the storage modulus (G') first decreased until a pH of 5.2 and then increased to its maximum when the pH was further lowered to 4.7, followed by a decrease at lower pH. At a pH lower than 5.2, the gels behaved like acid casein gels, showing a maximum storage modulus at a pH approaching the pI of casein. Once the pH was raised above 5.2, the gels had characteristic properties of rennet-induced casein gels.

Processed cheese is a category of dairy products manufactured from natural cheeses. During processing at an elevated temperature, the added inorganic salts, e.g., phosphates, chelate calcium in paracasein, which is then disintegrated into much smaller structures (*15*, *16*). Because the exact internal structure of casein micelles is still unknown, these smaller structures could be "submicelles" (*15*) or self-assembled aggregates of individual casein molecules. In this work, we use the term "protein micelles" to represent these smaller structures to distinguish them from casein micelles, paracasein, or casein submicelles used in dairy chemistry. An appropriate type and concentration of the inorganic salts may enable the complete hydration of paracasein (disassembled into smaller structures), which is otherwise insoluble in natural cheeses (*15*). As a result, caseins adsorb better at the fat/water interface, significantly improving the functional properties of processed cheese: less oiling-off during heating and better melting properties. The inorganic salts added thus improve the emulsifying properties of proteins and are traditionally named "emulsifying salts" even though these salts are technically not emulsifiers. Besides emulsifying salt types and concentrations, there are other formulation factors such as the pH and processing conditions that affect the quality of processed cheese.

Cooling is the final stage during processed cheese production, and the microstructure formed during cooling strongly impacts the final product quality and functionality. A slower cooling schedule results in a firmer cheese (*15*, *17*). During cooling, solidification (crystallization) of fat, protein−protein interactions, and protein−fat interactions are critical factors for forming a cheese matrix (*1*). A faster cooling rate has been shown to yield smaller fat crystals (*18*) and a higher solid fat content (*19*), and a firm cheese results from more smaller fat crystals (*20*). Protein, on the other hand, is the major component of the continuous network in processed cheese (*1*), but the cooling effects on protein network formation are poorly understood. An understanding of protein network formation with different cooling conditions may help unveil cooling rate effects within processed cheese and its analogues.

Foegeding and his co-workers (*21*−*23*) formulated a model processed cheese system to study the functional properties of processed cheese as affected by its formulation: emulsifying salt types and concentrations, pH, and addition of whey proteins. This cheese analogue was used as a base to study the effects of physicochemical variables on the functionality and microstructure of our model rennet casein system that excluded milk fat. We focus here on the process of protein gelation without fat present for the following reasons: (1) the protein forms a continuous network in processed cheese (*1*) and (2) little research has been directed toward a protein system containing emulsifying salts. In our earlier work, the rheology and microstructure of this model system were studied as a function of the cooling rate at pH 7.2 (*24*, *25*). In this paper, we extended our study on this model system to other pH values at different cooling rates.

The specific objectives of this part were to characterize rennet casein gelation during cooling and investigate how the pH and cooling rate affect the strength, interactions, and microstructure of the gel network. Because the pH of processed cheese is in the range between 5.0 and 6.5 (*15*), the model system was adjusted to pH 5.8 and 6.5. The model system was also investigated at pH 7.2 and 12.0 to provide comprehensive data for interpretation. Rheological tests probed the structure during cooling, and microscopy enabled the observation of the microstructure postcooling. The results were then interpreted by fractal aggregation concepts and colloidal interactions at different cooling rates and pH values. The knowledge gained may establish a framework for understanding the self-assembly of protein systems to benefit manufacturers of casein gelation products such as processed cheese.

MATERIALS AND METHODS

Materials. Rennet casein powder was purchased from New Zealand Milk Products USA, Inc. (Lemoyne, PA). The protein content of casein powder was determined by the Analytical Services Laboratory (Raleigh, NC) using a Perkin-Elmer PE 2400 CHN elemental analyzer (Perkin-Elmer Corp., Norwalk, CT). Casein powder had an 81.5% protein concentration calculated using a standard conversion factor (*26*). Food-grade monosodium and disodium phosphates were donated by Rhodia, Inc. (Cranbury, NJ), and salt (sodium chloride) was bought from a local store.

Gel Sample Preparation. Rennet casein gels were prepared by dispersing 15%, 16%, 17%, and 18% (w/w) protein, 2.5% Na_2HPO_4, 0.3% NaH_2PO_4, and 2.0% NaCl in deionized water. For samples without pH adjustment (pH 7.2), deionized water was heated to 80 °C followed by the dissolving of salts. Rennet casein powder was then dissolved into solution with a stir bar rotating at 350 rpm for 30 min. The sample appeared to be a viscous paste at this point and formed a gel upon overnight storage in a refrigerator at 5 °C. When the samples required pH adjustment (to 5.8, 6.5, and 12.0), 95% of the deionized water was used to dissolve salts, and the solution pH was adjusted by dropwise addition of 6 N HCl or 6 N NaOH. The remaining water was then

added, followed by heating the solution to 80 °C. Casein powder was then dissolved by continuous stirring at 80 °C for 30 min at 350 rpm, followed by a final adjustment of pH to the set value. After overnight storage in a refrigerator at 5 °C, the system appeared to be homogeneous with no observable particles.

Rheological Measurements. Small-amplitude oscillatory tests were performed using a couette geometry and a Bohlin VOR rheometer (Bohlin Reologica, Inc., Cranbury, NJ). The couette assembly included a serrated bob and cup with a cup inner diameter of 2.7 cm and bob outer diameter of 2.5 cm. Initially, 14 g of the gel sample was loaded into the cup, and the opening was then covered with Parafilm. After the rheometer reached 80 °C, the cup with the sample was loaded and incubated for 6 min at 80 °C to melt the gel. The film was removed, and the bob was lowered into the measurement position. Excessive sample was removed, a mineral oil layer was applied to the sample surface, and a sealed cap was placed on the cup to minimize moisture loss. The sample was equilibrated at 80 °C for 30 min and then cooled to 5 °C at four different rates (0.5, 0.1, 0.05, and 0.025 °C/min). A 1% strain and an oscillatory frequency of 1 Hz were used during cooling. Following oscillation, a strain sweep test was performed at a frequency of 1 Hz, or a frequency sweep test was performed at a 1% strain level after equilibration for 30 min at a temperature of 5 °C.

All samples involved in the experiments were weighed before and after the test to evaluate moisture loss. The data showed no statistical significance to the moisture loss effects for different cooling conditions. Furthermore, all strain sweep tests showed a limit of linear viscoelastic regime much greater than 1%, validating selected oscillatory parameters.

Microstructure Observation. Immediately following a rheological test, a small section of the sample was taken from the rheometer cup bottom and applied as a thin layer on a glass slide. The slide was observed by an Olympus Fluoview FV 300 confocal laser scanning biological microscope (Olympus, Tokyo, Japan) using a 100× immersion oil objective. The laser was operated at 488 nm and a scanning aperture of 3.0. The samples were observed at different horizontal and vertical positions. For the gels at pH 5.8, microscopy was performed on four protein concentrations cooled at four rates. For pH 6.5, the gels were studied at all four concentrations at a cooling rate of 0.5 °C/min, and an additional test was performed on a 15% gel cooled at 0.025 °C/min. For gels at pH 12.0, samples with 15% protein were studied at four cooling rates.

Image Analysis. The microscopy images were first converted to a gray scale using Adobe Photoshop 5.5 software (Adobe, San Jose, CA), and the aggregate size was measured by using Scion Imaging software (version Beta 4.0.2, Scion Corp., Frederick, MA). More than 200 random measurements were taken, and the results are presented with an average aggregate size with errors of 95% confidence intervals.

Water-Holding Capacity. Centrifugation tests were performed to quantify the amount of free water in the 15% casein gels at different pH values. Approximately 23 g of the gel sample was loaded into a plastic centrifuge tube, melted at 80 °C for 6 min in a water bath, and then cooled to 5 °C at 0.5 °C/min. Following the cool-down, the tubes with gels were centrifuged at 5 °C for 30 min at 26890*g* (rotor SS-34 at 15000 rpm) using a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Co., Wilmington, DE), and the test was performed in duplicate for each pH condition.

RESULTS AND DISCUSSION

Within the physical (temperature and cooling rates) and chemical (protein concentrations and pH) conditions studied, storage moduli (G') of rennet casein gels were generally very small above 40 °C and were thus not included in the data plots. The effects of the pH and cooling rate on the casein gel rheology and microscopy are presented and discussed separately for these two important parameters.

Rheological Data: Effects of pH. The rheological properties of 15% rennet casein gels during cooling are presented in **Figure 1**. Storage modulus development generally showed two regimes: a slow increase regime at a higher temperature region and a fast increase regime at a lower temperature region. At the high-temperature region, the rate of storage modulus increase was much smaller than that at the low-temperature region, suggesting distinct structure formation processes during cooling.

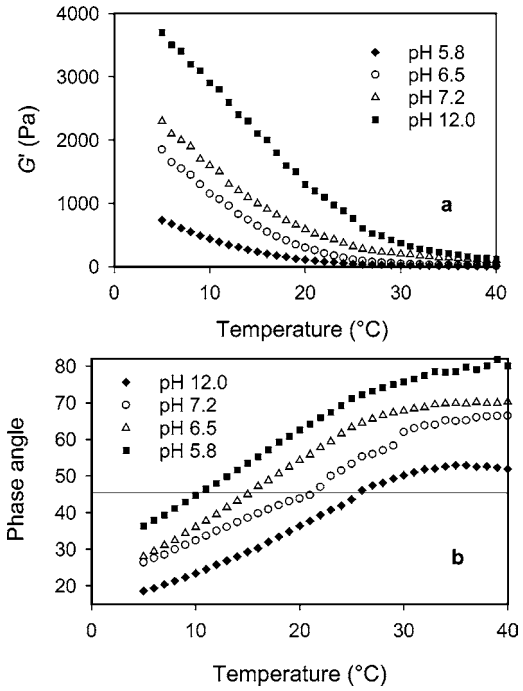

Figure 1. Rheological properties of 15% rennet casein gels at different pH values during cooling at 0.5 °C/min: (**a**) storage moduli and (**b**) phase angles.

The phase angle, the tangential function defined as the ratio of the loss modulus to the storage modulus, is a convenient way of monitoring the gelation process (*27*). A phase angle smaller than 45° indicates a storage modulus that dominates the loss modulus, and therefore, a system behaves more elastic- or solidlike, commonly referred to as a gel. The phase angle during cooling is presented in **Figure 1b** for 15% rennet casein gels at different pH conditions. At higher temperatures, the phase angles were higher than 45°, indicating the systems were fluidlike. Upon cooling, the phase angles decreased steadily, and the temperature corresponding to a phase angle of 45° was smaller for casein gels at a lower pH, indicating that rennet casein gelation occurred at a lower temperature for a lower pH system. A lower gelation temperature was also observed for caseinate-stabilized emulsions at a lower pH (*27*, *28*).

A higher storage modulus was observed for the casein gels of higher protein concentration at the same pH, shown in **Figure 2** for casein systems at pH 5.8 and 6.5. Upon cooling to 5 °C, strain sweep data showed that the systems were all well within a linear viscoelastic regime at the maximum rheometer strain limit of 20% (data not shown). For protein gels with a pH of 5.8 and 6.5, the mechanical spectra showed a crossing of the storage modulus (G') over the loss modulus (G'') at a frequency range between 0.01 and 10 Hz even at the highest (18%) protein concentration (**Figure 3a**). In contrast, G' always dominated at the same frequency range for gels at pH 7.2 and 12.0 (**Figure 3b**) even at the lowest protein concentration (15%). The gels were thus more elastic at a higher pH.

Rheological Data: Effects of the Thermal Rate. In contrast to the trend that G' was highest in gels cooled at the slowest schedule at pH 7.2 (*25*), the storage modulus did not show a monotonic increase with a slower cooling rate when the pH was 5.8. Instead, storage moduli were always highest at the fastest cooling rate (0.5 °C/min), exemplified for 15% and 18%

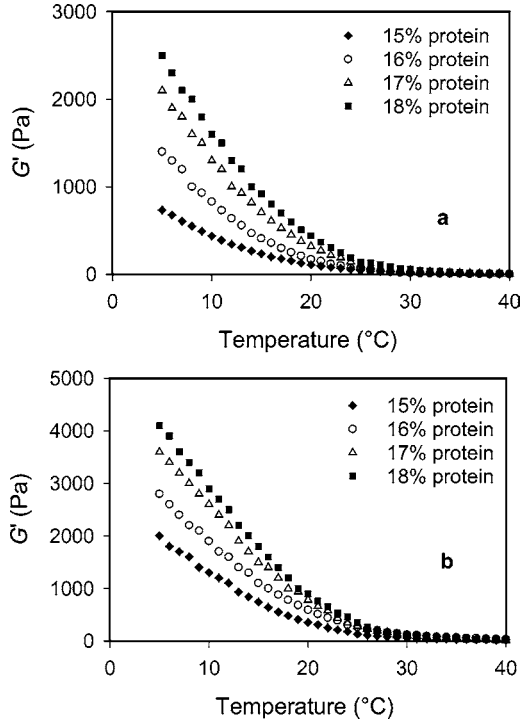

Figure 2. Storage moduli of 15–18% rennet casein gels during cooling at 0.5 °C/min: (**a**) pH 5.8 and (**b**) pH 6.5.

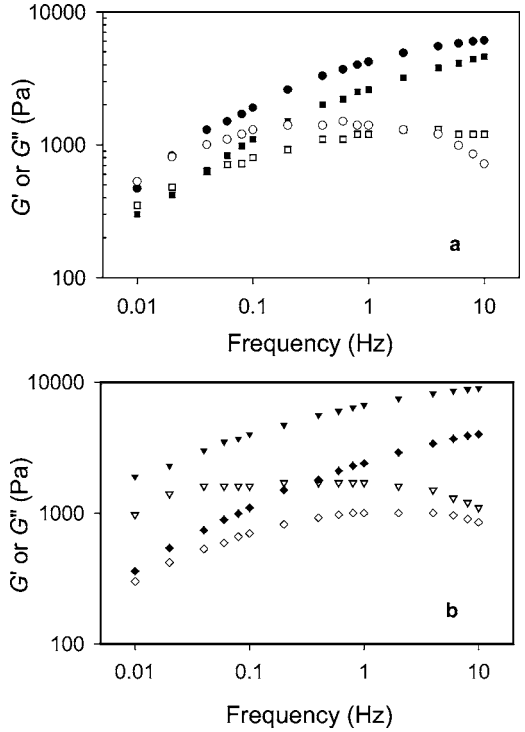

Figure 3. Mechanical spectra of rennet casein gels at 5 °C: (**a**) 18% protein (pH 5.8, squares; pH 6.5, circles) and (**b**) 15% protein (pH 7.2, tilted squares; pH 12.0, triangles). Filled symbols are for the storage modulus, and open symbols are for the loss modulus.

rennet casein gels in **Figure 4**. At the other three cooling rates, there was no identifiable trend: G' was second highest for 15% casein gels cooled at 0.025 °C/min and third highest for 18% casein gels cooled at the same rate. Rennet casein gels at pH 6.5 behaved similarly to those at pH 5.8: storage moduli were highest at the fastest cooling rate and did not show a trend at the other three cooling rates (**Figure 5**). Similar to treatments

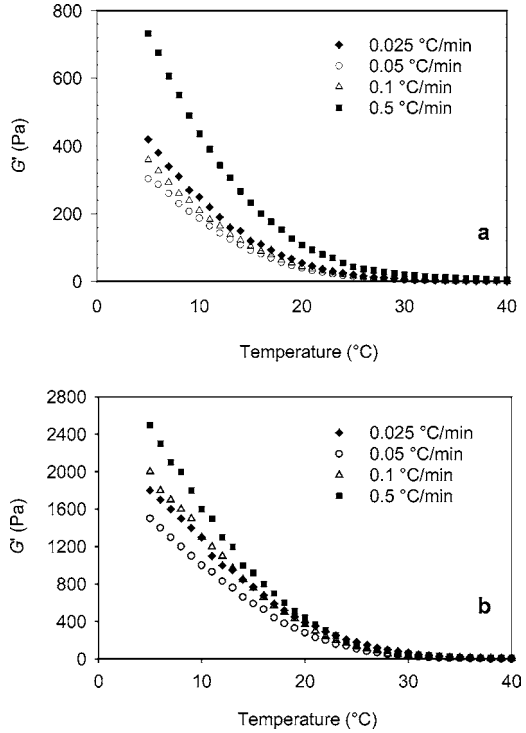

Figure 4. Storage moduli of rennet casein gels (pH 5.8) during cooling at different rates: (**a**) 15% protein and (**b**) 18% protein.

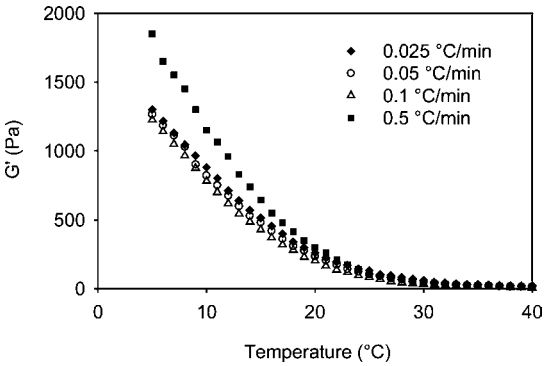

Figure 5. Storage moduli of 15% (protein) rennet casein gel (pH 6.5) during cooling at different rates.

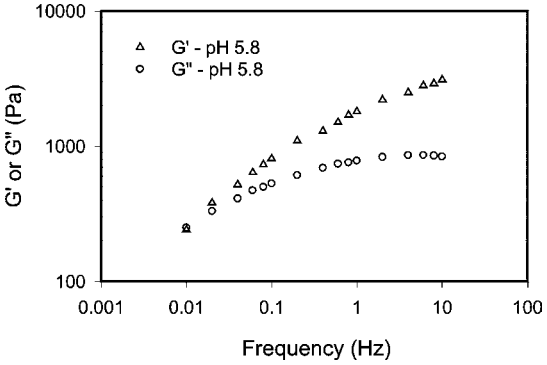

Figure 6. Mechanical spectra of 18% (protein) rennet casein gel (pH 5.8) at 5 °C after cooling at 0.025 °C/min from 80 °C.

at a cooling rate of 0.5 °C/min, a crossover of the storage modulus and loss modulus was also observed for all concentrations at the three other cooling rates for gels at pH 5.8 and 6.5, exemplified for 18% rennet casein gels at pH 5.8 after a cooling rate of 0.025 °C/min (**Figure 6**). For gels at pH 12.0, the cooling rate had effects on rennet casein gels similar to those on

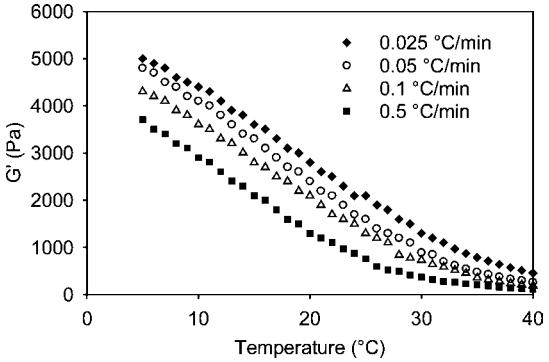

Figure 7. Storage moduli of 15% (protein) rennet casein gels (pH 12.0) during cooling at different rates.

processed cheese (*17*): a firmer gel formed at a slower cooling rate (see **Figure 7** for 15% protein), similar to the observations at pH 7.2 (*25*).

Summarizing the rheological data, the storage modulus was greater at a lower temperature and a higher pH at a fixed cooling rate and the same protein concentration. At pH 5.8 and 6.5, the gel systems were firmer at the fastest cooling rate, and there was no trend in the storage modulus at the other three slower cooling schedules. A monotonic increase in the storage modulus at a slower cooling schedule only occurred at pH 7.2 and above.

Microscopy Data. Confocal microscopy images of the protein aggregate structure are displayed in **Figure 8** for gels at pH 5.8, 6.5, 7.2, and 12.0 following cooling at 0.5 °C/min. Eighteen percent of the gels at pH 5.8 (**Figure 8a**) and 6.5 (**Figure 8b**) did not exhibit a continuous network, while a continuous network was created at the remaining pH values even at a protein concentration of 15% (**Figure 8c,d**). Similar discontinuous structures were observed for the samples cooled at the other three slower rates (results not shown). Because viscoelastic fluid systems usually demonstrate a crossover between storage and loss moduli (*29*), the discontinuous protein aggregates at pH 5.8 and 6.5 (**Figure 8a,b**) were consistent with the rheological data in **Figure 3a**, while dense, continuous aggregates at pH 7.2 and 12.0 (**Figure 8c,d**) matched the dominance of the storage modulus over the loss modulus in **Figure 3b**.

With the additional magnification provided by the confocal microscope, individual flocs were identified, as shown in **Figure 9b** for a 15% rennet casein gel at pH 5.8 cooled at 0.025 °C/min. The floc sizes, determined by averaging the data of more than 200 measurements, are presented in **Table 1** for rennet casein at pH 5.8 and 6.5. The average floc size increased slightly with cooling rate for two concentrations (15% and 18% protein) at pH 5.8, but showed no trends at other concentrations. Additionally, the floc size did not show a dependence on the protein concentration. The differences in the averages were all very small and within the experimental errors for all concentrations and cooling rates. The independence of the floc size on the casein concentration was also observed for caseins at pH 6.5 cooled at 0.5 °C/min. This correlates well with the results reported by Le Bon et al. (*30*). For 15% casein at pH 6.5, cooled at 0.025 °C/min, the average floc size was slightly greater than that when the cooling rate was 0.5 °C/min, but again the difference was within the experimental error. The dependence of the floc size on cooling rate was obvious at pH 7.2 and 12.0: a slower cooling rate generated smaller flocs (**Table 1**), even though the difference was not emphasized for gels at pH 12.0.

Water-Holding Capacity. After the 15% casein samples were centrifuged, approximately 0.15 g of free water was released for gels at pH 5.8. A slight amount of free water (~0.03 g) was observed for gels at pH 6.5, while no free water was noticeable at pH 7.2 and 12.0. Further centrifugation for 30 min did not yield more water. Even though the amount of free water was very small, the trend was in agreement with visual inspection of the microscopy images (**Figure 8**), suggesting more free water was present in the gels at a lower pH because of the discontinuity of the protein aggregate structure.

Possible Structure Development Processes during Cooling. The self-association behavior of individual casein molecules has been studied extensively (*31*, *32*). Caseins are phosphoproteins and have distinct hydrophobic and hydrophilic regions. The α_{s1}-, α_{s2}-, and β-caseins are calcium sensitive, while κ-casein is stable even at a high calcium concentration (*2*). Two mechanisms of casein self-assembly are described in the literature. The α-caseins associate into wormlike micelles with an extended rigid structure driven by hydrophobic interactions for α_{s1}-caseins and electrostatic attraction for α_{s2}-caseins (*33*−*35*). The β- and κ-caseins have been widely studied. They form core−shell structure micelles, similar to normal surfactants. The number of monomers in a micelle and the micelle size are a function of temperature (*31*, *32*, *36*−*38*), and micelle formation was observed at a temperature as high as 70 °C (*36*, *37*). The diameters of β- and κ-casein micelles are approximately 10 nm.

As discussed in the Introduction, the action of emulsifying salts (e.g., phosphates) in a processed cheese system leads to disassembly of paracasein into protein micelles. These protein micelles have a diameter between 20 and 30 nm, observed with high-resolution tunnel electron scanning microscopy, and associate into structures similar to fractal colloidal aggregates in processed cheese matrixes (*15*). Again, the exact composition of these protein micelles is still to be unveiled, but the self-assembly nature of individual casein molecules (now with calcium chelated after the addition of emulsifying salts) would most likely explain the similar size between measurements of single-component β- or κ-casein micelles from light scattering and those from microscopy. Chances do exist for the formation of protein micelles with more than one type of casein (*39*).

The rennet casein network formation during cooling is proposed to follow a sequence of steps similar to those of a whey protein system (*30*, *40*−*42*) except that only physical forces are involved in our system. At a high temperature, protein micelles have a high thermal energy and are not associated. Under complex interactions between these particles, they could form strand- or floclike aggregates that are associated into the structures shown in **Figure 8**. Further magnifying the structures in **Figure 8** 10 times, the aggregates of protein micelles are floclike, exemplified in **Figure 9b** for the case of 18% casein at pH 5.8. These flocs consist of many protein micelles whose identity cannot be resolved with our confocal laser scanning microscopy. The casein structure in our system can now be represented by fractal aggregates illustrated in **Figure 10**. The gelation starts when aggregation of the casein flocs is initiated upon cooling. At the higher temperature region (**Figures 1**, **2**, **4**, and **5**), the flocculation rate was small, and the rate of storage modulus development was thus small. At a lower temperature, the flocculation was irreversible as the temperature decreased, the structure was steadily developed, and the storage moduli increased at a much larger rate. The regimes of fast storage modulus rates were all within the ranges where the phase angles were smaller than 45° (cf. parts **a** and **b** of **Figure 1**).

Physical Explanation of the pH Effects. Protein micelles may be represented by **Figure 11a** with a structure similar to

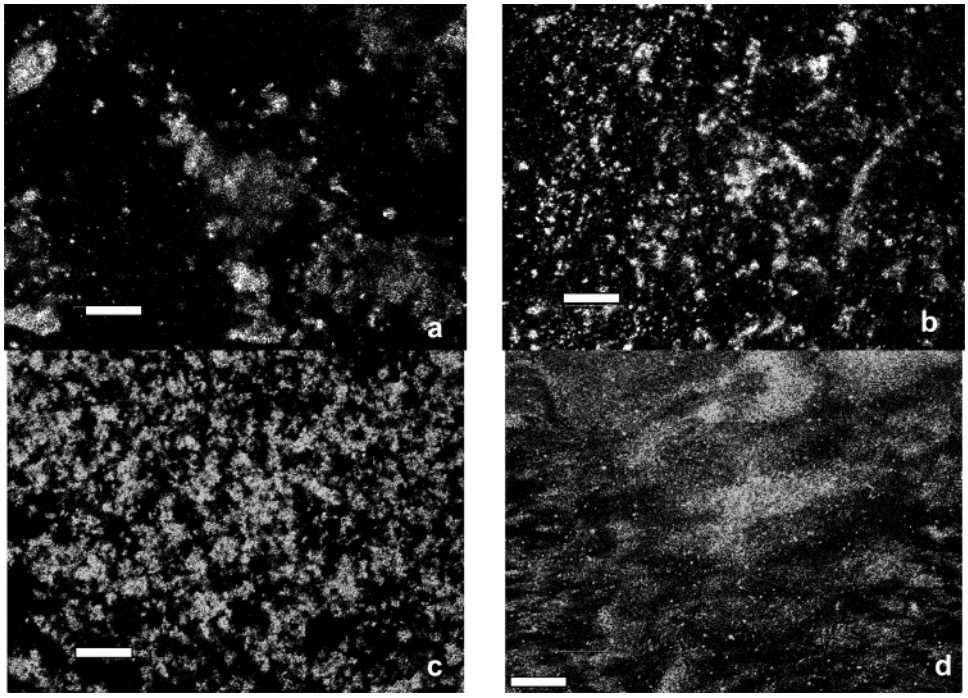

Figure 8. Microstructure of rennet casein gels observed by confocal microscopy: (**a**) 18% protein at pH 5.8, (**b**) 18% protein at pH 6.5, (**c**) 15% protein at pH 7.2, and (**d**) 15% protein at pH 12.0. The scale bars represent 20.0 μm.

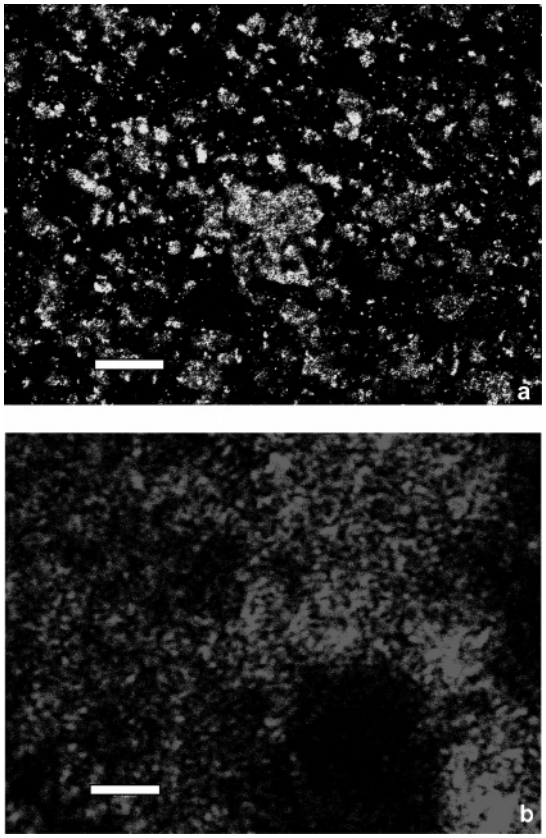

Figure 9. Microstructure of 18% (protein) rennet casein gels (pH 5.8) cooled at 0.025 °C/min: (**a**) lower magnification and (**b**) higher magnification. The scale bars represent 20.0 μm (**a**) and 2.0 μm (**b**).

Table 1. Cooling Rate Effects on the Floc Size for the Model Rennet Casein System at pH 5.8, 6.5, 7.2 (*24*), and 12.0

pH	protein concn (%, w/w)	cooling rate (°C/min)	floc size (μm) (av ± 95% confidence interval)
5.8	15	0.025	0.276 ± 0.033
		0.05	0.277 ± 0.031
		0.1	0.292 ± 0.034
		0.5	0.2950 ± 0.035
	16	0.025	0.302 ± 0.035
		0.05	0.330 ± 0.034
		0.1	0.331 ± 0.036
		0.5	0.326 ± 0.038
	17	0.025	0.295 ± 0.037
		0.05	0.315 ± 0.033
		0.1	0.277 ± 0.033
		0.5	0.319 ± 0.036
	18	0.025	0.277 ± 0.035
		0.05	0.286 ± 0.036
		0.1	0.304 ± 0.033
		0.5	0.315 ± 0.034
6.5	15	0.025	0.326 ± 0.038
		0.5	0.319 ± 0.038
	16	0.5	0.291 ± 0.037
	17	0.5	0.314 ± 0.032
	18	0.5	0.274 ± 0.032
7.2	15	0.025	0.260 ± 0.033
		0.05	0.309 ± 0.033
		0.1	0.348 ± 0.039
		0.5	0.425 ± 0.042
12.0	15	0.025	0.246 ± 0.030
		0.05	0.262 ± 0.030
		0.1	0.273 ± 0.031
		0.5	0.292 ± 0.032

that of conventional ionic surfactant micelles. The hydrophobic portion of caseins (dotted curve) forms the core, and the hydrophilic portion (solid curve) forms the shell with most charges. At a higher pH (above pI), individual casein molecules become more negatively charged and are separated further from each other due to a stronger electrostatic repulsion (part **a** vs part **b** of **Figure 11**, 1 vs 3 charges per molecule, for example). This could straighten the hydrophilic portion of the caseins to minimize contact (system free energy) and increase the size of the protein micelles. Additional physical forces affecting casein structure formation may include hydrophobic interactions between hydrophobic portions of the caseins. Hydrophobic interactions are stronger at a pH closer to pI (*8*), which brings

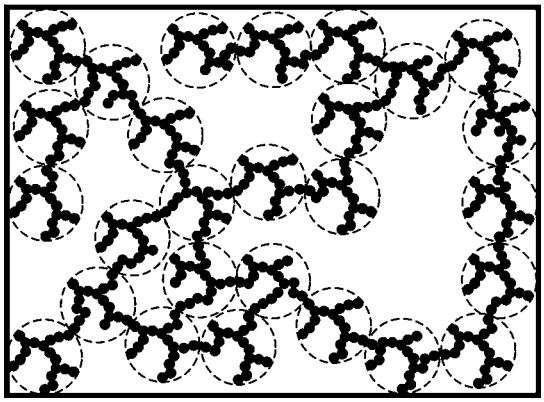

Figure 10. An illustration of a rennet casein network. Smaller filled circles stand for protein micelles, while bigger dashed open circles are arbitrary boundaries for flocs.

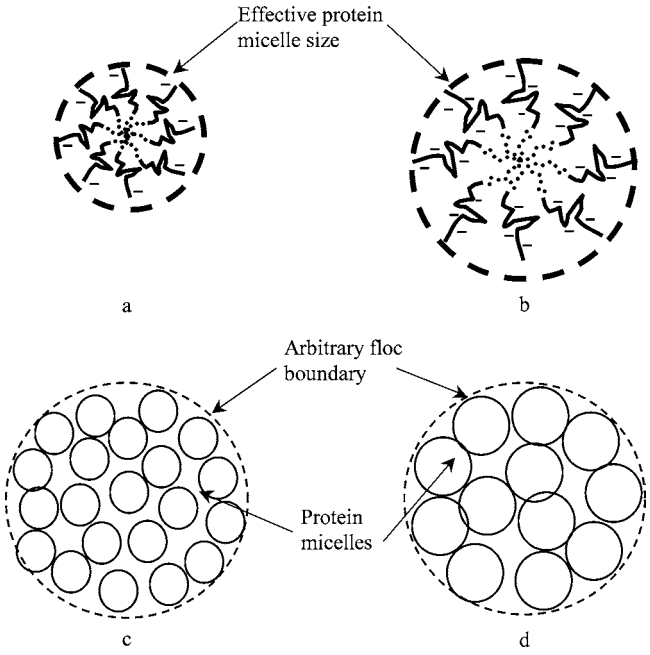

Figure 11. Structures of protein micelles and flocs as a function of pH. Protein micelles are smaller at a lower pH (**a**) than at a higher pH (**b**). For a similar floc size, the fractal dimension is greater at a lower pH (**c**) than that at a higher pH (**d**). Dotted and solid curves in protein micelles represent hydrophobic and charged, hydrophilic portions of casein molecules, respectively.

casein molecules in protein micelles closer. Both electrostatic and hydrophobic interactions indicate smaller protein micelle sizes at a lower pH, assuming the same number of casein molecules in protein micelles at all pH conditions. When these protein micelles form flocs during cooling, flocs of similar sizes will have a smaller amount of protein micelles at a higher pH (part **c** vs part **d** of **Figure 11**), in other words, a smaller fractal dimension.

With the fractal nature, the radius of a single casein floc, R_f, can be scaled to the number of protein micelles (N_p) by (*43*, *44*)

$$N_p \approx (R_f/a)^{D_f} \quad (1)$$

where a is the radius of protein micelles and D_f is the fractal dimension of the casein flocs. To accommodate all protein micelles in a network, eq 1 was rewritten as

$$N_{p,tot} \approx N_{f,tot}(R_f/a)^{D_f} \quad (2)$$

where $N_{p,tot}$ is the total number of protein micelles and $N_{f,tot}$ is the total number of flocs.

Analyses from microscopy images (e.g., **Figure 9b**) enabled the measurement of floc sizes (R_f) at different pH conditions, and the results did not show a changing trend in floc size with pH (**Table 1**). The analysis of the fractal dimension can follow the scaling theory of Shih et al. (*45*), detailed in our earlier publication (*25*). Basically, the fractal dimension of our casein systems can be estimated according to rheological data from different colloidal particle (protein micelle) concentrations:

$$G' \propto \phi^{(3+x)/(3-D_f)} \quad (3)$$

where x, varying between 1.0 and 1.3, is the backbone fractal dimension of the flocs, ϕ is the volume fraction, and D_f is the fractal dimension.

A fractal dimension of 2.35 was estimated for rennet casein gels at pH 7.2 after cooling to 5 °C (*25*). Storage moduli of 15%, 16%, 17%, and 18% rennet casein gels at pH 12.0 after cooling to 5 °C at 0.5 °C/min were measured to be 3700, 4800, 6500, and 9800 Pa, respectively. When fitting the exponent in eq 3 on the basis of these data, the fractal dimension was estimated to be 2.22. Because the aggregates were discontinuous at pH 5.8 and 6.5 (**Figure 8**), the applicability of eq 3 was questionable. The fractal dimensions of casein gels at these two pH values were not estimated even though the exponents were larger, indicating a higher fractal dimension. The above analysis was qualitatively in accordance with the earlier analyses based on molecular interactions, illustrated in **Figure 11**.

The model system had an identical amount of casein molecules and protein micelles at the same casein concentration before and after cooling. For a given amount of protein micelles, the amount of flocs had a greater dependence on the exponent (fractal dimension) than the base (R_f/a) in eq 2. At a higher pH, the diameter of the protein micelles was greater as a result of stronger electrostatic and weaker hydrophobic interactions, and the fractal dimension was smaller. On the other hand, there was no trend in the estimated floc size as a function of pH (**Table 1**). All three aspects translated into a greater amount of flocs ($N_{f,tot}$) at a higher pH, as supported by the microscopy images (**Figure 8**). At a lower pH, the number of flocs was insufficient to form a continuous network, while more cross-links among the large number of flocs generated a stronger gel at a higher pH. Phase angle evolutions for gels at different pH values (**Figure 1b**) clearly showed that the gelation occurred at a higher temperature for gels at a higher pH, resulting from more flocs at a higher pH that simplified floc cross-linking.

Significance of the Cooling Rate on Floc Formation and Aggregation into Networks. On the basis of dairy chemistry and microscopy evidence, the system can be described similarly to the formation of fractal structures in colloidal dispersions. Physical bases of fractal floc formation can be explained by doublet formation as the first step (*46*) followed by the addition of the remaining colloidal particles into established doublets (*24*). The colloidal particles in our rennet casein systems are protein micelles, and doublets refer to two flocculated protein micelles. The flocculation of protein micelles is determined by complex interactions, some of which are still difficult to quantify. Despite the challenges involved in quantitative analysis of such a complex system, qualitative speculations based on

casein interactions have been provided in some studies reported in the literature. It is known that caseins are easier to aggregate at a pH slightly above the pI (*3*), possibly because the weaker electrostatic repulsion cannot oppose stronger hydrophobic attraction (*8*). A steric hydration force, resulting from casein protein segments extruding from the protein micelle surface interacting with surrounding water molecules, was speculated to be a repulsive force enabling the stability of casein protein micelles (*24*). On the other hand, hydrophobic interactions, measured from β-casein (*32*), increase from 0 to ~30 °C and then decrease with temperature above 30 °C. Therefore, the "sticking" of protein micelles occurred once the steric hydration force was weaker than the attractive forces (hydrophobic and van der Waals), and thermal motion of the extruding protein segments became weaker during cooling from 80 °C, i.e., a shorter effective range of the force.

Regardless of the origins of the forces, the combined action of stronger, attractive hydrophobic interactions, weaker repulsive electrostatic interactions, and similar attractive van der Waals interactions (*3*) results in an overall larger attractive interaction energy between casein protein micelles at a lower pH for similar temperatures. It is likely that, at a higher pH, the flocculation process was slower due to a more repulsive overall interaction energy, making cooling schedules an effective means to affect the protein gelation process. On the other hand, the flocculation process was much faster at a lower pH, and the change of cooling rate, by controlling casein interactions, was not fast enough to compete for the time for sticking of protein micelles. The ultimate formation of flocs at pH 5.8 and 6.5 was thus not significantly affected by the cooling rate, and there was no apparent trend in the size and size distribution of the floc population.

Significance of Structure Rearrangement at Different pH Conditions. For rennet-induced casein gels, Mellema et al. (*9*) described possible rearrangements of structure at four different length scales: fusion of individual particles, rearrangement of particles, rearrangement of particle clusters, and macroscopic syneresis that leads to a separation of the liquid phase over a semisolid gel phase. In their work (*8*, *9*), the particles have been referred to as paracasein, and structure rearrangement has been reported more significant at a lower pH (between 5.3 and 6.65). The consequences of structure rearrangement included the increased particle size, partial disappearance of the fractal structure, and formation of straightened (partially broken) strands, leading to weakening of the gel strength.

The rennet casein gels at processed cheese conditions studied here were different from those at the natural cheese conditions studied by Mellema et al. (*8*, *9*). However, it was possible that rearrangement of the casein floc structure occurred in our system at pH 5.8 and 6.5 analogously to that of paracasein in rennet-induced casein systems. At the fastest cooling rate, the system experienced the shortest time after network formation, the network was weakened to the least extent, and the gel was thus strongest at the same temperature. Floc sizes were similar at different cooling conditions (**Table 1**); thus, fusion from individual flocs did not occur that would otherwise significantly increase the floc size. No observable free water (macroscopic syneresis) could be registered in the samples at any combination of pH, protein concentration, and cooling rate. The differences among rheological properties might have originated from rearrangement of casein flocs, or clusters of flocs, within the gel structure. When microscopy images of casein gels at a similar pH and protein concentration but at different cooling rates were compared, it was seen that casein clusters were more uniformly distributed after cooling at a slower rate, demonstrated for gels of 18% protein at pH 5.8 in **Figure 8a** versus **Figure 9a**. The same trend was observed at other protein concentrations at pH 5.8 and 6.5 (data not shown). At a faster cooling rate, the clusters are not given time to rearrange and are kinetically trapped in the less uniformly distributed structure.

At pH 7.2, a floc size change, i.e., particle fusion described by Mellema et al. (*9*), and floc and cluster rearrangements were not observed when the floc size and network of gels cooled at 0.5 °C/min were compared with those initially cooled at 0.5 °C/ min and then incubated at 5 °C until the total time for a cooling schedule of 0.025 °C/min was reached (*24*). The difference in the floc size, protein network, and rheological properties at this pH value was solely caused by the cooling rate. At pH 12.0, the protein networks were dense, and no strong conclusion could be made regarding the structure rearrangement as a function of cooling rate.

Concluding Remarks and Directions for Future Work. In summary, the pH, similar to that of other casein gels, dramatically changed the rheology and microstructures of our rennet casein systems targeted toward improving our understanding of processed cheese products. The storage modulus increased significantly as the pH was increased from 5.8 to 12.0. Both rheology and microscopy demonstrated a discontinuous structure at pH 5.8 and 6.5 and a continuous network at pH 7.2 and 12.0. The monotonic increase in storage modulus with pH was interpreted on the basis of protein interactions and fractal concepts. It was hypothesized that protein micelles are smaller at a lower pH due to weaker electrostatic and stronger hydrophobic interactions, resulting in a higher fractal dimension. With the estimated floc sizes from microscopy results and fractal dimensions from rheological measurements, our analyses further demonstrated that fewer flocs and thus fewer cross-links formed for the system at a lower pH, which corresponded to a weaker gel and a more porous aggregate structure.

The cooling rate is another important factor for rennet casein gel properties, and cooling effects were observed to have two regimes depending on the gel continuity. At pH 5.8 and 6.5, the storage modulus was highest at the fastest cooling schedule and did not show a trend with remaining cooling rates. Networks were discontinuous at these two pH values for all the conditions studied. A more uniform distribution of casein clusters formed at a slower cooling schedule, possibly due to floc or floc-cluster rearrangement weakening the structure. At pH 12.0, a slower cooling rate created a stronger gel, similar to the results reported previously at a pH of 7.2. The effect of the cooling rate on rennet casein systems at different pH values was hypothesized to have been modulated by the overall interactions between casein protein micelles. At a higher pH, the overall interactions were more repulsive, and the flocculation of protein micelles was slower and subsequently affected by the cooling rate. On the other hand, flocculation was fast at pH 5.8 and 6.5, and the cooling rate was not the controlling factor on the floc size and population.

The results also point out some interesting directions for future investigations. Discrepancies in cooling rate effects were observed between the rheological data from processed cheese (*17*) and those from rennet casein systems at the pH range (5.0−6.5) of processed cheese products (**Figures 4** and **5**). Rheological data from processed cheese demonstrated two regimes during cooling, and the transitions between these two regimes occurred at a higher temperature during a slower cooling schedule (*17*). The changing patterns for the transition temperature were the same as those of the fat crystallization temperature during

cooling at different rates, and the transition temperatures at cooling rates of 0.1 °C/min (~20 °C) and 0.5 °C/min (~18 °C) were almost the same as those at the second exotherm from differential scanning calorimetry (*18*). Our current effort is focused on incorporating milk fat into this model system to illustrate cooling mechanisms in processed cheese.

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