Examination of whole milk powder by confocal laser scanning microscopy

By ANTHONY B. McKENNA
New Zealand Dairy Research Institute, Palmerston North, New Zealand

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Summary. Confocal laser scanning microscopy was used to examine components of whole milk powder. Powder was dispersed in a glycerol suspension containing a fat-soluble fluorescent dye which was excited by laser light to produce images of the fat at the surface of and inside the whole milk powder particle. Lactose crystals, produced in the milk prior to spray drying, were seen to be in the diamond-like, α-hydrate form. The surface coverage and the stability of a wetting agent sprayed on to the surface of whole milk powder particles were examined. The wetting agent was shown to be located as a thin surface layer that partly migrated into creases and folds on the particle surface during storage at 37 °C.

Milk powders, particularly those sold directly to the consumer, are required to disperse rapidly in water and to be quickly and completely soluble, i.e. form a stable colloidal suspension of fat and protein leaving little or no visible residue suspended in the water or coated on the container surface. These requirements have led to a need for the determination of ingredient interactions and the localization of ingredients within or on the powder particle. Visualization of these components by microscopy can be a powerful tool for enabling the spatial distribution and interaction of fat, protein and crystals within or on the surface of the powder to be assessed. The techniques that have been used for powders include light microscopy (King & Shimmin, 1961), scanning electron microscopy (Buma & Henstra, 1971) and various forms of transmission electron microscopy, namely freeze fracturing (Buchheim, 1982) and thin sectioning of embedded powders (Muller, 1964). These have generally been laborious and/or have produced limited useful information.

A relatively new form of light microscopy, confocal laser scanning microscopy (CLSM), may be useful for the examination of milk powders. The problems normally associated with observing thick food specimens such as milk powder particles by light microscopy, i.e. a blurred image produced from a large depth of focus, can be overcome in CLSM as image formation does not depend on transmitting light through the specimen. In CLSM, a scanning laser illuminates a layer within the specimen at a specific focal plane and removes out-of-focus information by the use of a confocal pinhole, thus providing images of greater resolution than those from light microscopy. For a more detailed description of CLSM, see Heertje *et al*. (1987) and Brooker (1991, 1995). In CLSM, images of the components such as proteins and lipids are produced by using the laser light to excite a selective fluorescent dye introduced to the food system during manufacture or to the surface of the food after manufacture (Brooker, 1995). Modern CLSM instruments, many of which contain

combined krypton–argon lasers, produce light at 488, 568 and 614 nm, allowing the use of multiple dyes that excite at these different wavelengths. Therefore dyes specific to fat and protein can be examined within the same sample. As well as fluorescence, CLSM allows images of the sample to be produced using the reflection and transmission modes.

The advances in microscopy provided by CLSM may provide advantages in the examination of whole milk powder (WMP). Sample preparation that has been difficult may be simplified by the addition of specific dyes to an appropriate mounting medium. Optically thin sections should allow for the non-invasive production of high resolution images of the WMP particles. This paper describes the sample preparation and use of CLSM for the examination of a number of components in WMP.

MATERIALS AND METHODS

Three different types of WMP samples were examined by CLSM: standard WMP with 280 g fat/kg for fat localization, WMP containing high concentrations of crystalline lactose, and a WMP on to which had been sprayed a fluorescently labelled phospholipid (Table 1).

Fat localization

The study of fat in aqueous emulsions by CLSM is best achieved using the stain Nile blue (Brooker, 1991). This contains trace amounts of fluorescent Nile red (Nile blue A oxazone) dye which diffuses into the oil phase and then becomes strongly fluorescent when excited in the range 450–500 nm.

A number of solutions for mounting WMP in preparation for CLSM were examined. The requirements were that it must allow the solubilization of the dye Nile blue and its diffusion to the fat on the surface and within the WMP particle, that it must inhibit or allow only very slow dissolution of the WMP, and that its use must result in little or no background fluorescence.

Three commercially available fluorescent mounting media were evaluated for their suitability: Citifluor (UKC Chemical Laboratory, Canterbury CT2 7NH, UK), Dako (Dako Corporation, Carpinteria, CA 93013, USA), and Fluoprep (BioMérieux, F-69280 Marcy-l'Etoile, France). These fluids were all glycerol based and contained anti-fading agents. The Fluoprep and Dako media also contained a chemical agent that caused the fluid to set into a gel within minutes of attaching the cover slip. The powders were also evaluated in propylene glycol and glycerol.

The non-lecithinated, agglomerated WMP of 280 g fat/kg (New Zealand Dairy Board, Wellington, New Zealand) was mixed with the mounting medium containing Nile blue dye (added as a powder at 10 mg/l) and placed on a microscope slide.

The fat stained with Nile blue in the WMP was fluoresced using the fluoroscein isothiocyanate filter block (excitation at 488 nm) of a Leica confocal laser scanning microscope (model TCS 4D; Leica Lasertechnik, D-69120 Heidelberg, Germany).

WMP was prepared for transmission electron microscopy (TEM) by dispersing the powder into propylene glycol (1:9 ratio), mixing 1 part of this solution with 1 part of a solution of low-temperature-gelling agarose (30 g/l), cubing the set gel, fixing in glutaraldehyde (30 ml/l), post fixing in osmium tetroxide, staining in uranyl acetate and dehydrating in a graded ethanol series. The cubes were then embedded in an epoxy resin, cured at 60 °C, microtomed and the resulting thin sections were examined using a Philips 201 transmission electron microscope (Philips, NL-5600 MD Eindhoven, The Netherlands).

Table 1. *Summary of materials and methods for evaluation of whole milk powders*

Study	Whole milk powders	Stain/filter
Fat localization	280 g fat/kg, obtained from different sources	Nile blue/fluoroscein isothiocyanate filter (488 nm)
Lactose crystal localization	280 g fat/kg; 50 % of the lactose in crystalline form	Nile blue/fluoroscein isothiocyanate filter (488 nm) or reflection
Surface phospholipid localization	280 g fat/kg	Fluorescently labelled phosphatidylcholine and fat blend sprayed on to powder surface. Rhodamine filter (568 nm)

Lactose crystal localization

The WMP evaluated for this work (experimentally produced at the New Zealand Dairy Research Institute, Palmerston North, New Zealand) contained ~ 50 % of its lactose in crystalline form.

The WMP was simply dispersed into immersion oil (for examining crystalline lactose alone in the reflection mode) or into a glycerol-based mounting medium with added Nile blue (for imaging the fat and lactose), mounted on a slide and presented to the microscope.

Surface phospholipid localization

A fluorescently labelled phospholipid (Bopidy 3806, phosphatidylcholine with excitation and emission maxima at 581 and 591 nm respectively; Molecular Probes Inc., Eugene, OR 97402, USA) was added at 1 mg/kg to a 1:1 blend of anhydrous milk fat and a commercially available soya lecithin. This blend (1 g) was heated to 60 °C and sprayed (using a small pressurized air gun) on to 100 g of prewarmed (60 °C) standard agglomerated WMP of 280 g fat/kg (New Zealand Dairy Board) while being mixed in a Kenwood cake mixer. The resulting 'instantized' WMP and the unmodified standard WMP were evaluated 2 d and 6 months (storage was at 20 °C for 2 months, 37 °C for 1 month and 20 °C for 3 months to mimic conditions while shipping the powder to a consumer) after addition of the phosphatidylcholine.

The wetting time (the time required for all the particles of an instantized WMP to become wetted, i.e. sink below the surface or assume a wet appearance in cold water (International Dairy Federation, 1979)) and the location of the phospholipid fat blend were determined. The latter was examined by dispersing the WMP into immersion oil, mounting on a slide and presenting this to the confocal microscope. A combined image was produced by examining first the Bopidy-stained phospholipid using the rhodamine filter block (568 nm) and then the whole particle using the reflection mode of the confocal microscope.

RESULTS AND DISCUSSION

Fat localization

Use of all the commercial mounting media gave images with excellent signals from the fat and little background signal from the mounting medium itself. The use of Dako and Fluoprep media (gel-setting) gave the added benefit of minimizing streaming of the WMP particles during observation. However, the agent used for gel-setting may have been responsible for some disruption of the fat globules prior to gel

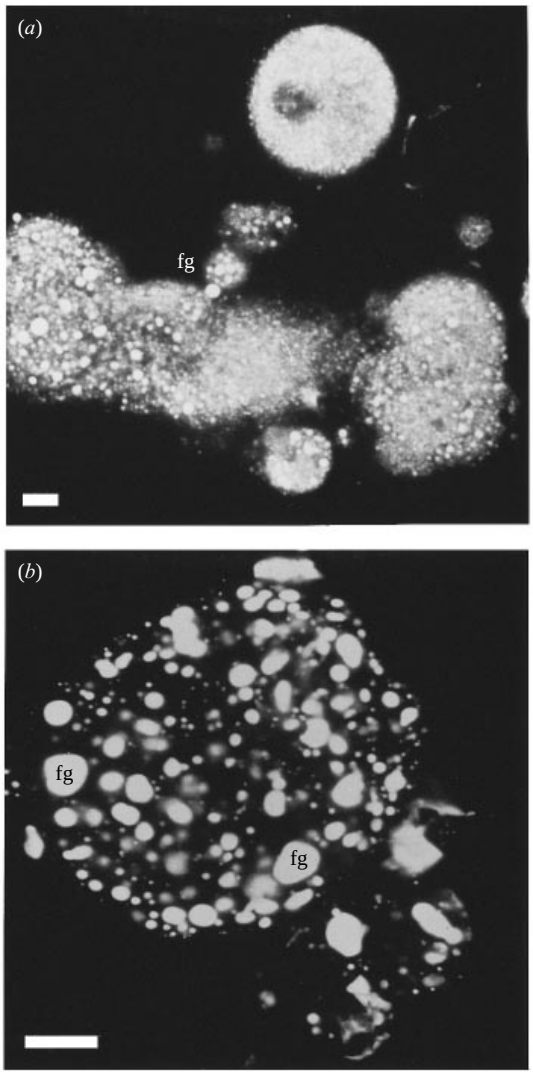

Fig. 1. Confocal micrograph of a whole milk powder particle stained with Nile blue. (*a*) Powder with fat globules (fg) no larger than 5 μm in size; scale bar, 10 μm. (*b*) Powder with most fat globules (fg) in the 5–15 μm size range; scale bar, 20 μm.

formation. The use of glycerol resulted in the gradual dissolution of powder and gave some background fluorescence in the presence of dye Nile blue. Propylene glycol was by far the least viscous of the mounting media and as a result the powder streamed extensively although dissolution was slow.

CLSM of WMP was clearly able to show the location and size of individual fat globules distributed through an optical section of the powder particle (Fig. 1). Powders that contained different fat globule size distributions could easily be compared. Fat globule size may be a factor in influencing the functional properties of the WMP in applications such as milk chocolate. Large fat globules may provide more easily extractable fat for reducing plastic viscosity during chocolate manufacture (Haylock, 1995).

The occasional presence of relatively large regions of fat on the surface and within

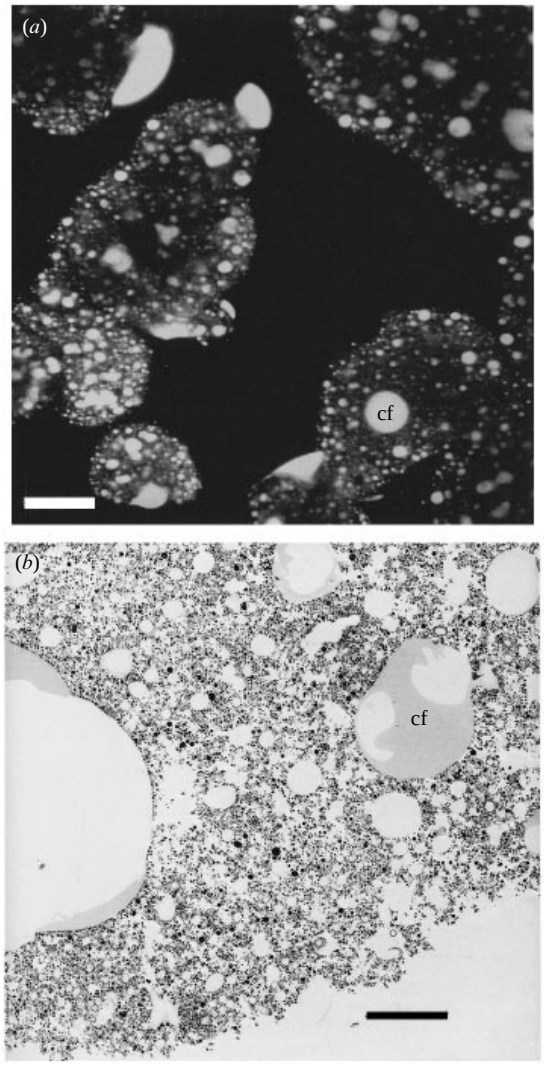

Fig. 2. Regions of coalesced fat (cf) within a whole milk powder particle (*a*) as examined using confocal laser scanning microscopy, scale bar, 20 μm, (*b*) as examined using transmission electron microscopy, scale bar, 4 μm.

some powder particles was identified using CLSM (Fig. 2*a*). These regions of coalesced fat globules were also observed within the same samples using TEM (Fig. 2*b*). However the coalesced fat globules were not as easily distinguished from air bubbles by TEM as they were by CLSM.

Regions of surface fat could be clearly observed using CLSM. It is evident that they appeared to pool at the joining points of agglomerated powder particles. They are likely to influence the flow properties and solubility of the powder particles (Buma, 1968, 1971*a*, *b*). Reconstructions by merging a series of optical sections taken from two agglomerated powders with completely different properties showed the presence or absence of surface fat (Fig. 3*a*, *b*).

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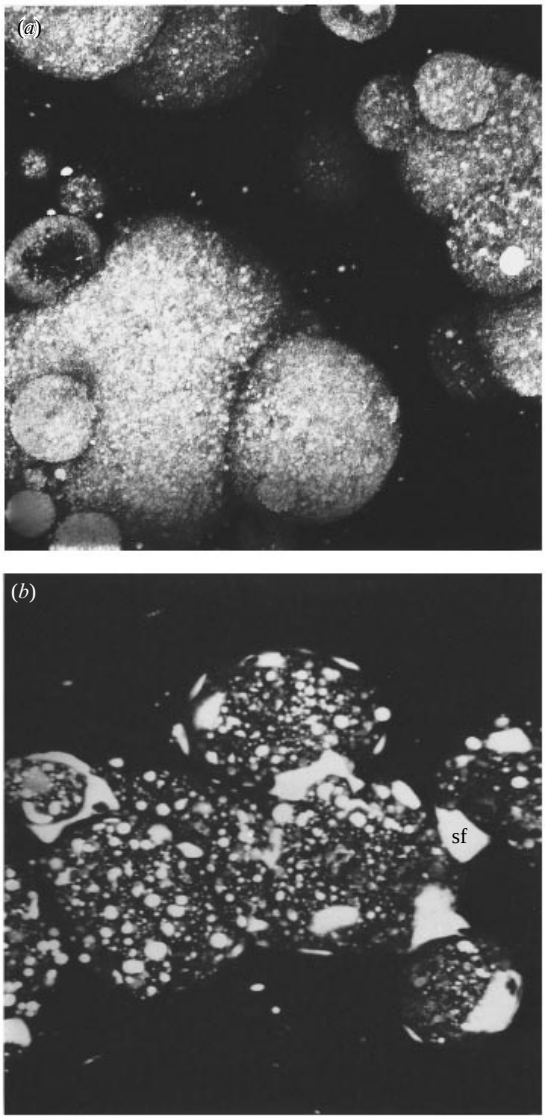

Fig. 3. Reconstruction from a series of optical sections showing the surface of a whole milk powder particle. (*a*) No surface fat was observed on this powder particle. (*b*) Regions of surface fat (sf) are shown.

Lactose crystal localization

Lactose crystals in WMP have been examined previously by the use of Heinz solution which disperses the powder and leaves the lactose crystals intact for microscopic examination (Saito, 1985), although there is a possibility that some larger lactose crystals were formed by the mounting procedure. The use of CLSM for the examination of lactose crystals is very simple and non-invasive (no change to the structure of the WMP). When stained for fat, the entire powder particle could be seen with the lactose crystals in diamond-like lactose α-hydrate form clearly evident by negative contrast (Fig. 4*a*). When operating the microscope in the reflection mode (Pawley, 1990) the crystals were also clearly visible (Fig. 4*b*). It was notable that the majority of reflecting signal in the powder at this magnification was from fat globules

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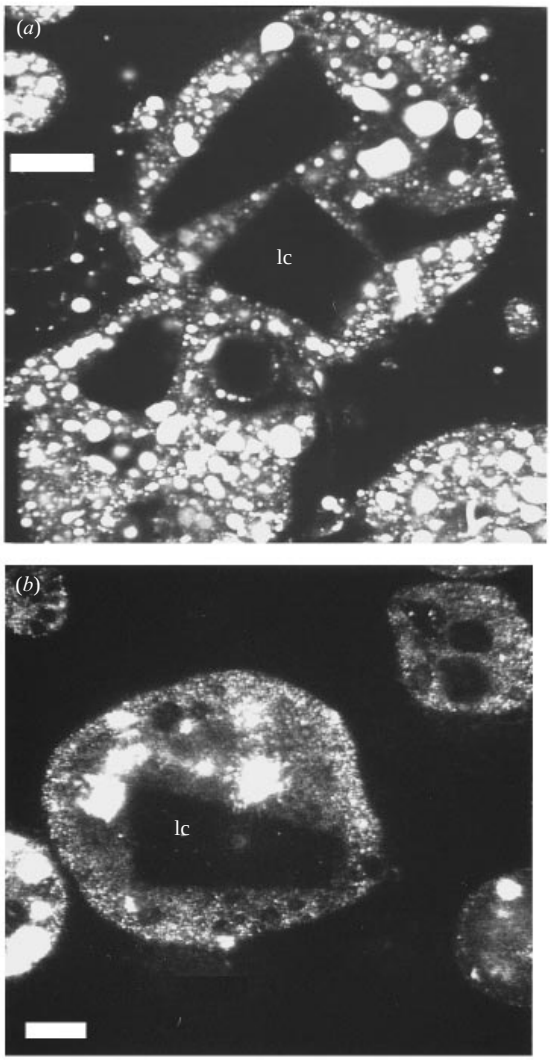

Fig. 4. Lactose crystals (lc) within a whole milk powder particle (*a*) as observed by negative contrast when the fat was examined in the fluorescent mode using confocal laser scanning microscopy, scale bar, 20 μm, (*b*) as examined in the reflection mode using confocal laser scanning microscopy, scale bar, 10 μm.

and air bubbles and that the lactose crystals were only visible by negative contrast. This is explained by the thickness of these crystals and that only scanning at the crystal surface produced a reflection of the laser light.

It has previously been reported that crystallization of lactose in the milk concentrate prior to drying results in the tomahawk (or diamond) lactose α-hydrate crystals whereas crystallization of the lactose in dry milk powder results in the needle-like lactose β-anhydride crystals (Caric & Kalab, 1987).

Surface phospholipid localization

The quantity of phospholipid in the powders was measured. The addition of the phospholipid–fat blend resulted in 1·5 g phospholipid/kg in the experimental powder whereas only 0·1 g phospholipid/kg was present in the control powder.

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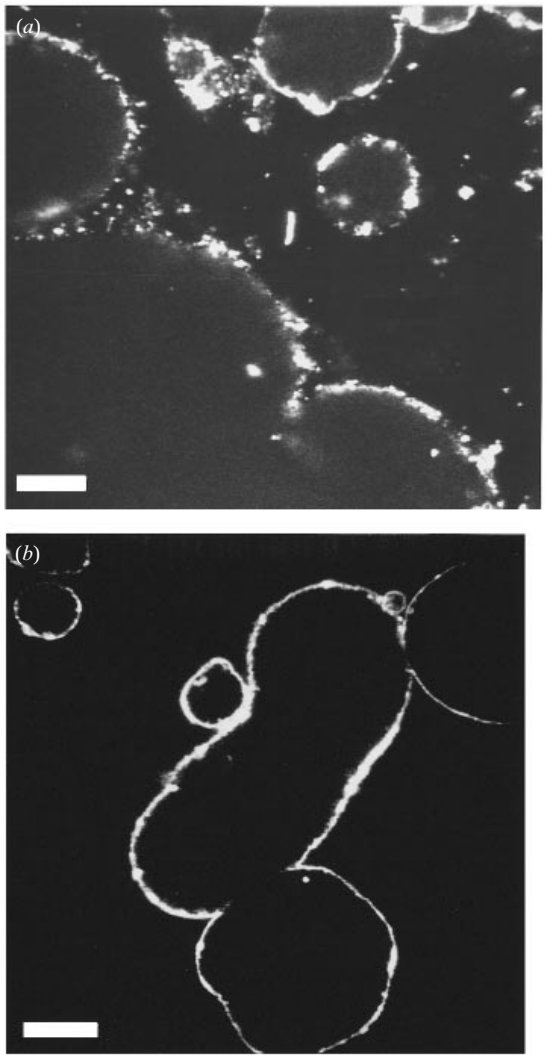

Fig. 5(*a*) and (*b*). For legend see facing page.

Initially, the WMP containing the added phospholipid wetted faster (16 s) than the same powder without the added phospholipid (64 s). After storage, the wetting times were similar for both powders (> 90 s).

The use of a glycerol-based mounting medium for examination of the surface phospholipid layer resulted in this layer being stripped off the surface of the WMP particle (Fig. 5*a*). The use of immersion oil resulted in a clear image of the fluorescent phospholipid around the surface of the powder particle (Fig. 5*b*), showing that a completely non-aqueous mounting medium was necessary for this layer to be retained. Storage of treated powder for 6 months, including 1 month at 37 °C, resulted in the pooling of this surface layer at the junction of agglomerated particles (Fig. 5*c*). The fluorescent phospholipid also appeared to become absorbed into the WMP, probably through cracks during high temperature storage. It is likely that storage of milk powder at temperatures that allow the wetting agent to partly melt and migrate around the surface of the WMP particle may explain the reduction in

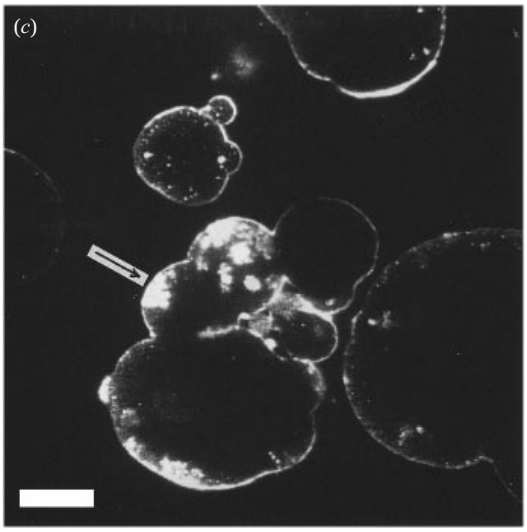

Fig. 5. Whole milk powder particle with fluorescent phospholipid layer (*a*) examined in a glycerol-based mounting medium, (*b*) examined in immersion oil (note the good surface coverage of the fluorescent layer on the powder particle) and (*c*) after 30 d storage at 37 °C; the arrow shows a region where the fluorescent phospholipid appears to have entered into the powder particle. All scale bars, 20 μm.

powder wettability. At 37 °C, nearly all (> 99 %) of the fat in anhydrous milk fat is in the liquid state (MacGibbon & McLennan, 1987) and is therefore able to move to regions of lower surface tension, i.e. the junction of agglomerated particles or into cracks on the particle surface. This may reduce the amount of surface phospholipid (which is presumably carried with the fat) that is available to act as an amphiphilic wetting agent. Storage at lower temperatures would reduce the amount of liquid fat, thus reducing its mobility, and may improve the wettability of the powder over longer storage periods.

The application of CLSM to WMP overcomes many difficulties associated with the microscopic examination of milk powder, such as inability to focus on a thin focal plane (light microscopy), inability to locate some of the components (scanning electron microscopy) and long preparatory techniques (TEM). CLSM requires only simple sample preparation and then allows non-invasive preparation of optical sections from below the surface of the WMP sample. However, for examining detailed protein–protein and protein–fat interactions in WMP only TEM will suffice.

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