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Measurement of Casein Micelle Size in Raw Dairy Cattle Milk by Dynamic Light Scattering

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Ivan Mehandgiiski and Georgi Radoslavov

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Abstract

The particle size of milk influences its microstructure and defines the qualities of dairy products, such as colloidal stability and texture. Moreover, differences in casein micelle size may affect milk processing, especially cheese making. Hence, the size of casein micelle is an important characteristic of raw milk and determines the yield of dairy products. The aim of the present research is to estimate the casein micelle size in the raw milk of dairy cattle by dynamic light scattering. The obtained results may be used for genetic elaboration of the breed, as well as to increase the competitiveness of the milk industry by selection of animals with higher casein micelle size.

Keywords: dairy cattle, casein micelle size, dynamic light scattering, DLS, cow milk

1. Introduction

It is well known that the casein fraction of bovine milk exists as polydisperse, large, roughly spherical colloidal particles, 50–600 nm in diameter (average ~150 nm), called "casein micelles" [1]. The size, form, and structure of the casein micelle are of great importance for the milk industry especially for cheese making, yellow cheese, etc. [2]. Casein micelle contains an average of 3.4 g H_2O per gram dry matter, which consists approximately of 93% protein and about 7% of inorganic component (Ca^{2+}), formed phosphoprotein complexes. The casein micelle in milk consists of four caseins: αs1- (CSN1S1), αs2- (CSNS2), β- (CSN2), and k-casein (CSN3). The latter is important for the stability and properties of the casein micelle. Although k-casein is in a relatively low amount of the casein system (12–15% of whole casein), it is soluble in the presence

of Ca^{2+}, whereas the remaining 85% of casein are precipitated by Ca^{2+}. CSN3 stabilizes up to 10 times its weight of the Ca^{2+}-sensitive caseins via the formation of micelles [3] (**Figure 1**).
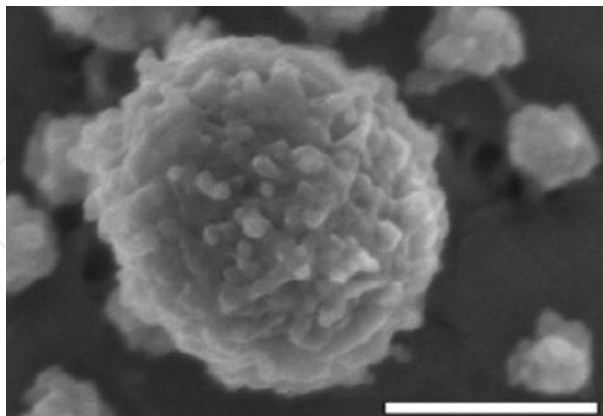

Figure 1. Electron micrograph of an individual casein micell [4].

One of the most important issues concerning the casein micelle is how the casein particles (micelles) in milk are stabilized and destabilized by the action of various agents and conditions. Thus, various hypotheses of casein micelle structure are proposed.

2. Casein micelle structure

2.1. Coat-core models

The first model of casein micelle structure was proposed by Waugh and Nobel in 1965 [5]. This model is based on the casein solubility in Ca^{2+} solutions. The model describes the formation of low-weight-ratio complexes of αs1- and k-caseins in the absence of calcium. The monomers of αs1- or β-caseins interact with a charged phosphate loop (**Figure 2**), then begin to aggregate
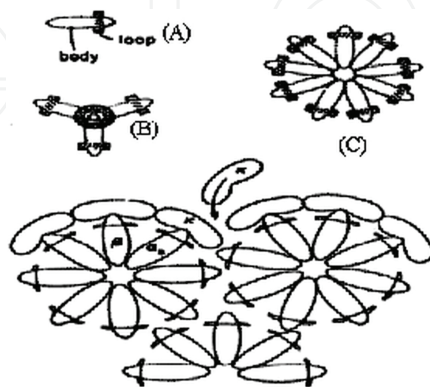
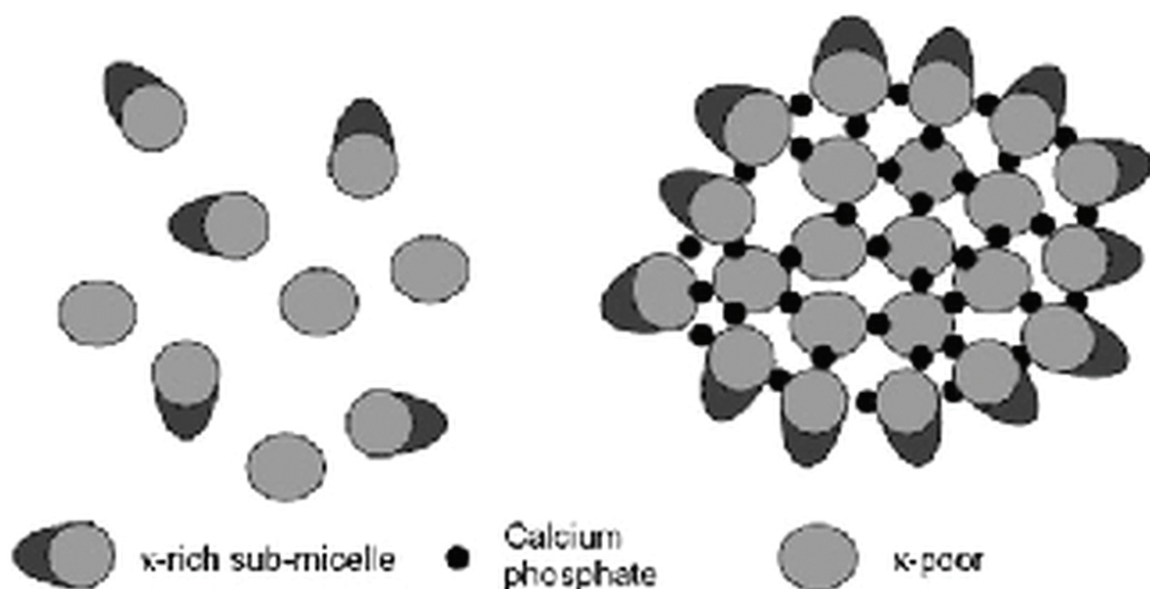**Figure 2.** Structure of casein micelle proposed by Waugh [6]. (A) Monomer model for αs1- or β-caseins with charged loop. (B) A tetramer of αs1-casein monomers. (C) Planar model for a core polymer of αs1- and β-caseins [7].

to a limiting size (the caseinate core) while the calcium ions are added. Precipitation of the caseinate is stopped after a monolayer of the low-weight αs1-k-casein complexes is formed. This coat complex has the k-casein monomers spread out completely on the surface [8].

2.2. Sub-micelle (subunit) models

The first sub-micelle model was proposed by Morr in 1967 [9]. Morr considered that αs1-, β-, and k-casein monomers form small uniform submicelles. The submicelles, estimated by sedimentation velocity studies, are stabilized by hydrophobic bonding and calcium caseinate bridges, and the submicelles are aggregated and held together by colloidal calcium phosphate linkages with a micelle structure covered by αs1- and k-casein [7, 10].

The model, described by Slattery and Evard [11] and Slattery [12], falls in the last category. In this model, the caseins first aggregate via hydrophobic interaction into subunits of 15–20 molecules each. The pattern of interaction is such that it brings about a variation in the k-casein content of these submicelles. The k-casein congregate on the micelle surface, those submicelles poor (like αs and β-casein) or totally deficient in k-casein are located in the interior of the micelle (**Figure 2**). This model does not explain what provokes the segregation of the k-casein or why k-casein molecules, having preferred to associate with their own kind to form aggregate patches in the k-rich submicelles, should then associate with the other caseins to complete the building of the submicelle. Another criticism of this model is the late entry of calcium phosphate into the assembly process by Slattery and Evard [11]. Separating the caseins from calcium and phosphate until this point in the assembly process is not really possible, since both calcium and phosphate are involved in the phosphorylation of the protein chain which occurs post-translation [13] immediately and presumably before the association of the chains into submicelles.


Figure 3. Representation of the casein micelle dual binding model proposed by Horne [16].

2.3. Internal structure models

The last category of models is based on the properties of the isolated casein constituents, causing or directing the formation of the internal structure of the casein micelle [7].

The first internal structure model was suggested by Rose in 1969 [14]. He assumed that β-casein monomers begin to self-associate into chain-like polymers. Subsequently, αs1-caseins molecules are attached to the β-casein polymers, while k-caseins interact with αs1-caseins, forming aggregates of limited size. Upon forming the micelle structure, colloidal calcium phosphate acts as a stabilizing agent and cross-links the network. In addition, these micelle networks are oriented in such a way that β-casein is directed internal, while the k-casein is directed external [8].

An alternative internal structural model is proposed by Holt [15] with the help of transmission electron micrographs. This model emphasized the role of hydrophobic interactions in giving rise to submicelles, the Holt model relies solely on the interactions between the caseins and calcium phosphate to hold the micelle together. In this model, the calcium phosphate is in the form of nanoclusters and the interaction sites on the caseins are the phosphoseryl clusters of the calcium-sensitive caseins (**Figure 3**). Because aS1- and aS2-caseins have more than two such clusters, arguably in the case of aS1-casein, they are able to cross-link the nanoclusters into extended 3-dimensional network structures. The dual-binding in Holt models is in the size of the nanocluster and in the number of phosphate clusters (or casein molecules) that the surface of the nanocluster can accommodate (**Figure 4**).
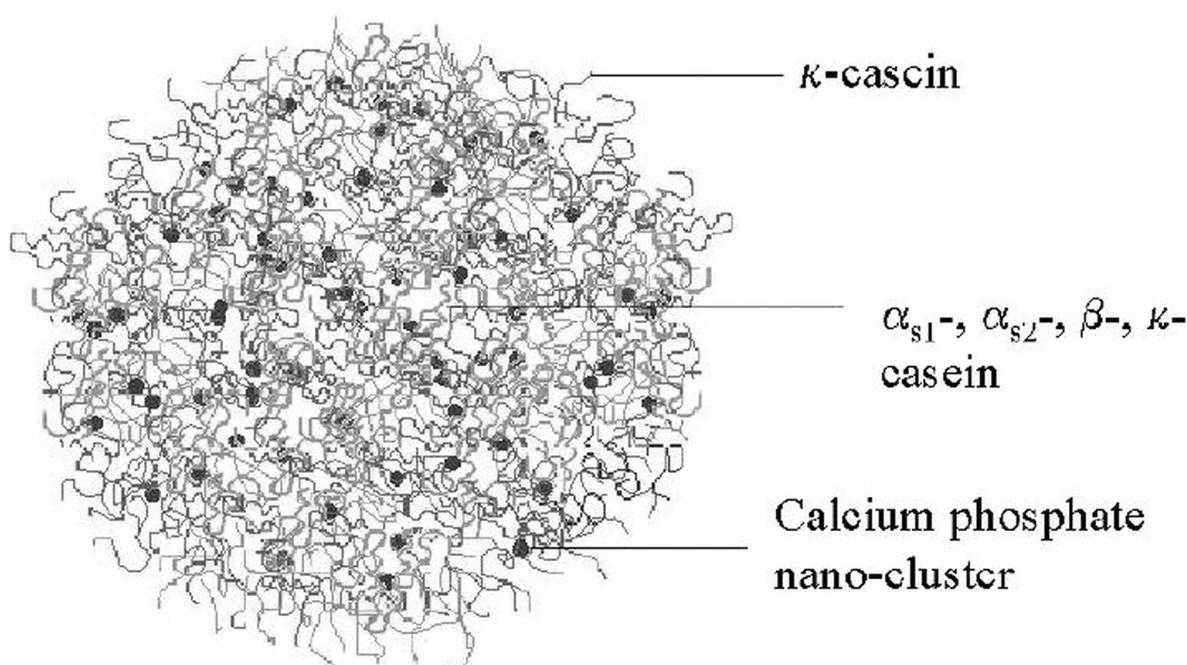

Figure 4. Schematic representation of Holt's model. The calcium phosphate formed nanocluster as interact with phosphoseryl residue of caseins [16].

3. Dynamic light scattering

Dynamic light scattering (DLS), sometimes referred to as quasi-elastic light scattering (QELS) or as photon correlation spectroscopy (PCS), is a non-invasive, well-established technique for measuring the size and size distribution of molecules and particles typically in the submicron region, and with the latest technology lower than 1 nm.

A laser or any other monochromaric light source is shot through a polarizer and into a sample. The scattered light is then sent through a second polarizer where it is collected by a photo-multiplier. The resulting image is then projected onto a screen and this phenomenon is known as speckle pattern (**Figure 5**) [17].
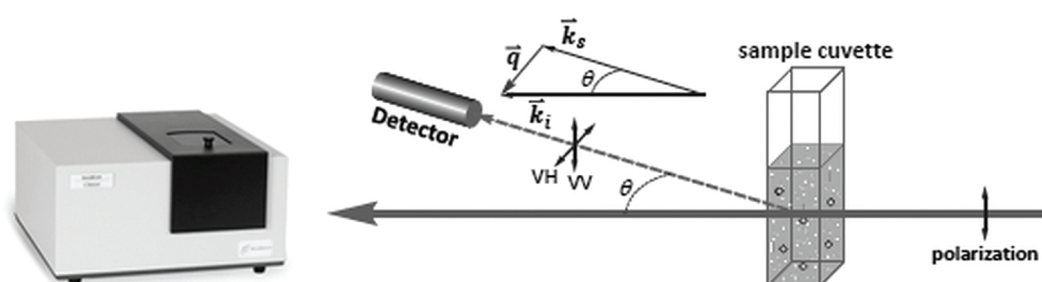
Figure 5. Principle of dynamic light scattering. www.brookhaveninstruments.com.

All of the molecules in the solution are being hit with the light and all of the molecules diffract the light in all directions [18]. The diffracted light from all of the molecules can either interfere constructively (shown as light regions) or destructively (shown as dark regions). The process is repeated at short time intervals. The resulting set of speckle patterns is then analyzed by an autocorrelator in order to compare the intensity of light at each spot over time. The polarizers that are being used can be set up in two geometrical configurations. One of the polarizers is set in a vertical/vertical (VV) geometry, while the second polarizer allows light through which is in the same direction as the primary polarizer. On the contrary, in vertical/horizontal (VH) geometry, the second polarizer allows light which is not in same direction as the incident light.

3.1. Practical realization

DLS is used for characterization of the various particle sizes (PSs), including proteins, carbo-hydrates, micelles, polymers, and nanoparticles. If the system is not dispersing in size, the average effective diameter of the particles can be determined [19]. This measurement depends on many factors as size of the particle core, size of surface structures, particle concentration, and the type of ions present in the medium. The diffusion coefficient of the particles can be determined since DLS typically measures fluctuations in scattered light intensity due to diffusing particles. DLS software of commercial instruments displays the distribution of particle population at different diameters [20]. In a monodisperse system, there should be only one population, while a polydisperse system would exhibit multiple particle populations. If a sample contains more than one size population, then either the CONTIN analysis should be

applied for PCS instruments or the power spectrum method should be applied for Doppler shift instruments [21]. DLS can be used for convenient stability studies. Conducting periodical DLS measurements of a sample can demonstrate whether the particles aggregate over time. This can be achieved by checking for an increase in the particle hydrodynamic radius. If particles actually aggregate, a larger population of particles with a larger radius should be observed. In addition, some DLS machines can analyze stability depending on temperature by controlling the temperature in situ.

4. Measurement of casein micelle size by dynamic light scattering (DLS)

The average casein micelle size varies widely between milk samples of individual cows. Casein micelle size is also variable and can range between 154 and 230 nm in milks from individual cows [22]. Moreover, micelles as small as 125 nm and as large as 487 nm have been reported in fractionated bulk milk [23]. Casein micelle size may be influenced by cow genetics, e.g., casein protein variants [24–26], protein post-translational modification involving phosphory- lation and glycosylation of casein molecules, and the levels of mineral compounds, such as calcium, in the milk [27]. Micelle size may also be influenced by farming and environmental factors such as feed [28] and season [29]. On the other hand, casein micelle size is of an important significance concerning influence the renneting behavior and the texture of manufactured dairy products [30]. The main used techniques for the analysis of PS and particle size distribution (PSD) are DLS, nanoparticle tracking analysis (NTA), scanning electron microscopy (SEM), size exclusion chromatography (SEC), cell electrophoresis, analytical ultracentrifugation (AUC), etc. Various analytical techniques may give different results [31– 34]. DLS is the most user-friendly and it gives relatively accurate and consistent results of protein samples which can be obtained in a short period of time [35]. The size of casein micelle is estimated in raw milk samples, pasteurized milk, bulk milk, milk powder, etc. Here we will highlight determining of casein micelle size in raw milk by the DLS method.

Research of Bijl et al. [25] have revealed the casein micelle size in raw milk in 50 Holstein- Friesian cows and 54 Montbéliarde cows. Initially, all animals were genotyped for the k-casein gene. On the basis of average casein micelle size, the authors defined two types of micelles: small (170.6 ± 9.1 nm) and large (206.7 ± 5.0 nm). The results showed no significant differences between the two types of micelles and caseins concentration in milk. On the other hand, there was a positive influence between k-casein and casein micelle size. The mean casein micelle size in CSN3 AA cows was 203.5 ± 14.6 nm), while in cow with AB genotype, it was 173.1 ± 5.4 nm. This study was the first to report a correlation between casein micelle size and glycosylation of k-CN. The authors concluded that changes in the structure of k-casein clusters caused by glycosylation can influence micellar stabilization during or after casein micelle formation in the mammary gland, and thereby influence casein micelle size.

Another study by de Kruif and Huppertz [22] has revealed the casein micelle size in individual milk samples in 18 Holstein-Friesian cows by DLS measurement. Data from **Figure 4** showed clear differences between hydrodynamic radius (Rh) between cows but do not vary as a

function of stage of lactation. To study the effect of different lactations on casein micelle size, the authors have collected the individual milk samples from 68 cows.

The results revealed that Rh did not change during milking, lactation, or even over a period of 3 years. This suggests that casein micelle size is strongly genetically determined and is extremely constant in the milks of individual cows. In this study, casein composition of each individual milk samples was determined by RP-HPLC. The results showed that first there were no significant differences between casein composition, as a function of stage of lactation, and second, casein composition did not influence on the casein micelle size (**Figure 6**).
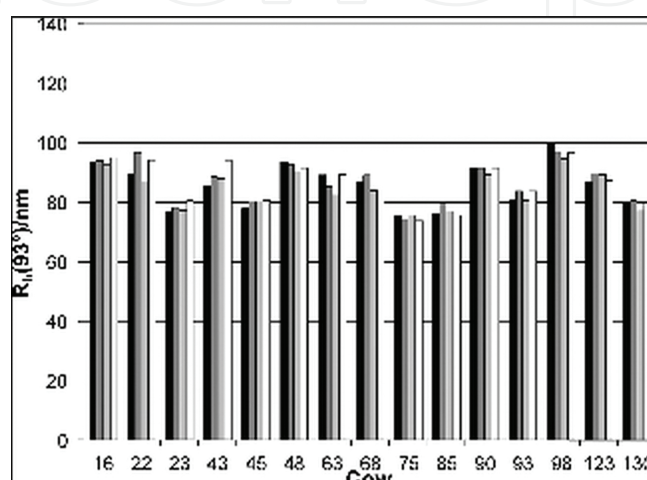
Figure 6. Hydrodynamic radius at a scattering angle of 93° in the milk of 15 individual cows taken at 4 different time points during a single lactation [22].

The study of Hristov et al. [26] aimed to compare the size of casein micelle in individual milk samples in dependence of kappa casein (CSN3) genetic polymorphism in 16 cows of Bulgarian Rhodopean cattle breed. The three defined kappa casein genotypes AA, AB, and BB were used for determining the casein micelle size by DLS. On the other hand, individual milk samples of each cow were assessed for protein and fat content. The results showed differences in the size and polydispersity of the casein micelles in milk from cows with different genotypes. The hydrodynamic radii of micelles at a scattering angle of 90°C varied from 80 to 120 nm and polydispersity varied from 0.15 to 0.37. The authors concluded that the casein micelle size of CSN3 AA cows (~120 nm) exceeds in about 60% of cows with AB (~80 nm) and BB genotype (~70 nm). In contrast, protein and fat content in milk cannot be correlated to casein micelle size. The obtained results could be useful for improving technological properties of milk and the yield of milk products (**Figure 7**).

Beliciu and Moraru [36] have analyzed the effect of the solvent on the accuracy of casein micelle PS determination by DLS at different temperatures and how to establish a clear protocol for these measurements. DLS analyses were performed at 6, 20, and 50°C in raw and pasteurized skimmed milk as sources of casein micelles. 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 PS measurements. The authors

concluded that when an ultrafiltered permeate was used as a solvent, the PS and polydispersity of casein micelles decreased as temperature increased. The effective diameter of casein micelles from raw skimmed 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. 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.

A similar investigation was carried out by Mootse et al. [37]. The authors aimed to study casein micelle size in individual Estonian Holstein dairy cows during 1-year period by DLS. The main results of this study can be summarized as follows: 1) The average mean intensity (mode) of casein micelle particle size distribution (CM PSD) in raw milk of Estonian Holstein dairy cows was 171.13 nm and its variation (range 135–210 nm) resembled statistically normal distribution. 2) There was a weak correlation between average mode and its variation in milk samples of individual cows which may refer to the possible influence of cows' physiological status, disease incidences, and stages of lactation, etc., which will be studied in a further research.

Devold et al. [28] have screened the influence of genetic milk protein variants on mean size of native and heated casein micelles in 58 cows of Norwegian red cattle breed. The results showed that the mean size of native and heated casein micelles was significantly influenced by the following parameters: group of cows (different feeding regime), genotype of αS1-casein (native mean size only) and k-casein, pH and the content of casein, whey protein, and casein number (**Table 1**).
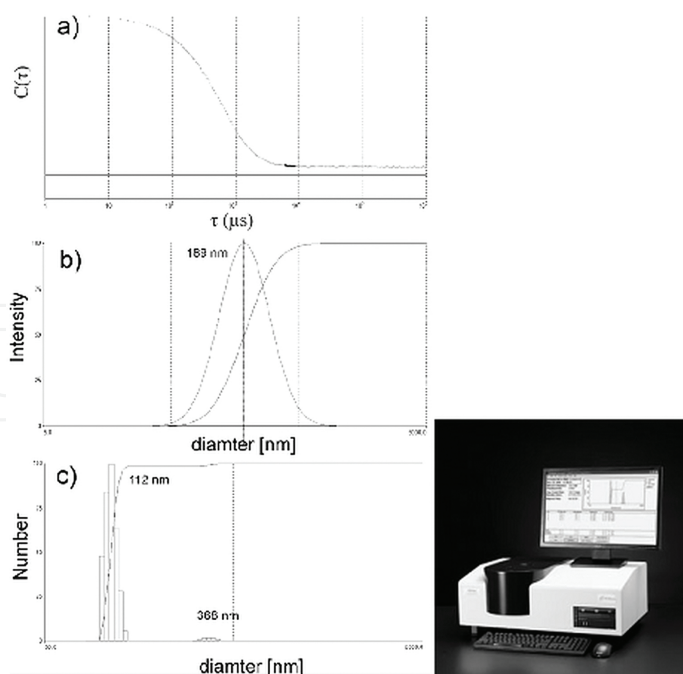
Figure 7. Representative DLS distributions in raw milk sample from heterozygous AB (CSN3) cow: a) correlation function, b) intensity particle size distribution, and c) multimodal number particle size distribution [26].
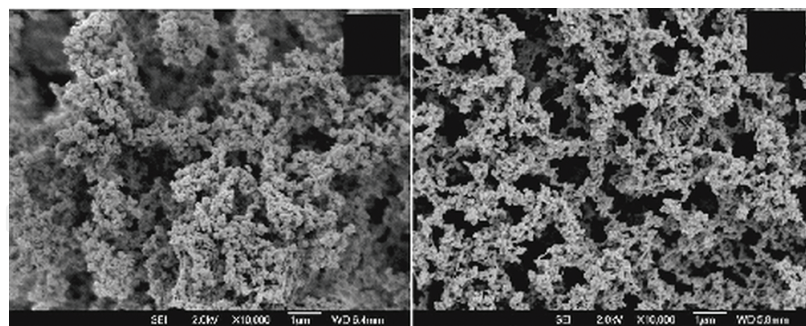
Variable	Mean size of casein micelles		
	Native	Heated	Change
αs1-CN	(BB > BC)[a]	c	c
β-CN	c	c	c
α-CN	(AA, AE > AB)[b]	(AA, AE > AB)[a]	c
β-lg	c	c	c
Fed. reg.	(Gr.3E > Gr.1)[a]	(Gr.3E > Gr.1)[a]	c
W.O.Lact.	c	c	c
Casein (%)	b	b	c
WP (%)	b	b	c
Casein no.	b	a	c
pH	a	a	c
Ca^{2+}	c	c	a
Ca	c	c	c
Mg	c	c	c
Citrate	c	c	c

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] N.S. = non-signifcant [28].

Table 1. Variation for mean size of native and heated casein micelles and for heat-induced change in size.

Figure 8. Scanning electron microscopy (SEM) imaging of the casein gel network produced from left, small casein micelle, and right, b large casein micelle milks. Images are at 10,000× magnification, scale bar = 1 μm [30].

Logan et al. [30] investigated the combined effects of milk fat globule size (MFG) and casein micelle size in bulk milk on the onset of gelation and the maximum rate of gelation. The results showed that casein micelle size is the major determinant of rennet gel strength. Milk fat globules could conceivably enhance the rheological properties of the rennet gel depending on their size in relation to the pore size of the casein micelle network. When the gels were formed with milks of large casein micelle, the size of MFG did not affect the gel properties, probably

because both large and small MFG were smaller than the pore size of the large casein micelle network. On the other hand, when the gels were formed with milks of small casein, the large MFG enhanced the gel firmness more than the small MFG. It is possible that when the size of large MFG fits well with the pore size of the casein micelle network, a synergistic effect on rennet elastic gel network is provided (**Figure 8**).

Finally, the effect of gross composition, protein composition, total and ionic calcium content, phosphorous content, and casein micelle size on chymosin-induced gelation was determined in milk from 98 Swedish Red cows by Gustavsson et al. [36]. The obtained results showed that protein content, ionic calcium concentration, total calcium content, and casein micelle size were the most important factors explaining the variation of gelation properties in this sample set of Swedish red cows. Furthermore, it was shown that composite effect of beta and kappa casein genetic variants have an effect on casein micelle size and it was suggested that this could be the reason for previously published differences in gelation properties between the composite genotypes in the present data set. Non-coagulating milk is a problem in Swedish red cows and the present study showed that non-coagulating milk is more common in cows in their first parity than in cows in their second parity.

5. Conclusion

Many products in the food industry, including milk, ice cream, mayonnaise, jam, etc., are colloids or have been produced via colloids. Dispersed particles determine, through their concentration, distribution, size, and structure, the physicochemical and organoleptic quality properties of a product. Additionally, the complex interactions between colloidal particles and with other ingredients will contribute to the rheology, texture, stability, appearance, and many other food characteristics. Techniques to measure the particle properties of the colloid systems allow for better understanding of these complex relations and further improvement food processing techniques and recipes. Furthermore, the implementation of such sensing-technologies in a food plant could enable online monitoring of the colloids or the derived products during the production process and promote early detection of an altering product quality.

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