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Milk fat globule membrane: formation and transformation

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Invited Review

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Abstract

The milk fat globule membrane (MFGM) is formed by complex cell biological processes in the lactating mammary epithelial cell which result in the release of the milk fat globule (MFG) into the secretory alveolus. The MFG is bounded by a continuous unit membrane (UM), separated from the MFG lipid by a thin layer of cytoplasm. This unique apocrine secretion process has been shown in all of the mammary species so far investigated. Once the MFG is released into the alveolus there is a considerable transformation of the UM with its attached cytoplasm. This is the MFGM. The transformation is stable and expressed milk shows the same transformed MFGM structure. Again, this transformation of structure is common to all mammalian species so far investigated. However, the explanation of the transformation very much depends on the method of investigation. Transmission electron microscope (TEM) studies suggest a literal breakdown to a discontinuous UM plus cytoplasm in patches and strands, whereas more recent confocal laser scanning light microscopy (CLSM) studies indicate a separation, in a continuous UM, of two phases, one liquid ordered and the other liquid disordered. This review is designed to show that the TEM and CLSM results show different views of the same structures once certain deficiencies in techniques are factored in.

Introduction

The success of the cell biological mechanism which results in the secretion of milk fat globule (MFG) is fundamental to dairy research and industry. Surprisingly, there is still no scientific consensus as to the details of the MFG secretion nor the structure of the milk fat globule membrane (MFGM) on the secreted MFG. Part of the problem may be that some early, crucial evidence is not available online (Wooding, 1977) or is located in non-dairy journals (Wooding, 1971a, 1971b), but another factor is the apparent difficulty of believing that transmission electron microscopy (TEM) can produce significant results given the difficulty of stabilising the lipid core of the cytoplasmic lipid droplet (CLD) or MFG. This paper is an attempt to provide a useful scientific comparative review of the various suggestions and to carefully consider the pros and cons of all scenarios, hopefully to indicate avenues of future research to resolve the differences.

Mammary lipid secretion

Secretion of lipid from the mammary epithelial cell (MEC) is generally considered to be apocrine in nature (Mather and Keenan, 1998; Monks and Mather, 2022; Wooding, 2023). In all species so far adequately investigated it produces a milk fat globule (MFG) surrounded by a thin layer of cytoplasm bounded by a unit membrane (UM: Figs 1, 2a). The interplay between the cytoplasmic organelles to produce this has evolved a system to minimise the loss of MEC cytoplasm, so as to maintain synthetic capacity throughout lactation. The most direct way to elucidate the cytoplasmic interactions which are the basis of this apocrine secretory process is by TEM. Recent reviews (Mather *et al*., 2019; Monks and Mather, 2022) claim that to infer a continuous process from 'static' TEMs is unacceptable. However, TEMs can be arranged in a sequence to illustrate the entire process in much greater detail than any other technique (Figs. 1a to d). Examples of such sequences are available for all mammalian species so far investigated using adequately fixed material. 'Adequate fixation' is essential in this context and probably requires initial glutaraldehyde perfusion fixation. After microdissection this must be followed by use of osmium tetroxide and uranyl acetate staining which produces organelles whose structure is predictably recognisable, circular lipid droplets and clear UM resolution. Such fixation indicates a close interaction between Golgi vesicles (GV) and cytoplasmic lipid droplets (CLD) as the basis for constitutive lipid secretion. The evidence for and against this assumption has recently been comprehensively reviewed (Wooding, 2023).

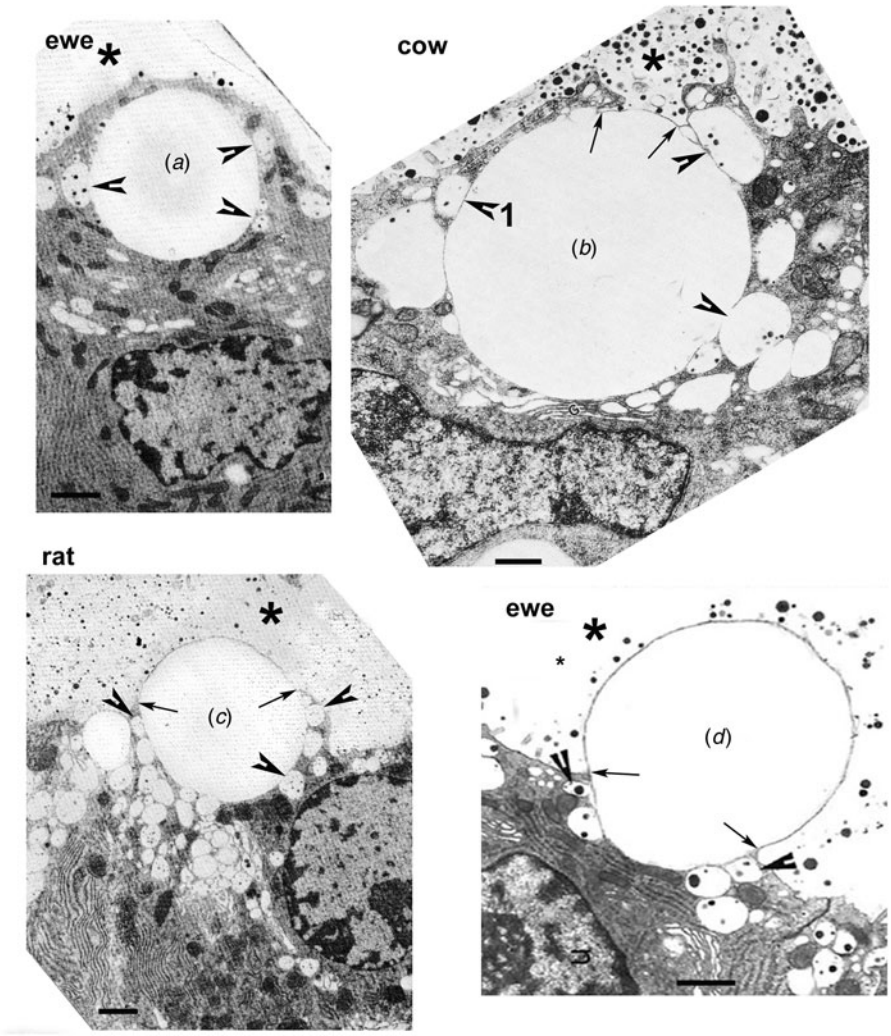

Figure 1. Suggested sequence (Figs 1–d) illustrating the gradual release of an MFG into the alveolus (asterisks) which produces the MFGM. This is largely driven by suitably positioned Golgi vesicles (arrowheads). The MFGM has formed between the arrows on Figs 1b–d. If the Golgi vesicle marked by arrowhead number 1 in Fig. 1b were next to exocytose, the cytoplasmic area between arrowhead 1 and the nearest arrow might eventually form part of a 'crescent'. In my experience an equivalent series can be assembled for any one species. The similarity of the sequences is emphasised by the use here of three different species. Bar lines: 1 μm

MFGM formation

These processes produce the MFGs, 90% of which can be recognised as a CLD surrounded by a uniformly thin layer of cytoplasm bounded by a UM which is referred to as the MFGM (Figs. 2a, c). Some 3–8% of the MFG, dependent on species, do show a local expansion of cytoplasmic envelope containing recognisable cytoplasmic organelles, known as 'crescents' (Figs. 1b, see legend and 2d, asterisk) which clearly establishes the apocrine nature of MFG secretion (Wooding *et al.*, 1970, 1971a, 1971b, 2023, Huston and Patton, 1990).

The MFGM structure is important in stabilising the milk emulsion and also for providing a variety of essential nutrients to the newborn and nursing offspring (Gallier *et al.*, 2015; Raza, 2021; Brink and Lönnerdal, 2020; Chai *et al.*, 2022). There is increasing evidence that isolated MFGM, produced by churning cream and collecting the MFGM fragments by centrifuging the buttermilk, has important commercial relevance as a nutraceutical addition to a variety of milk products (Spitsberg, 2005; Silva *et al.*, 2021; Chai *et al.*, 2022). It is, therefore, important to understand how the structure of the MFGM may contribute to the practical usefulness of milk and dairy products. Unfortunately, different types of light and electron microscope investigations produce very different conclusions. TEM indicates a comprehensive transformation of the alveolar MFGM structure (Wooding, 1971b, 1977) whereas confocal laser scanning microscopy (CLSM) provides evidence that the MFGM UM remains continuous even on freshly expressed MFG in milk (Lopez, 2011, Lopez *et al.*, 2019).

TEM evidence

With TEM, all species so far adequately investigated show the same remarkable transformation on release of the originally continuous MFGM from the MEC into the alveolus (Wooding, 1971a, 1971b, 1977, 2023). Early work (Wooding, 1971b) indicated that on transverse sections of the MFG the UM became discontinuous but later studies of serial sections of the MFG surface, giving a more 3D image (Wooding and Mather, 2017), show that the continuous UM has transformed into patches and strands forming the primary MFGM (Figs. 2b to e, arrows). This is seated on a continuous dense line, usually referred to as the secondary MFGM (Figs. 2b to e, arrowheads), equivalent in position to the boundary of the original cytoplasmic CLD. Again, in all species adequately investigated the primary MFGM cytoplasmic layer increases in electron density (Figs. 2b, c, 5b, detailed in legend) and in many species shows evidence of a quasi-crystalline reorganisation of content on sections (Figs. 2b, 3d to f, 5b, detailed in legend). This has never been seen around the forming MFG prior to release from the MEC. These MFGM structures are

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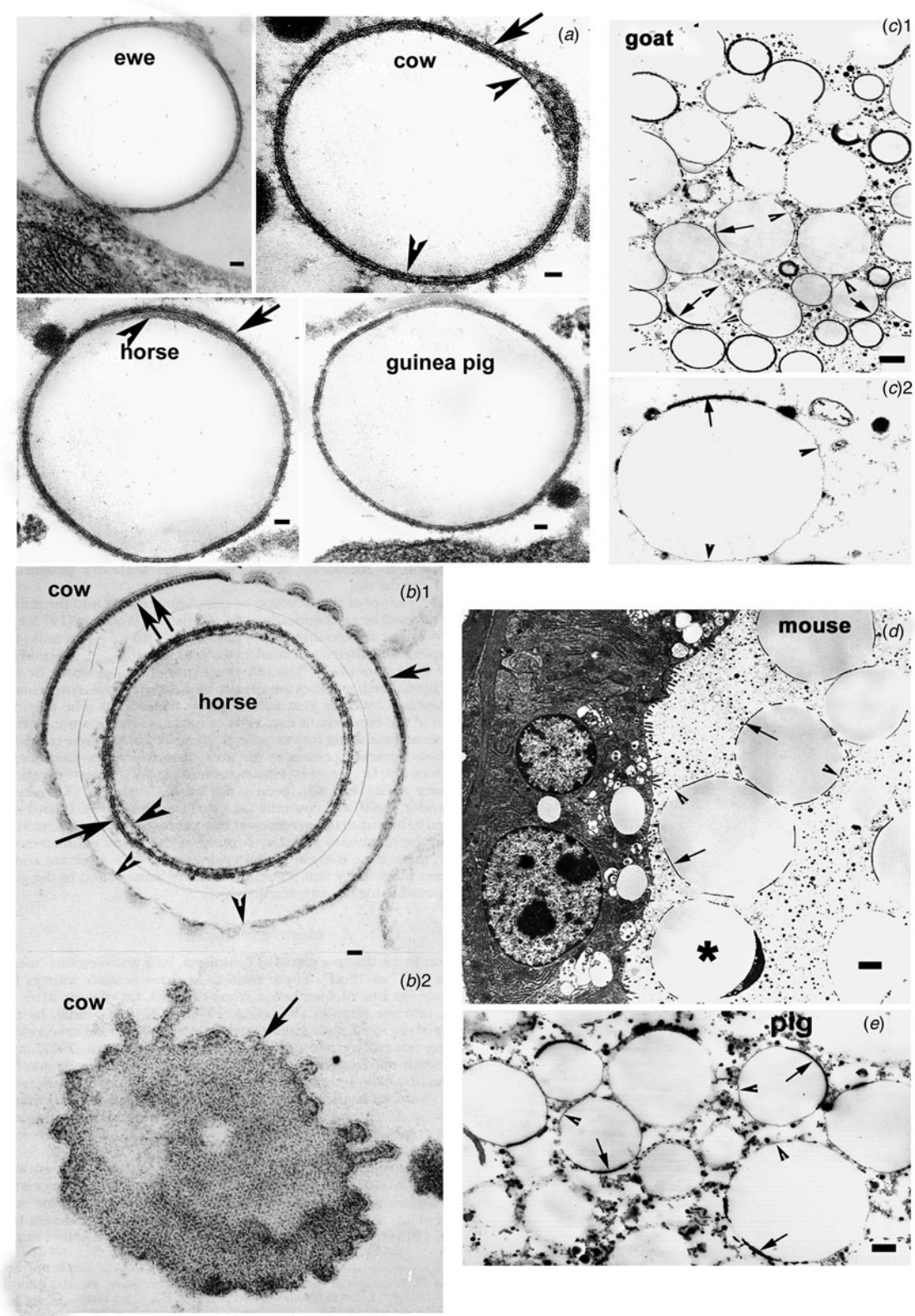

Figure 2. Figure 2a: MFG immediately before (ewe) and immediately after (cow, horse, pig) release from the MEC. All show a cytoplasmic layer (arrowheads) under a continuous UM (arrows). Bar lines: 25 nm. Fig. 2b1: Transverse section illustrating the change in MFGM structure from intact (horse) to transformed patches of primary MFGM (arrow, cow) on a continuous secondary MFGM (arrowheads). One patch shows evidence of a crystalline array (double arrow). Bar lines: 25 nm. Fig. 2b2: Grazing section of the surface of an MFG showing a crystalline array *en face* as distinct from the transverse section on 2b1. Fig. 2c1: Low power section of goat alveolar MFG, illustrating examples of primary MFG (arrows) and secondary MFG (arrowheads). Bar line: 2 μm. Fig. 2c2: Higher magnification example of a goat MFG, primary MFGM at arrow, secondary at arrowheads. Figs. 2d, e: Sections of MFG in mouse and pig alveoli, primary MFGM at arrows, secondary at arrowheads. Note also the section of a mouse MFG with a crescent of cytoplasm (asterisk). Bar line: 1 μm.

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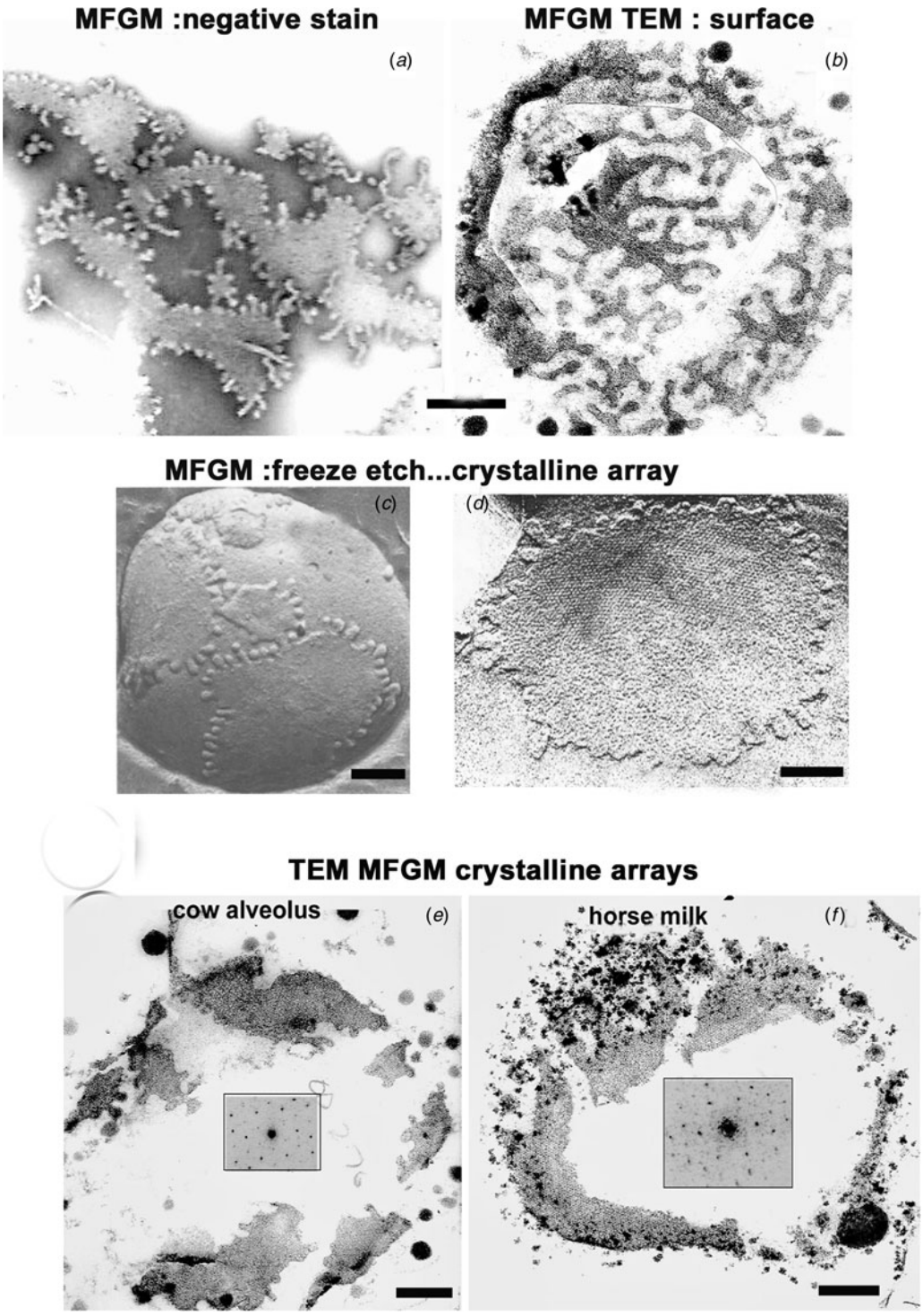

Figure 3. In Figures 3a, b the similarity of the primary MFGM structure after negative staining of fixed delipidated MFGM (a) and on the TEM surface (b) is clear. Freeze etch of unfixed MFG (Figs 3c, d) also shows equivalent patches of MFGM. The crystalline pattern visible on the Fig. 3d patch of MFGM is equivalent to the crystalline arrays on the TEM surface of alveolar cow MFG (Fig. 3e) and the expressed horse MFG (Fig. 3f). Bar lines: 2 μm

found on the MFG in the alveolus (Fig. 3e) and can also be seen on sections of fixed expressed MFG (Fig. 3f) Also, TEM of fixed negatively stained patches of delipidated cream MFGM show similar outlines and similar crystalline structures on their surface (see Wooding and Mather, 2017). Two of the components of the crystalline structure have been identified by EM immunocytochemistry (Wooding and Mather, 2017) as xanthine oxidase (XO) and butyrophilin 1A1 (BTN), both molecules essential for normal secretion of the MFG from the MEC. When the TEM section includes a grazing section of the top of an MFG the quasi-crystalline structure can be seen *en face* in the patches of the primary MFGM (Figs. 2b and 3e, f, detailed in legend). The patches also show a characteristic 'finger fringed' outline similar to the MFGM after negative staining (Fig. 3a).

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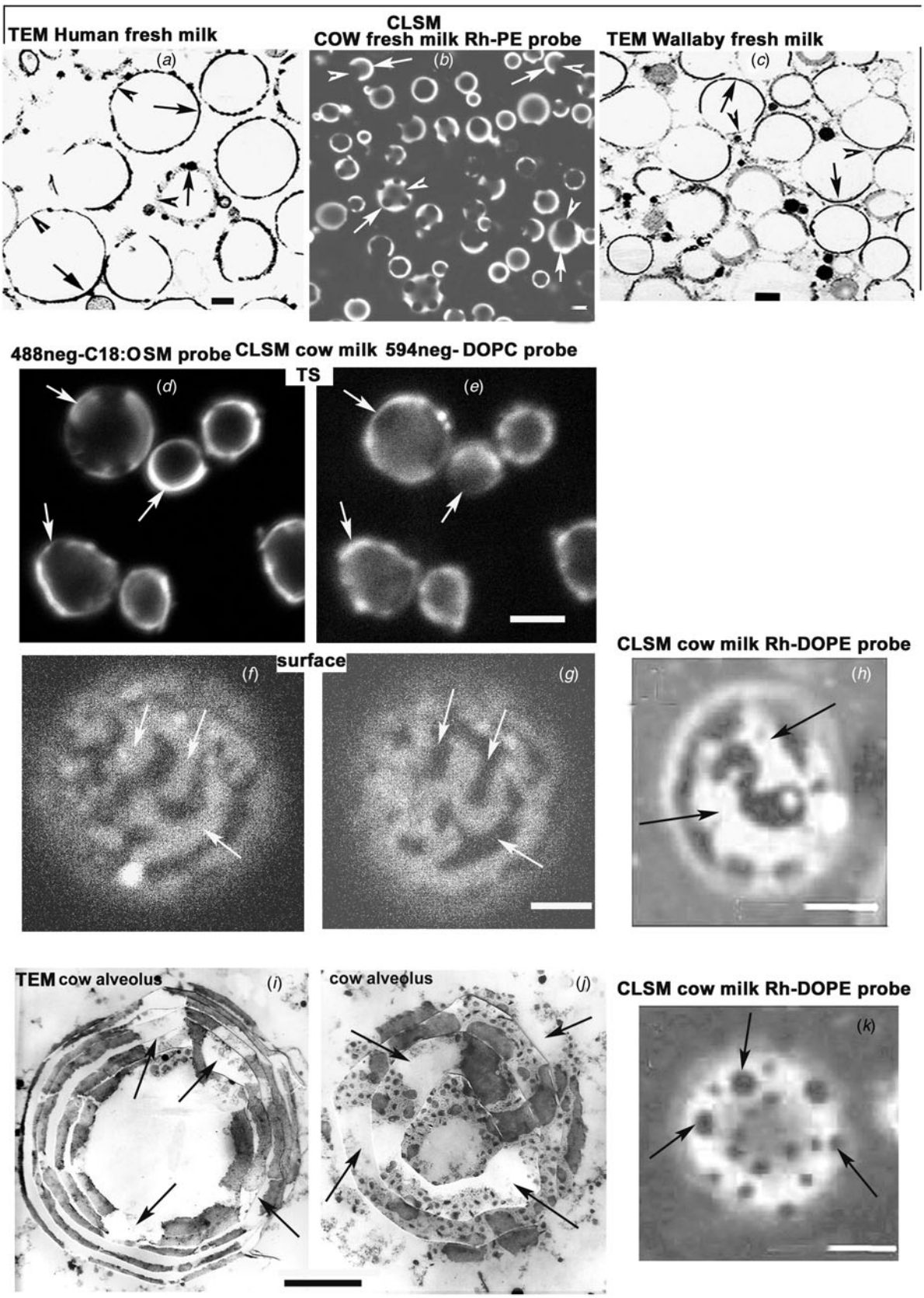

Figure 4. Figure 4a–c compares the transverse sections of MFG using TEM or CLSM. The similarities are emphasised by the arrows indicating primary MFGM patches and the arrowheads indicating secondary MFGM. Bar line: 5 μm. Fig. 4b courtesy of Professor Lopez. Fig. 4d–g: CLSM of cow MFG showing the complementarity of the patterns of fluorescence defining the ordered (sphingomyelin) and disordered (dioleoylphosphatidylcholine) lipid phases. Bar line: 5 μm. All courtesy of Professor Kinoshita. Fig. 4h–k: TEM and CLSM surface views of cow MFG, showing the similarities (arrows) of the patterns of the primary MFGM TEM with the fluorescence of the lipid disordered phase areas. Bar line: 5 μm. Figures 4h, k courtesy of Professor Lopez

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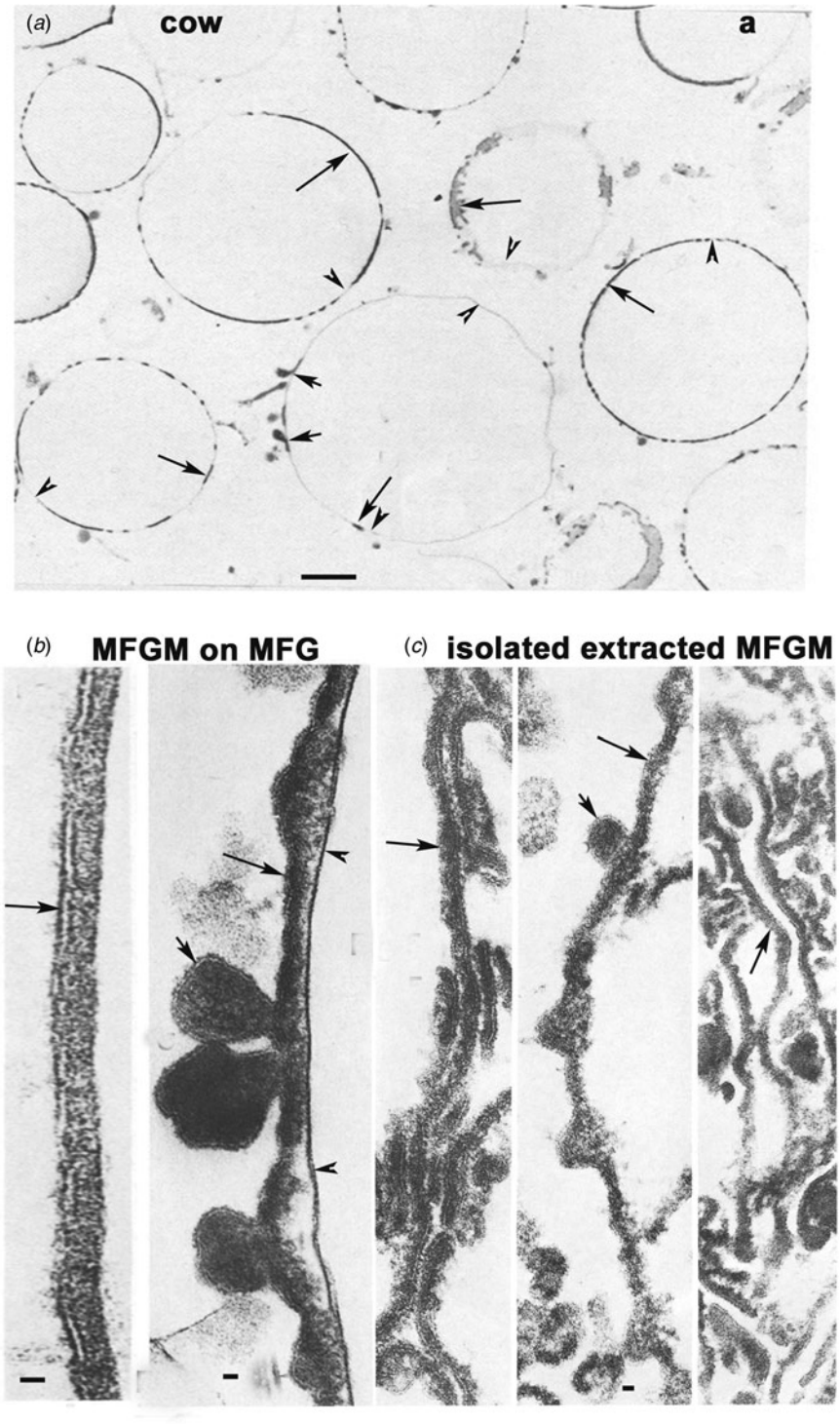

Figure 5. 5a.; TEM of expressed cow MFG. Note the primary MFGM (long arrows) and secondary MFGM (arrowheads). There is also an example of primary MFGM vesiculating (short arrows) into the milk. Bar line 1 μm. Fig. 5b: Three high magnification examples of primary MFGM. Figure 5b1, from an MFGM close to the alveolar surface with attached cytoplasm beginning to darken. Figure 5b2, an MFGM which has developed a crystalline organisation of the cytoplasmic protein coat. Figure 5b3, a primary MFGM with fully darkened cytoplasm (arrow) which is separating from the secondary MFGM (arrowhead) and producing vesicles (short arrow). Bar lines 5 nm. Fig. 5c: Three examples of isolated primary MFGM which have been comprehensively lipid extracted, only the left hand one fixed before extraction. Bar lines 5 nm. The MFGM structures (long arrows) are virtually identical to the unextracted MFGM, including the vesiculations (short arrows), establishing that most of the primary MFGM consists of protein.

From the TEM evidence, once the transformation of MFGM has occurred, the stability of the MFG is dependent on the continuous secondary MFGM. This secondary MFGM is in the equivalent position to the cytoplasmic CLD but is appreciably thicker. It is sufficiently robust to withstand TEM preparation and milk expression and has been clearly shown around fixed milk MFG since the earliest and subsequent studies (Henson *et al.*, 1971; Wooding, 1971b, 1977). Unfortunately, the Henson and Gallier papers only show individual MFGs with very little primary MFGM, and the Wooding, 1971b paper claimed that '*most* of the (primary) MFGM is lost into the milk plasma by a process of vesiculation'. That claim has subsequently been proved wrong since sections through cream aggregates/pellets of a variety of species (Figs. 2c, d, e, 4a, c, 5a) show that most of the MFG have at least 50% or more of their transverse sections covered by primary MFGM (see also Wooding, 1977, Wooding and Mather, 2017, Wooding, 2023).

The material comprising the secondary MFGM must derive from the cytoplasmic layer underlying the UM of the MFGM. TEM indicates that the primary MFGM can lift off the secondary

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on a few MFG, producing a structure similar to isolated MFGM (Fig. 5b, detailed in legend) and TEM immunocytochemistry indicates that the secondary MFGM contains no BTN or XO (Wooding and Mather, 2017). It would be interesting to investigate with other antibodies to establish the chemical composition of the secondary MFGM which from the TEM results is the source of stability of the milk emulsion. All TEM images are produced after chemical fixation of the MFG, but similar results are produced by three very different initial fixatives (glutaraldehyde, acrolein/dichromate or osmium). Also, freeze fracture images from *unfixed*, fast frozen milk show similar 'finger fringed' patches with similar crystalline organisation (Fig. 3c, d, Buchheim, 1986).

CLSM evidence

However, CLSM results are equally unequivocal and common to a variety of species. Using fluorescent markers which insert into the UM bilayer, the outcome is patches and strands of fluorescence over most of the *unfixed* MFG (Figs 4b, 4c to h, 4k; Lopez, 2011, 2020). The boundaries of the patches are sharp with no indication of 'finger fringing' but this could be the lack of resolution of the fluorescence as compared with the TEM (Figs 4b, h, k). The similarities between the 'patches and strands' demonstrated by both the TEM and CLSM results suggest that the two methods produce similar depictions of the reality of the MFGM transformation.

Developers of the CLSM method initially accepted the TEM indication of an absence of a UM between the patches and strands (Evers *et al.*, 2008). An identical fluorescent pattern produced by using a fluorescent antibody to the membrane associated proteins in the patches reinforced this conclusion. However, recent CLSM work (Lopez, 2011, 2020) has emphasised these sharp boundaries as an indication of phase changes in the lipid packing in the UM (Figs 4h, k). CLSM studies indicate that the MFGM is flexible and dynamic, with the non-fluorescent areas capable of diffusing, fusing and changing shape but probably not their total area. The areas can also modify in structure and shape dependent on the temperature at which the micrographs are taken (Et-Thakafy *et al.*, 2017; Lopez, 2020) The UM is claimed to be as continuous around fresh milk globules as when the globule was released from the MEC with the non-fluorescent areas interpreted as a UM with a high concentration of sphingolipids and cholesterol. The discontinuities in the primary MFGM alveolar TEM results are presumably considered to be artefacts of fixation, since they are unable to preserve the UM in the SM lipid and cholesterol non-fluorescent areas (see below). However, the TEM primary and secondary MFGM distinction is never discussed in CLSM papers which use the MFGM term where convenient, for example, allowing recognition of the primary MFGM when methods of isolation of this valuable nutritional resource are compared. Unfortunately, the dense line (secondary MFGM) around MFG in bovine milk has also been described as MFGM (see Fig. 1, 'bovine milk' in Lopez *et al.*, 2017). From the TEM work, the structure of the primary MFGM is clear: a unit membrane with an attached sheet of protein (Fig. 5b). Protein comprises 40–70% of the total MFGM depending on species. Extraction of isolated MFGM with chloroform-methanol and/or acetone removes >95% of the polar lipid of the MFGM but does not change the TEM appearance of the MFGM significantly (Fig. 5c and Wooding and Kemp, 1975a, 1975b). CLSM investigations of the phase changes in the MFGM UM *in situ* are relevant only to the UM lipids and are independent of the protein.

The CLSM results are interpreted as segregation of particular lipids in the UM in liquid ordered areas surrounded by patches and strands of different lipids in liquid disordered areas recognised by the fluorescent probe (usually Rh-DOPE) which is sterically excluded from the ordered areas (Lopez, 2011, 2020). Because the liquid ordered non-fluorescent areas in CLSM MFGM images are frequently circular, an analogy is drawn with the lipid rafts formed for specific purposes in normal cells. Such rafts have a characteristic composition with large amounts of sphingolipids (SM) and cholesterol. In the Lopez (2011) and (2020) reviews the liquid ordered, nonfluorescent, areas of the MFGM are therefore *interpreted* as being composed of SM and cholesterol although there is no direct pictorial evidence for this. However, recently developed fluorescent SM probes (Kusumi *et al.*, 2012, 2020; Kinoshita et al. 2017) have produced preliminary evidence using cow MFG of a complementary pattern of SM (liquid ordered) and Rh-DOPE (liquid disordered) fluorescent phases (Figs 4d to g), confirming the interpretation. These SM analogue probes have a fluorescent marker attached by a chemical spacer to the head of the SM molecule. This avoids the possible complication of steric exclusion of the fluorescent SM analogue from the UM (Kinoshita *et al.*, 2017).

Phase changes in unit membranes

A considerable amount of work has been published on liquid phase changes of lipids using artificial giant unilamellar vesicles (GUV) and Langmuir bilayers which consist of various percentages of suitable lipid mixtures but no transmembrane or other proteins (Bagatolli and Gratton, 2000; Cornell *et al.*, 2020). SM and/or cholesterol inclusion produces characteristic areas/phases which can be shown to vary in thickness, on a nanometre scale, by electron tomography, atomic force microscopy and X-ray diffraction. MFGM fragments have been isolated and polar lipid extracts (with no protein) used to make GUV. The patterns of fluorescence with liquid disordered probes are equivalent to those from the intact MFGs, confirming the interpretation of the lipid-phase separation in the MFGM UM (Lopez, 2011, 2020).

MFGM proteins

This raises a problem. The MFGM proteins, centred on a close association between transmembrane BTN and XO and adenosine diphosphate-heptose (ADPH) have been shown to be necessary for MFG secretion. (Monks and Mather, 2022; Wooding, 2023). They are still present as a major part of the primary MFGM as shown by TEM immunocytochemistry on sections as well as isolated negatively stained MFGM. They presumably form the bulk of what is visible after complete extraction of the polar lipids of the MFGM (Fig. 5c; Wooding and Kemp, 1975a, 1975b). According to the CLSM results the glycosylated transmembrane BTN would be excluded from the liquid ordered SM-cholesterol areas together with its associated and other proteins (Lopez, 2020). With no protein backup the SM-cholesterol UM domain would be very fragile. Also, SM lacks unsaturated lipids which would interact better with the fixatives to stabilise membrane structure. It seems likely that fixation for TEM is not capable of preserving the SM-cholesterol UM areas in recognisable form. The TEM results thus only produce the patches and strands of what was protein backed liquid disordered primary MFGM.

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Post-secretion phase changes

This raises the question of how the μm sized areas interpreted, and now verified in this paper, as SM-cholesterol UM (as demonstrated by CLSM) are formed, from what was originally the normal apical UM of the MEC. Evidence from living cells indicates that the SM containing, and other, UM rafts, which may control many cellular activities are tiny, from 5 to 70 nm (Simons and Ikonen, 1997; Simons, 2016; Sezgin *et al.*, 2017). This essential functional and structural microheterogeneity of any normal plasma membrane is reliant on connection to the actin cytoskeleton immediately under the plasma membrane (Kusumi *et al.*, 2019; Sezgin, 2022). There is no evidence that the MFGM retains an actin cytoskeleton although the MEC certainly does have one as shown by the vital imaging observations in mice MEC (Mather *et al.*, 2019). There is no biochemical evidence reported for actin in the isolated MFGM proteome, either (Reinhardt and Lippolis, 2006). Presumably, the complex processes utilising BTN, XO and ADPH which have evolved to produce MFG continuously through lactation (Wooding, 2023) displace the MEC actin cytoskeleton during secretion.

The CLSM results could be explained by a coalescence of the small SM-Cholesterol rafts by self-association once the UM of the MFG is released from the MEC and hence from cellular/cytoplasmic constraints including the actin cytoskeleton. Studies with GPMV have shown that actin removal is necessary before the liquid phase changes of lipids into ordered and disordered areas can form (Simons, 2016). The TEM results indicate that after release from the MEC, the MFGM proteins in the liquid disordered phase, the primary MFGM, modify to form crystalline arrays. There is remarkable uniformity among the different species in this respect (cow, goat, horse and human) confirmed by optical diffraction of the crystalline arrangement (Figs 3e, f; Wooding and Mather, 2017) .The formation of a crystalline array implies a reduction in area. This could be the basis for the apparent break-up of the MFGM into patches and strands shown by several preparation methods which all used chemically fixed MFGM (Henson *et al.*, 1971; Wooding, 1971b). Rapid freezing prior to freeze fracture of *unfixed milk* also produces evidence of similar patches with crystalline arrays (Figs. 3c, d) with no sign of any membranous connection between them (Buchheim, 1986). Presumably, the rapid freezing produces the same effect on SM UM structure as the fixatives.

Conclusions

The evidence from both CLSM and TEM indicates that there is a considerable transformation of the MFGM after it is released from the MEC into the alveolus. Only the TEM can provide evidence of the alveolar change. This indicates a breakdown of the initially continuous MFGM into patches and strands, which pattern persists in the expressed MFG in milk. The transformation must start after the release of the MFG from the MEC, for no MFG with intact MFGM have been reported from the alveolar central area. The CLSM evidence from expressed milk suggests that the transformation of the MFGM is based on a separation into μm sized disordered or ordered lipid phase areas in the MFGM UM. This would require a drastic modification of the nanometre scale microheterogeneity of the SM and other rafts in in the MEC PM into the two-phase simplicity shown by the CLSM of the MFGM. This presumably results from an aggregation of the SM/cholesterol micro-rafts into μm sized areas found on the MFG. The forces which could produce such an aggregation are unclear. Whether the lack of TEM evidence for the presence of the SM/cholesterol phase areas can be attributed to the inadequate fixation of such UM is also unclear. Future studies with the newly available SM fluorescent probes and EM immunocytochemistry of sections may well help to resolve these problems.

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