DOI: https://doi.org/10.21323/2618-9771-2023-6-4-547-553

Received 11.09.2023
Accepted in revised 30.11.2023
Accepted for publication 06.12.2023

Available online at https://www.fsjour.com/jour
Original scientific article
Open access

THE KINETICS OF MILK GEL STRUCTURE FORMATION STUDIES BY ELECTRON MICROSCOPY

Igor T. Smykov

All-Russian Scientific Research Institute of Butter- and Cheesemaking, Uglich, Yaroslavl Region, Russia

KEY WORDS:
milk, casein micelle, gelation mechanism, electron microscopy, microstructure, cheese production

ABSTRACT

The aim of this study is to enhance the comprehension of the mechanism of enzymatic gelation in milk by visualizing the evolution of its microstructure through transmission electron microscopy. In order to minimize the potential for artifacts during the preparation process and eliminate any possible difficulties in interpreting the resulting images, three distinct methods were employed in the research: shading the surface topography with vacuum deposition of heavy metal, negative staining of the specimen with a heavy metal solution and replicating a cleavage of a quick-frozen sample. The selection of time intervals for sampling the gel during its evolution is determined by the most probable significant modifications in the resulting gel. Based on the research, it has been shown that natural milk is a nonequilibrium system from the perspective of statistical thermodynamics. A notable observation is that the glycomacropeptides forming the hair layer on the surface of casein micelles are unevenly distributed, leading to the formation of micelle dimers and trimers. It has been determind that during the initial stage of enzymatic gelation in milk, clusters of loosely bound micelles are formed in areas with the highest concentration. The formation of micelle chains is absent at this stage due to the non-anisometric nature of micelles and the energetic disadvantage of their formation. It has been found that under the influence of enzymatic gelation near the gel point, a hierarchical process involving the transformation of the milk's protein component is activated. The trigger mechanism for this process is a cooperative conformational transition in clusters of casein micelles, which initiates a chain of more energy-intensive reactions in the following sequence: *hydro-phobic interactions* → *hydrogen bridges* → *electrostatic interactions* → *calcium bridges*. The result is the conversion of loosely bound micelle clusters into denser aggregates, predominantly contributing to the formation of milk curd. It is worth noting that gelation in milk can be regarded as a process that reduces the free energy of the dispersed system. Understanding the correlation between the decrease in the free energy value during gelation and the physical properties of the finished cheese and other dairy products continues to be a relevant area of research.

FUNDING: The article was published as part of the research topic No. FNEN-2019-0010 of the state assignment of the V. M. Gorbatov Federal Research Center for Food Systems of RAS.

Поступила 11.09.2023
Поступила после рецензирования 30.11.2023
Принята в печать 06.12.2023

https://www.fsjour.com/jour
Научная статья
Open access

ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКИЕ ИССЛЕДОВАНИЯ КИНЕТИКИ СТРУКТУРООБРАЗОВАНИЯ МОЛОЧНОГО ГЕЛЯ

Смыков И. Т.

Всероссийский научно-исследовательский институт маслоделия и сыроделия, Углич, Ярославская область, Россия

КЛЮЧЕВЫЕ СЛОВА:
молоко, мицелла казеина, механизм гелеобразования, электронная микроскопия, микроструктура, производство сыра

АННОТАЦИЯ

Цель этой работы заключается в улучшении понимания механизма ферментативного гелеобразования в молоке за счёт визуализация эволюции его микроструктуры с помощью просвечивающей электронной микроскопии. Для снижения возможности возникновения артефактов препарирования и устранения проблем достоверной интерпретации получаемых изображений в работе были использованы три различных способа подготовки препаратов к исследованиям: оттенение топографии поверхности вакуумным напылением тяжелого металла; негативное окрашивание препарата раствором тяжелого металла; репликация скола быстрозамороженного образца. Выбор моментов времени отбора образцов геля в процессе его эволюции обусловлен наиболее вероятными существенными изменениями в образующемся геле. В результате исследований показано, что с точки зрения статистической термодинамики натуральное молоко является неравновесной системой. Отмечено, что волосковый слой казеиномакропептидов на поверхности мицелл казеина распределён неравномерно, что способствует образованию димеров и тримеров мицелл. Определено, что в первичной фазе ферментативного гелеобразования в молоке образуются слабосвязанные кластеры мицелл в местах их наибольшей концентрации. Образования цепочек мицелл на этом этапе не наблюдается, что объясняется отсутствием анизометрии мицелл и энергетической невыгодностью их образования. Установлено, что при ферментативном гелеобразовании в молоке вблизи гель-точки запускается иерархический процесс преобразований белковой составляющей молока. Спусковым механизмом этого процесса является кооперативный конформационный фазовый переход в кластерах мицелл казеина, который вызывает цепочку более энергоёмких реакций в последовательности: *гидрофобные взаимодействия* → *водородные связи* → *электростатические взаимодействия* → *кальциевые мостики*. Результатом чего является преобразование слабосвязанных кластеров мицелл в более плотные агрегаты, преимущественно из которых окончательно формируется молочный сгусток. Отмечено, что в целом гелеобразование в молоке можно рассматривать как процесс, снижающий величину свободной энергии, а определение того, как уровень снижения величины свободной энергии молока при гелеобразовании связано с физическими свойствами готового сыра и других молочных продуктов остаётся актуальной темой исследований.

ФИНАНСИРОВАНИЕ: Статья подготовлена в рамках выполнения исследований по государственному заданию № FNEN-2019-0010 Федерального научного центра пищевых систем им. В. М. Горбатова Российской академии наук.

FOR CITATION: **Smykov, I. T.** (2023). The kinetics of milk gel structure formation studies by electron microscopy. *Food Systems*, 6(4), 547-553. https://doi.org/10.21323/2618-9771-2022-6-4-547-553

ДЛЯ ЦИТИРОВАНИЯ: **Смыков, И. Т.** (2023). Электронно-микроскопические исследования кинетики структурообразования молочного геля. *Пищевые системы*, 6(4), 547-553. https://doi.org/10.21323/2618-9771-2022-6-4-547-553

1. Introduction

The central aspect in the technological process of cheese manufacture, one of main dairy products, resides in the milk gel formation. Numerous researchers from different countries dedicated their works to studying this process and defining optimal methodologies for regulating the gelation process in milk.

The process of rennet milk coagulation, according to the prevailing current opinion, can be subdivided into two partially overlapping main phases: the primary (enzymatic) phase, which involves the hydrolysis of κ-casein micelles resulting in the formation of paracasein and the partial aggregation of destabilized micelles; and the secondary (non-enzymatic) aggregation phase, which encompasses the complete aggregation of micelles into a cohesive spatial gel structure and its densification.

The enzymatic phase mechanism has been thoroughly explained in [1,2], with a quantitative understanding of its interaction with various environmental factors. In [3], it is demonstrated that during gelation, stronger bonds tend to substitute weaker bonds. This occurs due to the denaturation of micelle structures, which exposes new reactive centers according to a hierarchical order: *hydrophobic interactions* → *hydrogen bonds* → *electrostatic attraction* → *calcium bridges*. Additionally, the investigation highlights the ongoing relevance of determining how individual casein micelles contribute to the microstructure of milk curds.

In previous studies [4,5], researchers observed that enzymatic destabilization of casein micelles leads to their transformation into a "molten globule" state, accompanied by micelle swelling, which is visually clear through an increase in their size. It signifies the initial stage of subsequent conformational changes.

The initial aggregation of rennet-destabilized casein micelles during the enzymatic phase has been confirmed by electron microscopic photographs obtained by various researchers [6,7]. Furthermore, it is observed that cooperative transitions take place in casein molecules towards the end of the enzymatic phase. These transitions are a result of conformational changes that substantially alter the properties of casein micelles and their subsequent restructuring [8,7]. It is important to note that while these processes occurring with proteins in the gelation phase are often referred to as denaturation, caseins are rheomorphic proteins, meaning that their conformation depends on the surrounding environment. Therefore, it is more correct to describe the changes as conformational rather than denaturation.

The conformational changes in milk proteins and their kinetics, which play a crucial role in determining the characteristics of the final product, have been a subject of interest for many researchers [9,10]. However, the conformational changes of proteins within casein micelles induced by different milk-clotting enzymes and their specific effects on the properties of the resulting milk curd are still not sufficiently studied.

The understanding of the state of milk protein system near the gel point, specifically the transition mechanism from the primary gelation phase to the secondary phase, is still limited and not fully comprehended. At this stage, clusters of micelles or their aggregates reach a state where they are no longer actively engaged in random motion known as Brownian motion. Nevertheless, there must be a mechanism facilitating intensive interactions between these clusters, leading to the formation of a common structure.

In the secondary phase of rennet coagulation, which typically takes place under conditions of relative rest of the milk, there is continued aggregation of micelles that have undergone conformational changes. This aggregation process ultimately results in the formation and densification of a milk curd [11,12].

The mechanism of micelle aggregation in this phase can be described by the von Smoluchowski theory for diffusion-limited aggregation (DLA), according to some authors [13,14]. The theory suggests that the rate of aggregation is limited by particle diffusion, and it occurs through random collision and joining of particles of destabilized micelles [15].

The percolation model of gelation or the fractal model of cluster-cluster aggregation offer a broad understanding of the process, but they do not fully clarify how clusters of micelles from the primary phase interact with one another to form a unified spatial structure.

Researchers are increasingly recognizing the secondary gelation phase as including two substages: micellar aggregation and densification of the milk curd, resulting in the formation of a three-dimensional structure. In this context, paper [16] focuses on the primary descriptive models of gelation in milk. The findings reveal that the majority of these models solely address the micellar aggregation substage, neglecting the densification substage. However, more recent models are applicable to a broader range of conditions and accurately distinguish both substages.

Electron microscopy is extensively applied in numerous studies regarding the mechanism and kinetics of milk gel formation. Its usage enables the visualization of specific moments during these processes and enhances our comprehension of gelation mechanism. Different methods of electron microscopy, such as transmission, scanning, and high resolution, are employed in these studies. In an early work [17], dedicated to electron microscopy analyses of milk proteins and dairy products, it was highlighted that a deeper understanding of how microstructure influences the properties of the final product enables the development of production processes focused on making high-quality products.

Modern researchers [18] confirm it and emphasize that analyzing the microstructure of cheeses at various stages of their production enhances the comprehension of manufacturing processes and the connection between cheese microstructure, product quality, and functionality. This understanding ultimately enables the optimization of production methods.

In [19], researchers examined the impact of high-pressure treatment on the microstructure of casein micelles in milk using electron microscopy. The findings revealed three phenomena associated with increasing pressure and processing time: the reduction in size of casein micelles, deformation and loss of their round shape, and the formation of agglomerated micelle fragments with more intense treatment (higher pressure and/or longer duration). This agglomeration resulted in an overall increase in the average diameter of micelles.

The microstructure of milk curd samples was examined [20] using transmission and scanning electron microscopy. The results of this study prove a notable interaction between coagulation temperature and the coagulation characteristics of milk protein, which requires certain modifications in cheesemaking procedures. It is worth mentioning that reducing the coagulation temperature significantly alters the microstructure of the milk curd, leading to a decreased tendency for syneresis during curd cutting.

Exciting advancements in research methodologies are becoming more prevalent, enabling us to get supplementary knowledge regarding the intricate transformations that occur within protein microstructures during the manufacture of dairy products. Consequently, to investigate the impact of endogenous milk protein additives on the microstructure and properties of milk gels, a super-resolution microscopy technique called Stimulated Emission Depletion (STED) was used [21].

The effect of temperature on the structure of casein micelles and the gelation process in milk was examined through the use of transmission electron microscopy and field emission scanning electron microscopy (FESEM) in [22]. The findings revealed a clear correlation between the temperature, microstructure of casein micelles, and the gelation characteristics of cow's milk.

The impact of pH on the microstructure of casein micelles was studied in [23] using direct Stochastic Optical Reconstruction Microscopy (dSTORM). dSTORM-visualization of casein micelle aggregates at a pH of 4.5, which was below the isoelectric point of caseins, demonstrated that β-casein dispersed throughout the protein network and revealed minuscule pores within the structure at the nanoscale level.

Super-resolution microscopy is a powerful method for analyzing the microstructure of complex colloidal systems in conditions that closely resemble their natural state. In a study cited as [24], Coherent Anti-Stokes Raman Scattering microscopy (CARS) showed that the use of fluorescent dye during the preparation of milk gel does not affect the visualization of its microstructure, while offering exceptionally high resolution.

In relation to electron microscopy, diverse techniques are used to prepare specimens for research. The researcher's selection of a particular area of interest within the sample also holds significant importance. Nevertheless, the process of choosing microscopy methods and preparing specimens unavoidably gives rise to preparation artifacts and challenges in accurately interpreting the resulting images.

In [25], three techniques were applied to prepare specimens for studying milk proteins using transmission electron microscopy, aiming to assess and eliminate preparation artifacts while gaining supplementary insights. Research findings indicate that utilizing the ultra-fast freeze-chip method, as opposed to fixation with glutaraldehyde, enables the visualization of finer structures within milk's casein micelles.

Even with the multitude of techniques available for investigating the microstructure of milk gel and the diverse specimen preparation methods used, it is notable that the majority of studies focus solely on either the morphology and microstructure of casein micelles or the microstructure of the completed milk curd, encompassing its initial and final states. Intermediate states are typically depicted solely through theoretical models. A comprehensive depiction of the development of milk gel microstructure during enzymatic gelation, substantiated by (electron) microscopic images confirming this evolution, is currently missing.

The primary objective of this study is to enhance comprehension of the process of enzymatic gelation in milk and to visually depict the evolution of the microstructure of rennet milk gel through the application

of transmission electron microscopy and a range of specimen preparation methods.

2. Objects and methods

2.1. Materials

The research used the following:

- milk sourced from AgriVolga LLC, the village of Burmasovo, Uglich district, Yaroslavl region, Russia, the Yaroslavskaya cow breed, with a raw milk fat content ranging from 4% to 4.2% and protein content between 3.4% and 3.7%;
- milk-clotting enzyme of Chy-max® M 1000 Liquid, manufactured by Chr Hansen A/S, based in Hoersholm, Denmark.

2.2. Methods

2.2.1. Milk preparation

The milk underwent pre-treatment, which involved heating to 40±1 °C and standardizing it by adjusting the casein/fat ratio to 0.7. The milk pasteurization was carried out in a tank at a temperature of 68±1 °C for 10 minutes, followed by cooling to a temperature of 36±1 °C. Enzymatic coagulation was then initiated by adding the enzyme at a ratio of 0.0025–0.0075% and stirring the milk for 2 minutes. The milk coagulation process took place at a temperature of 35±1 °C.

2.2.2. Electron microscopy

Electron microscopic examinations of the microstructure of milk components and milk gel were conducted using an EM-410 transmission electron microscope (Philips, the Netherlands). To minimize distortions caused by the microscope's electron beam on the sample, investigations were performed using the microscope's electron beam at a minimal current and a voltage of 40 kV.

Three different methods were used for preparing specimens for electron microscopy:

I. Shading of surface topography by vacuum deposition of heavy metal (MS)

Milk samples, obtained from a common reservoir at specific time intervals after adding rennet, were fixed at room temperature using a 2% glutaraldehyde solution in 0.1 M Sorenson phosphate buffer with a pH of 7.2 for 20 minutes (0.1 ml milk to 1 ml fixative). Subsequently, the fixed samples were diluted with distilled water at a ratio of 1:100. A 5 µl drop of the diluted, fixed sample was then transferred using a pipette onto a 400-mesh copper grid coated with nitrocellulose film (parlodium) and held for 2 minutes. Afterward, excess solution was removed from the grid's surface with filter paper, and the grid was dried in a vacuum chamber at room temperature. To enhance sample contrast, a 3–5 nm thick layer of platinum (Pt) was deposited on the sample's surface at a 24° angle using vacuum metal sputtering in a vacuum chamber [26]. The thickness of the applied metal was monitored using a quartz sensor. The completed sample, featuring shadows reflecting its surface topography due to the metal deposition, was then ready for examination in the electron microscope.

II. Negative staining of the preparation with a heavy metal solution (NS)

Samples of milk were collected from a common reservoir at specific time intervals after adding the rennet enzyme. All selected specimens were fixed at room temperature using a 2% glutaraldehyde solution in 0.1 M Sorenson phosphate buffer at pH 7.2 for 20 minutes (at a ratio of 0.1 ml of milk to 1 ml of fixative). Subsequently, the fixed samples were diluted with distilled water at a ratio of 1:100.

A 5 µl drop of the fixed sample was pipetted and transferred onto a copper grid coated with a nitrocellulose film (parlodium) and held for 2 minutes to ensure dispersed particles adhered to the film's surface. To enhance contrast, negative staining was employed by pipetting a 2 µl drop of a 4% uranyl acetate solution onto the fixed sample on the grid and holding for 4 minutes [27,28]. Excess solution was removed with filter paper, and the grid was dried in a vacuum chamber at room temperature. The resulting sample was transferred to an electron microscope for analysis.

III. Frozen Fracture Replication (FFR)

Gel samples were collected at distinct stages of its formation. The samples were carefully taken from the milk gel as minimally deformed parallelepipeds and placed into specially prepared tanks measuring about 2×2×8 mm made of aluminum foil.

To freeze the samples, a precisely controlled process was employed using a polished copper block cooled with liquid nitrogen to a temperature of –180 °C. The freezing rate was maintained at no less than –100 °C s^{-1} to prevent the formation of ice crystals in the sample. Subsequently, the frozen sample was placed in a cold vacuum chamber, where it was split crosswise at a temperature of –170 °C and held for one minute to remove surface ice.

To obtain a contrasting replica of the prepared cleavage surface of a milk gel sample, a 3–5 nm thick layer of platinum (Pt) was applied to the sample's surface at a 24° angle and a temperature of –130 °C in a vacuum chamber using thermal vacuum metal sputtering. Subsequently, to obtain a durable replica, a layer of carbon (C) with a thickness of 20–30 nm was also applied at a 90° angle. The thickness of the applied material was controlled by a quartz sensor.

After the replica was applied to the cold cleaved sample, it was promptly removed from the vacuum chamber and separated from the sample using a heated sodium hypochlorite (NaOCl) solution and then washed with toluene, acetone, and ethanol. Finally, it was rinsed with distilled water. The resulting finished replica of the cleavage surface of the milk gel sample was placed on a 400-mesh copper grid and transferred to an electron microscope for research.

Images of the microstructure of the prepared specimens, captured at various magnifications using an electron microscope, were then analyzed. At each stage of the study, at least ten obtained images were analyzed, and the most representative ones were selected for further examination and analysis.

3. Results and discussion

Electron microscopy provides a means to visually observe changes in casein micelles and their interactions during the gelation process in milk. This can be achieved using different specimen preparation methods for microscopy. However, the selection of research methods and specimen preparation techniques for microscopy invariably introduces the possibility of preparation artifacts and challenges in interpreting the resulting images.

In this study, the use of three distinct specimen preparation methods allowed for a reduction in the influence of preparation artifacts on research result interpretation. Consequently, it enhanced our understanding of the intricate mechanism underlying enzymatic gelation in milk.

For microscopy specimen preparation, milk samples were obtained at specific time points denoted by letters in Figure 1, which represents the gelation kinetics of milk due to enzymatic activity. These time points were selected based on previous literature highlighting significant changes in the resulting gel during milk gelation.

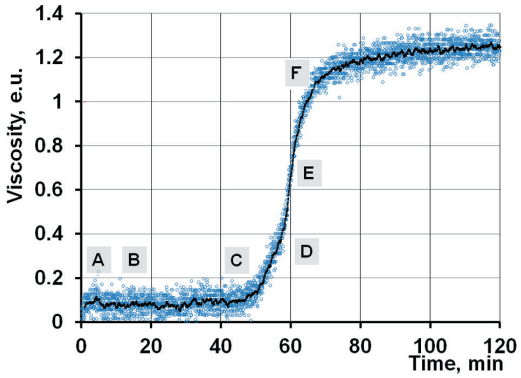

Figure 1. **Kinetics of enzymatic gelation in milk**

Рисунок 1. **Кинетика ферментативного гелеобразования в молоке**

In Figure 2, the electron microscopy images captured at time point (A) depict casein micelles in the original pasteurized milk. Image (a) in this figure was generated using heavy metal shading (MS), which effectively reveals the micelles as spherical structures of different sizes. This observation aligns with the widely accepted notion of their typical shape.

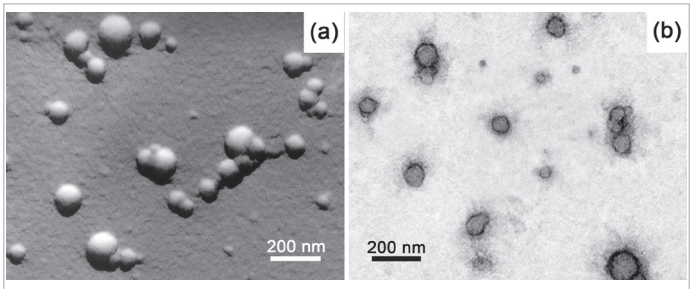

Figure 2. **Casein micelles in pasteurized milk. Methods: (a) — MS, (b) — NS**

Рисунок 2. **Мицеллы казеина в пастеризованном молоке. Методы: (a) — MS, (b) — NS**

The utilization of the negative contrast (NS) technique with uranyl acetate in the specimen allowed for the generation of image (b), which reveals that the ideal spherical shape of the casein micelles is not accurate and demonstrates the existence of a specific shell (hydrophilic hairbrush), on the micellar surface, that in general is not uniform. In a previous study [15], the presence of a casein macropeptide brush on the micellar surface was noted, although it was depicted as uniform. This unevenness indicates that different areas of the micelles have various levels of hydrophilicity. However, this unevenness specifies that in the original milk, as clear from both images, dimers and trimers of micelles, formed due to weak hydrophobic interactions, may also be present.

However, both methods of specimen preparation mentioned have a notable drawback: they require preliminary chemical treatment and strong dilution of the solution. These processes can potentially introduce preparation artifacts and prevent an accurate assessment of the micelles' native state and volume distribution in milk. The presence of dimer and trimer micelles observed in the images could possibly be attributed to such artifacts, thus additional research using a different, non-destructive method is required. Figure 3 presents an image that demonstrates the distribution of micelles across a microvolume of milk. This image was generated using the ultra-fast freezing-fracture method (FFR) for specimen preparation, which enables the preservation of the micelles' native state and volume distribution.

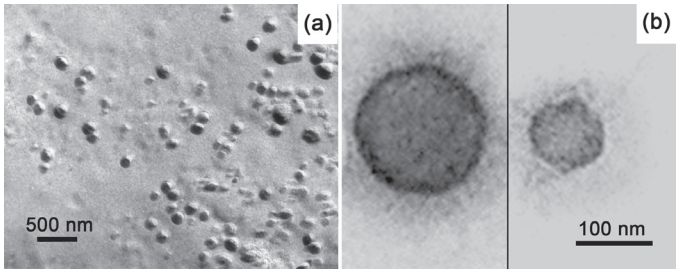

Figure 3. **Distribution of micelles over a microvolume of milk (a) — FFR method; (b) — enlarged images of casein micelles — NS method**

Рисунок 3. **Распределения мицелл по микрообъему молока (a) — метод FFR; (b) -увеличенные изображения мицелл казеина — метод NS**

The image presented in Figure 3(a) provides evidence supporting the presence of dimers and trimers of casein micelles in milk, as also observed in Figure 2; perhaps they are weakly bound by hydrophobic interactions, but they are not regarded as mere preparation artifacts. Though there may be debates among researchers regarding the existence of these dimers and trimers in natural milk. Figure 3(a), despite its clear simplicity, necessitates careful analysis and addresses a significant question. In the realm of statistical thermodynamics, most researchers view milk as an equilibrium disperse system. This implies that the dispersed particles (casein micelles) should be uniformly distributed throughout the entire volume, i. e., the level of fluctuations in their location should be minimum from a thermodynamic perspective. However, as evident from the image and several similar ones we have obtained, the distribution of casein micelles in reality is highly uneven across the milk volume. Some areas contain a considerable number of micelles, while others exhibit an absence of micelles altogether. Importantly, this is not a result of preparation artifacts. These observations lead to two conclusions. Theoretical conclusion: milk can be considered as a nonequilibrium system; and practical one: gelation primarily initiates in areas where casein micelles accumulate, forming primary clusters of micelles. These findings contribute to a deeper understanding of the gelation mechanism in milk.

Figure 3(b) displays high-magnification images of two casein micelles with different sizes. It is evident that both micelles show a complex internal structure, similar to the images described in [29]. While these images are occasionally interpreted as evidence of submicellar structure within casein micelles, it is more probable that they signify the complex nature of the overall micelle structure. Furthermore, the image clearly shows a halo surrounding the micelles. However, this image clearly shows a halo, framing the micelles, but these are not glycomacropeptides, which are much smaller in size, but the area of their interaction with the environment.

The widely-held belief among researchers is that the structure of rennet milk gel can be described as a spatial (three-dimensional) network of casein micelle chains. This notion is often supported by repeated artistic illustrations (models), suggesting that individual micelles form chains during the initial stages of enzymatic gelation. However, experimental data from various researchers have revealed contradictions to the traditional views on the gel structure. In our opinion, this prevailing belief is erroneous for two reasons. Firstly, systems inherently strive to minimize free energy, and the formation of chains becomes energetically unfavorable when glycomacropeptides are uniformly removed from the micelle surface. Instead, it is more plausible that these glycomacropeptides are randomly clustered together. Secondly, the formation of primary chains would require an anisometric arrangement of hydrophobic areas on the micelle surface, which is unlikely, unsupported by observations, and lacks confirmation from any evidence. The photographs in Figure 4 illustrate the formation of small, loosely bound clusters of micelles during the initial phase of enzymatic gelation, represented at time point (B) in Figure 1.

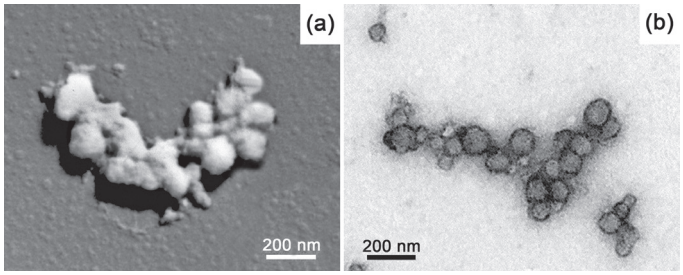

Figure 4. **Formation of clusters of casein micelles in the primary gelation phase. Methods: (a) — MS, (b) — NS**

Рисунок 4. **Образование кластеров мицелл казеина в первичной фазе гелеобразования. Методы: (a) — MS, (b) — NS**

At this moment, the milk-clotting enzyme molecules eliminated the glycomacropeptides of κ-casein from the surface of some micelles and formed a hairy layer with hydrophilic properties on the surface of the micelles. Consequently, the surface of the micelle (or a part of it) turned into a hydrophobic surface, which is not energetically favorable for the dispersed system. To reduce its free energy, the system aims to minimize the size of free hydrophobic areas by connecting micelles into clusters through hydrophobic interactions. Figure 4(a) and Figure 4(b), obtained using different methods, clearly show the formation of these loosely bound micelle clusters. However, the presence of any chains is not evident.

Figure 5 displays images of large clusters of casein micelles observed at time point (C) in Figure 1, marking the end of the initial stage of enzymatic gelation. By this time, approximately 80–90% of the glycomacropeptides attached to the micelles' surface were removed by the milk-clotting enzymes. Images obtained through various techniques reveal that the majority of micelles are arranged in large, loosely connected clusters. It's important to mention that despite these changes, the viscosity of the milk remains relatively unchanged throughout the primary gelation phase. This can be attributed to the well-known Einstein formula (1), which states that the viscosity η of a dispersed system is solely dependent on the volume fraction of the dispersed phase, rather than the sizes of non-interacting particles.

$$\eta = \eta_0 (1 + 2{,}5\varphi) \qquad (1)$$

where η_0 is the viscosity of the dispersion medium, φ is the volume fraction of the dispersed phase.

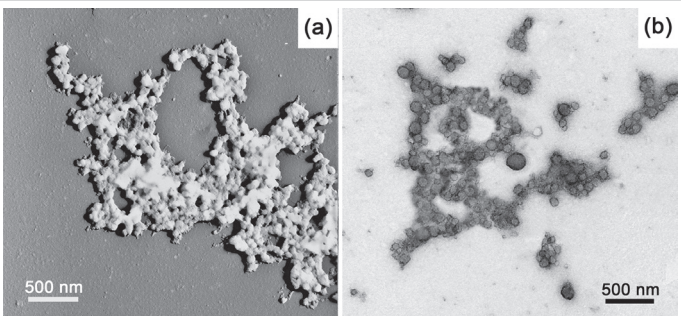

Figure 5. **Clusters of casein micelles at the end of the primary gelation phase. Methods: (a) — MS, (b) — NS**

Рисунок 5. **Кластеры мицелл казеина в конце первичной фазы гелеобразования. Методы: (a) — MS, (b) — NS**

The electron microscopic studies of the gelation process at this stage yield results that are consistent with earlier findings in [30,31] obtained through different methods and indicate the occurrence of a clustering process of casein micelles as a preliminary step prior to coagulation.

Regarding the timeframe, the formation of large clusters of casein micelles occurs around the "gel point", which refers to the moment when the viscosity of the dispersed system rapidly increases. Consequently, investigations were conducted on the microstructure of the resulting gel immediately after reaching the gel point (time point D — Figure 1). Figure 6 presents electron microscopy images depicting the state of casein micelle clusters at this specific time.

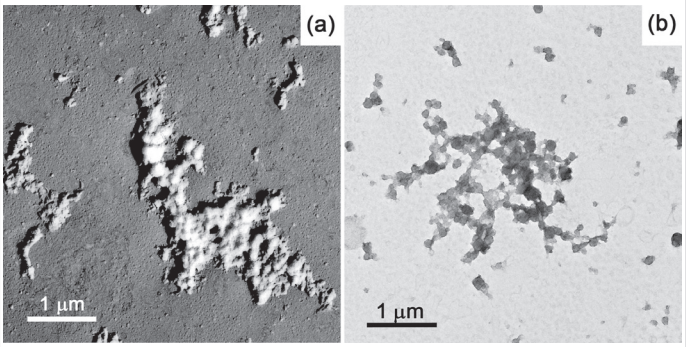

Figure 6. **Clusters of casein micelles at the beginning of the secondary gelation phase Methods: (a) — MS, (b) — NS**
Рисунок 6. **Кластеры мицелл казеина в начале вторичной фазы гелеобразования Методы: (a) — MS, (b) — NS**

Based on the photographs and a comparison with Figure 5, it is evident that both the micelles within the clusters and the clusters themselves experienced notable structural alterations after reaching the gel point. Prior to the gel point, the micelles maintained a spherical shape, and the bonds between them in the clusters were relatively weak (hydrophobic). However, after the gel point, only a few spherical micelles remained within the clusters, with some disintegrating, unfolding, and forming multiple intermicellar bonds.

Our observation suggests that during the final stage of the primary gelation phase, there is a cooperative conformational transition similar to a second-order phase transition in casein molecules, forming micelles and clusters of micelles. This transition has two main effects: first, the increase in micelle size due to enhanced hydration and volume fraction, resulting in a slight viscosity increase, and, secondly, the significant alteration of physicochemical properties of the micelles, facilitating the formation of a cohesive milk gel structure. Loosely bound micelle clusters transform into coherent aggregates of remaining micelles and protein particles after the conformational transition. These aggregates gradually become denser through fluctuation, oscillatory, and rotational movements of casein micelles and protein particles within the aggregate, leading to the formation of additional intermicellar bonds. Subsequently, these aggregates serve as the starting materials for the formation of the three-dimensional spatial structure of the milk gel in the secondary phase. This viewpoint is supported by the analysis of a series of gel state images at the end of the secondary gelation phase. Figure 7 displays photographs capturing the progression of casein micelles clustering into a cohesive structure. The photographs vividly show the formation of small micelle aggregates, their mutual interactions, and the subsequent emergence of a unified structure.

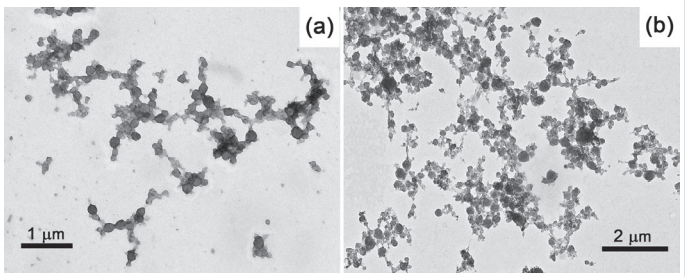

Figure 7. **Interaction of aggregates of casein micelles during the formation of an integral structure. Methods: (a), (b) — NS**
Рисунок 7. **Взаимодействие агрегатов мицелл казеина при образовании целостной структуры. Методы: (a), (b) — NS**

The studies conducted prove a notable drawback despite their apparent clarity. The method employed for direct transmission electron microscopy involves the deposition of dispersed particles on a flat substrate, resulting in significant deformation of the particles. Consequently, the three-dimensional structure of clusters and aggregates can only be inferred from their two-dimensional representations in photographs. Furthermore, this method proves inadequate for assessing the evolution of gel structure during coagulation and syneresis, as the structural deformations become catastrophic in nature. Therefore, alternative methods are necessary to examine these changes. In [32], scanning electron microscopy was used to capture images of the structure of the final milk gel. The findings clearly illustrate that the gel primarily consists of aggregates (flakes) formed by micelles and protein particles rather than chains of micelles, which aligns with our own results.

Our investigation into the microstructure of the milk gel using the ultrafast freeze-cleavage transmission electron microscopy method showed similar results (Figure 8(a)). If the image obtained by scanning electron microscopy provides a comprehensive view with a large depth of field, allowing for a quasi-three-dimensional assessment of the structure, transmission electron microscopy presents an image of a random cross-sectional cleavage of the structure of studied sample. It is highly unlikely that such a random cleavage would consistently pass through chains of micelles. Thus, the photograph presented above depicts a cross-sectional cleavage of the spatial structure formed by aggregates of micelles. It is important to note that the gel's characteristic size can be influenced by the size of the gel's unit cell, which varies based on the properties of the milk and the processing techniques used. In this case, the cells have an average size of approximately 5 μm, which is similar to the characteristic sizes of micelle clusters near the gelation point. Evidently, this size affects the gel's permeability and, accordingly, the rate of syneresis.

Figure 8(b) illustrates the location of the milk fat globule within the milk gel structure. This photograph demonstrates that the fat globule, as it undergoes the gelation process, gets a secondary protein shell, which serves as a protective measure for the fat against lipolysis during the storage of cheese.

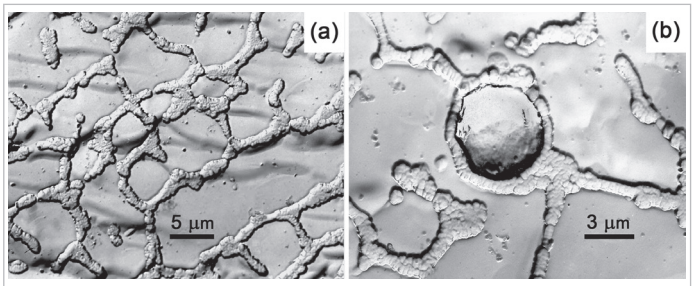

Figure 8. **Structure of milk gel. FFR method**
Рисунок 8. **Структура молочного геля. Метод FFR**

Therefore, the experimental confirmation of the hypothesis that the composition of rennet milk gel consists of sequential chains of distinct casein micelles is absent, thus rendering it an inadequate description of the gel structure. Instead, it can be proposed that milk gel structuring occurs on two levels: localized structuring within clusters of micelles and overall structuring of a pre-existing cluster system. It is highly probable that the final gel structure is formed during the coagulation process through the aggregation of pre-formed aggregates of casein micelles.

To complete the description of the milk gel structure, it is crucial to elucidate its topological characteristics. The key aspect to consider is that the topology of the resulting structure should minimize the energy of the phase interface during the sol-gel transition. During enzymatic gelation, para-κ-casein, which has a notable hydrophobic nature, is formed. Namely, an additional phase interface appears, leading to an increase in free (excess) interphase energy. According to the Gibbs-Helmholtz principle, this energy tends to spontaneously decrease in dispersed systems. As a result, the entropy of the system decreases due to the increased proportion of ordered entities within the surrounding water structure. The hydrophobic interactions between para-κ-casein molecules and water molecules contribute to an increase in entropy and the system transition to a more favorable energetic state. Furthermore, the combination of micelles into a single, compact structure is advantageous in terms of energy dynamics for the system. This combination minimizes contact of hydrophobic areas with water, and, consequently, the interaction of identical hydrophobic areas also contributes to a reduction in the system's overall free energy. The process of diminishing interfacial energy is further facilitated through the aggregation of micelles, which goes with a decline in free surface energy as a consequence of decreased specific surface area of dispersed phases upon intermolecular contacts among the micelles' hydrophobic areas. Additionally, the formation of a curd at specific stages involves the participation of ionic, hydrogen, and van der Waals bonds.

During the process of gelation, milk undergoes a transition from a stable dispersed state to a metastable state. The phase separation of the metastable system does not occur through the emergence of nuclei of two finished phases followed by competitive growth, as assumed in the chain model. Instead, it is attributed to the initial formation of numerous small nonequilibrium areas (clusters) within the metastable system, which show slight variations in concentration and have the ability to gradually merge and approach an equilibrium state. Consequently, the transformation of the dispersed system (milk) into a structured one (milk curd) occurs as a result of this process.

From the perspective outlined, the milk curd can be characterized within the realm of scientific discourse as a bicontinuum system. There one continuum corresponds to whey, while the other continuum relates to a structured protein matrix. These continua permeate each other mutually. The main condition for the emergence, existence, and development of this system lies in the organization of its topology, prioritizing the minimization of the interface between the continua. The conversion of milk into a milk curd occurs under the influence of deterministic and stochastic factors. Deterministic processes dictate the fundamental nature and properties of the curd's topology, whereas stochastic processes govern their distortion or dispersion within a specific confidence interval.

4. Conclusion

Based on the conducted research, the following conclusions can be drawn.

From the perspective of statistical thermodynamics, natural milk shows characteristics of a non-equilibrium system, as proved by the uneven distribution of casein micelles throughout its volume.

The uneven distribution of a glycomacropeptide hair layer on the surface of casein micelles in milk contributes to the generation of dimer and trimer micelles.

During the initial phase of enzymatic gelation in milk, loosely bound clusters of micelles form at areas of the highest micelle concentration. Due to the weak nature of these hydrophobic bonds, these clusters have minimal impact on the viscosity changes observed in milk. The formation of micelle chains does not occur at this stage, which can be attributed to the absence of micelle anisometry, and the energetic disadvantage associated with their formation.

During the enzymatic gelation process in milk near the gel point, a hierarchical transformation process of the protein component of milk is activated. The trigger mechanism for this process is a cooperative conformational phase transition in clusters of casein micelles, leading to a sequential chain of increasingly energy-intensive reactions in the order: *hydrophobic interactions* → *hydrogen bonds* → *electrostatic attraction* → *calcium bridges*. The result is the conversion of loosely-bound micelle clusters into more densely packed aggregates.

Ultimately, the resulting milk curd predominantly consists of individual aggregates and constitutes a bicontinuous system. In this system, one continuous phase is the whey while the other continuous phase is a structured protein matrix. These two phases permeate each other mutually.

Generally, the gelation phenomenon in milk can be conceptualized as an energy-reducing process. Investigating the correlation between the reduction in milk's free energy during gelation and the physical characteristics of the final cheese and other dairy products continues to be an ongoing subject of scientific inquiry.

REFERENCES

1. Lucey, J. A. (2011). Rennet-induced coagulation of milk. Chapter in a book: Encyclopedia of Dairy Sciences. Oxford: Academic Press. 2011.
2. Fox, P. F., Guinee, T. P., Cogan, T. M., McSweeney, P. L. H. (2017). Fundamentals of Cheese Science. Springer, New York. 2017.
3. Amaro-Hernández, J. C., Olivas, G. I., Acosta-Muñiz, C. H., Gutiérrez-Méndez, N., Rios-Velasco, C., Sepulveda, D. R. (2022). Chemical interactions among caseins during rennet coagulation of milk, *Journal of Dairy Science*, 105(2), 981–989. https://doi.org/10.3168/jds.2021-21071
4. Ptitsyn, O. B. (1995). Molten globule and protein folding. Chapter in a book: Advances in Protein Chemistry. 1995. https://doi.org/10.1016/S0065-3233(08)60546-X
5. Farrell Jr., H. M., Qi, P. X., Brown, E. M., Cooke, P. H., Tunick, M. H., Wickham, E. D. et al. (2002). Molten globule structures in milk proteins: Implications for potential new structure-function relationships. *Journal of Dairy Science,* 85(3), 459–471. https://doi.org/10.3168/jds.S0022-0302(02)74096-4
6. Fox, P. F., Guinee, T. P. (2013). Cheese science and technology. Chapter in a book: Milk and Dairy Products in Human Nutrition. Oxford, Wiley Blackwell.2013.
7. Smykov, I. T. (2015). Kinetics of Milk Gelation. Part I. Coagulation Mechanism. Chapter in a book: Rheology: Principles, applications and environmental impacts. New York: Nova Science Publ. 2015.
8. Surkov, B. A., Klimovskii, I. I., Krayushkin, V. A. (1982). Turbidimetric study of kinetics and mechanism of milk clotting by rennet. *Milchwissenschaft*, 37, 393–395.
9. Mosler, A. B., Shaqfeh, E. S. G. (1997). The conformation change of model polymers in stochastic flow fields: Flow through fixed beds. *Physics of Fluids*, 9, 1222–1234. https://doi.org/10.1063/1.869262
10. Markoska, T., Vasiljevic, T., Huppertz, T. (2020). Unravelling conformational aspects of milk protein structure-contributions from nuclear magnetic resonance studies. *Foods*, 9(8), Article 1128. https://doi.org/10.3390/foods9081128
11. Horne, D. S., Lucey, J. A. (2017). Rennet-Induced Coagulation of Milk. Chapter in a book: Cheese: Chemistry, Physics and Microbiology, Oxford: Academic Press. 2017. https://doi.org/10.1016/B978-0-12-417012-4.00005-3
12. Fox, P. F., Guinee, T. P., Cogan, T. M., McSweeney, P. L. H. (2016). Enzymatic Coagulation of Milk. pp.185–229. Chapter in a book: Fundamentals of Cheese Science, Springer Nature, New York. 2013. https://doi:10.1007/978-1-4899-7681-9_7
13. Tuszynski, W. B. (1971). A kinetic model of the clotting of casein by rennet. *Journal of Dairy Research,* 38(2), 115–125. https://doi.org/10.1017/S0022029900019233
14. Witten Jr., T. A., Meakin, P. (1983). Diffusion-limited aggregation at multiple growth sites. *Physical Review B*, 28(10), 5632–5642. https://doi.org/10.1103/PhysRevB.28.5632
15. De Kruif, C. G., Holt, C. (2003). Casein micelle structure, functions and interactions. Chapter in a book: Advanced Dairy Chemistry — 1 Proteins. Springer, Boston, MA. 2003.
16. Salvador, D., Acosta, Y., Zamora, A., Castillo, M. (2022). Rennet-induced casein micelle aggregation models: A review. *Foods.* 11(9), Article 1243. https://doi.10.3390/foods11091243
17. Kalab, M. (1979). Microstructure of dairy foods. 1. Milk products based on protein. *Journal of Dairy Science,* 62(8), 1352–1364.
18. Ong, L., Li, X., Ong, A., Gras, S. L. (2022). New insights into cheese microstructure. *Annual Review of Food Science and Technology,* 13, 89–115. https://doi.org/10.1146/annurev-food-032519-051812
19. Serna-Hernandez, S. O., Escobedo-Avellaneda, Z., García-García, R., Rostro-Alanis, M. D. de J., Welti-Chanes, J. (2022). Microscopical evaluation of the effects of high-pressure processing on milk casein micelles. *Molecules,* 27, Article 7179. https://doi.org/10.3390/molecules27217179
20. Panthi, R. R., Kelly, A. L., Sheehan, J. J., Bulbul, K., Vollmer, A.H., McMahon, D. J. (2019). Influence of protein concentration and coagulation temperature on rennet-induced gelation characteristics and curd microstructure. *Journal of Dairy Science*, 102(1), 177–189. https://doi.org/10.3168/jds.2018-15039
21. Li, R., Ebbesen, M. F., Glover, Z. J., Jæger, T. C., Rovers, T. A. M., Svensson, B. et al. (2023). Discriminating between different proteins in the microstructure of acidified milk gels by super-resolution microscopy. *Food Hydrocolloids*, 138, Article 108468. https://doi.org/10.1016/j.foodhyd.2023.108468
22. Zhang, Y., Liu, D., Liu, X., Hang, F., Zhou, P., Zhao, J. et al. (2018). Effect of temperature on casein micelle composition and gelation of bovine milk. *International Dairy Journal,* 78, 20–27. https://doi.org/10.1016/j.idairyj.2017.10.008
23. Foroutanparsa, S., Brüls, M., Tas, R. P., Maljaars, C. E. P., Voets, I. K. (2021). Super resolution microscopy imaging of pH induced changes in the microstructure of casein micelles. *Food Structure,* 30, Article 100231. https://doi.org/10.1016/j.foostr.2021.100231
24. Glover, Z. J., Ersch, C., Andersen, U., Holmes, M. J., Povey, M. J., Brewer, J. R. et al. (2019). Super-resolution microscopy and empirically validated autocorrelation image analysis discriminates microstructures of dairy derived gels. *Food Hydrocolloids*, 90, 62–71. https://doi.org/10.1016/j.foodhyd.2018.12.004
25. Karlsson, A. O., Ipsen, R., Ardo, Y. (2007). Observations of casein micelles in skim milk concentrate by transmission electron microscopy. *LWT — Food Science and Technology,* 40(6), 1102–1107. https://doi.org/10.1016/j.lwt.2006.05.012
26. Matsko, N., Letofsky-Papst, I., Albu, M., Mittal, V. (2013). An analytical technique to extract surface information of negatively stained or heavy-metal shadowed organic materials within the TEM. *Microscopy and Microanalysis,* 19(3), 642–651. https://doi.org/10.1017/S1431927613000366
27. Kavanagh, G. M., Clark, A. H., Ross-Murphy, S. B. (2000). Heat-induced gelation of globular proteins: 4. Gelation kinetics of low pH β-Lactoglobulin gels. *Langmuir,* 16, 24, 9584–9594. https://doi.org/10.1021/la0004698
28. Hayat, M. A. (2000). Principles and techniques of electron microscopy: Biological applications. Cambridge University Press. 2000.
29. Dalgleish, D. G., Spagnuolo, P., Goff, H. D. (2004). A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *International Dairy Journal,* 14(12), 1025–1031. https://doi.org/10.1016/j.idairyj.2004.04.008
30. Casanova, H., Chen, J., Dickinson, E., Murray, B. S., Nelson, P. V., Whittle, M. (2000). Dynamic colloidal interactions between protein stabilized particles — experiment and simulation. *Physical Chemistry Chemical Physics,* 2(17), 3861–3869. https://doi.org/10.1039/b004023l
31. de Kruif, C. G. (1998). Supra-aggregates of casein micelles as a prelude to coagulation. *Journal of Dairy Science,* 81(11), 3019–3028. https://doi.org/10.3168/jds.S0022-0302(98)75866-7
32. Shamsi, K. (2008). Effects of Pulsed Electric Field Processing on Microbial, Enzymatic and Physical Attributes of Milk and the Rennet-Induced Milk Gel. RMIT University, Melbourne. 2008.

AUTHOR INFORMATION	СВЕДЕНИЯ ОБ АВТОРАХ
Affiliation	**Принадлежность к организации**
Igor T. Smykov, Doctor of Technical Sciences, Chief Researcher, Department of Physical Chemisry, All-Russian Scientific Research Institute of Butter- and Cheesemaking 19, Krasnoarmeysky Boulevard, Uglich, 152613, Yaroslavl Region, Russia Tel.: +7–48532–9–81–21 E-mail: i_smykov@mail.ru ORCID: https://orcid.org/0000-0002-5663-3662	**Смыков Игорь Тимофеевич** — доктор технических наук, главный научный сотрудник, Отдел физической химии, Всероссийский научно-исследовательский институт маслоделия и сыроделия 152613, Ярославская область, Углич, Красноармейский бульвар, 19 Тел.: +7–48532–9–81–21 E-mail: i_smykov@mail.ru ORCID: https://orcid.org/0000-0002-5663-3662
Contribution	**Критерии авторства**
Completely prepared the manuscript and is responsible for plagiarism.	Автор самостоятельно подготовил рукопись и несет ответственность за плагиат.
Conflict of interest	**Конфликт интересов**
The author declares no conflict of interest.	Автор заявляет об отсутствии конфликта интересов.