1993

Practical Aspects of Electron Microscopy in Dairy Research

Miloslav Kalab

Follow this and additional works at: http://digitalcommons.usu.edu/foodmicrostructure

Part of the Food Science Commons

Recommended Citation
PRACTICAL ASPECTS OF ELECTRON MICROSCOPY
IN DAIRY RESEARCH

Miloslav Kaláb
Centre for Food and Animal Research
Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6

Abstract

Milk products are based mostly on casein micelles, fat globules, and whey proteins. The former two constituents are corpuscular and whey proteins become corpuscular when coagulated. Structural changes in these basic constituents during processing have been studied by electron microscopy. This review discusses the structures of yoghurt, curd, cheeses (hard cheeses, mould-ripened cheeses, cream cheeses, and process cheese), cream, milk powders, and nontraditional dairy products. Defects and deviations from traditional structures of these products are explained where the causes are known. Examples of such causes are foaming of milk, presence of unusual ingredients (bacterial polysaccharides, whey protein concentrates), and alterations in manufacturing procedures (temperature regimens, ultrafiltration, or microparticulation).

The review emphasizes the importance of electron microscopy alone and also in conjunction with X-ray microanalysis and image analysis. Data obtained by structural studies facilitate understanding of sensory properties of the products and help to develop new foods with desired properties. The review is illustrated with 29 micrographs and supported by 165 references.

Key Words: Casein micelles, cheese, cream, dairy products, electron microscopy, fat globules, microstructure, milk, milkfat, milk powder, review, yoghurt.

Introduction

Chemical and physical analyses of raw materials and intermediate and finished products are important to the dairy industry. Microscopy is one of physico-chemical analytical procedures used because it shows the spatial distribution of corpuscular components and the overall structure of the ingredients and products. Specific staining techniques reveal chemical composition of the structures, particularly the major groups of substances such as proteins, fats, ribonucleic acids, and calcium salts. Optical microscopy is commonly used as part of microbiological analyses.

The properties of light used in optical microscopy limit the resolution of this technique to approximately 0.5 μm and the depth of focus is very shallow. This means that, e.g., casein micelles, fat globule membranes, bacteriophages, and other minute particles may not be seen under the optical microscope. In contrast, electron microscopy extends the resolution to several nanometers (1 nm = 1x10⁻⁹ m) and in the transmission electron microscopy mode makes it possible to study in detail even the smallest objects mentioned. A considerable depth of focus characterizes scanning electron microscopy (SEM), which is used to visualize details of three-dimensional objects such as the protein networks in yoghurt and cheese or lactic acid bacteria. In the cryo-SEM mode, food samples may be examined in the frozen state. This is particularly useful in the studies of high-fat products such as whipped cream, frozen foods such as ice cream, and microorganisms, e.g., in mould-ripened cheeses. Because of the high cost of the microscopes and ancillary equipment, electron microscopy is not yet used as part of routine analyses in the food industry. Used in research and development, however, it helps to solve many problems which cannot be resolved by other analytical methods and contributes to structural characterization of the materials under study. The potential of electron microscopy in studies of correlations between structure and physical properties of foods has been recognized by the industry which now introduces electron microscopes as a part of the normal research functions. The objective of this presentation is to summarize some of the practical aspects of electron microscopy in dairy research.
Practical aspects

Earlier reviews show that the use of electron microscopy in dairy research has a relatively long tradition [15]. The reasons for using it are numerous: high resolution providing details several nanometers in diameter, ability to study surfaces as well as internal structures [24, 82]; ability to examine samples in the frozen hydrated state [25, 140]; possibility to compare results and confirm them using different procedures [21]; possibility to carry out elemental X-ray microanalysis of samples on an electron microscopy scale [18], and immunological microanalysis using gold-labelled antibodies [73, 147].

Scientific papers on electron microscopy of milk products may be found in various journals, particularly Food Structure, Journal of Dairy Science, Milchwissenschaft, and Journal of Dairy Research. Reviews on the structure of milk and milk products [15, 81, 84, 86, 87, 122, 161] and on electron microscopy in dairy science [1, 82, 83, 85], a book presenting micrographs of a great variety of dairy products, many of which show three-dimensional images [155], and a cumulative index [72] of papers on the structure and texture of foods published before 1991 provide additional information on the subject.

Milk

From the electron microscopic viewpoint, milk consists of constituents which are corpuscular and have dimensions making them visible (fat globules, casein micelles, submicellar casein) and constituents which are in solution as individual molecules and are, therefore, too small to be seen in their native state (whey proteins, lactose, and minerals). A report on electron microscopy of α-lactalbumin and β-lactoglobulin was published by Schmidt and Buchheim [144].

Casein micelles are one of the most important components of milk which make it possible to produce a variety of milk products ranging from yoghurt to hard cheeses. They undergo visible changes under the effects of heat, proteolytic enzymes, and pH.

In fresh unheated milk, casein micelles are present in the form of globules, 100-300 nm in diameter (Fig. 1). They consist of submicelles [142], 10-20 nm in diameter, which are linked with each other by calcium phosphate bridges [109].

The fact that only casein micelles and fat globules may be seen in milk under the electron microscope was used as a basis for the detection of reconstituted dried milk in fluid milk [128]. The milk under study is centrifuged at a low speed and the pellet is freeze-fractured and replicated. The emphasis is on detecting undissolved milk powder particles or their parts such as characteristic aggregates of casein micelles and fat globules and not on preserving the original structure of the pellet. It is permissible, therefore, to impregnate the pellet with 30% glycerol as a cryoprotective agent before freeze-fracturing. Glycerol prevents ice crystal formation on freezing and the development of associated artifacts. Cryoprotective agents are not used when the original structure of a food product is examined.

Detection of buttermilk solids added to skim milk is another example where electron microscopy has proved to be useful. Skim milk solids are part of meat binders. Replacement, even partial, of the skim milk solids by buttermilk solids is undesirable. Qualitative composition of both products is similar and, therefore, chemical analysis of skim milk which is suspected of being adulterated with buttermilk could be difficult or even impossible. There is, however, an important structural difference between buttermilk and skim milk: fat globule membrane fragments resulting from the churning of cream are present only in buttermilk but not in skim milk. These membrane fragments make it possible to detect by electron microscopy as little as 15% of buttermilk solids added to skim milk, even if the milk solids are part of the meat binders. In this case, coarse constituents are removed from the binders by low-speed centrifugation; milk solids are isolated by subsequent preparative ultracentrifugation and the pellet obtained is embedded in a resin and sectioned [88] (Fig. 2).

A different kind of membrane may be found in milk which was subjected to severe foaming. The membrane develops at the air-serum interface in the form of an electron-dense layer, 5 nm thick, and casein micelles attach to it (Fig. 3). The air-serum interface consists of a mixture of globular whey proteins and soluble caseins [3, 16]. Electron microscopy has thus explained a phenomenon which had been observed as early as in 1923 by Hekma and Brouwer (mentioned in reference 16) who concluded that separator slime consisted mostly of collapsed milk foam. Association of casein micelles with collapsed milk foam was also demonstrated by Mulder and Walstra [122]. These findings have practical implications in that bubble ghosts resulting from foam collapse may be falsely detected by electronic counters as bacterial cells in milk [71].

Casein micelles consist of several caseins (αs-1, β-, and κ-casein) [68, 116]. Following several attempts to localize κ-casein using various methods [37, 73], κ-casein is now generally recognized to be concentrated at the surface of the casein micelles. Intact κ-casein and the presence of calcium phosphate have been shown to be essential for the integrity of the casein micelles.

Calcium phosphate may be removed from the casein micelles in various ways, either by sequestering calcium (e.g., by phosphates) or by dissolving calcium phosphate by acidification; as a result, the micelles disintegrate either partially or fully depending on the amount of calcium phosphate removed. The availability of casein micelle calcium for reaction with low-methoxyl pectin was used on a commercial scale several years ago as a base for one kind of instant pudding. Mixing of a fruit-flavoured low-methoxyl pectin jelly with milk produced the pudding. However, no pudding was formed when chocolate or cocoa powders were present in the pectin jelly prior to its reaction with milk. Electron microscopy of milk in which cocoa powder was dispersed showed partly disintegrated casein micelles. This disintegration evidently resulted from the sequestering of calcium in the micelles by phytin present in cocoa [80]. Preliminary addition of calcium gluconate or lactate as a compensation for calcium bound by phytin to the milk used to make the puddings preserved its ability to form gels with low-methoxyl pectin even in the presence of chocolate or cocoa.
Fig. 1. Casein micelles isolated from heated goat milk visualized by metal shadowing and TEM.

Fig. 3. Protein membranes (small arrows) develop in a milk foam at the air-serum interface to which casein micelles (large arrows) become attached (16). (Thin section. Courtesy of BE Brooker).

Fig. 2. Fat globule membrane fragments (arrows) found among casein micelles indicate that buttermilk solids were present in the sample (88). (Thin section).

Fig. 4. Casein micelles which had been heated above 85°C form chains on coagulation (93). (Rotary shadowing, TEM).
Changes detectable by electron microscopy in the structure and dimensions of casein micelles were recorded in ultra-high temperature short-time sterilized (UHTST) skim milk and sterile concentrated milk which gelled during storage [4, 65, 70, 131, 133, 139, 141]. "Tendrilis" or "thread-like tails" were reported to develop at casein micelle surfaces before the casein micelles aggregated into pairs or triplets and formed a gel. In gelled milk, the micelles formed a continuous three-dimensional network which consisted of interconnected chains.

In milk heated at a temperature above 85°C, β-lacto-globulin of the whey proteins interacts with κ-casein [165] and the resulting complex changes the topography of the casein micelle surface. In thin sections [4, 65], the casein micelles appear to have "tendrilis" or "hairs" but the freeze-fracturing [140, 146] and rotary shadowing [93] techniques show minute globular clusters rather than sharp "spikes" attached to the casein micelle surfaces. These findings have important implications for structure formation in products made from unheated or heated milk.

The unaltered surface of casein micelles in unheated milk makes it possible for the micelles to aggregate under the effects of proteases or acidification into large clusters. The formation of the β-lactoglobulin-κ-casein complex on the surfaces of casein micelles in heated milk leaves only a limited number of points at which the micelles can contact. The resulting aggregates are thus in the form of chains rather than clusters (Fig. 4). This finding is in agreement with the hypothesis by Knoop and Peters [106] postulating that denatured β-lactoglobulin either alone or in the complex with κ-casein in heated milk may inhibit the fusion of casein micelles.

Heating of milk at temperatures above 100°C did not confirm the hypothesis of Hostettler et al. [74] that casein micelles disintegrate into submicelles on heating and reaggregate on cooling. Heating at 130°C or 140°C induced the formation of appendages [121] on casein micelle surfaces in milk at pH below 6.7 but not above 7.0. Distortion and aggregation of casein micelles was also found in milk heated at 200°C for 3 min and reacted with glutaraldehyde (fixed) at this temperature [66].

In contrast to former assumptions that casein micelles aggregate on acidification, Heertje et al. [68] showed that the micelles start to disaggregate as pH of milk is decreased below 5.9. This disaggregation is the result of the solubilization of calcium phosphate. The release of calcium phosphate leads to the dissociation of weakly bound β- and κ-caseins but the αs1-casein matrix of the micelles apparently remains intact. Reassociation of the caseins starts as pH is further lowered to 5.2. At pH 4.8, β-casein become positively charged and reaggregate with the negatively charged αs1-casein.

Whey proteins cannot be visualized by electron microscopy as long as they are in their native state dissolved in the milk serum. Their coagulation, however, leads to the formation of aggregates which in most cases may be visualized under the electron microscope [11, 28, 61, 62, 76]. Depending on the ionic strength and protein concentration of β-lactoglobulin solutions, heating at 90°C causes no effect or induces the formation of gels or precipitates [60].

**Fig. 5.** Yoghurt matrix (63) consists of casein particle chains. (SEM).

**Fig. 6.** Mucogenic bacteria (arrows) in yoghurt prepared for conventional SEM appear to be attached by filaments to the protein matrix (150).

**Fig. 7.** Minute fat globules (arrows) resulting from homogenization become part of the protein structures in the milk coagulum (151). (Thin section).

**Fig. 8.** Pits (arrows) in the protein structures of a coagulum obtained from homogenized milk are the result of extracting fat from the samples in preparation for conventional SEM.

**Fig. 9.** Curd made from unheated skim milk consists of casein micelle clusters (93). (SEM).

**Fig. 10.** Fat globules (asterisks) in a rennet curd obtained by ultrafiltration of whole milk (45). Postfixation using osmium tetroxide preserved the fat globules for SEM examination.

Green et al. [54] examined heat-induced gels from whey protein powders and found that the tensile strength and impact force to fracture increased with increased β-lactoglobulin concentration and, as showed by SEM, with the compactness of the matrices.

Electron microscopy plays an important role in the studies of milk films developing on surfaces of the milk processing equipment [113] including ultrafiltration membranes [6, 7].

**Yoghurt**

A dairy product very popular in North America and Europe is produced by fermenting milk using bacteria. Initially an ethnic food, yoghurt is now produced widely in a variety of textures and flavours and is prized for its nutritional quality [12].

Yoghurt is made from milk that had been heated at a temperature above 85°C, held at this temperature for some time, and subsequently cooled to approximately 40°C; the milk is then inoculated with thermophilic lactic acid bacteria, particularly *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. These bacteria metabolize lactose and oxidize it to lactic acid, which lowers the pH of the mixture and turns it into a gel. Yoghurt was made traditionally from milk concentrated by boiling but now is made on commercial scale from partially or fully skimmed milk which may be fortified with milk solids; a smaller amount of yoghurt is produced from homogenized whole milk.

Electron microscopy shows that the protein matrix in yoghurt consists of casein micelles forming short interconnected chains [39, 94] (Fig. 5). Consequently, the liquid phase is immobilized in the interstitial spaces in the protein network, the dimensions of which depend on the casein concentration in the yoghurt milk: the higher the protein concentration, the smaller the interstitial spaces [63]. The structure of the network is one of the most
important factors which affect the susceptibility of the products to syneresis, i.e., separation of the liquid phase from the protein network. Modifications of the network structure are conveniently examined by electron microscopy.

Fortification of yoghurt milk with whey proteins markedly alters the structure of the finished product [119]. Casein micelles in the chains are bound to each other by some strains of bacterial cultures produce extracellular mucus or slime which increases the viscosity of yoghurt [14, 138, 150, 158]. The mucus consists of polysaccharides and binds water, thus reducing the need to use thickening agents and stabilizers in the yoghurt to achieve a similar water-binding effect. In micrographs of samples which had been dehydrated prior to electron microscopic examination, the mucus appears in the form of filaments (Fig. 6). This appearance is an artifact; since the polysaccharides cannot be fixed chemically, they shrink on drying and form filaments.

Straining of yoghurt reduces its moisture content. The resulting product is called labneh [151]. Yoghurt is strained through a cheese cloth in the traditional manufacturing procedure, but alternatively the total solids may be increased in labneh by ultrafiltering fresh yoghurt or by culturing milk having a high total solids content [151, 153]. The structure of labneh is denser than that of the initial yoghurt. Homogenization of the strained product breaks the initially coherent protein matrix into minute particles. Void spaces occupied by lactic acid bacteria in the initial yoghurt also break and the bacteria are distributed evenly in labneh [153]. Fat is not dispersed freely in this product made from whole homogenized milk but is part of the matrix in the form of minute globules associated with the agglomerated protein particles (Fig. 7) and increases the firmness of the product. Extraction of the fat from the samples as part of preparation for SEM produces minute pits in the protein structures (Fig. 8).

Curd

Coagulation of milk using rennet or other proteases of animal or microbial origin is the basis of cheesemaking. Fresh or pasteurized milk has traditionally been used for this purpose. The heat treatment of the milk during pasteurization, e.g., heating at 63°C for 30 min [120], does not affect the structure of the casein micelles because it is insufficient to induce the formation of the β-lactoglobulin – κ-casein complex. The micelles, thus, form large interconnected clusters (Fig. 9) instead of chains seen in yoghurt. Changes in the microstructure of curd during cheesemaking were also studied [49-51, 54, 56, 107].

Ultrafiltration (UF) of milk, which has been introduced into cheesemaking, markedly alters the composition of the starting material for the manufacture of curd. Milk retentates obtained by UF have higher protein and fat contents than milk whereas the concentrations of lactose and minerals are similar to those in milk or are lower; these latter constituents are particularly severely decreased if diafiltration was also used. (Diafiltration consists of adding water to the retentate followed by further ultrafiltration in order to reduce the contents of low-molecular mass components).

The increase in the concentration of protein reduces the mean free path of casein micelles from about 3 micelle diameters in the original milk to less than 1 micelle diameter in 4-fold concentrated milk [52, 143]. It is, therefore, conceivable that the retentates may behave in a different way from milk on coagulation.

Ultra-high-temperature (UHT) treatment of UF milk markedly affected the appearance of the casein micelles. Yousif et al. [163] suggested that either an irreversible denaturation of α-lactalbumin or an additional change in the structure of denatured β-lactoglobulin took place in milk which was heated to 123°-140°C. Aggregation of these proteins and submicellar casein on casein micelle surfaces markedly retarded rennet coagulation of the milk compared to unheated milk.

A study of curd obtained from homogenized and nonhomogenized UF milk retentates using rennet and proteases isolated from microorganisms showed a good correlation between firmness and microstructure: firm curd produced by rennet or Mucor miehei protease consisted of interconnected robust casein particle chains and clusters whereas a soft curd made by Bacillus polymixa protease showed smaller casein clusters and a less extensive branching [45].

Curd made from homogenized retentate was firmer than curd made from nonhomogenized retentate. Electron microscopy also explained this difference. Fat globules disintegrated into considerably smaller fat particles by homogenization. These fat particles deprived of their membranes reacted with proteins in the retentate and became an integral part of the protein matrix during coagulation. A similar development in labneh has been described in the preceding section. In nonhomogenized retentates, the original fat globules were dispersed as separate entities in the coagulum and as inclusions weakened rather than strengthened the curd structure. Using imidazole-buffered osmium tetroxide [2], the fat globules were preserved also for examination by conventional SEM (Fig. 10).

Milk coagulum that has been cut by wire knives into cubes and subsequently cooked, forms the base of Cottage cheese. The casein matrix of the coagulum expels whey and becomes compacted as the result of cooking. Microscopical examination of the curd granules showed their surface to be of irregular porosity but no signs of a "skin" which was believed to form on the granule surfaces was reported [46, 47, 81]. This hypothetical skin was considered to prevent rapid draining of whey from the curd granules in some cases of defective Cottage cheese. However, only good-quality Cottage cheese was the subject of the electron microscopic examinations. Cottage cheese suffering from various defects may probably produce different images.

A defect in Cottage cheese characterized by the development of a thick layer of sludge at the bottom of the vat was studied by Brooker [17]. The acidity of the curd failed to drop below pH 5.2 so that the curd-cutting stage was not reached. Electron microscopy showed that the lactic acid bacteria were infected by bacteriophage which markedly reduced their viability and production of lactic acid. The structure resembled a precipitate rather than a gel.
Electron Microscopy in Dairy Research

Coagulation of hot milk using glucono-δ-lactone or an acid such as citric or hydrochloric acids to pH 5.5 results in a curd which has a characteristic core-and-shell [58, 59, 64] ultrastructure of the protein particles (Fig. 11). This ultrastructure is characterized by compact cores surrounded by proteinaceous shells. The cores and the shells are separated by a void annular space 60-80 nm wide. Several conditions must be met [58, 59, 64] for the core-and-shell structure to develop: (a) the milk must be heated to a minimum of 85°C; (b) the presence of β-lactoglobulin, casein micelles, and the milk salt system is essential; (c) the final pH of the coagulum must be 5.5±0.1. These conditions are usually met in the production of White [90], whole-milk Ricotta [118], and Paneer cheeses [96].

The development of the core-and-shell structure is assumed to proceed in two stages. Firstly, the β-lactoglobulin–κ-casein complex appears in the form of "spikes" on casein micelle surfaces and then additional protein is aggregated at the ends of the spikes thus forming a continuous shell around the core. This ultrastructure is stable under a variety of conditions including frying [96] or cheese processing [35, 98].

Hard cheeses

There is a great variety of cheeses [110, 114] produced by the cheese industry. Electron microscopy has markedly contributed to the understanding of the structure in some of them [53, 54, 57, 79, 89, 105, 107, 108, 124, 130, 133, 157].

Traditionally, cheeses have been made from unheated milk. As has been mentioned above, the resulting coagulum obtained by rennet or another milk-curdling protease and a bacterial starter culture rapidly releases whey on cooking and forms curd granules. The curd is formed of casein whereas the whey proteins are lost in the whey. Heating of milk to >85°C would preserve the whey proteins in the curd but alters the properties of the gel in such a way that the release of whey is reduced. Experimental manufacture of cheese from heated milk and from ultrafiltered milk is already in progress [110, 115] but there are few structural studies [50, 54].

Curd granule junctions, which may be seen by a naked eye as characteristic patterns [112] in some cheeses, have been found to form by the fusion of granules, the superficial areas of which are depleted of fat [43] when the coagulated milk is cut with wire knives. The casein micelle clusters are then transformed into a uniform protein mass by pressing and ripening of the curd. Fat globules retain their membranes and can be seen as single entities or in the form of clusters interspersed in the protein matrix [108] (Fig. 12).

Various texturizing procedures are used in the manufacture of cheeses such as Mozzarella, Provolone, or Cheddar, which consequently attain fibrous structure. It results from mechanical elongation of the curd granules and is also reflected by a fibrous microstructure [108] (Fig. 13).

In low-fat Cheddar cheese, the contrast between the fat-free junctions and the low fat concentration in the granule interior is reduced making the junctions more difficult to distinguish. In cheese made from homogenized milk, the smaller: dimensions of the fat globules reduce the thickness of the zone depleted of fat at the granule surface and make the junctions thinner. Thus, in both instances, the curd granule junction patterns are not as easily noticeable as in regular cheese [43].

Brooker et al. [22] observed crystalline inclusions in ripening and mature Cheddar cheese which they identified as a variety of calcium salts, particularly phosphates and lactates. Their location in spaces between the fat and casein phases in the cheese suggests that they develop from the pockets of residual whey. Bottazzi et al. [13] found similar crystals in Grana cheese and confirmed the presence of calcium and phosphorus in them using X-ray microanalysis.

The lack of calf rennet has led to the use of various substitutes such as chicken, bovine, or porcine pepsin [41, 42, 149]. Cheddar cheese made with bovine or porcine

Fig. 11. Core-and-shell structure of casein particles in Paneer cheese (96). (Asterisk: Core. Arrow: Shell. Thin section).

Fig. 12. Casein micelles are transformed into a compact protein mass (asterisk) in young cheese (89), in which fat globules with their membranes (short arrows) are dispersed. (Long arrow: Bacterium. Thin section).
pepsin was more compact than cheese made with rennet. In addition, Eino et al. [42] concluded that the fibrous microstructure of the cheeses made with either pepsin vanished after 8 months of storage compared to cheese made with rennet.

Cheese used as an ingredient in pizza is exposed to high heat on baking. The heat treatment is more severe in a conventional than a microwave oven [125] but neither treatment reduced the nutritional quality of the cheeses as concluded from their amino acid analysis. Their microstructure, however, was altered by melting during baking. The changes were more easily noticeable in a high-fat cheese than in low-fat cheese. Least structural changes developed in a process cheese.

Mould-ripened cheeses

Moulds, i.e., fungi rather than bacteria are used to ripen soft cheeses. The moulds grow [55, 57] either inside the cheese (like in the blue-veined Blue, Gorgonzola, Roquefort, and Stilton cheeses) or on its surface (Camembert cheese). The hyphae of Penicillium camemberti, P. roqueforti, P. glaucum, or Mucor ramosus penetrate the protein matrix but the fruiting bodies called sporangia develop at surfaces (Fig. 14). The blue-veined cheese are porous on the macroscopical scale; the presence of pores containing air enhances the carbon dioxide-air exchange during mould growth and development of the sporangia. In the production of Blue cheese, air passages are bored into a salted curd made from milk which had been inoculated with P. roqueforti spores. Curd made from homogenized milk or cream recombined with skim milk is usually more porous than curd made from non-homogenized milk and is, therefore, particularly useful in the manufacture of Blue cheese [100, 101]. The mould grows in the pores and produces the blue veination.

Fig. 13. Fibrous structure of a stretched Mozzarella cheese. (Asterisks: Void spaces originally occupied by fat before the sample is prepared for conventional SEM).

Fig. 14. Fruiting bodies of Penicillium camemberti develop at the surface of Camembert cheese. (Cryo-SEM. Courtesy of P Allan-Wojtas).
Electron Microscopy in Dairy Research

In Camembert cheese, *P. camemberti* spores germinate on the cheese surface and penetrate the curd whereas a thick white mat of sporangia develops on the cheese surface [129]. During ripening, calcium phosphate becomes concentrated near the cheese surface. Brooker [18] based his explanation of this phenomenon on electron microscopic observation and X-ray microanalysis: deamination of amino acids by the mould at the surface increases pH of the cheese and results in the precipitation of calcium phosphate from the aqueous phase. The precipitation of calcium and phosphate results in the development of a gradient between the surface and the interior of the cheese which is followed by migration of both ions to the surface and their precipitation. Thus, the gradient operates like a pump which concentrates calcium phosphate at the cheese surface.

**Cream cheese**

Cream cheese is a spreadable soft cheese which differs structurally from the other cheeses mentioned by the lack of a compact protein matrix and a relatively high (up to 55%) moisture content. The cheese is made from cream and its major structural component is fat (~33%). Depending on the manufacturing procedure, Cream cheese consists of fat globule clusters interspersed with milk proteins. This corpuscular structure contributes to spreadability of the product.

The traditional manufacturing procedure involves coagulation of cream, heating the curd, and draining the whey. Changing the formulation of the raw materials by increasing the total solids content of the cream mix before culturing makes it possible to avoid draining of the resulting Cream cheese. In both cases, the casein and fat present in cream are coagulated and processed together at the same time.

The most recent development in Cream cheese spread manufacture [120] is homogenization of a mixture of high-fat (60-70%) cultured cream with coagulated milk proteins such as Ricotta or White cheese [118]. If the curd used had been obtained by acid-induced coagulation of milk at 90°C, the resulting cream cheese spreads show the characteristic core-and-shell structure [90] already mentioned in the section on milk. Milk curd may be replaced in these spreads by acid-heat-coagulated whey proteins [91].

Differences in Cream cheese manufacturing processes are reflected in the structures [92] of the products. In traditional Cream cheese, fat globule clusters are coated with protein (Fig. 15) or the protein is embedded in the fat globule clusters. In contrast, in a newly formulated product, fat is present mostly in the form of large particles and protein is aggregated around minute fat droplets.

SEM was used to explain the origin of minute hard particles which caused grittiness [120] in a low-fat cheese spread made by homogenizing mixtures of curd (85%), high-fat cream (10%), and sugar (5%). It was deduced from their microstructure, which consisted of compacted protein and contained lactic acid bacteria (Fig. 16), that the curd had been made from insufficiently heated milk and contained many casein micelle clusters common to cheese curd rather than chains. These clusters are known to be susceptible to collapse and release of whey, which is important in flashf
cheese manufacture. Grittiness was avoided by increasing the heat treatment of the milk used to make the curd.

Cream cheese having a low fat content may be made either as Neufchâtel cheese (20% fat and 65% moisture) or low-fat dairy imitation Cream cheese (12.5% fat) made from a mixture of regular traditional Cream cheese and Cottage cheese. The microstructures of both products differ correspondingly: Neufchâtel cheese is uniform whereas in the Cream cheese-Cottage cheese mixture, the high resolution of electron microscopy makes it possible to distinguish between the components [92]. However, the differences in the organization of the fat-protein complexes can be noticed only in the interior of such complexes. The thin-sectioning or freeze-fracturing and replication modes of transmission electron microscopy (TEM) are, therefore, the only methods suitable for this purpose.

**Process cheese**

Processing was initially invented in order to utilize (recycle) natural cheese which would otherwise be difficult to sell. Shredding it and melting with calcium-sequestering salts such as sodium citrate or phosphates yielded a new product which could be formed in various shapes, filled with various ingredients such as vegetables, spices, or meats. In addition, a variety of other milk solids such as milk and whey powders may also be included in process cheese.

Processing changes the microstructure of natural cheeses and results in the development of a new microstructure (Fig. 17) having characteristic features [35, 36, 67, 95, 104, 126, 127, 137, 152, 154].

![Fig. 17. SEM shows fat globules in the process of emulsification (asterisks), imprints of melting salt crystals (small arrows), and calcium phosphate crystals (large arrows) in a particular sample of process cheese (95).](image)

![Fig. 18. Presence of the core-and-shell structure (arrow for the shell and asterisk for the core) in process cheese indicates that acid-coagulated White cheese was used as an ingredient in the cheese blend (98). (Arrowheads: Fat particles. Thin section).](image)

Curd granule junctions disappear and fat particles become reemulsified; during this process, the original fat globule membranes disintegrate and new membranes are formed from the cheese proteins on the fat particle surfaces. Crystals of the melting salts used to restore the emulsification capability of cheese proteins become dissolved during processing but their remains may be still visible in the finished product (Fig. 17). Restoration of the emulsifying capability is achieved by sequestering calcium from the calcium-caseinate complex using the melting salts which consist mostly of sodium citrate or various sodium phosphates. Consequently, insoluble calcium salt crystals develop in the process cheese.

Electron microscopy makes it possible to register differences in the ultrastructure of the protein matrices in various process cheeses. Thus, some
process cheeses were found to contain minute protein strands [35, 67] whereas some other cheeses consist of protein in the form of minute individual particles. In the opinion of Taneya et al. [154], the strands contribute to the retention of the shape of the process cheese upon heating.

Excessive heating increases viscosity of melted process cheese to the point that the cheese hardens. This hardening is accompanied by the development of osmiophilic areas [95] of submicroscopic dimensions in the process cheese. Using freeze-fracturing, Klostermeyer and Buchheim [104] found compacted protein in heated process cheese made with no creaming (melting time of 4 min). The dimensions of these compact areas were reduced with mild creaming and were absent in cheese made with optimal creaming (melting time of 9 min).

Effects of partially replacing natural cheese with other dairy ingredients such as a cheese base made from reconstituted skim milk powder or unripened White cheese on some properties of the resulting process cheese were also studied and electron micrographs were obtained. The cheese base treated with added protease had an open structure compared to the compact structure of the untreated base [152], but the microstructures of the resulting process cheeses, which contained up to 42% of either cheese base, were similar.

White cheese prepared by coagulating milk at 90°C with citric acid was added to the process cheese blends in amounts of up to 33%. When prepared at pH 5.5, the White cheese has a unique core-and-shell structure as was already mentioned earlier. This structure is stable during processing and, thus, the presence of White cheese in process cheese may be detected using electron microscopy [98] (Fig. 18).

Total replacement of ripened cheese with rennet or acid caseins [137] resulted in acceptable process cheeses. However, there were marked differences in meltability between process cheeses made using rennet or acid caseins depending on the melting salts used. The relationship between meltability and microstructure found agrees with that of Rayan et al. [127]; a more advanced emulsification resulted in poorer meltability.

**Cream**

Cream is a milk product consisting of concentrated fat globules and contains, in addition, all other milk components. Being buoyant in milk, the fat globules are concentrated by centrifugation. Because of its nature, cream in its various forms including
whipped cream and ice cream is most conveniently studied by TEM using the freeze-fracturing and replication procedure [25, 30] or by cryo-SEM [23, 30, 145]. The structure of fat globules has been extensively reviewed [26, 27, 122, 161].

Over 90% of all fat globules in cow's milk [122] have diameters between 1 and 8 μm. The surface of the globules is smooth in fresh milk. It is formed by the fat globule membrane which is susceptible to structural changes on cold storage and mechanical action. Heating leads to deposition of aggregated protein on the fat globule surfaces.

Homogenization breaks the fat globules into smaller droplets, the combined surface of which is 5 to 6-fold as large as the original fat globule surface. This newly formed fat surface becomes coated with surface-active proteins from the milk serum [38]. Interactions of this kind may lead to the formation of fat-protein complexes which would sediment rather than float on centrifugation. Products made from homogenized milk may have the minute fat droplets encapsulated in the protein matrix as was mentioned above in the section on labneh.

Homogenized cream with 10-20% milkfat is a popular coffee whitener, particularly in Europe. This coffee cream should disperse evenly in hot coffee irrespective of the brewing conditions and the temperature of the coffee. Coagulation of the cream in contact with the hot coffee results in a defect called feathering. Electron microscopy showed that fat globule clusters were formed in the defective cream as the result of instability of the milk proteins present [30]. Heat-denaturable whey proteins were found in the interfacial layers and also casein micelles were found in the form of aggregates. The resistance of coffee cream to feathering may be improved by increasing the heat stability of the milk proteins and avoiding fat globule clustering. Addition of trisodium citrate was found to be one of the remedies [27].

Whipping turns cream into whipped cream. It is a foam which consists of 3 phases: water, fat, and air. Because of the delicate balance of all three components, only rapid freezing is able to fix whipped cream for electron microscopic examination. Using cryo-SEM, Schmidt and van Hooydonk [145] observed a decrease in the dimensions of air cells in whipped cream during whipping until maximum foam strength was reached. The air cells were smaller in whipped cream made from homogenized cream, where also the fat globules were smaller than in nonhomogenized cream. Overwhipping leads to the disruption of the fat globule membranes and partial coalescence of the fat globules. As overwhipping progresses, butter grains are formed, air cells disappear, and the foam collapses. The proportion of ruptured fat globule membranes is higher in the case of homogenized cream, where the transition from optimal whipping to overwhipping is less clearly noticeable. Considerably longer time is required to reach maximum strength by whipping homogenized than nonhomogenized cream. In well-whipped cream, the fat globules protrude into the air cells [23, 27, 111, 145] (Fig. 19).

The structure of ice cream is further complicated by the presence of other ingredients such as sugar, emulsifiers, fruits and, in particular, ice crystals compared to whipped cream; ice cream contains 40-50% of air by volume. Emulsifiers are an important ingredient. In their absence, fat globules in the ice cream mix would be coated with thick layers of aggregated protein which would prevent them from adsorption at the air cells. Emulsifiers remove this obstacle [27]. Their effects on the structure and other properties of ice cream were reviewed by Berger [9]. Electron microscopy has played an important role in the studies of ice cream structure, particularly concerning the introduction of new ingredients such as vegetable oils and various emulsifiers [10, 33, 48].

At -5.5°C, about one half of the water present in the ice cream is present in the form of ice crystals and the rest is liquid water. As the temperature is decreased, this proportion changes and at -10°C over 70% of the water is frozen. Dimensions of the ice crystals (Fig. 20) markedly affect the texture of ice cream: in smooth ice cream, they are less than 20 μm long [9].

**Milk powders**

Milk is dried for several reasons [34] such as preserving milk for the time of lower supply or for use in a variety of products.

There used to be two different milk drying processes: roller-drying and spray-drying. Each of them produces milk powders in a different form.

Roller-dried powders develop by drying a thin layer of preconcentrated milk on the hot surface of a drum. The resulting thin sheet of dried milk is scraped off from the drum and is pulverized in a hammer mill; this procedure yields powder particles having irregular shapes and sharp edges (Fig. 21). The contact of milk proteins with the hot drum surface leads to an extensive denaturation of the whey proteins and reduces the solubility of the powders on reconstitution of the milk. Now, this type of powder is used almost exclusively only in bakery products and roller-drying is being replaced by spray-drying.

Spray-dried powder is obtained by dispersing (atomizing) preconcentrated milk into droplets 10 to 250 μm in diameter and drying them in a stream of hot air. The process takes place in a spray-drying chamber. The temperature of the inlet air may be as high as 195°C and the temperature of the outlet air may vary from 70° to 105°C.

The earliest electron microscopical studies of milk powders [40, 123] showed the distribution of casein micelles and fat globules in milk powder particles.

---

Fig. 21. Roller-dried milk powder particles have sharp edges and irregular shapes (34). (SEM).

Fig. 22. Spray-dried buttermilk: smaller particles (arrow) embedded in larger particles are surrounded by rims (34). (SEM).

Fig. 23. Spray-dried ultrafiltration milk retentate (97): large particles are dimpled (arrows). (SEM).

Fig. 24. Instant milk powders consist of agglomerated primary particles (34). (SEM).

Fig. 25. Spray-dried permeates (99) obtained from the ultrafiltration of milk consist of globular (asterisks) and crystal-like particles (arrows). (SEM).
Buma [31] and Buma & Henstra [32] completed the most comprehensive study of spray-dried milk structure by SEM and explained many phenomena taking place in dried milk such as the particle structure and size distribution, fat globule size distribution in the powder particles, and the occurrence of fat in four forms in the powder particles. Buma's findings [31] were confirmed by Buchheim [24], who dispersed milk powder particles in polyethylene glycol, freeze-fractured the resulting suspensions in liquid nitrogen, replicated the fractures with platinum and carbon, and examined the replicas by TEM. This procedure may be used to study the structure and size distribution of the milk droplets during drying provided that the samples may be obtained in the form of a suspension in a medium which does not alter them.

Electron micrographs may help to explain some of the processes which occur in the spray-drying chamber. In some powders such as spray-dried buttermilk, small particles surrounded by rims were observed on the surfaces of larger particles whereas shallow depressions in particles surfaces were observed in spray-dried retentates obtained from the ultrafiltration of milk [97]. These differences have been explained [87] as follows: small droplets dry and solidify sooner than the larger droplets. If these small droplets collide with the larger particles while these latter particles are still viscous, the small particles become embedded in the larger particles and the impact forms the rim around the small particles (Fig. 22). If, however, the larger particles are no more sticky but are not yet hard, the collisions with the small hard particles result in the formation of depressions (Fig. 23) while the small particles rebound after the collisions.

Most powder particles contain air in the form of vacuoles. Their development and prevention were studied by Verhey [159, 160].

Spray-dried milk powders dissolve in water with difficulty; they foam and form lumps. This problem has been solved by instantization of the powders. It consists of agglomerating the primary powder particles (Fig. 24) by controlled crystallization of amorphous lactose and formation of α-monohydrate lactose crystals.

The effects of a humid atmosphere on crystallization of amorphous lactose in spray-dried whey powder were studied by Saltmarch and Labuzza [136] using SEM. At 40% relative humidity (atmosphere over a saturated zinc nitrate solution), lactose in the whey powder crystallized after one week storage at 25°C. Crystallization of lactose in milk and whey powders was recently studied using electron microscopy by Saito [134, 135].

Permeates obtained from the ultrafiltration of milk have a very high lactose concentration of 84-90% in the total solids but also contain relatively high concentrations of mineral salts (7.5-8.1%), the salt concentration in the total solids may be reduced by electrodialysis [117] to 0.5%. The spray-dried permeates consist of globular and crystal-like particles (Fig. 25), the latter evidently being the result of preliminary crystallization of lactose prior to spray-drying. Demineralization reduces the proportion of the globular particles, which were shown by X-ray microanalysis to consist of a mixture of minerals and very small lactose crystals in contrast to the crystal-like particles which are almost pure lactose [99].

Electron microscopy was also used to characterize the structures of spray-dried milk and other milk products such as cheeses (Cheddar, Blue, Provolone, Swiss, Parmesran, and Romano), casein, whey and whey protein concentrates [103]. This study provides data which may be important if there is a suspicion that a certain spray-dried milk powder had been adulterated with a lower price product. In studies of the properties of spray-dried, roller-dried, and freeze-dried heat-coagulated whey proteins [77], the water-holding capacity was highest in the freeze-dried product; numerous ice crystals which developed in the whey proteins during freezing left the product porous after freeze-drying; in contrast, a compact superficial shell which developed in roller-dried particles markedly reduced the rate of their hydration.

A review on milk powder structure [34] was published recently.

Nontraditional dairy products
There are many new food products being developed for the market by the dairy industry. They are of particular interest to the electron microscopists because they make it possible to compare their structures and other properties with other traditional products. However, products which are new to one part of the world may have been considered traditional elsewhere. Yoghurt [93] from the Balkans and whey-based cheeses [75] from Scandinavia are two such examples. Regulations concerning new foods and dietetic traditions, both varying from country to country, markedly affect the introduction of new foods on the market. Whey-based drinks and cheeses, cheeses and process cheeses in which milk fat has been replaced by vegetable oils [54] and casein micelles have been replaced by caseinate [8] or soy proteins [156, 162], process cheeses made from ingredients other than ripened natural cheese [137, 152], whippedable emulsions containing vegetable oils [29], low-fat butter spreads [27, 44], whey protein stabilized oil-in-water emulsions as egg white substitutes [78], coffee whiteners [27] (Fig. 26), cream liqueurs [27] (Fig. 27), or fat substitutes based on whey proteins or casein micelles wrapped in denatured egg white [147, 148] may be considered as a few examples of nontraditional foods developed in North America and Europe. Cream cheese spreads obtained by homogenizing rennet or acid casein curd with high-fat cream may also be considered to be new products. These foods are nontraditional either because they contain unusual ingredients or because they are made by nontraditional processes.

The microstructure of a low-fat (37%) dairy spread [44] developed in 1979 resembles low-fat Cream cheese and also consists of clusters containing protein and fat. In contrast, a completely new microstructure was produced by Singer and Dunn [147]. These authors noticed that particles similar in dimensions to milkfat globules, i.e., smaller than 3 μm in diameter, are perceived as cream in the mouth.

A microparticulation process patented earlier [148] was used to make a product consisting of casein micelles or whey proteins wrapped in denatured egg white. These
aggregates have a mean diameter of approximately 1.5 \mu m and successfully imitate fat. The patented product called Simplesse has been used as a fat substitute in products such as frozen desserts, salad dressings, cheeses, and cheese cakes, i.e., in applications which don't involve baking or frying. The microparticulation process was found to be safe in food production [5]. Similar sensory attributes were obtained [148] for an ice cream made with Simplesse (fat content <1\%) and a super premium ice cream (16\% fat) but the electron microscopy images were markedly different. However, they had one aspect in common and that is similar dimensions of the fat globules in the super premium ice cream mix (Fig. 28) and the protein aggregates in the fat-free ice cream (Fig. 29).

Fig. 26. In a coffee whitener, vegetable fat droplets of uniform dimensions (arrows) are dispersed in a sodium caseinate base (27). (Freeze-fracturing, replication, TEM. Courtesy of W Buchheim).

Fig. 27. Cream liqueurs are dairy emulsions in which the fat droplets (arrows) are very small (27). (Freeze-fracturing, replication, TEM. Courtesy of W Buchheim).

Fig. 28. A super premium ice cream mix (147) contains fat globules \( \sim 1 \mu m \) in diameter (arrows). (TEM. Courtesy of NS Singer).

Fig. 29. Milk and egg white protein aggregates (arrows), \( \sim 1 \mu m \) in diameter, replace the fat globules in a fat-free ice cream mix containing the Simplesse fat substitute (147). (TEM. Courtesy of NS Singer).
Conclusions

Examples presented in this review have demonstrated the useful role which electron microscopy plays in dairy research. It may be anticipated that the interest in the structure of the products will further increase, particularly with respect to the replacement of fat with newly developed fat substitutes. Microscopical analysis of frozen foods and microwavable foods will also be extended. Although several analytical procedures different from microscopy may be used to characterize other aspects of the milk products under study, electron microscopy shows their three-dimensional structure and the ultrastructure of the ingredients at a high resolution which will be surpassed only by atomic force microscopy. Data from X-ray microanalysis [18-20, 99] which provide information about elemental composition of the sample on a submicroscopical scale and digital image analysis data [132] on quantitative evaluation of the micrographs complement electron microscopic examinations. Jointly with other techniques such as confocal scanning laser microscopy [69] and fluorescence microscopy [69] and fluorescence microscopy [164], electron microscopy is an important tool in dairy research.

Acknowledgments

The author thanks Dr. B. E. Brooker, Dr. W. Buchheim, Miss K. B. Caldwell, and Dr. N. Singer for providing micrographs to illustrate this review, Dr. D. H. Goff and Dr. H. W. Modler for useful suggestions, and Miss Gisèle Larocque for skillful technical assistance. Electron Microscopy Unit, Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 91-25 from the Centre for Food and Animal Research.

References

Electron Microscopy in Dairy Research


Electron Microscopy in Dairy Research


131. Ruegg M, Blanc B. (1978). Influence of pasteurization and UHT processing upon the size distribution of


