In Situ Observation of Casein Micelle Coagulation

KENNETH W. RUETTIMANN* AND MICHAEL R. LADISCH†

Laboratory of Renewable Resources Engineering, *School of Chemical Engineering and †Department of Agricultural Engineering, Purdue University, West Lafayette, Indiana 47907

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Milk proteins occur in aqueous media as colloidal particles which are calcium-dependent, water-containing structures referred to as casein micelles. In situ studies of casein micelles using dark-field microscopy allow direct observation of micellar motion and interaction during coagulation induced by the enzymatic hydrolysis of the A-casein component of the micelles. The evolution of floe structure can be clearly visualized with the dark-field method. It is seen that flocs form irreversibly from only hydrolyzed micelles during the coagulation in a stagnant fluid. Coagulation patterns show that flocs from filamentous clusters incorporating micelles which may have several surface reaction sites. The techniques which make it possible to directly observe the coagulation reaction as it is occurring in an aqueous environment in a manner which provides insights into the mechanisms of the reaction are described.

INTRODUCTION

Caseins are polyepitides of specific amino acid residue numbers: 169 (αs-casein), 209 (β-casein), 207 (α„casein), and 199 (αs-casein) (1). These milk proteins naturally occur in aqueous suspension in the form of colloidal particles known as casein micelles. Casein micelles are spherical aggregates of the casein proteins, having a size range of 20 to 600 nm and a mean diameter of 140 nm (2, 3). One model for the structure of the casein micelle postulates that the micelle consists of smaller units known as submicelles which are bridged or cross-linked by calcium phosphate. The proteins are arranged in the micelle so that the more hydrophobic α- and β-caseins are in the micelle interior and the less hydrophobic αs-casein is present on the micelle surface (2). Native casein micelles form a stable colloidal dispersion which can be destabilized by enzymatic action. Enzymatic cleavage of the αs-casein at the Pro356-Met357 bond results in the release of a glycomacropeptide into solution; the fragment remaining in the micelle is known as para-α-casein. Coagulation of the protein particles then begins when the surfaces of the micelles have been sufficiently hydrolyzed (4).

This enzymatically initiated coagulation process forms the basis of cheese making; subsequent steps further process the curd formed by the micelle coagulation into the final product. Until now it has been difficult to observe the details of the coagulation process noninvasively since established noninvasive methods give limited information. We are seeking the mechanistic basis of this process on a microscopic level so that kinetic and transport equations which describe this reaction in a quantitative manner can be formulated and applied to the design of future cheese-manufacturing processes. The first task is to observe the process firsthand and obtain a quantitative representation of the micelle floe patterns which form. This is important since the final properties of the coagulated protein product depend in part on the floe structures which are developed during the coagulation process. For example, the physical properties of the resulting gel matrix would differ if the clusters (or aggregating units) making up the gel are globular as opposed to chain-like.
A variety of physical methods has been used to study the coagulating casein micelle system. These methods include changes in turbidity, light scattering, viscosity, and electron microscopy (4). Unfortunately, each of these methods has its drawbacks. Turbidity, light scattering, and viscosity data only give average particle size information over a limited size range. Electron microscopy may yield more detailed information, but cannot be performed while the coagulation is occurring in its native environment and may introduce artifacts due to sample preparation. The ideal method would allow for the measurement of the particle size distribution and the flocc conformation as the coagulation proceeds in its native environment. There is a need for this detailed information because one must know the events occurring at the micellar level in order to develop realistic coagulation models. The most unambiguous way of obtaining this information is by direct observation of the micellar behavior. Differing models which have been developed in an effort to describe the coagulation reaction could be reconciled if such detailed information was available. To this end we have developed dark-field microscopy as a method for observing the micelles in an aqueous medium.

Dark-field illumination is a method of increasing the image contrast, and it therefore increases the detecting power of the microscope. Under ideal illumination conditions, high-contrast particles as small as 5 nm can be detected (5). The sample is illuminated with light rays that do not enter the objective lens unless they are scattered by particles in the sample. Particles present in the sample are then seen as bright scattering sources against a dark background. An advantage of dark-field microscopy is its wide range of utility. Particles and structures from submicrometer size to hundreds of micrometers can be imaged, an advantage not shared by any of the other in situ methods.

Dark-field microscopy has been a useful technique for studying colloidal systems in the past. The forerunner of the modern version of the dark-field microscope was known as the ultramicroscope and was used in the early 1900s by Zsigmondy in his studies of colloids (6, 7). The dark-field microscope's ability to detect small colloidal particles can be used to determine diffusion coefficients and zeta potentials of colloidal particles as well as to study their coagulation (8, 9). Dark-field microscopy has also been used as a quantitative method of sizing latex particles (10). In more recent work, dark-field microscopy has been used to study bacterial flagella (11, 12) and other microscopic biological structures (13, 14).

MATERIALS AND METHODS

A Zeiss IM inverted microscope, fitted with a dark-field condenser (1.2/1.4 NA), 100-W mercury arc illuminator, and a 100× plan apochromatic oil-immersion objective lens (0.8–1.3 NA), was used for dark-field observation of the casein micelles. The path of light rays through the dark-field condenser is schematized in Fig. 1: no light enters the objective lens unless scattering sources are present in the sample. Thus, dark-field microscopy is essentially a light-scattering technique. The dark-field images can be observed in three ways: seen through the eyepieces, photographed on either 35-mm or 4 × 5 Polaroid film, or mon-

![Diagram of a microscope setup](image)

**Fig. 1.** Illustration of the path of light rays through a dark-field condenser (15).

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stored with a video camera. Images can be documented photographically, recorded with a VCR, or stored in digitized form.

The use of dark-field illumination results in images of low overall light intensity, albeit of high contrast. Furthermore, for our system, the casein micelles are constantly moving in aqueous solution due to Brownian motion. Therefore, fast films are required to provide exposure times short enough to stop the motion photographically. For video observations, a camera with a low lag time is needed to prevent blurring of the image. A Dage-MTI Series 68 video camera with a Newicon image tube was used in this work. This camera provides a reasonable compromise between sensitivity, lag, and resolution.

Clean slides are required in dark-field microscopy since contaminants on the slide surface add to the background optical noise and also may be mistaken for particles. The dark-field technique vividly illuminates these undesired contaminants. Commercially available precoated slides can be used after being wiped with a Kimwipe moistened with ethanol followed by rinsing with colloidal-free water. More rigorous cleaning methods are recommended to remove the majority of the contaminants from the glass (10, 16). Coating precoated slides and No. 1½ coveslips were used in this work after an additional chromic acid wash.

The casein micelles in these experiments were obtained from reconstituted low-fat, spray-dried, skim milk supplied by Kraft, Inc. (Glencoe, IL). The milk was reconstituted with deionized water and calcium chloride, to a final concentration of 10% w/v and 5 mM added CaCl₂, by gentle stirring at room temperature for a period of 1–2 h. The reconstituted milk was then equilibrated at 4°C for at least 12 h. Dried milk, which is readily stored, was used instead of fresh milk to assure uniformity from one sample to the next since the properties of fresh milk may vary among different batches. Nonfat milk was used in order to simplify the experimental system, removing any interfering effects due to fat globules. The low-heat drying process minimizes any protein degradation so that the properties of the reconstituted milk are similar to those of fresh milk. Since the larger micelles are more easily observed under dark-field conditions, the micelles were fractionated by centrifugation in a microfuge for 10 min. The pellets were then resuspended to a concentration of 0.8% casein (w/v) (by comparison, fresh milk is approximately 2.6% casein) in an imidazole buffer (10 mM imidazole, 5 mM CaCl₂) at the desired pH and allowed to equilibrate for several hours at 4°C. The Z-average size of the fractionated micelles was determined to be 280 nm by photon correlation spectroscopy (Malvern Zetasizer 3). This is about 50% larger than the value of 190 nm determined for unfractonated micelles. A variety of enzymes can be used to initiate the coagulation reaction, including calf rennet, chymosin (purified from calf rennet), and porcine pepsin. The coagulations presented in this paper used pepsin as the enzymatic agent. After addition of the enzyme to a diluted micelle solution, approximately 30 μl of the reaction mixture was applied to a clean glass slide and the coverslip was sealed with silicone grease to prevent evaporation. The micelles were then observed under dark-field illumination.

Pepsin activity was assayed using the method of Rajagopalan et al. (17), which is based on the hydrolysis of hemoglobin and the measurement of TCA-soluble peptides by absorbance at 280 nm. Milk protein concentration was determined using a dye-binding assay (18). Hydrolyzed, but uncoagulated, micelles were prepared at 0°C by cold hydrolysis of skim milk with pepsin. After complete hydrolysis, the pH of the solution was adjusted to 8.0 for 10 min, which destroys most of the pepsin activity. The pH was then readjusted to 6.5 and the hydrolyzed micelles were combined with unhydrolyzed micelles in the desired proportions. The extent of hydrolysis was determined by densitometric scanning of the para-casein band obtained by electrophoresis of hydrolyzed milk on a 0.1% (w/v) sodium dodecyl sulfate, 6 M urea, 15% acrylamide gel stained with colloidal Coomassie G-250.
RESULTS AND DISCUSSION

The primary advantage afforded by dark-field microscopy is the increase in image contrast created by the method of illumination. Bright-field and dark-field images of the same sample are compared in Fig. 2. Figure 2A is a photomicrograph of 1.14-μm polystyrene microspheres in an aqueous suspension under bright-field illumination. Figure 2B is the same sample as seen with dark-field illumination. The increase in contrast is readily apparent; it is this effect that enables the observation of small, low-contrast particles such as casein micelles which contain 80% water and exist in an aqueous medium. Very small particles can be detected in dark-field microscopy if the refractive index difference between the particles and the medium is large. We have observed particles as small as 5-nm colloidal gold particles with this instrument. Figure 3 is a photomicrograph of 10-nm protein-coated colloidal gold particles adsorbed to the surface of a glass slide. Note that in dark-field microscopy, the observed image size depends upon such factors as the light-scattering characteristics of the particles and the intensity of the illumination, and in photomicrography the exposure and printing conditions also affect the perceived image size.

The typical time course of a coagulating casein micelle system containing 0.15% casein is illustrated in Figs. 4A to 4C. Figure 4A shows the native casein micelles before the addition of the enzyme. Those particles which are not in the focal plane appear as out-of-focus blurs. The protein particles are constantly moving about due to Brownian motion and exist as a stable dispersion of single micelles. The addition of the enzyme to the system destabilizes the micelles as the enzyme hydrolyzes the κ-casein. Figure 4B shows the state of the system approximately 5 min after the enzyme has been added. There is a lag time after enzyme addition before the first doublets are observed. This lag time is presumably the time required for a sufficient degree of hydrolysis to take place before coagulation can begin. It has been estimated that 90% of a micelle’s κ-casein must be hydrolyzed before it can aggregate (19). Figure 4B shows that the system now consists mainly of single micelles and small clusters. Successful collisions between micelles result in permanent contact between the particles or clusters—the coagulation is an irreversible process. As the coagulation proceeds, the clusters grow in size consuming the single micelles and smaller clusters. The flocs, or large clusters, eventually reach a limiting size and no longer move about in solution, settling to the slide surface. The coagulation is effectively ended. Figures 4C and 4D show this final state for systems containing 0.15 and 0.20% casein. The conformation of the flocs is not chain-like, nor are they globular; instead they are filamentous structures which may contain regions where several micelles are packed together, and may be described as branching chains of clusters. Indeed, these structures are reminiscent of fractal clusters generated by computer simulation (20). Although these experiments are conducted with diluted micelle solutions, the results are representative of the events occurring in more concentrated solutions. Coagulation tests using fractionated micelles over a concentration range of 0.08 to 0.8% (w/v) casein showed the basic floc structures to be similar. An image of a floc from the coagulation of a system containing 0.8% casein after coagulation is shown in Fig. 4E.

An important consideration in understanding the micelle coagulation reaction is the degree of hydrolysis necessary for a successful collision between two particles. Must both particles be fully hydrolyzed before they aggregate or is some critical degree of hydrolysis sufficient? Another question is whether a hydrolyzed micelle can coaggregate with an unhydrolyzed micelle. Experiments combining hydrolyzed and unhydrolyzed micelles can address these questions. Hydrolyzed but uncoagulated micelles were prepared and combined with unhydrolyzed micelles in varying proportions. Dark-field microscopy results showed that the proportion of micelles which coagulated in each sample was qualitatively
equal to the proportion of hydrolyzed micelles in that sample. The state of the system after no further coagulations is illustrated in Fig. 5. Figure 5A is a sample containing 20% hydrolyzed and 80% unhydrolyzed micelles while Fig. 5B shows a sample with 80% hydrolyzed micelles. The relative floc sizes and free-micelle content observed in these types of experiments indicate that only the hydrolyzed micelles participate in the coagulation reaction. Representative photographic documentation of these types of events is often difficult, especially when the fast-moving, relatively dim single micelles are present. A photographic print of a system containing both single micelles and clusters is necessarily a compromise between the printing conditions which would give the best image for the single micelles and those providing the best image for the cluster alone (due to the large increase in the light intensity of the clusters compared with that of the single micelles).

This work, to the best of our knowledge, is the first time the casein micelle coagulation has been visually observed and documented as it is occurring in its native environment. The observation of the behavior of the micelles under various conditions gives needed insight into their properties and the mechanisms driving their association and can aid in vali-

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**FIG. 2.** Comparison of bright-field (A) and dark-field (B) images of 1.14-μm polystyrene microspheres. The particles (Pandex, Mundelein, Ill.) were diluted from 5 to 0.8% w/v and trapped between the slide and coverslip for photography. Scale bar is equal to 10 μm.
dating proposed models. A variety of models has been proposed to describe the enzymatic coagulation of casein micelles. Such models, usually attempt to couple the enzymatic hydrolysis reaction with the physical coagulation reaction since the coagulation is dependent on the course of the hydrolysis. An example of this type of model is that proposed by Dalgleish (19) which proposes a mechanism based on three concerns: the proteolysis of \(\kappa\)-casein, the probability that a micelle with a given proportion of hydrolyzed \(\kappa\)-casein will aggregate, and the aggregation of micelles via a Smoluchowski mechanism. The end result of this analysis give the weight-average molecular weight as a function of time:

\[
M_c(t) = M_0 \left( \frac{\int \left( \sum_i i^2 \frac{dn_i}{dt} \right) dt}{\int \left( \sum_i i \frac{dn_i}{dt} \right) dt} \right) + \frac{2k_s}{\int \left( \sum_i i \frac{dn_i}{dt} \right) dt} \times \left( \int \left( \sum_i i \frac{dn_i}{dt} \right) dt \right)^2 \left( \frac{dn_i}{dt} \right) dt.
\]

Here, \(M_0\) is the molecular weight of a casein monomer, \(i\) is the number of monomers in the particles which are added to the reaction mixture at rates \(\frac{dn_i}{dt}\), and \(2k_s\) is the rate constant of Smoluchowski aggregation. Based on fits of this model to experimental data,

**Fig. 4.** Dark-field photomicrographs of the casein micelle system during coagulation. (A) Before enzyme addition, (B) approximately 5 min after enzyme addition, and (C) after the coagulation is complete, approximately 30 min after enzyme addition. (D) A second example of a typical floc after the coagulation has ended. (E) A floc resulting from a more concentrated solution of casein micelles. For (A), (B), and (C), the casein concentration was 0.15%. Pepsin was added to a concentration of 0.25 units/ml to initiate the coagulation. For (D), the casein concentration was 0.2%, and the pepsin activity was 0.39 units/ml. For (E), the casein concentration was 0.8%, and the pepsin activity was 0.25 units/ml. Fractionated casein micelles were used, and the coagulation was carried out at room temperature and pH 6.5. Scale bar is equal to 10 \(\mu\) m.
Fig. 5. Dark-field photomicrographs of (A) a system containing 20% hydrolyzed micelles and 80% unhydrolyzed micelles, and (B) 85% hydrolyzed micelles and 15% unhydrolyzed micelles. Scale bar is equal to 10 μm.
Dalgleish found that more than 90% of a micelle's κ-casein must be hydrolyzed before it will coagulate.

Another class of models is that which presume or predict some degree of specific interaction between micelles. An example of this type of model is presented by Carlson et al. (21). This is a site-limited aggregation model, where micelles are limited to aggregation only at certain "patches" on their surface. Such active sites reportedly may arise if the distribution of κ-casein is uneven or through the presence of charged groups on the surface of the micelle. A kinetic rate equation describing the concentration of such sites leads to the result,

\[
\frac{n}{n_0} = 1 + \frac{1}{2} \left( \frac{1}{n_0 + 2k_s n_d} \right) \frac{\Delta n}{n_0},
\]

where \(n_0\) and \(\Delta n\) are the initial concentrations of particles and patches, respectively, \(n\) is the number of particles at time \(t\), and \(k_s\) is a second-order rate constant. A second example of a model which presumes some kind of surface site availability is the branching process model explored by Green and Morant (22). Here, micelles are again considered to have a limited number of reaction sites, described as the functionality of the micelle, \(f\).

The utility of the dark-field microscopy method lies in its ability to provide a visual means of evaluating such models through comparing observed micellar interactions with those predicted by theory. Carlson's model resulted in an average number of active sites per micelle, \(S_0/n_0\), of 1.18 at 30°C while Green and Morant found the average functionality of micelles to be less than 2 in all the cases they studied. Both of these results are rather unrealistic since the average patch concentration of functionality must be greater than 2 for any extensive network formation. It is also clear from our own dark-field microscopy results that the micelles are not severely limited in their interaction sites. This is evidenced by the complex branching structures that develop in the micelle flocs. Indeed, this point was further supported by observed floc conformations when an external force was applied, i.e., the shear force on a floc induced by the aspiration of solution through a pipette tip. In this case, the external shear allowed for the rearrangement of the floc by bringing more surface sites in contact with each other, resulting in more globular structures. The conditions of the coagulation reactions reported here, however, were stagnant, with floc conformations being determined by diffusion and interparticle forces. It thus appears that the micelles are not site limited in their aggregation ability to such an extent as has been proposed, although it is possible that site limitations may become apparent with micelles of different size or micelles in certain environmental conditions, and dark-field microscopy could be used to explore such situations.

This dark-field microscopy work is somewhat similar to the work of Green et al. (23–24), which was a study of the micelle coagulation reaction using electron microscopy. The course of the coagulation was studied by an examination of the micrographs of seven samples fixed during the reaction. The results were similar to those presented here in that the development of the system from a population of single particles to a more complex network was observed. Electron microscopy has the potential for high resolution, but a major disadvantage of electron microscopy methods lies in the sample preparation. Artifacts may easily be introduced in the fixation and dehyd ration of biological samples. In the case of Green et al. (23), the milk samples were mixed with glutaraldehyde for fixation and mixed again with agar before staining and dehydration. Shear forces from such mixing probably change the conformation of the floc network. The rearrangement of flocs due to shear forces has been observed in our own work. Dark-field microscopy allows the interactions of the micelles and the process of floc assembly to be visually observed as they occur in an aqueous medium. The technique is noninvasive, so the reaction occurs without the influence of external forces, and high-contrast
images can be obtained during the course of the reaction.

SUMMARY

The dark-field microscopy techniques described here may be applied to other systems of interest where the particles are too small or of too low contrast to be seen with conventional light microscopy but where an in situ visual observation is needed. We have examined liposomes and surfactant micelles under dark-field conditions. These structures, although submicrometer in size and having a small refractive index difference compared with the suspending medium, were successfully observed with the dark-field technique. Dark-field observations could complement the study of these systems by more traditional techniques, such as light-scattering methods.

This work with dark-field microscopy has resulted in a new technique to study the casein micelle system. By seeing what could not be seen before, new insights into the behavior of the system and the mechanism of the coagulation reaction can be gained, thus forming the foundation for more complete and useful models. The dark-field technique allows for qualitative visual verification of postulated mechanisms; current work with a coupled image analysis system is being developed as a means of obtaining quantitative data for model building. The behavior of the micelles under various conditions can be correlated to their physicochemical properties for a more fundamental understanding of the events occurring on the microscopic level and how they affect the macroscopic properties of the coagulated protein.

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REFERENCES