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Changes in the Ultrastructure of Emulsions as a Result of Electron Microscopy Preparation Procedures

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Abstract

Various methods of preparing emulsions for electron microscopy were examined with peanut oil/protein and ice cream mix emulsions. For transmission electron microscopy (TEM), fresh peanut oil/bovine serum albumin emulsions were mixed with 2% agar, fixed in phosphate-buffered (pH 7.0) 4% glutaraldehyde solution and postfixed in phosphate-buffered (pH 7.0) 1% osmium tetroxide; alternatively, the glutaraldehyde-fixed samples were briefly rinsed in acetone prior to postfixation. Both preparations yielded satisfactory fat globule preservation. Similar emulsions were prepared on loops and suspended over vapors of 25% glutaraldehyde and 1% osmium tetroxide. This preparation resulted in angular fat globules surrounded by a heavy protein precipitate.

Ice cream mix emulsions were prepared for TEM study by mixing with 4% agar, mixing with 2% agarose or using agar tubes. After fixation in phosphate-buffered 4% glutaraldehyde (pH 7.0) solution, the samples were postfixed in either phosphate/imidazole buffered (pH 7.0) or phosphate-buffered (pH 7.0) 1% osmium tetroxide. Mixing with 2% agarose and postfixing in imidazole/phosphate buffered osmium tetroxide yielded the best results. A clearly visible fat membrane and well-delineated fat crystals were observed.

Scanning electron microscope (SEM) studies of peanut oil/casein emulsions mixed with 4% agar yielded good results whereas in ice cream mix emulsions, the results were inconclusive.

Introduction

There has been a great deal of interest in the microstructure of food emulsions in recent years but because of variations in emulsion properties, standard preparation methods for electron microscopy study may have to be modified. It is well known that changes in preparation procedures for electron microscopy study can alter the specimen (Hayat, 1970; Ericsson et al. 1965; Sjostrand, 1967). Chabot et al. (1979) discussed these effects on the ultrastructure of white bread; Khoo et al. (1975) experimented with vapor fixation of dough in order to eliminate artifacts caused by dehydration but discarded this method as unsatisfactory and adopted a more standard fixation method. Carroll et al. (1968) found that glutaraldehyde preserved micelle structure better than osmium tetroxide or formaldehyde. Parnell-Clunies et al. (1986) noted that electron dense particles were not observed when yoghurt was fixed in glutaraldehyde alone whereas the addition of osmium tetroxide as a postfixative in conjunction with glutaraldehyde as a primary fixative resulted in the appearance of dense particles. In a study of muscle tissue, Colquhoun and Rieder (1980) found that the relatively minor change of initiating the dehydration step at 75% ethanol rather than 30% ethanol caused an increase in contrast of EM images in phosphate buffer samples whereas cacodylate buffer specimens were unaffected.

Fluid multiphase samples, such as peanut oil/protein or ice cream mix emulsions present additional difficulties because they cannot be prepared as simply as semi-solid or solid samples. The use of freeze-etch (Buchheim and Precht, 1979; Buchheim, 1974) and cold stage SEM (Kalab, 1985) to study liquid samples may not provide the type of information needed or simply may not be available. In addition, some specimens are better preserved using the traditional critical point drying method rather than the cold stage for SEM (Kalab et al., 1986). The purpose of this research was to evaluate different electron microscopy preparation procedures for studying the ultrastructure of emulsions.
Specimen changes that occurred using peanut oil/protein and ice cream mix emulsions were examined.

Materials and Methods

TEM - Peanut Oil/Bovine Serum Albumin (BSA)

Peanut oil/BSA emulsions were prepared as described by Haque and Kinsella (1988). The fresh emulsions were mixed with warm 2% agar (3 parts emulsion:1 part agar), mixed gently, with a wooden applicator stick and allowed to set. The resultant gel was cut into 1mm³ pieces and fixed in 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) overnight. After several brief rinses in phosphate buffer, the samples were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.0) for 2h or rinsed briefly in acetone between the glutaraldehyde and osmium step, dehydrated in a graded series of acetone, embedded in Spurr resin and polymerized in a vacuum oven overnight at 70°C.

Loops were prepared as follows: Wire loops approximately 2 mm in diameter were fashioned from nichrome wire. The loops were dipped into the peanut oil/BSA emulsions causing the formation of an emulsion droplet within the loop by virtue of surface tension. The emulsions were then suspended over vapors of 25% glutaraldehyde for 1/2 h followed by suspension over 1% osmium tetroxide vapors overnight. After this process, the samples were brittle enough to be removed from the loop, dehydrated and embedded as described above. In some cases, the droplet did not retain its integrity within the loop and there was no material left to embed.

TEM - Ice Cream Mix

Ice cream mixes were prepared as follows: 10% milkfat, 11% milk solids not fat, 10% sucrose, and 5% corn syrup solids. Fresh cream, skim milk, and nonfat dry milk were used as the sources of milk solids. The 8kg mixes were blended, pasteurized at 74°C for 30 minutes, homogenized at 17.2 MPa (2500 psi), 3.6 MPa (500 psi) second stage, cooled to 5°C, and aged 2h. Following aging, ice cream mix emulsions were warmed to 15°C and combined with either 2% agarose (22°C), an ultra low gelling temperature agarose derived from agar (Sea Prep Agarose, FMC Marine Colloids Div., Rockland, ME) which remains liquid at room temperature if stirred constantly or with warm 4% agar in the proportion of 3 parts sample:1 part agar or agarose. The 2% agarose was prepared as follows: Deionized, distilled water was heated to 95°C. The agarose was added slowly until it dissolved while the water was constantly stirred. The agarose solution was allowed to cool to 22°C with constant stirring. If stirring was stopped, the solution became quite viscous. The samples were gently mixed with a wooden applicator stick and allowed to gel overnight at 4°C.

Agar microtubes were prepared as follows: the flame sealed thin portion of a Pasteur pipette was repeatedly dipped into warm 4% agar and allowed to harden until a thick film had adsorbed, forming an agar tube. After cutting the distal end of the tube with a razor blade, the emulsion was drawn into the tube by dipping the pipette into the emulsion and partially withdrawing the pipette. The agar tube containing some of the emulsion was placed on a glass slide and the pipette was removed. After trimming and sealing the ends of the agar tube with warm agar, the tube was momentarily dipped in warm 4% agar to complete sealing and prevent leakage. The tube was then allowed to gel. This method is a variation of the one described by Allan-Wojtjes and Kalab (1986b). The three sample types were cut into approximately 1 mm³ pieces and fixed in 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) overnight.

In addition, the 2% agarose mixtures were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.0). The samples were briefly rinsed in several changes of 0.1M phosphate buffer (pH 7.0) and postfixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.0) (1:1, v/v) 1h. The agar mixtures were also postfixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.0). After dehydration in a graded series of ethanol, the samples were embedded in Spurr resin and polymerized in a vacuum oven at 70°C.

Sectioning was done on a Sorval Portermo ultramicrotome. The sections were picked up on carbon-coated Formvar grids, stained with uranyl acetate followed by Reynolds's lead citrate and examined with a Phillips 300 TEM at 80 kV.

SEM - Peanut Oil/Casein

Peanut oil/casein emulsions were prepared as described by Haque and Kinsella (1988). They were mixed with 4% warm agar. A spatula was used to lift the gelled emulsion, causing several free fractures and exposing fresh surfaces for examination. The samples were treated as described in the TEM section (peanut oil/BSA) until the dehydration step. The SEM samples were dehydrated in a graded series of ethanol and critical point dried in a Tousimis Auto-Dri critical point drier. The fresh surfaces of the samples were oriented for viewing, mounted on aluminum stubs covered with double sticky tape, and coated with gold/palladium in a Balzers Union Sputter Coater.

SEM - Ice Cream Mix

Ice cream mix emulsions mixed with 4% agar, 2% agarose or encapsulated in agar tube were prepared as described in the TEM section (ice cream mix) up to and including the ethanol dehydration step. Fresh surfaces of the 4% agar and 2% agarose mixtures were obtained as described in SEM section (peanut oil/casein). The agar tube samples were sliced lengthwise with a razor blade after critical point drying. Specimen samples were taken from areas untouched by the razor blade. Samples near the center of the tube rather than the edge were selected in order to avoid the areas most exposed to heat. The samples were critical point dried in a Tousimis Auto-Dri critical point drier, mounted on aluminum stubs covered with double-sticky tape.
and coated with gold/palladium in a Balzer's Union sputter coater.
All SEM samples were examined with an AMRay 1000 SEM at 10 kV.

Results and Discussion

The first emulsions studied, peanut oil/BSA, were mixed with warm 2% agar, fixed in 4% glutaraldehyde and postfixed in 1% osmium tetroxide. This preparation resulted in fat globules that were well-preserved, regular in shape, and surrounded by a fine, evenly distributed protein precipitate (Figure 1).

Figure 1. TEM micrograph of peanut oil/BSA emulsion mixed with agar. No acetone treatment. F = well-preserved fat globule. FP = fine protein precipitate. Bar = 1 μm.

The same emulsions were prepared as described above except for a brief acetone rinse between the glutaraldehyde and osmium step. This step was included as a result of the observation by Henstra and Schmidt (1970) that in milk, the saturated fat which is not fixed with osmium tetroxide was extracted during the dehydration, leaving an empty space surrounded by a well-stained fat globule membrane. Since peanut oil is composed of unsaturated fat which is well fixed with osmium tetroxide, a brief acetone rinse before the osmium step was tested in order to extract the unsaturated fat, possibly enhancing TEM visualization of the protein film at the lipid interface. The acetone treated emulsion also yielded well-preserved fat globules which had a regular shape, but the protein precipitate surrounding the globules was denser and less homogeneous in size. The distribution of the precipitate around the globules was uneven (Figure 2). The fat globules in the acetone treated samples did not appear to be less densely stained than those in the non-acetone treated samples, suggesting that the acetone rinse may have been too brief to extract the fat.

Figure 2. TEM micrograph of peanut oil/BSA emulsion mixed with agar. Acetone treatment. F = globule. P = protein precipitate. Bar = 1 μm.

Loops were tested in order to avoid the sample dilution and agar network visualization associated with the above methods as noted by Kalab (1981). Loop preparation yielded fat globules with an angular shape surrounded by very dense protein precipitates (Figure 3). It was possible that surface tension changes within the emulsion suspended in the loop caused angular distortion of the fat globules. Brooker (1985) and Brooker et al. (1986), working on milk foams and whipped cream suspended in loops over fixative vapors, obtained excellent results. However in this study, the loop method was time consuming, tedious and did not always yield usable material since the emulsion droplet sometimes fell out of the loop after a period of time.

Figure 3. TEM micrograph of peanut oil/BSA emulsion mixed with agar. Loop method. F = well-preserved fat globule with angular shape. DP = dense protein precipitate. Bar = 1 μm.
It was concluded that the simple procedure of mixing the emulsions with agar (or agarose discussed below) without acetone treatment gave the most satisfactory results.

SEM samples of peanut oil/casein emulsions mixed with warm 4% agar, fixed in 4% glutaraldehyde and postfixed in 1% osmium tetroxide yielded well-preserved fat globules with round, regular shapes. Size distribution was well-illustrated (Figure 4). Occasionally an agar film covered the emulsion, but with careful scanning of the field it was possible to find an unobstructed area (Figure 5).

Since the micrographs of peanut oil/protein emulsions mixed with agar were satisfactory, the first samples of ice cream mix emulsions were prepared using the same method (agar mix, 4% glutaraldehyde fixation, 1% osmium tetroxide post-fixation). However, since saturated fatty acids were a major component in the ice cream mix samples, osmium tetroxide was buffered in phosphate/imidazole in addition to phosphate buffer alone. Angermüller and Fahimi (1982) found that imidazole-buffered osmium tetroxide stained lipid droplets more intensely than aqueous or cacodylate buffered osmium tetroxide. They suggested that unsaturated fatty acids, particularly linolenic, oleic and linoleic acid reacted intensely with imidazole-buffered osmium tetroxide. Allan-Wojtas and Košelj (1984a) found that the liquid matrix of the fat globule assumed to be composed of unsaturated fatty acids such as oleic and linoleic acid were deeply stained when postfixed with osmium tetroxide in veronal-acetate/imidazole buffer. The lightly stained fat crystals composed of saturated fatty acids contrasted sharply with the well-stained unsaturated fatty acids.
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Figure 8. TEM micrograph of ice cream mix emulsion prepared in agar microtubes illustrating fat globule distortion, clearly evident fat globule membrane and melted fat crystals. F = fat globule, M = fat globule membrane, C = melted fat crystals. Bar = 1 μm.

Figure 9. TEM micrograph of ice cream mix emulsion mixed with agarose fixed in 4% glutaraldehyde illustrating well-preserved fat globule, clearly evident fat globule membrane and well-delineated fat crystals. F = fat globule, M = fat globule membrane, C = fat crystals. Bar = 1 μm.

Figure 10. TEM micrograph of ice cream mix emulsion mixed with agarose fixed in 2% glutaraldehyde illustrating well-preserved fat globule, clearly evident fat globule membrane and well-delineated fat crystals. C = fat crystal adjacent to distorted membrane. F = fat globule, M = fat globule membrane. Bar = 1 μm.

Figure 11. Light micrograph under oil immersion of diluted ice cream mix emulsion in depression slide. Arrow indicates a distorted area in the fat globule. Bar = 10 μm.

Ice cream mix emulsions postfixed in phosphate-buffered osmium tetroxide contained fat globules that appeared distorted and disrupted. There were no visible fat globule membranes and no fat crystals were evident (Figure 6). The emulsions postfixed in phosphate/imidazole buffered osmium tetroxide contained fat globules whose shape appeared somewhat less disrupted but the fat globule membranes were clearly visible (Figure 7).

It was concluded that the presence of imidazole in phosphate-buffered osmium tetroxide improved the image of the fat globule membrane (Figure 6 vs. Figure 7). It appeared that unsaturated fatty acids present in the fat globule membrane reacted with the imidazole, producing a well-stained membrane. A similar reaction was noted between the fat globule matrix of yoghurt and imidazole (Allan-Wojtas and Kalab, 1984a; Kalab, 1985) discussed earlier in this section. In addition, this reaction may have resulted in a less delicate membrane. This could have contributed to fat globule stability causing less disruption to globular shape (Figure 6 vs. Figure 7).

In order to improve on these results, a number of different procedures were studied. The ice cream mix emulsions were prepared in agar tubes. This procedure drastically reduced the amount of time the samples were in contact with heat, which could cause distortion of fat
globule shape. Henstra and Schmidt (1970) and Jewell (1981) used variations of this technique introduced by Salyaev (1968) for the preparation of milk and orange juice for TEM. Allan-Wojtas and Kalab (1984b) used this method for preparation of yoghurt for SEM. After preparation of samples in the agar tubes, they were fixed in 4% glutaraldehyde and postfixed in 1% osmium in phosphate/iodoacetate buffer. The membranes of the fat globules were well-defined with this preparation (Figure 8). However, gross distortion of many fat globules was evident and the fat crystals seemed to have melted, losing their definition (Figure 6). Therefore, it appeared that ice cream mix emulsions were very sensitive to temperature changes. The brief exposure to heat when sealing the agar tubes appeared to have caused melting of some of the fat crystals and fat globule distortion.

In order to circumvent heat induced alterations, the ice cream mix emulsions were mixed with 2% agarose (Sea-Prep) which is gelatine at room temperature. To process single cells for TEM, Yuan and Gulyas (1981) used an agarose preparation (Sea Plaque Agarose, Marine Colloids Inc., Biomedical System, Rockland, ME) that gels at 30°C. However, Strausbauch et al. (1985) used the newly introduced Sea Prep agarose for TEM preparation of cell suspensions. They found that 2% agarose solutions were best suited for this purpose. Concentrations of less than 1% did not give consistent gelling while higher concentrations were difficult to prepare.

The samples were fixed in 2% or 4% glutaraldehyde and postfixed in 1% osmium tetroxide in phosphate/iodoacetate buffer. Ice cream mix emulsions fixed in 4% glutaraldehyde showed well-preserved fat globules which had minimal irregularities in shape compared to the fat globules in Figures 6, 7 and 8. The membrane was clearly evident and the fat crystals were well-delineated (Figure 9). Fixation in 2% glutaraldehyde was compared to 4% glutaraldehyde and seemed to have had no noticeable effect. The membrane was clearly evident and the fat crystals were well delineated (Figure 10). There were some fat globules that were more regular in their shape than others. Some of the fat crystals appeared to cause distortion of the fat globule membrane (Figure 10). In order to determine whether fat globules were distorted before preparation for TEM study, light micrographs were taken of diluted ice cream mix emulsion (1 drop emulsion: 10 drops distilled, deionized H2O) in a depression slide under oil immersion. Light microscopy illustrated that some fat globules were distorted before preparation for TEM study (Figure 11). Van Boekele et al. (1981) hypothesised that crystals in an oil-in-water emulsion may protrude through the membrane into the aqueous phase. Darling (1982) suggested that fat crystals pierce the film between two approaching interfaces in dairy emulsions. The TEM and light microscopic data indicated that in some cases this appeared to be the mechanism that caused fat globule distortion.

SEM of ice cream mix emulsions proved unsatisfactory. The fat globules were obscured by either agar or an undefined precipitate probably composed of proteins. This occurred regardless of whether the emulsions were mixed with agar, agarose or in agar microtubes.

It was concluded that for TEM study of liquid emulsions, especially those sensitive to heat such as ice cream mix emulsions, mixing with agarose was preferable to mixing with agar or preparation in agar tubes. This technique minimised fat globule distortion and prevented melting of the fat crystals. Postfixation in phosphate/iodoacetate buffered osmium tetroxide resulted in well-preserved fat globules, clearly defined membranes and well-delineated fat crystals. These results correlate well with those reported by Allan-Wojtas and Kalab (1984a) and Kalab (1985). Fixation in 2% or 4% glutaraldehyde produced similar results.

Further study on ice cream mix emulsions using cold stage SEM on frozen, hydrated specimens may produce useful results.

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References


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Discussion with Reviewers

M. Kalab: Do the authors have any explanation why the "loop method" was unreliable? I used Brooker's loop procedure too and obtained excellent results with various foams.

Authors: It appeared that the surface tension between the loop and some of the emulsions was not strong enough to allow the emulsions to remain intact until the completion of EM preparation procedures. Many times the emulsions destabilized and "fell out" of the loop several hours after preparation resulting in a complete loss of sample.

R. Martin: How do you know the absence of fat crystals is due to heat and not a fixation artefact?

Authors: Samples mixed with 4% agar (Figure 7), encapsulated in agar tubes (Figure 8) and mixed with 2% agarose (Figure 9) were fixed (4% glutaraldehyde) and postfixed (1% osmium tetroxide in phosphate/imidazole buffer) in a similar manner. Since there was no exposure to heat while preparing samples with 2% agarose, we believe that this was the variable that affected preservation of fat crystals. In addition, there appeared to be a temperature dependent progression of fat crystal preservation. Samples mixed with agar were subject to the most heat and had few, if any, fat crystals. Samples encapsulated in agar tubes thereby subject to less heat than the agar mix exhibited fat crystals but in many cases they appeared to have melted. The samples mixed with agarose exhibited the most satisfactory fat crystal preservation.

R. Martin: Are these fat crystals or merely the location where fat crystals were once present?

Authors: We believe that these are fat crystals because they are stained, albeit very slightly, compared to the background. However, without conducting chemical analyses of the residues after fixation, post fixation and dehydration, it cannot be stated with certainty what proportion, if any, of the naturally occurring fatty acids were washed out. Allan-Wojtas and Kalab (1984a) and Kalab (1985) observed fat crystals in yoghurt very similar in appearance to those we observed.

B.E. Brooker: Perhaps some explanation might be given to account for the great difference in appearance of the fat globule membranes in Figures 1 and 2.

Authors: It is possible that protein membrane surrounding the fat globule is very fragile, even after fixation with glutaraldehyde. The acetone appears to have caused the protein to coagulate but additional work on this aspect of sample preparation is needed.

B.E. Brooker: Are the authors implying a connection between crystal melting and globule distortion? If not, will they please comment on the possible cause of fat globule distortion.

Authors: We believe that the primary cause of
fat globule distortion is due to heat, although fat globule melting could be a contributing factor. Preliminary experiments with emulsions composed solely of unsaturated fatty acids and protein (devoid of fat crystals) also exhibited globule distortion. The minimal fat globule distortion noted in the 2% agarose/emulsion might be explained by the hypotheses of Darling (1982) and Van Boekel and Walstra (1981) stated briefly in the text.