Novel Analytical Techniques for Studying the Milk Fat Globule Membrane

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Jacobus Meindert Evers

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Abstract

Fat in milk and cream is present as tiny droplets, which are each enveloped in a thin membrane, called the milk fat globule membrane (MFGM). The MFGM can easily be damaged by factors such as pumping the milk and applying other forms of agitation. MFGM damage is believed to reduce processing efficiency and compromise the quality of manufactured products.

A comprehensive review of the literature showed that our understanding of changes occurring in the MFGM post secretion of the fat globule by the mammary secretory cell is still rudimentary. Furthermore, it was found that a fundamental understanding of MFGM damage in raw milk is lacking. Hence, this study sought to develop analytical techniques for studying the MFGM.

Fluorescent probes were identified that associated with the MFGM (bovine, ovine, human) in one of two ways: either by embedding in the phospholipid bilayer (lipophilic probe) or by binding to carbohydrate moieties of glycosylated chains in the glycocalyx (lectin probes). The use of these probes, in combination with either conventional fluorescence microscopy or confocal laser scanning microscopy, allowed 2-D images and 3-D images of fat globules to be made. Application of water-soluble lipophilic probes and the lectin wheat germ agglutinin (WGA) directly to milk allowed the staining of the MFGM in its native environment. Variable distribution patterns of the probes in the MFGM were observed, which suggests that the MFGM of fat globules in harvested milk is structurally and chemically heterogeneous both within and among globules from the same species and between species, and even among fat globules within the milk of an individual animal. Furthermore, the binding behaviour of WGA to the MFGM of native fat globules (in bovine milk) and washed fat globules (in model systems) following heat treatment implicated β-lactoglobulin, α-lactalbumin, immunoglobulin M and/or the glycosylated proteins Periodic acid Schiff 6/7 in the disappearance of fat globule aggregation upon elevated heat treatment of milk. The results of the current study showed that the use of membrane-specific fluorescent probes, particularly in combination with confocal laser scanning microscopy, has significant potential for providing real time structural and chemical information about the MFGM in matrices such as harvested milk and milk products.

In addition to the fluorescence microscopy techniques, development of other techniques was also conducted. Flow cytometry was shown to have significant potential for the quantitative determination of various properties of fat globules and their membranes. Although no suitable sample preparation technique could be developed in this study, atomic force microscopy is believed to have significant potential for studying structural and physical properties of the MFGM. Selective harvesting of individual fat globules was shown to be possible by using a micromanipulator. In future work, this technique is expected to be used in combination with fluorescence microscopy, or atomic force microscopy.

The present study has shown that the development and application of novel analytical techniques has advanced, and in the future will further advance, understanding of the MFGM.

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The great Creator of the universe of which I was privileged to study a tiny, tiny part. Delving into the microscopic world I found this to be true, "For the invisible things of him from the creation of the world are clearly seen, being understood by the things that are made, even his eternal power and Godhead" (Romans 1:20). Marvellous are thy works; and that my soul knoweth right well (Psalm 139:14b).

Take my life, and let it be
consecrated, Lord, to thee;
take my intellect, and use
every power as thou shalt choose.[1]

[1] Abridged from Frances Ridley Havergal (1874).

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List of abbreviations

5,5'-Ph_2-$DiIC_{18}(3)$	1,1'-Dioctadecyl-5,5'diphenyl-3,3,3',3'-tetramethylindocarbocyanine chloride
AFM	Atomic force microscope/microscopy (depending on context)
BSA	Bovine serum albumin
BTN	Butyrophilin
CD36	Cluster of differentiation 36
CLSM	Confocal laser scanning microscope/microscopy (depending on context)
ConA	Concanavalin A
DiA	4-(4-(Dihexadecylamino)styryl)-*N*-methylpyridinium iodide
$DiIC_{18}(3)$	1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
$DiIC_{18}(3)$-DS	1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-5,5'-disulfonic acid
$DiOC_{18}(3)$	3,3'-Dioctadecyloxacarbocyanine perchlorate
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
D109	5-Dodecanoylaminofluorescein
FFA(s)	Free fatty acid(s)
FFMR	Free frozen milk fat for recombining
FITC	Fluorescein isothiocyanate
FM1-43	*N*-(3-Triethylammoniumpropyl)-4-(4-(dibutylaminostyryl)pyridinium dibromide
FM1-84	*N*-(3-Triethylammoniumpropyl)-4-(4-(dipentylaminostyryl)pyridinium dibromide
FM4-64	*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)-pyridinium dibromide
GlcNac	*N*-Acetyl-D-glucosamine
GMP	Glycomacropeptide
Ig(G, M)	Immunoglobulin (G, M)
MDCK cells	Madin-Darby canine kidney cells
MFGM	Milk fat globule membrane
MUC1	Mucin 1
NANA	*N*-Acetylneuraminic acid
NMR	Nuclear magnetic resonance
N316	6-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid
PAS 6/7	Periodic acid Schiff 6/7
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SP- $DiIC_{18}(3)$	1,1'-Dioctadecyl-6,6'-di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine
SP- $DiOC_{18}(3)$	3,3'-Dioctadecyl-5,5'-di(4-sulfophenyl)-oxacarbocyanine, sodium salt
TEM	Transmission electron microscopy
UHT	Ultra-high temperature
WGA	Wheat germ agglutinin
WPI	Whey protein isolate
XO	Xanthine oxidase

1 General introduction, objectives and format of the thesis

1.1 General introduction

Fat in milk and cream is present as tiny droplets, which are each enveloped in a thin membrane, called the milk fat globule membrane (MFGM). This membrane protects the fat inside the droplet and also prevents the fat from separating from the milk as an oily layer. However, the MFGM can easily be damaged by factors such as pumping the milk and applying other forms of agitation. In turn, MFGM damage is believed to cause practical problems, resulting in reduced processing efficiency (e.g., through fouling of manufacturing equipment) and compromised quality of manufactured product (e.g., off-flavours in certain products). Such problems significantly affect business profitability for milk processors.

Although some progress has been made over the last 40 years, understanding of the molecular structure of the MFGM is still rudimentary. Moreover, a fundamental understanding of MFGM damage in raw milk or cream is lacking. This lack of fundamental information: (1) significantly hampers efforts to make efficiency gains in milk and cream processing, and (2) prohibits the building of the understanding needed to underpin the development of new products.

1.2 Objectives

Given the lack of fundamental understanding of the composition and structure of the MFGM, and the need for analytical methodology for studying changes in the MFGM, the present study focused on developing a range of analytical techniques for measuring compositional and structural changes qualitatively (i.e., kind of change) and, if possible, quantitatively (i.e., degree of change) in model and field studies.

The supposition underpinning this work was that the MFGM of individual fat globules is not homogeneous, and differs between different fat globules. The challenges then were: (1) to develop non-invasive and non-damaging analytical techniques to investigate intrinsic heterogeneity within and between the MFGMs of milk from cows and from other species, and (2) to apply these techniques to monitor changes in the MFGM resulting from mechanical, heat and/or other treatments. Such changes may lead to altered characteristics of milk and/or milk products. The current study focused mainly on the first challenge; the second challenge should be addressed in future work.

The intermediate objectives included the following:

(1) To summarise and critique current knowledge on changes in the MFGM from the secretory cell to the harvested and treated milk;

(2) To summarise and critique published methodology for studying aspects of, or changes to, the MFGM;

(3) To advance the "analytical toolbox" by developing a range of new physico-chemical methods that either would provide new insights into the structure or composition of the MFGM, or that could be used to measure changes to it;

(4) To demonstrate the potential of the developed methods for advancing knowledge of the structure and/or composition of the MFGM.

1.3 Format of the thesis

An initial assessment of published papers on the subject of the MFGM revealed a significant gap in the scientific literature: although the origin and secretion of fat globules, as well as the effects of milk handling and treatment of harvested milk, were well covered, a systematic overview of the *changes occurring in the MFGM* was lacking in the literature. The literature review of the current study aimed to fill this gap. In addition, considering that the overall aim of the current study was to develop new analytical techniques, a thorough review and critique of analytical methods used in MFGM research was undertaken. Hence, the overall literature review resulted in two distinct, yet related, parts, which are incorporated in this thesis as Chapter 2 and Chapter 3 respectively[1].

Chapters 4–6 describe the development and application of fluorescent probes that stain the MFGM. Chapter 4 outlines the probe screening programme and describes the development of 2-D and 3-D fluorescence microscopy techniques. Chapter 5 describes the validation and application of the lectin wheat germ agglutinin (WGA). This chapter differs from Chapters 4 and 6 in two ways. First, Chapters 4 and 6 focus on the study of the structure and composition of the MFGM in its *native environment* (i.e., milk). However, to study the interactions between the MFGM, WGA and milk plasma components, several experiments in Chapter 5 involved the use of *model systems*. Secondly, part of the work in Chapter 5 is described chronologically, because of the scoping nature of the work. This was done to make it easier for the reader to understand the background of, and rationale for, the various experiments. Chapter 6 provides a preliminary evaluation of structural and compositional features of the MFGM as revealed by fluorescence microscopy.

Chapter 7 details development work on three additional analytical techniques, namely atomic force microscopy (AFM), a micromanipulation technique and flow cytometry. One of the main aims of this chapter is to sketch potential future developments in analytical techniques for MFGM research. The work described in this chapter will serve as the basis of such future work.

Finally, Chapter 8 assesses the outcomes of this study in the context of MFGM research and recommends directions for future work using the developed techniques.

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1 These chapters have been published as two separate papers (Evers, 2004a, b). Relevant literature published since 2004 has been discussed in Chapters 4–8.

2 The MFGM — compositional and structural changes post secretion by the mammary secretory cell

The content of this chapter was published in the International Dairy Journal (2004), 661–674.

2.1 Introduction

Fat in milk is present as more or less spherical globules. These fat globules are enveloped in a layer of surface-active material, which is called the MFGM. The MFGM consists of many different compounds, mainly phospholipids, glycolipids and proteins, many of the latter being enzymes. The origin, composition and structure of the MFGM have been the subject of many literature reports, and these have been reviewed at various times (e.g. reviews published in the last three decades include Mulder & Walstra, 1974; Anderson & Cawston, 1975; Patton & Keenan, 1975; Patton & Jensen, 1976; Keenan *et al.*, 1983a; McPherson & Kitchen, 1983; Walstra & Jenness, 1984; Keenan & Dylewski, 1985; Keenan *et al.*, 1988; Kanno, 1990; Keenan & Dylewski, 1995; Keenan & Patton, 1995; Mather & Keenan, 1998; Danthine *et al.*, 2000; Keenan, 2001; Keenan & Mather, 2002; Ollivier-Bousquet, 2002).

The MFGM is believed to change post secretion by the mammary secretory cell, but little is known about the factors that cause such changes (Keenan & Mather, 2002). Consequently, this review does not aim to provide an updated version of the above-cited reviews, but rather focuses on how the MFGM may change post secretion by the mammary secretory cell.

It is useful to distinguish between two different stages in which changes in the MFGM can occur. The first stage is when the fat globules are still in the mammary gland. This is discussed in Section 2.2. Section 2.2.1 summarises current knowledge of the origin and secretion of milk fat globules. The main purpose of this section is to provide a brief background for the subsequent discussions on changes in the MFGM. For a more in-depth overview of the origin and secretion of milk fat globules, the reader is referred to the above-quoted reviews and papers cited therein. Section 2.2.2 discusses changes in the MFGM during and after secretion by the mammary secretory cell, but before extraction of the milk from the mammary gland.

The second stage concerns changes in the MFGM as a consequence of milk harvesting and subsequent milk handling and treatment. Aspects of this (refer to Table 2.1 for a summary) are discussed in Section 2.3.

2.2 Formation and secretion of milk fat globules by the mammary secretory cell

2.2.1 Origin of milk fat globules

Intracellular fat globule precursors appear to originate from the endoplasmic reticulum (Zaczek & Keenan, 1990) and to assemble into globules of various sizes ranging from less

than 0.2 μm to greater than 8 μm (Mather & Keenan, 1998; Ollivier-Bousquet, 2002), which migrate through the mammary secretory cell to the apical plasma membrane by, as yet, unidentified mechanisms[1] (Keenan, 2001; Ollivier-Bousquet, 2002). The intracellular fat globule is surrounded by a diffuse interfacial layer, the composition of which includes phospholipids, glycosphingolipids, cholesterol and proteins (Keenan *et al.*, 1970; Hood & Patton, 1973; Keenan *et al.*, 1983a; Kanno, 1990; Keenan & Dylewski, 1995), but not β-carotene[2] (Keenan *et al.*, 1970). The distribution of cholesterol between the fat globule core lipid and the fat globule interfacial layer has not been established (Keenan & Dylewski, 1995).

When the fat globule approaches the plasma membrane, a dense-staining layer of 10–20 nm between the fat globule and the plasma membrane is seen by electron microscopy (Wooding, 1971a). This layer consists mainly of protein, including xanthine oxidase (XO), butyrophilin (BTN), adipophilin (Keenan *et al.*, 1982; Mather & Keenan, 1998) and possibly a class of low molecular mass guanosine triphosphate-binding proteins (Keenan & Dylewski, 1995; Ollivier-Bousquet, 2002). The generally accepted mechanism for the excretion of the fat globule from the cell (a process that is sometimes called "budding") is via progressive envelopment of the fat globule by the apical plasma membrane of the secretory cell. The latter is a true bilayer membrane.

Hence, the MFGM originates from several distinct layers with total thickness varying between approximately 10 and 20 nm (Walstra *et al.*, 1999). As viewed from the lipid core outwards, there is first an inner surface-active layer that surrounds the intracellular fat droplet, then a dense proteinaceous coat located on the inner face of the bilayer membrane and finally a true bilayer membrane (Keenan & Mather, 2002). In electron micrographs of globules that were in the process of being secreted by the cell (Henson *et al.*, 1971; Wooding, 1971a; Bauer, 1972; Freudenstein *et al.*, 1979), the dense coat and the innermost interfacial layer could not be distinguished from one another. Whether this was due to limitations in electron microscopy technology, to a merging of the two layers into one coat or to loss of membrane material is not clear (Keenan & Mather, 2002).

As the bilayer membrane of the MFGM is derived from the apical plasma membrane of the secretory cell, the most widely accepted model for this type of membrane would be the fluid mosaic model (Figure 2.1). Some corroborating evidence for the applicability of the fluid mosaic model to the MFGM may be derived from nuclear magnetic resonance (NMR) studies, which indicate that MFGM proteins have a highly ordered structure (Chandan *et al.*, 1972). Further, the very low interfacial tension between the fat globule core and the milk plasma that results from the presence of the MFGM (Phipps & Temple, 1982) is indicative of a somewhat ordered membrane (van Boekel & Walstra, 1989).

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1 A proteomics approach may substantially progress the knowledge in this area (Wu *et al.*, 2000).

2 The MFGM of fat globules in secreted milk is also devoid of β-carotene (Patton *et al.*, 1980a).

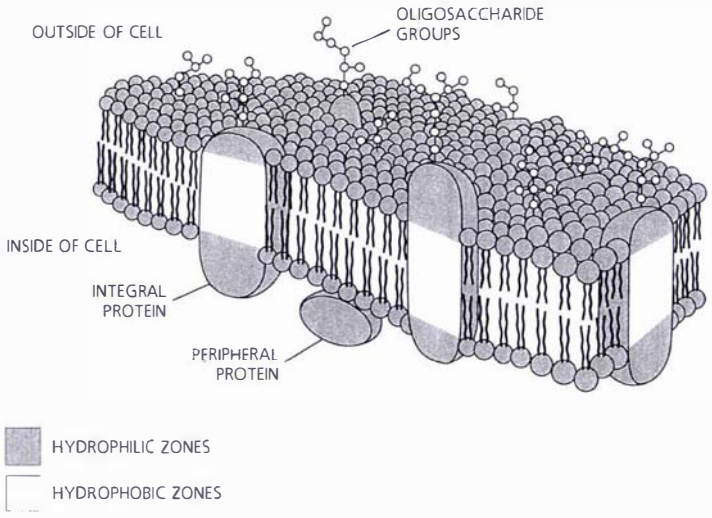

Figure 2.1 Schematic of the fluid mosaic membrane according to Singer & Nicolson (1972). This model suggests that the phospholipid bilayer serves as the backbone of the membrane, which exists in a fluid phase. Peripheral membrane proteins are partially embedded or loosely attached to the bilayer. Transmembrane proteins extend through the lipid bilayer. Figure reproduced from Parola (1993) with permission, Wiley-VCH, Weinheim, Germany.

There is evidence that the proteins of the MFGM are arranged asymmetrically in the MFGM (Mather & Keenan, 1975; Kanno, 1990). Some membrane enzymes are known to have their active sites accessible from one face, but not from both faces, of the phospholipid bilayer (e.g., 5'-nucleotidase: outer face; Mg^{2+}-adenosine triphosphatase: inner face; reviewed by Keenan *et al.*, 1983a). Likewise, several membrane proteins are predominantly exposed either on the inner face (e.g., XO; Keenan & Patton, 1995) or on the outer face (e.g., periodic acid Schiff (PAS) 6/7; Mather & Keenan, 1998) of the membrane. Other proteins are likely to be transmembrane proteins (e.g., BTN; Jack & Mather, 1990). Carbohydrate moieties appear to be uniformly distributed over the external membrane surface as shown both morphologically, using transmission electron microscopy (Horisberger *et al.*, 1977), and biochemically (references cited by Keenan *et al.*, 1983a).

On the basis of knowledge of various cellular membranes, phospholipids, like proteins, are also expected to be asymmetrically arranged. However, concrete evidence for this in the MFGM has not yet been forthcoming (Keenan & Mather, 2002).

Table 2.1 Summary of various factors and their effects on the bovine MFGM after the milk leaves the udder.

Factor	Effect	Comments/Reference(s)
Air bubbles	Substantial loss of membrane material	Walstra & Jenness (1984)
Cooling	Loss of copper	Mulder & Walstra (1974)
	Loss of phospholipids	Baumrucker & Keenan (1973); Patton *et al.* (1980b)
Heating	Adsorption of copper	Mulder & Walstra (1974)
	Adsorption of whey proteins	Iametti *et al.* (1997)
	Aggregation of BTN and XO	Ye *et al.* (2002)
	Loss of PAS 6/7	Lee & Sherbon (2002)
	Loss of phospholipids (?)	Koops & Tarassuk (1959); Greenbank & Pallansch (1961); Houlihan *et al.* (1992)
Ageing	The effects depend on temperature	Little is known about how the MFGM is affected by ageing as a function of temperature
Agitation	Depends on the degree of air incorporation. High shear forces are required to change the MFGM in the absence of air	Mulder & Walstra (1974); Stannard (1975); Te Whaiti & Fryer (1975); Miller & Puhan (1986b)
Bacterial growth	Production of lipases, phospholipases, proteinases or glycosidic hydrolases may change the MFGM	McPherson & Kitchen (1983)
Stage of lactation/season	Affect the amount of membrane material, fat globule size distribution and probably relative distribution of membrane components	Few controlled studies investigating the effects of stage of lactation, season and other factors have been reported in the literature

2.2.2 Changes in the MFGM during and after secretion by the mammary secretory cell

At the present time, very few facts are available regarding changes that might occur in the MFGM post secretion from the mammary secretory cell. Reported observations are based mainly on microscopy techniques, although some biochemical data are also available. Electron microscopy observations indicate that MFGM isolated from the fat globules of harvested milk tends not to vesiculate, in contrast to isolated plasma membrane, which tends to vesiculate (Keenan *et al.*, 1970). The former phenomenon is presumably due to the presence of the dense layer at the inner face of the phospholipid bilayer (McPherson & Kitchen, 1983). This and other observed morphological differences, such as a lack of intramembranous particles (probably membrane proteins; Peixoto de Menezes & Pinto da Silva, 1978; Hui & Boni, 1991) in the plasma membrane at locations where fat globules are budding (Peixoto de Menezes & Pinto da Silva, 1978; Zerban & Franke, 1978), suggest that a re-arrangement of constituents within the apical plasma membrane/MFGM occurs upon secretion of the fat globule by the mammary secretory cell. The clearing of intramembranous particles in the membrane during budding may increase local membrane elasticity (Zerban & Franke, 1978; Freudenstein *et al.*, 1979), but the phenomenon is not consistent, as the MFGM of some extracellular fat globules does contain intramembranous particles, albeit at a reduced density (Banghart *et al.*, 1998). The reason for the inconsistency is not known.

Similarly, the process of loss of material from the membrane after secretion of the fat globule by the secretory cell is unclear. Morphological observations of excreted fat globules suggest that gradual loss or restructuring of membrane material occurs during the sojourn of the globule in the secretory alveolus and its passage into expressed milk (Henson *et al.*, 1971; Wooding, 1971a, b; Bauer, 1972). At least some loss appears to occur through either dissolution or vesiculation (i.e., the formation of small, microsome-like particles that are subsequently dislodged from the fat globule, a phenomenon called blebbing) (Wooding, 1971b; Zerban & Franke, 1978). However, on the basis of biochemical analysis of harvested milk, the validity of these interpretations has been questioned and the possibility that the supposed losses of membrane material may, at least partly, have been artifacts of the electron microscopy fixation process used should be considered (Bauer, 1972; Baumrucker & Keenan, 1973; Patton, 1973; Keenan *et al.*, 1983a; Mather & Keenan, 1998). Nevertheless, as the apical plasma membrane of the mammary secretory cell is a highly dynamic membrane, the fat globule secretion process is expected to involve many biochemical and biophysical changes. Hence, there will be significant rotational and lateral diffusion, and possibly trans-layer movement (flip-flop), of membrane compounds. Furthermore, there will also be changes in the curvature of the bilayer membrane (the degree of which will depend on the size of the fat globule), as well as changes in electrostatic properties, which are caused mainly by the changed environment of the inner bilayer leaflet. All these changes suggest a highly dynamic process at the molecular level.

These binding and re-arrangement processes may continue for some time after the fat globule has been secreted into the alveolus, but could also be rapid. The extent of change will depend on a balance of energy and entropy (van der Meer, 1993) and will be affected by such factors as local surface tension, the kind of interaction and bonding between membrane constituents, and the diffusion/mobility rates of individual components in the different media (i.e., core fat, different membrane layers and milk plasma).

Although, at the present time, membrane blebbing should be considered a possible process of post-secretion changes in the MFGM, both in the alveolar lumen and in the harvested milk (Horisberger *et al.*, 1977; Zerban & Franke, 1978; Pinto da Silva *et al.*, 1980), confirmation of the phenomenon should be sought by alternative microscopy techniques to corroborate the interpretations that have been based on electron microscopy observations alone.

In general, the sometimes conflicting opinions of authors (e.g., Kobylka & Carraway, 1973; Patton, 1973; Shimizu *et al.*, 1979; Keenan *et al.*, 1983a) and the paucity of reliable data regarding the MFGM composition and the structure of fat globules post secretion by the mammary secretory cell, as a function of time and other variables, make this area ripe for further study. Future research, using techniques such as confocal laser scanning microscopy (CLSM), atomic force microscopy (AFM) and fluorescence methods, should shed new light on these aspects and contribute to settling existing controversies.

2.3 Changes in the MFGM and fat globule during and after milk harvesting

Changes in the MFGM during and after milk harvesting are affected by a number of factors, which may be divided arbitrarily into three groups, viz. physiological, chemical/enzymic and physical/mechanical (Kirst, 1996). Such changes may manifest themselves as loss of membrane components, adsorption of milk plasma components and chemical or enzymic reactions (Walstra & Jenness, 1984), which, in turn, may affect the stability of the fat globule.

Physiological factors include the diet of the cow, breed, fat globule size and stage of lactation (Anderson & Cawston, 1975; McPherson & Kitchen, 1983). These affect the stability of the fat globule (Te Whaiti & Fryer, 1975; Kirst, 1996; Deeth, 1997; Walstra *et al.*, 1999), but the relationship between these factors, the MFGM composition and fat globule stability is extremely complex. The present review limits itself to a discussion of fat globule size distribution and stage of lactation only.

Physical and mechanical factors arise in milk handling during and after milk harvesting. Pre-factory milk handling involves air inclusion, agitation of the milk (pumping and stirring), changes in temperature and changes in time (ageing of the milk). Handling of the milk at the factory involves ageing, agitation, air inclusion and temperature changes; deliberately applied treatments or processes include separation, heat treatments (e.g., pasteurisation), homogenisation and changes in water content (McPherson & Kitchen, 1983).

Like the physiological factors, these physical factors may effect compositional and structural changes in the MFGM and hence the stability of the fat globules. Some of these factors are discussed in more detail below. However, it is noted that there has been little research in this area dealing specifically with the MFGM, because of the complex variables involved (Keenan & Patton, 1995).

The effects of heat treatment on enzymic changes and on chemical changes in the MFGM have been reviewed by Houlihan (1992) and by van Boekel & Walstra (1995) respectively, and are not reviewed separately here. Instead, because enzymic and chemical reactions are often affected significantly by physical or mechanical factors, comments are included in the discussions on physical and mechanical factors where relevant.

2.3.1 Some physical and mechanical factors affecting the stability of fat globules

This section comprises a discussion of changes in the MFGM as a consequence of milk handling and treatment. Aspects of what is commonly called "fat globule damage" in relation to specific measurement methods are discussed in Chapter 3.

It is noted that much of the knowledge about the structure and molecular organisation of the MFGM of fat globules in harvested milk (Figure 2.2) has been obtained using morphological or biochemical techniques applied to isolated membranes. In many studies, MFGM material was obtained from milk by either physical separation techniques (e.g., churning, repeated freezing and thawing) or chemical techniques, such as those using surfactants to destabilise the fat globules, or washing techniques using water, sucrose solutions and/or buffers followed by a separation technique (usually centrifugation). Therefore, in most cases, the results obtained concerned fat globules that had undergone substantial mechanical treatment and whose external environment had been substantially changed. Different results were obtained depending on the isolation techniques and conditions used (Anderson & Brooker, 1975; Bhavadasan & Ganguli, 1976, 1977; Mather *et al.*, 1977; Yamauchi *et al.*, 1978; Keenan *et al.*, 1988). Hence, the results reported on structure or composition may only partially represent the membrane present on fat globules in their native environment, i.e. milk plasma (Wooding, 1971b; McPherson & Kitchen, 1983; Walstra, 1985). This significantly complicates the interpretation of the results reported in the literature comparing the MFGM of fat globules in harvested milk with that of extracellular fat globules in the mammary gland. Consequently, although models have evolved over time (for a comparison of different models, see Danthine *et al.*, 2000), the structure of the MFGM still remains a poorly understood aspect of the milk fat globule (Walstra *et al.*, 1999).

Another confounding factor is that the MFGM composition may vary significantly between milks of different animals and even between different milkings of the same animal (Walstra & Jenness, 1984), and therefore the same mechanical treatment of milk from different origins does not necessarily effect the same changes in the MFGM or in the fat globules (Miller &

Puhan, 1986b). This further complicates the comparison of results of different studies that investigated the effect of the physical or mechanical factors on the stability of the fat globule, such as those discussed below.

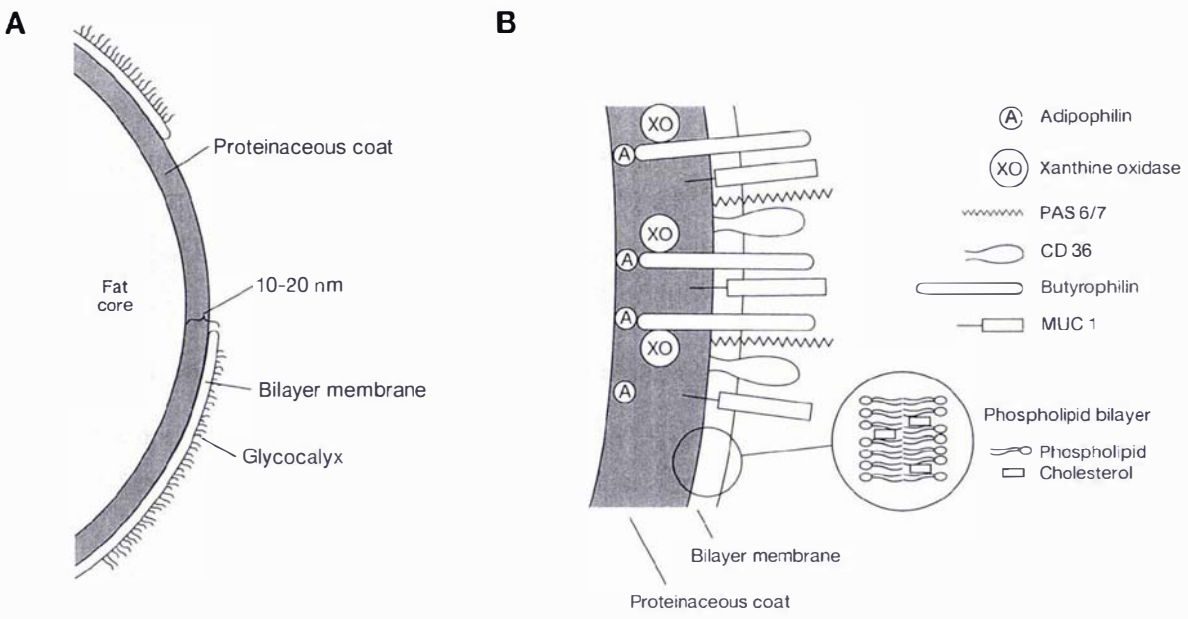

Figure 2.2 Overviews of the structure of the MFGM. Highly schematic. Drawings not to scale. (A) Schematic showing the core fat, the proteinaceous coat, the bilayer membrane and its glycocalyx (a macromolecular layer formed by the oligosaccharides of the glycolipid head groups and the branched polypeptide/oligosaccharide head groups of the glycoproteins). (B) Schematic of predicted topologies of some proteins. Adapted from Mather & Keenan (1998) and Michalski *et al.* (2001b). MUC 1 (Mucin 1) = heavily glycosylated mucin-like glycoproteins. CD (cluster of differentiation) 36 = Integral MFGM protein, heavily glycosylated.

Gas bubbles

Air is incorporated into milk at various points during milk handling and processing. Sometimes, the presence of air is deliberate and wanted (e.g., in buttermaking), but usually it is deemed to be undesirable. Mixing of milk or cream with air, or any gas (Stannard, 1975; Tolle & Heeschen, 1975), can significantly reduce the stability of the fat globules (Te Whaiti & Fryer, 1975; Miller & Puhan, 1986b). In this process, it is envisaged that, when a milk fat globule and an air bubble come in contact with each other, the MFGM is ruptured. Consequently, the membrane material and (part of) the core fat will spread over the air/milk plasma interface (Figure 2.3) and will be released into the milk plasma when air bubbles collapse or coalesce (Walstra & Jenness, 1984; van Boekel & Walstra, 1989).

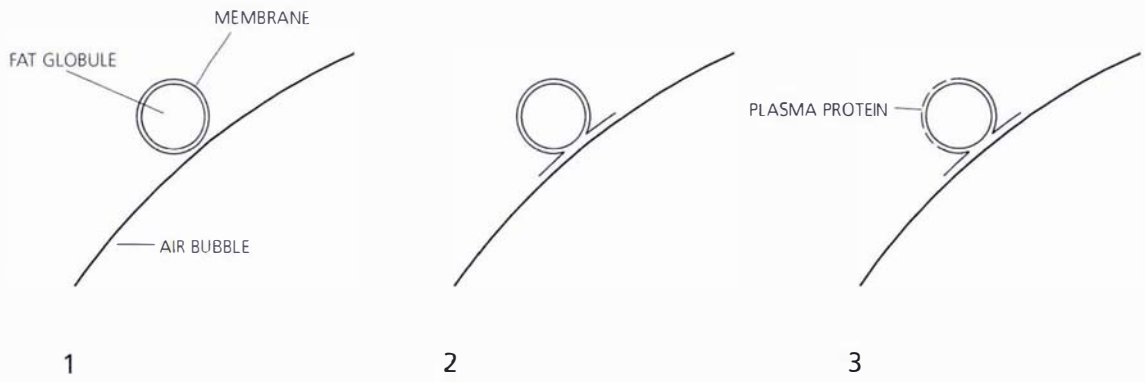

Figure 2.3 Schematic overview of fat globule membrane damage by air (adapted from Mulder & Walstra (1974). (1) A fat globule and an air bubble make contact. (2) Shearing of membrane material and consequent spreading of the material over the milk plasma/air interface. (3) Adsorption of milk plasma protein to MFGM-depleted areas of the fat globule. Refer to Walstra & Jenness (1984) for a discussion of the theoretical aspects of the physico-chemical phenomena involved.

Significant changes in the MFGM caused by mixing milk and air can occur readily on the farm. Most of these changes occur in milking machines in which air is used as the transport medium for the milk (e.g., Salvatierra *et al.*, 1978; Kirst, 1981; Evers & Palfreyman, 2001). Rough treatment of the milk may be evident visibly by the presence of foam on top of the milk in the farm bulk tank (Deeth & Fitz-Gerald, 1976; Flückiger, 1987). Although indicative of MFGM damage, this does not necessarily result in significantly increased concentrations of free fatty acids (FFAs) by lipolysis (Evers & Palfreyman, 2001), because this is also dependent on other variables, such as the activity of the native lipase (see Chapter 3).

Agitation

The effect of agitation (stirring and pumping) is dependent on other factors such as temperature, the presence of air and the fat content. At temperatures below 40°C, fat crystals start to form in the fat core of the globule. Upon deformation of the fat globule, such fat crystals can cause local structural changes to the membrane, for example by piercing it. This can lead to fat globule aggregation and partial coalescence (Hinrichs, 1994; Walstra *et al.*, 1999). Studies in which the volume of incorporated air was reduced or eliminated showed that the presence of air is by far the most significant factor causing changes in the MFGM (Mulder & Walstra, 1974; Stannard, 1975; Te Whaiti & Fryer, 1975; Miller & Puhan, 1986b). In turn, this suggests that little change in the MFGM occurs by agitation in the absence of air, unless high shear forces are present.

Large fat globules are more susceptible to shear stress than smaller fat globules (Hinrichs & Kessler, 1995; Wiking *et al.*, 2003). For disruption, this can be readily derived from the ratio of viscous stress to Laplace pressure for a fluid particle,

$G \eta d / \gamma$

where G is the velocity gradient (s^{-1}), η is the viscosity (Nsm^{-2}) of the milk plasma, d is the diameter (m) of the fat globule, and γ is the interfacial tension (Nm^{-1}) (Walstra & Jenness, 1984). This shows that an increase in size of the fat globule increases this ratio. Disruption of fat globules occurs when approximately $G \eta d / \gamma > 0.5$, which requires high velocity gradients (Walstra & Jenness, 1984). In practice, high shear forces can occur in poorly designed pumps, pipelines and other equipment (Lehman, 1982) and they result in the adsorption of plasma protein, notably casein, to the fat globule surface (Michalski *et al.*, 2001b). Wiking *et al.* (2003) showed that the resistance of fat globules against coalescence during pumping is determined by a combination of factors including fat globule size, fat content, temperature of the milk and shear rate.

Following harvesting and storage of the milk, agitation of the milk by pumping from the farm bulk tank to the milk tanker can, in principle, cause further damage to the fat globules. However, with the use of properly designed equipment, changes in the MFGM and fat globules, as measured by FFA levels and fat globule size distribution, should be limited (Evers *et al.*, 2001b). Transport of the milk by truck or train to the factory would be expected to effect further changes in the MFGM, but, as no experimental data appear to be available in the literature, the extent of these changes is unknown.

Temperature and ageing

In the dairy industry, manipulation of the temperature of milk is employed to safeguard the quality of milk and to influence the properties of processed milk products. For example, to improve its keeping quality, milk is cooled at the farm and heat treated at the factory. Further heat treatment at the factory is performed to aid processing and to manufacture products having a certain water content. However, heating, cooling and ageing of milk can effect physical changes in the MFGM and fat globules, and these are discussed below.

Temperature effects on adsorption of components from the milk plasma and desorption of membrane components

The composition of the MFGM can change by adsorption of surface-active milk plasma constituents and selective, or non-selective, desorption of membrane components (Anderson *et al.*, 1972; Buchheim, 1986; van Boekel & Walstra, 1989; Houlihan, 1992). In turn, this may affect other properties of the fat globule such as the electrokinetic, or zeta (ζ), potential (Walstra, 1983) and stability.

The rate of adsorption or desorption will vary with temperature, the medium (fat, MFGM or milk plasma) as well as the concentration and type of compound. The adsorption of surface-active components from the milk plasma on to fat globules that are wholly or partially denuded is estimated to be particularly fast, i.e. about 0.01 s (Walstra, 1995).

Cold storage of fresh raw milk at 8°C resulted in about 10% loss of phospholipids from the MFGM over 96 h, but no loss of 5'-nucleotidase or adenosine triphosphatase activity was detected (Baumrucker & Keenan, 1973). The ageing of bovine milk at 2–4°C for 24 h caused, on average, no change in the cholesterol content of the skim milk (Patton *et al.*, 1980b). These authors concluded that this did not prove that on average no cholesterol was lost from the MFGM, because there could have been an equilibrium between membrane cholesterol and milk plasma cholesterol. However, this hypothesis remains to be tested. Furthermore, although these authors did discuss the release of cholesterol into the skim milk phase, they did not consider the possibility of cholesterol partitioning from the MFGM into the core fat of the fat globules. It would be useful if a study were conducted in which the distribution of cholesterol between the core fat, the MFGM and the milk plasma upon cold ageing was determined. Additionally, Patton *et al.* (1980b) reported an average increase of 18% in skim milk phospholipid content under the same experimental conditions, but large differences in individual milks were evident (range: from –4 to 514%), indicating highly variable responses of the individual milks. Although there are indications that cooling changes the structure and composition of the MFGM, from these studies it is not clear which of the two factors (cooling or ageing) has the greater effect. This aspect deserves further investigation.

Cooling of milk appears to induce the migration of copper from the MFGM to the milk plasma, whereas heating of milk has the reverse effect (Mulder & Walstra, 1974). The latter phenomenon may contribute to the oxidation of unsaturated fatty acids of membrane phospholipids (McPherson & Kitchen, 1983; van Boekel & Walstra, 1995). This appears to be so particularly for phosphatidyl ethanolamine, which contains 40–60% unsaturated fatty acids, of which about one-third is polyunsaturated, and which strongly binds copper (Allen & Humphries, 1977; Deeth, 1997). Oxidation of MFGM constituents may thus be expected to affect the fluidity and stability of the MFGM. However, considering that all processing equipment in modern dairy factories is made of stainless steel, rather than copper, it is questionable whether the effect of copper is a significant factor in changing the MFGM.

Losses of glycoproteins and/or glycosphingolipids appear to occur upon cooling, heating or pressurising milk, as these treatments resulted in a significant decrease in total carbohydrate in the MFGM of buffalo milk with large reductions (46–80%, depending on the treatment) being observed in sialic acid content (Bandyopadhyay & Ganguli, 1975). It appears that similar studies have not been performed on bovine milk and this would deserve attention in future investigations.

In cream, pasteurisation (exact temperature and time conditions not reported) resulted in the association of casein and, to a lesser extent, whey protein with the MFGM (Iametti *et al.*, 1997). However, in this study, the heating effect may have been confounded with the effect of mechanical damage through pumping and circulation through the pasteurisation plant (Iametti *et al.*, 1997). Other authors (Dalgleish & Banks, 1991) concluded that the presence of casein is the result of mechanical treatment, not heat treatment.

Pasteurisation of cream also increased the β-lactoglobulin content of the MFGM, which is not surprising as the surface hydrophobicity of β-lactoglobulin is known to transiently increase when heated even at temperatures lower than those required for its insolubilisation or for its association with other milk components (Iametti *et al.*, 1997; Macej *et al.*, 2002). Also, at 60°C, a temperature lower than the denaturation temperatures of the whey proteins, BTN and XO started to aggregate, probably by forming intermolecular disulfide bonds (Ye *et al.*, 2002). At 65°C, serum proteins were reported not to interact with the MFGM in one study (Dalgleish & Banks, 1991), but it was demonstrated that they did in another study (Corredig & Dalgleish, 1996). At higher temperatures (70–90°C), significant association of serum proteins, particularly β-lactoglobulin, occurred (Dalgleish & Banks, 1991; Corredig & Dalgleish, 1996). For heating at 80°C similar results were found in other studies (Houlihan *et al.*, 1992; Lee & Sherbon, 2002). Furthermore, heating at 80°C resulted in the total loss of PAS 6 and partial loss of PAS 7 protein from the MFGM, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), but had no effect on the size and surface area of the fat globules (Lee & Sherbon, 2002).

Conflicting results have been reported regarding the effect of heating on phospholipids. Heating milk at 80°C for 20 min resulted in statistically significant losses of triacylglycerol, but not of phospholipid[3], from the MFGM (Houlihan *et al.*, 1992). This contrasts with earlier studies where heating at 80°C for 15 min, followed by cooling and separation, was reported to result in the loss of about 20% of phospholipids; at 90°C for 15 s, the figure was about 14% (Koops & Tarassuk, 1959). These results agree with the conclusion of Greenbank and Pallansch (1961) that loss of phospholipids is both temperature and time dependent. However, there is no agreement as to what degree phospholipids are lost from the MFGM upon either heating or cooling and further work should be conducted. This should include a systematic investigation of the effect of the separation technique used, as this appears to affect the degree of loss of phospholipids from the MFGM (Anderson & Brooker, 1975).

Few morphological results on the effect of the temperature and the ageing of milk on the structure of the MFGM have been reported in the literature. However, electron microscopy observations suggested that heat treatment, including ultra-high temperature (UHT)

3 The contention of the authors and van Boekel and Walstra (1995) that phospholipids migrated from the MFGM to the milk plasma was not supported by the reported data, as the results were not statistically significant at the significance criterion quoted ($P < 0.01$).

treatment, did not result in the release of the native membrane from the fat globule, which was contrary to the effects observed for cooling and stirring (Buchheim, 1986). Future morphological studies, using alternative microscopy techniques, should verify these observations.

Temperature and stability of fat globules

Cooling of milk can have a pronounced effect on the stability of the fat globules (Ismail *et al.*, 1972b; Anderson & Cheeseman, 1975; Deeth & Fitz-Gerald, 1978; Flückiger & Hänni, 1980; Miller & Puhan, 1986b; Kirst, 1996). However, in most studies reported in the literature, the process of cooling involved ageing as well as some form of agitation. The effects of these additional factors could have confounded the results and make it very difficult to establish the true effects of cooling on the MFGM.

Studies on the effect of holding milk at 4°C suggested that cold ageing affects the structure of the MFGM in a way that renders the membrane more susceptible to mechanical damage during membrane isolation steps (Anderson *et al.*, 1972) and thus, presumably, during milk harvesting and further milk handling. Furthermore, increased FFA levels upon cold storage of raw milk (Evers, 2003) are indicative of a changing MFGM. A reduced stability of the MFGM upon cold storage may be caused by the partially selective loss of certain proteins from the MFGM (Anderson & Cheeseman, 1975).

Heat-induced changes can either improve or impair the stability of the fat globules (van Boekel & Walstra, 1989), but results are not always in agreement. Fink (1986) and Fink & Kessler (1985a, b; 1986c), using 30% unhomogenised cream, concluded that the MFGM became more permeable after UHT treatment at 115–135°C. However, van Boekel & Folkerts (1991), using milk (4% fat) and two creams (of approximately 30% fat and 38% fat), could not reproduce Fink & Kessler's results and claimed that natural fat globules are remarkably stable against coalescence during UHT heating.

It is noted that, like the studies on the effect of cooling, the results of studies investigating the effect of heat treatment should be interpreted with care, as heating may be accompanied by some mechanical treatment such as agitation, which may lead to changes in fat globule size owing to coalescence or disruption. Furthermore, besides the intensity of heating, the temperature effect will also depend on the presence or absence of air (van Boekel & Walstra, 1995).

Te Whaiti and Fryer (1973) found that gelling[4] of cream was temperature dependent. These authors also reported an interaction between the aeration of milk and temperature[5] (Te

4 The phenomenon of gelling is called re-bodying by Walstra *et al.* (1999) and involves partial coalescence. For this to happen, warming to a temperature at which approximately 60% of the fat crystals melt is crucial.

5 Tests were carried out in the presence and absence of an air bubble by oscillating or shaking closed tubes at standardised temperatures.

Whaiti & Fryer 1974, 1975). In general, it was concluded that free fat, which was considered to be a measure of fat globule stability, was most readily released in the temperature range 15--40°C. Similar results for untreated and cooled raw milk were reported by Herbst *et al.* (1984), Lehman (1988b) and Kessler & Fink (1992).

The stability of the fat globules against coalescence is affected by the liquid:solid fat ratio, which in turn is affected by the temperature. Partial coalescence can occur readily provided that at least part of the fat is present in crystalline form (Mulder & Walstra, 1974). In the absence of crystals, full coalescence can occur, but this seldom happens in practice (Walstra *et al.*, 1999). Both partial coalescence and full coalescence result in a decreased fat globule surface area and hence the shedding of MFGM constituents into the milk plasma (Walstra, 1983). At low temperatures (< 7°C), the fat globules contain a high proportion of solid fat and are much more resistant to shear stress (Miller & Puhan, 1986b; Hinrichs, 1994; Hinrichs & Kessler, 1995). Also, the residual liquid fat is then retained in the pores of the crystal network. In this case, no liquid fat is available to act as a "glue" to hold the globules together in a granule upon milk fat globule damage (Walstra *et al.*, 1999).

Besides affecting the liquid:solid fat ratio of the core fat of the fat globules, the temperature would also be expected to affect the crystallisation behaviour of the various membrane constituents. Little is known about the relationship between the physical state of MFGM constituents and membrane stability, and this area deserves further attention in future research.

Temperature and fat globule size distribution

The effect of heating on the fat globule size distribution appears not to have been studied systematically. Fink & Kessler (1985a) reported that the fat globule size distribution changed when cream was heated above 90°C, but van Boekel & Folkerts (1991) claimed that heating did not affect the fat globule size distribution. Agitation is a confounding factor (van Boekel & Walstra, 1989) and the results of the study of Corredig and Dalgleish (1996), comparing indirect heating and direct steam injection, suggest that agitation has a greater effect than temperature. Hence, further work is required to elucidate the effect of temperature on the fat globule size distribution of milk and cream as a function of temperature and time in the absence of mechanical agitation.

2.3.2 Bacteriological quality and mastitis

Harvested milk contains bacteria from both the cow's udder and the external environment. Bacteria in milk may produce enzymes such as lipases, phospholipases, proteinases and glycosidic hydrolases. These could affect the properties and composition of the MFGM (Shimizu *et al.*, 1980; McPherson & Kitchen, 1983), and may lead to flavour defects such as rancid milk or bitty cream (reviewed by Deeth & Fitz-Gerald, 1995). It may be assumed that

significant changes in the MFGM occur only when such bacteria are present in sufficiently high numbers (i.e., in poor quality milk).

Mastitis may also result in the production of enzymes; mastitic milk has been found to have higher levels of acid hydrolases, which could change the surface charge of the MFGM. Furthermore, the MFGM of fat globules in mastitic milk appears to contain less phospholipid, more protein and two additional protein components compared with milk from healthy udders (Anderson & Cawston, 1975). Nevertheless, the effects of mastitis on the MFGM are not well known as little research has been carried out in this area (McPherson & Kitchen, 1983); further work would be warranted.

2.3.3 Stage of lactation and season

From a study on bovine milk from 3 to180 days post partum, Bitman and Wood (1990) concluded that the relative amounts of the five major phospholipid classes (phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine and sphingomyelin) remained constant. This constancy contrasts with results obtained for buffalo milk, which showed significant changes in relative levels of phospholipids during lactation. Sphingomyelin was found to be the predominant phospholipid in early lactation (Hofi *et al.*, 1977). Furthermore, during the last 2 months of lactation, phosphatidyl choline and sphingomyelin were reported to decrease significantly in bovine milk (Kinsella, 1970).

The total phospholipid and cholesterol contents of bovine milk at 180 days of lactation were lower than those in early lactation (Bitman & Wood, 1990). This would indicate a decreased quantity of MFGM material on fat globules if the mean fat globule size remained constant or decreased during that period (Bitman & Wood, 1990). Furthermore, these authors found that the fatty acid compositions of individual phospholipids changed throughout lactation. This could result in changes in MFGM fluidity and hence fat globule stability.

In buffalo milk, major differences in the carbohydrate contents of the MFGM were observed when comparing colostrum and mid-, early- and late-lactation milk. Sialic acid, hexose and hexosamine levels tended to be lower in colostrum milk than in the other milks. SDS-PAGE showed eight major protein bands in colostrum milk compared with six bands in the other milks. Total phospholipid levels did not change significantly from the day of parturition to the final day of lactation. Nitrogen results were variable, but tended to increase somewhat in late lactation. Similarly, the number of protein bands identified by isoelectric focusing increased with the advancement of lactation (Singh & Ganguli, 1976). Evaluation of the recoveries of the enzymes alkaline phosphatase, XO, 5'-nucleotidase, glucose-6-phosphatase, thiamine pyrophosphatase and γ-glutamyl transpeptidase in the cream of buffalo milk suggested that the composition of the MFGM had changed during lactation (Singh *et al.*, 1977). In another study on buffalo MFGM (Hofi *et al.*, 1977), the amount of both neutral lipids and phospholipids (expressed as per unit volume of milk or per unit fat globule mass) decreased

from colostrum to a minimum at about the third month of lactation and then increased continuously until the end of lactation. Membrane protein levels decreased from the maximum levels observed in colostrum to rather constant levels for most of the remainder of lactation, although there tended to be a slight increase towards the end of lactation. For bovine milk, from both Holstein and Jersey cows, the levels of BTN and XO, both of which are major membrane proteins, were high in early lactation, decreased as lactation progressed to its midpoint and then rose towards the end of lactation. However, the molar ratio BTN:XO (about 4:1) remained constant (Mondy & Keenan, 1993). These trends in MFGM protein contents were confirmed in a recent study (Ye *et al.*, 2002) (breed: Friesian; Singh, personal communication, 2003), except that the BTN:XO ratio was estimated to be about 3:1. The PAS 7 content was higher in mid season than in either early or late season (Ye *et al.*, 2002). The total ganglioside content appeared to vary in much the same fashion as the contents of BTN and XO, i.e. it was high in colostrum, decreased in early lactation and tended to increase in late lactation (Martin *et al.*, 2001). The pattern of individual gangliosides varied with the stage of lactation (Martin *et al.*, 2001).

In buffalo milk, the total membrane material (g/100 g fat) increased during summer months and reached a maximum in September (Asker *et al.*, 1978). As this coincided with late lactation, the increased membrane material may have been due to an increase in smaller fat globules, which have relatively more membrane material per unit volume of core fat than larger globules (Hofi *et al.*, 1977; Asker *et al.*, 1978). However, the fat globule size distribution was not measured in these studies. Nevertheless, it is known that the average fat globule diameter is affected by the stage of lactation. According to Mulder & Walstra (1974), the fat globule diameter, being at a maximum in early lactation, decreases throughout lactation. However, from the data shown, the average decrease appears to be minimal after about 25–30 weeks of lactation. Furthermore, recent results suggest that no significant change in the fat globule size distribution occurs after mid-lactation (Ye *et al.*, 2002). As the latter study involved only three sampling points during lactation, confirmation of the observations on the fat globule size distribution is required, including comparisons between different breeds. In contrast to the fat content, the fat globule size distribution does not appear to change significantly during milking (Guinard-Flament *et al.*, 2001). This would mean that a representative sample for the measurement of the fat globule size distribution can be taken at any time during individual milkings. However, these results should be interpreted with caution as previous studies of the changes in fat globule size distribution during single milkings were inconclusive (Keenan *et al.*, 1988).

When considered together, the above results suggest that the quantity of membrane material is lower in mid lactation than in either early or late lactation. This could indicate that the fat globules in mid-lactation are less stable than those at either the beginning or the end of lactation. This hypothesis needs to be tested, in particular because it is in disagreement with

earlier conclusions that late-lactation milk fat globules are more unstable, presumably because of a reduced quantity of membrane material (Kinsella, 1970).

A shortcoming of most studies is that sampling was not continued for a full season or lactation period and that the fat globule size distribution was not measured. Furthermore, in most studies, sampling was carried out intermittently. This makes it difficult to compare the effect of any long term changes with short term variation. Future studies should involve regular and frequent sampling intervals.

Generally, it is difficult to positively identify the factors responsible for variation in the composition of the MFGM when comparing different studies. In most studies, the experimental design did not allow for an evaluation of the separate effects of stage of lactation and season. Hence, it is difficult to discern which of these variables are responsible for the observed results and to what degree. Future experiments would require a controlled feeding regime, as factors other than stage of lactation, such as feed (the quality of which can be season dependent), may have confounded the results obtained in previous studies. Furthermore, the results of the study on buffalo milk suggest that mammary gland development plays a significant role (Singh & Ganguli, 1976), particularly in the days immediately post partum. On the basis of the fact that large compositional changes occur in the first few days post partum (Anderson & Cheeseman, 1975), it is postulated that significant changes in fat globule stability take place during this period.

It may be concluded that the structure and the composition of the MFGM do change throughout lactation. The relationship of these changes to the fat globule size distribution is not clear and further studies are required to provide a fundamental understanding of the phenomena involved. Although the relationship between total membrane material and the stability of the fat globule needs to be further explored, it is postulated that the stability of the fat globule is affected by stage of lactation and/or season. This postulation agrees with that of others (Kessler & Fink, 1992), but neither the extent nor the direction (increased or decreased stability) is known with certainty, as concrete experimental data are still lacking.

2.4 Conclusions

The MFGM is subject to changes in composition and structure from the moment the fat globule leaves the mammary secretory cell. Changes to the MFGM while the fat globule is still in the mammary gland appear to involve re-arrangement of membrane components and subsequent loss of some of the membrane material. However, the phenomena involved are not well understood and further research to obtain a fundamental understanding is required.

Upon milk harvesting and further milk handling, further changes to the MFGM take place. Depending on the type and degree of treatment, this may involve different physico-chemical interactions between various membrane components, the loss of membrane components and/or adsorption of components from the milk plasma. However, the effects appear to be

variable and dependent on physiological (cow) factors, and much remains to be learned about the phenomena on a molecular level.

Future research should increasingly focus on studying the milk fat globule in its native environment (i.e., milk plasma) and in real time. Such studies will contribute significantly to the understanding of the MFGM and how it changes post secretion by the mammary secretory cell. This knowledge will ultimately lead to improved milk-handling practices, more efficient manufacturing processes and better dairy products.

3 The MFGM — methodologies for measuring milk fat globule (membrane) damage

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3.1 Introduction

In contrast to the suckling calf, which ingests milk straight from the cow's udder, the use of bovine milk for human purposes generally involves various milk-handling and treatment steps between the moment of milk extraction and that of consumption. These steps may affect substantially the composition and characteristics of the milk. Chapter 2 discussed aspects of changes in the MFGM both prior to and after milk harvesting. The present chapter focuses on methodology used to determine changes in the MFGM that may result from milk-handling practices from the point of milk extraction until processing at the factory. A summary of the techniques discussed in this review is presented in Table 3.1.

3.2 Principles and application of methodology for measuring MFGM damage

3.2.1 Definitions of milk fat globule damage and MFGM damage

Reports in the literature often use the term "fat globule damage" to denote that changes have occurred in the fat globules as a consequence of milk handling and treatment. However, what is meant by the word "damage" is not always clear from the terminology used; neither is it clear whether the phenomena discussed relate specifically to the MFGM, the fat globule as a whole, or both. Thus, it appears that terminology has generally been used in an intuitive sense only and that no attempts have been made to define it. Hence, clarity in terminology is needed.

Firstly, the question "What actually is the MFGM?" should be answered. At first glance, it is tempting to answer this question from both a compositional perspective and a structural perspective as follows.

Definition 1:

"The MFGM is a layer, consisting of many different compounds (mainly polar and neutral lipids, proteins and enzymes), that surrounds a more or less spherical core that predominantly consists of neutral lipids. The MFGM originates from three sublayers, namely, an innermost layer that existed on the intracellular fat droplet, an outer bilayer membrane that originated from the apical plasma membrane and a proteinaceous coat located at the inner leaflet of the bilayer membrane that presumably originated in part from the cytoplasm of the secretory cell."

Although this definition may be criticised for being incomplete and lengthy, it would be a very useful summary of what constitutes the MFGM. However, another approach is possible. One could focus on the intended ultimate purpose of the fat globule, i.e. digestion by the suckling young. In this case, the definition of the MFGM could be formulated as follows.

Table 3.1 Summary of the main techniques reported in the literature for measuring MFG(M) damage.

Technique	Comments	Selected references	Potential for future research
"Free fat"	Two different techniques: (1) temperature cycling and centrifugation; (2) solvent extraction	Deeth & Fitz-Gerald (1978); Halter *et al.* (1978); Fink & Kessler *et al.* (1983); Evers *et al.* (2001a)	Not recommended. Methods produce highly variable results
Free fatty acids (FFAs)	Wide range of methods	Deeth & Fitz-Gerald (1995); Evers & Palfreyman (2001); Evers *et al.* (2001b)	Can be a useful indicator of MFGM damage when the limitations of the method used are understood
Lipolysable fat	Similar technique to that for measuring FFAs, but controlling some variables. May involve the use of an external lipase	Deeth & Fitz-Gerald (1975, 1978); Miller & Puhan (1986a); Iametti *et al.* (1997); Giangiacomo & Bonomi (1993)	Has potential. Requires tight control of method parameters
Total "free fat"	Defined as the sum of free fat and FFAs	Halter *et al.* (1978); Kirst (1980b, c, 1981, 1996)	Not recommended
Desorption of MFGM components	Desorption techniques measure the loss of one or more MFGM constituents; adsorption techniques measure the adsorption of components from the milk plasma onto the MFGM	Baumrucker & Keenan (1973); Stannard (1975); Patton *et al.* (1980b); Keenan *et al.* (1983b)	Has potential. Simultaneous measurement of the distribution of a range of different components will improve the reliability of the technique
Interfacial properties	Techniques used include measurement of surface charge, surface hydrophobicity	Phipps & Temple (1982); Fink & Kessler (1985a); Giangiacomo & Bonomi (1993);	Good potential. Techniques, as applied to measurement of MFGM damage, are still

	and interfacial tension	Michalski *et al.* (2001b)	in stage of infancy
Fat globule size distribution	Various techniques have been used, including light microscopy, turbidity measurement, laser light scattering, electronic counting and electroacoustics	Horvath(1974); Lehman (1988c); Michalski *et al.* (2002b)	Good potential
Microscopy	Techniques used include light microscopy, electron microscopy and confocal laser microscopy.	Buchheim (1986); Hinrichs & Kessler (1995); J. M. Evers & R. Hirst (unpublished results, 2001; Figure 3.1)	Good potential. Advances in microscopy techniques should progress the fundamental understanding of changes occurring to the MFGM
Sieving and viscosity	Measures fat globule aggregation	Kammerlehner & Kessler (1980); Hinrichs (1994)	Sieving – Not recommended as instrumental techniques provide more information. Viscosity – Limited potential
Infrared spectroscopy	Measures C=O ester bond	Rudzik (1987)	Has potential. Technique as applied to measurement of MFG(M) damage is in very early stage of infancy
Creaming or separation	Employs the difference in density between fat globules and milk plasma	Aule & Worstorff (1975); Lehman (1988b); Liang *et al.* (2000)	Limited potential. Fat globule size distribution may provide more informative data

Definition 2:

"The MFGM is a layer around a small globular mass of fat that effects a stable emulsion of this fat in an aqueous medium for the purpose of facilitating ingestion and digestion of the fat by the suckling young."

A third approach could be based on the assumption that the layer surrounding the fat globule is, on a molecular level, in a state of constant flux (the rate of change depending on circumstances; Chapter 2). Against the background of a molecularly ever-changing membrane, it would be useful to define the MFGM with respect to its status at a given point in time with which any future changes might be compared. From this perspective, the following definition would naturally follow.

Definition 3:

"The MFGM constitutes those parts of the fat globule that envelope the lipid core immediately after secretion by the mammary secretory cell."

In fact, yet further definitions are possible and the wording of any of these would depend on the context of the discussion in which the definition was given. For each of these definitions, it would need to be realised that the composition and structure of the MFGM will vary between individual globules and probably even between different locations on the same globule. It will also be clear that, failing the ability to measure exactly the composition and structure of the MFGM, any such definition can be used only in a conceptual sense.

For the purpose of the ensuing discussion of changes in the MFGM after excretion from the mammary gland, definition 3 is used in this review.

Secondly, on the basis of this definition of the MFGM, the term "MFGM damage" would have to exclude those changes that occur naturally, either in the mammary gland or in the suckling young's digestive system, but would include those changes that are inflicted by milk handling and treatment during and after extracting bovine milk for human use. Hence, in the context of the current review, MFGM damage is defined as:

"MFGM damage constitutes those changes to the composition or structure of the MFGM that occur as a consequence of milk handling and treatment during or after milk extraction, such as those caused by mechanically induced motion of fat globules, changes in temperature, microbial growth and ageing, and any biochemical reactions resulting therefrom."

It should be noted that this definition does not distinguish between the different degrees of damage that are possible, or whether these are measurable in practice. However, it does include changes in the MFGM that are caused by enzymes produced by bacteria after extraction of the milk from the udder.

Thirdly, for future discussions, it is proposed here to distinguish between MFGM damage and milk fat globule damage. The former relates to changes occurring in the MFGM alone, whereas the latter relates to changes occurring in the whole fat globule, including its size and shape.

When the fat globule as a whole is changed, i.e. when the changes go beyond those occurring in the MFGM, it is proper to speak about "milk fat globule damage", and this could be defined as:

"Milkfat globule damage constitutes those changes in the milk fat globule, additional to those occurring in the MFGM alone, that occur as a consequence of milk handling and treatment during and after milk extraction, such as those caused by mechanically induced motion of fat globules, changes in temperature, microbial growth and ageing, and any biochemical reactions resulting therefrom."

From the above definitions of milk fat globule damage and MFGM damage, it is evident that milk fat globule damage is always accompanied by MFGM damage, but that the converse is not necessarily true.

Two major types of milk fat globule damage may be distinguished: (1) where the average fat globule size is reduced, and (2) where the average fat globule size is increased through partial, or full, coalescence. The former effect results in the adsorption of surface-active compounds on to the fat globule-milk plasma interface, whereas the latter effect results in the shedding of membrane material from the MFGM into the milk plasma.

3.2.2 Methodologies used for measuring milk fat globule (membrane) damage

"Free fat"

"Free fat" is a term used in the literature to denote a particular parameter that has been claimed to correlate with the degree of damage to, or stability of, fat globules. However, the term is ambiguous and various definitions of what constitutes "free fat" have been given. These include: (1) fat inside damaged globules (Fink & Kessler, 1983), or fat that is insufficiently enclosed by an undamaged membrane[1] (Kessler & Fink, 1992); (2) fat that has leaked out of damaged globules (Fink & Kessler, 1983); (3) "the proportion of fat extracted by centrifugation at 60°C", when a centrifugation method was used (Te Whaiti & Fryer, 1975); and (4) "solvent-extractable fat" when an extraction method was used (Deeth & Fitz-Gerald, 1978). The last two definitions define free fat as a method-dependent parameter. Method-dependent approaches to terminology are also used in standard methods issued by organisations such as the

1 This interpretation appears to be based on that of Lagoni & Peters (1959) and denotes partly or completely denuded fat. However, the existence of such denuded fat for times beyond a fraction of a second is considered to be impossible by Walstra and co-workers (e.g., see van Boekel & Folkerts, 1991; Walstra, 1995).

International Dairy Federation (IDF) and the International Standards Organisation (ISO)[2] and such definitions help to avoid ambiguity. However, the use of method-dependent definitions has the disadvantage that results produced by different methods cannot be compared. Hence, great care in interpretation of results is needed when the same term is used but different methods have been employed. Indeed, comparisons of extraction and centrifugation methods have shown that these methods do not give comparable results (Fink & Kessler, 1986a, b). The following sections review these two techniques of free fat determination in some detail.

"Free fat" centrifugation methods

The free fat centrifugation method is said to make use of a difference in density between fat that has escaped from the fat globule and fat that is still surrounded by a native membrane (Halter *et al.*, 1978; Fink & Kessler, 1983; Kessler & Fink, 1992). The method generally involves warming the sample (milk or cream) in a bottle having an elongated neck, raising the surface of the contents so that it is located in the neck of the bottle and centrifuging to separate the so-called free fat into the neck of the bottle. Repeated heating, cooling and centrifugation steps may be applied. The height of the separated "free fat" column is estimated and used as a measure of the degree of fat globule damage or the fat globule stability. A number of different versions of this method have been reported (Webb & Hall, 1935; Rothwell, 1962; Dillier-Zulauf & Wirasekara, 1971; Ismail *et al.*, 1972a, b; Te Whaiti & Fryer, 1975).

Halter *et al.* (1978) employed a specially developed free fat butyrometer, and centrifuged the diluted sample at 65°C in the presence of the fat-soluble dye Sudan III. This method enabled the authors to measure as little as 0.002 g free fat. Nevertheless, the method appeared to be prone to error[3], and was subsequently standardised for cream by Fink & Kessler (1983; 1986a). The latter authors found that the degree of dilution, the centrifugation time and the pH of the diluent all significantly affected the free fat values. Using the standardised method, the error of the method was reported to be ± 0.03% free fat for 30% cream samples.

Free fat centrifugation methods have been used to study the effects of various factors associated with fat globule damage in milk, including fat content, cow breed, fat globule size distribution, stirring, cooling, storage temperature and mechanical stress (Te Whaiti & Fryer, 1976a; Herbst *et al.*, 1984). However, the performance of the method has been reported to be unsatisfactory in some studies; using fresh milk from a Friesian herd, Deeth & Fitz-Gerald (1978) could not obtain meaningful results with this method. For cream, free fat centrifugation methods have been used to determine the degree of fat globule damage occurring in cream crystallising silos (Palfreyman, 1988), or to identify points in a cream processing line where fat globule damage occurred (Williams, 1986). The effects of freezing, heating, stirring, pumping

2 For example, the definition of the fat content determined by the Röse Gottlieb method for milk is defined as follows by IDF and ISO standards: "The mass fraction of substances determined by the procedure specified in this International Standard" (e.g., IDF, 1996).

3 The Te Whaiti & Fryer (1975) free fat method has also been reported to be prone to large errors (Palfreyman, 1988).

and homogenisation on fat globule stability in cream have also been investigated (Webb & Hall, 1935; Kammerlehner & Kessler, 1980; Fink & Kessler, 1985a). Fink & Kessler (1985b, 1986b) concluded that the centrifugation method could detect mechanical damage caused through pumping, but not by homogenisation or heating if the method was applied immediately after these treatments. Only when homogenised or heated cream had been stored for a prolonged period of time (3–4 weeks) could the centrifugation method detect changes.

"Free fat" extraction methods

Free fat extraction methods involve the mixing of the sample with an organic solvent followed by gravimetric determination of the extracted fat. It has been claimed that the solvent dissolves the fat that is outside the membrane (as in the centrifugation method), but also penetrates damaged fat globules that have become permeable, and thus dissolves the fat from the core of such globules (Fink & Kessler, 1983, 1986b). As in the case of the free fat centrifugation method, various versions of the method have been reported.

Lagoni & Peters (1959) and Kirst (1980a) extracted the sample three times with a mixture of diethyl ether and petroleum ether, and thus essentially used the Röse Gottlieb principle (Gottlieb, 1892) except for the digestion step, whereas Aule & Worstorff (1975), Deeth & Fitz-Gerald (1978) and Lehman (1988b) used either hexane or petroleum ether as the solvent. Furthermore, significant variations in sample size, solvent volume and other conditions were used. A notable variation of the free fat extraction method involved passing low fat or diluted milk (fat content < 3.0 g 100 mL^{-1}) through a chromatographic column containing silica gel, washing the column with distilled water, eluting the absorbed fat with chloroform and gravimetrically determining the extracted fat (Aristova *et al.*, 1974).

It has been reported that the extraction method could not detect damage caused by pumping, supposedly because the amount of fat globule damage done by pumping was too little to be detected given the error of the test (± 0.2%; Fink & Kessler, 1986b). However, the extraction method was reported to be able to detect changes in the stability of the MFGM caused by heating and homogenisation (Fink & Kessler, 1985a, c, 1986c).

Critique

It is evident from the literature that the term "free fat" is controversial. The existence of free fat has been disputed repeatedly by some authors who say that fat cannot be free (van Boekel & Walstra, 1989, 1995; van Boekel & Folkerts, 1991; Walstra, 1995; Walstra *et al.*, 1999). Although parts of the original membrane may be removed from the fat globule, these denuded areas are said to be quickly covered by surface-active material present in the milk plasma (i.e., in about 0.01 s; Walstra, 1995).

Owing to the poor robustness, free fat methods tend to exhibit poor repeatability. Hence, various authors (Aule & Worstorff, 1975; Deeth & Fitz-Gerald, 1978; Fink & Kessler, 1983, 1986b; Fang,

1998; Walstra *et al.*, 1999) have pointed out that the conditions of the methods need to be tightly controlled in order to obtain repeatable results. Another shortcoming of the techniques is that damage to the membrane that does not result in the release of free fat during the application of the method cannot be detected (Stannard, 1975).

Furthermore, a detailed study of a number of variables of the free fat extraction method showed that the method itself damages fat globules, thereby magnifying, or even generating, the extractable fat result (Evers *et al.*, 2001a). Similar conclusions may be drawn from a study by Ismail *et al.* (1972b) regarding the free fat centrifugation method. In this study, the free fat content of cream, aged at room temperature for 4 days, increased after 1 day, but thereafter steadily decreased to zero over the following 3 days. It is improbable that free fat was formed initially and subsequently became covered by a native membrane upon standing of the cream. It is more likely that the centrifugation method generates free fat during the test. If so, then the results of this study may be explained by the fat globules showing a changing degree of stability against coalescence upon ageing. Thus, the concepts underlying the free fat methods, as defined by Fink & Kessler (1986a, b), are questionable. The free fat methods should probably be considered as some form of fat globule stability test to which the sample is subjected, rather than the measurement of an analytical parameter (van Boekel & Walstra, 1989).

Free Fatty Acids

When the MFGM is damaged, native milk lipase can come into contact with the fat and lipolyse the triacylglycerols of the fat globule core, resulting in the formation of free fatty acids (FFAs), a process termed lipolysis. Consequently, in various studies, the FFA concentration has been measured to determine the degree of MFG(M) damage (Te Whaiti & Fryer, 1976b; de Boer & Nooy, 1980; Flückiger & Hänni, 1980; Kirst, 1980a, b, c, 1981, 1996; Flückiger *et al.*, 1981; Herbst *et al.*, 1984; Rudzik, 1987; Evers *et al.*, 2001b; Wiking *et al.*, 2003). The FFA methods may be classified into three broad categories, viz. (1) titrimetric, (2) colorimetric and (3) chromatographic (IDF, 1991). The principles of FFA development and the methods used to determine the concentration of FFAs in milk and milk products have been reviewed in detail elsewhere (IDF, 1975, 1980, 1991; Joshi & Thakar, 1994; Collomb & Spahni, 1995; Deeth & Fitz-Gerald, 1995) and are not further discussed here.

Interpretation of FFA results to determine the degree of MFG(M) damage is difficult, because a number of variables influence the development of FFAs, including the type and duration of agitation, temperature (which affects the activity of the lipase and the degree of crystallisation of the milk fat) (Deeth & Fitz-Gerald, 1977) and the amount of lipase (Deeth & Fitz-Gerald, 1995). In addition, the initial FFA concentration of the sample affects the extent of increase of FFA levels in treated samples (Lehman, 1988a). This is probably a consequence of the inhibition of the lipase by accumulation of FFAs at the fat globule/plasma interface (Anderson, 1983). Furthermore, different FFA methods give different FFA recoveries (e.g., IDF, 1991; Evers, 2003). Finally, differences in the susceptibility of individual milks, which depend on the

vulnerability of the MFGM and the balance of activating factors and inhibiting factors, have been observed (Deeth & Fitz-Gerald, 1995; Evers, 2003). The above reasons would explain why, in some studies, differences between repeated measurements of the same treatment were larger than those between different treatments (Kirst, 1980a).

Although the measurement of FFAs can be a valid tool for assessing the organoleptic quality of milk, it has only limited use in the assessment of MFGM damage (Deeth & Fitz-Gerald, 1978), because the development of FFAs is a consequence of MFGM damage, and relies on a second variable (i.e., activity of the native lipase). One must have a proper understanding of the principle of the method used and the effects of the milk-handling step (Lehman, 1988b). Even then, particular care in the interpretation of the results is required.

Lipolysable fat

The above-mentioned problems with FFA analysis as an indicator of MFG(M) damage may be partly overcome by applying the method under strictly standardised conditions. Deeth & Fitz-Gerald (1975) incubated samples at a fixed temperature (5°C) for a fixed period of time (20 h). This method was modified by Miller & Puhan (1986a), who incubated the sample at 37°C for 48 h after adding hydrogen peroxide to preserve the sample. The latter authors defined the fraction of the milk fat that can be hydrolysed by the native milk lipase, as a consequence of mechanical treatment of the milk, as "lipolysable fat". The increase in the FFA level was determined by calculating the difference between the FFA levels of samples taken before and after the mechanical treatment. The method was claimed to be particularly suitable as an indicator of the degree of disruption (disruption increases the availability of substrate for the native lipase because of an increased fat globule surface area[4]). Although this approach overcomes some of the problems mentioned above for FFA analysis, it does not address the fact that different milks may exhibit different changes (increases) in FFA level when subjected to the same treatment. Hence, the method would be of limited use for determining the effect of mechanical damage quantitatively.

The problem of a different response of the native lipase in different milks may be overcome by adding a foreign lipase to the milk followed by incubation under standardised conditions. Hemingway *et al.* (1970) adjusted the pH of 10 mL of milk to pH = 8, added 1.0 mL of porcine pancreatic lipase (approximately 0.3 units/mL) and incubated the sample at 37°C (incubation time not given). FFAs were titrated with NaOH (1.25 M) in a pH-stat mode[5]. A similar approach was taken by Iametti *et al.* (1997) and Fang (1998), although the latter author incubated the treated sample for 24 h and titrated the FFAs directly rather than by using a pH-stat method.

4 The lipase is believed to be attached to the casein micelles (Deeth & Fitz-Gerald, 1995). When casein micelles "repair" damaged fat globules, the lipase is brought into contact with the fat globule core fat.

5 In this method, a small quantity of a certain lipase is added to a pH-adjusted substrate (e.g., milk, cream etc.), which may have been diluted with water. The volume of an alkali titrant required to keep the pH constant for a fixed period of time is then measured (Stewart *et al.*, 1975; Iametti *et al.*, 1997).

Deeth and Fitz-Gerald (1978) added a fungal lipase (from *Candida cylindraceae*[6]), which does not attack intact fat globules (Deeth & Fitz-Gerald, 1995; J. M. Evers, unpublished results, 2001), and incubated the samples at 5°C for 20 h. Net lipolysis by the enzyme was determined by subtracting the FFA content of controls incubated with 0.5 mL of water in place of the lipase.

Lipolysable fat methods have been used both in model experiments and in practice (Deeth & Fitz-Gerald, 1978; Miller & Puhan, 1986a; Fang, 1998). Factors investigated include the effect of air incorporation, temperature, fat content and susceptibility to damage of milk from different cows. Meaningful results appear to have been produced.

A practical problem with these methods is the potential for the growth of bacteria, which can produce significant concentrations of lipase, phosphatase or proteinase during incubation of the sample. These may attack the intact MFGM, resulting in inflated lipolysable fat results being produced. Furthermore, lactic acid produced by bacteria during the incubation of the sample interferes with the FFA analysis in an FFA-level-dependent way (no lactic acid is produced when FFA levels are high; J. M. Evers, unpublished results, 2001). Hence, the growth of bacteria must be inhibited in a manner that does not interfere with the action of the native or added lipase, or with the FFA determination. The pH-stat method approach (Iametti *et al.*, 1997) may overcome some of these problems as the assay time is of the order of a few minutes. FFA concentrations measured with this technique were positively and significantly correlated with the degree of homogenisation of the samples, indicating an increase in enzyme-accessible surface area upon homogenisation (Iametti *et al.*, 1997). It remains to be determined whether this technique is robust and sensitive enough to determine the effects of less severe mechanical treatments than that inflicted by homogenisation.

Total "free fat"

The amount of the so-called total "free fat" has been calculated from the sum of the free fat and FFA contents of the same sample (Halter *et al.*, 1978). This parameter has been used in some other studies as well (Kirst, 1980b, c, 1981, 1996; Herbst *et al.*, 1984). Considering the difficulties associated with the free fat and FFA methodologies as mentioned above, the value of the total "free fat" parameter is questionable.

Desorption of MFGM components

Handling and treatment of milk can cause the release of certain MFGM components, or entire membrane pieces, into the milk plasma (see Chapter 2). The distribution of MFGM components has been used as a tool for correlating the severity of a mechanical treatment of milk with MFGM damage. The methods may address components of either the lipid fraction or the protein fraction, as discussed in the next two sections, respectively.

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6 Currently called *Candida rugosa*.

Lipid fraction

The change in distribution of the phospholipids between the fat phase and the milk plasma of milk and cream after certain treatments (heating, cooling, centrifugation and homogenisation) has been used as an indicator of MFGM damage (Koops & Tarassuk, 1959; Greenbank & Pallansch, 1961; Anderson *et al.*, 1972; Patton *et al.*, 1980b). Total phospholipid concentrations were typically calculated from measured phosphorus contents of fat extracted by means of the Röse Gottlieb procedure. However, none of these studies reported the use of a modified version of the Röse Gottlieb method (Walstra & de Graaf, 1962) to maximise phospholipid recovery. Hence, it must be assumed that the reported phospholipid results were based on incomplete phospholipid recoveries. In contrast, Keenan *et al.* (1983b) employed an extraction using a mixture of chloroform and methanol (2:1 v/v), which is known to be an efficient solvent for phospholipid recovery. The results of Greenbank & Pallansch (1961) have been questioned because the preparation of the samples for analysis did not include steps to remove non-lipids from lipid extracts (Keenan *et al.*, 1983b). These observations do not necessarily mean that the conclusions reached in the studies concerned were invalid, but at least they should be considered as tentative. The distribution of individual phospholipids, if measured, was determined using thin layer chromatography (Patton *et al.*, 1980b; Keenan *et al.*, 1983b).

In addition to measuring the distribution of the phospholipids, Patton *et al.* (1980b) and Keenan *et al.* (1983b) also measured the distribution of cholesterol using colorimetric methods. Modern gas chromatographic techniques would offer increased accuracy (Richardson *et al.*, 1994) and would also allow for simultaneous determination of other sterols. Alternatively, cholesterol may be determined enzymically (Graham & Higgins, 1997).

Protein fraction

Various authors have attempted to infer the degree of MFGM damage from the activity of certain enzymes that transfer to milk plasma, but that are normally associated with the MFGM. The MFGM markers selected include XO (Reuter *et al.*, 1975; Stannard, 1975; Wiking *et al.*, 2003), alkaline phosphatase (Stannard, 1975; Deeth & Fitz-Gerald, 1978; Salvatierra *et al.*, 1978), 5'-nucleotidase (Baumrucker & Keenan, 1973; Keenan *et al.*, 1983b), adenosine triphosphatase (Baumrucker & Keenan, 1973), phosphodiesterase I (Keenan *et al.*, 1983b) and acid phosphatase (Keenan *et al.*, 1983b). XO activity has been determined using polarographic, radioenzymic or colorimetric methods (Kirst, 1980a). Deeth & Fitz-Gerald (1978) found that the release of alkaline phosphatase into the milk plasma increased with increasing time of agitation.

Critique

The measurement of desorption of MFGM components as a way of measuring the degree of MFGM damage is conceptually attractive, but may not be a straightforward matter. For example, the desorption of XO also depends on factors other than mechanical activation (e.g., trace element content and temperature) and, therefore, this enzyme has been considered to be of

only limited use for the determination of the degree of mechanically induced changes in the MFGM (Kirst, 1980a). Wiking *et al.* (2003) found that their assay for measuring the XO activity in milk plasma was not sensitive enough to detect the effect of mechanical stress inflicted on fat globules by pumping. Measuring the activity of XO can be misleading because XO is redox sensitive; measurement of the distribution of the actual mass of XO would probably provide more meaningful results (Keenan & Patton, 1995).

In general, a number of aspects deserve attention. First, the ease with which the release of the constituent from the membrane occurs will depend on the type of component (sterol, lipid, protein), its association with other membrane components (type of chemical and/or physical bonding) and its location in the membrane (inner or outer bilayer leaflet or trans-membrane). Second, the membrane composition varies between the fat globules of different milks, and even between fat globules of the same milk sample (Walstra & Jenness, 1984). It is not known how this might affect the overall release of the membrane components. Third, the thickness of the membrane of individual globules is not uniform (Walstra *et al.*, 1999). This, in turn, indicates that there is no such thing as a uniform membrane composition. It is therefore not known how representative the release of a component is for the bulk of the milk fat globules. Fourth, the release of membrane components may be expected to be temperature dependent, and thus the handling/storage history of the milk prior to testing may significantly affect the measurement response. Furthermore, heat treatment may alter the activity of the enzyme of interest, thereby interfering with the measurement response.

Considering the above-mentioned aspects, it would seem to be risky to base conclusions about MFGM damage on the results of measuring the release of just one component. It would seem to be more desirable to measure the release of a range of components, both lipids and proteins, as this would improve the ability to draw conclusions regarding MFGM damage, both quantitatively and qualitatively.

Measurement of interfacial properties

Changes in the MFGM potentially result in changes in the interfacial properties of the fat globules. This phenomenon may be utilised to correlate the latter changes with the former changes.

Surface charge

At physiological pH values, fat globules carry a negative charge because of the presence of a range of phospholipids, which contain a negatively charged phosphate group, and other membrane components such as glycosphingolipids containing sialic acid.

Changes in the composition or structure of the membrane (caused, for example, by adsorption of components from the milk plasma on to the fat globule interface) may be accompanied by changes in the overall charge of the fat globules. The latter changes can be estimated by

measuring the change in mobility of the fat globules in an electric field (Fink & Kessler, 1985a; Fink, 1986). Although this technique does provide a tool for determining whether changes have occurred on the surfaces of the fat globules, electrophoretic mobility is not necessarily a linear function of the surface coverage of the fat globules by milk plasma proteins (Dalgleish & Banks, 1991).

An extension of this technique, using laser Doppler electrophoresis, involved the calculation of the apparent ζ potential from the electrophoretic mobility (Michalski *et al.*, 2001b). This technique was applied to milk, cream and washed milk fat globules in buffer. The apparent ζ potential thus calculated was correlated with the degree of fat globule membrane damage upon homogenisation. There was a sigmoid relationship between the fraction of membrane covered by plasma proteins and the increase in the absolute value of the ζ potential. Additionally, the method appeared to be suitable for measuring the degree of damage inflicted by other mechanical treatments, such as ultrasonication, high speed mixing, shearing at high shear rate and pumping. The method may also be suitable for determining the effect of heat treatment and moderate mechanical treatment, and is capable of detecting changes to the MFGM when no significant change in fat globule size distribution has occurred (Michalski *et al.*, 2001b).

Surface hydrophobicity

The properties of the MFGM of fat globules in milk or cream have also been studied by measuring the change in colour resulting from the binding of a lipophilic pH indicator (Neutral Red) to the MFGM (Giangiacomo & Bonomi, 1993; Iametti *et al.*, 1997; Nielsen *et al.*, 2000). Hydrophobic patches or positive charges on the fat globule surface were shown to stabilise the orange–yellow, unprotonated, form of the indicator, whereas hydrophilic regions or negatively charged parts stabilised the purple–red, protonated, form. Tristimulus colorimetry was used to detect changes in the colour of the cream/Neutral Red mixtures. It was claimed that this method could be useful for discriminating between the effects of relatively mild treatments of cream and, in combination with other approaches, could be helpful in understanding and quantifying the nature and extent of alterations taking place at the molecular level in the MFGM of processed cream (Giangiacomo & Bonomi, 1993).

Interfacial tension

An experimental method for measuring the interfacial tension, γ, of individual fat globules involved the suction of a single globule into a capillary tube connected by water-filled tubing to a water reservoir the height of which could be adjusted to vary the suction pressure (Phipps & Temple, 1982). The Laplace equation (Equation 1) was used to calculate γ, with ΔP being the pressure difference between the inside and outside of the capillary tube, $R1$ being the radius of the fat globule and $R2$ being the radius of the inside of the capillary tube.

$$\Delta P = 2\,\gamma\,(1/R1 - 1/R2) \qquad (1)$$

Although the results obtained had a relatively large error, an increase in γ could be discerned after fat globule damage was inflicted by shaking a sample of milk at room temperature. This indicated that some surface-active material had been lost from the MFGM. It was found that the change in γ partly reversed upon standing, indicating the adsorption of surface-active matter. Disadvantages of this method were: (1) the tedious nature of the test, (2) the need to attain considerable skill in the construction of pipettes, and (3) the need to attain considerable skill in the manipulation of droplets under a microscope (Phipps & Temple, 1982). Another drawback of the method was that no results could be obtained for globules with a diameter of less than about 2 µm. It is currently assumed that smaller fat globules possess the same type of membrane as larger fat globules (Keenan & Dylewski, 1995), and hence it must necessarily be assumed at the present time that the γ of these smaller globules is not significantly different from that of larger globules (> 2 µm). It is noteworthy that, despite the importance of the data produced, the results of the study of Phipps & Temple (1982) have never been verified experimentally by other investigators. A more rapid and convenient method of determining the γ of individual fat globules could prove to be an important tool in the study of MFGM damage.

Fat globule size distribution

Fat globules in milk vary in size from < 0.1 µm to about 10 µm. The distribution curve of fat globule sizes, when plotted as a volume frequency distribution, is approximately Gaussian (Mulder & Walstra, 1974). A change in the fat globule size distribution is indicative of milk fat globule damage (Horvath, 1974; Fink & Kessler, 1985c, 1986d, e; Lehman, 1988c; van Boekel & Folkerts, 1991).

The fat globule size distribution has been determined using various techniques, such as various forms of microscopy, turbidity measurements, electronic impulse counting methods and electroacoustics (Kirst, 1980a; Michalski *et al.*, 2001a). The results are dependent on whether a casein-dissolving agent is used, and, if so, which agent (van Boekel & Folkerts, 1991; McCrae & Lepoetre, 1996). If no casein-dissolving agent is used, fat globule aggregate formation through casein interactions may cause an apparent increase in the average globule diameter.

Currently, the fat globule size distribution is usually estimated from laser light scattering measurements (e.g., McCrae & Lepoetre, 1996; Michalski *et al.*, 2001a, 2002a, c; Wiking *et al.*, 2003). The technique has been used successfully to correlate changes in the fat globule size distribution of milk or cream with the severity of mechanical treatment such as blending (J. M. Evers, unpublished results, 2001), pumping (Michalski *et al.*, 2002b; Wiking *et al.*, 2003), homogenisation (McCrae & Lepoetre, 1996; Michalski *et al.*, 2002b), shearing and ultrasonication (Michalski *et al.*, 2002b). A novel method of analysing the particle size distribution data, which involves dividing the size distribution plot into zones and calculating the area under the curve in each zone, has been reported to be particularly useful in assessing the extents of mechanically induced disruption and coalescence of fat globules (Downey *et al.*, 2002).

Microscopy

Microscopy has been used mainly to determine the degree of MFG(M) damage qualitatively. However, it could also be used to measure fat globule damage quantitatively if a statistical morphometric approach is used (Keenan & Patton, 1995). Samples of treated milk or cream have been investigated using normal light microscopy (King, 1951; Fink & Kessler, 1986d, e; Hinrichs & Kessler, 1995), electron microscopy (Darling & Butcher, 1978; Keenan *et al.*, 1983b; Buchheim, 1986) and CLSM (Fang, 1998; J. M. Evers & R. Hirst, unpublished results, 2001, Figure 3.1). Although it is a general truth that microscopy images must be interpreted with care, this is particularly so when the fat globules are first isolated and then subjected to fixation steps, as is done, for example, in the case of electron microscopy (Caroll *et al.*, 1972; Oortwijn *et al.*, 1977). Nevertheless, microscopy can detect changes in the MFGM upon milk handling that are difficult or impossible to determine otherwise. A combination of freeze-fracture surface replication and negative staining may be particularly suitable for studying changes in interfacial layers of fat globules in milk (Schmidt & Buchheim, 1992). Significant progress in knowledge of the ultrastructure of MFGM damage will be made when microscopy techniques are able to follow changes in the MFGM in its native environment and in real time. Future research will probably increasingly involve the use of AFM and CLSM. AFM in particular holds promise as it has a similar resolution to that of electron microscopy and has the capacity for studying the surface nanostructure of interfacial films in real time in an aqueous environment (Yang *et al.*, 1993; Gunning *et al.*, 1996; Kolb *et al.*, 1999; Dufrêne & Lee, 2000).

Viscosity and sieving

MFG(M) damage may result in the formation of fat globule aggregates, which causes an increase in viscosity. This phenomenon has been used as a tool for measuring fat globule damage under controlled experimental conditions (Hinrichs, 1994; Hinrichs & Kessler, 1995). In practice, the method might be useful for identifying operations in the milk-handling chain that cause significant fat globule aggregation.

A direct method for determining the degree of fat globule aggregation involved the sieving of fat globule agglomerates using a filter of pore size 0.19 mm (Kammerlehner & Kessler, 1980). This method does not seem to be particularly sensitive and fat globule size distribution measurements using instrumental techniques would provide more useful information.

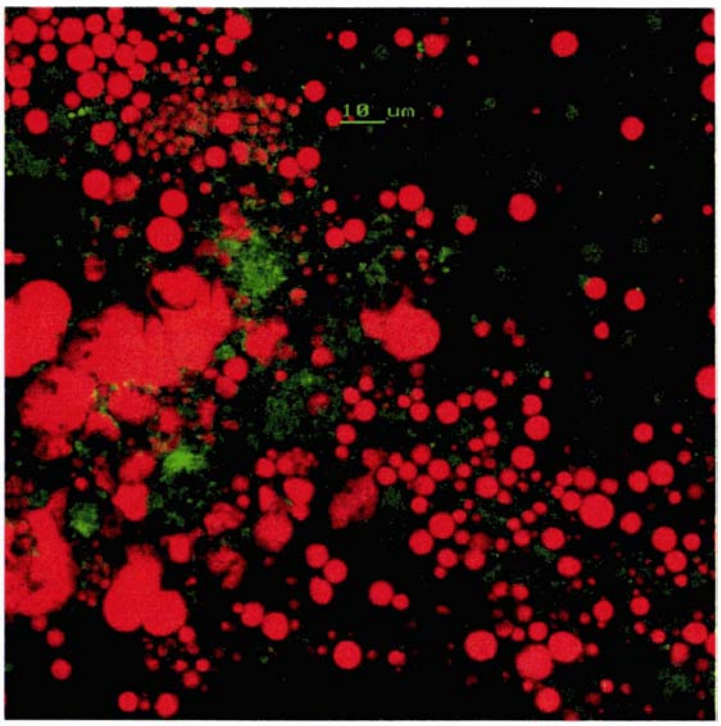


Figure 3.1 Confocal laser scanning micrograph of a milk sample that was damaged by blending for 15 s at 40°C using a kitchen blender, and incubated with *Candida rugosa* lipase for 24 h at 40°C. Close-up micrograph of an aggregate showed extensive fat globule coalescence, the presence of non-globular fat and a protein network. Confocal laser scanning microscopy was performed using dual staining. Fat was stained with Nile Blue (excitation wavelength: 488 nm) and protein was stained with Fast Green FCF (excitation wavelength: 568 nm). Micrograph colours: red = fat; green = protein. Bar = 10 µm. (J. M. Evers & R. Hirst, unpublished results, 2001).

Infrared spectroscopy

A novel way of measuring MFGM damage involved measuring the absorbance of the milk in a selected portion of the infrared spectrum (1800–1700 cm^{-1}; C=O ester bond) (Rudzik, 1987). The breaking of the ester bond of fat was assumed to be associated with loss of fat. A difference spectrum of the treated milk compared with the original untreated milk was used as a qualitative indicator of the degree of fat globule damage. The same technique was used to determine changes in proteins by measuring the shift in frequency of three peaks in the amide region (1600–1500 cm^{-1}) (Rudzik, 1987). However, the technique was used in model experiments on milk that was severely damaged by repeated pumping. Whether it is sufficiently sensitive to detect smaller changes that occur in practice remains to be tested. However, the use of modern Fourier transform infrared instruments may be expected to improve the potential of the technique, as indicated by model studies investigating the molecular basis of interactions between β-lactoglobulin and phospholipid bilayers (Lefèvre & Subaride, 2000).

Separation and creaming methods

Measurement of the percentage of fat in the skim milk separated from agitated milk using a laboratory separator provides an estimate of the extent of dispersion or homogenisation of the fat globules (Aule & Worstorff, 1975; Deeth & Fitz-Gerald, 1978; de Boer & Nooy, 1980; Lehman, 1988b). Generally, the fat content of the separated skim milk increases with increased intensity or duration of the mechanical treatment suffered by the original milk. To obtain meaningful results, the same test conditions (temperature of the milk, flow rate to the separator, cream fat content and type of separator) must be maintained within a series of experiments (Lehman, 1988b).

Creaming methods measure the rate of creaming of fat globules. Disruption of larger globules would result in a reduced creaming rate, whereas formation of fat globule aggregates or the presence of (partially) coalesced fat globules would result in an increased creaming rate (Walstra, 1995). Creaming tests may be performed using a centrifuge and the rate of creaming may be determined photographically by measuring the turbidity gradient over the length of the centrifuge tube (Liang *et al.*, 2000).

3.3 General discussion

The use of the term "milk fat globule damage" has been criticised, because it is deemed to be a very loose term (Walstra, 1995). Indeed, findings reported in the literature may seem to be in conflict with each other, because of different assumed meanings of the term fat globule damage. For example, on the basis of shear stress data, Hinrichs (1994) reported that, at a low temperature (5°C; high proportion of solid fat), the fat globule is more stable to damage than at higher temperatures. In contrast, Miller & Puhan (1986b), using a lipolysable fat method and investigating the effect of cooling and stirring, reported that the fat globule damage increased with decreasing temperature. In fact, the results of these experiments do not necessarily contradict each other, when it is realised that the authors measured different forms of fat globule damage (i.e., different phenomena) by using different methods. Hence, it may be concluded that, when different methods are used to measure fat globule damage, and when the principles of the respective methods are not fully understood, it must be assumed that these different methods actually measure the results of different phenomena.

Thus, method-specific terminology is required to avoid confusion and to be able to compare like with like. This observation is consistent with that of Deeth & Fitz-Gerald (1978), who concluded that different methods measure different consequences of agitation treatments, and that each of these gives results that vary in different ways with temperature, duration and severity of agitation.

Although the terms "MFGM damage" and "milk fat globule damage" are not specific from a fundamental science perspective, they can have a proper place in communications with dairy industry personnel who have some understanding of the milk fat globule, but who do not

possess an in-depth physico-chemical understanding of the MFGM. In discussions with such personnel, the terms “MFGM damage" and “milk fat globule damage" may be used to convey concepts of changes to the MFGM and the milk fat globule as a result of milk handling and treatment.

The fact that knowledge of the MFGM structure, and particularly the molecular arrangement of the components in the MFGM, is still in its infancy is a major inhibiting factor in the development of fundamentally understood methods that can be used in a routine fashion in practice. Hence, increasing knowledge of the molecular arrangement of MFGM constituents and the membrane's general structure would appear to be key objectives of future research. It is envisaged that, ultimately, techniques that will be able to accurately correlate milk-handling and treatment steps with the kind and degree of changes (“damage”) occurring to the MFGM will be developed. Such techniques will probably first be developed in a research environment. However, they may form the basis on which routine methods, suitable for application in the field, can be developed. In turn, this will allow predictions to be made regarding the effects of MFGM damage on downstream processing of the milk and the properties of finished products.

4 Development of fluorescence microscopy techniques for studying the MFGM

Chapter 3 concluded that significant progress in knowledge of the ultrastructure of intact MFGM and damaged MFGM will be made when microscopy techniques are able to follow changes in the MFGM in its native environment and in real time. Several authors have used fat-soluble fluorescent dyes such as Nile Red or Nile Blue to stain milk fat globules in milk and milk products, using either conventional fluorescence microscopy or CLSM[1] (McKenna, 1997; Sutheerewattananonda *et al.*, 1997; Herbert *et al.*, 1999; Everett & Olson, 2003; Trachoo, 2003; Evers, 2004b; Michalski *et al.*, 2004a; Ye *et al.*, 2005; Lopez *et al.*, 2007; Michalski *et al.*, 2007; Ye *et al.*, 2007). However, as the dyes used in these studies do not locate in the MFGM, but penetrate through it and dissolve into the fat core of the globules, they provide no information about the MFGM (Figure 4.1).

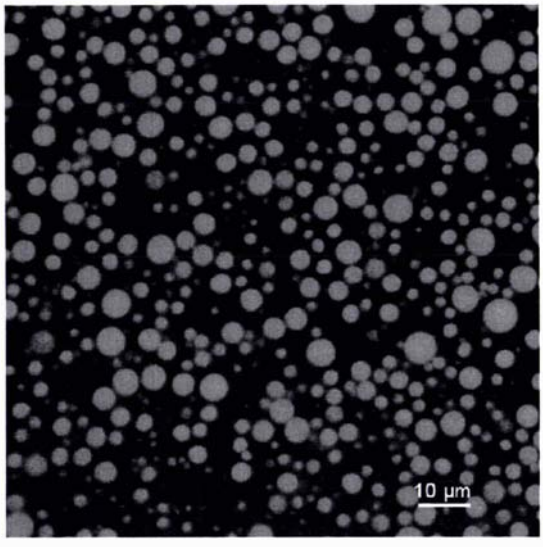


Figure 4.1 Human milk fat globules stained with Nile Red and imaged using CLSM. The dye reveals the core fat of the fat globules, but fails to show membrane features.

The fluorescent dye Acridine Orange (Figure 4.2) has been used to visualise fat globules (King, 1958; Scolozzi *et al.*, 2003), or cytoplasmic crescents associated with fat globules (Janssen & Walstra, 1982; Patton & Huston, 1988), but this dye is presumed to stain cytoplasmic matter rather than the MFGM itself (Scolozzi *et al.*, 2003).

Klebanov *et al.* (1972) studied conformational properties of the MFGM by measuring autofluorescence of MFGM proteins (more specifically, that of the tryptophan amino acid residue) using spectrofluorometry. However, these authors isolated the fat globules from the milk and, additionally, separated the lipids from the membrane to study re-suspended

1 The abbreviation "CLSM" is used in this thesis for either "confocal laser scanning microscopy" or "confocal laser scanning microscope". The context determines which term applies.

membrane fragments. Consequently, no information about the MFGM of fat globules in their native environment was obtained.

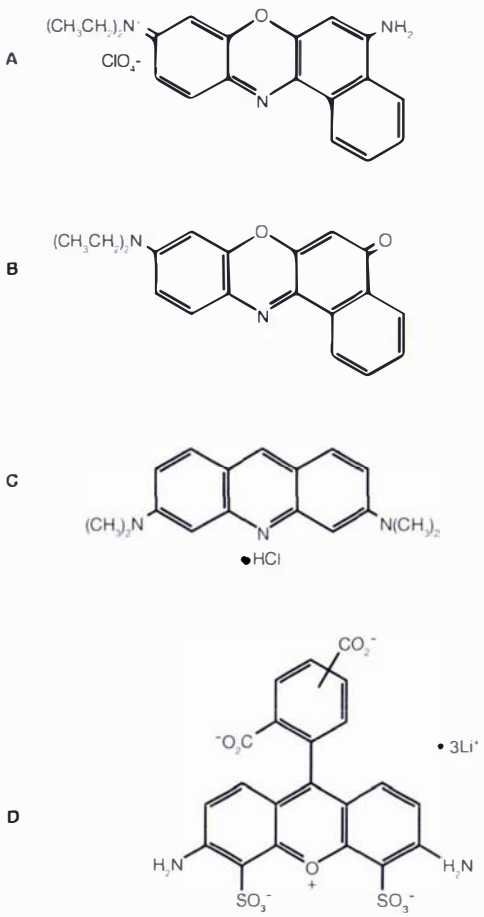


Figure 4.2 Molecular structures of selected fluorescent probes reported in the literature as having been used for staining milk fat globules. (A) Nile Blue. (B) Nile Red. (C) Acridine Orange. (D) Alexa Fluor® 488 conjugate.

Hence, it appears that no studies have been reported in which fluorescent dyes were used to study the MFGM of fat globules in their native environment. Furthermore, an enquiry by the author at one of the major manufacturers of fluorescent dyes (Molecular Probes, Eugene, OR, USA) revealed that this company did not know of the use of any of its dyes to stain the MFGM.

It was hypothesised that the molecular structure of some commercially available fluorescent probes would favour the binding to, or association with, the MFGM rather than the fat core of milk fat globules. It was further hypothesised that fluorescent probes could be selected that possessed a molecular structure such that they could be added to milk and stain the fat globules in their native environment to reveal novel structural and/or compositional details of the MFGM.

This chapter describes the screening of potential probes and development of fluorescence microscopy techniques for studying aspects of the MFGM of fat globules in their native environment. Figure 4.3 shows a flow diagram of the experimental strategy.

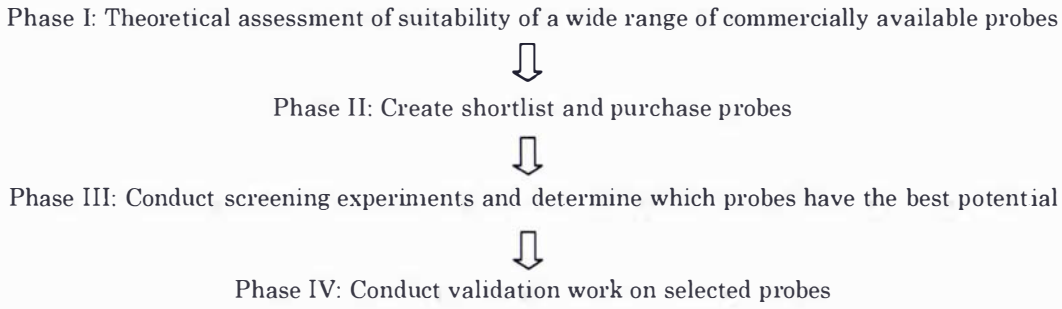


Figure 4.3 Flow diagram of planned experimental approach for developing microscopy techniques for investigating the MFGM using fluorescent probes.

Chapter 5 describes further validation work for one particular probe, the lectin wheat germ agglutinin (WGA). Chapter 6 focuses on the significance of the staining patterns of selected probes applied to milk of different species.

4.1 Theoretical assessment of the potential suitability of fluorescent probes for staining the MFGM

Without the availability of published applications detailing the use of fluorescent probes for staining the MFGM specifically, it was decided to study the molecular structures of a large range of commercially available fluorescent probes and to assess the probability (on physico-chemical grounds) that these compounds would locate in, or bind to, the MFGM rather than diffuse into the fat core of the globule, or remain exclusively in the aqueous milk plasma phase. In addition, the ease of use of these probes, from a practical perspective, was assessed. The following criteria were considered of importance:

- class of compound (e.g., fatty acid, phospholipid, sphingolipid, lipopolysaccharide, organic dye, lectin, etc.);
- Excitation spectrum and emission spectrum (i.e., whether the microscope filters of available microscopes – for both conventional fluorescence microscopy and CLSM – allowed imaging of the fluorescent probe);
- The degree of fluorescence in water (ideally this should be as low as possible so that there is no background fluorescence of the milk plasma caused by a portion of the probe molecules that is not bound to the MFGM);
- The price of the fluorescent probe;

- Storage and handling requirements (e.g., some probes require storage at temperatures below –40°C; others can be stored at room temperature);

- Solubility in commonly used solvents[2], viz. water, ethanol, dimethylformamide (DMF) and dimethylsulfoxide (DMSO) as well as uncommon solvents;

- Toxicity data.

About 40 potentially useful probes were identified (Appendix 1). Maximum potential for success was then determined by selecting probes that could be used in both conventional fluorescent microscopy and CLSM (using the instruments available at the Fonterra Research Centre and Massey University), that were not too expensive, or that would not present storage difficulties. As a cost-saving strategy, a sampler kit of lipophilic probes (classes: oxacarbocyanine (DiO), aminostyryl (DiA), indocarbocyanine (DiI); Table 4.1) was purchased, which contained 1 mg each of nine different probes (kit price NZ$ 474[3]). These, plus some others, were purchased for screening experiments. Selected data for all tested probes are shown in Table 4.1. (Some probes from the lipophilic sampler kit were not tested because the available microscope wavelength filters were not suitable). Full product names and molecular structures of these probes are shown, where available, in Table 4.2.

4.2 Initial screening experiments

4.2.1 Health and safety

A hazard analysis was performed for each of the probes. There appeared not to be any particular health and safety risk associated with the lectins and it was decided that standard laboratory safety practices would be adequate for dealing with these compounds. For the other probes, no toxicity data or health hazards data were available. To eliminate any risk, it was decided that, in addition to standard laboratory safety practices, these compounds be treated with caution, i.e. to use nitrile gloves (resistant to penetration of organic compounds) and to wear a dust mask when weighing out the chemical.

4.2.2 Solvent for making up the stock solution

Except for the lectins and styryl probes (which are water-soluble), initial screening experiments were performed using ethanol (96%, Merck, Darmstadt, Germany) as solvent. The principal reason for using ethanol was that this solvent has a lower toxicity compared with that of DMF or DMSO.

2 As recommended by the manufacturer of the fluorescent probes (Molecular Probes).

3 2004 price.

Table 4.1 Selected data for fluorescent probes that were purchased for initial screening.

Code[1]	Class	Abbreviation	Excitation/Emission maxima[2] (nm)	Cost[3] NZ$ (unit size in mg)
C11252	Lectin	ConA488[4]	495 / 519	227 (5)
D109	Organic dye	NA[5]	497 / 519[6]	210 (100)
D275	Oxacarbocyanine	$DiOC_{18}(3)$	484 / 501	425 (100)
D282	Indocarbocyanine	$DiIC_{18}(3)$	549 / 565	425 (100)
D3883	Aminostyryl	DiA	491 / 613	366 (25)
D7776	Indocarbocyanine	$DiIC_{18}(3)$-DS	555 / 570	425 (5)
D7777	Indocarbocyanine	SP-$DiIC_{18}(3)$	556 / 573	425 (5)
D7778	Oxacarbocyanine	SP-$DiOC_{18}(3)$	497 / 513	425 (5)
D7779	Indocarbocyanine	5,5'-Ph_2-$DiIC_{18}(3)$	576 / 599	425 (5)
N316	Fatty acid	NA[5]	467 / 539	168 (100)
T3166/ T13320[7,8]	Styryl dye	FM4-64	Depends on pH and type of membrane	609 (1)
T3164[7]	Styryl dye	FM1-84	Depends on pH and type of membrane	557 (1)
W11261	Lectin	WGA488[9]	495 / 519	372–459 (5)
W11262	Lectin	WGA594	590 / 617	
W32466	Lectin	WGA647	650 / 665	
W32465	Lectin	WGA680	679 / 702	

1 Molecular Probes catalogue code.

2 According to the manufacturer.

3 Approximate prices; based on 2005/2006 price lists.

4 Concanavalin A; "488" is a 3-digit code reflecting the excitation maximum of the Alexa Fluor® conjugate.

5 Not applicable.

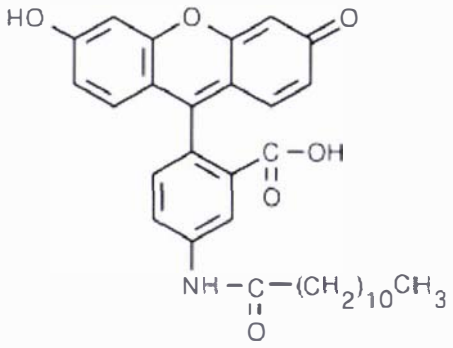
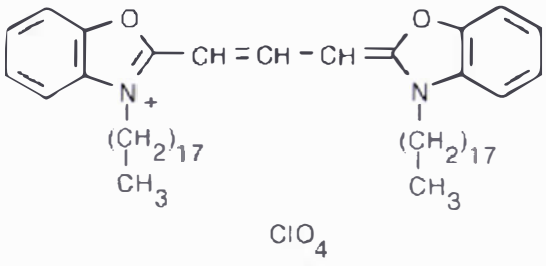
6 Spectra of this product are pH-dependent; these data are for basic solutions prepared in methanol containing a trace of KOH.

7 This probe was suggested, because of its known water solubility, by Molecular Probes experts during a meeting at their office in Eugene (OR, US) where the author discussed initial results obtained with some of the other probes. Hence, this probe was screened about a year after the others.

8 Same product as T3166, but different packaging. As the T13320 was purchased, this product code will be referred to hereafter as FM4-64.

9 Wheat germ agglutinin; the 3-digit code reflects the approximate excitation maximum of the Alexa Fluor® conjugate.

Table 4.2 Product codes, product/chemical names and structures (where available) for the screened fluorescent probes[4].

Abbreviation ConA488 **Product name** Concanavalin A, Alexa Fluor® 488 conjugate	Structure of this fluorescent probe was not available. For structural detail of ConA refer to e.g. Becker *et al.* (1976) and Sharon & Lis (2003). For a discussion of the preparation of fluorescent ConA, refer to Mallucci (1976). Refer to Figure 4.2 for the structure of the Alexa Fluor® 488 conjugate.
Product code D109 **Product name** 5-Dodecanoylaminofluorescein	 **Figure 4.4** Molecular structure of D109.
Abbreviation $DiOC_{18}(3)$ **Product name** 3,3'-Dioctadecyloxacarbocyanine perchlorate	 **Figure 4.5** Molecular structure of $DiOC_{18}(3)$.

4 Data obtained from Molecular Probes; reproduced with permission.

Abbreviation

$DiIC_{18}(3)$

Product name

1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

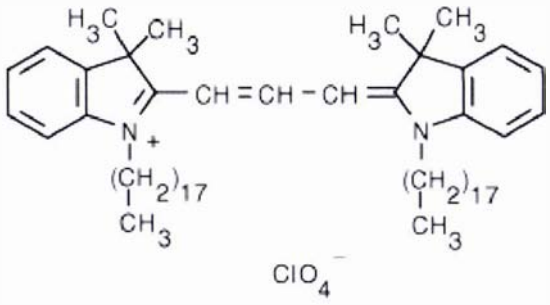


Figure 4.6 Molecular structure of $DiIC_{18}(3)$.

Abbreviation

DiA

Product name

4-(4-(Dihexadecylamino)styryl)-*N*-methylpyridinium iodide

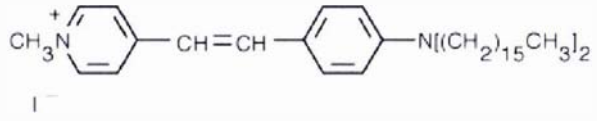


Figure 4.7 Molecular structure of DiA.

Abbreviation

$DiIC_{18}(3)$-DS

Product name

1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-5,5'-disulfonic acid

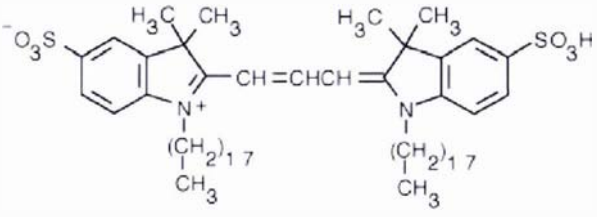


Figure 4.8 Molecular structure of $DiIC_{18}(3)$-DS.

Abbreviation

SP-$DiIC_{18}(3)$

Product name

1,1'-Dioctadecyl-6,6'-di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine

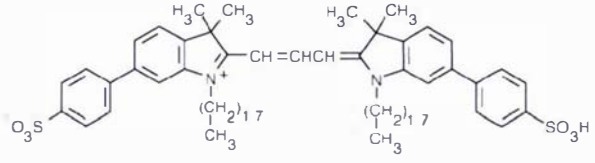


Figure 4.9 Molecular structure of SP-$DiIC_{18}(3)$.

Abbreviation

SP-DiOC$_{18}$(3)

Product name

3,3'-Dioctadecyl-5,5'-di(4-sulfophenyl)-oxacarbocyanine, sodium salt

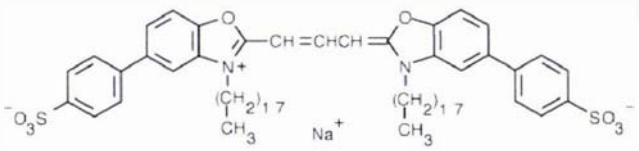


Figure 4.10 Molecular structure of SP-DiOC$_{18}$(3).

Abbreviation

5,5'-Ph$_2$- DiIC$_{18}$(3)

Product name

1,1'-Dioctadecyl-5,5'diphenyl-3,3,3',3'-tetramethylindocarbocyanine chloride

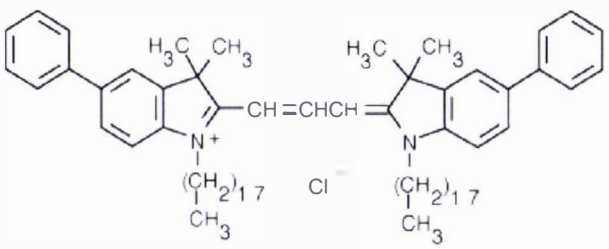


Figure 4.11 Molecular structure of 5,5'-Ph$_2$- DiIC$_{18}$(3).

Product code

N316

Product name

6-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid

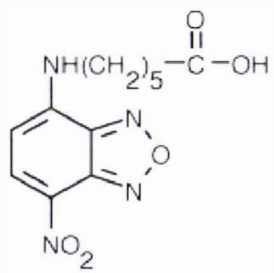


Figure 4.12 Molecular structure of N316.

Abbreviation

FM4-64

Product name

N-(3-Triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl)pyridinium dibromide

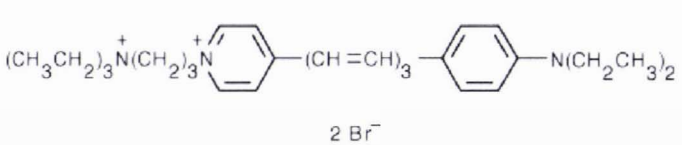


Figure 4.13 Molecular structure of FM4-64.

Abbreviation

FM1-84

Product name

N-(3-Triethylammoniumpropyl)-4-(4-(dipentylamino)styryl)pyridinium dibromide

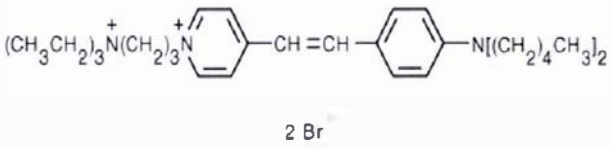


Figure 4.14 Molecular structure of FM1-84.

Abbreviation

WGA488/594/647/680

Product name

Wheat germ agglutinin, Alexa Fluor® 488/594/647/680 conjugate;

For structural detail of WGA refer to Sharon & Lis (2003). Refer to Figure 4.2 for the structure of the Alexa Fluor® 488 conjugate. For other Alexa Fluor® conjugates, refer to Molecular Probes.

4.2.3 Materials and equipment

Bovine milk was obtained from Massey University No. 1 Dairy Farm (Palmerston North). The herd consisted of Friesian cows of mixed age and lactation (town milk supply). The vast majority of samples was taken from the cooled bulk tank. In some cases, samples were obtained from the milk line after the plate heat exchanger. The milk was analysed and found to be of normal composition. If not used immediately, samples were stored at 4°C until analysis. Human milk was donated by a healthy volunteer over a period that included the third and part of the fourth year of continuous lactation, and was generally used on the day of sampling.

Depending on instrument availability, conventional fluorescence microscopy was performed using one of the following instruments: (1) Zeiss Axiophot (Carl Zeiss, Göttingen, Germany), fitted with an RT colour SPOT camera; (2) Zeiss Axiokop 2 Plus, fitted with an Axiocam MRC5 camera; (3) Olympus BX51 (Olympus Australia, Mt Waverley, Australia) fitted with an Optromics camera.

From the available microscope filters for conventional fluorescence microscopy, the filter combination that gave the best brightness of fluorescence emission was selected.

Fluorescent probes were purchased from Molecular Probes.

4.2.4 Preparation of stock solutions

Probe stock solutions of about 1mg/mL were prepared. This concentration is typical for non-dairy applications and was deemed to be a useful starting point for staining fat globules in milk.

Using this concentration, the following probes readily dissolved in ethanol: N316, D109 and DiIC18(3). Using sonication, the following probes fully dissolved within 1h: DiA and $DiIC_{18}(3)$-DS. In contrast, $DiIC_{18}(3)$, SP-$DiIC_{18}(3)$, SP-$DiOC_{18}(3)$ and 5,5'-Ph_2- $DiIC_{18}(3)$ did not fully dissolve even after 1 h of sonication. For these probes, the supernatant was used for experiments, which means that the exact concentration of the probes in the milk was not known.

ConA and WGA were dissolved in phosphate buffered saline (PBS; pH 7.4) or water, and styryl probes (FM4-64, FM1-84) were dissolved in water, using 5 min of sonication where necessary.

All stock solutions were protected from light by wrapping vials in aluminum foil and were stored at temperatures in accordance with the manufacturer's recommendations.

4.2.5 Preparation for microscopy imaging

Probe stock solutions, or supernatants, were added to milk samples typically at a ratio of 1:100 (v/v), unless stated otherwise. For lipophilic probes, this approach is consistent with the labelling technique for plasma membranes which is typically done by injection of an ethanol-dissolved solution of the probe into the aqueous phase (Wolf, 1988). This resulted in final probe concentrations of 10 µg/mL milk for the probes that were fully dissolved in the stock solution solvent. At all times, stained milk samples were protected from light by wrapping vials in aluminum foil.

A small aliquot (5–10 µL) of the stained milk was placed on a microscope slide and covered by a cover slip (either 20x 20 or 25 x 25 mm). It was found that the placement of the cover slip could cause damage[5] to the fat globules if it was placed on the microscope slide with one edge touching the slide, and at an angle of about 30–45°, and then allowed to drop onto the sample under the force of gravity. The best procedure was found to be to gently lower the cover slip keeping it parallel to the microscope slide, until it touched the sample, and then releasing the cover slip, allowing it to settle by gravity. In general, this procedure prevented damage to the fat globules in most instances. However, as a precaution, conclusions about the state of the fat globules were not made on the basis of observing fat globules in the middle of the slide, as this area tended to be prone to showing damaged fat globules. Presumably, there was more pressure at the centre than in the off-centre areas under the cover slip.

4.2.6 Microscopy results

Table 4.3 summarises the findings of the initial screening experiments.

5 Damage was visually evident from irregularly shaped fat globules and extensive fat globule coalescence.

Table 4.3 Summary of initial screening results for a range of fluorescent probes as observed by conventional fluorescence microscopy.

Probe	Bovine milk	Human milk
ConA488	Most fat globules appeared not to have a stained membrane. The appearance of the fluorescence was of a particulate nature (i.e., not smooth along the periphery of the fat globule). ConA 488 seemed to promote aggregation of fat globules. Fluorescence intensity appeared to be greatest where fat globules touched each other. A typical example is shown in Figure 4.15.	Weak staining of membranes of a significant proportion of fat globules. Fluorescence seemed to fade relatively rapidly.
D109	Probe appeared to be located in the MFGM, but faded rapidly making imaging difficult.	Not tested.
$DiOC_{18}(3)$	A small portion (< 5%) of fat globules fluoresced, but fluorescence was bright.	A small portion (< 5%) of fat globules fluoresced. However, fluorescence intensity was variable; some fat globules fluoresced brightly, whereas the fluorescence of others was relatively faint. Fluorescence within a single MFGM appeared to be variable.
$DiIC_{18}(3)$	A small portion (< 5%) of fat globules fluoresced, but fluorescence was bright.	A small portion (< 5%) of fat globules fluoresced. However, fluorescence intensity was variable; some fat globules fluoresced brightly, whereas the fluorescence of others was relatively weak. Fluorescence within a single MFGM appeared to be variable.
DiA	A small portion (< 5%) of fat globules fluoresced, but fluorescence was bright.	A small portion (< 5%) of fat globules fluoresced, but fluorescence was bright. Fluorescence within a single MFGM appeared to be variable.
$DiIC_{18}(3)$-DS	A small portion (< 5%) of fat globules fluoresced, but fluorescence was bright.	A small portion (< 5%) of fat globules fluoresced, but fluorescence intensity was variable; some fat globules fluoresced brightly, whereas the fluorescence of others was relatively weak.

Probe	Bovine milk	Human milk
SP-$DiIC_{18}(3)$	A small portion (< 5%) of fat globules fluoresced. Fluorescence was bright, but faded relatively rapidly.	Not tested.
SP-$DiOC_{18}(3)$	A small portion (< 5%) of fat globules fluoresced. Fluorescence was weak. Available wavelength filter may not have been optimal.	Not tested.
5,5'-Ph_2-$DiIC_{18}(3)$	A small portion (< 5%) of fat globules fluoresced. Fluorescence was bright, but faded relatively rapidly.	Not tested.
N316	Fluorescent rings were observed around fat globules. However, the milk plasma also fluoresced. Hence, it was difficult to determine whether the MFGMs were actually stained or whether this was an artifact due to diffraction from the milk plasma. Fluorescence faded rapidly.	Not tested.
FM1-84	Virtually no fat globules were stained after 4 h incubation at room temperature. A staining protocol involving 2.5 h incubation of the milk/probe mixture at 40°C did not result in a significant improvement. Significant background interference occurred, presumably because components in the milk plasma interacted with the probe.	Fat globule membranes were stained after 1 h and 4 h incubation time at room temperature, but fluorescence was weak. Substantial staining of the milk plasma occurred. Fluorescence faded rapidly.
FM4-64	Most fat globules were stained after 4 h incubation at room temperature, but fluorescence was very weak and faded relatively rapidly. However, available wavelength filter may not have been optimal.	Most fat globules were stained after 3.5 h incubation at room temperature, but fluorescence was weak and faded relatively rapidly. However, available wavelength filter may not have been optimal.
WGA488/ WGA594	Effective staining of the membranes of almost all fat globules.	Effective staining of the membranes of almost all fat globules.

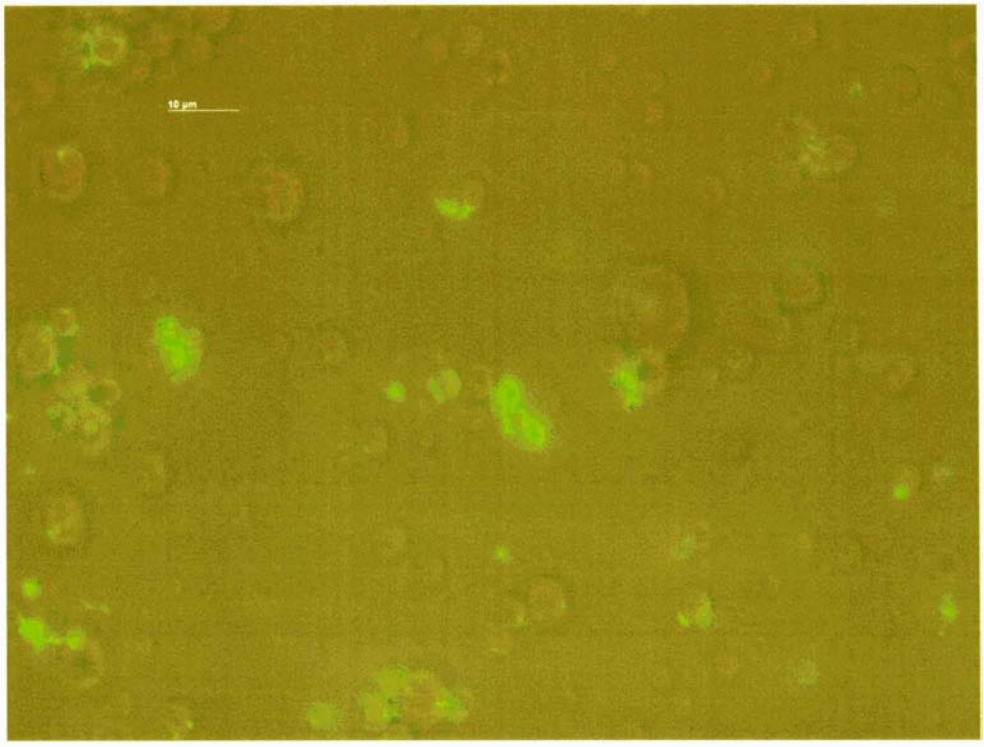


Figure 4.15 Conventional microscopy image (combined phase contrast and fluorescence) of a bovine milk sample stained with ConA 488 (green). Note that ConA fluorescence seems to be interspersed between fat globules in fat globule aggregates. Most fat globules were not stained by ConA 488.

4.2.7 Discussion

The results in Table 4.3 showed that the lectin ConA 488, and the lipophilic probes D109, SP-DiIC$_{18}$(3), SP-DiOC$_{18}$(3), 5,5'-Ph$_2$-DiIC$_{18}$(3), N316 and FM1-84 appeared to have limited potential for studying the membranes of bovine milk fat globules either because of poor staining of the MFGM, high background fluorescence, or poor photostability of the probe (rapid fading of fluorescence upon excitation). Hence, in some cases, the probes were not tested on human milk.

As the lectins WGA488 and WGA594 stained the membranes of most fat globules and the lipophilic probes DiOC$_{18}$(3), DiIC$_{18}$(3), DiA and DiIC$_{18}$(3)-DS brightly stained at least a proportion of the fat globules in both bovine milk and human milk, these probes were selected for further study. Although FM4-64 stained fat globules only weakly, it was decided to further study its suitability as sub-optimal wavelength filter settings had been used in the screening experiment.

4.3 Lipophilic probes — Ethanolic stocks: effect of concentration

As mentioned above, the screening experiments showed that for the lipophilic probes DiOC$_{18}$(3), DiIC$_{18}$(3), DiA and DiIC$_{18}$(3)-DS only a small proportion of fat globules were stained when using an ethanolic probe stock solution. This was thought to be either the result of

different fat globules possessing different physico-chemical properties (allowing some fat globules to be stained, but not others), or insufficient probe being present for the number of fat globules in the sample. Hence, different ratios of probe stock solution to milk sample were examined using conventional fluorescence microscopy (for DiA:milk the ratios were 0:100, 1:100, 2:100, or 4:100; for $DiIC_{18}(3)$:milk the ratios were 0:100, 1:100, 3:100, or 5:100 v/v).

Increasing the volume of the probe stock solution in the milk increased the proportion of stained fat globules. However, the background fluorescence interference tended to increase also. An increasing volume of $DiIC_{18}(3)$ stock solution in the milk also increased the number of red fluorescent particles, presumably undissolved particulate probe; they could not be small fat globules because stained fat globules were yellow, not red. The ratio 4:100 for DiA stock to milk sample resulted in all fat globules being stained, although the majority were only weakly fluorescent (Figure 4.16).

In summary, these observations suggested that the mechanism of fat globule staining was a function of the concentrations of the probe and ethanol in the milk. Presumably, at ratios of 1:100 or 2:100 (v/v) the initial squirt of ethanolic stock solution into the milk sample created locally an environment conducive to diffusion of the probe into the fat globules. However, as the probe stock solution became progressively diluted as it diffused further in the milk sample, the ratio of ethanolic probe stock to milk sample changed such that the conditions for the uptake of probe molecules by the membranes of the fat globules became unfavourable. Two experiments provided further support for this explanation. In the first experiment, a combined ethanolic probe stock, containing $DiOC_{18}(3)$ and $DiIC_{18}(3)$-DS, was added to bovine milk, which resulted in the staining of a proportion of fat globules by either $DiIC_{18}(3)$-DS or by both probes. However, when the combined stock was first added to the skim fraction of this milk, followed by addition of the cream fraction and mixing, virtually none of the larger fat globules (i.e., from the added cream) were stained. In the second experiment, an aqueous $DiIC_{18}(3)$-DS stock was added to a human milk sample. No fat globules appeared to be stained after 20 min incubation at room temperature. However, fluorescence of fat globules was apparent when ethanol was added at 10% (v/v) and increased with additional ethanol in the same sample (final concentration 25% v/v).

Hence, for fat globule staining to occur using these probes, ethanol must be present at relatively high concentrations. However, ethanol is known to denature the MFGM proteins[6], and therefore assessing milk samples that contain significant amounts of ethanol was not compatible with the objective of studying the MFGM in its native environment. Further, concerning the application of fluorescent probes for studying dairy products, these results reinforce the importance of recognising the potential effects of probe solvents on the sample matrix. Although this has been recognised in some studies (Gunasekaran & Ding, 1999; Auty *et al.*, 2001), this has not always

6 In fact, this phenomenon is utilised in the Röse-Gottlieb method for determining the fat content of milk to destabilise the MFGM and render the fat in the globules available for extraction by a solvent (Evers & Hughes, 2002).

been the case (e.g., Herbert *et al.*, 1999 ; Lopez *et al.*, 2007). Thus, caution is required in interpreting the results of studies in which organic probe solvents have been used but their effect on the sample matrix has not been reported.

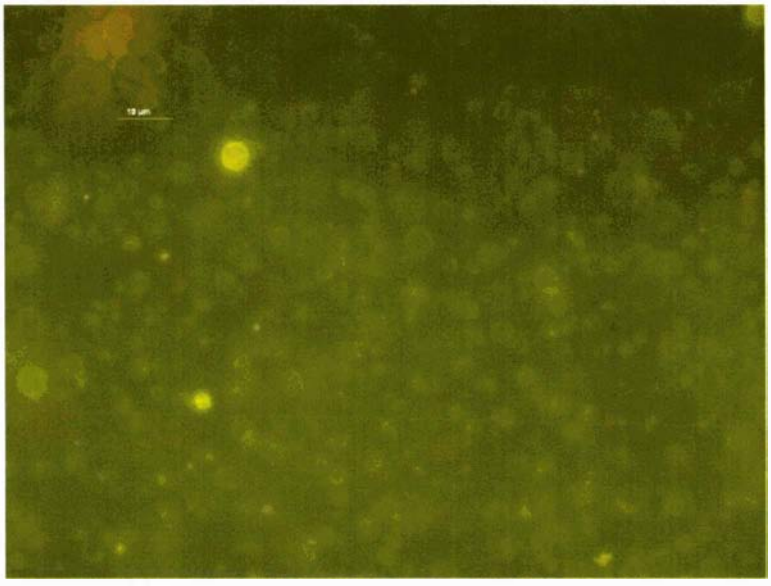


Figure 4.16 Conventional fluorescence microscopy image of DiA-stained (40 µg/mL milk) bovine fat globules.

4.4 Lipophilic probes — Effect of probe stock solvent

It was hypothesised that use of a different solvent from ethanol for the probe stock solution might overcome the problem of incomplete staining of fat globules in the milk, while not adversely affecting the environment of the fat globules. Two types of solvent were tested. Type 1 solvents were non-polar solvents that carried the suspended probe into the milk. In this case, the exchange of the probe from the carrier solvent to the MFGM was expected to rely on collisions between droplets of the non-polar stock containing the suspended probe and the fat globules themselves. Type 2 solvents were polar solvents that completely dissolved the probe and that were completely miscible with water (to allow mixing of the probe stock solution with the milk sample). In this case, staining of the MFGM was expected to rely on the diffusion of dissolved probe molecules into the MFGM.

Concentrations of the probes in these stock solutions were 1 mg/mL for all experiments, unless stated otherwise. As the type of stock solvent may affect the ability of a probe to stain the MFGM, some of the probes that were previously shown to have little potential for staining fat globules when added as an ethanolic stock to the milk sample (Table 4.3) were tested again.

4.4.1 Type 1 (non-polar) solvents

Hexane

Hexane (Merck, ≥ 98.0%) was tested as a solvent for DiA. DiA did not dissolve in hexane, but a suspension was obtained after 5 min sonication at 40–50°C. The addition of this stock to milk at a ratio of 1:100 (v/v) resulted in significant fat globule damage as evidenced by partial coalescence of fat globules and the presence of non-spherical fat globules. Very few of these were stained by DiA. Addition of this stock to milk at a ratio of 2:100 (v/v) increased the degree of fat globule damage as evidenced by large pools of free fat. Hence, hexane was not considered a suitable probe solvent. Incidentally, as these pools of fat were not stained by DiA, it may be concluded that DiA does not dissolve in neutral fat, or, if it does, exhibits no substantial fluorescence emission upon partitioning into this lipid environment.

FFMR

Free frozen milk fat for recombining (FFMR) (Fonterra, Palmerston North, New Zealand) was tested as a probe stock solvent for DiA. DiA could not be dissolved in FFMR (which also explains the failure of DiA to stain the free fat in the hexane experiment), but a suspension was obtained after 5 min sonication at 40–50°C. Mixing the FFMR probe suspension and milk (at a ratio of either 1:100 or 2:100 v/v) by inversion of the sample vial resulted in only very few fat globules being stained. The ability of the probe to stain the fat globules would have been affected by the frequency of collisions between FFMR droplets and fat globules as well as the rate of exchange of DiA between FFMR droplets and fat globules during each collision. From the very low number of fat globules being stained, it appeared that the number of collisions was very low and/or the effectiveness of the exchange of the probe from the FFMR to the fat globule was very poor. However, the fat globules that were stained looked the same as those that were stained by the ethanolic DiA stock. This suggested that the probe-globule interaction in a milk environment containing some ethanol (1–2% v/v) was similar to that in an environment without ethanol. This might mean that low concentrations of ethanol (e.g., 1% v/v) could be safely used in milk. Further work would be required to determine this. However, as the addition of somewhat higher ratios of ethanolic probe stock to milk (5:100 v/v) caused partial coalescence of some fat globules, there would seem to be a risk that a lower concentration (1% v/v) of ethanol in the milk might still effect some changes to the MFGM structure by denaturing some membrane proteins even if these changes were not severe enough to (partially) destabilise the membrane.

4.4.2 Type 2 (polar) solvents

DMF

DMF[7] (minimum 98.5%, M&B Ltd., Dagenham, UK) readily dissolved the DiA probe. When this stock solution was added to milk at a ratio of 1:100 (v/v), virtually no fat globules were stained. A large number of red particles were observed, which in an additional experiment were shown to

7 One of the solvents recommended by Molecular Probes for preparing stock solutions of various lipophilic probes.

be undissolved DiA. Hence, the mixing of DMF-dissolved DiA with milk resulted in DiA coming out of solution and aggregating into particles that were visible under the microscope. Using DMF in the milk at concentrations greater than 1% (v/v) resulted in significant destabilisation of fat globules (burst globules).

DMF also fully dissolved $DiOC_{18}(3)$. In contrast to DiA, when this probe stock was added to bovine milk, a number of fat globules were stained (about the same proportion as that of a milk sample stained with an ethanolic $DiOC_{18}(3)$ stock solution). However, fading of fluorescent fat globules appeared to be more rapid than when an ethanolic $DiOC_{18}(3)$ stock was used. Together, these results showed that DMF was not a suitable probe solvent.

DMSO

DMSO (GPR grade, minimum 98%; BDH, Poole, UK) readily dissolved $DiIC_{18}(3)$ and $DiOC_{18}(3)$ (the latter required 5 min sonication). The use of this stock solvent did not appear to damage fat globules (i.e., there was an absence of coalescence of fat globules). However, the staining efficacy of $DiIC_{18}(3)$ carried by this solvent into the milk sample was poor in bovine milk and human milk. Furthermore, milk stained by DMSO-$DiIC_{18}(3)$ stock showed a significantly greater number of undissolved probe particles than that stained by ethanolic $DiIC_{18}(3)$.

In contrast to DMSO-dissolved $DiIC_{18}(3)$, using DMSO-dissolved $DiOC_{18}(3)$ resulted in most fat globules in human milk being stained. However, the large majority of fat globules was only very weakly fluorescent, although some fat globules were brightly fluorescent. As had been observed for DMSO-dissolved $DiIC_{18}(3)$, many small fluorescent particles were seen. In human milk, ethanolic $DiOC_{18}(3)$ resulted in very few undissolved particles even though about the same proportion of fat globules was stained as compared with the DMSO-dissolved $DiOC_{18}(3)$. For bovine milk, no difference was observed between the milk stained with ethanolic $DiOC_{18}(3)$ stock or DMSO $DiOC_{18}(3)$ stock in terms of the proportion of stained fat globules or undissolved probe particles. Hence, DMSO offered no advantages over ethanol as a probe stock solvent.

CitiFluor

CitiFluor (a mixture of glycerol and PBS; Agar Scientific Ltd., Stansted, UK) was tested as probe solvent for DiA, N316 and D109. DiA did not dissolve, but a suspension could be made after 5 min sonication at 40–50°C. Application of this stock to milk, at either 1% or 2% (v/v), resulted in virtually no fat globules being stained. This suggested that DiA in an undissolved state is not taken up by the MFGM. Most of the D109 could be dissolved at a concentration of 1.45 mg/mL after sonication for 30 min at 40–50°C. Results for D109 were similar to those obtained with ethanolic D109 probe stock solution, i.e. rapid fading of stained MFGM (Table 4.3). N316 dissolved well at 1 mg/mL with the aid of sonication. Staining of the fat globule membranes was similar to that observed when using the ethanolic N316 (Table 4.3), except that background (i.e., milk plasma) fluorescence interference was much reduced compared with that obtained

when using the ethanolic N316 probe stock solution. These results showed that, overall, CitiFluor was not a suitable solvent for these probes.

Water

Milli-Q water was tested as a probe stock solvent for DiA, $DiOC_{18}(3)$ and $DiIC_{18}(3)$. DiA could not be dissolved (even after 25 min sonication), but a uniform suspension was obtained. This suspension was filtered through a 17 µm disc filter to remove large probe aggregates. When applied to milk this probe stock failed to stain any fat globules. Undissolved probe aggregates were abundant (showing up as small red particles). The addition of ethanol to the stained milk sample (1:100 v/v) did not increase the number of fluorescent fat globules. This supports the earlier observation (section 4.3) that when adding an ethanolic stock of DiA to milk, the squirt of stock solution into the milk momentarily creates an environment conducive to the interaction of the probe with the membranes of the fat globules (i.e., a relatively high ratio of ethanol:milk sample). Both $DiOC_{18}(3)$ and $DiIC_{18}(3)$ seemed to be as insoluble in water as DiA was. Additionally, these probes had poor suspension properties; even after 65 min sonication only a small proportion appeared to be suspended.

Milli-Q water was also tested as a probe stock solvent for $DiIC_{18}(3)$-DS at a concentration of 3 mg/mL. In contrast to DiA, $DiOC_{18}(3)$ and $DiIC_{18}(3)$, a significant proportion, but not all, of the $DiIC_{18}(3)$-DS probe dissolved. Addition of this stock to human milk and bovine milk samples resulted in staining of fat globules. Differences in staining efficacies between human and bovine fat globules, both in terms of intensity of MFGM fluorescence and the total number of fat globules being stained within a given time interval, suggested significant differences in the MFGM composition and/or structure between these species. Because of the high potential of using aqueous $DiIC_{18}(3)$-DS, it was decided to conduct a detailed study to investigate the effect of time and temperature on its MFGM staining efficacy (see section 4.5).

4.5 Lipophilic probe $DiIC_{18}(3)$-DS (aqueous stock) – Optimising staining conditions

Initial experiments had shown that $DiIC_{18}(3)$-DS completely dissolved in ethanol at a concentration of 1 mg/mL, but that using this stock resulted in only a small proportion of fat globules being rapidly stained (< 5 min); these fat globules were brightly fluorescent. Furthermore, initial experiments using aqueous dissolved $DiIC_{18}(3)$-DS showed that this probe could stain fat globules, but apparently at a much slower rate than that obtained when using the ethanolic stock (hours rather than minutes at room temperature).

A series of experiments were conducted with the aim of optimising the MFGM staining conditions using aqueous $DiIC_{18}(3)$-DS stock.

4.5.1 Effect of concentration

An aqueous stock was prepared that contained 3 mg $DiIC_{18}(3)$-DS/mL, part of which was not dissolved. The staining efficacy was assessed qualitatively (relative degree of fluorescence

intensity of stained fat globules) after 1–1.5 h incubation at room temperature for two ratios of stock:human milk (0.33:100 and 1:100 v/v, respectively). At the ratio 0.33:100 (v/v) few fat globules appeared to be stained, whereas at the ratio 1:100 (v/v) most fat globules were stained. Using conventional fluorescence microscopy, the fluorescence intensity across the membranes appeared not to be homogeneous as some areas of the membrane were shown to be less fluorescent than the surrounding membrane or even non-fluorescent (Figure 4.17). A number of fat globules contained a few mainly large (relative to the size of the fat globule) non-fluorescent areas, which tended to be roughly circular (arrows). In contrast, other fat globules contained a relatively large number of small non-fluorescent areas (double arrows).

Bovine milk samples stained at a ratio 1:100 (v/v) and observed after 3.5 h incubation at room temperature, yielded proportionally less stained fat globules compared with human milk. Also, on average, the fluorescence intensity of the fat globules that were stained was less than that of the fat globules in human milk. Furthermore, non-fluorescent patches were not observed, although membrane fluorescence intensity did appear to be variable on some fat globules.

4.5.2 Effect of incubation temperature and time

It was observed that fat globules of human milk samples stained at a stock:sample ratio of 2:100 (v/v) using aqueous DiIC$_{18}$(3)-DS stock (3 mg/mL) and incubated overnight at 5°C exhibited membranes that were fluorescing at much greater intensity than the same samples incubated at room temperature for 20 min or 1.5 h. Furthermore, the average membrane fluorescence intensity after 1.5 h incubation was greater than that after 20 min incubation. These results strongly indicated that there was a time-temperature relationship that affected the staining efficacy of fat globule membranes. A series of experiments were conducted to optimise the staining protocol using aqueous DiIC$_{18}$(3)-DS stock.

Effect of incubation time at room temperature followed by overnight incubation at 5°C

Bovine and human milk samples were stained using DiIC$_{18}$(3)-DS stock (aqueous; 3 mg/mL) at two levels (the stock:sample ratios were 1:100 and 5:100 (v/v), respectively). The intensity of the membrane fluorescence was assessed using conventional fluorescence microscopy after 0.4, 1, 2, 3, 5 and 7 h incubation at room temperature and again after 1 d following overnight incubation at 4°C. The camera exposure time was kept constant at 215 ms.

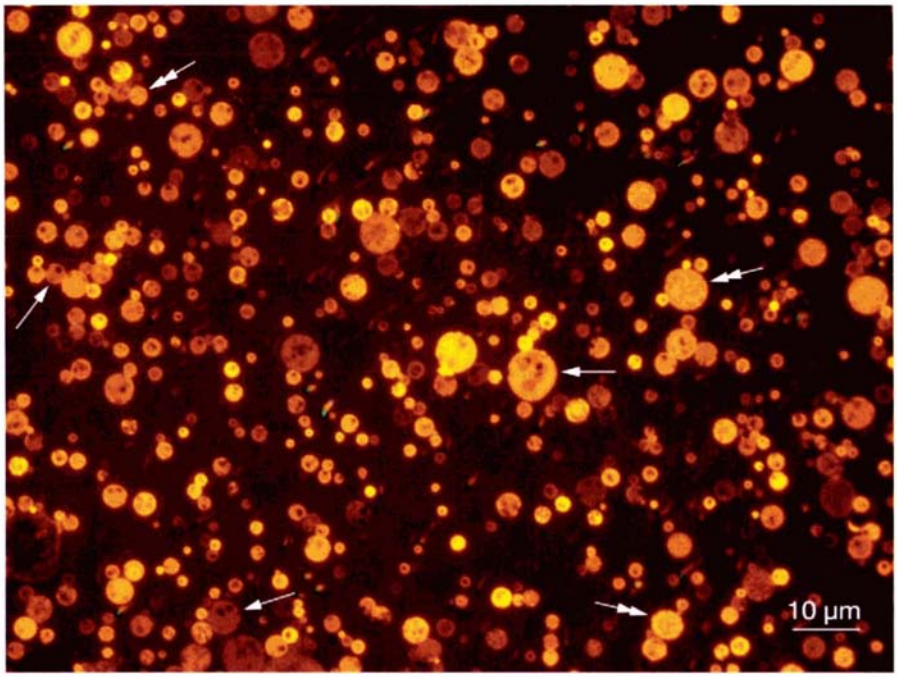


Figure 4.17 Human fat globules in milk stained using $DiIC_{18}(3)$-DS (aqueous stock) and imaged using conventional fluorescence microscopy. Arrows indicate fat globules showing relatively large non-fluorescent areas, whereas double arrows indicate fat globules containing relatively small non-fluorescent areas.

For human milk, fat globule membranes were clearly stained after 3 h incubation, although after 5 h incubation staining of the membranes was, on average, brighter. Overnight incubation at 4°C resulted in bright staining of fat globules for both concentrations. However, significant differences in the degree of membrane fluorescence between different fat globules of the same sample were observed irrespective of the incubation time. This suggests a different rate of diffusion of $DiIC_{18}(3)$-DS into human fat globule membranes for different fat globules within the same milk.

For bovine milk, on average, fat globule membranes did not fluoresce as brightly as those in human milk for comparable incubation times. Furthermore, it appeared that $DiIC_{18}(3)$-DS associated with milk plasma components in bovine milk, which resulted in significant background interference. This made it difficult to distinguish fat globules from the background. This phenomenon was not observed with human milk, probably because the protein concentration in human milk is 3–4 times smaller than that in bovine milk, and/or bovine milk may perhaps contain a relatively high proportion of tiny fat globules. Differences in membrane fluorescence intensity as a function of incubation time were less pronounced than those for human milk. Little improvement in staining efficacy seemed to occur beyond 3–5 h incubation time. Dark (non-fluorescent) patches, as seen on human milk fat globules, were not observed on bovine milk fat globules. Overnight storage of the bovine milk sample at 4°C resulted in bright staining of all fat globules for both concentrations. This suggests that, given time,

$DiIC_{18}(3)$-DS will diffuse into all bovine fat globule membranes, and that, as observed above for human milk, the rate of diffusion of $DiIC_{18}(3)$-DS into membranes is different for different fat globules within the same milk. Hence, these results suggest differences in membrane structure/composition between species as well as between fat globules within the same sample.

The number of small fluorescent particles (undissolved $DiIC_{18}(3)$-DS) in the milk samples was clearly significantly greater for the stock:sample ratio of 5:100 (v/v) than for the ratio of 1:100 (v/v). On the one hand, the presence of undissolved probe in the stock increases the background (i.e., milk plasma) fluorescence, but on the other hand it increases the effective concentration of dissolved $DiIC_{18}(3)$-DS in the milk sample resulting in a slightly better membrane staining efficacy.

$DiIC_{18}(3)$-DS staining efficacy using unfiltered and filtered stocks

To investigate whether the background interference caused by the undissolved $DiIC_{18}(3)$-DS particles could be reduced, the staining efficacy[8] of unfiltered $DiIC_{18}(3)$-DS (1 mg/mL) stock, containing a large number of undissolved probe particles, and a portion of the same stock, filtered through a 0.02 µm disc filter (virtually free from undissolved probe particles), were compared using conventional fluorescence microscopy.

Filtered $DiIC_{18}(3)$-DS stock was added (ratio 2:100 v/v) to both bovine milk and human milk samples and its membrane staining efficacy was evaluated after about 2 h and 5 h incubation at room temperature and again after overnight storage at 4°C. It was evident that the staining efficacy of the filtered $DiIC_{18}(3)$-DS was significantly lower than that of the unfiltered parent stock. Human milk fat globules were somewhat stained after 5 h but not significantly after 2 h. Bovine milk fat globules appeared not to be stained at all even after overnight incubation.

Further experiments, using fresh and aged (2 days at 4°C) bovine milk, confirmed the time-temperature relationship with regard to staining efficacy of the fat globules as well as the effect of ageing. Increasing incubation times (10, 30, 60 and 120 min) at 40°C[9] resulted, on average, in greater fluorescence intensity of fat globule membranes. However, significant differences between fat globules within a sample were observed; some fat globules were very brightly stained, whereas the majority was less intensely stained and some appeared to be stained very weakly or not at all. At all incubation times, the fluorescence intensity of the fat globules in the aged milk appeared to be greater than that of the fresh milk. Fluorescence of fat globules was more intense, on average, for the samples stained using the unfiltered stock compared with samples stained with the filtered stock.

8 Assessed qualitatively by comparing the relative degree of fluorescence intensity of fat globules.

9 The temperature of 40°C is close to the physiological temperature. Furthermore, at 40°C all the fat in the fat globules is liquid ensuring free exchange (if any) between components of the core fat and the MFGM. The incubation time was not extended beyond 120 min in order to minimise the risk of bacteriological spoilage of the sample.

An additional experiment, using fresh bovine milk, compared the staining effects of unfiltered stock with that of the filtered stock at both 40°C and 50°C at different incubation times (30, 60, 90 and 120 min). Although longer incubation times at 40°C improved the staining effect, even after 120 min fat globules were generally only weakly stained. Staining of fat globules was better at 50°C than at 40°C, with 120 min incubation time yielding the best staining results. At this point, experiments using incubation temperatures greater than 50°C were not conducted as it was known that the MFGM changes at higher temperatures (e.g., BTN and XO start to aggregate at 60°C (Ye *et al.*, 2002) and at 65°C, serum proteins start to interact with the MFGM (Corredig & Dalgleish, 1996).

The above results for different staining rates indicated differences in MFGM structure /composition between the human MFGM and the bovine MFGM as well as a changing membrane during ageing. From a review of the literature it is clear that cold ageing does affect the MFGM composition (Chapter 2), and the current staining results confirm this, but it is not clear *how* the MFGM changes upon cold ageing. Hence, it is difficult to explain why the fluorescence intensity of DiIC$_{18}$(3)-DS-stained fat globules is greater in cold-aged milk than that in fresh milk. This should be explored in future work. Furthermore, it appeared that when the unfiltered stock was used, some of the initially undissolved DiIC$_{18}$(3)-DS dissolved in the milk plasma thereby increasing the effective concentration of DiIC$_{18}$(3)-DS in the milk sample and providing quicker visible staining of the fat globules. Alternatively, individual undissolved particles might have interacted with membranes directly resulting in a transfer of probe to the membrane. The former explanation seems to be more likely.

4.6 Lectin probes

A significant advantage of using the lectin probes over most of the lipophilic probes was the ability to make up stock solutions using buffer or water as the solvent. This allowed for mixing of the stock with the sample in the knowledge that the stock solvent did not directly alter the MFGM. However, for both ConA and WGA, the application of the stock solutions to milk samples at a ratio 1:100 (v/v) resulted in the onset of clustering of fat globules (for ConA, see Figure 4.18). The rapid clustering of fat globules observed in this work agreed with results from previous investigations (Keenan *et al.*, 1974; Patton & Hubert, 1983) which showed that ConA binding to MFGM is nearly complete within 10 min for both bovine and caprine milk. Although the fat globule clustering phenomenon was evidence for the desired binding of the lectin with the MFGM, it was an undesirable side-effect from the perspective of microscopy imaging. For WGA, limited fat globule clustering was observed during the first 5–10 min after mixing of the probe stock and the milk sample. Hence, the staining protocol developed for WGA probe involved imaging the samples as soon as possible after the addition of the stock to the milk sample.

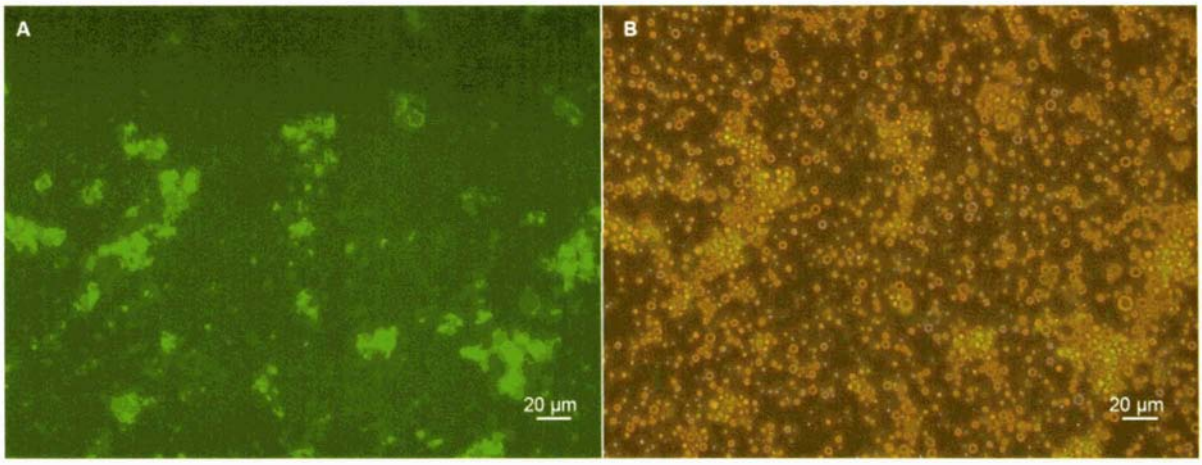


Figure 4.18 Conventional microscopy image of bovine milk stained with ConA 488 showing the presence of a significant number of fat globule clusters. (A) Fluorescence image. (B) Combined bright field and fluorescence image. Note the presence of a significant number of fat globule clusters (compare with corresponding fluorescence image).

4.7 CLSM

4.7.1 Aims

Conventional fluorescence microscopy allows imaging of objects with relatively little effort as the operation of the instrument is very similar to that used for bright field microscopy. The technique is particularly useful for relatively quick assessments of large numbers of fat globules. However, a disadvantage is that, at the magnification needed to detect the detail, fluorescent parts of the globule and its surroundings that are out-of-focus produce unwanted light which is collected by the microscope objective, thus reducing the contrast of the signal from the region of focus (Inoué, 1995). To produce better quality images and to obtain a greater degree of resolution of parts of the membranes of fat globules in their native environment, the suitability of CLSM was investigated. This microscopy technique allows imaging of optical sections even below the surface of a specimen (refer to Appendix 2 for an explanation of the principle of the CLSM). In contrast to conventional fluorescence microscopy, the operation of the instrument requires special training, particularly in operating the rather extensive software package. Such training was undertaken by the author to obtain sufficient expertise to routinely use the CLSM for the imaging of fat globules.

CLSM has been used before to image fat globules in milk (Fang, 1998; Herbert *et al.*, 1999; Evers, 2004b), but the dyes employed stained the fat core of the globules rather than the MFGM. Hence, at the time the work reported in this thesis was conducted there were no known reports of the application of CLSM to studying the MFGM specifically. Furthermore, there were

no known reports of studies in which the membranes of fat globules in their native environment had been studied by using three-dimensional (3-D) imaging[10].

Therefore, the first aim was to develop a CLSM protocol for making two-dimensional (2-D) images of fat globules through staining the MFGM. The second aim was to develop a protocol for generating 3-D images of fat globules through staining the MFGM. The third aim was to develop CLSM protocols for observing multiple staining (i.e., using two or more probes) of fat globules using either 2-D or 3-D imaging techniques. Imaging was performed using a Leica DMRBE TCS CLSM (Leica Microsystems GmbH, Wetzlar, Germany).

4.7.2 CLSM — 2-D and 3-D imaging of fat globules using single stains

The staining protocol and slide preparation technique developed for conventional fluorescence microscopy were also found to be suitable for CLSM, except that a sample volume of 6.5 µL was typically used (for conventional microscopy the typical sample volume was 7.5 µL). With this smaller volume, the fat globules became immobile more quickly on the microscope slide. The absence of any motion during imaging was found to be critically important for obtaining good quality CLSM images[11]; when using a sample volume of 7.5 µL most fat globules often kept on moving for several hours. Even if there were only very small movements, good quality images could not be made. Figure 4.19[12] shows an example of a poor quality image as a consequence of a minute rocking motion of the fat globule.

A general staining protocol is described in Appendix 3 and optimised CLSM parameters for selected probes are summarised in Appendix 4.

10 In addition, expert advice obtained in New Zealand prior to the current work suggested that it would not be possible to generate 3-D images of fat globules.

11 This comment applies to the CLSM used in this study, which was 10 years old. Preliminary experience with a modern CLSM suggested that, owing to the very rapid scanning capability of modern instruments, some movement in fat globules during scanning at low zoom can be tolerated in certain scanning modes.

12 For some of the following images, a scale bar is not available. Unfortunately, because at the end of this project the CLSM broke down beyond repair, the scale bars could not be retrieved. Typically, the fat globule diameters would have been within the range 4–8 µm.

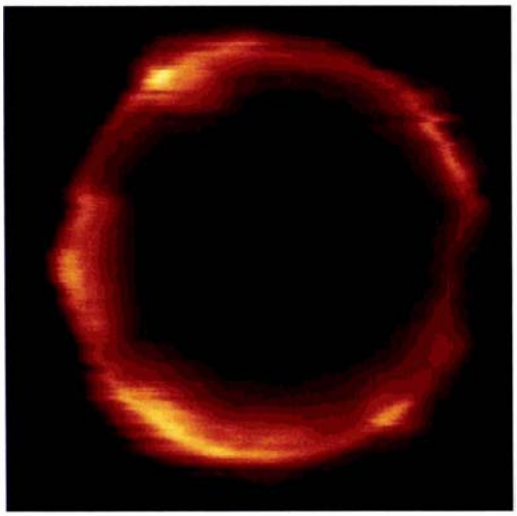


Figure 4.19 CLSM image of a bovine fat globule the membrane of which was stained using $DiIC_{18}(3)$-DS (ethanolic stock). This image shows that even a very slight rocking motion, probably with an amplitude as small as 100 nm, resulted in a blurry image of the MFGM. Exact size of fat globule not known (probably about 3–4 µm).

2-D imaging

The developed 2-D CLSM imaging technique was shown to highlight successfully aspects of the fat globules using a range of fluorescent probes (see Figure 4.20, and Figure 4.24). The fluorescence intensities in the images, based on a grey scale, were given false colours which varied from black (no fluorescence) to red (optimum) to white (high intensity) to blue (overexposure). In this thesis, where colour printing did not enhance the image, images are printed in grey scale. Prior to imaging, the correct laser level setting was estimated by pre-illuminating the fat globule(s) and varying the detector voltage level and, if necessary, the laser output level. This process was more an art than a science and required experience to strike a good balance between avoiding underexposing areas that had a low concentration of fluorescent probe and overexposing areas that contained a high concentration of fluorescent probe.

3-D imaging

To construct 3-D images, the CLSM takes sequential depth scans of the specimen and then the microscopist reconstructs these images by using the CLSM software to generate a 3-D image. Hence, the microscopist must first determine the maximum width and the length as well as the full depth of the object to be imaged.

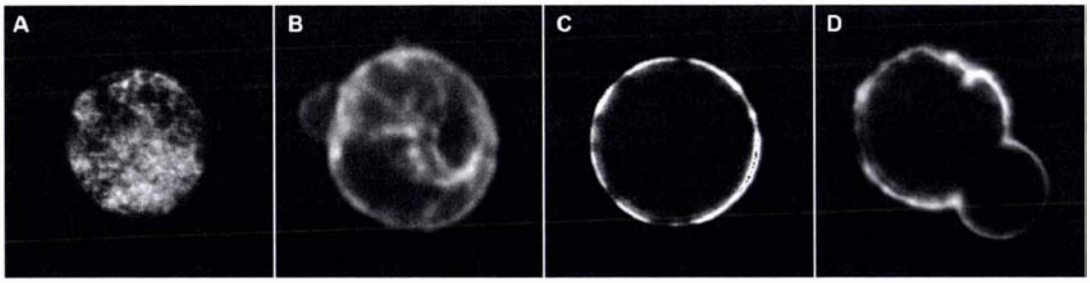


Figure 4.20 2-D images of human fat globules. (A) Stained with $DiOC_{18}(3)$ (ethanolic stock). Optical section of the middle of the fat globule. The MFGM is somewhat stained (see position between 11–2 o'clock). The core of the fat globule contained some fluorescent probe, but it was not homogeneously stained. (B) Stained with $DiIC_{18}(3)$ (ethanolic stock). Optical section of the middle of the fat globule. In addition to the MFGM, a part of the fat globule core was also stained, but fluorescence was not homogeneously distributed. A fluorescent bulge (denoting membranous matter) was observed at a position of about 10 o'clock. (C) Stained with DiA (ethanolic stock). Optical section of the middle of the fat globule. The MFGM was highly fluorescent, but fluorescence intensity varied significantly. The core of the fat globule was essentially non-fluorescent. (D) Stained with $DiIC_{18}(3)$-DS (ethanolic stock). The MFGM of fat globules was fluorescent, whereas the cores of the fat globules were non-fluorescent. True partial coalescence is evident from the absence of a stained membrane at the place where the two globules are joined together.

To obtain 3-D images of single fat globules in milk, the following protocol was used. First, after adjusting the microscope stage to bring the fat globule of interest to the desired position (as viewed on the screen), the microscope zoom was adjusted to ensure the desired magnification of the fat globule was obtained, as viewed on the screen. Then the microscope stage was adjusted along the Z-direction (up and down) and the computer was instructed as to which Z-position corresponded with the top of the fat globule and which with the bottom. This defined the total depth of the scan area. Next, the amount of laser light required to obtain optimum fluorescence was determined by scanning the fat globule through this depth and varying the laser output. Depending on the fluorescence parameters of the probe used and the number of line scans[13] desired, in practice the level of excitation light was often chosen such that some parts of the top of the fat globule were slightly overexposed. This reduced the risk of total loss of fluorescence for the lower parts of the fat globule as a consequence of photobleaching of the

13 The line scan number *n* determines how many times a particular line in the defined X-Y raster pattern is scanned, and thus also affects the time required to complete the scan. The line scan number that can be selected follows the formula $n = 2^x$, where *x* is an integer, and where the quality of the scan generally increases with an increasing value of *x*. The increase in the quality, if any, of each depth scan (and hence the final 3-D image) must be weighed up against the extra time needed to perform the scan.

probe. (The fat globule is repeatedly illuminated by the laser light during subsequent depth scans. Hence, photo-bleaching effects become more pronounced with both an increasing line scan number and number of depth scans). Finally, the computer was instructed to execute the entire depth scan. The imaging could take up to 20 min depending on the number of depths scans and the line scan number. Using this protocol, it was shown that 3-D images could successfully be made of fat globules stained using a range of fluorescent probes (Figure 4.21 and Figure 4.22). Stereo glasses are required (located on the inside of the back cover) to obtain the 3-D effect. Factors that improved the chances of obtaining good quality 3-D images of fat globules were: (1) a highly fluorescent membrane (this allowed the laser be set at low output, thus minimising photobleaching); (2) a minimum fat globule diameter of about 3–4 µm, as this reduced the risk of movement during imaging and improved the chance of identifying different distribution patterns of the probe if these existed. For probes that stained the MFGM only, the 3-D images often showed gaps between the optical sections because of selecting suboptimal sectioning parameters (Figure 4.22A). These gaps could be artificially smoothed out by using image processing software (Figure 4.22B). In the current study it was decided not to do this, but instead to rely on the human mind to imagine the connecting membrane patterns. However, with newer versions of the CLSM, modern 3-D graphical software and improved scanning experience, it is envisaged that the 3-D image quality of stained fat globules will be significantly enhanced in the future.

4.7.3 CLSM — 2-D imaging of fat globules using dual stains

Multifluorescence experiments involve specimens in which more than one target is labelled or the same target is labelled by different probes. Dual or triple labelling techniques for staining different components (e.g., fat, protein and whey) in milk and milk products have been reported in the literature (Herbert *et al.*, 1999; Auty *et al.*, 2001; Evers, 2004b; Michalski *et al.*, 2004a). However, there appear to be no published studies in which more than one probe has been used to stain fat globules. Hence, the aim of this part of the project was to develop fluorescence microscopy techniques using two, or if possible, three different probes for staining fat globules, and particularly the MFGM.

Initial work focused on using conventional fluorescence microscopy. Results obtained from experiments in which different lipophilic probes (using ethanol stocks) were added sequentially to a milk sample seemed to suggest that different probes associated with different fat globules, and not with the same fat globules (Figure 4.23).

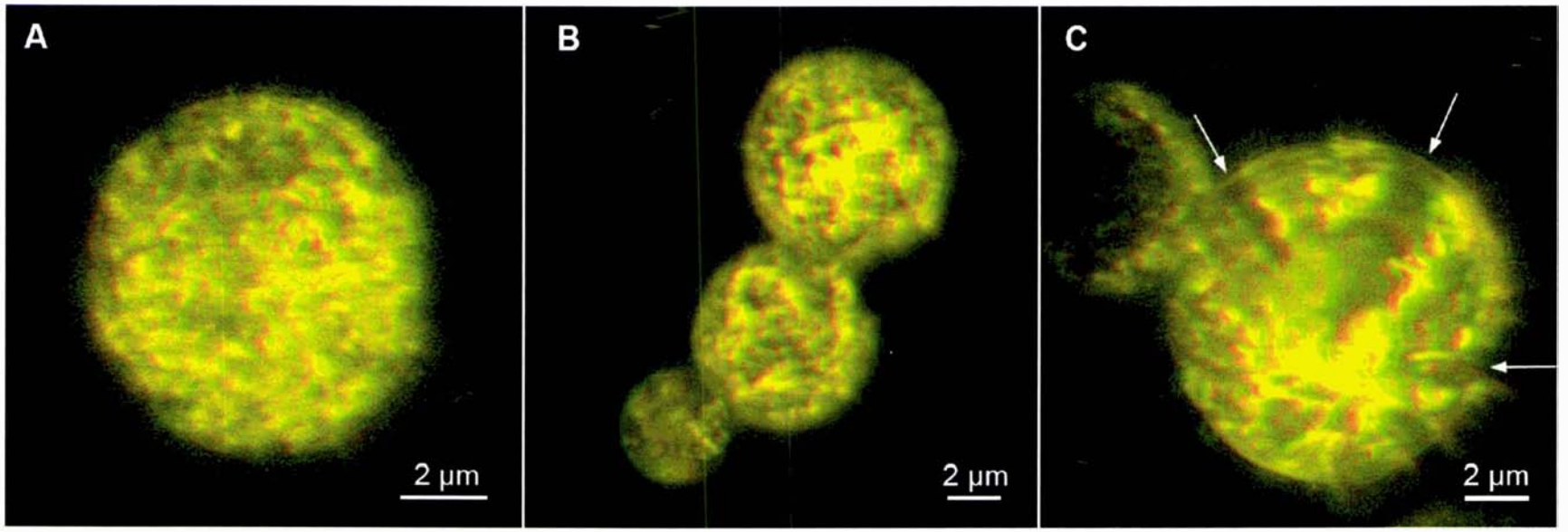


Figure 4.21 (A) 3-D image of a human fat globule stained by using $DiOC_{18}(3)$ (ethanolic stock). The probe appears to be distributed throughout the fat globule in a "spotty" manner. The scale bar is not entirely accurate (probably up to 10% too large) as it was copied from another image of very similar magnification. Note that the depth of the 3-D effect is affected by the distance the viewer is from the image: the further away from the image, the greater the 3-D effect. Distortions occur either way from the optimum: if the viewer is too close to the image, the fat globule will look somewhat squashed, whereas when the viewer is too far away, the fat globule looks somewhat elliptical rather than globular. (B) 3-D image of three DiA-stained (ethanolic stock) human fat globules that were touching each other. The smallest fat globule was about 4 µm in diameter, the largest about 7µm. Note the spotty distribution of the probe on the fat globule surface. (C) 3-D image of a human fat globule stained by using $DiIC_{18}(3)$ (ethanolic stock). The probe appears to be mainly located in the MFGM, but is also partly located in the fat core. Some non-fluorescent patches appear to be elongated (arrows).

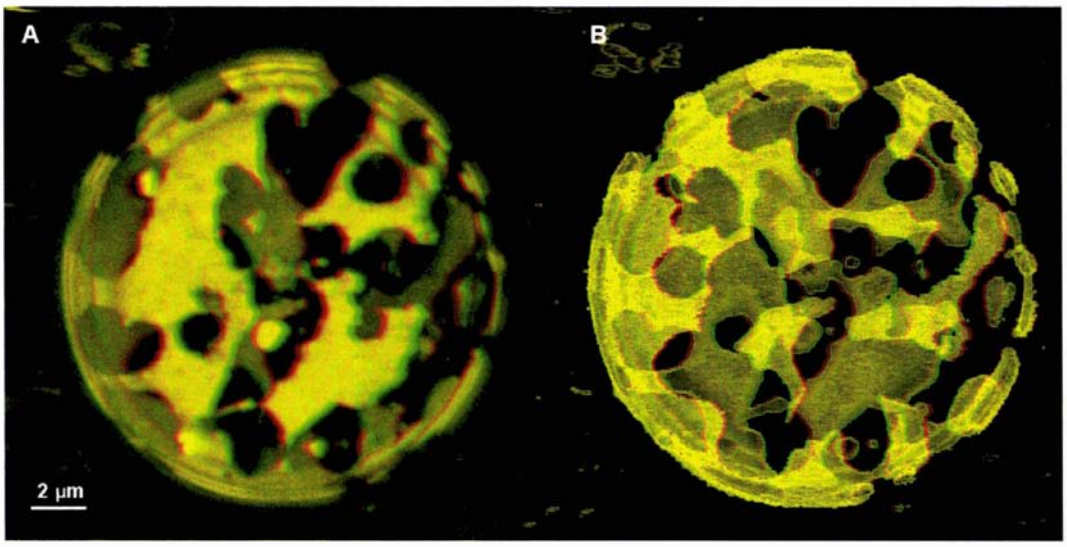


Figure 4.22 WGA488-stained bovine fat globule in milk that was heat treated (75°C, 5 min) and shaken (30 s). (A) 3-D image showing gaps between the stack of optical sections. Note that it is difficult to see the staining pattern of the bottom of the fat globule (at the back in the image) as the view is partially obscured by the staining of the top of the fat globule (front of the image). (B) The gaps between the optical sections of Figure 4.22A have been smoothed out using ImageSpace image processing software (version 5.1, Molecular Dynamics, Silicon Valley, US). This 3-D rendering of the fat globule shows two levels of fluorescence intensity. High intensity results from "overlapping" stained areas at the top and bottom of the fat globule. Low intensity means there is no overlapping of the area stained at the top of the globule by an area at the bottom of the fat globule or vice versa. Note, that this rendering allows better visualisation of the staining pattern at the bottom of the fat globule, but makes it more difficult to see the staining pattern at the top of the fat globule.

However, a subsequent experiment in which two lipophilic probes (DiIC$_{18}$(3)-DS and DiOC$_{18}$(3)) were added to the milk in a combined probe stock solution (in ethanol) yielded some fat globules that were stained by both probes. Hence, when ethanolic stocks are used, the probes must be combined in the same stock. It would seem good practice to also use a combined-probe stock when using aqueous probe stocks. For example, when staining fat globules using different fluorescent conjugates of the same marker (e.g., lectin) a combined stock would need to be used, because sequential addition of the probes would result in the first probe binding to available sites on the MFGM, and thus competing "unfairly" with the second probe.

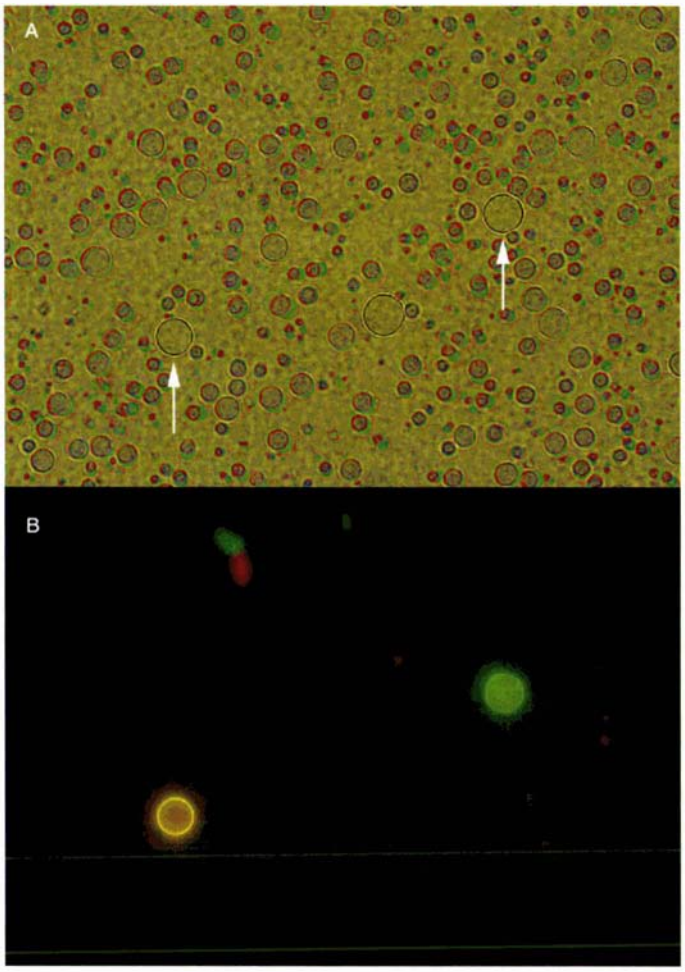


Figure 4.23 Conventional microscopy image of human milk fat globules to which DiA stock (ethanolic) and $DiOC_{18}(3)$ stock (ethanolic) had been added sequentially. (A) Bright field image. Arrows denote the fat globules that were stained in the corresponding fluorescence image. (B) Corresponding fluorescence microscopy image of Figure 4.23A. In this image, one fat globule was stained by DiA (yellow) and one by $DiIC_{18}(3)$ (green). Note that the majority of fat globules were not stained. The size of the stained fat globules is not known (probably about 10 µm).

$DiIC_{18}(3)$-DS in combination with WGA488, WGA594, WGA680 or Nile Red

Dual staining using the lipophilic probe $DiIC_{18}(3)$-DS (aqueous stock) and the lectin WGA488 was not successful because the emission spectra overlapped. Dual staining using $DiIC_{18}(3)$-DS and WGA594 was not successful as $DiIC_{18}(3)$-DS was excited by both the 488 nm laser and the 568 nm laser (the latter laser being used also to excite WGA594). Hence, although $DiIC_{18}(3)$-DS emission could be collected without interference from WGA594, imaging of WGA594 stained objects was compromised by overlap of fluorescence emission from $DiIC_{18}(3)$-DS in its spectrum. Rather than compensating digitally for the $DiIC_{18}(3)$-DS interference, it was considered to be more appropriate to combine $DiIC_{18}(3)$-DS with WGA having a different Alexa Fluor® conjugate to avoid overlap of emission spectra.

As the CLSM had three excitation wavelengths, namely 488, 568 and 647nm, it was thought that the use of a WGA conjugate that emits in the red region of the visible spectrum could be used in combination with $DiIC_{18}(3)$-DS. Hence, WGA680 was purchased and its suitability regarding staining the MFGM was examined.

Using the 647 nm laser and a LP 665 nm filter, it was shown that there was no autofluorescence of either human fat globules or bovine fat globules in the emission region of WGA680. However, the use of WGA680 was found not to be suitable for staining the membranes of either human or bovine fat globules, because of the low fluorescence intensity of the stained membranes and extremely high rate of photobleaching. This resulted from the need to use a high laser power to make the stained membrane visible, as the laser (647 nm) did not excite the probe at its optimum excitation wavelength (680 nm). Although there is a shoulder at 647 nm in the excitation wavelength curve of Alexa Fluor® 680, the absorption at that wavelength is only about 60% of that at 680 nm. It appears that this is not sufficient for practical purposes when imaging MFGM. To verify that this was the correct explanation, samples from the same bovine milk were stained with WGA488. Only a low laser power was required to obtain good images. This demonstrated that the carbohydrate-moieties (Figure 5.1) were available to WGA, i.e. the low fluorescence intensity of WGA680-stained membranes was not due to a lack of carbohydrate residues in the MFGM.

Combining $DiIC_{18}(3)$-DS with Nile Red was not successful because of an overlap of the respective emission spectra that could not be separated with the available filters.

FM4-64/WGA488

Because of its broad emission spectrum (significant above 500 nm; Du *et al.*, 1998), Nile Blue could not be combined with FM4-64. However, human milk was successfully stained using a combined stock solution containing FM4-64 and WGA488 (concentration of each probe was 0.5 mg/mL PBS (pH 7.4); the ratio probe stock:milk sample was 1:50 (v/v) to compensate for the lower probe concentration in the stock). CLSM filter settings were optimised (Appendix 4) and dual scanning of the sample was performed. Although background fluorescence was high, images made of fat globules were adequate for qualitative assessment of the staining pattern (i.e., absence, presence, or co-location of the probes).

For two different fat globules, Figure 4.24 1A and 1B, and 2A and 2B, respectively, show examples of the images obtained in the two channels. These images were then overlaid to study co-location of the probes in the same fat globule (Figure 4.24, 1C and 2C, respectively) and both WGA488 and FM4-64 were given a false colour (green and red, respectively).

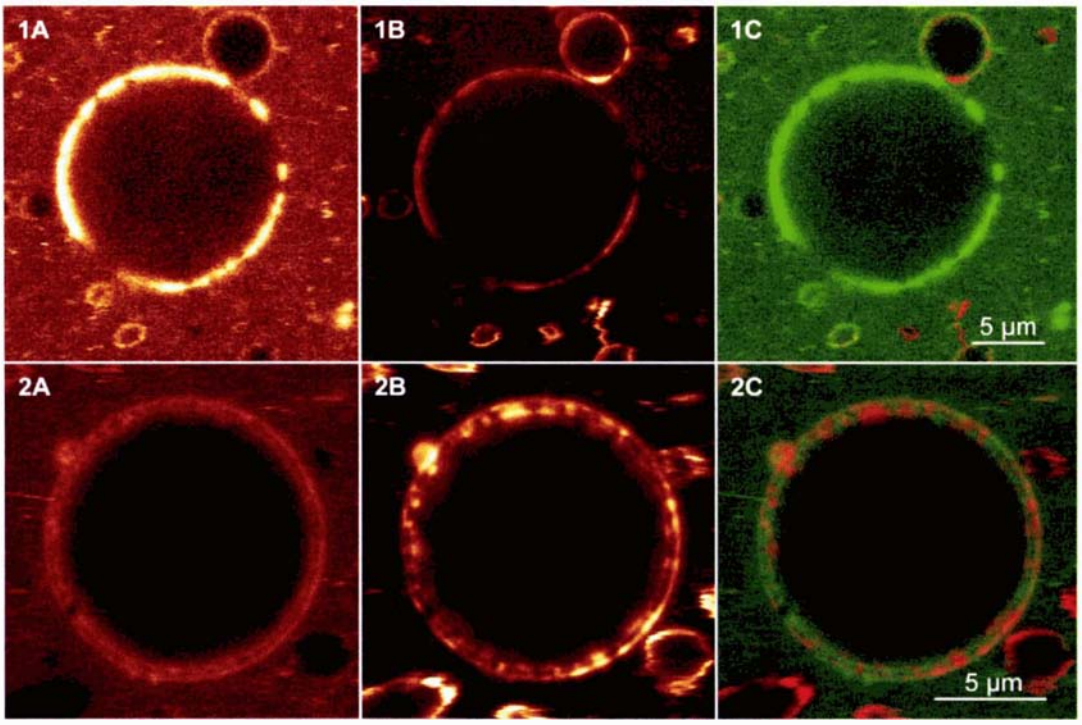


Figure 4.24 Dual stained human fat globules. Images labelled "A" show WGA488 fluorescence in the CLSM channel 1. Images labelled "B" show FM4-64 fluorescence in the CLSM channel 2. Images labelled "C" show the overlaid images of "A" and "B". Fluorescence from each probe was given a false colour (green = WGA488; red = FM4-64). (1A) WGA488 staining showed that the fat globule cores were non-fluorescent. Some fat globules contain non-fluorescent patches on their membranes. Distorted globules at the bottom of the image were the result of these globules moving during imaging. (1B) FM4-64 staining showed that the MFGM of fat globules fluoresced, whereas the cores of the fat globules were non-fluorescent. Fat globules contained non-fluorescent patches on their membranes. Distorted globules at the bottom of the image were the result of these globules moving during imaging. (1C) Fluorescence intensity of WGA488 was much higher than that of FM4-64 and hence the large globule appears as if it is stained with WGA488 only. (2A) WGA488 staining is clearly visible but not intense. (2B) FM4-64 staining intensity is variable across the membrane. (2C) Bright fluorescent patches of FM4-64 (red) show co-location with WGA488 (green).

Comparing 1A with 2A highlighted the fact that the degree of fluorescence of fat globules when WGA-stained can vary considerably; the fluorescence intensity of the membrane of the large fat globule in 1A was significantly greater than that of the fat globule in 2A. The same phenomenon also applied to the lipophilic probe, but in reverse order (the fat globule in 2B was more fluorescent than that in 1B). Consequently, there were significant differences between the overlay images. In 1C, the WGA488 fluorescence was much greater than that of FM4-64, and

hence the MFGM appeared green. The presence of FM4-64 could only be detected upon close examination of the electronic image, where a yellow line could be identified at some positions within the green ring thus revealing the location of FM4-64. In contrast, in 2C highly fluorescent membrane parts stained by FM4-64 (red) were clearly visible; where there was a relatively low concentration of FM4-64 the membrane appeared green as WGA488 fluorescence was dominant there. From these two examples the following was apparent:

- The absence of both probes in the same location resulted in non-fluorescent parts of the membrane in the overlay images. Hence, these results demonstrated that the use of dual staining could provide more information about the MFGM than the use of single fluorescent probes.

- Using FM4-64 resulted in very low background fluorescence, whereas using WGA488 resulted in high background fluorescence.

- The phenomenon of two separately stained concentric rings within a single fat globule (2A-C) had been observed before with other probes and is discussed in detail in Chapter 6.

4.8 General discussion and conclusions

4.8.1 Hypotheses

The work described in this chapter supported the hypotheses that the molecular structure of some commercially available fluorescent probes would favour the binding to, or association with, the MFGM rather than the fat core of milk fat globules. On the basis of the results obtained using water-soluble probes, the hypothesis that fluorescent probes could be selected, that possessed a molecular structure such that they could be added to milk and stain the fat globules in their *native* environment, to reveal novel structural and/or compositional details of the MFGM also appears valid.

4.8.2 Screening programme

A range of commercially available probes was found to stain the membranes of fat globules in their native environment (milk). However, several probes (D109, SP-DiIC$_{18}$(3), 5,5'-Ph$_2$-DiIC$_{18}$(3), N316) appeared to be unsuitable for routine analysis because of poor photostability. These probes were not considered for further microscopy work. However, these probes may be useful in combination with selected instrumental techniques (e.g., flow cytometry; refer to Chapter 7).

Several lipophilic probes (DiOC$_{18}$(3), DiIC$_{18}$(3), DiA, and DiIC$_{18}$(3)-DS) stained a small proportion of fat globules in a sample of milk when added as an ethanolic stock at a ratio of 1–2:100 (v/v) in the milk. These probes require a particular ratio of ethanolic stock:milk sample for association of the probe with the fat globule (membrane) to occur. This ratio appears to be

optimal when the probe stock is just added to the sample. As the probe stock progressively diffuses into the milk sample, the stock becomes progressively diluted and further staining of fat globules does not occur. The staining of fat globules is also affected by the type of solvent. For most of the lipophilic probes, ethanol was shown to be a more useful stock solvent than CitiFluor, DMF, DMSO, FFMR, hexane or water as judged from the staining results or solvent toxicity data.

Adding ethanol to milk carries the risk of denaturing membrane proteins of some fat globules, which may affect the association of the probe with, or binding of the probe to, the MFGM and thereby create artifactual results. Although the final concentration of 1% ethanol in the milk (as in the current application) may have little effect, some fat globules will be exposed to much higher concentrations and, at the present time, it is impossible to distinguish between fat globules exposed to a high concentration of ethanol and those that were exposed to a low concentration of ethanol. It is further noted that a relatively low concentration of ethanol (5%, v/v) is known to have a significant membrane-destabilising effect. Nevertheless, in this preliminary work the staining patterns of fat globules stained by ethanolic $DiIC_{18}(3)$-DS were not obviously different from those in milk stained by aqueous $DiIC_{18}(3)$-DS. Hence, further work investigating the effect of using ethanolic stocks on the staining of fat globules is warranted. As a precaution, most further validation work focused on probes that were at least partially soluble in water. These included $DiIC_{18}(3)$-DS, WGA (conjugated with different fluorophores) and FM4-64. Nevertheless, it is recommended that future work should also focus on $DiOC_{18}(3)$, $DiIC_{18}(3)$ and DiA, or suitable analogues, as these probes have promising potential for staining either the fat globule core or the MFGM. In particular, the oxacarbocyanine $DiOC_{18}(3)$ and the indocarbocyanine $DiIC_{18}(3)$ yielded intriguing results by diffusing inhomogeneously, but in different patterns[14], into the core fat of the globules (Figure 4.20) (refer also to section 4.8.3).

Water was shown to be a suitable solvent for a limited range of probes. It appeared that for the lectin probes adding a 1mg/mL stock solution to milk at a ratio of 1:100 (v/v) resulted in a sufficient degree of staining. For WGA, further validation work on the effect of probe concentration is described in Chapter 5.

$DiIC_{18}(3)$-DS was found to not fully dissolve in water at 1mg/mL. It appeared that part of the suspended $DiIC_{18}(3)$-DS in the stock solution dissolved in the milk after adding the probe stock to the milk sample. The contribution of the undissolved stock particles to the staining efficacy was significant when compared with a sample that did not contain undissolved probe, but increasing the volume of probe stock (whether dissolved or undissolved) to the milk gave only a marginal improvement in membrane staining efficacy. Furthermore, the *staining pattern* of the

14 $DiOC_{18}(3)$ is distributed in a spotty pattern, whereas $DiIC_{18}(3)$ is distributed in a streaky manner. Furthermore, $DiOC_{18}(3)$ appears to be poorly distributed in the MFGM (notice the absence of a defined border-line around the fat globule in Figure 4.20A, whereas $DiIC_{18}(3)$ is clearly present in the MFGM as shown by the defined border-line around the fat globule in Figure 4.20B).

MFGM appeared not to be affected (as determined by visual assessment) by the final probe concentration, except when it was too low; membrane features did not then show up or were difficult to see.

Specific targets for future work using these probes include making improvements to the staining protocols for $DiOC_{18}(3)$, $DiIC_{18}(3)$ and DiA to ensure that the composition or structure of the MFGM is not affected (see also the discussion on the effect of ethanol in section 4.8.3), while ensuring that all fat globules that are stainable are indeed stained by the probe. The results from the experiments using DiA indicate that the probe must be dissolved to stain the fat globules and this probably applies to other probes as well. (In non-food applications, sometimes the direct application of probe crystals is a successful technique for staining the specimen (e.g., Ozaki & Snider, 1997; Kubota et al., 2004), but in the current study, this technique was shown not to work well for fat globules in milk).

4.8.3 Lipophilic probes — Molecular structure and location/orientation in fat globules

On the basis of information published by the manufacturer of the screened probes, the styryl probes (DiA, FM1-84 and FM4-64), oxacarbocyanine probes ($DiOC_{18}(3)$ and SP-$DiOC_{18}(3)$), and indocarbocyanine probes ($DiIC_{18}(3)$, $DiIC_{18}(3)$-DS, SP-$DiIC_{18}(3)$, 5,5'-Ph_2-$DiIC_{18}(3)$) were expected to locate in the phospholipid bilayer of the MFGM as shown in Figure 4.25.

Styryl probes

The location of DiA in fat globules was inconsistent. In some images this probe appeared to locate solely in the MFGM, whereas in other images the probe appeared to also penetrate the MFGM and diffuse into the core fat of the globules (results not shown). The latter contrasts with the observation that solid DiA did not dissolve in FFMR (section 4.4.1). However, in the experiment involving FFMR *solid* DiA was added to the FFMR, whereas the results that showed that DiA was also present in the core fat were obtained after addition of dissolved DiA (in ethanolic stock) to the milk sample, and this may explain why in the latter case some DiA partially diffused into the fat core. In the light of these results, replacing the methyl group on the pyridinium ring of DiA by a carboxylated or sulfonated alkyl chain of suitable length would make the compound more soluble in water as well as promoting its location in the MFGM. The effect of this modification and other potential modifications to the molecular structure might be explored in future investigations.

Amphiphilic styryl dyes such as FM4-64 insert in the outer leaflet of membranes and have been widely used as fluorescent reporters of vesicle trafficking and organelle organisation in living animal and fungal cells (Fischer-Parton *et al.*, 2000) and are increasingly used in studies of living plant cells (Bolte *et al.*, 2004). The current study showed that FM4-64 can also be used in a food (i.e., milk). In contrast to the inconclusive results regarding the location of DiA, the FM4-64 probe was found to locate solely in the MFGM.

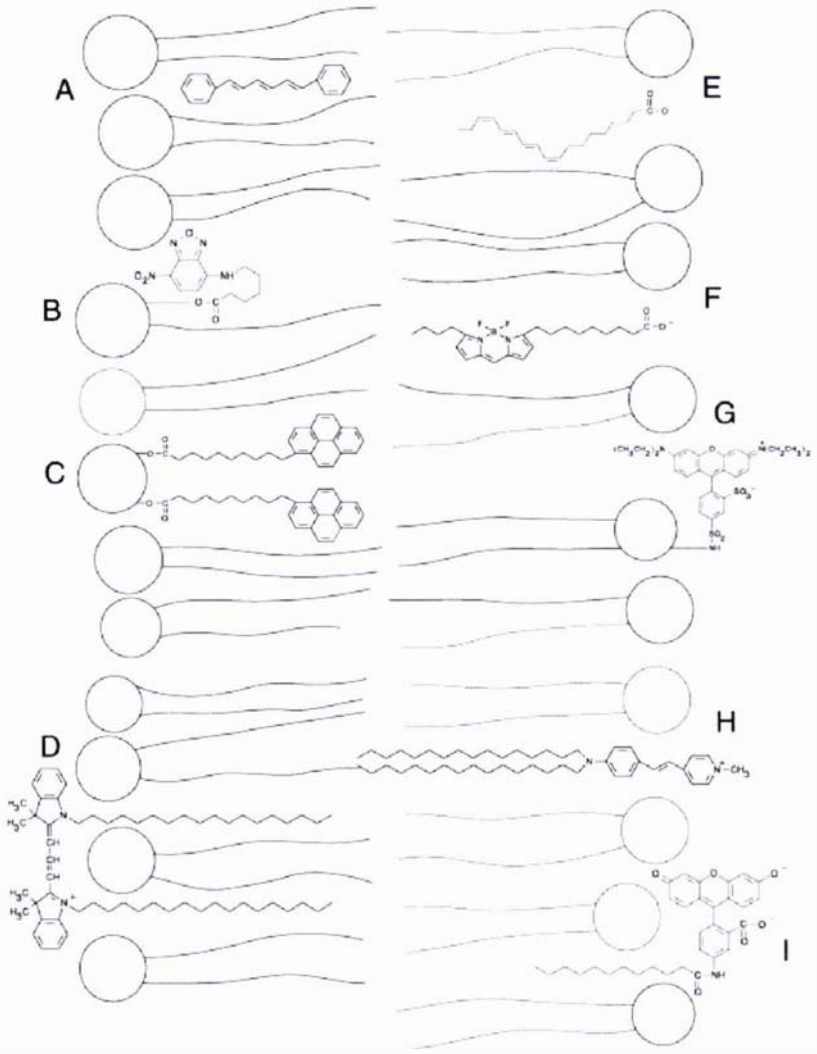


Figure 4.25 Expected location and orientation of different types of fluorescent membrane probes in a phospholipid bilayer. (A) Diphenyl. (B) & (C) Phosphocholine. (D) DiIC$_{18}$(3). (E) Fatty acid. (F) BODIPY[15] fatty acid. (G) Rhodamine glycerophosphoethanolamine. (H) DiA. (I) C_{12}-fluorescein (D109). Highly schematic. Not to scale. Reproduced with permission from Molecular Probes.

The part of the molecular structure mainly responsible for membrane association is the dialkylaminophenyl group (the so-called "tail" of the molecule (Betz *et al.*, 1996)). Hence, the observation that FM4-64 locates in the MFGM, and not in the fat globule core, suggests that shorter dialkyl chains in the tail promote location of styryl probes in the MFGM rather than in the fat globule core (note that the tail of DiA contains $(-CH_2)_{15}CH_3$ (i.e., long dialkyl) chains). It remains to be elucidated in future studies what caused the differences in fluorescence intensity of the probe in the MFGM. Possibly, areas of high fluorescence intensity could denote high concentrations of FM4-64 in domains rich in basic phospholipids (bright patches). However,

15 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (Boldyrev *et al.*, 2007).

domains rich in basic phospholipids might result in such high concentrations of FM4-64 that self-quenching occurs and thus dark patches in the membrane result (Fishov & Woldringh, 1999).

Carbocyanine probes

The images obtained for $DiIC_{18}(3)$-DS-stained fat globules showed that the location of this probe corresponded with the expected location, as it was totally absent from the fat core and visible only in the MFGM. In contrast, the images obtained for $DiOC_{18}(3)$-stained fat globules and $DiIC_{18}(3)$-stained fat globules showed that these probes were found to be mainly or partly located in the fat core of the globules. This meant that the latter probes could either penetrate the phospholipid bilayer membrane or enter the fat core via bilayer-denuded areas (if present) on the fat globule. Contrary to Nile Red or Nile Blue, which are homogeneously distributed, $DiOC_{18}(3)$ and $DiIC_{18}(3)$ are distributed inhomogeneously in the fat globule core (Figure 4.20). It is unlikely that the use of ethanolic stock caused these probes to locate in the fat core, for instance by ethanol diffusing into the fat core and thus carrying the probe along. This was demonstrated through confirmatory experiments by staining bovine milk with a combined stock solution containing both $DiOC_{18}(3)$ and $DiIC_{18}(3)$-DS. In dual-stained fat globules, $DiIC_{18}(3)$-DS was distributed in the MFGM whereas $DiOC_{18}(3)$ was distributed in the core of the fat globule (Figure 4.26).

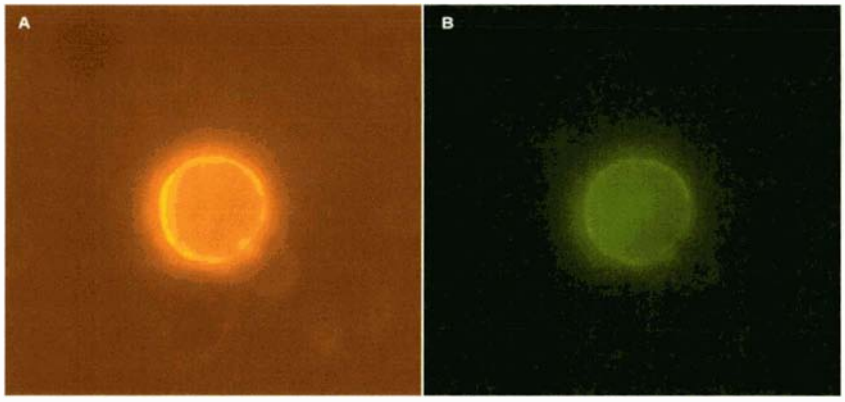


Figure 4.26 Conventional fluorescence microscopy images of a fat globule dual-stained with $DiIC_{18}(3)$-DS and $DiOC_{18}(3)$ using a combined probe stock. (A) $DiIC_{18}(3)$-DS staining shown using an excitation/emission filter 520–570/570–640 nm (included the emission from $DiIC_{18}(3)$-DS, but excluded the emission from $DiOC_{18}(3)$). $DiIC_{18}(3)$-DS was located in the MFGM. (B) $DiIC_{18}(3)$-DS and $DiOC_{18}(3)$ shown by using an excitation/emission filter 450–490/LP 515 nm which captured both $DiIC_{18}(3)$-DS emission, although at reduced intensity[16] (yellow; located in the MFGM), and $DiOC_{18}(3)$ emission (green; located inhomogeneously in the core).

16 Hence, brightly fluorescing fat globules were observed at reduced intensity compared with that seen using the other filter, and weakly fluorescing fat globules were not observed (note that the fat globules with faint MFGMs in (A) are not seen in (B)).

Hence, the different distribution patterns of $DiOC_{18}(3)$ and $DiIC_{18}(3)$ in the fat globule, and the fact that the use of ethanolic stocks of $DiIC_{18}(3)$-DS (an analogue of these probes) as well as of other lipophilic probes do not result in these probes diffusing into the fat core, argue against the supposition that the stock solvent carried the probe into the fat core. Probably, the hydrophilic/lipophilic balance of $DiOC_{18}(3)$ and $DiIC_{18}(3)$ is such that it allows association with neutral fat (triacylglycerols), possibly analogous to the tocopherols (for structures see Figure 4.27). Conceivably, their distribution is related to the state of the fat (liquid or crystalline). For example, if the probe dissolved in either the liquid fat or if it aligns itself with the crystalline phase, then the absence or presence of fluorescence within stained fat globules could potentially be used as indicators of the melting range of individual fat globules. Recently, an increasing fat globule diameter was shown to be associated with a higher crystallisation temperature (Michalski, 2004; Michalski *et al.*, 2004a). However, this study involved four relatively broad size classes (average volume/surface mean diameter of the particles diameters $d_{4,3}$ were 0.96, 3.30, 6.03 and 7.34 µm) and thus provided no information about *individual* fat globules. If the distribution of some fluorescent probes into the fat core is indeed related to the physical state of the fat, then the use of selective fluorescent probes combined with flow cytometry (see Chapter 7) would potentially allow the study of fat melting/crystallisation properties of individual fat globules as a function of fat globule size. As some flow cytometry instruments allow the separation and collection of different classes of particles, it should be possible to perform subsequent analyses, such as small-angle X-ray diffraction and nuclear magnetic resonance (NMR) spectroscopy, to study the crystalline properties of such classes of fat globules.

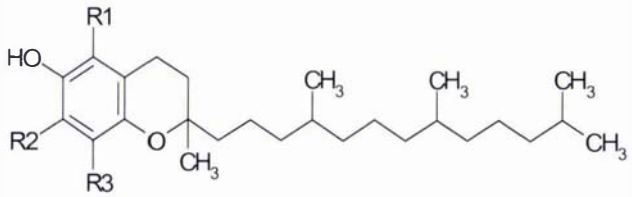


Figure 4.27 Chemical structure of tocopherol (vitamin E); (i) α-tocopherol: R1, R2 and R3 are CH_3; (ii) β-tocopherol: R1 and R3 are CH_3; (iii) γ-tocopherol: R2 and R3 are CH_3; (iv) δ-tocopherol: R3 is CH_3. Tocopherols are located in the core fat of the globule (Mulder & Walstra, 1974).

On the basis of the above results, some initial conclusions can be made regarding the relationship between the molecular structure of the lipophilic probes and their location in fat globules. Considering that $DiIC_{18}(3)$-DS is a sulfonated analogue of $DiIC_{18}(3)$, and that $DiIC_{18}(3)$ tended to partly stain the core fat of the globule, whereas $DiIC_{18}(3)$-DS stained only the MFGM, it can be concluded that the sulfonic acid groups at the 5,5' positions of the indocarbocyanine rings (Figure 4.8) make the molecule less lipophilic. In turn, this apparently prevents $DiIC_{18}(3)$-DS from penetrating the MFGM and diffusing into the core fat of the globule. Therefore, a 5,5' sulfonic acid analogue of the oxacarbocyanine $DiOC_{18}(3)$ should similarly locate in the MFGM only and not diffuse into the core fat. This analogue is conceptual at the present time, but may

be able to be synthesised on a commercial scale. Such a probe may have a different distribution in the MFGM than that of $DiIC_{18}(3)$-DS and may thus allow the study of different aspects of the MFGM composition or structure. Additionally, the fluorescence quantum yield of such a sulfonated membrane-bound probe would be expected to be greater than that of its parent molecule as the negatively charged sulfonate groups reduce the tendency of the probe to aggregate in membranes[17] (Molecular Probes). Furthermore, like $DiIC_{18}(3)$-DS, this analogue would be expected to be soluble in water, at least to some degree, allowing an aqueous stock solution to be used instead of an ethanolic stock, thereby eliminating the potential risk of altering the membrane by the use of ethanol as a stock solvent.

An alternative way of rendering these carbocyanine probes less lipophilic would be by reducing the length of the 18-carbon alkyl chains. Changing the length of the alkyl chains does not significantly affect the spectral properties of these probes as these are determined by the heteroatoms in the terminal ring systems and the length of the unsaturated connecting bridge (Molecular Probes). However, the length of the alkyl chain does affect the phase preference (fluid or gel) of the probe in co-existing membrane domains, and indocarbocyanines having a C18 alkyl chain appear to prefer the gel phase (Wolf, 1988). Hence, indocarbocyanines having appropriately (short) alkyl chains may potentially be used to reveal information about the MFGM phase behaviour as a function of temperature.

Changing the double bond bridge (-CH=CH-CH=) in $DiIC_{18}(3)$-DS between the aromatic rings would be expected to result in spectral changes (and thus affect the colour of the probe). The excitation and emission wavelengths are expected to become longer as the number of double bonds increases. This is due to a higher degree of conjugation between the aromatic rings and double bonds, resulting in a lower transition energy between the ground state and the excited state (Betz *et al.*, 1996). Hence, by changing the length of the double bond bridge in $DiIC_{18}(3)$-DS, it should be possible to obtain analogues that have the same, or a very similar, affinity for the MFGM as that of $DiIC_{18}(3)$-DS, but that posses different excitation and emission wavelength spectra. This is an important option to explore when developing multiple-staining protocols as $DiIC_{18}(3)$-DS has been found not to be compatible with some probes because of emission spectrum overlap.

4.8.4 Binding of ConA to the MFGM

Lectins are a class of proteins that bind carbohydrates, agglutinate cells and precipitate polysaccharides and glycoproteins (Sharon & Lis, 2003). Several studies have shown that a variety of lectins bind to MFGM oligosaccharides of either isolated or intact MFGM (Appendix 5). It appears that of the studies cited in Appendix 5 only one (Patton & Hubert, 1983) involved the binding of a lectin (ConA) to caprine milk fat globules in their native environment. However, in this study the actual measurements were carried out on washed isolated fat globules and no

17 Probe aggregation is a major cause of fluorescence quenching (Molecular Probes, The Handbook).

fluorescence microscopy was used. All studies that did involve fluorescence microscopy (Welsch *et al.*, 1988; Buchheim *et al.*, 1988b; Welsch *et al.*, 1990) were conducted using washed fat globules or MFGM fragments (i.e., fat globules were not studied in their native environment). Furthermore, none of these studies involved either bovine milk or the use of CLSM.

The results of the screening study suggest that, for either bovine milk or human milk, fluorescent ConA binds inhomogeneously to some fat globules. A significant proportion of fat globules appeared to be not stained at all; for other fat globules only parts of the membrane were stained (Figure 4.15). These results agree with those obtained by electron microscopy (Horisberger *et al.*, 1977), but contrast with those obtained using fluorescence microscopy (Buchheim *et al.*, 1988b). However, the latter study did report that ConA fluorescence was weaker than that of WGA. Although no studies have been reported in the literature in which the binding of ConA to intact bovine or human fat globules in their native environment was studied, some general observations can be made if the different techniques used in the different studies are kept in mind.

The findings listed in Appendix 5 show that ConA binding has been variably reported as exhibiting weak to strong binding to membranes of fat globules of different species (bovine, human, ovine, caprine, equine, rat). For none of these species has a total lack of ConA binding to MFGM been reported. This means that glycoconjugates in the MFGM of these species share a commonality in that they contain mannose and/or glucose residues. The binding of *Lens culinaris* agglutinin (Appendix 5) provides further evidence for the presence of either or both of these residues in bovine MFGM and human MFGM.

Concerning their location, the glycoconjugates containing the mannose and/or glucose residues appear to be externally disposed in the MFGM (Keenan *et al.*, 1974). ConA has been reported to be bound by several electrophoretically separable glycoproteins from the bovine MFGM bilayer as well as from the proteinaceous internal coat (i.e., that part of the membrane that was insoluble[18] in 1% Triton X-100 in 10 mM Tris-HCl, pH 8) (Murray *et al.*, 1979). This means that some of the externally located residues may be linked to transmembrane proteins that are anchored in the proteinaceous coat.

Using affinity chromatography, bovine PAS 1[19, 20], PAS 2, PAS 3, PAS 4, PAS 5, PAS 6, but not PAS 7, glycoproteins were retained by ConA (Kanno, 1986). Hence, these proteins may be involved in the binding of ConA to MFGM in its native environment. Although PAS 7 does not contain glucose it does contain mannose (Seok *et al.*, 2001) and therefore might have been expected to bind to ConA. The reason why PAS 7 did not do so in the study of Kanno (1986) is

18 1 h, 37°C.

19 PAS 1 is a historical name for MUC1 (Patton *et al.*, 1995).

20 Although ConA binds to bovine MUC1, ConA does not bind to human MUC1, probably because of the absence of significant levels of mannose, or a lack of accessibility (Patton *et al.*, 1995).

not clear, but it could be an issue of accessibility (at neutral pH ConA exists as a tetramer of about M_r 110,000 (Liener, 1976; Welsch *et al.*, 1983)). The fact that in the present study ConA was found not to bind to most fat globules could be related to a lack of accessibility by the fluorescent version of ConA to these residues[21]. Additionally, the fact that ConA has less dye per molecule (Molecular Probes, personal communication, 2005) would also contribute to a much lower fluorescence intensity of fat globules than that found when using WGA.

Binding of ConA to MFGM appears to occur rapidly, i.e. it is substantially complete within 10–30 min (Keenan *et al.*, 1974; Patton & Hubert, 1983). This rapid binding was found to be a significant practical problem in the current study as microscopy images frequently showed extensive fat globule aggregation. In principle, the degree of aggregation could form the basis of an agglutination assay (using the rate of agglutination as the response factor, indicating the degree of glycosylation of the MFGM). However, as fat globules have a natural tendency to aggregate in a milk sample on a microscope slide covered by a cover slip, and because this aggregation does not appear to occur in a repeatable fashion, it would be difficult to separate the agglutination action of ConA from the natural tendency of fat globules to aggregate. Furthermore, studies of ConA binding to caprine MFGM showed that soluble glycoproteins in the milk serum as well as other membranous matter (cells, tissue debris) also bind to ConA (Patton & Hubert, 1983). Considering that ConA promotes agglutination of cells, it may be speculated that, in milk, ConA also promotes aggregation of MFGM with non-MFGM matter (e.g., milk plasma membrane and somatic cells). In some of the images obtained (not shown) there was some indication that this might indeed be the case. This would be an interfering factor in an agglutination assay. Hence, the possibility of developing an agglutination assay was not further pursued.

In the light of the above, it was concluded that the use of ConA488 in combination with conventional fluorescence microscopy techniques would have only limited potential in the study of the properties or structure of the MFGM. Hence, no further work using ConA was performed.

4.8.5 Binding of WGA to the MFGM

In contrast to ConA, WGA stained almost all fat globules and thus showed a high potential for successful use in the study of the MFGM. Further validation and application of this lectin is described in Chapters 5 and 6.

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21 This notion appears to agree with reports from the literature (Horisberger *et al.*, 1977; Horisberger & Tacchini-Vonlanthen, 1982) in which the effect of gold granule size to which ConA was conjugated was investigated. These authors found that ConA bound to the membrane of red blood cells only when the size of the conjugated gold granule was less than 12 nm (in contrast, for WGA a gold granule size of up to 50 nm still allowed binding of WGA to red blood cell membrane). These results suggested that ConA binding sites are close to the bilayer and that access is masked by the glycocalyx (Horisberger & Tacchini-Vonlanthen, 1982). From the results of the present study it would appear that the same may apply to the MFGM.

5 WGA-staining of the MFGM

5.1 Introduction

Chapter 4 described the screening programme performed for identifying suitable fluorescent MFGM probes. WGA was found to be a promising probe for staining the MFGM of at least human milk and bovine milk. This chapter describes further validation work conducted to better understand the limitations and potential of WGA as a MFGM probe. With the exception of testing the effect of the concentration of the WGA on its staining efficacy, no work was done on human milk as it was not available at this stage of the experimental work. Therefore, all experiments in this chapter involving milk samples were performed using bulk bovine raw milk, unless specified otherwise. Although the main aim of the current work was to study fat globules in their native environment, it was recognised that it might be difficult to study interactions between fat globules and other milk components in their native environment (i.e., milk), because milk is a complex matrix. Studying stained fat globules in model systems reduced the number of possible confounding factors that might have been present when studying fat globules in their native environment.

Where relevant, the individual sections in this chapter include short discussion/conclusion statements to aid the reader in comprehending key findings. However, the overall significance of the results is discussed in the general discussion (section 5.8).

5.2 Control experiments

5.2.1 Verification that stained objects are fat globules

Addition of WGA stock to milk showed that many objects in the milk were stained (Chapter 4). To confirm that the identity of the WGA-stained objects were indeed fat globules, and not, for example, cells, a control experiment was conducted in which Nile Blue was used as a counter stain. Nile Blue is known to stain neutral fat; hence it stains the core of the fat globules only.

Nile Blue (4 µL, saturated aqueous stock; filtered), was added to milk (1 mL) conditioned to either 4°C or 20°C. After about 2 h, WGA488 stock (1 mg/mL; 5 µL) was added just prior to assessment using a conventional fluorescence microscope. Nile Blue staining of fat globules was observed using filter set 2 (excitation maximum 365 nm; emission: LP 420 nm) that excluded WGA488 emission, whereas WGA488 staining was observed using filter set 3 (excitation: BP 450–490 nm; emission: LP 515 nm). All the objects stained by WGA488 were also stained by Nile Blue demonstrating that WGA-stained objects were fat globules and not cells (the latter would not be stained by Nile Blue, because they have an aqueous, rather than a lipid, interior).

5.2.2 Degree of non-specific binding of WGA488 to MFGM

The valid use of WGA as a specific MFGM stain demanded the absence of non-specific binding of WGA to fat globules. WGA-fat globule binding experiments were conducted using fat globules that had been washed using deionised water (refer to Appendix 6 for the washing procedure) to ensure that binding of WGA to fat globules could be studied in an environment free from competing binding sites for WGA488 (e.g., glycosylated matter in the milk plasma). WGA488 stock was added to a phosphate buffer[1] containing 0.2 M *N*-acetylneuraminic acid (NANA; min. 95%, Sigma, St. Louis, MO, USA) (Figure 5.1) at a ratio of 1:100 (v/v) and incubated for 0.5 h at room temperature, before addition of washed fat globules (suspended in deionised water) to give a final concentration of fat similar to that of milk. The degree of fluorescence of fat globules was assessed visually using conventional fluorescence microscopy after 60–90 min and again after about 5 h. Repeat experiments were conducted in which the concentration of the WGA488 stock in the sugar-containing buffers was increased up to a ratio of 6:100 (v/v). Further binding specificity experiments were carried out as described above, but using free *N*-acetyl-D-glucosamine (GlcNAc) (min. 99.0%, Sigma) (Figure 5.1) instead of free NANA. Calculations showed that the inhibiting sugar was present in excess[2] (10^6 times greater; calculations performed on molar basis) compared with the WGA concentration. A control sample consisted of washed fat globules added to NANA- and GlcNAc-free phosphate buffer containing WGA488. Typical results for the control sample, the NANA sample and the GlcNAc sample after 60–90 min of incubation are shown in Figure 5.2.

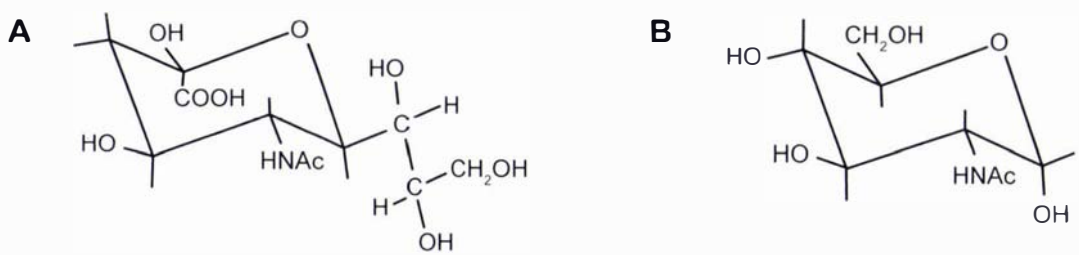

Figure 5.1 Chemical structures of sugars specifically bound by WGA. (A) *N*-acetylneuraminic acid. (B) *N*-acetyl-D-glucosamine.

In the presence of free NANA no significant binding of WGA488 with MFGM occurred even when the ratio of WGA stock:buffered sugar solution was increased up to 6:100 (v/v), i.e. up to six times greater than that typically used (1:100 v/v). Furthermore, there appeared to be no time effect for at least 5 h as there was no difference in results after 5 h. Hence, it can be concluded that for the association constants (Ka), relating to the binding of WGA488 with its substrates (free NANA, bound NANA and bound GlcNAc), $Ka_{NANA\text{-}free} >> Ka_{NANA\text{-}bound}$ and also $Ka_{NANA\text{-}free} >> Ka_{GlcNAc\text{-}bound}$.

1 Phosphate buffer (KH_2PO_4, 0.001 M; Na_2HPO_4, 0.01 M) was adjusted to pH 7.3 using 0.1 M HCl.

2 If the ratio inhibiting-sugar:lectin is not high enough proper blocking of the binding sites of the lectin may not occur (Johnsen *et al.*, 2000).

Figure 5.2 Testing the degree of non-specific binding of WGA488 to MFGM. (A) Control sample (sugar-free phosphate buffer with WGA-stained fat globules). (B) Washed fat globules in the presence of NANA and WGA488. (C) Washed fat globules in the presence of GlcNAc and WGA488. Bar = 20 µm.

In the presence of free GlcNAc, some WGA488 binding to fat globules occurred. The average fluorescence intensity of fat globules appeared to increase with the concentration of WGA488 and with incubation time. Judged by the lower fluorescence intensity of the fat globules compared with that of the control, it may be concluded that this binding was generally significantly less than that of WGA488 to fat globules in the absence of free GlcNAc. Hence, it appears that WGA488 was inhibited incompletely by free GlcNAc, but essentially inhibited completely by free NANA. From the latter results, it is concluded that WGA does not bind in a non-specific manner to MFGM.

5.2.3 WGA binding to MFGM — Effect of Alexa Fluor® conjugate

The manufacturer of the fluorescent WGA conjugates markets a wide range of Alexa Fluor® conjugates. In principle, this allows the selection of a suitable WGA conjugate when a staining protocol is desired that combines WGA with (an)other probe(s). To verify that WGA binding to MFGM is not hindered or promoted by its fluorescent conjugate, a dual staining experiment was conducted using two different WGA conjugates. Samples were assessed visually using conventional fluorescence microscopy.

Preliminary work, using individual probes, showed that the available filter sets were not suitable for WGA680-stained fat globules, and this probe was not used. However, WGA488-stained fat globules could be observed with filter set 3; no bleed-through was observed into filter set 4 (excitation: BP 532.5–557.5 nm; emission: BP 570–640 nm). The emission of WGA594-stained fat globules could be observed[3] with filter set 4, with minimum bleed-through into filter set 3 (only the very brightly fluorescent WGA594-stained fat globules could be observed with filter set 3).

Raw milk was stained with a combined stock (1 mL) containing WGA488 and WGA594 (each 0.5 mg/mL) and a subsample was observed using both filter set 3 and 4. The fluorescence

3 The fluorescence of WGA594-stained fat globules was somewhat weak compared with that of the WGA488-stained fat globules, because use of filter set 4 resulted in suboptimal excitation (about 50%) of the probe.

emission of WGA594, as seen by using filter set 4, was somewhat weak compared with that of WGA488 using filter set 3, because the excitation filter of filter set 4 corresponded to a shoulder of the absorption curve at about 50% of the absorption maximum (589 ± 5 nm). Despite this, the emission of WGA594 was sufficient for visual assessment. In general, there was a good correlation between the relative intensity of stained fat globules as seen when using filter set 3 (WGA488) and that when using filter set 4 (WGA594) (Figure 5.3). This suggested that binding of WGA to MFGM was not conjugate-dependent.

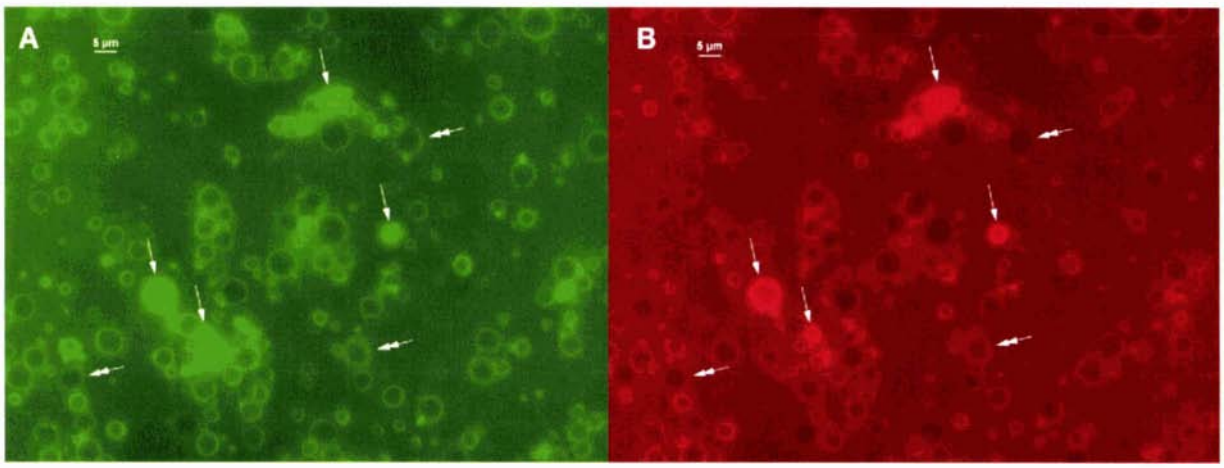

Figure 5.3 Milk dual-stained with WGA488 (A) and WGA594 (B). Comparing the relative fluorescence intensity of fat globules within each channel showed that the same fat globules were brightly or weakly stained (single arrows are examples of bright fat globules; whereas double arrows are examples of relatively weakly fluorescent fat globules). Note that, because of the time required to switch between filter sets and the time taken to capture the images, some fat globules have moved so that the fat globules in these two images are not necessarily present at identical positions.

5.2.4 Heat stability of WGA

Heat treatments are used in the dairy industry to safeguard the quality of the milk and to influence the properties of processed milk products (Evers, 2004a). Preliminary experiments involving heat treatment of WGA-stained milk indicated that the fluorescence intensity of fat globules was affected by heating temperature and time (i.e., fluorescence decreased with increasing temperature and time; results not shown). To elucidate whether the heat treatments caused a change in the MFGM, or whether WGA was heat-labile a series of experiments was conducted.

5.2.5 Heat treatment of milk at 55°C and 90°C

Milk samples (350 µL) were heated and subsequently cooled (1 min under cold running tap water) either before or after addition of the probe to the sample (Table 5.5.1). Subsamples of the stained milk were assessed using a conventional fluorescence microscope. The microscope

camera exposure time was kept constant at 215 ms for all samples. Two heat treatment levels were chosen: mild (55°C) and high (90°C).

Table 5.5.1 Experimental conditions in the heat stability experiment for WGA488.

No.	Temperature (°C)	Holding time (min)	Treatment[1]
1	55	1	HP
2	55	1	PH
3	55	10	HP
4	55	10	PH
5	55	15	HP
6	55	15	PH
7	55	90	HP
8	55	90	PH
9	90	1	HP
10	90	1	PH
11	90	10	HP
12	90	10	PH
13	90	20	HP
14	90	20	PH
Control	Room temperature	–	Probe added to sample at room temperature

[1] HP = The sample was heated and cooled, and then the probe was added; PH = the probe was added to the sample, and then the sample was heated and cooled.

It was found that at

- 55°C, fat globule clusters were observed irrespective of whether the probe was added before or after the heat treatment for holding times of up to 90 min;
- 90°C, no fat globule clusters were observed irrespective of whether the probe was added before or after the heat treatment for holding times of up to 20 min;
- 55°C, increasing the holding time when the probe was added after the heat treatment (i.e., the HP treatments) appeared to result in brighter fat globules;
- 55°C, when the probe was added prior to heating the sample, the fluorescence of fat globules heated for 10, 15, or 90 min was less than that of the sample heated for 1 min;
- 90°C, the HP treatment yielded fluorescent fat globules irrespective of holding time;

- 90°C, the PH treatment only yielded fluorescent fat globules when the sample was heated for 1 min. Heating for 10 or 20 min resulted in the fat globules becoming virtually non-fluorescent.

5.2.6 Heat treatment of WGA488 stock

Aliquots of WGA488 stock (3.5 µL) were pipetted into 4 mL vials and held for 1, 10, or 20 min at 90°C and cooled for 1 min under running cold tap water (the temperature of the sample after the cooling step was typically about 20°C as measured with a thermocouple thermometer[4]). Milk (350 µL) was then added and the fluorescence of fat globules was compared with that of an unheated control sample (WGA488 added to the milk at room temperature).

The fluorescence intensity of fat globules was found to be in the order: control > 1 min heated > 10 min heated > 20 min heated. The fluorescence of fat globules of the samples heated for 10 or 20 min was very weak and faded very quickly , i.e. after 1 s of illumination by the excitation source.

5.2.7 Heat treatment of WGA488-stained milk followed by addition of WGA488

WGA488 stock (3.5 µL) was added to milk (350 µL). The stained sample was allowed to stand at room temperature for 6 min to allow the WGA to bind to the sugar residues, and the sample was then heated at 90°C for 10 min and cooled under cold running tap water for 1 min. A subsample (7.5 µL) was evaluated under the conventional fluorescence microscope after 15 min (PH treatment). Subsequently, WGA stock (3.5 µL) was added to this heat treated sample and a subsample (7.5 µL) was evaluated (PHP treatment). A control sample (3.5 µL stock to 350 µL milk at RT) was also evaluated. No fluorescent fat globules were observed in the PH sample. Significant fluorescence of fat globules was observed in the PHP sample. In the PHP sample, no fat globule clustering was observed, whereas in the control sample this was observed (as before).

5.2.8 Discussion & conclusions

The results of the experiments in sections 5.2.5–5.2.7 showed conclusively that the loss of fluorescence of fat globules in pre-stained heat treated milk was caused by WGA being heat labile. The heat stability of WGA was found to be both time and temperature dependent. This means that in experiments involving heat treated milk, WGA must be added to the milk sample *after* the heat treatment.

4 Similarly, cooling of samples under running cold tap water for 1 min after heating at 75°C brought the temperature down to 20°C.

Furthermore, the phenomenon of fat globule clustering, which is observed in WGA-stained milk at room temperature[5], disappears evidently as a consequence of heat treatment of the milk, not by denaturing WGA. The effect is rapid, i.e. < 1min at 90°C.

It was considered desirable to undertake further work to better understand the interactions between milk plasma components, WGA and the MFGM. However, considering the complex interactions possible between different serum components and the MFGM as a function of temperature and time, experiments were conducted using model solutions so that complexity would be reduced.

5.3 Reconstitution, heat treatment and staining of washed fat globules

As a prerequisite to model studies investigating the effect of individual milk plasma components on WGA staining of fat globules, first it had to be established whether washed fat globules would stay intact upon reconstitution in an aqueous medium and heat treatment prior to staining with WGA.

Washed cream (50 µL; pH = 7.13, i.e. within the pH range in which the fluorescence was shown not to be affected), obtained using the protocol described in Appendix 6, was diluted with milli-Q water (450 µL)[6] and WGA488 stock was added (5 µL) to unheated or heat treated samples (75°C, 5 min, cooled for 1 min under cold running tap water). Images were captured using a constant camera exposure time for all images.

It was found that although some fat globules were non-spherical, most were spherical. This showed that, in general, the washing procedure did not destabilise the fat globules. Furthermore, the fat globules were stained with WGA. Variation of fluorescence intensity of fat globules was observed between different areas on the same slide. Hence, to obtain a good impression of the fluorescence of fat globules on the slide, multiple images needed to be made. However, the number of replicates was limited by logistical factors, i.e. the number of images that could be made per unit time (it took 15–30 min generally to assess the slide and to take four images).

Not surprisingly, the washing procedure significantly reduced the proportion of small fat globules. Therefore, most fat globules were probably > 0.5 µm. It was assumed that this would not adversely affect the general outcomes of further studies in which washed fat globules were used in model systems.

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5 Recall that fat globule aggregation was also observed at 55°C.

6 This ten-fold dilution of cream appeared to reduce the number of fat globules on-slide to below that typical for milk. In subsequent experiments a five-fold dilution was used, which gave a concentration of fat globules on-slide that was similar to that in milk. A practical advantage of using the five-fold dilution factor was that fat globules immobilised more quickly on the microscope slide (most in-focus fat globules needed to have become immobile before an image could be captured, otherwise moving fat globules would result in blurry lines in the image).

In conclusion, this experiment showed that it was possible (from a practical perspective) to isolate fat globules from their native milk plasma environment by repeated washing using milli-Q water, and to stain these washed fat globules using fluorescent WGA, even when they were heat treated.

Considering the possible variation in fluorescence intensity of fat globules on the same slide (as determined visually), small differences in fluorescence intensity between different treatments could not be detected with certainty. However, gross differences (little/weak fluorescent, medium fluorescent, or brightly fluorescent) could be detected and the visual evaluation method was based on this classification system. Similar categorisation of fluorescence microscopy results has been used in other studies (e.g., Johnsen *et al.*, 2000). Nevertheless, more subtle differences were sometimes observable, and, where appropriate, these were taken into account, although the weighting of such results in formulating final conclusions was significantly smaller than that of results that showed gross differences.

5.4 Effect of milk plasma proteins on fluorescence intensity of WGA-stained MFGM

To study the contribution of plasma proteins on the fluorescence of WGA-stained MFGM, a series of experiments was conducted using solutions of isolated plasma proteins, and their effect on the fluorescence of washed fat globules was investigated.

5.4.1 Caseins

Of the caseins in mature milk, only κ-casein is glycosylated[7] and contains NANA moieties[8] (Swaisgood, 1995), and thus can interact with WGA.

κ-Casein

Isolated κ-casein (gift from S. Anema, Fonterra Research Centre) was dissolved in milli-Q water. The solubility of κ-casein was uncertain, but from experience it was known that the isolated κ-casein did not completely dissolve in water (S. Anema, personal communication, 27 July 2006). κ-Casein (28.4 mg) was added to water (2.70 mL) and after 2 h stirring an aliquot of the solution was filtered through a 0.45 µm disc filter, which yielded a visually clear filtrate. Gravimetric analysis (drying at 102°C to constant weight) showed that the concentration of the dissolved κ-casein was 0.65 g/100 g, which is about 2x its concentration in mature bovine milk[9].

Washed cream (60 µL) was diluted with milli-Q water or κ-casein solution (240 µL) and WGA488 (3.5 µL) was added to an unheated solution or after heat treatment (75°C, 5 min). The microscope camera exposure time was kept constant at 148 ms for all images, and the

7 However, in mature milk the non-glycosylated form is the major component (~ 85%).

8 κ-Casein glycosylation in colostrum is more complex than that in mature milk and contains GlcNAc also (Swaisgood, 1995).

9 Typical κ-casein concentration in milk is 0.35 g/100 g (Jensen *et al.*, 1995).

fluorescence intensity of fat globules in the different samples was compared visually. Raw milk samples (heat treated and unheated) served as the controls. Replicate images from different points were captured for each slide.

The results showed:

1. Fading of fluorescence.
   Some fading of fat globule fluorescence intensity was observed during assessment of the sample. Hence, the effect of duration of excitation on fat globule fluorescence was systematically studied through a time-series experiment (total 130 s, steps of 10 s). This clearly showed that fading was significant within 10 s intervals. Hence, the sample had to be exposed for as little time as possible to the excitation light prior to capturing an electronic image. Note that switching the microscope from viewing the sample through the eye-piece to viewing the image through the camera, as projected on the computer screen, required refocusing the microscope. If this was done while the microscope was in fluorescence mode significant fading would occur. As the total exposure time would then be different for different images (sometimes refocusing can be quick, at other times it can be slow) the ability to compare the fluorescence intensity of fat globules on different images could be severely compromised. To prevent the latter from happening in this study of the effect of individual plasma proteins on fluorescence intensity of WGA-stained MFGM, a best-practice evaluation and image-capturing technique was developed as described in Appendix 7. This technique was used for subsequent experiments.

2. Motion of fat globules on the slide.
   Fat globules tended to keep moving for much longer times than usually observed when assessing a milk sample. It appeared that the sample matrix (milk plasma or aqueous protein solution) affected the motion of fat globules on a microscope slide. In several cases, the fat globules that came to rest and that touched one another coalesced. As individual fat globules kept on moving around (and thus could not be imaged) in many cases the only option was to take images of the coalesced fat globules. Note that such coalescence was not representative of the fat globules in the parent sample.

3. The effect of κ-casein on the fluorescence intensity of washed fat globules.
   The fluorescence of washed fat globules re-suspended in milli-Q water was very weak. Heating did not affect the fluorescence intensity, but effected a change in the fat globules so that they did not coalesce. Similarly, the fluorescence of washed fat globules re-suspended in κ-casein solution was also very weak but somewhat greater than that of fat globules re-suspended in milli-Q water. No significant change in fluorescence intensity upon heat treatment was observed.

Sodium caseinate

Na-caseinate (Alanate 180, Fonterra Co-operative Group Ltd) was dissolved in milli-Q water (0.5 g/100 g)[10] and a clear filtrate was obtained by filtering through a 0.45 µm disc filter. Fat globule fluorescence was assessed visually after addition of WGA488 stock (3.5 µL) to washed fat globules in milli-Q water or in the Na-caseinate solution (240 µL). Re-suspended samples were either unheated or heat treated (75°C, 5 min) prior to addition of the WGA488. Controls consisted of WGA488-stained protein solutions (i.e., solutions containing no fat globules).

In contrast to the κ-casein results, washed fat globules re-suspended in Na-caseinate were found to be, on average, more fluorescent than those in milli-Q water. Heating of washed fat globules in the presence of Na-caseinate seemed to result in an increase of "loose" fluorescent filamentous matter and fluorescent "bits". These could be caseinate (aggregates?) and/or parts of MFGM (identifying the origin of these was deemed to be outside the scope of the current study). Thickened fluorescent areas (some were very bright) were seen on several fat globules (Figure 5.4).

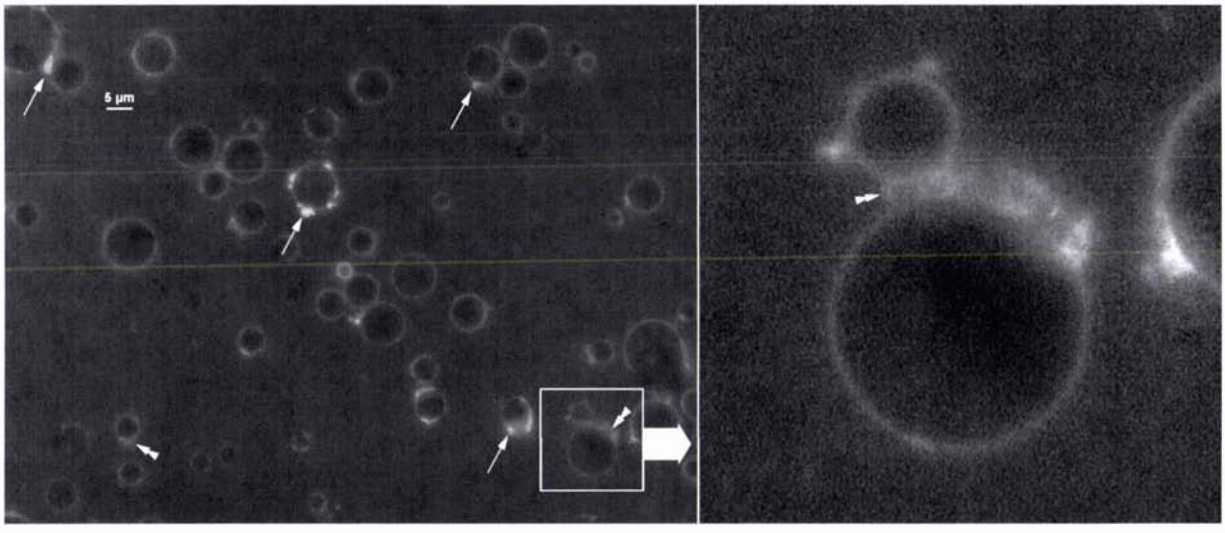

Figure 5.4 Washed fat globules re-suspended in Na-caseinate solution and stained with WGA488. Some fat globules contain bright fluorescent spots (arrows), whereas thickened deposits are seen on other fat globules (double arrow heads). Inset (contrast enhanced): note how the thickened matter acts as a "glue" between the two fat globules (double arrow head).

5.4.2 Whey protein isolates

To simulate the effect of whey proteins in milk on fat globule staining, whey protein powder was reconstituted in milli-Q water. Two different whey protein isolates were used (both made from cheese whey), one not containing glycomacropeptide (GMP) (WPI 8972, Fonterra Co-operative Group Ltd; obtained by ion exchange) and one containing GMP (WPI 8945, Fonterra Co-

10 This concentration is about 1/6th of the casein content of milk. However, in order to compare the effect with that of β-lactoglobulin (see section 5.4.3), the same concentration as that used in the β-lactoglobulin experiment was used.

operative Group Ltd; obtained by ultrafiltration). Solutions in water were made with protein contents of 0.5 g/100 g and 0.6 g/100 g[11] respectively (corresponding to a typical whey protein level in bovine milk of 0.5–0.7 g/100 g). The two solutions were slightly cloudy; clear solutions were obtained by filtering through 0.2 µm and 0.45 µm disc filters respectively.

Washed cream (60 µL) was diluted with water or whey protein solution (240 µL) and WGA488 (3.5 µL) was added to an unheated or heat treated (75°C, 5 min) solution. The microscope camera exposure time was kept constant at 148 ms for all images. The unheated sample and the heat treated counterpart were placed next to one another on the same microscope slide to allow quick comparison of the fluorescence intensity of the unheated sample and heat treated sample. Raw milk samples (heat treated and unheated) served as the controls. Replicate images from different points were captured for each slide. The experiment was repeated on the next day. The stained samples of the repeat experiment were stored at 5°C for 3 days protected from light, and evaluated again by conventional fluorescence microscopy.

The results showed that:

1. the fluorescence intensity of washed fat globules re-suspended in whey protein solutions was relatively high in the unheated samples compared with that of fat globules re-suspended in milli-Q water, but no significant difference in fluorescence intensity was observed between the fat globules re-suspended in the solutions of the two different types of whey protein;

2. the re-suspended fat globules in the heat treated whey protein solutions appeared to be, on average, slightly brighter than those in the unheated samples. This agreed with the heat treated *vs* the unheated milk experiments, except that in milk only a proportion of fat globules appeared to become brighter, whereas fat globules re-suspended in WPI solutions all tended to become more intensely fluorescent;

3. the WGA-bound fat globules in both unheated and heat treated stained samples, and those in the stained raw milk controls, remained intact and fluorescent upon 3 days cold storage. The main effects observed prior to cold storage were all observed in the cold-storage samples;

4. fat globules in heated samples were consistently much less prone to aggregation, and thus coalescence, on the microscope slide, than fat globules were in unheated samples. Hence, in heat treated samples, repulsive forces exceeded attractive forces between fat globules and this remained so for at least 3 days upon cold storage.

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11 To compensate for the GMP, 0.6 g/100g equates to about 0.5 g/100 g whey proteins. Note that the addition of the cream effectively reduced the concentration of the protein in the final sample by 1/5th; this comment applies to all subsequent experiments in which 60 µL cream was added to 240 µL diluent.

5.4.3 β-Lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulin G

As the experiment using WPIs showed that whey proteins affected the fluorescence intensity of washed fat globules, the next aim was to investigate the effect of *individual* whey proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA) and immunoglobulin G (IgG)), on the staining of fat globules by WGA488 in model systems. Furthermore, for one major whey protein (β-lactoglobulin) and one minor whey protein (IgG)), the effect of buffer was compared with that of water as the solvent for the proteins.

Experiments involving PBS

Isolated β-lactoglobulin (Sigma; this contains both β-lactoglobulin A and β-lactoglobulin B) was dissolved in PBS or water to obtain solutions that contained 0.50 g β-lactoglobulin/100 g (bovine milk contains 0.18–0.5 g β-lactoglobulin/100 g (Hambling *et al.*, 1992)). The upper level was chosen to ensure that if there was an effect it would be observed. Similarly, IgG (Sigma) was dissolved in PBS or water to obtain solutions that contained 0.072 g IgG/100 g (typical level in bovine milk is about 0.07 g total IgG/100 g; Larson, 1992). Washed cream (60 µL) was diluted with water, whey protein solution (240 µL), or PBS, and WGA488 (3.5 µL) was added to an unheated or heat treated (75°C, 5 min) solution. Assessment by microscopy was as described before for WPIs.

Experiments not involving PBS

BSA (> 99%, Sigma) was dissolved in milli-Q water (0.04 g/100 g; the typical BSA concentration in bovine milk is 0.04 g/100 g (Grappin & Ribadeau-Dumas, 1992)). Isolated α-lactalbumin (gift of M. Li, Fonterra Research Centre) was dissolved in milli-Q water (0.20 g/100 g)[12]. Addition of washed cream and WGA and further experimental details were as described above for the experiment involving β-lactoglobulin and IgG (i.e., *Experiments involving PBS*).

Results

The results are summarised in Table 5.5.2.

Fat globule clustering

Staining of washed fat globules re-suspended in PBS (pH 6.6) resulted in extensive clustering of fat globules in all samples containing PBS (samples 2a, 2b, 4a, 4b, 9a and 9b). In contrast, fat globule clustering was essentially absent in all samples in which milli-Q water only was used as the solvent.

12 Typical α-lactalbumin concentrations in bovine milk have been reported as 0.2 g/100 g (Andrews, 1992) and 0.12 g/100 g (Grappin & Ribadeau-Dumas, 1992). In the current study, the higher concentration was chosen to ensure that if there was an effect it would be observed.

Table 5.5.2 Summary of results obtained for various model systems and heat treatments investigated for studying the effects of protein and buffer on WGA binding to washed fat globules.

Sample	Model system	Results
1a[1]	β-Lactoglobulin in milli-Q water	No significant fat globule clustering was observed. The fluorescence of fat globules was similar to that of those in the milli-Q control sample (8a).
1b[2]	β-Lactoglobulin in milli-Q water	Results were very similar to those of sample 1a. However, dark patches on the fat globule membranes could be discerned clearly, whereas this was not the case for fat globules in sample 1a.
2a	β-Lactoglobulin in PBS	Many fat globule clusters seen immediately after the sample was placed on the slide. Clusters were highly fluorescent. Background (i.e., solution) was dark in contrast to e.g. milk where the background was greenish. The majority of fat globules appeared to have weakly fluorescent membranes, but these were clearly visible against dark background.
2b	β-Lactoglobulin in PBS	Results were very similar to those of sample 2a.
3a	IgG in milli-Q water	No fat globule clusters observed. The fluorescence of fat globules was highly variable; a significant proportion (~ 10%) did not have a fluorescent membrane.
3b	IgG in milli-Q water	The fluorescence of fat globules appeared to be weaker than that of those of sample 3a. Non-fluorescent fat globules were observed, but not as extensively as in sample 3a.
4a	IgG in PBS	Many fat globule clusters were observed. Dark background. Fat globules became immobile quickly.
4b	IgG in PBS	Many fat globule clusters were observed. Dark background. Fat globules in cluster appeared to be somewhat less fluorescent than those of sample 4a.
5a	α-Lactalbumin in milli-Q water	No fat globule clustering was observed. Fat globule fluorescence was relatively high (i.e., comparable to that of the raw milk control sample, 7a). A significant proportion of very bright fat globules was observed. Dark patches were observed on the membranes.
5b	α-Lactalbumin in milli-Q water	No fat globule clustering was observed. Fat globule fluorescence was relatively high, but appeared on average to be less than that of sample 5a. Also, the number of very bright fat globules appeared to be smaller than that of sample 5a.
6a	BSA in milli-Q water	Mild tendency to form fat globule clusters was observed. The fluorescence of fat globules was weak (similar to those of the milli-Q water control sample, 8a).
6b	BSA in milli-Q water	Mild tendency to form fat globule clusters was observed. The fluorescence of fat globules was highly variable.
7a	Raw milk[3]	Many small fat globule clusters were observed immediately after placing the sample on the slide. Background and fat globules appeared to be "fluffy" (i.e., small fluorescent particulate matter/strands).
7b	Raw milk[3]	No fat globule clusters were observed. Dark patches on fat globule membranes could be discerned clearly on both highly fluorescent fat globules (minority) and weakly fluorescent fat globules (majority). The proportion of bright fluorescent fat globules appeared to be larger in this sample than in sample 7a.
8a	Milli-Q water	Virtually no fat globule clusters were observed. Most fat globules were weakly fluorescent, although few were bright. After a few minutes, significant fat globule coalescence occurred on-slide.
8b	Milli-Q water	No fat globule clusters were observed. Fluorescence of fat globules on slide was highly variable.
9a	PBS	Extensive fat globule clustering was seen immediately when the sample was placed on the slide.
9b	PBS	On average, this sample appeared to contain fewer bright fat globules than sample 9a. Extensive fat globule clustering was seen immediately when the sample was placed on the slide.

[1] "a" denotes unheated sample.
[2] "b" denotes heat treated sample.
[3] This was not the diluent, but the control sample.

Fluorescence intensity of washed fat globules

The results for washed fat globules re-suspended in milli-Q water (samples 8a and 8b) were similar to those obtained for washed fat globules re-suspended in milli-Q water in the κ-casein, sodium caseinate and WPI experiments (i.e., very weak fluorescence of fat globules and no difference between the heat treated and unheated samples).

The fluorescence intensity of fat globules re-suspended in the β-lactoglobulin in milli-Q water solution (samples 1a and 1b) was, on average, significantly greater than that of those in milli-Q water alone (samples 8a and 8b), but there was no discernable difference between the unheated and heat treated samples (1a and 1b). This suggested that heating was not a major factor for fluorescence intensity of fat globules, but the presence of protein was. This agreed with the results of the WPI experiment, but also raised the question whether it was just whey protein or any protein, and whether the concentration of the protein played a role.

The presence of the IgG in milli-Q water (samples 3a and 3b) appeared to result in an increase in the fluorescence of the fat globules, but an increase somewhat less than that observed when β-lactoglobulin was present. The fat globules in the heat treated sample (3b) appeared to be somewhat less fluorescent compared with those in the unheated sample (3a). The same was observed in the corresponding PBS samples (4a and 4b). Although this result should be interpreted with caution, it can at least be concluded that heat treatment in the presence of IgG did not result in the fat globules becoming *more* fluorescent. Hence, the effect observed in milk upon heating (i.e., increased fluorescence intensity of some fat globules) is unlikely to be caused by (denatured) IgG. Note that the initial denaturation temperature of IgG is 72°C (Singh & Havea, 2003).

Fat globules re-suspended in the α-lactalbumin solutions (both unheated (5a) and heat treated (5b)) were significantly fluorescent, whereas those in the BSA solutions (both unheated (6a) and heat treated (6b)) were only weakly fluorescent. The heat treated α-lactalbumin solution (5b) seemed to result in less brightly stained fat globules compared with those in the unheated α-lactalbumin solution (5a). Within the BSA solutions the degree of fat globule fluorescence varied drastically, and ranged from virtually non-fluorescent to reasonably fluorescent. A similar phenomenon had been observed for fat globules re-suspended in IgG solution (in milli-Q water; samples 3a and 3b) and indicated that the concentration of protein was an important factor (in both cases the protein concentrations were < 0.1 g/100 g).

Although in the heat treated α-lactalbumin solution (5b) the average fluorescence intensity of the fat globules appeared to be less than that in the unheated sample (5a), on balance it was concluded that in neither the α-lactalbumin solution (sample 5) nor the BSA solution (sample 6) did heating appear to have had a large effect on fat globule fluorescence. Hence, as for IgG, this suggested that if there is any effect in milk caused by heating (increased fluorescence

intensity of some fat globules) then it is unlikely to be caused by (denatured) α-lactalbumin[13] or BSA.

Additional α-lactalbumin control samples (consisting of α-lactalbumin solution plus WGA, but without fat globules; results not listed in Table 5.5.2) showed the presence of fluorescent particles. The size of the particles varied from almost "dot-like" to "strand-like" such as those seen in raw milk or sometimes in washed fat globules. These were not abundant and were probably undissolved α-lactalbumin or impurities in the α-lactalbumin source. They were not caused by the heat treatment as they were observed in both the unheated and heat treated controls, and therefore could not be heat-induced α-lactalbumin aggregates such as those observed when heating whey (Kulozik *et al.*, 2003). Such fluorescent particles were not observed in β-lactoglobulin solutions. In the Na-caseinate control samples, abundant fluorescent particles were observed much like that in the plasma of raw milk, but no filamentous matter was observed. It is therefore likely that WGA binds to casein micelles (presumably through κ-casein of which 15% is glycosylated) and that this is a major contributing factor to the background (i.e., milk plasma) fluorescence of raw milk. This conclusion corroborates observations by Welsch *et al.* (1988) who showed that ferritin-labelled WGA bound to casein micelles in equine milk. An experiment in which casein micelles are dissociated, or alternatively in which casein is precipitated and removed by filtration from raw bovine milk, could further confirm this explanation. These fluorescent particles were also observed in the heat treated control sample, but there appeared to be fewer particles (only the bright particles could be seen), and this would be consistent with κ-casein being shielded through interactions of whey proteins with casein micelles upon elevated heating (Macej *et al.*, 2002).

5.5 Effect of the protein concentration on the fluorescence intensity of WGA488-stained fat globules

The previous experiments (section 5.4) suggested that protein concentration *per se* could potentially affect the fluorescence of WGA-stained fat globules, and this was further investigated as described below.

5.5.1 β-Lactoglobulin

Isolated β-lactoglobulin (3 g/100 g) in globular form (obtained by the procedure of Manderson *et al.* (1998); gift of T. Considine, Fonterra Research Centre) was diluted using milli-Q water to obtain a series of solutions having β-lactoglobulin concentrations of 0.05, 0.3 or 0.5 g/100 g and washed cream was added as described in section 5.4.3. WGA488 stock (3.5 µL) was added to unheated or heat treated samples (75°C, 5 min). Control samples consisted of WGA488-stained protein solutions (0.5 g protein/100 g). Evaluation of the fluorescence intensity of the fat

13 The initial denaturation temperature of α-lactalbumin is 68°C (Singh & Havea, 2003).

globules was performed using a conventional fluorescence microscope, within 30 min of the addition of WGA stock to the relevant protein solution.

The fluorescence of the fat globules in the unheated solutions decreased with decreasing concentration of β-lactoglobulin.

The heat treated samples for the two highest β-lactoglobulin concentrations (0.3 and 0.5 g/ 100 g, respectively) clearly had a greater proportion of brightly fluorescent fat globules than their unheated counterparts. This contrasted with results reported for β-lactoglobulin in section 5.4.3 in which heat treatment was not observed to have a significant effect on fat globule staining by WGA488. As the β-lactoglobulin globulin from the latter experiment was reconstituted from lyophilised material, whereas in the current experiment the β-lactoglobulin used was in solution and globular, this suggested that *globular* β-lactoglobulin interacts with the MFGM of some (but not the majority of) fat globules and suggested different fat globules behave differently in terms of their interaction with whey proteins. Hence, this result strongly suggested β-lactoglobulin playing a role in the increased occurrence of bright fat globules for heat treated milk samples.

Irrespective of the heat treatment, the fluorescence of washed fat globules re-suspended in the β-lactoglobulin (0.5 g/100 g) solutions appeared to be less than that of fat globules in the α-lactalbumin (0.2 g/100 g) solutions, indicating a greater effect of α-lactalbumin than β-lactoglobulin on fat globule fluorescence by WGA.

Using either the 40x objective or the 100x objective, dark (i.e., non-fluorescent) patches were clearly seen on fat globules in the heat treated samples for the two highest β-lactoglobulin concentrations (0.5 and 0.3 g/100 g, respectively). Although dark patches could also be seen on fat globules in the unheated counterparts, these were much more difficult to see and were also of a different shape (Figure 5.5). In the unheated samples, dark patches tended to be relatively large, round and few per globule, whereas in the heat treated samples they tended to be larger and more elongated, probably as a result of the round non-fluorescent patches becoming larger and fusing upon heating. This confirmed earlier observations that heating affects the distribution of WGA (by affecting the distribution or accessibility of NANA and GlcNAc residues) on the MFGM.

Irrespective of heat treatment, the fluorescence of fat globules in the solution containing 0.05 g β-lactoglobulin/100 g varied significantly in different places on the same slide. This agreed with the IgG and BSA observations (section 5.4.3) and suggested that once the concentration of protein in solution is below a certain level (about 0.1 g/100 g?) overall binding of WGA to MFGM is significantly affected.

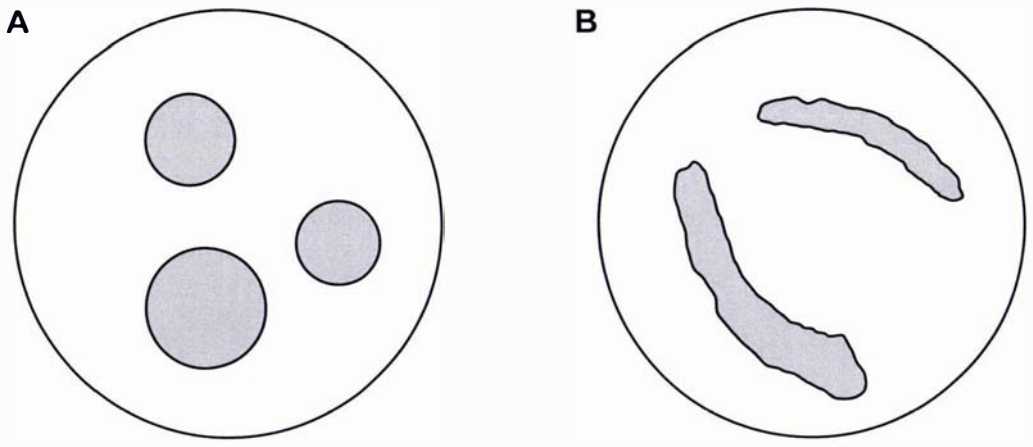

Figure 5.5 Illustration of typical shapes of dark patches observed by conventional fluorescence microscopy of WGA488-stained washed fat globules re-suspended in β-lactoglobulin (globular) solution. (A) Unheated solutions. (B) Heat treated (75°C, 5 min) solutions.

5.5.2 IgG and BSA

To verify the findings of section 5.5.1, washed fat globules were re-suspended in milli-Q water solutions of IgG and BSA (Sigma) at either the typical level of these proteins in milk or a "high-concentration" level. The "high-concentration" level was equal to the typical level of β-lactoglobulin in milk (0.5 g/100 g). A solution of globular β-lactoglobulin (0.5 g/100 g) as well as a raw milk sample were used as controls. Samples were either heat treated (75°C, 5 min) or unheated (see Table 5.5.3). Fat globule staining using WGA488 was performed as described above.

Samples were assessed using conventional fluorescence microscopy. Each sample was evaluated by using both the x40 objective and the x100 objective. To ensure valid conclusions were made with regard to the protein concentration effect, samples with different protein concentrations were put on the same slide next to one another so that rapid comparisons could be made, thus minimising the subjective element in the assessment of the samples. Results are summarised in Table 5.5.3.

Increasing the concentration of either IgG or BSA in milli-Q water from the concentration equivalent to typical levels in milk (1a, 1b, 3a and 3b) to 0.5 g/100 g (2a, 2b, 4a and 4b) resulted, on average, in greater fluorescence intensity of WGA488-stained fat globules. Hence, this confirmed that protein concentration *per se* is a significant factor in WGA binding to washed fat globules in milli-Q water. Furthermore, at 0.5 g/100 g (2a, 2b, 4a and 4b), the fat globules in BSA solution or in IgG solution appeared to be, on average, as fluorescent as those in the β-lactoglobulin solution. This supported the conclusion that the protein concentration *per se* was a significant factor.

Table 5.5.3 Model system, protein concentration and treatment of washed fat globules.

Sample	Model system	Protein (g/100 g)	Summary of results
1a[1]	IgG	0. 07	Fluorescent particles and strands were observed.
1b[2]	IgG	0. 07	Significant variation in fluorescence intensity of fat globules was observed. Fluorescence intensity was weaker than that of sample 2a. Fast moving small fluorescent particles were observed.
2a	IgG	0. 50	Fat globule fluorescence intensity was greater than that of sample 1a. Also there were more brightly fluorescent fat globules than in sample 1a. Fast-moving small fluorescent particles and strands similar to those in sample 1a were observed.
2b	IgG	0.50	No significant difference in fluorescence intensity of fat globules compared with that of sample 1b. Fast-moving small fluorescent particles were observed.
3a	BSA	0.04	For fluorescence intensity, see comments on sample 4a. Very few fast-moving small fluorescent particles were observed.
3b	BSA	0.04	Fat globule fluorescence intensity was similar to that of sample 3a and clearly weaker than that of sample 4b. Very few fast-moving small fluorescent particles were observed.
4a	BSA	0.50	Fat globule fluorescence appeared to be greater than that of sample 3a. Some bright fat globules were observed; these were not observed in sample 3a. Very few fast-moving small fluorescent particles were observed.
4b	BSA	0.50	Overall, the fluorescence of fat globules appeared to be slightly more intense than that of sample 4a, but it was difficult to discern whether the fluorescence intensity of fat globules in this sample was actually different from that of those in sample 4a. Sample contained some brightly fluorescent fat globules. Fast-moving small fluorescent particles were clearly seen.
5a	Control 1 β-Lactoglobulin	0.50	Sample contained some brightly fluorescent fat globules.
5b	Control 2 β-Lactoglobulin	0.50	Sample contained some brightly fluorescent fat globules. Overall, the fluorescence of fat globules appeared to be slightly more intense than that of sample 5a.
6a	Raw milk[3]	3.53	Small clusters of fat globules were observed.
6b	Raw milk[3]	3.53	No fat globule clusters were observed.

[1] "a" denotes unheated sample.
[2] "b" denotes heat treated sample.
[3] This was not a model system, but a control sample.

As the initial thermal denaturation temperature for IgG is 72°C (Singh & Havea, 2003), it is probable that at least part of the IgG became denatured and associated with the MFGM. Yet,

heat treated fat globules in IgG solution tended to be less fluorescent than those in the unheated counterpart, which agreed with previous IgG experiments. Hence, it was concluded that, in the presence of IgG, elevated heat treatment of washed fat globules results in less fluorescent fat globules.

Heat treatment of washed fat globules re-suspended in a solution containing a low concentration of BSA (0.04 g/100 g) (3b), on average, did not appear to affect the fluorescence intensity of the WGA-stained fat globules. This agreed with results of previous BSA experiments. However, when fat globules were heated in a solution containing a high concentration of BSA (0.5 g/100 g) (4b), no clear effect of heating was observed.

In the IgG solutions, at both low concentrations (samples 1a and 1b) and high concentrations (2a and 2b), many tiny fluorescent fast-moving particles were observed as well as fluorescent filamentous matter. In the heat treated IgG solutions, the fast-moving small particles were also observed. In the BSA samples, small fluorescent particles were observed to some degree only in the heat treated sample at high protein concentration (4b). In the other BSA samples (3a, 3b and 4a), only very few of such particles were observed. It was unlikely that the small particles were tiny fat globules, as (1) the cream washing procedure results in the loss of the majority of small fat globules from the cream to the serum, and (2) if these particles were tiny fat globules they should also have been observed in other samples. However, they were mainly observed in IgG solutions whereas in the BSA solutions only a few were observed. This would suggest that these particles are stained protein (aggregates?), which is the more probable explanation considering that IgG is glycosylated and BSA is not; this would explain the general lack of these small particles in the solutions containing the latter (the few small particles that were observed in BSA solutions may have been a few small fat globules).

5.6 Fat globule aggregation — What promotes it?

Fat globule aggregation was observed to be much more extensive when fat globules were re-suspended in PBS and stained with WGA488 (section 5.4.3) than that typically observed in WGA-stained milk. Further work was conducted to attempt to understand the factors promoting fat globule aggregation.

5.6.1 Effect of PBS and WGA488

Fat globule aggregation was assessed in samples containing re-suspended washed cream in milli-Q water, in PBS alone or in PBS to which WGA488 was subsequently added. No fat globule aggregation was observed in the absence of WGA488 either in milli-Q water or PBS (Figure 5.6A), whereas for the PBS solution extensive fat globule aggregation was observed in the presence of WGA488 (Figure 5.6B). This showed that an interaction between MFGM, WGA and PBS promoted fat globule aggregation.

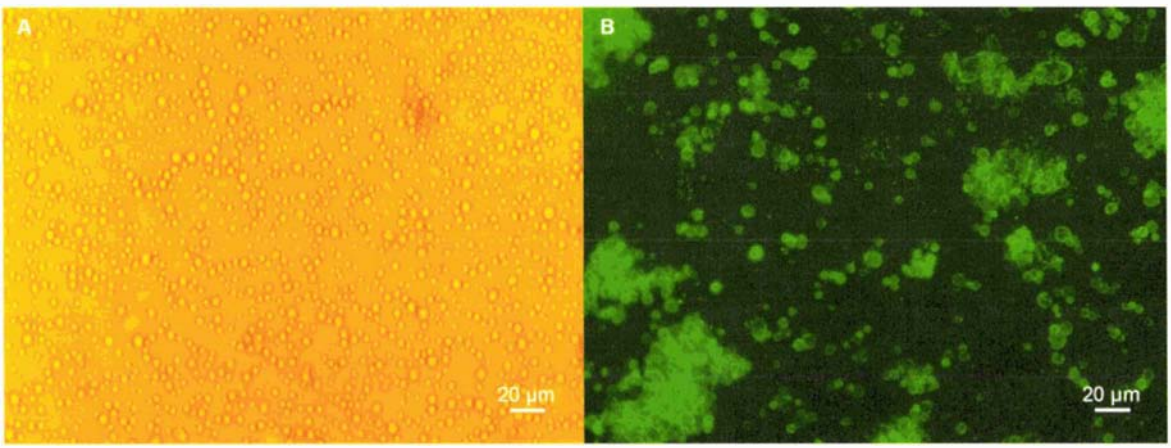

Figure 5.6 (A) Bright field image showing absence of aggregation of washed fat globules re-suspended in PBS in the absence of WGA488. (B) Fluorescence image showing extensive aggregation of washed fat globules re-suspended in PBS in the presence of WGA488. To make it easier to see the aggregates, a fluorescence image is shown rather than a bright field image.

5.6.2 Effect of pH

Fat globule aggregation was assessed in washed cream re-suspended in PBS having different pH values (5.73, 6.60, or 7.35) in the absence or presence of WGA488. No fat globule aggregation occurred in any of the samples that did not contain WGA488, whereas significant fat globule aggregation occurred in all samples containing WGA488. This showed that, within this range, the pH was not a factor promoting aggregation of fat globules by WGA488 in PBS.

5.6.3 Effect of NaCl concentration

As chloride was the main ionic species in PBS, its effect on fat globule aggregation was investigated. The effect of PBS (Table 5.5.4) was compared with that of phosphate buffers (Table 5.5.4 and Table 5.5.5).

Table 5.5.4 Composition of buffers (molar concentrations).

Buffer	KH_2PO_4	Na_2HPO_4	NaCl	KCl
PBS	0.001	0.01	0.137	0.0027
Phosphate buffer	0.001	0.01	–	–

Aliquots (about 30 mL) of phosphate buffer were pH-adjusted using 0.1 M HCl to obtain buffers as shown in Table 5.5.5.

Table 5.5.5 Volume of HCl (0.1 M) added to 30 mL of buffer stock, calculated chloride concentration and final pH.

Buffer	HCl added (mL)	Chloride concentration (M)	Ionic strength	pH
PBS	1.5	0.145	0.341	6.60
Phosphate (stock)	–	0.000	0.061	8.02
Phosphate buffer, pH adjusted A	0.6	0.002	0.064	7.40
Phosphate buffer, pH adjusted B	1.9	0.006	0.069	6.61
Phosphate buffer, pH adjusted C	2.9	0.010	0.073	5.36
Milli-Q water	–	0.000	0.000	7.10

Washed cream (60 µL) was added to 240 µL buffer or milli-Q water and a subsample was transferred to a microscope slide and examined for fat globule aggregation by bright field microscopy immediately after placing the cover slip onto the sample on the slide. WGA stock (3.5 µL) was then added to the sample in the vial and a subsample was examined as before.

Samples that did not contain WGA488 were evaluated using bright field microscopy, whereas samples containing WGA488 were evaluated using both bright field microscopy and fluorescence microscopy. The camera exposure time for fluorescence microscopy was kept constant for all samples.

In agreement with previous observations, no fat globule aggregation was observed in the PBS buffer in the absence of WGA488, but extensive fat globule aggregation (large aggregates) was observed in the presence of WGA488. Furthermore, as observed previously, no significant aggregation was observed for fat globules re-suspended in milli-Q water irrespective of whether WGA488 was present or not. In the absence of WGA488, little fat globule aggregation was observed in any of the phosphate buffers. In the presence of WGA488, many small aggregates were observed in the phosphate buffer pH adjusted C (pH 5.36), but not in the other phosphate buffers, indicating that when chloride levels increased to above 0.006 M fat globule aggregation was starting to become noticeable; as in the previous experiment it was shown that the pH did not affect fat globule aggregation, the concentration of chloride could be the only factor.

Furthermore, the degree of fluorescence of the fat globules in the phosphate buffers was high and comparable to that of those in the PBS (section 5.6.2). This suggested that the presence of the phosphate salts alone was sufficient for promoting a high degree of fluorescence of WGA488-stained washed fat globules, or alternatively that even relatively low concentrations of chloride in the phosphate buffers contributed significantly to fluorescence of the fat globules.

However, considering there was little difference between the fluorescence intensity of the fat globules in the three phosphate buffers, the variation in chloride concentration probably had no significant influence on fluorescence intensity.

To further study the effect of chloride on fat globule aggregation of WGA-stained washed fat globules the following experiments were conducted.

1. Added NaCl was dissolved in raw milk (final concentration 0.134 M, i.e. similar to that in PBS). Fat globule aggregation was assessed both in the absence and presence of WGA488. The control consisted of a WGA488-stained raw milk.

2. Washed cream was re-suspended in NaCl solution (final NaCl concentration after addition of washed cream: 0.107 M). Fat globule aggregation was assessed both in the absence and presence of WGA488.

In the presence of WGA, no major differences were observed in the degree of fat globule aggregation between the unsalted raw milk and the salted raw milk. For fat globules re-suspended in NaCl solution, no significant aggregation was observed in the absence of WGA488, but extensive aggregation was observed in the presence of WGA488. These results showed that: (1) the presence of relatively high levels of NaCl (0.134 M) in raw milk does not effect fat globule aggregation irrespective of whether WGA is present or not; (2) washed fat globules in NaCl solution do not aggregate unless WGA is added. In turn, this means that in aqueous solutions, aggregation of washed fat globules is related to a combination of ionic strength and the presence of WGA488; (3) judging from the absence of fat globule aggregation or coalescence in the salted milk and in the WGA448-free NaCl solution, high ionic strength alone does not destabilise the MFGM. The replacement of the milk plasma (containing suspended and dissolved proteins as well as various salts) with water during the washing procedure may have rendered the environment of the fat globules more susceptible to the effect of added salt. A more detailed study by using other salts (multiple charges) would show whether salt concentration or ionic strength was the major factor in fat globule aggregation.

5.7 Effect of WGA concentration on fat globule staining efficacy

5.7.1 Effect of different concentrations of WGA488 on the staining of fat globules

WGA488 stock was added to milk at ratios 1:100, 2:100 and 3:100 (v/v) to obtain samples containing concentrations of 9, 17, or 28 μg WGA488/mL milk, respectively. Using conventional fluorescence microscopy, the effect of each concentration was evaluated for both unheated or heat treated (75°C, 5 min) samples. The camera exposure time was kept constant to ensure valid comparisons.

Increasing the WGA488 concentration resulted in increased fluorescence of fat globules as well as that of the background, but did not affect the degree of fat globule aggregation in either the

unheated or the heat treated samples. For both unheated milk and heat treated milk at 28 μg WGA488/mL milk, the fluorescence signal was too high when using a 100x objective: images could not be made because overexposure occurred at the exposure time selected. Hence, the field of view first had to be subjected to excitation light for up to 30 s so that photobleaching could occur to the point that images could be captured. Nevertheless, despite the increased average fluorescence intensity, differences in fluorescence intensity between different fat globules were still observed (Figure 5.7).

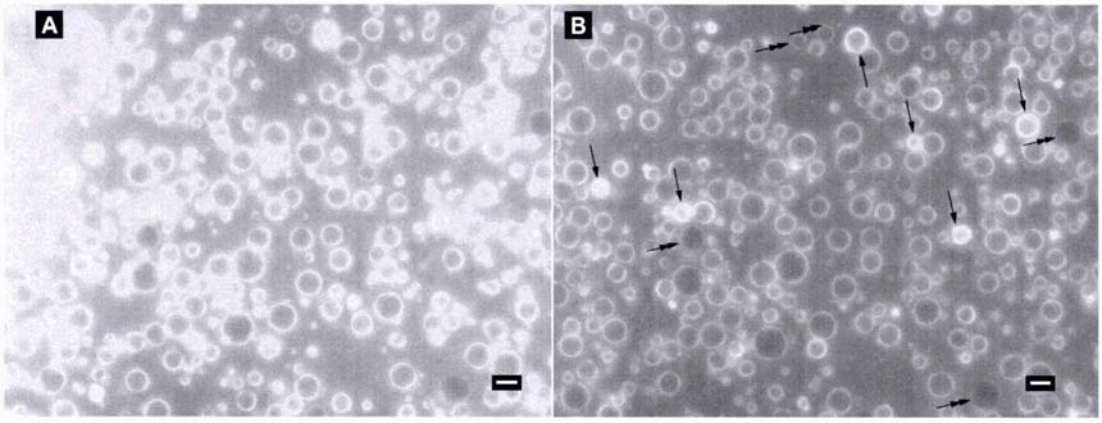

Figure 5.7 Heat treated (75°C, 5 min) bovine milk stained with WGA488 (final concentration 28 μg/mL) and viewed by conventional fluorescence microscopy using a 100x objective. Bar = 5 μm. (A) Image captured after photobleaching for 10 s. Significant overexposure was still evident. (B) Image captured after photobleaching for 30 s. Some individual fat globules are still overexposed, but the majority is not. Differences in fluorescence intensity between individual fat globules were still observed even at this elevated concentration of WGA488 in the milk. For example, some fat globules were highly fluorescent even after 30 s photobleaching (arrows), whereas others were weakly fluorescent (double arrows). Note that although generally most fat globules are in the same position compared with that in image A, some may have moved during the time that elapsed between the image capturing of A and the capturing of B.

In unheated milk, no dark patches were observed on the MFGM, whereas in all heated samples dark patches were observed. This phenomenon had been observed several times before when conducting other experiments involving heat treated milk. Thus, heat treatment of milk (75°C, 5 min) appeared to affect the distribution of WGA488 on the MFGM. Furthermore, with an increasing concentration of WGA488 the dark patches became more clearly distinguishable on the "less bright" fat globules, because of the greater contrast between stained and unstained patches.

Increasing the WGA488 concentration to 28 μg/mL did not affect the degree of fat globule aggregation in either unheated milks or heat treated milks.

Fluorescent strands were observed particularly clearly in the unheated sample containing the highest WGA488 concentration. Some fat globules were connected by strands (Figure 5.8). The observation that upon movement of the two fat globules the strand moved with the fat globules was evidence that the strand was indeed attached to both fat globules, and not a microscopy artifact.

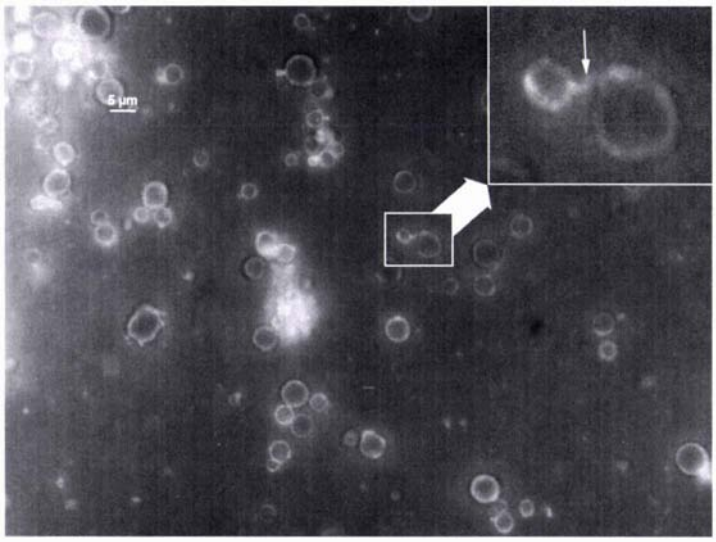

Figure 5.8 Heat treated (75°C, 5 min) bovine milk stained with WGA488 (final concentration 28 µg/mL) and viewed by simultaneous bright field and fluorescence microscopy using a 100x objective. This imaging technique positively identifies the round objects as fat globules and the strand to be filamentous in nature (i.e., not globular). Inset: magnification of two fat globules connected to each other by a strand (arrow). The strand is fluorescent and thus must contain glycosylated components.

As had been observed many times before, heat treatment (75°C, 5 min) caused the fluorescent filamentous matter associated with fat globules to disappear. Hence, the filaments were not WGA aggregates as WGA was added after the heat treatment. They probably were milk plasma components stained by WGA. It thus appears that, at room temperature, fat globules associate with some glycosylated matter present in the milk plasma, a phenomenon that has not been reported in the literature before. It is not yet clear what this matter is. It is unlikely that these filaments involve whey proteins as they are present in a solubilised state in the milk plasma. Potentially, the filamentous matter could be casein micelles linked together by WGA (which has multiple binding sites; Wright, 1992), but this explanation would require that upon heat treatment (75°C, 5 min) κ-casein binding sites for WGA would no longer be available. As outlined above, this is improbable. Furthermore, it would also be peculiar that such WGA-associated casein micelles would form filamentous strands and not large aggregates for example, such as is the case for fat globules. Direct linkages between casein micelles can also be excluded as an explanation for the filamentous matter. Although casein micelles do form strands, rather than aggregates, this does not occur at room temperature but only upon heating at temperatures >

85°C (Kaláb, 1993). A more probable explanation is that the filamentous matter comprises previously existing membranous material in the milk plasma.

Finally, overnight storage of samples (5°C) did not appear to affect the binding of the WGA to the fat globules, as there was no observable difference between the unheated sample containing 28 µg WGA488/mL assessed within 5–10 min of addition of WGA and the same sample after 1 day cold storage. This confirmed earlier observations that WGA binding to the MFGM is rapid (<10 min) and that the relative distribution of WGA on the membranes of the fat globules remains stable even upon cold storage.

5.7.2 Addition of washed cream to WGA488 stock

To check whether the dark patches seen on stained fat globules could be the result of insufficient WGA in the sample, washed cream (10 µL) was added to a large excess of WGA488 stock (40 µL). Subsamples were evaluated by both bright field microscopy and fluorescence microscopy. No fat globule clustering was observed. An extremely high background fluorescence was observed and the fluorescence of the stained fat globules was high as well. Extreme overexposure resulted at the camera, and in order to "see" the fat globules in the image, the camera exposure time needed to be reduced from 148 ms to 6 ms. Even then it was difficult to tell whether the MFGM was stained or whether the green rings (Figure 5.9) arose from diffraction such as that seen around fat globules under bright field microscopy. However, dark patches on immobilised fat globules could clearly be observed through the microscope eye-piece.

Figure 5.9 Fluorescence image of WGA488-stained fat globules upon adding washed cream to WGA488 stock and reducing the microscope camera exposure time from 148 ms to 6 ms.

In conclusion, the dark patches were not caused by a lack of WGA in the sample. Furthermore, in terms of relative contrast of stained fat globules against their background, it appeared that

there is no advantage in having a very high WGA concentration in the sample. The use of lower concentrations of WGA was found to be better as these gave a better contrast between the stained MFGM membrane and the stained milk plasma.

5.7.3 Effect of Alexa Fluor® conjugate

The available filter sets of the conventional fluorescence microscope were found not to be suitable for capturing the emission of WGA680- or WGA647-stained fat globules, and the effect of the concentration of these probes was tested using the CLMS. This required the use of the 647 nm excitation line. Using a LP 665 emission filter, autofluorescence of milk in this region of the spectrum was not observed.

For WGA680-stained fat globules, a high laser power was required to see the fat globules, because the excitation wavelength was sub-optimal for the probe. Consequently, photobleaching of WGA-stained human fat globules and bovine fat globules was rapid. Dubert-Ferrandon *et al.* (2006) reported that Alexa Fluor® 594 was more sensitive to photobleaching than Alexa Fluor® 488. The current study thus confirms that the photobleaching effect increases with increasing excitation wavelength of the fluorophore. Under these conditions, the probe WGA680 was considered not to be suitable.

The fluorescence of bovine milk samples stained with WGA647 was weak and faded quickly when the ratio of WGA647 stock to milk was 1:100 (v/v). Increasing the WGA647 concentration, by using a ratio of WGA647 stock to milk of 6:100 (v/v), provided sufficient contrast between the stained fat globules and the background, but photobleaching was still a concern. Hence, attempts to make 3-D images or high zoom images of WGA647-stained fat globules without using an anti-fade agent will result in a compromised image quality owing to photobleaching.

5.8 General discussion

5.8.1 Assessing fat globules in WGA-stained milk using fluorescence microscopy

Verifying that stained objects are fat globules

Bacteria, cells and fat globules constitute the main membranous objects in milk, and WGA could potentially bind to all such objects. This raises the question whether microscopy evaluation of stained milk samples allows conclusions to be drawn with regard to fat globules alone. In milk of healthy cows, the number of bacteria is typically $< 3 \times 10^5$/mL (Jensen *et al.*, 1995), of cells[14] $< 5 \times 10^5$/mL (Walstra & Jenness, 1984), and of fat globules $> 10^{10}$/mL (Huppertz & Kelley, 2006). Considering the low number of bacteria per unit volume, compared with the number of fat globules, their generally small size compared with the larger fat globules (>1 µm), and their generally non-spherical shape it is highly unlikely that bacteria would interfere in the microscopy

14 Usually reported as somatic cell count, which consists of leukocytes (98%) and epithelial cells (2%) (Jensen *et al.*, 1995).

assessment of fat globules in WGA488-stained milk. In contrast, because of their size[15], cells stained by WGA could potentially be mistaken for fat globules. However, the control experiments in which WGA488-stained objects were counterstained with the neutral fat dye Nile Blue, failed to detect such cells. Apparently, any cells present on the slide sediment onto the microscope slide, whereas the fat globules rise to locate underneath the cover slip. Hence, the cells would generally be outside the microscope focus when the fat globules are imaged. This conclusion agrees with findings of other researchers, who studied the fluorescence of ovine fat globules by light microscopy (Scolozzi *et al.*, 2003). Therefore, for image analysis of WGA-stained milk sandwiched between a microscope slide and cover slip, it may safely be concluded that the stained objects are fat globules. For assessment of milk containing a high somatic cell count it might be desirable to identify these cells by using a selective stain. This option was not further explored in the current work.

Non-specific binding of WGA to fat globules

Control experiments, using solutions of inhibiting free sugars, showed that free NANA was a potent inhibitor of WGA488 as washed fat globules were non-fluorescent in a solution free from competing substrates for WGA488 (i.e., glycosylated milk plasma components). This demonstrates that WGA488 does not associate in a non-specific manner with the MFGM. In contrast, the inhibiting action of free GlcNAc was found to be lower than that of free NANA, and to be dependent on time and WGA488 concentration (even when the free sugar was in large excess[16]). It should be noted that, in the context of milk fat globules, conflicting results have been reported in the literature regarding WGA control studies using GlcNAc. Agglutination studies involving bovine milk fat globules and human milk fat globules washed and re-suspended in PBS showed that GlcNAc completely inhibited WGA binding (Farrar *et al.*, 1980). Similarly, non-specific binding of gold-labelled BSA-cross-linked WGA to bovine MFGM and human MFGM in the presence of *N*-acetylchitopentaose (i.e., $(GlcNAc)_5$) was reported to be practically absent (Horisberger *et al.*, 1977). In contrast, in equine milk, GlcNAc had no inhibiting effect on fluoresceinisothiocyanate-labeled WGA (Welsch *et al.*, 1988). No studies appear to have been performed in which WGA binding to milk fat globules involved control studies using *both* NANA and GlcNAc (see Appendix 5); this is interesting, as since 1979 WGA has been known to recognise both residues (references cited by Debray *et al.*, 1981). Furthermore, the GlcNAc results of the present study agree with those obtained for cells in that free GlcNAc was shown to be a poor inhibitor for WGA (i.e., 10–40 times less inhibitory than chitobiose and 100 times less than chitotriose; Debray *et al.*, 1981; Gallagher *et al.*, 1982). Hence, the time-dependent and concentration-dependent binding of WGA to MFGM observed in the presence of

15 About 10 μm (Mulder & Walstra, 1974).

16 That is, 10^6 x that of WGA. Furthermore, a free GlcNAc concentration of 20–50 mM is generally considered sufficient for fluorescence microscopy inhibition studies of nuclear and cytoplasmic glycoproteins (Berlin & Hanover, 1993). Recall that in the current work a much higher concentration (200 mM) was used.

free GlcNAc is probably due to the MFGM-bound GlcNAc oligomers[17] and/or terminal NANA successfully competing for WGA over time rather than to non-specific binding of WGA with MFGM.

Use of Alexa Fluor® dyes

Alexa Fluor® dyes (a series of sulfonated coumarin dyes and sulfonated rhodamine dyes) are the brightest and most stable low molecular weight fluorescent probes available (Panchuk-Voloshina *et al.*, 1999) and thus offer significant practical advantages over other dyes in single-dye fluorescence microscopy applications. Furthermore, Alexa Fluor® dyes cover the entire spectrum from ultraviolet to red[18], and thus are ideal for multiple labelling experiments. The fluorescence intensity of Alexa Fluor® dyes has been reported to be unaffected by pH in the range pH 4.0 to pH 9.0 (Panchuk-Voloshina *et al.*, 1999). The current study corroborated the pH-insensitivity in a narrower range (5.7–7.4) for Alexa Fluor® 488 conjugated to WGA, which was bound to MFGM. This pH-insensitivity of the fluorophore provides the possibility of studying the binding of WGA to the MFGM in milk at different pH values, although any pH-dependency of WGA binding to MFGM would obviously still need to be taken into account. The current study also showed that WGA access to, or binding with, the MFGM is not affected by the Alexa Fluor® conjugate. This permits the use of different WGA conjugates in multiple labelling studies (see Chapter 6). However, WGA488 was shown to be heat-labile, heat-lability being a function of both holding time and temperature. This prevents the use of this probe (and presumably that of other WGA conjugates) in heat treatment studies involving elevated temperatures in which it is desired to add the probe to the sample *prior to* the heat treatment. Nevertheless, the effect of heat treatment can be studied if the sample is stained post heat treatment.

5.8.2 Conventional fluorescence microscopy allows accurate detection of gross differences in fluorescence intensity of WGA-stained fat globules

In model systems, several factors affect the perceived fluorescence intensity of the fat globules. These include the time elapsed between exciting the fluorophore and capturing an image (i.e., the degree of fading), the number of fat globules on the slide, the darkness of the background, the degree of fat globule aggregation and the position of fat globules on the slide (fat globules aggregating at the cover slip edges should not be taken into account as they look much brighter

17 It is known that the affinity of WGA for GlcNAc oligosaccharides is greater than that for free GlcNAc (Sharon & Lis, 2003) and also than that of bound NANA (Hughes, 1983). Note also that K_a for WGA binding to bound $(GlcNAc)_3$ is about 2x greater than that for bound $(GlcNAc)_2$, which in turn is 13x greater than that for bound $(GlcNac)_1$. Although the non-reducing residue of the oligosaccharide usually occupies the primary combining site of the lectin, having the same contacts as that of the free monosaccharide, the additional contacts to the binding site provided by the other residues of the oligosaccharide result in a greater binding constant for linear oligosaccharides compared with that of the corresponding free sugar (Hughes, 1983; Sharon & Lis, 2003).

18 Molecular Probes markets the following Alexa Fluor® dyes: 350, 405, 430, 488, 514, 532, 546, 555, 568, 594, 610, 633, 635, 647, 660, 680, 700, 750, where the numbers refer to the approximate excitation maximum of the dyes. Not all of these are available as WGA conjugates.

than those further away from the edges). Hence, the use of a standardised imaging protocol such as outlined in Appendix 7 is a necessary prerequisite for ensuring that valid conclusions can be drawn from the observed images. When such precautions are taken, the fluorescence microscopy technique developed here permits the detection of gross differences between samples. For example, the difference between a sample containing weakly fluorescing fat globules and a sample containing strongly fluorescing fat globules is easily and accurately detected (e.g., Figure 5.2). Additionally, visual assessment of images may identify smaller differences between samples with regard to the fluorescence intensity of fat globules. However, considering the various factors that can affect the apparent fluorescence intensity of stained fat globules, when small differences are observed one would need to be very cautious in drawing conclusions on the basis of microscopy results alone. Some confirmatory analysis would then be highly desirable.

5.8.3 Fat globule aggregation

In addition to showing gross differences in average fluorescence intensity of WGA-stained fat globules, conventional fluorescence microscopy also showed differences in the degree of fat globule aggregation. Note that this concerns aggregation *induced* by the pipetting of a small subsample of the sample (about 7.5 µL) onto a microscope slide and covering it by a cover slip. This phenomenon is hereafter called "on-slide aggregation". At the point of pipetting the subsample onto the slide and applying the cover slip, fat globule aggregation is absent. However, as the fat globules subsequently move around (generally towards the edges of the cover slip), some fat globules, particularly the larger ones, become immobile and other fat globules come into contact with such fat globules, which may or may not result in the formation of aggregates.

Using model system studies, it has been shown that WGA was the main causative factor in milk fat globule aggregation. This should not be surprising considering that WGA is oligovalent and allows cross-linking between cells (Sharon & Lis, 2003). As the MFGM is derived from the mammary secretory cell membrane, its composition, at least in terms of its containing glycolipids and glycoproteins, is similar to that of the originating cells and thus WGA may be expected to cross-link milk fat globules also.

Depending on the sample matrix (model systems or milk), there appear to be different mechanisms for on-slide aggregation, and these are explained below.

On-slide fat globule aggregation in model systems

In whey protein solutions (β-lactoglobulin or IgG) containing PBS, on-slide aggregation of washed fat globules is observed irrespectively of whether these fat globules have been heated or not. Thus, heat treatment is not a causative factor for on-slide aggregation. By inference, any heat-induced changes in the MFGM do not affect the formation of fat globule aggregates in the

presence of WGA. Therefore, this also rules out the MFGM as a causative factor for on-slide aggregation in model systems.

Washed fat globules re-suspended in NaCl solution form aggregates in the presence of WGA. In contrast, washed fat globules re-suspended in milli-Q water (i.e., low ionic strength), irrespective of whether either β-lactoglobulin or IgG is present, do not aggregate to any extent in the presence of WGA. Furthermore, when the NaCl concentration in PBS is significantly reduced (thereby essentially obtaining a low-salinity phosphate buffer) a decreased tendency for washed fat globules to aggregate in the presence of WGA is observed. It was further shown that the pH was not a factor either in on-slide aggregation. Finally, for all model systems, no fat globule aggregation occurred in the absence of WGA. Hence, by elimination of possible factors, the causative factor must be the interaction between WGA and ionic strength. A possible explanation for the aggregation mechanism in model systems is given in Figure 5.10. The scheme depicted in Figure 5.10 does not agree with observations on washed human fat globules; in 0.1 M phosphate buffer the high molecular weight glycoproteins appeared to become short (folded), whereas in a 0.001 M phosphate buffer these glycoproteins became extended, similar to those in distilled water (Buchheim *et al.*, 1988b). However, the glycocalyx of human fat globules is quite different from that of bovine fat globules (Buchheim *et al.*, 1988b), and it remains to be determined whether in the presence of WGA these high molecular weight glycoproteins in human milk fat globules behave in the same way as glycoproteins of lower molecular weight found on bovine milk fat globules. Furthermore, the behaviour of glycosylated compounds in buffered solutions may not be in accordance with expectation. For example, contrary to the authors' expectation, in model system studies of GM_1[19] monolayers, the mean molecular area of GM_1 was higher in the presence of buffer than in pure water (Na^+ counter-ions would have been expected to screen the negatively charged polar head groups thus reducing electrostatic repulsion between the head groups; Heywang *et al.*, 2001). Possibly, the presence of buffer ions may have changed the organisation and orientation of the polar head groups at the interface thus affecting the orientation of alkyl chains and the packing of molecules (Heywang *et al.*, 2001). This shows that the effect of ionic strength on the orientation of the alkyl chains and packing of glycosylated molecules can be complex.

19 A sialylated negatively charged glycolipid

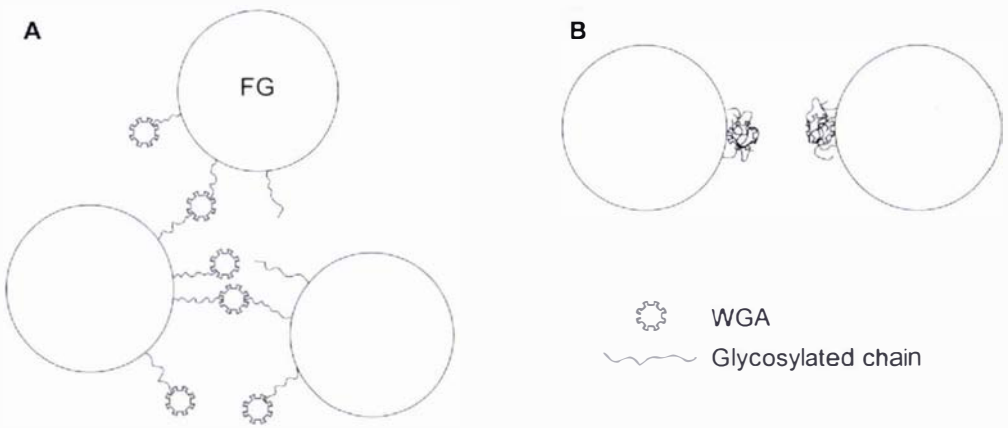

Figure 5.10 Possible mechanism for on-slide bovine fat globule aggregation in model systems consisting of washed fat globules re-suspended in an aqueous environment. Highly schematic. Not to scale. FG = fat globule. To preserve clarity, glycosylated chains are only shown on part of the fat globule surface. In reality, fat globules could either be entirely or partially covered by these compounds. Oligovalency of WGA is denoted by multiple square pits at its surface. (A) At "high" ionic strength, glycosylated compounds extend from the MFGM and repel each other. WGA binding is optimal, resulting in high fluorescence intensity and, as WGA can bind more than one glycosylated chain, promoting the formation of fat globule aggregates. (B) At low ionic strength, glycosylated branches get tangled up with each other (Warren, 1994), providing limited binding opportunities for WGA and resulting in a low fluorescence intensity of fat globules and a low occurrence of fat globule aggregates.

On-slide fat globule aggregation in milk

In unheated WGA-stained milk, some on-slide fat globule aggregation was always observed. Although literature reports of studies involving WGA-stained milk are silent on fat globule aggregation, the fluorescence microscopy image of WGA-stained fat globules in Buchheim *et al.* (1988b) clearly shows on-slide fat globule aggregation, thus confirming the phenomenon. In the present study, addition of NaCl (0.134 M)[20] to the milk would have increased the ionic strength and lowered the pH of the milk by about 0.1 pH unit (Grufferty & Fox, 1985; Gaucheron *et al.*, 2000)[21], but neither the increased ionic strength nor the change in pH resulted in observable changes in the degree of fat globule aggregation. Consequently, it is improbable that the stage of lactation is a factor in on-slide fat globule aggregation (with advancing lactation sodium and

20 When the concentration of added NaCl is < 0.2 M, no change in viscosity of the milk is observed. Furthermore, NANA levels in the milk plasma would have remained constant, indicating no increase in the milk plasma κ-casein content (Grufferty & Fox, 1985).

21 Several mechanisms contribute to the lowering of the pH, including displacement of casein-bound H^+ into the serum by added Na^+, and increased dissociation of ion pairs at higher increased ionic strength. The latter mechanism appears to be predominant as added NaCl in ultra filtrate permeate results in pH reductions similar to that of milk (Huppertz & Fox, 2006).

chloride levels in the milk increase (Grufferty & Fox, 1985)). Evidently, the main factor affecting (i.e., decreasing) the degree of on-slide aggregation is the heat load (i.e., combination of temperature and time). Changes in the MFGM when milk is heated from 37 to 50°C are believed to be minor (Keenan & Patton, 1995). The finding in the current study that fat globule clustering of WGA-stained milk was still observed at 55°C supports this contention. However, total absence of on-slide fat globule aggregation was observed when the milk was heated at elevated temperatures (e.g., 75°C for 5 min, or 90°C for 1 min). The changes in the milk effected by such heat treatment appear to be irreversible upon cooling, at least within the experimental time frame, as the addition of WGA *after* such heat treatment no longer effects on-slide aggregation. As fat globules are still well-stained by WGA following such heat treatment, it follows that glycosylated material on the MFGM is still available for binding to WGA. The question arises, "What changes occur in the milk, as a consequence of heat treatment, that allow WGA to bind to individual fat globules but prevent the formation of fat globule aggregates?" An additional heat treatment experiment (heating bovine milk samples at 20–80°C, in steps of 10°C for 1 min or 5 min) indicated the involvement of serum proteins: the critical temperature was within the range 60–80°C. After heating at 60°C, both 1 min- and 5 min-heated samples showed on-slide aggregates. After heating at 70°C, the 1 min-heated sample showed aggregates, but the 5 min-heated sample did not. After heating at 80°C, neither the 1 min- nor the 5 min-heated sample showed aggregates. These values are exactly within the temperature range in which whey protein denaturation occurs (60–90°C; Walstra & Jenness, 1984; Macej *et al.*, 2002). Furthermore, they closely correlate with the heat-induced association of whey proteins with the MFGM (Dalgleish & Banks, 1991; Corredig & Dalgleish, 1998; Ye *et al.*, 2004b). In contrast, casein micelles are much more heat stable than whey proteins (heat-induced coagulation of casein micelles at 100°C takes 12 h (Macej *et al.*, 2002)). This rules out casein micelle heat instability as a factor in the mechanism of the loss of on-slide aggregation in WGA-stained milk as such loss is observed at temperature-time combinations (e.g., 5 min at 70°C) well below that required for casein micelle coagulation. The observations that at ≤ 80°C little or no association of (kappa-)casein with the MFGM takes place (Dalgleish & Banks, 1991; Corredig & Dalgleish, 1996; Lee & Sherbon, 2002; Ye *et al.*, 2004b) support this conclusion.

Upon heat treatment, whey proteins bind quickly (within 105 s) and strongly to the MFGM (Corredig & Dalgleish, 1996). The heat-induced association of β-lactoglobulin with the MFGM has been repeatedly demonstrated (Dalgleish & Banks, 1991; Houlihan *et al.*, 1992; Kim & Jimenez-Flores, 1995; Corredig & Dalgleish, 1996; Corredig & Dalgleish, 1998; Lee & Sherbon, 2002; Ye *et al.*, 2004a, 2004b). The association occurs through disulfide bonds and starts to take place at temperatures below the denaturation temperature of β-lactoglobulin (Ye *et al.*, 2004b). The maximum amount of β-lactoglobulin associated with the MFGM makes up about 10–15% of the total MFGM proteins, but this represents only about 1% of total β-lactoglobulin in milk (Ye *et al.*, 2004a, 2004b). Conflicting results have been reported in the literature for α-lactalbumin in terms of it associating with the MFGM at temperatures below 80°C. Dalgleish &

Banks (1991) found no evidence for it; Corredig & Dalgleish (1996) reported it did occur when milk was heated, but not when cream was heated (Corredig & Dalgleish, 1998). However, at 80°C, low levels of α-lactalbumin were associated with the MFGM (Lee & Sherbon, 2002), and Ye *et al.* (2004b, 2004a) demonstrated that low, but measurable, quantities of MFGM-associated α-lactalbumin were observed at temperatures ≥ 70°C. Similarly to β-lactoglobulin, α-lactalbumin association with the MFGM plateaus at about 0.8% of total α-lactalbumin in the milk (Ye *et al.*, 2004b). However, because of the lower concentration of α-lactalbumin in milk compared with that of β-lactoglobulin, the quantity of MFGM-associated α-lactalbumin was only about 1/5th that of β-lactoglobulin. In conclusion, the involvement of α-lactalbumin in the on-slide fat globule aggregation phenomenon cannot be ruled out.

In addition to association of β-lactoglobulin, and possibly α-lactalbumin, with the MFGM, immunoglobulins are likely to be involved. IgM, but not IgG or IgA, is known to promote fat globule aggregation by binding to both MFGM carbohydrate moieties (including NANA) and milk plasma membrane at temperatures below 37°C (Euber & Brunner, 1984). In the present study, IgM would have been bound to the fat globules, because the unheated milk was obtained from a cooled bulk farm tank. Consequently, it is probable that the fluorescent fluffy matter that was often associated with the membranes of a proportion of fat globules comprised IgM-milk plasma membrane complexes. Such complexes are large enough to be clearly seen at the resolution of the light microscope, and are glycosylated (i.e., they are stained by WGA). Furthermore, immunoglobulins are denatured at the temperature-time combinations studied (Walstra & Jenness, 1984; Walstra *et al.*, 1999; Fox, 2002), but not at milder heat treatments (e.g., 50°C). All these characteristics agree with the observations in the current study as on-slide fat globule aggregation was observed when milk samples were heated at temperatures up to about 60°C, but not when heated at 70–75°C (5 min).

Heating of milk also induces changes in the MFGM proteins. In the absence of milk plasma components, such changes were shown not to affect aggregation of washed fat globules (see section "*On-slide fat globule aggregation in model systems*" above). However, in milk (i.e., presence of plasma components) the situation may be different. Houlihan *et al.* (1992) showed that in milk heated at 80°C, the concentration of SDS-PAGE band 12 MFGM protein was not affected, whereas significant losses were observed for bands 3, 15 and 16. These proteins can be identified as BTN, XO, PAS 6 and PAS 7[22] by comparing with the nomenclature reported by Mather (2000). XO is not glycosylated and, as it does not interact with WGA, it is unlikely to be involved in on-slide aggregation. In contrast, the peripheral proteins PAS 6/7 are glycosylated and contain NANA and GlcNAc. In addition, these proteins have adhesive properties, and may mediate adhesive interactions with other cells (Mather, 2000), and thus, presumably, also with

22 In fact, PAS 6/7 is the product of a single gene, but gives rise to a doublet on SDS-PAGE owing to variable glycosylation of an identical polypeptide chain (Keenan & Mather, 2006). Here, we will consider them as separate proteins.

fat globules. In simulated milk ultrafiltrate (i.e., in the absence of serum protein), PAS 6/7 were shown to be stable at 70°C (40 min), but after heating at 80°C (10 min) they tended to form aggregates via intermolecular disulfide bonds (Ye *et al.*, 2002). As in the present study fat globule aggregation was no longer observed after heating at 70°C for 5 min, aggregate formation by PAS 6/7 is probably not a factor in the on-slide fat globule aggregation phenomenon in milk. However, besides PAS 6/7 aggregation, simply loss of these proteins from the MFGM may be a factor. Ye *et al.* (2004b) showed that, in milk, heating results in substantial losses of PAS 6 and PAS 7 from the MFGM with increasing heating temperature and time; at 75°C heating for 10 min, losses might be about 40–50% and > 85% for PAS 6 and PAS 7 respectively.

With regard to other MFGM proteins, it is unlikely that the glycosylated protein MUC 1 is a factor, as this protein is readily dissociated from the fat globules when milk is cooled and agitated (Singh, 2006). As in the present study milk was obtained from cooled farm bulk tanks it is assumed that most of the MUC 1 would have partitioned into the serum phase prior to experimental work. The major MFGM protein BTN potentially may play a role, not through loss from the MFGM (the concentration of BTN does not change upon heating; Ye *et al.*, 2004b), but potentially through heat-induced aggregation with itself or other MFGM proteins, which already starts at 60°C (Ye *et al.*, 2002). Nevertheless, it would be expected that even after such aggregation, the glycosylated part of BTN would still remain located in the glycocalyx and thus be available for WGA binding. This would agree with the results of the present study that after heat treatment significant WGA binding to fat globules still occurs.

In conclusion, there are strong indications that β-lactoglobulin, immunoglobulins and/or PAS 6/7 may somehow be involved in the disappearance of on-slide fat globule aggregation upon elevated heat treatment of milk. α-Lactalbumin may also be involved. More conclusive data about the involvement of β-lactoglobulin could be obtained if the studies of Ye *et al.* (2004a, 2004b) were repeated on human milk or rodent milk, as milk from these species does not contain β-lactoglobulin (Hambling *et al.*, 1992). Indeed, in the present study (see Chapter 6, section 6.5) completely opposite results were observed for WGA staining between β-lactoglobulin-free (human) milk and β-lactoglobulin-containing (bovine) milk: after heating (75°C, 5 min), human milk fat globules could not be stained by WGA, whereas bovine fat globules were stained and some of them were highly fluorescent (see also section 5.8.4 regarding the effect of heating fat globules re-suspended in an aqueous solution of globular β-lactoglobulin).

Having established the components most likely to be involved in the on-slide aggregation phenomenon in milk, the underlying mechanism of on-slide fat globule aggregation is of interest. Direct binding between (partially) denatured whey proteins and MFGM proteins through intermolecular disulfide bonds and/or hydrophobic interactions is one of the possible mechanisms of incorporation of whey proteins onto the MFGM during heating (Dalgleish & Banks, 1991; Ye *et al.*, 2004b). An alternative explanation that denatured whey proteins,

particularly β-lactoglobulin, displace MFGM proteins (e.g., PAS 6 and PAS 7; Kim & Jimenez-Flores, 1995; Corredig & Dalgleish, 1998) has been disputed by a later study, which showed that the amount of β-lactoglobulin in the MFGM increased upon holding time, whereas the loss of MFGM protein was not significant and was far smaller than the amount of β-lactoglobulin incorporated (Lee & Sherbon, 2002). In any case, it appears that PAS 7 is removed from the MFGM upon heating before it interacts with other membrane proteins *via* sulfydryl-exchange reactions (Ye *et al.*, 2004b). It should be noted that PAS 6/7 may play a bigger role than suspected from the literature because reported protein loss studies involved MFGM protein analysis of *washed* and *isolated* MFGM. It is well known that peripheral proteins may be lost on washing and membrane isolation, the degree of loss being dependent on the procedures employed (Ye *et al.*, 2002; Evers, 2004a)[23]. Thus, the differences reported in the literature for PAS 6/7 contents in the MFGM as a function of heat treatment may be smaller than is the case in reality, considering that in the control milk these proteins would have been lost also due to the washing and isolation procedures used. Hence, as in this part of the present study fat globules were studied in their native environment, more weighting is given to the involvement of these proteins in the aggregation mechanism than otherwise might have been expected from the literature reports. Figure 5.11 proposes possible mechanisms.

In contrast to the mechanism shown in Figure 5.11, in which the denatured whey proteins deposit onto many fat globules, a different scenario should also be considered. Ye *et al.* (2004a) and Singh (2006) concluded that it is not known why the heat-induced association between MFGM proteins and β-lactoglobulin/α-lactalbumin reach limiting values. In the light of the fact that a maximum of only about 1% of total whey protein becomes associated with the MFGM when milk is heated in the range 65–95°C (Corredig & Dalgleish, 1996; Ye *et al.*, 2004b, 2004a), the observation in the present study that only a small proportion of fat globules becomes highly fluorescent upon heating of bovine raw milk could indicate that only a selected proportion of fat globules interacts with whey proteins, particularly β-lactoglobulin, upon heating. This would then mean that the bulk of fat globules apparently have a membrane composition/ structure which prevents them from interacting in a similar way. Hence, marked differences in the composition/structure between individual fat globules may well be the answer to the question of Ye *et al.* (2004a) and Singh (2006). This explanation has some support from the results of the model systems experiments in which heating (75°C, 5 min) resulted in an increased number of bright fluorescent fat globules in β-lactoglobulin solutions, but a decrease in α-lactalbumin solutions. In the studies of Ye *et al.* (2004a, 2004b) and in the present study, the samples were taken from bulk milk from Massey University farms (i.e., very similar sources of milk). If the above explanation is correct, it raises the question of whether fat globules from some individual cows show a greater propensity for interacting with β-lactoglobulin than do fat globules from other cows.

23 However, Ye *et al.*, 2004b claimed that their washing procedure did not result in loss of MFGM protein.

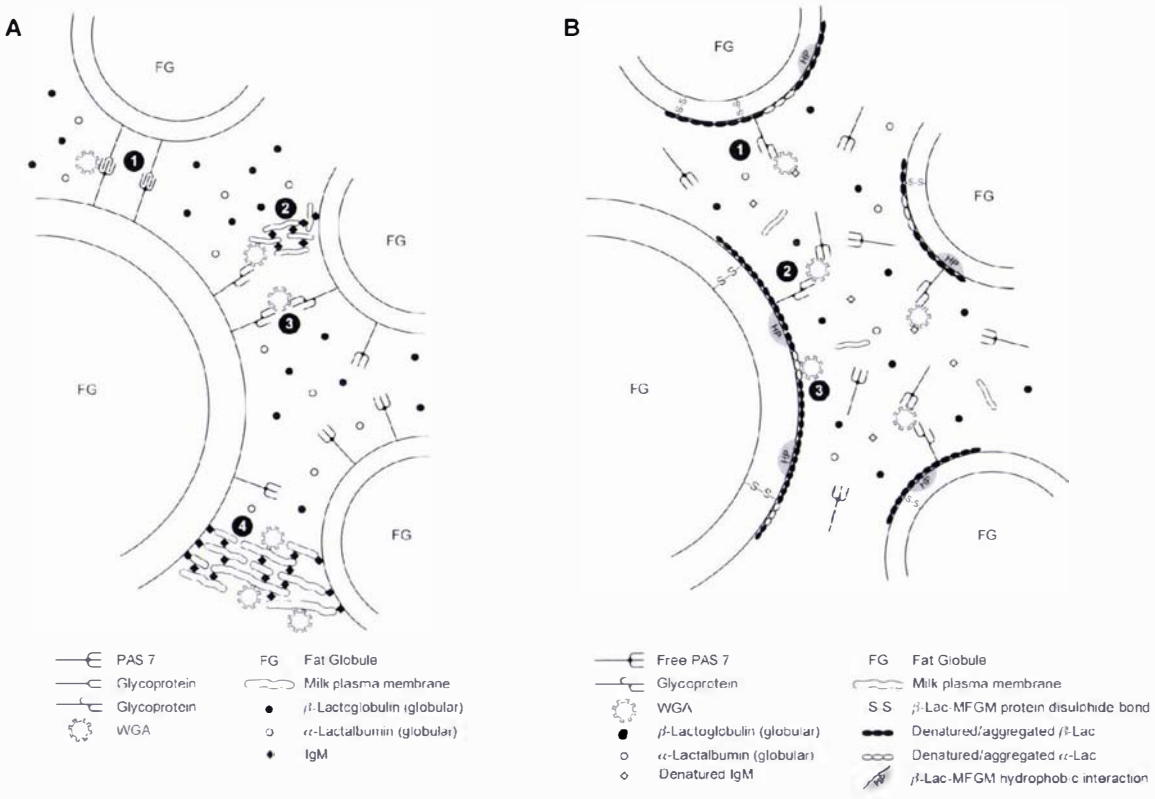

Figure 5.11 Possible mechanisms for the loss of fat globule aggregation properties, in the presence of WGA, as a result of elevated heat treatment of bovine milk. Highly schematic. Not to scale. For clarity, casein micelles are not shown. (A) Raw bovine milk sampled from a cooled farm bulk tank. WGA is added to a sample at room temperature. PAS 7 is bound to the MFGM. Some immunoglobulins (mainly IgM) have associated with milk plasma membrane[24]. WGA binds to glycosylated matter from the MFGM. Fat globule aggregation is promoted by: [1] adhesive properties of PAS 7; [2] & [3] WGA agglutinating different fat globules through binding to glycosylated compounds from the MFGM and/or IgM-milk plasma membrane complexes; [4] IgM-milk plasma membrane complexes. (B) Raw bovine milk which received a heat load that resulted in partial and/or full unfolding of whey proteins, but that did not affect κ-casein. IgM is denatured, hence IgM-milk plasma membrane complexes no longer exist. PAS 7 is lost from the MFGM. A small proportion (~ 1%) of total β-lactoglobulin and α-lactalbumin, probably evenly spread out over the surface, is associated with the MFGM through disulfide bonding with MFGM proteins or hydrophobic interactions. [1] & [2] WGA binds to glycosylated MFGM matter, but is capped by free denatured immunoglobulins and/or PAS 7, and is thus prevented from cross-linking with glycoproteins of other fat globules. [3] About 10% of α-lactalbumin is glycosylated (Brew & Grobler, 1992) and WGA could thus bind to MFGM-associated α-lactalbumin.

24 These are believed to be lipoprotein particles (Huppertz & Kelley, 2006).

5.8.4 Factors influencing the degree of fluorescence of stained fat globules in model systems

In model systems, fat globules were still fluorescently labelled even though the fat globules had been washed. This conclusively shows that the MFGM of washed fat globules contained glycosylated moieties that bound to WGA. Fluorescence intensity of fat globules re-suspended in buffer was variable, which suggests that in washed cream some fat globules have little NANA and/or GlcNAc residues, some have an intermediate content and others have a high content. This is exactly what is found for fat globules in their native environment (milk; see Figure 5.3, Figure 5.7) and could thus suggest that washing cream (40°C, 3 washes) results in retention of the *relative* distribution of these residues on fat globule membranes. Furthermore, because these fat globules retain these glycosylated moieties after repeated washing, it is highly probable that these residues are associated with integral membrane proteins (or lipids). These include BTN and CD36 (Mather, 2000). Different washing procedures are known to affect the MFGM composition differently (Evers, 2004a). In future work, a combination of a membrane-analytical approach (i.e., determining the average composition of the glycosylated compounds of the MFGM) and a microscopy approach (i.e., assessing the degree of membrane surface covered by glycosylated compounds) as a function of different washing techniques, would elucidate which proteins are lost upon using a particular washing technique and where these were situated on, or in, the MFGM.

Studies of washed fat globules re-suspended in casein solutions (either sodium caseinate or κ-casein) indicated that the fluorescence intensity of WGA-stained fat globules, on average, was greater than that of WGA-stained fat globules in milli-Q water, but less than that of native fat globules in stained raw milk. Similarly, WGA-stained washed fat globules in whey protein solutions were more fluorescent than those in milli-Q water, but less fluorescent than native fat globules in stained raw milk. At the typical concentration of α-lactalbumin in milk, α-lactalbumin in aqueous solution caused significant fluorescence intensity of WGA-stained washed fat globules, whereas BSA, at concentrations that are typical for milk, had virtually no effect on the fluorescence intensity of WGA-stained washed fat globules. The presence of glycomacropeptide did not affect the degree of fat globule fluorescence intensity either. In contrast, the concentration of protein *per se* was shown to be a major factor affecting the degree of fluorescence of WGA-stained fat globules; within the ranges tested, the fat globule fluorescence intensity increased with increasing protein concentration. Similarly, the presence of phosphate salts (phosphate buffer) resulted in an increased degree of fluorescence intensity of WGA-stained fat globules compared with that of WGA-stained fat globules in milli-Q water. These results support the model depicted in Figure 5.10 regarding the conformation of glycosylated MFGM compounds, with the exception that protein solutions and phosphate buffer (but PBS excepted) do not significantly promote aggregation of WGA-stained fat globules.

It can thus be concluded that, in aqueous solutions, WGA binding to NANA and/or GlcNAc residues on the MFGM is improved by changes in the conformation of glycoproteins,

presumably by extending the amino acid backbone. In the absence of plasma proteins, or (sodium or potassium) ions, NANA residues (negatively charged) may associate with positive charges on the protein backbone amino acids, thus becoming unavailable for interaction with WGA. Such ionic interactions are presumably ruptured when the medium contains ionic species or proteins.

Effect of heat treatment

Heat treatment of fat globules re-suspended in milli-Q water does not affect the fluorescence intensity of the fat globules. This suggests that heating fat globules in the absence of plasma proteins does not affect the access of the WGA to the NANA or GlcNAc residues. Hence, in heated samples, any denaturation and/or aggregation of MFGM proteins do not affect the access of WGA to the MFGM carbohydrate moieties.

The results of the heat treatment experiments, where WGA-stained fat globules in solutions of milk plasma proteins were observed, suggest that κ-casein[25], α-lactalbumin and IgG do not cause the increased fluorescence of some fat globules upon elevated heat treatment of milk. This does not necessarily mean that these proteins do not associate with the MFGM upon elevated heat treatment – only that if they do, there is no observable effect as "measured" by WGA488 binding to the MFGM. Further insight may be obtained by comparing the contradictory results obtained in the heat treatment experiments using β-lactoglobulin from different sources. When β-lactoglobulin powder is used then heating is without effect, whereas heating of a solution containing globular β-lactoglobulin results in an increase in the proportion of fluorescent WGA-stained fat globules, thus reflecting what is observed in raw milk. Consequently, this supports the alternative explanation mentioned above that upon elevated heat treatment (75°C, 5 min) β-lactoglobulin in aqueous solution binds to a subpopulation of fat globules, but not to all fat globules (recall that for α-lactalbumin in aqueous solution, heating (75°C, 5 min) resulted in a *decrease* of the proportion of brightly fluorescent fat globules). Furthermore, it may also be concluded that β-lactoglobulin (solid) reconstituted in water appears to assume a conformation that does not represent that of β-lactoglobulin in milk. This is an important finding as this means that reconstituted β-lactoglobulin powder is not a good model for studying the interactions of β-lactoglobulin with the MFGM as a function of heat load.

5.8.5 Effect of WGA concentration on the staining of fat globules in milk

In unheated milk, increasing the concentration of WGA results in increased average fluorescence of both the stained fat globules and the background. Using conventional fluorescence microscopy, good contrast between the stained fat globules and the background is obtained for a ratio of WGA488 stock:milk of 1:100 (v/v) (i.e., final concentration, 9 μL WGA/mL

25 Recall that the concentration used was nearly double that of κ-casein in milk.

milk). Using higher concentrations may result in overexposure of images, but may also better show some features such as filamentous matter and dark patches on the MFGM.

For each concentration tested, comparison of the images obtained using different concentrations showed that fluorescence intensity differences existed between fat globules, and that heating caused non-fluorescent parts of the membrane to become more pronounced. Hence, these features are not artifacts of the staining procedure. Nevertheless, differences in the fluorescence intensity of the fat globules can in principle be caused, at least in part, by probe concentration gradients that occur when the probe is added to the milk sample, and this should be taken into account when interpreting results. However, the observations that, in some heat treatment experiments involving model systems, either decreases or increases in the proportion of brightly fluorescent fat globules occurred as a result of heat treatment argue against the WGA concentration gradient being the major reason for the existence of a small proportion of brightly fluorescent fat globules. Instead, it points to real changes in the availability of glycosylated material on the membrane of some fat globules. Nonetheless, future work should further address the effect of probe concentration gradient.

The optimum concentration of the probe will depend on the Alexa Fluor® conjugate used, type of sample (e.g., washed fat globules re-suspended in a certain aqueous medium, or milk), source of fat globules (i.e., species), and type of microscope. Hence, optimum concentrations for different applications will need to be determined empirically.

5.9 General discussion and conclusions

Alexa Fluor® conjugates of WGA have been shown to be novel tools for studying structural or compositional features of the MFGM using either conventional fluorescence microscopy or CLSM. Gross differences in the degree of fluorescence between WGA-stained fat globules, and in the distribution of fluorescence within the membrane of individual fat globules, provide insight into the glycosylation profiles of the MFGM between fat globules and within the fat globules. In addition to the fluorescence of Alexa Fluor® conjugates of WGA, the agglutinating property of WGA can be utilised to extract supplementary information, particularly in heat treatment studies. The successful use of WGA conjugates in studying the structure and composition of the MFGM as described in this chapter should serve as a template for further studies of the MFGM using fluorescent conjugates of different lectins. Indeed, all lectin studies of the MFGM reported in the literature (Appendix 5) were performed in the period prior to routine use of advanced versions of the CLSM. Combining the resolving power of the modern CLSM with the specificity of binding of lectins will no doubt significantly progress our understanding of the structure and composition of the MFGM and its interactions with other matrix components. For example, recently the effect of heating on the structure of gels was studied by CLSM using Alexa Fluor® conjugates of whey proteins and casein (Dubert-Ferrandon *et al.*, 2006); in future work, it would be logical to extend

the current studies by multiple staining studies in which both the fat globules and the milk plasma proteins (whey protein and casein) are fluorescently labelled.

Another useful extension of this work would be to develop a multi-staining protocol that resulted in the staining of both fat globules and other sources of membrane, including cells. The staining of the latter might possibly be achieved by using a nucleotide stain. However, as outlined in Chapter 4, the staining protocol may be affected by the choice of stock solvent (depending on the presence or absence of interactions between the stock solvent and the milk sample matrix), and this needs to be considered when developing new staining protocols.

A summary of effects of several factors relating to on-slide fat globule aggregation is given in Table 5.6.

Table 5.6 Effects of several factors relating to on-slide fat globule aggregation.

Phenomenon	**Factors believed to have an effect**	**Factors believed to not have an effect**
Fat globule aggregation in model systems	• Interaction between WGA and ionic strength	• Heat treatment • pH
Fat aggregation in milk	• WGA • Heat load • β-Lactoglobulin • α-Lactalbumin • IgM • PAS 6/7	• Increasing ionic strength by addition of NaCl (01.34 M) • MUC 1 • BTN

6 Structural and compositional features of the MFGM as revealed by fluorescence microscopy — a preliminary evaluation

6.1 Introduction

Much of the current knowledge of the structure and molecular organisation of the MFGM has been obtained using morphological techniques such as (immuno)electron microscopy (e.g., Wooding, 1971b, Henson *et al.*, 1971, Monis *et al.*, 1975, Horisberger *et al.*, 1977, Sasaki & Keenan, 1979, Pinto da Silva *et al.*, 1980, Franke *et al.*, 1981, Buchheim, 1982, Welsch *et al.*, 1988, Schmidt & Buchheim, 1992, Mather *et al.*, 2001, Robenek *et al.*, 2006) applied to fat globules that had been isolated from the original milk sample. In these studies, the results obtained applied to fat globules that might, or might not, have undergone substantial physical and/or chemical modification, and whose environments had been drastically changed. Hence, the degree to which these results represent the membranes of fat globules in the original sample remains uncertain. Thus, the development of alternative microscopy techniques was highlighted as a prerequisite for progressing knowledge in this field (Chapter 2).

The fluorescence microscopy techniques developed in the current study can be used to study in real time the structural and compositional details of the MFGM in its native milk environment in a non-destructive manner. This chapter discusses various structural and compositional phenomena of the MFGM as observed by using selective MFGM probes. Chemical and structural heterogeneity were evident at the micron and submicron scale in the MFGM, not only of individual globules, but also among globules. The results reported in this chapter confirm, complement, and substantially extend results reported in the literature, particularly those that were based on electron microscopy, but also raise further questions. Each section stands on its own and therefore includes a discussion section. General conclusions are presented at the end of this chapter.

6.2 Double fluorescent rings

Initial work involving the CLSM for optical sectioning of human fat globules stained with lipophilic probes repeatedly, but not consistently, showed for middle sections two distinct fluorescent layers at the perimeter of the fat globule. This phenomenon is called a "double ring". The double ring was not always observed in optical sections above or below the middle section of the fat globule. Expert microscopists from three different organisations[1] believed the double ring to represent a real membrane feature rather than a microscopy artifact, because in several images the non-fluorescent gaps in the inner and outer rings did not match (Figure 6.1). Measurements

1 Personal communication: Elizabeth Nickless, Massey University, 7 June 2005; Robyn Hirst, Fonterra Research Centre, 13 June 2005; Colin Green, Auckland University, 8 August 2005.

showed that the non-fluorescent gap between the two fluorescent rings (at least 60–100 nm) significantly exceeded the width of the MFGM bilayer (< 20 nm; Mulder & Walstra, 1974; Walstra *et al.*, 1999), which eliminated the possibility that the two rings represented the two leaflets of the phospholipid bilayer[2]. In turn, this meant that if the double ring phenomenon represented a real feature, there would have to be two lipophilic layers surrounding the fat globule one of which was the MFGM bilayer and another as yet unidentified layer (membrane?). However, the presence of a double membrane on fat globules has never been reported in the literature. These considerations prompted further experiments to be performed in an attempt to elucidate whether the observations represented real features or microscopy artifacts.

6.2.1 Microscope objective

The images showing the double ring phenomenon had all been obtained using a 100x (1.4 numerical aperture) oil-immersion objective. To verify that the objective did not cause the double ring phenomenon, a series of images were made using a x63 oil-immersion objective of human fat globules stained with $DiIC_{18}(3)$-DS (ethanolic stock). Each of these confirmed the presence of the double ring.

Electronic zoom

The double ring phenomenon had been observed in images in which high electronic zoom was used. To verify whether using high electronic zoom could cause the double ring as an artifact, human fat globules stained with $DiIC_{18}(3)$-DS (ethanolic stock) were imaged using the 100x objective at low electronic zoom. For assessment, very large fat globules were selected as at low magnification, the microscope resolution was not sufficient to see whether or not a double ring was present in fat globules of < 10 µm in diameter. The double ring phenomenon was also observed at low electronic zoom (Figure 6.2).

To further verify that features seen at high electronic zoom were not microscopy artifacts, 3-D images of human fat globules stained with $DiIC_{18}(3)$-DS (ethanolic stock) were made at low zoom $(1.2)^3$ and subsequently at high zoom (5.0) (example in Figure 6.3). Owing to the fact that more optical sections can be taken with increasing zoom, structural features in high zoom images were better defined than in low zoom images. Nevertheless, the same structural features were observed as judged from the staining pattern of the fluorescent probe.

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2 In addition, note that the resolution of the microscope was not sufficient to resolve objects that were that close together.

3 1.0 = no electronic zoom.

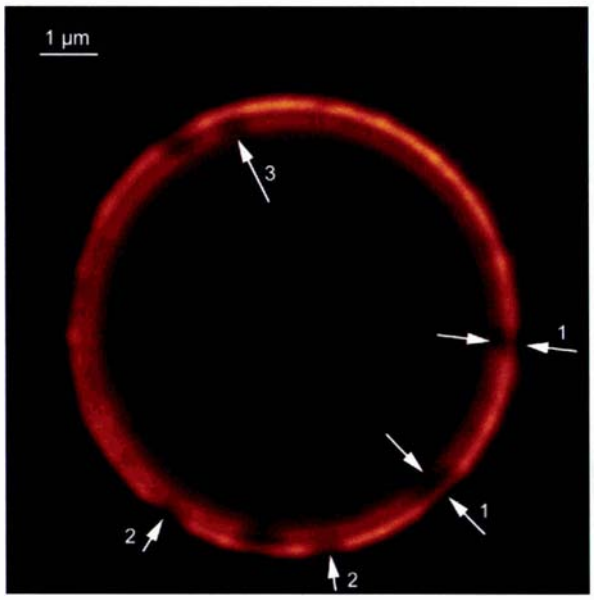

Figure 6.1 Optical CLSM section of a human milk fat globule stained with $DiIC_{18}(3)$-DS (ethanolic stock), showing the double ring phenomenon. Gaps in the outer and inner rings do not always match. Sometimes the gap is present in both the inner ring and the outer ring (opposing arrows 1). Sometimes there is a reduced thickness or gap in the outer ring, but not in the inner ring (short arrows 2). Sometimes there is a gap in the inner ring, but not in the outer ring (single arrow 3).

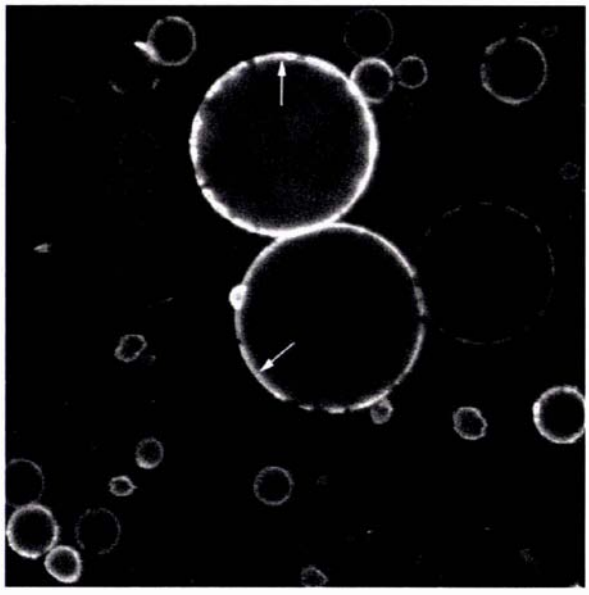

Figure 6.2 Human milk fat globules imaged using low electronic zoom (< 2). Double rings are observed for the larger fat globules (arrows). A scale bar was not obtained for this image; however, from comparison with another image the diameters of the two largest fat globules are estimated to have been about 20 µm.

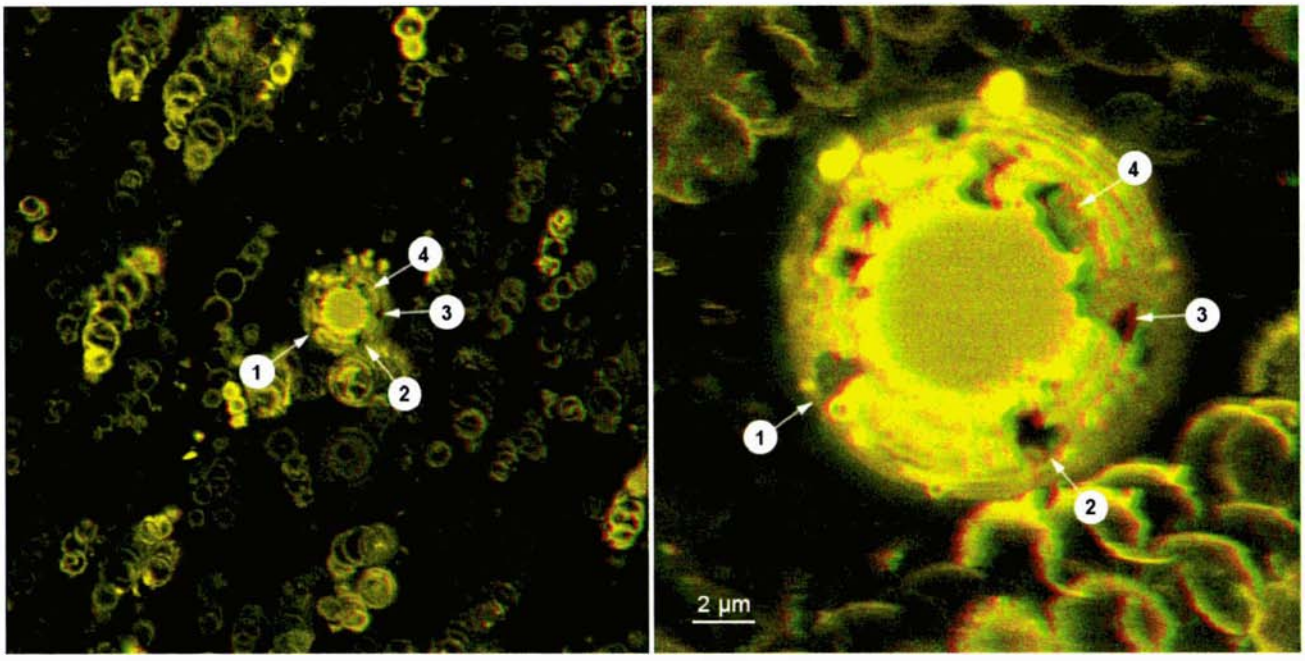

Figure 6.3 3-D images of a human fat globule stained with $DiIC_{18}(3)$-DS. (A) Low electronic zoom (13 optical sections were used to image the fat globule). (B) The fat globule in the middle of Figure 6.3A was re-imaged at high electronic zoom (21 optical sections were used). Structural features of the fat globule in the high zoom image were the same, although better defined, as those in the low zoom image (e.g., compare the non-fluorescent patches denoted by arrows with the same number).

6.2.2 Fluorophore

Different probes were examined to determine whether the double ring phenomenon was observed only in $DiIC_{18}(3)$-DS-stained human fat globules. It was found that the double rings were also occasionally, but not so clearly, observed in human milk stained with $DiIC_{18}(3)$ or DiA. In contrast, the double rings could be clearly observed in human fat globules dual stained with FM4-64 and WGA488. The images obtained revealed that the two rings were not necessarily mirror images (Figure 6.4B).

6.2.3 Pinhole size

As explained in Appendix 2, the detector pinhole is a pivotal design feature of the CLSM, allowing optical sectioning of the sample to take place by eliminating out-of-focus light. Reducing the pinhole size improves contrast by excluding a greater proportion of out-of-focal-plane light (Murphy, 2001) and reduces the thickness of the optical plane, thereby allowing higher resolution in optical sectioning, which is essential for obtaining high quality 3-D images. The latter feature was employed to investigate whether the pinhole size usually used somehow caused the double ring phenomenon.

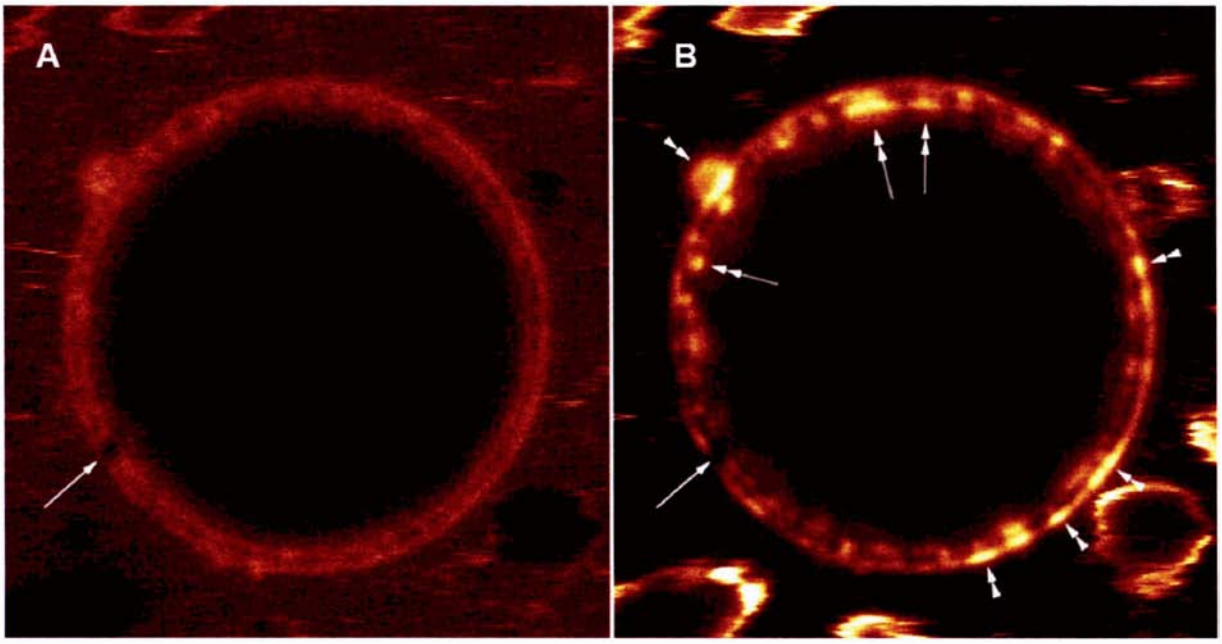

Figure 6.4 Dual stained milk fat globule. (A) WGA488. (B) FM4-64. Both probes show a double ring. The patch not stained by WGA488 was also not stained by FM4-64 (arrow). Note that the FM4-64 fluorescence intensity at some places in the outer ring is greater than in the inner ring (double arrow-heads) and vice versa (double arrow). Membrane areas with high fluorescence intensity indicate local differences in membrane composition.

A WGA488-stained human fat globule was imaged using a pinhole size of 100 µm (as determined by the CLSM software) and re-imaged using a pinhole size of 50 µm (i.e., a significant reduction) (Figure 6.5). In the "100" image, the double membrane was clearly visible in both large and some smaller fat globules. In the "50" image, the double ring phenomenon could be observed in the two larger fat globules, albeit less clearly than in the "100" image. The main reasons were that reducing the pinhole size also reduces the proportion of light emitted from the specimen that reaches the detector, resulting in a weaker signal, and that repeated scanning causes some photobleaching of the probe. Therefore, the fluorescent features in the "50" image were less clearly defined.

6.2.4 Sample matrix

Species

To verify whether the double ring phenomenon was observed only in human milk, bovine milk and ovine milk were stained with WGA488 and assessed using CLSM. The double ring phenomenon was observed in some fat globules in milk of these latter two species.

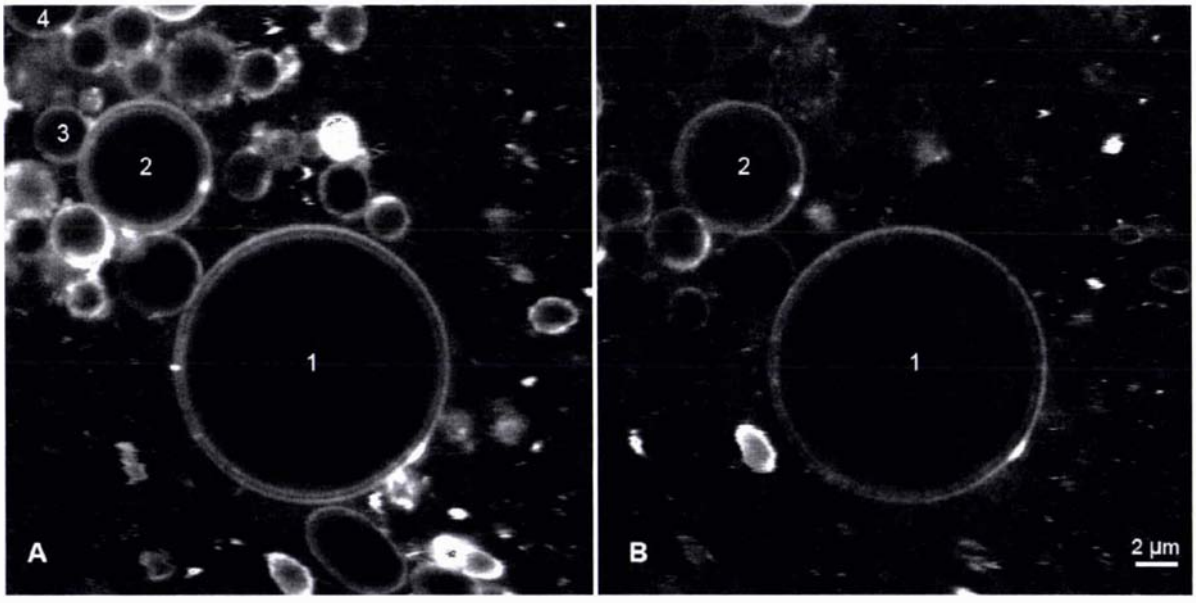

Figure 6.5 WGA488-stained human fat globules. Double rings are observed irrespective of pinhole size. (A) Pinhole size: 100 µm. Double rings can be seen in larger and smaller fat globules (1, 2, 3, & 4). (B) Pinhole size: 50 µm. Double rings can be seen in the two largest fat globules (1 & 2), but fluorescence intensity is reduced owing to less light reaching the detector, and photobleaching.

Washed fat globules resuspended in phosphate buffer

Washed bovine cream and probe stock (either WGA488, WGA647, or $DiIC_{18}(3)$-DS (aqueous stock)) were added to phosphate buffer (pH = 8.02). CLSM filter settings, laser output, detector voltage settings, line settings and zoom were optimised for each imaging attempt. The double ring phenomenon was observed, at least for some fat globules, for all probes used.

6.2.5 Effect of heating

$DiIC_{18}(3)$-DS-stained human milk was heat treated (75°C, 5min) and then evaluated by CLSM. The double ring phenomenon was observed.

6.2.6 Multiple rings

Although in the majority of cases only two fluorescent rings were observed, occasionally more than 2 fluorescent rings were observed (Figure 6.6). However, usually the extra rings appeared to be flare from the two dominant rings.

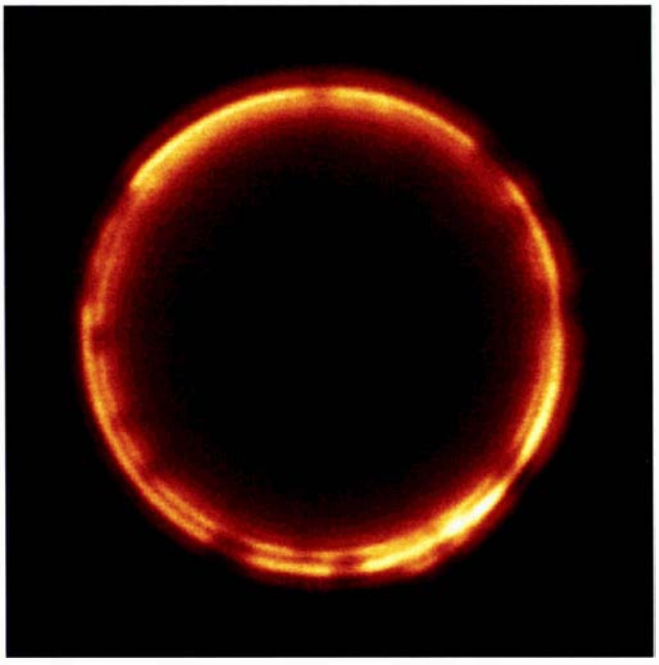

Figure 6.6 Human fat globule stained with DiIC$_{18}$(3)-DS (ethanolic stock), showing four rings in some places. Two rings were dominant in terms of fluorescence intensity, and the diffuse outermost and innermost rings appeared to be flare from these two dominant rings. The fat globule diameter was estimated to be approximately 8 µm.

6.2.7 Discussion

Attempts to determine whether the double ring phenomenon was a microscopy artifact or reflected a real membrane feature yielded inconclusive results. Apart from testing whether the phenomenon could also be reproduced on another CLSM[4], which indeed was the case (results not shown), there was no opportunity in the current study to pursue a resolution of the phenomenon beyond the experiments described above. Nevertheless, it is deemed important that such work be conducted in future studies as the phenomenon concerns either a spectacular artifact or the discovery of a structural feature of the MFGM that hitherto was unknown. Further work should determine which of these possibilities is correct. The current discussion will serve as a useful introduction to this work.

A range of possible microscopy variables, such as the use of different objectives, high electronic zoom, and pinhole size, were shown not to cause the phenomenon. Furthermore, as the phenomenon was observed in fat globules from different species (human, bovine and ovine[5]) in

4 Leica TCS SPE DM2500. CLSM software: Leica Application Suite Advanced Fluorescence 1.5.1 build 869 (this used frame averaging rather than line averaging). A 63x lens was used. This instrument used a diode laser rather than an argon-krypton gas laser and thus the output radiation was much lower than that of the Leica DMRBE used in all other experiments.

5 Bulk sheep milk was collected from a commercial sheep farm, transported cooled by ice packs to the laboratory and stored at 5°C until testing.

their native environment (milk) as well as in washed fat globules that had been re-suspended in phosphate buffer, it is improbable that interference from the sample matrix was a causative factor. The phenomenon was also not related to the laser excitation wavelength or the fluorophore emission wavelength range as it was observed when using different excitation lasers (488 or 647 nm) and with different emission spectra (e.g., for WGA488 the spectrum was 515–580 nm and for WGA 647 the spectrum was > 665 nm). Finally, the phenomenon was not a chemical artifact caused by the use of ethanol (probe stock solvent), as it was also observed when using probes made up with water (e.g., DiIC$_{18}$(3)-DS (aq), FM4-64 and WGA).

Evaluation of many images showing the double ring revealed that the outer ring, on average, was more intense than the inner ring (e.g., Figure 6.1) for optical sections up to the middle of the fat globule. In optical sections between the middle and the bottom of the fat globule the inner ring tended to be more fluorescent than the outer ring. These findings suggest some kind of diffraction effect. The observation that sometimes three or more rings were seen, lends some further support for a diffraction artifact.

In contrast, other observations are difficult to reconcile with a diffraction phenomenon. For example, the non-fluorescent patches in the two rings clearly do not always match (e.g., Figure 6.1 and Figure 6.6). Similarly, the highly fluorescent patches in the two rings also do not always match (e.g., Figure 6.4B). These observations would seem to indicate that structural and compositional differences exist between the two rings. It is possible at least partly to reconcile these observations with current knowledge of the MFGM. First, the double ring is observed with different lipophilic probes. It can be imagined that lipophilic probes stain the phospholipid bilayer and perhaps the surface-active layer that existed on the intracellular fat droplet. The latter layer is sometimes called the "monolayer" (Robenek *et al.*, 2006). However, WGA-stained fat globules also showed the double ring. This would mean that the two lipophilic layers would have to be similarly glycosylated. It is difficult to rationalise from the current model of the MFGM (a triple-layered membrane; Keenan & Mather, 2006) how there could be two glycosylated layers of similar thickness; one would expect the majority of glycosylated chains to be disposed externally to the bilayer, but the current scenario would require the inner glycosylated layer to be sandwiched between two lipophilic layers. Although the current study (see section 6.3) indicated that this might be the case, thus agreeing with similar findings reported in the literature (Freudenstein *et al.*, 1979; Sasaki & Keenan, 1979), the prevailing model of the MFGM (which is primarily based on electron microscopy studies), does not include the presence of glycosylated chains between the bilayer and the fat core. Maybe this is an anomaly that needs correcting. Furthermore, crude measurements in the current study showed that the two rings are at least 50–100 nm apart. This is generally larger than the distance between the bilayer and the monolayer as shown in electron microscopy images (10–50 nm). This discrepancy may conceivably be reconciled if it is assumed that (1) the CLSM measurements do not correctly represent the true distance between the two layers (because the fluorescent rings around the fat globule microscopy are relatively thick, whereas the stained structures they represent have a

thickness much below the resolution of the light microscope), or (2) the current model of the MFGM based on electron microscopy data has provided an incorrect distance between the bilayer and the monolayer. It is important to realise that the CLSM data were obtained on fat globules in their native environment, i.e. fully hydrated. In contrast, electron microscopy requires extensive sample pre-treatment, often involving dehydration of the specimen[6]. The use of dehydrating agents could perhaps reduce the thickness of the stained area resulting in incorrectly small distances. On the basis of freeze-fracturing electron microscopy Robenek *et al.* (2006) claim that variable amounts of cytoplasm are often entrained between the inner monolayer and the outer bilayer. The possibility of a thin layer of entrained cytoplasm has previously been shown by thin-section electron microscopy (Wooding, 1977) and freeze-fracture electron microscopy (Pinto da Silva *et al.*, 1980). Furthermore, images of milk stained with Acridine Orange (Patton & Huston, 1988) show that fat globules not containing cytoplasmic crescents were still weakly stained and this could be interpreted as indicating the presence of a very thin layer of cytoplasm (Scolozzi *et al.*, 2003). Nevertheless, a contention that fat globules contain entrained cytoplasm cannot be upheld if it is meant to be interpreted to be applicable to fat globules universally. Clearly, in electron microscopy images, cytoplasm layers, and in CLSM images the double rings, are observed in some fat globules, and then only in localised places, but not in all. Thus, for a proportion of fat globules, the presence of an entrained cytoplasm layer could provide an explanation for: (1) the distance between bilayer and monolayer as indicated by CLSM, and (2) the presence of glycosylated matter in the monolayer as the glycosylated chains would extend into the aqueous cytoplasm phase located between the bilayer and the monolayer. Consequently, the current model of the MFGM, as being a compact three-layered membrane, would need to be supplemented by a liposome-type model, which is applicable only to a certain proportion of fat globules. In this type of membrane, fluorescent probes associate with the bilayer, but also can reach and stain the monolayer, presumably through transfer via the entrained cytoplasm layer. The electron microscopy dehydration effect would then raise questions also about the protein layer seen during budding of fat globules from the secretory cell. This has been reported to be a uniform 10–20 nm (Wooding, 1971a), but may in reality be thicker.

Nevertheless, the above explanation arguing for a liposome type membrane is unlikely to be correct. The main factor arguing against this is probably that the inner ring has been shown in the current study to contain protein, because it is stained by WGA. Such a separate protein-containing layer should have been visible in electron microscopy images. Although transverse sections by thin section microscopy occasionally have shown a stained inner layer that is clearly separated from the bilayer (Figure 10 in Wooding, 1971b; inset of Figure 34 in Wooding, 1977), and double membranes were visible in clear vesicles in the pellet obtained from washed cream

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6 Note that surface views of specimens can be obtained with little or no prior preparation using field emission cryo scanning electron microscopy (Auty, personal communication, 5 October 2007).

(Anderson & Brooker, 1975), or in vesicles from the skim milk fluff layer[7] (Wooding, 1974), double membranes have never been shown to exist in fat globules. Furthermore, it is peculiar to observe a double ring in a FM4-64-stained fat globule, considering that the dicationic head of FM4-64 should prevent passage across membranes unless assisted by flippase activity (Fischer-Parton *et al.*, 2000; Torralba & Heath, 2002). Although the possibility of flippase-mediated translocation of FM dyes across the lipid bilayer cannot be excluded (Read & Kalkman, 2003), to date no evidence for such a mechanism has been reported (Parton *et al.*, 2001; Bolte *et al.*, 2004). Thus, it would seem that FM4-64 staining of an inner MFGM layer would require access to the inner layer by some other means than by penetrating the bilayer. It is difficult to envisage what mechanism, other than some "hole" in the bilayer membrane providing direct access from the milk plasma to the inner layer, could account for this. Further work would be needed to investigate this possibility, but, on balance, it would seem that it is more likely that the double ring phenomenon is some artifact rather than a real structural feature of the MFGM.

Irrespective of whether the double ring represents a real structural feature or is an artifact, it has little effect on the 3-D renderings of stained fat globules, because the phenomenon generally becomes observable only in optical sections close to the point where the fat globule diameter is largest. However, in 3-D renderings, the top optical sections contribute by far the most to the 3-D impression as seen by the viewer, and the middle optical sections contribute only a little to the 3-D impression of the fat globule.

6.3 Heterogeneity within fat globules and between fat globules within species

Structural features

Morphological features of the MFGM as shown by electron microscopy suggest that the MFGM is heterogeneous and contains domains of different shapes (e.g., Buchheim *et al.*, 1988b; Robenek *et al.*, 2006). CLSM images obtained in the current study corroborated the electron microscopy data by confirming that a variety of distribution patterns exists for different fluorescent probes in the MFGM. Some fat globules were completely stained with a probe (e.g., Figure 6.5), whereas in membranes of other fat globules distinct stained and non-stained areas were observed. For WGA staining, this corroborates electron microscopy data which showed that carbohydrates were distributed either uniformly or in discrete patches over the fat globule surface (Sasaki & Keenan, 1979). Furthermore, freeze-fracture electron microscopy of human milk has shown circular physical structures of about 0.1–0.2 µm in diameter (Buchheim *et al.*, 1988b; Robenek *et al.*, 2006). In the current study, more or less circular non-fluorescent areas were frequently observed (Figure 6.7), some of which were at the submicron scale (i.e., < 0.5 µm; Figure 6.7B) and some at the micron scale (~1–2.5 µm). However, non-fluorescent areas of non-circular shape were also regularly observed (e.g., Figure 6.7A). The submicron non-

[7] The "fluff' layer was obtained by centrifuging fresh raw milk at 1000 *g* to obtain skim milk. The skim milk was centrifuged at 85,000 *g*, which yielded a fluffy layer on top of the compact casein pellet.

fluorescent patches cannot represent membrane blebbings (see Chapter 2) such as those seen in electron microscopy (Henson *et al.*, 1971; Wooding, 1971b; Wooding, 1972; Wooding & Kemp, 1975; Wooding, 1977; Pinto da Silva *et al.*, 1980) as those clearly were bounded by a bilayer membrane, and thus would have shown up as fluorescent patches in CLSM images. However, if membrane blebbing indeed does occur, then conceivably these submicron non-fluorescent patches could represent bilayerless patches on the fat globule surface caused by the blebs detaching from the MFGM. Figure 6.7A suggests that domains exist on the fat globule surface that are more intensely stained by the $DiIC_{18}(3)$-DS probe than the surrounding area. This was not an artifact of the use of an ethanolic probe stock as the use of an aqueous stock of another lipophilic probe (FM4-64) also showed that certain parts of the MFGM were stained more intensely than surrounding areas (Figure 6.8A and Figure 4.24). These domains could be the same as the plaque-like domains, which were reported to vary widely in size and shape, shown in freeze-etched electron microscopy images (Buchheim, 1982; Buchheim, 1986).

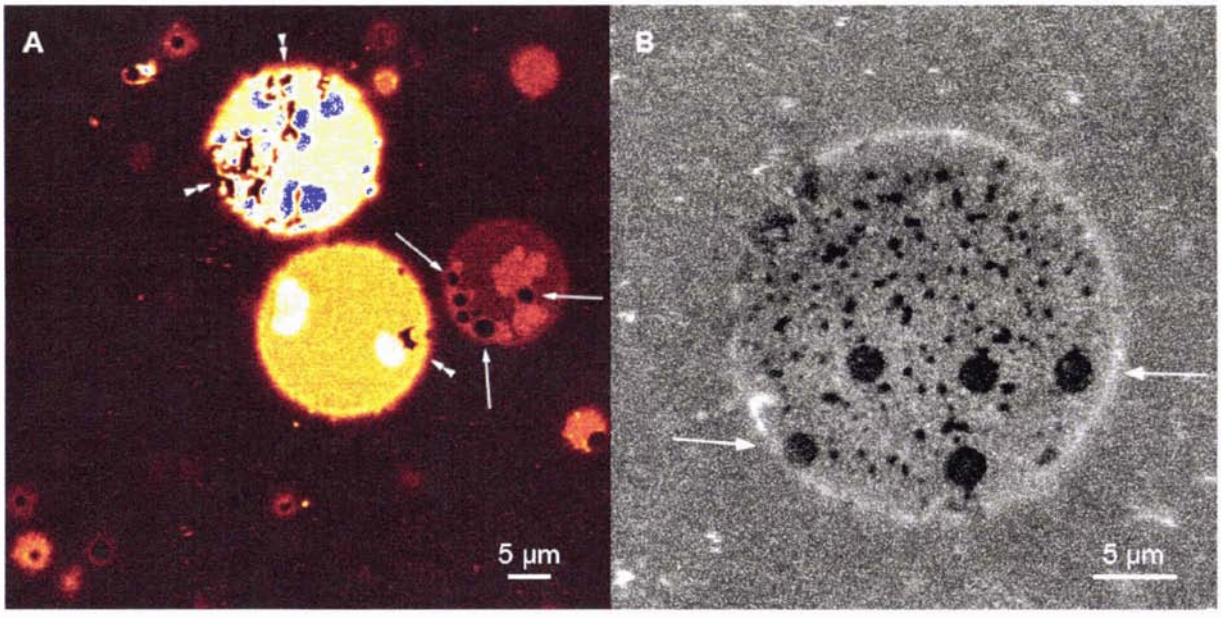

Figure 6.7 2-D CLSM images of the surfaces of fat globules showing a variety of probe staining patterns. (A) Human milk fat globules stained with $DiIC_{18}(3)$-DS (ethanolic stock) showing non-fluorescent patches that were circular (arrow) or non-circular (double arrow-heads). The scale bar for the original image was not available, but was estimated on the basis of that from an image with a similar electronic zoom. The actual error is estimated at < 25%, which permits concluding that the circular non-fluorescent patches were ~1–2 µm in diameter. (B) Ovine fat globule stained with WGA488. Large non-fluorescent patches are circular (arrows) with a diameter ~1.5–2.5 µm. Small non-fluorescent areas were typically < 0.5 µm.

The 2-D staining pattern of some fat globules in the current study (e.g., Figure 4.20C, Figure 4.24 1A & 1B, and Figure 6.2) show remarkable similarity to some of the membrane outlines

visualised by electron microscopy, such as that of globule 2 in Figure 14 published by Wooding (1971b). These show a substantially intact bilayer that is interrupted at a few places. Hence, this suggests that the non-fluorescent areas in the CLSM images represent bilayerless patches on the MFGM. 2-D dual staining results using FM4-64 and WGA647 further support this conclusion as these indicated that sections of the bilayer were absent, the MFGM being stained by neither the lipophilic FM4-64 nor the lectin WGA (Figure 6.8). The CLSM data, however, shed new light upon an existing controversy in the literature. On the basis of electron microscopy, it has been suggested that most of the bilayer (i.e., > 2/3rd) is lost from milk fat globules in bovine milk post secretion by the secretory cell (Henson et al., 1971; Wooding, 1971b; Bauer, 1972; Wooding, 1974). In contrast, biochemical data (i.e., bulk fat globule analysis) have suggested that the structure of the MFGM is largely preserved in secreted milk (Mather & Keenan, 1998). The CLSM dual staining results indicate that the truth may be somewhere in between, i.e., that most fat globules still possess a substantially intact bilayer, but that in a certain proportion of fat globules *part* of the bilayer has been lost. Although future work should establish quantitative data for the proportion of membrane surface that is bilayerless, it appears that, on average, it is substantially less than that proposed on the basis of electron microscopy results. Thus, this strongly supports the contention that electron microscopy fixation techniques may promote loss of the bilayer membrane (see Chapter 2, section 2.2.2)[8].

Degree of glycosylation as shown by WGA

WGA binds specifically to GlcNAc and NANA residues. MFGM proteins carrying GlcNAc and/or NANA residues include BTN, MUC1 and CD36 (Mather, 2000). WGA has been reported to bind strongly to both bovine and human MUCX and MUC1 (Patton *et al.*, 1995; Liu *et al.*, 2005). In these glycoproteins, binding of WGA appears to be associated with NANA residues, rather than GlcNAc residues, as treatment of the milk with neuraminidase significantly reduced or abolished WGA binding (Liu *et al.*, 2005). In milk, most of the NANA appear to be associated with glycoproteins rather than glycolipids (Neeser *et al.*, 1991) and are usually in terminal non-reducing positions (Gallagher *et al.*, 1982).

8 Note that the loss of the bilayer is much greater in chemically fixed and thin sectioned electron microscopy preparations than in freeze-etched electron microscopy samples, thus clearly indicating that the observed membrane loss as seen in electron microscopy images is caused to a significant extent by the sample preparation technique (Bauer, 1972).

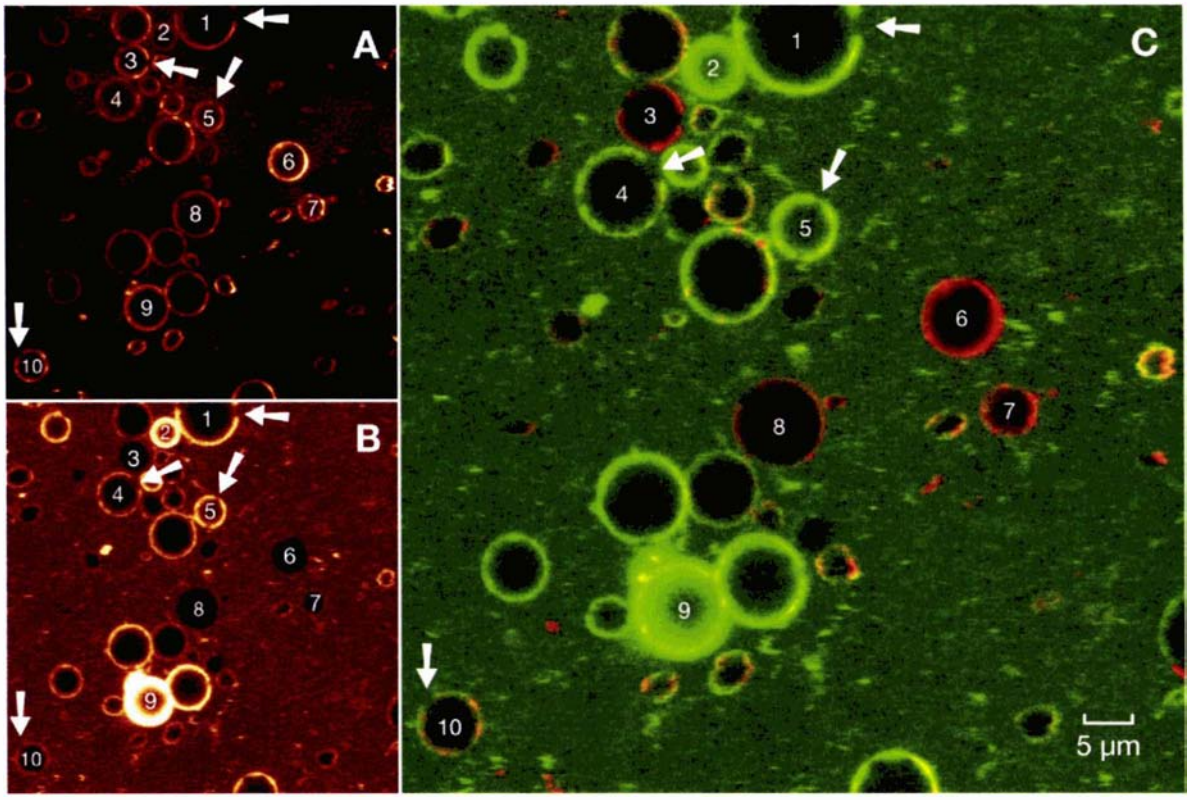

Figure 6.8 Dual staining of human fat globules. (A) CLSM image, channel 2, showing fat globules stained with the lipophilic styryl probe FM4-64. In some fat globules, the fluorescence of the MFGM was interrupted, indicating absence of the probe at these locations (e.g., 1, 3, 5, and 10). Variation in fluorescence intensity between different fat globules was less extreme than in the case of WGA488 (image B). The noncircular shape of some fat globules was the result of these fat globules moving during imaging. (B) CLSM image, channel 1, showing fat globules stained with WGA488. In some fat globules, the fluorescence of the MFGM was interrupted, indicating absence of the probe at these locations (e.g., 1, 4, 5, and 10). Significant variation in fluorescence intensity was observed among different fat globules, these ranging from weakly or non-fluorescent (e.g., 3, 6–8) to highly fluorescent (e.g., 2 and 9). Background fluorescence was caused by WGA488 binding to glycosylated compounds in the milk plasma. (C) Overlay of images A and B. FM4-64 and WGA488 were given false colours (red and green, respectively). A yellow/orange colour means co-location of the two probes, with fluorescence at about the same pixel intensity. Although some fat globules were stained by both probes, the much greater fluorescence intensity of one of the probes might mean that only one colour is seen in the combined image. Hence, for proper analysis, one needs to compare the individual channel images. Arrows indicate non-fluorescent areas on dual-stained fat globules.

Differences in fluorescence intensity between different WGA-stained fat globules suggest differences in degree of MFGM glycosylation between different fat globules, which is in agreement with electron microscopy results (Horisberger *et al.*, 1977). In addition, using dual staining, it was observed on some occasions that WGA488 was bound to areas on the MFGM surface lacking the lipid bilayer (i.e., to areas not stained by FM4-64; results not shown), indicating that the interface between the core fat and the milk plasma can be stabilised, at least partially, through glycosylated compounds. This finding corroborates an earlier observation of this phenomenon by electron microscopy (Horisberger *et al.*, 1977) and agrees with Murray *et al.* (1979) who found, for both bovine MFGM and human MFGM, that WGA bound to glycoproteins from the proteinaceous coat. This glycosylated matter will probably include BTN, as this glycoprotein is anchored into the proteinaceous coat (Keenan & Mather, 2006) and recently has been shown to be present in relatively high concentrations in the monolayer (Robenek *et al.*, 2006). On the other hand, it was observed that some fat globules that did contain a bilayer (i.e., stained by FM4-64) were virtually unstained by WGA488 (Figure 6.8). Again, this finding corroborates electron microscopy data in which fat globules were observed that for the most part were not stained by WGA (Horisberger *et al.*, 1977). Hence, glycosylation of the MFGM is variable, the variation being caused in part by variation in the proportion of the fat globule surface being covered by a bilayer membrane. The question of how this variation might arise in the secretory process has not yet been raised in the literature. However, a study of human breast luminal surfaces of duct and acinar cells, stained with horseradish peroxidase-conjugated WGA, showed variation in intensity of staining between globules within the same section (Walker, 1984). On the basis of this observation, it may be speculated that the differences observed on the fat globule membranes originate from compositional differences existing in the apical plasma membrane of secretory cells of different alveoli.

6.4 Similarities and differences between fat globules from different species

Degree and distribution of glycosylation

WGA has been reported to bind strongly to the membrane of washed fat globules of human, bovine, ovine or equine milk (references in Appendix 5), and, apart from the equine milk, this was corroborated in the current study for the Alexa Fluor® conjugates of WGA. Yet, differences between the species of milk were observed. Although human and bovine milk fat globules were generally adequately stained with WGA488 at 10 µg/ml milk, ovine milk required higher concentrations of WGA488 (30–50 µg/ml milk) to achieve a similar intensity of staining. Furthermore, the WGA load per globule (as determined by visual assessment of fluorescence intensity of fat globules) appeared to be more variable in human milk and ovine milk than in bovine milk. This was somewhat unexpected as NANA contents of complex carbohydrates of human milk have been reported to be higher than those in bovine milk (Neeser *et al.*, 1991). On the other hand, the human milk was obtained from a volunteer over a period that covered the entire 3rd year and first quarter of the 4th year of continuous lactation. Such long lactation

periods are unusual in Western society; consequently, virtually no data are available for milk composition at such long periods of lactation. However, levels of aminosugars (including NANA) are known to fall drastically with progressing lactation and to bottom out after about 28 weeks (Atkinson & Lönnerdal, 1995). The CSLM results suggest that such a decrease is not evenly spread over fat globules, but is rather highly variable as evident by the presence of fat globules that are either not or very lightly stained through to those that are very brightly stained.

Preliminary investigations of some ovine fat globules revealed that the distribution of WGA on these fat globules could be quite different from those observed in any of the human fat globules or bovine fat globules, as a number of distinct large or small non-fluorescent areas were observed on the same fat globule (Figure 6.7 and Figure 6.9). The large non-fluorescent areas possibly originate by fusion of smaller non-fluorescent areas (Figure 6.9) and, curiously, were observed at one occasion to be touching one another or be connected by narrow non-fluorescent "channels" (Figure 6.9). So far, such "channels" have not been observed in any of the human or bovine milk fat globules stained with WGA488.

In addition to differences in staining patterns of the MFGM, extra-globular differences were observed as well. As discussed in detail in Chapter 5, WGA-stained bovine raw milk showed glycosylated milk plasma components in the form of strands being associated with some fat globules . Tiny globular structures (presumably small fat globules or large casein micelles) were observed to be part of the strands. Strand-fat globule interactions were generally not observed in human milk. Hence, they did not represent mucins as these have been shown to be present on human fat globules, extending up to 1 µm from the membrane surface.

Together, these results are evidence for significant differences in both carbohydrate composition and distribution in MFGM of human, bovine and ovine origins. Future work should expand on this work by employing different lectins that are known to bind to MFGM (Chapter 4 and Appendix 5) and to conjugate these with selected Alexa Fluor® fluorophores to allow double or triple staining. This approach would, for example, quickly establish whether the non-fluorescent patches as shown by WGA are devoid of carbohydrate moieties altogether, or whether there is a specific grouping of other glycosylated matter at those domains. Furthermore, extending the study to include milk from species other than those used in the current study is of interest, as this could reveal further similarities or differences in MFGM composition and structure between groups of animals (e.g., ruminants *vs* non-ruminants).

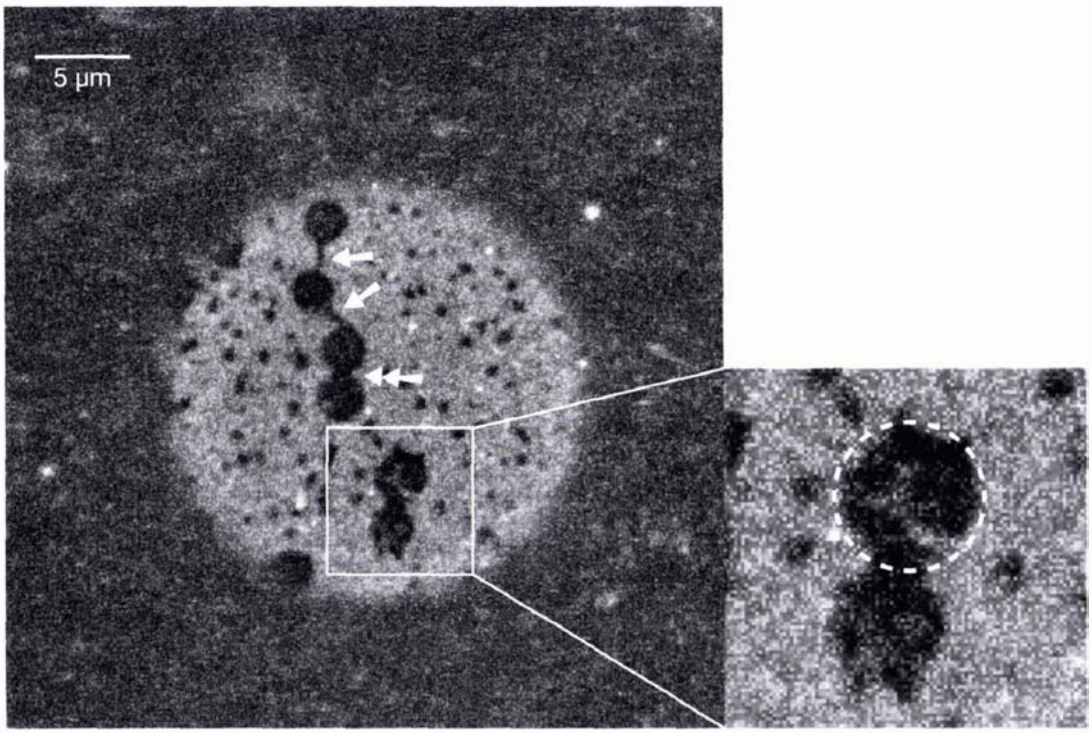

Figure 6.9 CLSM optical section showing part of the WGA488-stained membrane of an ovine fat globule (the same as that of Figure 6.7B, but opposite side of globule). Some large non-fluorescent areas were connected to each other by "channels" (arrows), or by sharing part of their boundaries (double arrow). Inset: smaller non-fluorescent areas appear to fuse to form larger non-fluorescent areas. The dashed circle denotes the extent of the expected final circular non-fluorescent area.

Lipophilic stains

Besides differences in the uptake of WGA, distinct differences in the uptake of each of the lipophilic probes $DiIC_{18}(3)$-DS and FM4-64 between fat globules from different species were observed. In contrast to the staining efficacy of WGA, ovine milk fat globules did not stain well with $DiIC_{18}(3)$-DS under the normal incubation conditions that were employed for human milk or bovine milk (irrespective of whether an ethanolic stock or aqueous stock was used), indicating a substantial difference in MFGM composition or structure compared with that of bovine and particularly human milk fat globules (the latter were stained more readily than either ovine or bovine fat globules). It should be noted, however, that both the ovine milk sample and the bovine milk sample were from pooled sources. In contrast, the human milk was from a single source. The composition of milk varies with stage of lactation, diet, etc. Hence, factors inherent in the milk other than the structure of the MFGM may play a role in the uptake of the probes by the fat globules.

Further work should elucidate which factors affect the uptake of the tested fluorescent probes by fat globules from milk of different species, as this will lead to a greater understanding of the

differences in structure and composition of the MFGM between species. Additionally, further screening of fluorescent probes should be conducted as it is conceivable that analogues of the fluorescent probes tested in this study possess superior staining properties for fat globules from certain species of milk.

6.5 Heat treatment of milk

Chapter 2 explained the importance of heat treatment in dairy processing. Although much is known about the effects of heat treatment on bovine milk, little is known about heat treatment of human milk as the latter is normally consumed directly upon expression. To determine whether heat treatment affects bovine fat globules (bulk tank sample) differently from human fat globules, the fluorescence intensity of fat globules stained with either DiIC$_{18}$(3)-DS (aqueous stock) or WGA488 was investigated after heating (75°C, 5 min) and compared with an unheated control. Samples were assessed using conventional fluorescence microscopy, using a constant camera exposure time to allow comparisons between samples. Staining experiments using DiIC$_{18}$(3)-DS were conducted using an unfiltered DiIC$_{18}$(3)-DS stock and were repeated using a filtered DiIC$_{18}$(3)-DS stock (i.e., undissolved DiIC$_{18}$(3)-DS particles were removed). For heat treated samples, the probe was added either before or after the heat treatment. The stained samples were allowed to stand 5–6 h before microscopy assessment.

The average fluorescence intensity observed when using the filtered DiIC$_{18}$(3)-DS stock was lower than that of the correspondingly treated samples stained with the unfiltered stock[9], but the results showed the same trends. An independent repeat experiment using only the unfiltered stock confirmed the findings. Hence, all these results are treated together as one set of results. The key findings were: (1) heating of milk prior to addition of the probe greatly *decreased* the average degree of staining for both human and bovine fat globules, compared with that of the respective unheated samples, and (2) heating of milk after addition of the probe greatly *increased* the average degree of staining for both human and bovine fat globules, compared with that of the respective unheated samples.

These DiIC$_{18}$(3)-DS staining results clearly showed that changes occur in the MFGM as a consequence of heating and cooling. Thus, the use of DiIC$_{18}$(3)-DS reveals a different aspect of the MFGM behaviour upon heat treatment than does WGA (compare with Chapter 5 and discussion below). The finding that the same trends occurred in both human fat globules and bovine fat globules points to DiIC$_{18}$(3)-DS revealing changes occurring in the MFGM bilayer. This conclusion was supported by the results of an additional experiment using bovine milk in which DiIC$_{18}$(3)-DS was added to a heat-treated sample. After incubation for 5 h and assessment by conventional fluorescence microscopy, showing the expected decrease in fluorescence intensity of fat globules, the same (i.e., now stained) sample was heated again

9 Recall that this suggests that undissolved probe introduced into the milk can dissolve in the milk and contribute to the staining of fat globules.

(75°C, 5 min). This yielded highly fluorescent fat globules, indicating that the factor(s) that prohibited the staining of fat globules after the first heat treatment were reversible. In turn, this indicated that the $DiIC_{18}(3)$-DS results did not represent the association of denatured whey proteins with the MFGM, but rather some reversible physical characteristic of the MFGM. Possibly, with increasing temperature, a much greater uptake of the probe and its increased mobility in the MFGM results in a higher concentration of $DiIC_{18}(3)$-DS and thus a greater fluorescence intensity than occurs at room temperature when the MFGM presumably is more rigid. Further support for this explanation was obtained by another experiment in which the heating time was reduced from 5 min to 1 min, and which still yielded highly fluorescent fat globules if $DiIC_{18}(3)$-DS was added prior to heating. Future work should investigate in more detail the effect of temperature-time combinations on the fluorescence intensity of fat globules stained with $DiIC_{18}(3)$-DS.

Thus, the use of this lipophilic probe revealed similarities between the human MFGM and the bovine MFGM in terms of bilayer behaviour upon heating at 75°C (5 min).

In contrast, the use of WGA488 yielded completely opposite results upon heating. After heat treatment, human milk fat globules were not fluorescent upon staining with WGA488, whereas for bovine milk not only did fat globules retain their fluorescence, but a small proportion (< 5%) actually became very highly fluorescent. The latter phenomenon has already been discussed in Chapter 5. Here the focus is on the difference between bovine and human milk. In addition, some bovine fat globules, that became highly fluorescent upon staining with WGA488 after heat treatment, also revealed dark patches similar to those seen on $DiIC_{18}(3)$-DS -stained fat globules.

The contrasting results for WGA488 staining of heat-treated human fat globules and heat treated bovine fat globules were most remarkable and unexpected. However, electron microscopy has shown the existence of high-molecular weight (> 400,000) glycoproteins in the human MFGM (Buchheim *et al.*, 1988a; Buchheim *et al.*, 1988b) and also in equine MFGM (Welsch *et al.*, 1988), which were rapidly lost on heat treatment (70–85°C; Buchheim, 1986; Buchheim *et al.*, 1988a; Welsch *et al.*, 1988). Such glycoproteins were not detected in bovine milk and this makes these glycoproteins logical candidates for explaining the observed phenomenon. In turn, this finding would indicate that essentially all WGA488 binding to human milk fat globules is via these high-molecular weight glycoproteins (which are known to contain NANA; Buchheim, 1986), whereas in bovine milk WGA-binding must occur via different glycoproteins. This conclusion is indirectly supported by the finding that WGA bound to all seven bovine glycoproteins identified by SDS-PAGE, whereas only two human glycoproteins showed intense binding to WGA (Murray *et al.*, 1979).

As mentioned above, conventional fluorescence microscopy of WGA488-stained fat globules also revealed dark patches similar to those seen on $DiIC_{18}(3)$-DS-stained fat globules. Follow-up

experiments using CLSM confirmed the presence of non-fluorescent patches on WGA488-stained fat globules following heat treatment. Non-fluorescent areas were pronounced, but of variable shape and size (Figure 6.10). At this stage of the development work, it has not been possible to perform quantitative analyses of average fat globule surface area stained before and after heat treatment. However, the impression obtained from the limited work performed to date is that elevated heat treatment results, on average, in more pronounced and larger non-fluorescent patches. In this respect, CLSM seems to be a better technique than electron microscopy, which apparently does not reveal changes in patches on fat globules which have been subjected to either low or elevated heat treatment, including UHT treatment (Buchheim, 1986). This is despite the fact that changes in the MFGM do occur, even upon low temperature pasteurisation, as has been shown in studies that revealed that the membrane of unheated fat globules was a strong barrier against lipase, whereas after low pasteurisation the fat became easily accessible to the lipase (cited by Buchheim, 1986). Clearly, the use of membrane-selective probes in combination with CLSM has significant more potential than electron microscopy for showing changes in the MFGM upon heat treatment. Hence, the study of heat treated milk by CLSM should become an area of interest in future work.

6.6 Cytoplasmic crescents

The secretion of fat globules appears usually to involve tight envelopment of the intracellular fat globule by the secretory cell plasma membrane. However, cytoplasm can sometimes be entrained between the membrane and the fat globule core, resulting in cytoplasmic crescents of various sizes. The proportion of fat globules that bear cytoplasmic crescents varies with species and individuals within species (Janssen & Walstra, 1982; Patton & Huston, 1988; Huston & Patton, 1990). Crescent-bearing fat globules can be shown morphologically using electron microscopy (e.g., Wooding, 1975; Wooding, 1977; Keenan *et al.*, 1983a; Keenan *et al.*, 1988; Patton & Huston, 1988; Huston & Patton, 1990) or fluorescence microscopy (Janssen & Walstra, 1982; Patton & Huston, 1988). Fluorescence microscopy studies invariably involved the use of the dye Acridine Orange. Furthermore, to date only 2-D images of crescent-bearing fat globules have been shown in the literature. In the current study, crescents were visualised using membrane-specific fluorescent probes and visualised in both 2-D and 3-D format using either conventional fluorescence microscopy or CLSM.

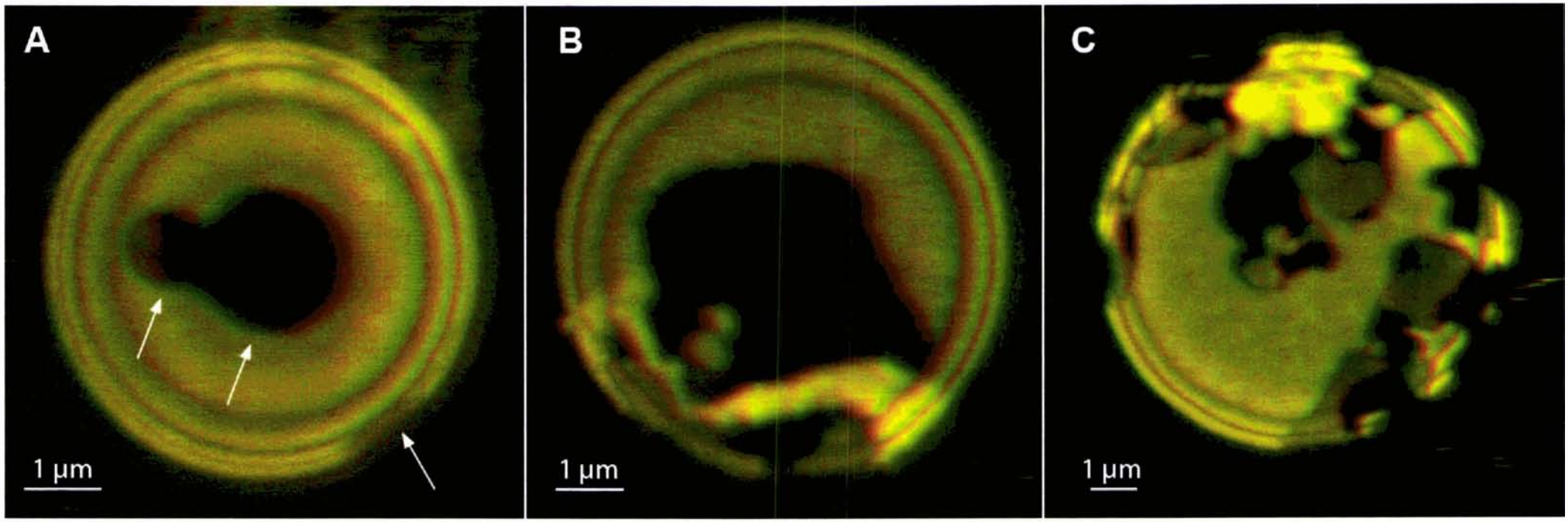

Figure 6.10 3-D images of bovine fat globules stained with WGA488 after heat treatment (75°C, 5 min). (A) A fat globule showing non-fluorescent patches that were mostly round (arrows). A large proportion of the fat globule surface is stained with WGA488. (B) A fat globule showing two non-fluorescent patches that were not round. A large proportion of the fat globule surface is not stained. (C) A fat globule showing many medium-sized non-fluorescent patches A large proportion of the fat globule surface is not stained.

Cytoplasmic crescents fluoresced brightly when milk was stained with either $DiIC_{18}(3)$-DS or WGA, and they were more frequently observed in human milk than in bovine milk, which agrees with Huston & Patton (1990). Using conventional fluorescence microscopy, dual labelling of heat treated milk, using $DiIC_{18}(3)$-DS and Nile Blue, allowed the relative sizes of the crescents to be easily compared with the size of the associated fat globule (Figure 6.11). This showed considerable variation in crescent size which supports claims that crescents can vary from as small as thin slivers to as large as exceeding the fat globule core volume (Heid & Keenan, 2005). Using CLSM, single staining using $DiIC_{18}(3)$-DS generally did not allow the full contour of the fat globule core to be seen (Figure 6.12). However, dual labelling of heat treated milk, using Nile Red and WGA488, revealed the contour of the fat globule core while also showing the shape of the crescent (Figure 6.13). Optical depth scanning of crescent-bearing fat globules stained with $DiIC_{18}(3)$-DS showed that crescents were always shaped like rounded sacs (Figure 6.14). In contrast, electron microscopy images of crescents frequently show crescents of non-rounded shapes, including pointy sacs or pointy protrusions (e.g., Wooding, 1975; Wooding, 1977; Keenan *et al.*, 1988; Patton & Huston, 1988; Keenan & Mather, 2006). Careful assessment of these images showed that sometimes pieces of membrane were missing from the crescent (image in Keenan *et al.*, 1988), or that the shape of the crescent or the fat globule was jagged (e.g., images in Wooding, 1975; Wooding, 1977; Patton & Huston, 1988) indicating that these shapes did not represent these crescents and fat globules in their native state. This strongly suggests that these pointy shapes were artifacts caused by the electron microscopy sample preparation technique.

The above results showed that $DiIC_{18}(3)$-DS-stained fat globule crescents were consistently stained much more brightly than the fat globule membrane itself. CLSM optical depth scan images showed that the greater fluorescence intensity of the crescent originated for the most part from stained matter inside the crescent. Additionally, the fluorescence quantum yield of the probe in a bilayer may be enhanced if there is an aqueous phase on both sides of the membrane, but this is not certain. The fluorescence within the crescent was found not to be homogeneously distributed. This pointed to the presence of both stainable and non-stainable matter being present within the crescent, which agrees with previous studies (Wooding, 1977; Patton & Huston, 1988). Electron microscopy has shown that the crescent is filled with intracellular membranous matter (Keenan & Mather, 2006), and this matter is expected to be stained by lipophilic fluorescent probes in a similar way to the MFGM. The fact that the internal matter of crescents is indeed stained by fluorescent probes suggests that the probes can actually penetrate the bilayer membrane, at least when the environment on the side of the inner leaflet is an aqueous environment. Incidentally, this lends some support to the above discussion (section 6.2) on the double ring phenomenon: should there indeed be a cytoplasm phase between the bilayer and the monolayer, then $DiIC_{18}(3)$-DS (and presumably other similar lipophilic probes) can indeed penetrate the bilayer and reach (i.e., stain) the monolayer.

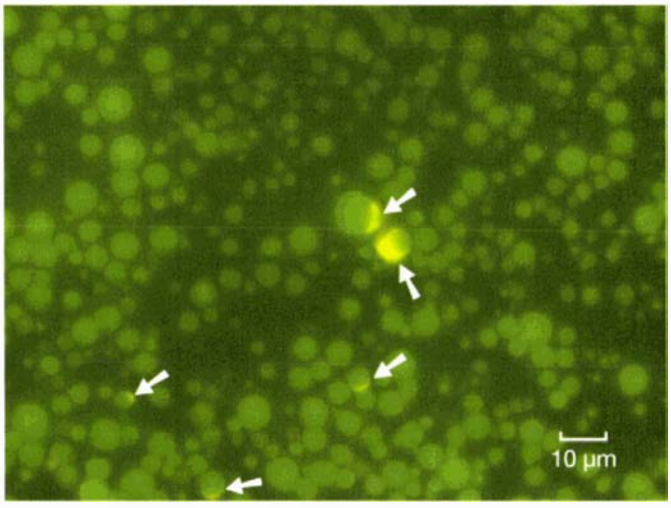

Figure 6.11 Conventional fluorescence microscopy image of heat treated (75°C, 5 min) human fat globules stained with DiIC$_{18}$(3)-DS (aqueous stock; added prior to heat treatment) and counterstained with Nile Blue (added after heat treatment). The image was acquired using a BP450–490 nm excitation filter and a LP515 nm emission filter. Green colour: Nile-Blue-stained neutral fat. Yellow colour: DiIC$_{18}$(3)-DS-stained crescents. The size of the crescents (arrows) varied from being relatively small to exceeding the volume of the fat globule.

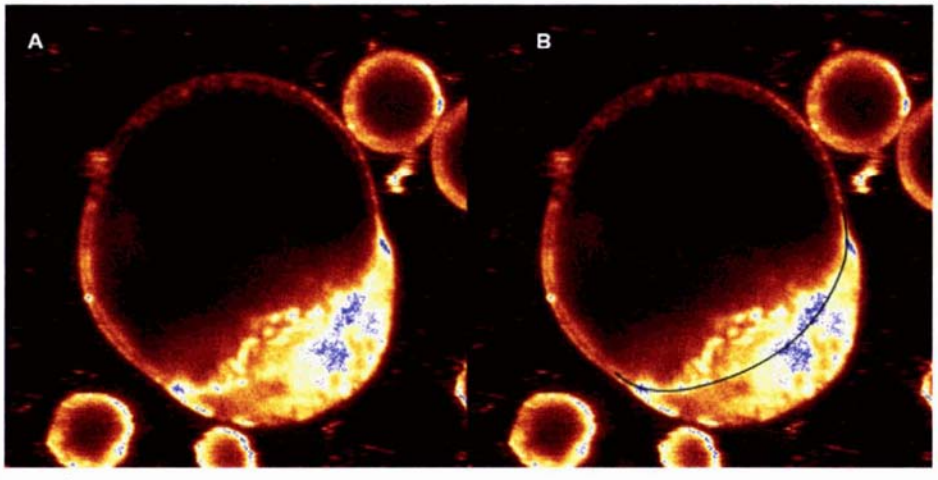

Figure 6.12 Heat treated human fat globule stained with DiIC$_{18}$(3)-DS (aq). Optical section showing the crescent (bright area) and the fat globule (dark area). (A) Original image. (B) Line drawn to denote the approximate contour of the fat globule core. Note the double ring around the fat globule core between about 7–3 o'clock.

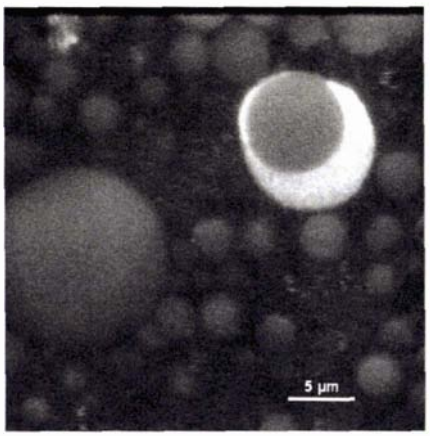

Figure 6.13 Heat treated (75°C, 5 min) human fat globules dual stained with Nile Red and WGA488 and imaged using CLSM. Note that the fat globules are not fluorescent as elevated heat treatment results in fat globules no longer being able to bind to WGA. In the fat globule containing the cytoplasmic crescent, the Nile Red perfectly shows the contour of the core fat, whereas the WGA488 shows the boundaries of the crescent, proving that the crescent contains glycosylated matter that is not removed by elevated heat treatment. Note that at the left of the fat globule the crescent narrows to a thin sliver, and covers the fat globule until about 12 o'clock.

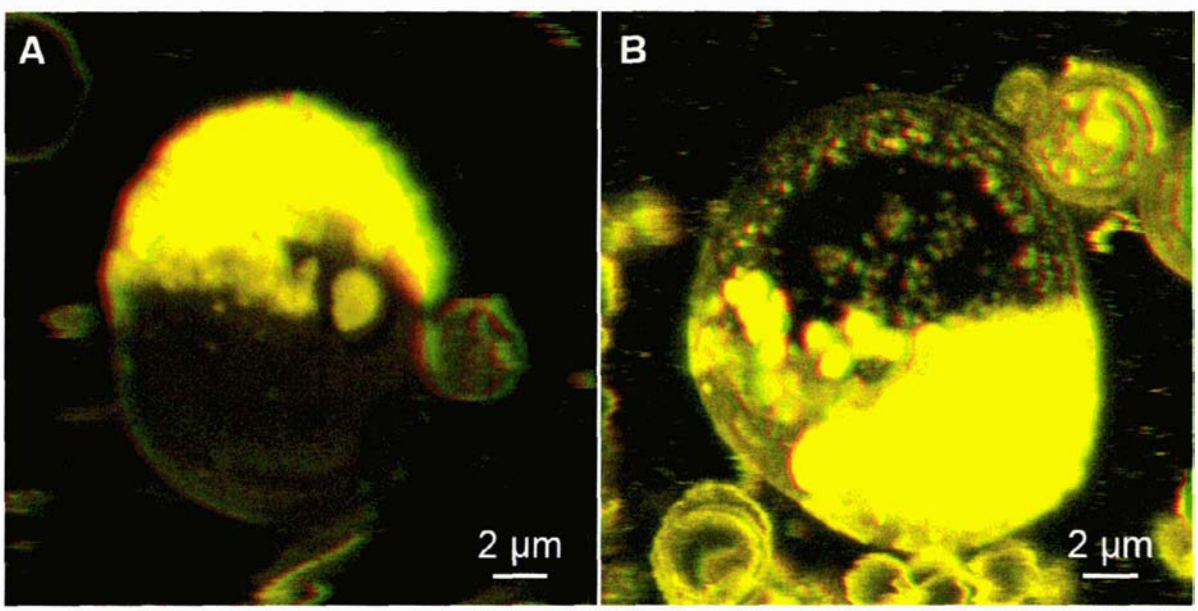

Figure 6.14 3-D images of heat treated human fat globules stained with $DiIC_{18}(3)$-DS. The crescents are of a rounded shape and sit on the fat globule like a large blister or sac.

It is believed that gaining an understanding of the molecular mechanism of crescent formation would advance the understanding of milk fat globule secretion in general (Heid & Keenan, 2005; Keenan & Mather, 2006). Electron microscopy is unlikely to be the tool that will deliver crucial kinetic data, considering that the process of fixation may disturb secretory events leading to promiscuous associations and interactions that are unrelated to normal secretory processes (Wooding, 1975; Mather & Keenan, 1998; Keenan & Mather, 2006). In contrast, use of membrane-specific probes, such as those presented in the current study, in combination with CLSM has significant potential for providing insightful data, as this novel technique should allow secretory processes to be studied in real time and under physiological conditions.

6.7 General discussion and conclusions

CLSM imaging of fat globules stained with selective membrane probes must be considered to be in an embryonic stage. Without doubt, future developments in sample preparation techniques and further advances in CLSM instrumentation will see this technique gaining increasing importance in the study of the MFGM in milk and milk products. However, at the present time, depending on the experimental situation, several practical problems may need to be dealt with including background interference, and the inability to create "smooth-looking" 3-D images.

6.7.1 Background interference and photobleaching

Background interference

The ratio of the fluorescence intensity of the stained membrane *vs* that of the milk plasma determines the contrast between the fluorescent membrane and its background. Obviously, the aim is to maximise this contrast as this will allow imaging of both strongly fluorescing fat globules and weakly fluorescing fat globules. Furthermore, in contrast to typical fluorescence microscopy procedures, in the current study unbound probe was not washed out from the specimen as the intention was to preserve the native environment of the fat globules. Consequently, background staining (caused by undissolved probe or by the probe staining milk plasma components) was observed to some degree with all probes. Although the contrast between the fluorescent membranes and the background generally was sufficient to allow imaging, the contrast between the stained fat globule and its background was sometimes compromised (e.g., when using WGA conjugates or FM4-64). Hence, in the current study only those fat globules that possessed a high fluorescence intensity were suitable for 3-D imaging by CLSM. This is a problem that may have to be contended with if one wants to study fat globules in their native environment, although the possibility should not be excluded that novel techniques could be developed that suppress background fluorescence arising from the milk plasma. Alternatively, the aims of certain studies will permit isolating fat globules and studying these in a “clean” liquid environment such as simulated milk ultra filtrate (Jenness & Koops, 1962) after either prior staining in the native environment or subsequent staining in the clean

environment. Background interference should then be much less of a problem. However, it would still need to be established whether washing stained fat globules alters the distribution of the probes in the MFGM. Finally, the effect of chemical fixation of fat globules prior to CLSM assessment has not been investigated and this should be given high priority in future work. Fixation techniques could potentially improve both the study of kinetic aspects (by comparing effects with those observed in unfixed fat globules) and removal of background interference (by transfer of fixed fat globules to a clean environment).

Photobleaching

A related problem to background interference is the fading of fluorescence upon excitation of the probe (i.e., photobleaching). Some probes photobleached relatively quickly, under either conventional fluorescence microscopy or CLSM, and are not deemed to be suitable for investigating structural features of the membranes of fat globules in their native environment. Firstly, conventional fluorescence microscope camera systems can take several seconds to several minutes (depending on the type of camera and on the amount of fluorescent light seen in the image) to capture the available light. During this exposure time the probe may fade to such an extent that a (very) poor quality image is obtained. This was the case for probes D109, N316, SP-DiIC$_{18}$(3), SP-DiOC$_{18}$(3) and 5,5'-Ph$_2$-DiIC$_{18}$(3), and these were not selected for further study (see Chapter 4). It should be noted that the use of an anti-fade, such as those marketed by Molecular Probes, could make the use of these probes acceptable. As expected, the WGA Alexa Fluor® conjugates were more photostable than some other probes (e.g., FM4-64). DiIC$_{18}$(3)-DS was also relatively photostable. Nevertheless, the performance of these probes may potentially be improved with the use of a suitable anti-fade, and this deserves to be investigated in future work. Obviously, this should take into account the requirement that the anti-fade must not interfere with the native sample matrix (milk).

6.7.2 3-D imaging

From observations of many stained fat globules it appears that probe association with the fat globule (membrane) is not related to, or dependent on, fat globule size. Nevertheless, 3-D imaging is limited to fat globules having a diameter greater than about 3 µm. However, too large a diameter[10] (i.e., > 10 µm[11]) can result in a partial compression of the top and bottom of the fat globule owing to limited space between the cover slip and microscope slide. In this context it is worth noting that in some 3-D images the front of the fat globule appears flattened; this probably is an optical, rather than real, problem as such fat globules appear to be perfectly spherical when viewed through the microscope eye piece.

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10 This is mainly an issue for sheep milk and to a lesser extent for human milk as milk of these species contains a significant proportion of large fat globules (> 10 µm). This problem is highly unlikely to occur in bovine milk.

11 Calculated as follows, 6.5 mm^3/(25 mm x 25 mm) ≈ 0.010 mm, where the value in the numerator is the sample volume and that in the denominator is the surface area of the cover slip. Assumption: the sample volume is evenly spread over the surface underneath the cover slip.

6.7.3 Do the probes perturb the membrane?

Microscopy techniques can create artifacts and this has been considered in detail for one phenomenon in section 6.2. However, artifacts may also arise from the fluorescent probes themselves perturbing the system under investigation. For milk, system perturbation can occur in two ways, (1) through the use of a non-polar probe stock solvent, or (2) by the probe itself affecting the composition and/or structure of the membrane locally or entirely. The former was discussed in Chapter 4; it is appropriate to consider the latter here.

WGA is a multivalent protein and its binding to monovalent sugar residues could in principle lead to glycoprotein and glycolipid aggregation, resulting in WGA-stained patches. However, several factors argue against WGA-induced membrane component aggregation:

1. It did not happen in the *apical membrane* of Madin-Darby canine kidney (MDCK) cells (Kovbasnjuk & Spring, 2000);

2. In the same sample that contained fat globules of which the membranes contained non-fluorescent patches (which could potentially be argued to be caused by glycoprotein/glycolipid aggregation), there were fat globules of which the *entire* membrane was evenly stained. This argues against WGA-induced glycoprotein/lipid aggregation as then all fluorescently labelled fat globules would be expected to show non-fluorescent patches.

3. Several different distribution patterns were observed on the MFGM. If WGA-induced aggregation occurred, the distribution of fluorescent *vs* non-fluorescent patches would be expected to be similar between fat globules, which evidently it is not;

4. Rather than observing WGA-stained patches, often patches were observed that were not stained by WGA, whereas the surrounding membrane was stained;

5. Robenek *et al.* (2006) suggest that the presence of patches of concentrated areas of MFGM proteins (i.e., areas of high fluorescence intensity) shown up by using fluorescent markers, most likely represent protein domains;

6. The dual staining results (Figure 6.8) suggest that, in some fat globules, the non-fluorescent areas of the membrane as revealed by the lipophilic probe FM4-64, and those as revealed by the lectin WGA, are the same. These non-fluorescent places presumably represent areas where a bilayer membrane is absent. It seems unlikely that the addition of the lipophilic probe causes the loss of significant sections of the MFGM, particularly considering that similar heterogeneous staining was observed using two very different lipophilic probes (i.e., the styryl probe FM4-64 and the indocarbocyanine probe $DiIC_{18}(3)$-DS).

   Considered together, these observations provide indirect support to the supposition that the staining patterns observed with WGA reflect the pre-existing, and not an artifactual,

membrane state. Future work should aim to strengthen this conclusion using a range of different probes and techniques.

6.7.4 Final considerations

The use of membrane-specific probes in combination with CLSM has corroborated some structural MFGM features as shown by electron microscopy, such as a structural heterogeneity (e.g., the presence of various domains on the submicron or micron scale) both within and between fat globules. Furthermore, the literature results were extended by providing physico-chemical information (i.e., presence/absence of bilayer or NANA/GlcNAc moieties). In addition, 2-D and 3-D imaging of crescent-bearing fat globules showed that some electron microscopy images of crescents reported in the literature probably showed artifactual shapes of crescents. It is believed that gaining an understanding of the molecular mechanism of crescent formation would advance the understanding of milk fat globule secretion in general (Heid & Keenan, 2005; Keenan & Mather, 2006). Electron microscopy is unlikely to deliver crucial kinetic data, considering that the process of fixation may disturb secretory events leading to promiscuous associations and interactions that are unrelated to normal secretory processes (Keenan & Mather, 2006; Mather & Keenan, 1998; Wooding, 1975). In contrast, the use of membrane-specific probes, such as those presented in the current study, in combination with CLSM has significant potential for providing insightful data, as this novel technique should allow secretory processes to be studied in real time and under physiological conditions.

Whether the observed double fluorescent ring is a microscopy artifact or reflects a true membrane feature remains unknown, and further work is required to elucidate this phenomenon.

WGA-Alexa Fluor® conjugates have been shown to stain fat globules of at least three species (human, ovine, and bovine). Species differences were evident from the rate of MFGM staining by the different probes, by staining pattern (e.g., features seen on ovine MFGM have not been observed on bovine or human MFGM) and from heating experiments. Because the nature of the association of WGA with the MFGM (binding to NANA and GlcNAc residues) is fundamentally different from that of the lipophilic probes (which embed in the phospholipid bilayer), and because a large range of fluorescent WGA Alexa Fluor® conjugates covering the wavelength range 400–700 nm are commercially available, WGA is an ideal probe for use in multiple staining techniques. Protocols for multiple staining techniques should be further developed. For example, this could include:

1. comparing the staining patterns obtained with WGA and that of a succinylated form of WGA, which binds to GlcNAc, but not NANA (Stegemann *et al.*, 1990), before and after treatment with either neuraminidase (specific for terminal NANA; Gottschalk, 1960) or with bovine milk galactosyltransferase (specific for terminal GlcNAc; Kelly & Hart, 1989); or,

2. dual staining techniques such as those performed on the apical plasma membrane of MDCK cells (Röper *et al.*, 2000), or on certain nerve cells (Minana *et al.*, 1998), involving WGA in combination with immunofluorescence probes specific for certain proteins to study molecular interactions between different membrane components and association of certain membrane components with membrane subdomains.

Similarly, the use of lipophilic probes has revealed non-fluorescent patches on many fat globules, indicating significant differences in MFGM composition and structure. This finding is highly relevant with regard to understanding the stability of fat globules to mechanical stress or enzymatic attack. It is not clear whether loss of the bilayer makes the fat globule more stable against mechanical stress or enzymatic attack, but it is highly probable that the stability of a fat globule fully covered with a bilayer membrane is different from one which is only partially covered by a bilayer membrane. Hence, the ability to quantitatively measure the proportion of fat globules with intact membranes would appear to be a key step in advancing our understanding of the effects of the various milk handling treatments used in the dairy industry. Furthermore, it is speculated that the variable proportion of fat globule surface covered by a bilayer membrane seen on different fat globules of the same milk, is a factor in spontaneous lipolysis, a phenomenon which currently is not understood.

In addition, it is evident that further improvements in both the theoretical understanding and practical staining protocols are possible and desirable. With such improvements the use of fluorescent probes is expected to become a highly effective tool for the study of milk fat globules. It is also evident that the novel technique developed has huge potential in the study of interactions of fat globules with other milk components.

7 Other techniques — the potential

To increase our understanding of the MFGM structure and composition, and how the MFGM changes as a consequence of milk handling and treatment, a suite of new analytical techniques for studying the MFGM is needed. The development of the fluorescence microscopy techniques constitutes a significant step forward, but additional techniques are required to provide information complementary to that obtained using fluorescence microscopy. This chapter describes exploratory work conducted for assessing the following techniques: (1) atomic force microscopy (AFM), (2) micromanipulation, and (3) flow cytometry. The potential of NMR spectroscopy was briefly investigated, but results were not encouraging (Appendix 8). The main purpose of this chapter is to demonstrate that besides fluorescence microscopy other techniques have significant promise for studying individual fat globules and their membranes, and that these techniques should be further developed in future work.

7.1 Atomic force microscopy

7.1.1 Introduction

The resolution of the light microscope is a function of the wavelength of the incident light. Using a high-quality conventional light microscope, the resolution is limited to about 200 nm, which is about 2 orders of magnitude above that required for assessment of morphological nanodetail (i.e., detail at the nanometer level) of the MFGM. Electron microscopy, which uses high-energy electrons instead of light as the incident beam, can be used to visualise nanometer detail. Different electron microscopy techniques have been used to produce high-resolution images of the MFGM, including transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (e.g., Horisberger *et al.*, 1977). However, a significant disadvantage of the electron microscopy technique is that extensive sample pre-treatment is generally required. This typically involves the isolation of the fat globule from the milk, fixation of the specimen and extraction with non-polar solvents to remove the fat. Besides the risk of producing artifacts, this means that the fat globule is not imaged in its native state. Hence, certain conclusions about the structure of the MFGM that have been based on electron microscopy observations have been questioned (e.g., Keenan *et al.*, 1983a; Mather & Keenan, 1998). A further disadvantage of electron microscopy is that this technique cannot be used to provide information about biophysical properties (e.g., elasticity) of the MFGM.

The AFM[1] is capable of imaging specimens with a resolution similar to that of the electron microscope[2]. In contrast to electron microscopy, with AFM the sample should not need to be stained, coated or frozen. Furthermore, AFM can be used to image surface topography in either

1 The abbreviation AFM either means atomic force microscope or atomic force microscopy. The context determines which of these applies.

2 A lateral resolution of less than 1 nm can be achieved under certain conditions, e.g. a resolution of 0.2 nm on cardiolipin molecules has been achieved using AFM (Muscatello *et al.*, 1996).

gaseous or liquid environments as well as to measure local physical properties (e.g., adhesion forces and elasticity). Another obvious advantage of AFM over electron microscopy is the minimal sample preparation needed (Elofsson *et al.*, 1997). Typically, the sample can be imaged on the day of preparation, whereas sample preparation may take several days or weeks for electron microscopy. As AFM produces images of the surface of the specimen, the information obtained should be complementary, and not necessarily identical, to that obtained by electron microscopy (Yang *et al.*, 1993; Russell *et al.*, http://www.veeco.com/pdfs.php/154).

In recent years, AFM has increasingly been used to study biomaterials, such as cells in a native environment (Dufrêne, 2004), as well as a nanotechnology tool in food science (Yang *et al.*, 2007). So far, the majority of applications of AFM in dairy-related research has focused on dairy proteins. Examples of dairy-related studies are listed in Table 7.1.

Table 7.1 Examples of dairy-related studies using AFM, as reported in the literature.

System	Reference
Interfacial films of BSA at a hexadecane-water interface	Gunning *et al.* (1996)
Interfacial films of β-casein at an air-water interface	Gunning *et al.* (1996)
β-Lactoglobulin and whey protein concentrates air-dried on mica	Elofsson *et al.* (1997)
Edible whey protein films on mica	Lent *et al.* (1998)
β-Lactoglobulin films at air-water interfaces	Mackie *et al.* (2000); Morris *et al.* (1999b, 2001); Morris (2004)
Mixed β-casein and β-lactoglobulin films at an air-water interface	Mackie *et al.* (2001)
High-melting fraction of milkfat	Narine & Marangoni (1999)
Heated β-lactoglobulin and WPI on mica under butanol	Ikeda & Morris (2002)
Heat-induced fibrilar aggregation of β-lactoglobulin	Arnaudov *et al.* (2003)
Heat-induced gelation of whey proteins	Ikeda (2003)
Mixes of κ-carrageenan and β-lactoglobulin on mica under butanol	Roesch *et al.* (2004)
WPI and β-lactoglobulin at the air-water interface on mica under butanol	Woodward *et al.* (2004)
Air-dried casein particles on mica	Merel-Rausch *et al.* (2007)

Considering the potential of AFM to provide high-resolution 2-D and 3-D images of surface topography, work was conducted with the aim of developing an AFM imaging protocol for the

evaluation of the MFGM. Significant practical problems were encountered which could not be overcome using the available instrumentation. This section of Chapter 7 first presents a brief introduction to the AFM technique, then outlines the experimental work conducted and concludes with a general discussion and directions for future work.

7.1.2 Principle of operation of the AFM[3]

The AFM belongs to the family of scanning probe microscopes. AFMs are not microscopes in the classical sense, as they do not generate images using an incident beam such as light or electrons. Instead, these instruments measure the physical interaction (attractive and repulsive forces) between a sharp tip and the sample. A typical set up is shown in Figure 7.1 and a photo of one of the AFMs in use at Massey University is shown in Figure 7.2.

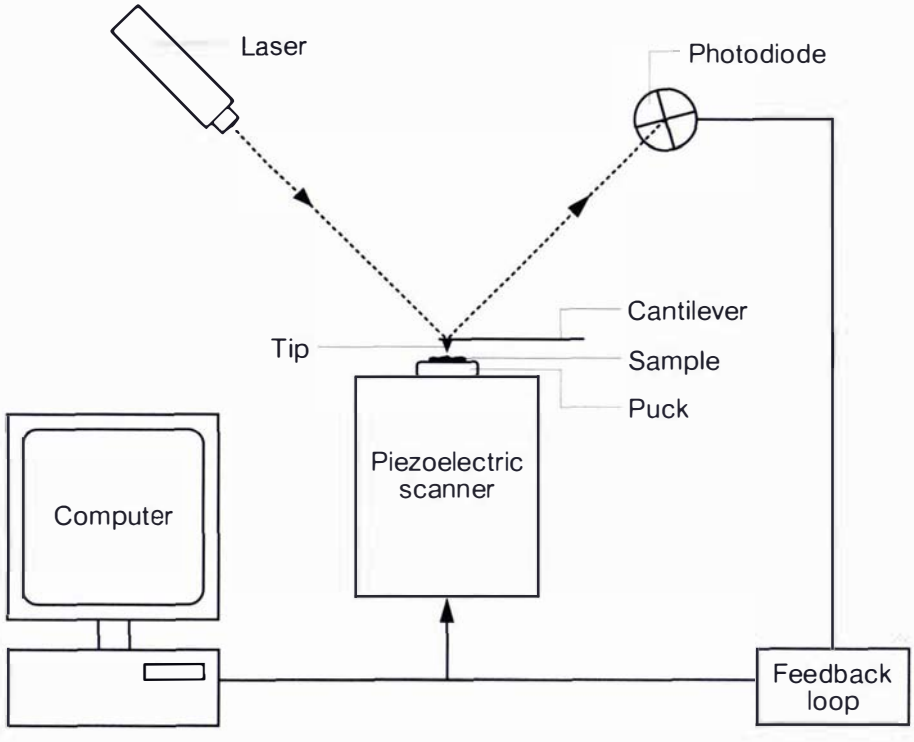

Figure 7.1 The principle of the atomic force microscope (adapted from Morris *et al.*, 1999a and Dufrêne, 2004).

The key elements of the AFM are a sharp probe (tip) that is attached to a cantilever, a laser beam, an optical detection system (photodiode), a piezoelectric scanner[4], which serves as the sample stage, and a computer. The probe is brought into close proximity with the surface of the

3 Information for this section was sourced from Morris *et al.* (1999a), Dufrêne (2004) and the Digital Instruments AFM instruction manual.

4 By applying an electrical signal, the piezoelectric transducer can be made to expand or contract with a precision of atomic dimensions. Hence, this provides the AFM with the accuracy required for positioning the tip.

sample by adjusting the z-position of the piezoelectric scanner. The interaction force[5] between the sample surface and the tip is measured through the deflection of the cantilever (which essentially acts as a spring), which in turn is monitored by the laser beam focused on the free end of the cantilever and reflected into a photodiode. Scanning of the sample can occur in the *x* and *y* directions with simultaneous displacement of the z-position of the piezoelectric scanner (constant force imaging), or by allowing the cantilever to bend in response to sample topography (variable deflection imaging).

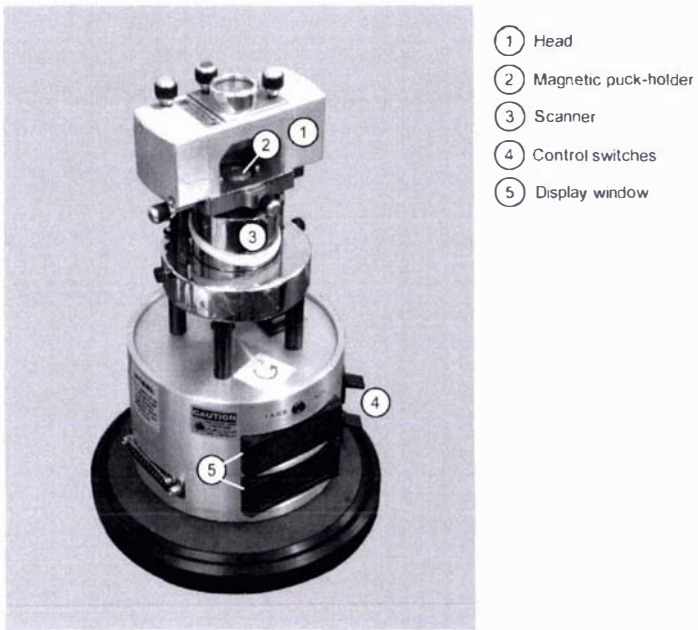

Figure 7.2 Photo of the AFM-2 (see "Materials and equipment – General").

7.1.3 Operating modes

Biological samples can be imaged using different AFM imaging modes. In the current work both the "contact mode" and the "tapping mode" were used and these are briefly explained below.

Contact mode

The tip is brought into direct contact with the sample surface. There is a repulsive interaction between the tip and the surface and this bends the cantilever. The cantilever deflection (and thus the imaging force) is preset by the operator to a certain value. As the sample surface is scanned, the AFM aims to maintain the preset cantilever deflection value and will move the piezoelectric scanner up and down as the sample is scanned in the x and y directions, thus generating a 3-D image.

5 These might involve van der Waals forces, electrostatic forces, capillary forces, adhesive forces (when the tip becomes contaminated with small amounts of sample), double layer forces (in aqueous media) (Morris *et al.*, 1999a).

Tapping mode

In tapping (or AC) mode, the probe tip is oscillated in the *z* plane[6] and taps the surface at kHz frequencies. In this mode, interactions between the tip and the sample are reduced[7]. Furthermore, as the tip touches the surface only when it is at the lower end of the oscillation, lateral shear forces are almost eliminated. Consequently, the risk of damage to the surface should be significantly reduced. Hence, this mode may produce better results than the contact mode when imaging soft surfaces or samples that are not tightly immobilised on a substrate.

7.1.4 Cantilevers/tips

Tips are typically made of silicon or silicon nitride as these materials are very hard (i.e., resistant to wear and suited to micro-fabrication). The cantilever spring constant[8] and the shape of the tip (short, long, narrow, wide) are fundamentally important parameters. The tip shape affects the lateral resolution of the AFM; the sharper the tip the better the resolution. However, for biological samples, dull tips may be the better choice as the pressure exerted on the sample is less than in the case of sharp tips. With the latter, membrane damage (rupture) is a significant risk. Tips can be functionally modified[9] by coating with certain materials (chemically: e.g., by silanisation; biochemically, e.g., by enzymes or antibodies) to confer desired interaction properties related to the surface under investigation.

7.1.5 Experimental techniques and results[10]

Materials and equipment — General

AFM-1 Asylum Research (Santa Barbara, CA, USA), model MFP (multi force probe)-3D in combination with a Nikon Eclipse TE 2000-E; MFP3D software running on an Igor Pro platform; laser 810–850 nm.

AFM-2 Digital Instruments (Santa Barbara, CA, USA) model E in combination with a JV (S/N 6328) piezoelectric scanner; Nanoscope software di5.12b49; laser 670 nm.

Milk Milk samples were obtained from Massey University No.1 Dairy Farm from either individual cows (collected from the milking machine receiving can) or from the farm bulk tank.

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6 Oscillations may be up to 100 nm in amplitude. For an animation of tapping mode refer to the video clip at http://www.veeco.com/library/resources_sub_type.php?sub_id=2; accessed July 2007.

7 In air, a sample is surrounded by an extremely thin film of water, which creates a capillary force that can trap the tip. The tapping mode prevents the tip from being trapped. In liquids, there is no capillary force present on the surface of the sample. Yet, the tapping mode may still be better than contact mode particularly when the sample has a poor affinity for the substrate (e.g., mica) and is at risk of being scraped off owing to the lateral force exerted by the tip.

8 Spring constants can vary from < 0.1 nN to > 100 nN.

9 See e.g. http://www.novascan.com/brochures/Novascan_AFM_Probes.pdf; accessed July 2007.

10 All AFM images shown are raw data with no processing.

Cleaving of mica[11]

In all cases where mica was used as the substrate, a freshly cleaved surface of mica (either glued onto a glass microscope slide or attached to a puck[12]) was obtained by sticking a piece of adhesive tape onto the mica and withdrawing the tape from the mica surface.

Fat globules cannot be imaged in liquid milk

Using AFM-1 in contact mode, no signal was received by the photodiode detector when the tip (CSG 11; ND-MDT Co., Moscow, Russia) was placed in either undiluted milk or 100x diluted (aq) milk. Apparently, the large number of casein micelles, fat globules and other insoluble matter present in the milk scattered the laser light to such a degree that no signal was received by the detector. Hence, AFM imaging of fat globules in liquid requires the use of a medium that is transparent to the laser light.

Although fat globules could not be imaged in their native environment, imaging the MFGM in *non-native* conditions at nanometer resolution was considered still to be very useful, and thus warranted further development work. This focused on developing a technique that immobilised fat globules onto a mica surface so that the surface could be scanned in dry conditions.

Scanning of dried milk

A few drops of raw milk (undiluted) or 10x diluted (aq) milk were allowed to air-dry at room temperature protected from dust and were subsequently scanned using the AFM-1 in contact mode. No image could be obtained for the air-dried raw milk sample, presumably because the surface was too rough (i.e., there was too much variation in height in the dried milk layer for the tip to cope with).

In contrast, images could be generated for the 10x diluted (aq) milk sample. Figure 7.3 shows the remainder of a fat globule cluster (this can be inferred from the diameter of the individual structures (range ~ 0.5–8 µm), which is typical of that of fat globules) surrounded by a layer of dried milk plasma components. The latter contained lumpy matter that, judged by their diameters (~ 100–400 nm), probably were casein micelles. However, fat globules in the cluster were not intact; only the footprints of the globules were visible. Surprisingly, inside the perimeters of many fat globule footprints, the bare mica surface (smooth surface) was visible, although inside the perimeters of some other fat globule footprints particulate matter was observed. To determine whether fat globules were intact *prior* to AFM scanning, an air-dried 10x diluted (aq) milk sample was evaluated by differential interference contrast microscopy. This indicated that some, but not all, globules in the fat globule clusters still contained fat after sample preparation (dark areas in Figure 7.4). The presence of fat-containing globules was

11 Mica is an atomically flat, cleavable aluminum silicate crystal (Elofsson *et al.*, 1997).

12 A round piece of steel (∅ 10 mm). The puck is held in place through magnetic forces exerted by the piezoelectric scanner head.

confirmed by fluorescence microscopy after Nile Red-staining of a 100x diluted (aq) milk sample (results not shown). Thus, the AFM tip appeared to have removed most of the globule structure, including the fat, from the substrate. Apparently, at room temperature the fat is too soft and the tip "ploughs" through the fat globules. It is not clear what the particulate matter inside the perimeter of some fat globules footprints represented. This could have been original MFGM or, alternatively, milk plasma components that sedimented onto the mica before the fat globule cluster did. However, the horizontal streaks seen in Figure 7.3A are artifacts caused by the tip mistracking or jumping[13], a common problem encountered when imaging relatively large structures in AFM (Morris *et al.*, 1999a).

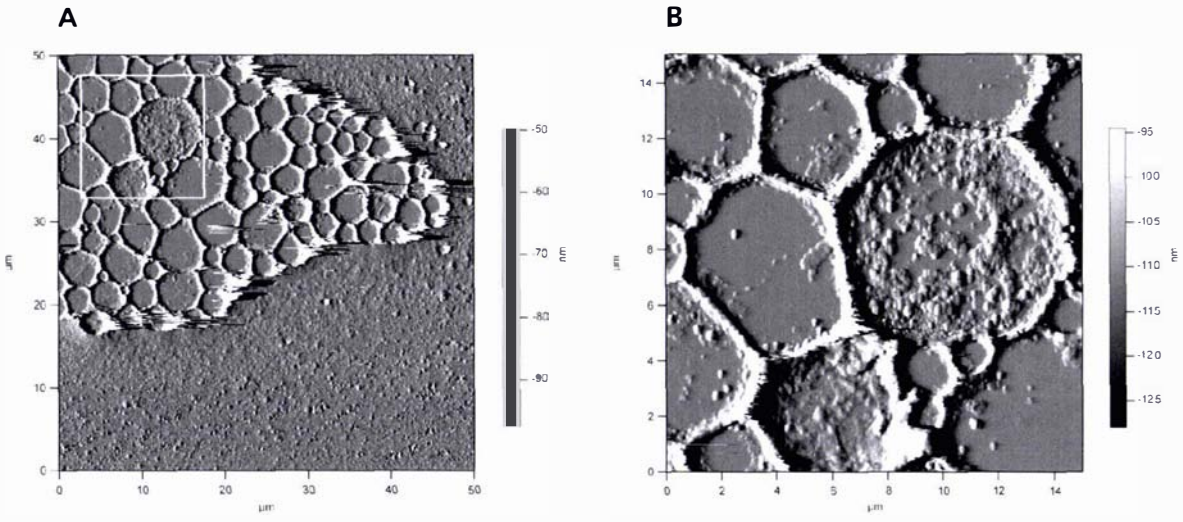

Figure 7.3 (A) AFM deflection image of air-dried 10x diluted (aq) milk showing the footprint of a cluster of fat globules. The hexagonal shape of the fat globule perimeters was caused by the dense packing of the fat globules. Outside the fat globule cluster, air dried milk plasma components are visible, showing numerous particulate structures that presumably represented casein micelles. Inside the cluster, either bare mica (smooth surface) or particulate matter can be seen. Streaks are artifacts caused by the tip "jumping" off the surface. (B) Re-scan of the boxed area in Figure 7.3A. This shows that rescanning the same area does not affect the structural features (i.e., the matter is stable upon scanning).

Scanning of washed fat globules

The results for the air-dried milk samples had identified two problems: (1) the presence of milk plasma components made it impossible to tell whether one was looking at the MFGM or at milk plasma components or both; (2) fat globules at room temperature appeared to be too soft for imaging with the available equipment.

13 The topography image (not shown) showed that the streaks exceeded a height of 400 nm.

To overcome the problem of the presence of plasma components, fat globules needed to be isolated from the milk. Washed fat globules were obtained by using the washing procedure described in Appendix 6. Light microscopy evaluation of the serum of the 3rd wash showed that it contained a low concentration of fat globules (∅ ~ 2–8 μm). This was considered advantageous, as fat globules would be expected to be well spaced out and easily identifiable upon drying of the serum.

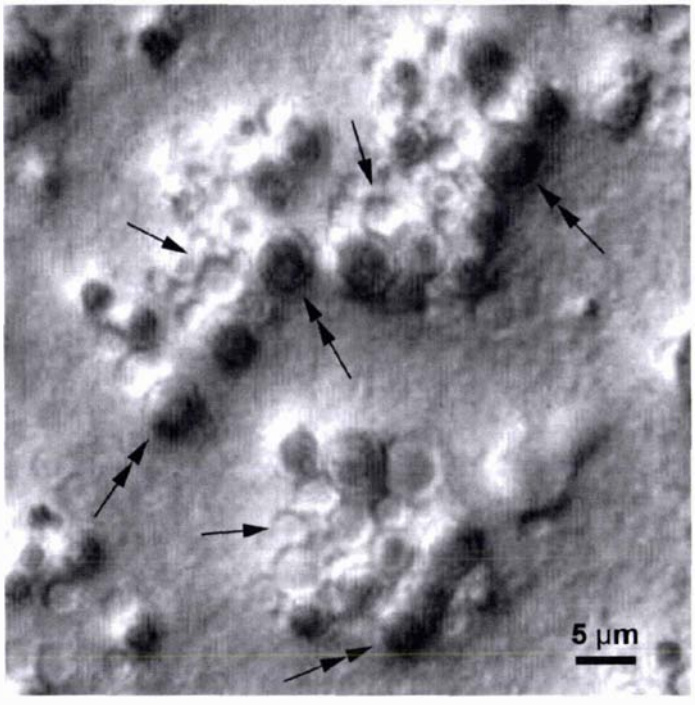

Figure 7.4 Differential interference contrast microscopy image of an air-dried 10x diluted (aq) milk sample. Footprints of what appeared to be fat globule clusters contain fat-filled fat globules (double arrows) as well as "empty" footprints (arrows).

Contact mode at room temperature

A subsample (7.5 µL) was placed on freshly cleaved mica at room temperature and milli-Q water (100 µL) was added on top so that the tip could be operated immersed in liquid. No image could be obtained, presumably because fat globules were not immobilised onto the mica, but freely moving in the liquid, thus interfering with the path of the laser light. Furthermore, no image could be obtained of an air-dried sample when using a soft Bio-lever tip (Olympus BL-RC 150 VB-C1, Tokyo, Japan) in direct contact mode in air. The tip "stuck" to the sample when it engaged with the sample surface, presumably because of strong capillary-induced forces. In contrast, images were obtained of an air-dried sample scanned at room temperature using a CSG 11 tip (force ~ 2 nN). However, similarly to images obtained for the air-dried milk, only "footprints" of fat globules, but not intact fat globules, were seen (results not shown).

Tapping mode at reduced temperature

As experiments had shown that at room temperature the sample was too soft for imaging, an attempt was made to increase the rigidity of the fat globules by storing the air-dried sample at 8°C after air-drying but prior to scanning in tapping mode in air. The scanning results obtained indicated that the fat globules remained at least partially intact (Figure 7.5).

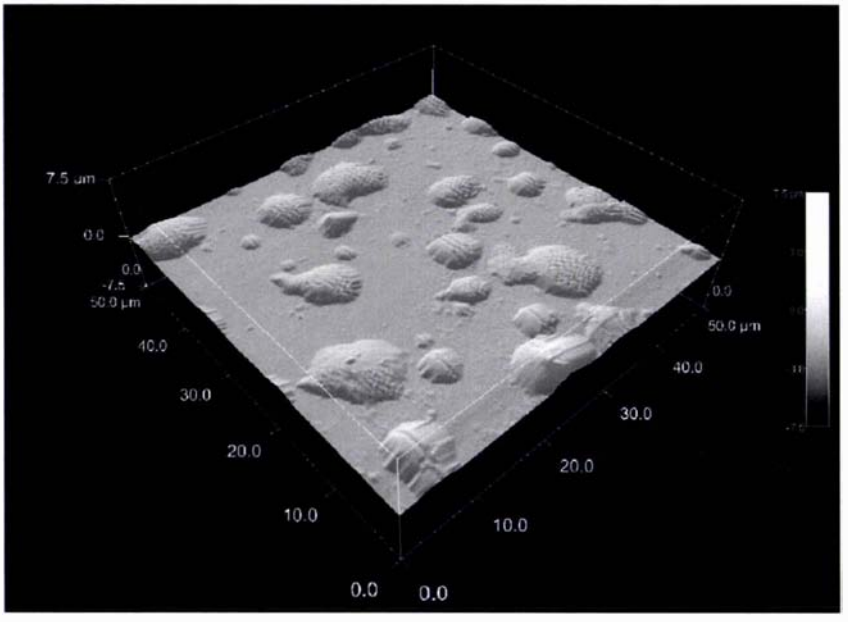

Figure 7.5 3-D AFM deflection image of air-dried fat globules, cooled to 8°C prior to scanning in tapping mode in air at room temperature. An impression of the fat globule structure is obtained, but the presence of rippling lines (artifacts) show that scanning conditions were not optimal. Varying the scanning conditions did not result in improved images being obtained.

Effect of drying temperature on the structure of the fat globules

The above pre-cooling results were encouraging in terms of maintaining the shape of the fat globules, and an experiment was conducted to better understand the effect of the drying temperature on fat globule structure upon drying. Samples containing washed fat globules were air-dried at room temperature or cold-dried according to a novel procedure as follows. The serum containing the washed fat globules was pre-cooled to 5°C. A subsample was placed on a microscope slide, which was then placed in a pre-cooled (5°C) desiccator, and the liquid was allowed to evaporate overnight in the desiccator while the temperature was maintained at 5°C. This yielded cold air-dried fat globules.

The structure of the fat globules was compared using light microscopy (Figure 7.6). The results showed conclusively that air-drying at room temperature caused many fat globules to burst open resulting in their core fat spilling out. Other fat globules were embedded in the pools of free fat that had been created by the burst globules (Figure 7.6A). In contrast, air-drying at 5°C

significantly improved the stability of the fat globules. Although there was some evidence that fat had escaped from a small proportion of fat globules, the majority of fat globules evidently had retained their spherical shape (Figure 7.6B). However, cold drying did promote the aggregation of fat globules in chain-like structures or as clusters. Nevertheless, many individual fat globules were present. Both the aggregated fat globules and the individual fat globules were considered to be suitable objects for AFM scanning.

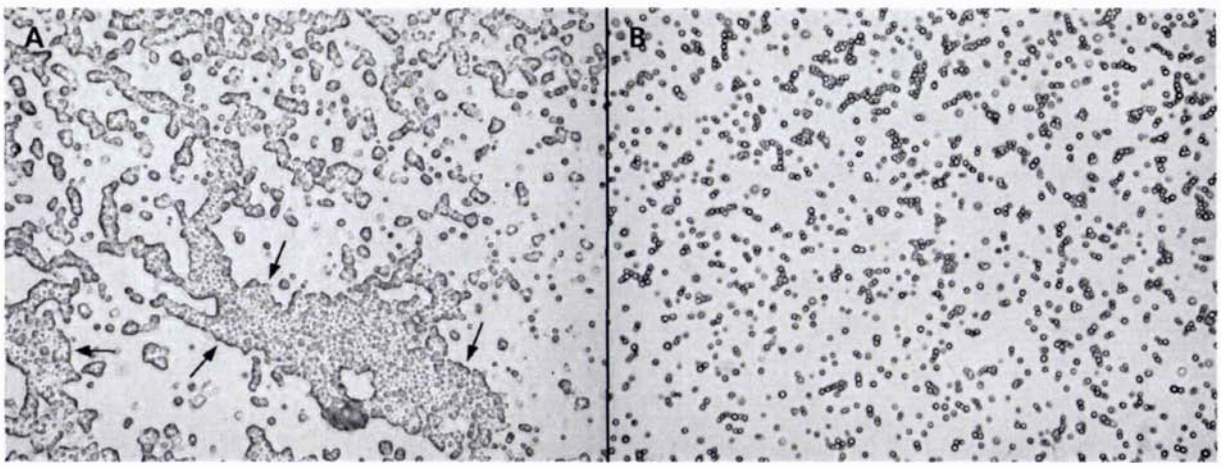

Figure 7.6 Effect of drying temperature of washed fat globules as examined by light microscopy. (A) Drying at room temperature caused fat globules to burst resulting in large pools of free fat (arrows) in which numerous intact, or partially intact, fat globules appear to be embedded (dark objects within the pools of fat). (B) Drying of washed fat globules in a desiccator at 5°C maintains the globular structure of fat globules although many fat globules aggregate into small chains or clusters.

Unfortunately, AFM imaging of cold-dried fat globules did not yield the expected images. The fat globules appeared to quickly warm up to room temperature when removed from the cold desiccator and subsequently lost their rigidity. To counteract this environmental warming of fat globules, a cold stage was required. This accessory was not available. Instead, a Peltier device[14] was constructed. To remove the generated heat, this device was placed on a heat sink (copper plate). Attempts at imaging cold air-dried fat globules placed on the cooled side of the Peltier device were unsuccessful as the heat sink was not effective enough. Hence, warming of the cold side of the Peltier device, and thus of the fat globules, could not be prevented and fat leaked from the fat globules.

The above experiments were conducted using the AFM-1 instrument. Pre-cooled air-dried fat globules imaged using AFM-2 at room temperature yielded similar results to those obtained

14 Peltier devices, also known as thermoelectric modules, are small solid-state devices that function as heat pumps. A "typical" unit is a few millimeters thick by a few millimeters to a few centimeters square. It is a sandwich formed by two ceramic plates with an array of small bismuth telluride cubes ("couples") in between. When a DC current is applied heat is moved from one side of the device to the other - where it must be removed with a heat sink (http://www.peltier-info.com/info.html, accessed August 2007).

using the AFM-1 (i.e., only the footprints of fat globules remained after scanning; Figure 7.7). As imaging of fat globules at controlled low temperatures could not be performed with either AFM, development work was not progressed further.

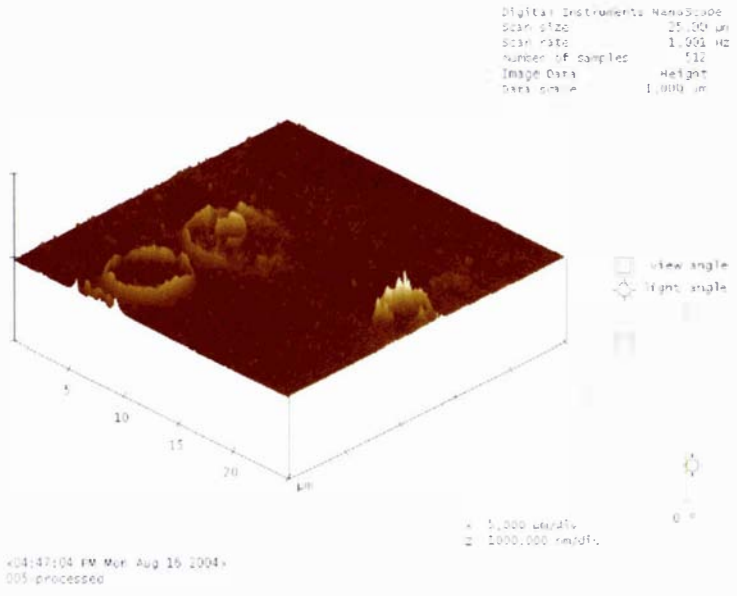

Figure 7.7 Image of cold air-dried fat globules on mica. Scan performed at room temperature using the AMF-2. Only footprints remained after scanning.

7.1.6 General discussion, conclusions and future work

The current work demonstrated that fat globules cannot be imaged by AFM in their native environment, because freely moving fat globules and casein micelles in the milk interfere with the path of the laser light and thus no signal is detected by the photodiode. Evidently, a medium that is transparent to the laser light is a pre-requisite for successful AFM. Furthermore, air-drying of a small aliquot of milk on a mica substrate for direct imaging was not successful for two reasons: (1) milk plasma components dried together with the fat globules and this raised question marks about what the sample image represented (it is likely that milk plasma components "contaminated" the fat globule surfaces), and (2) at room temperature, the fat in the globules was too soft, which resulted in the AFM tip destroying the fat globules during scanning. Destruction of the specimen by the AFM tip is not peculiar to fat globules alone. Small, IgG-containing, lipid vesicles (∅ ~ 200–300 nm) that were immunochemically immobilised on an anti-IgG-labelled substrate were destroyed under certain conditions during scanning (Shibata-Seki *et al.*, 1996) and so were phospholipid bilayers on freshly cleaved mica (Yang *et al.*, 1993). Thus, a soft fat globule interior is a disadvantage for AFM scanning of the MFGM. The problem of a soft support beneath the MFGM is somewhat analogous to the problem encountered with scanning cells and large membrane vesicles at room temperature under liquid; the membrane is basically floating between the interior fluid and the exterior solution (Yang *et al.*, 1993). Hence,

the membrane needs to be supported by a rigid structure underneath[15] (Yang *et al.*, 1993). Furthermore, because of the variation in height (several μm) the tip has to negotiate, imaging whole fat globules requires one to work at the very limits of the instrument (Dufrêne, personal communication, 2004; Morris and Gunning, personal communication, 2004). To date, no studies have been reported in the literature in which MFGM of intact fat globules has been imaged by AFM. Indeed, analogously to cells, fat globules may be considered as "troublesome large samples" (Morris *et al.*, 1999a). Apart from the work reported in this thesis, which was conducted in 2004, MFGM has apparently only been studied as monolayers spread on mica (Krommenhoek *et al.*, 2006[16]). Hence, research on the MFGM using AFM may initially need to focus on isolating the MFGM from the parent fat globules, and immobilising that onto some solid substrate.

In conclusion, AFM presented some practical problems that could not be overcome with the currently available resources. Despite the problems encountered in the current work, AFM holds significant promise for dairy-related research. Although the majority of previous dairy-related studies focused on the major milk proteins (Table 7.1), the successful application of AFM to the study of various biological samples, including cells, encourage the view that ultimately the MFGM could also be studied using AFM. In non-dairy related work, domain structures of lipid membranes have been visualised (cited in Yang *et al.*, 1993). Therefore, the domains visualised by the use of fluorescent probes (Chapter 6) might also be able to be visualised and studied by the AFM. Subtle structural and physical properties might be able to be discerned (see Dufrêne, 2004 for an overview of the application of AFM in microbiology; some of these applications might work for MFGM). In turn, this should yield additional information about the different MFGM domains. However, AFM images should be compared with electron microscopy images, as AFM does not identify all types of structures. For example, in MDCK cells, the numerous microvilli present at the surface were not visible in AFM images, whereas they were the most prominent feature seen by SEM (Hoh & Schoenenberger, 1994).

Future work should focus on developing both a wet-scanning technique and a dry scanning technique. In both cases, fat globules would have to be isolated from the parent sample. As freely-moving fat globules interfere with the laser beam, scanning in liquid environments is probably possible only if the fat globules are first immobilised on the substrate. A combined technique, in which the fat globules are first air-dried and subsequently placed in a suitable solution, similar to that performed for nucleic acids (Yang *et al.*, 1993), might prove to be useful.

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15 Fixation of cells with glutaraldehyde did not improve the situation in one study (Yang *et al.*, 1993), but had a beneficial effect in other studies (Hoh & Schoenenberger, 1994; Kolb *et al.*, 1999).

16 This reference contains only an abstract of the Experimental Biology meeting held in San Francisco, 2006. The full abstract reads, "The structure and function of the milk fat globule membrane (MFGM) from bovine milk was analyzed using a Langmuir-Blodgett trough and atomic force microscopy (AFM). Isotherms of bovine buttermilk powder were obtained at temperatures ranging from 17–37°C. A collapse was observed at 28 ± 1 mN/m at 19.7 ± 0.2°C. MFGM monolayers were deposited on cleaved mica at various surface pressures. Domains were observed."

Scanning in liquid has the advantage that the medium can be adapted to mimic milk plasma, or alternatively can be formulated to influence the MFGM in some way. In the latter case, the aim would be to investigate the effect of the medium, or a constituent thereof[17], on the MFGM. Furthermore, because scanning under liquid eliminates the so-called capillary forces, lower probe forces are achievable than when scanning in air (Yang *et al.*, 1993).

Scanning of dried fat globules could not be performed at room temperature as the fat globules were not rigid enough. Hence, cold drying of fat globules is required in combination with scanning at a low temperature (e.g., 2–5°C). The latter would require either an effective cold stage or conducting the AFM scanning in a temperature-controlled room. At the time the AFM work was carried out, neither of these options were available and thus these strategies could not be pursued. However, an effective cold stage has recently been installed and further work should be conducted in future studies.

Acknowledgements

I thank Assoc. Prof. R. Haverkamp for operating AFM-1 and constructing the Peltier device.

7.2 Micromanipulator

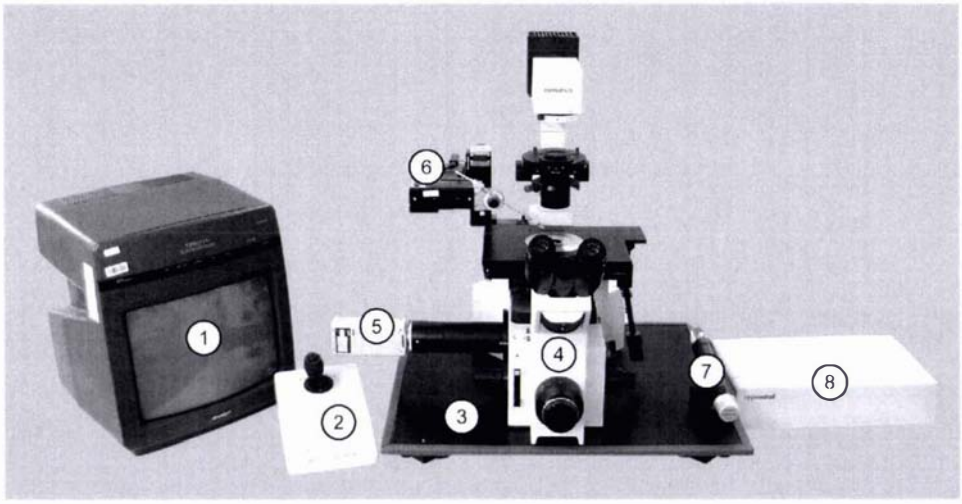
7.2.1 Introduction

In future research, the study of the properties of *individual* fat globules will likely be of increasing interest in order to understand the molecular structure of the MFGM. To conduct such research, one needs to be able to manipulate individual fat globules. This section describes a micromanipulation technique for harvesting and transferring individual fat globules.

7.2.2 Materials and equipment

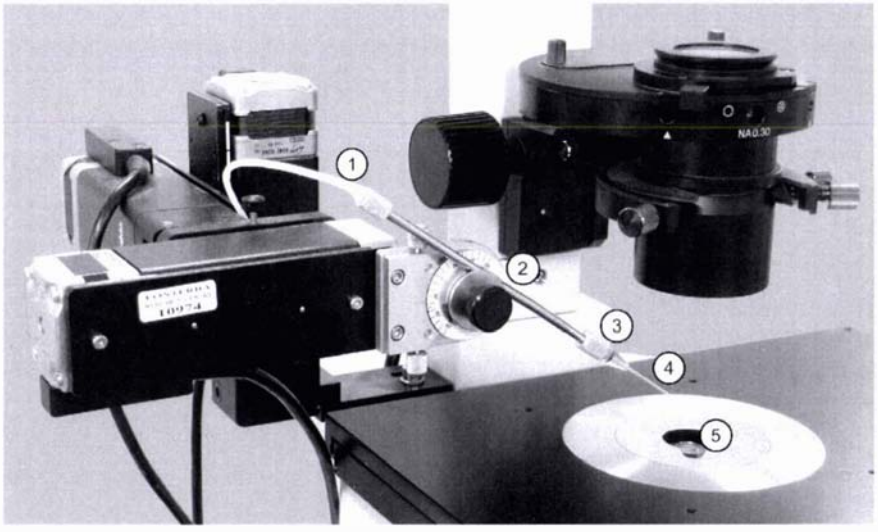
A micromanipulator (model 6540 R094, Eppendorf, Hamburg, Germany) was fitted to an inverted microscope (Olympus IX 70) and connected to a joystick (Eppendorf TransferMan NK). A colour video camera (model TK-C136OE, Victor Company of Japan Ltd., Yokohama, Japan) connected to the microscope allowed the microscope field of view to be seen and recorded in real time on a video screen/recorder. The glass capillary was connected to an oil-filled tube which in turn was connected to a device (CellTram® vario) that allowed manual regulation of the oil pressure. By varying the oil pressure, fluid from the sample could be sucked into, or expelled from, the capillary. The complete set up is shown in Figure 7.8. A close-up photo of the motorised device and capillary is shown in Figure 7.9.

17 For example, Clausen-Schaumann *et al.* (1998) used AFM to study the action of the lipolytic enzyme phosholipase A2 on supported phospholipid membranes after inducing local deformations by the AFM tip.



1 Video screen/recorder
2 Joystick
3 Anti-vibration pad
4 Inverted microscope
5 Video camera/lens
6 Motorised device
7 Celltram vario
8 Micromanupulator power/controller box

Figure 7.8 Photo of the complete set of equipment used to manipulate individual fat globules.



1 Oil tube
2 Graduated dial clamp
3 Capillary holder
4 Capillary
5 Microscope lens

Figure 7.9 Close-up photo of the motorised device which can be moved with micron precision in the *x*, *y*, and *z* planes. The capillary is connected to the capillary holder (the angle, relative to the microscope stage, of the latter can be adjusted manually by means of rotating the graduated dial clamp). The microscope lens is underneath the microscope stage.

7.2.3 Experimental techniques and results

Preparation of sample

Preliminary experiments showed that fat globules could not be harvested from undiluted raw milk as the tip of the capillary could not be seen when inserted into the sample. Hence, to allow the tip of the capillary to be seen when inserted in the sample, further experiments were conducted using washed fat globules which were obtained either by diluting washed bovine cream (5 µL) with milli-Q water (10 mL), or by using the serum of the 3rd wash obtained from the washing procedure described in Appendix 6. The reduced concentration of fat globules in these types of sample compared with that in milk was appropriate for harvesting purposes; if there are too many fat globules then it is very difficult to capture selected fat globules.

Capillary with a tip diameter of 4 µm

Using an Eppendorf capillary (∅ 4 µm), fat globules having a diameter < 4 µm could be harvested. Open the video file "4um Tip 2 Globules" (all video files are on the accompanying CD) to see an example of the harvesting of fat globules. In this video clip, first a small fat globule is harvested followed by a somewhat larger fat globule. The latter can be seen to be drawn towards the tip and finally is located at the opening of the tip. Note that only the very end of the tip is in focus. Small movements of the capillary (by operating the joy stick) often required refocusing the microscope (by operating the focus knob using the right hand) to ensure that the tip was in focus. If the tip was not in focus, it was very difficult to see the position of the target fat globule relative to the tip opening.

The transfer of harvested fat globules to another medium was shown to be possible by withdrawing the capillary from the sample, replacing the sample slide with a slide containing a drop of another medium (e.g., milli-Q water), manoeuvring the capillary into the drop of water and expelling the fat globules. Open file "4umTip Globule expel" to see fat globules being expelled into water (note the absence of fat globules in the medium). This video clip begins with showing the capillary tip already inserted into the water medium. A fat globule inside the capillary tip is first drawn backwards and then expelled and is followed by another fat globule.

However, the use of a capillary with a 4 µm tip diameter caused several practical problems.

- The tip was sensitive to significant vibration (this can be observed in the video clip "4um Tip 2 Globules"). The vibration was caused mainly by equipment in an adjacent room and was acceptable when the equipment in the adjacent room was not operating.

- The fat globules tended to rise to the top of the sample droplet. Hence, harvesting of fat globules became difficult after some time, because when the tip got too close to the surface the meniscus broke around the tip resulting in the tip being outside the sample.

- Most fat globules proved to be too large to be harvested with a 4 µm-diameter capillary tip opening. These large fat globules formed a cluster and blocked the tip opening preventing fat globules having diameters < 4 µm from being harvested. Open the video file "4um Tip Cluster" to see this happening. The first part of this clip shows the capillary (seen as a thin "shadow" extending from the left border) being moved towards, and then through, the surface of the sample droplet. Once the tip has penetrated the sample surface, capillary suction draws in sample fluid and consequently fat globules cluster together at the tip opening. A proportion, but not all, of the fat globules forming the cluster is then dispersed by expelling sample from the capillary. The remaining fat globules were tightly associated with the tip as neither expelling fluid from the capillary nor sudden movements of the capillary dislodged them.

- Sometimes, fat globules that were successfully sucked into the capillary strongly adhered to the outside of the tip when expelled into the receiving medium (milli-Q water). The adhesion forces were so strong that even rather abrupt movements of the capillary did not dislodge the globule from the tip.

The use of a capillary having a tip diameter > 4 µm was tested in an attempt to mitigate some of these practical problems.

Capillary with a tip diameter of 15 µm

An Eppendorf Transfer Tip-ES (∅ 15 µm) was purchased and fitted to the capillary holder. As only a very small proportion of bovine fat globules have a diameter > 10 µm (Mulder & Walstra, 1974) no blocking of the tip opening by fat globules was expected to occur.

Mainly because of the greater suction power, harvesting fat globules using the ∅ 15 µm tip proved to be easier compared with harvesting fat globules using a ∅ 4 µm tip. However, the use of the ∅ 15 µm tip created a greater drag in the fluid than did the ∅ 4 µm tip. This tendency to drag fat globules along when the tip moved was reduced by two actions: (1) by manoeuvring the tip at a slower pace through the sample, and (2) by manoeuvring the microscope stage (onto which the microscope slide containing the sample was placed) in the opposite direction of that of the tip. Hence, the selective harvesting of fat globules required the control of five different devices: (1) microscope stage; (2) microscope focus; (3) the CellTram® vario to regulate the oil pressure; (4) joystick to manoeuvre the capillary, and (5) video recorder which served as a visual "laboratory note book". Items 1–3 were operated using the right hand, whereas items 4 and 5 were operated using the left hand. Operating devices 1–4 was done "blind" as the effect of changing any of these needed to be observed while looking through the microscope eye piece. Because of the need to operate multiple devices, harvesting fat globules proved to be a slow process.

Examples of harvesting fat globules using the ∅ 15 µm tip are shown in the following video clips. In all clips, the capillary tip is already inserted into the sample when the clip starts. Only the very end of the tip is in focus.

"15umTip 2 Glob 1harvest"

Note the relatively large tip opening compared with the size of the fat globules elsewhere on the screen. The meniscus of the sample inside the tip can be seen just at the left of the tip opening (the dark area to the left of the meniscus is the air gap that separates the sample fluid from the CellTram® vario oil). To harvest fat globules, the oil pressure was reduced so that fluid was drawn into the tip. The oil pressure could be regulated very precisely; using the video clip timer at the bottom right of the window, observe that from 1–3 s the suction was very gentle, from 4–6 s, suction speed was somewhat increased, from 7–10 s suction was almost halted and at 11 s a sudden drop in oil pressure rapidly drew in sample fluid. All these pressure differences were controlled by manually rotating either the fine control dial or the coarse control dial of the CellTram® vario. The target fat globule was situated at about 10–15 µm above the tip opening and was first gently manoeuvred closer to the tip opening and subsequently drawn into the tip (at about 11 s video clip time).

Note that one fat globule was attached to the tip opening and remained there throughout the experiment, showing that for some fat globules the glass-fat globule adhesion forces were relatively strong as neither suction of fluid into the tip nor movement of the tip dislodged this fat globule. Note also that the tip was nearly free from vibration.

"15umTip 2 Glob 2 harvest"

The target fat globule was at about 10 µm to the right of the tip opening and was gently harvested at about 8 s video clip time. Note again that the fat globule attached to the tip opening remained in place during the harvesting of the target fat globule.

"15umTip 2 Glob 3 harvest"

This clip shows that fat globules can be selectively harvested even when other fat globules are in the vicinity. During the first 3 s the microscope focus was adjusted to ensure that the tip opening was in focus (observe that no fat globules were present inside the tip). The tip was subsequently manoeuvred backwards and upwards to position it for the target fat globule (positioned at about 5–10 µm to the right of the tip opening). The fat globule was then drawn into the tip. Note that a smaller fat globule that was further away from the tip moved inside the tip ahead of the target fat globule. However, as soon as the target fat globule (which can be seen fuzzily at about 15 µm to the left of the tip opening) was inside the tip, the oil pressure was stabilised so that other fat globules remained outside the tip. Note again that the fat globule attached to the tip opening remained in place during the process of harvesting the target fat

globule. Also, observe that moving the tip created fluid drag and that fat globules in the vicinity were dragged along. However, when the tip was moved away gently, the distance between the tip opening and the latter fat globules gradually increased, allowing another fat globule of choice to be harvested (not shown).

"15umTip 2 Glob 4 harvest"

This clip demonstrates that, after some practice, very fine control over the suction can be achieved. The targets were two fat globules positioned at about 7 µm to the right of the tip opening. At about 6 s video clip time, these fat globules were drawn towards the opening of the tip. Using very gentle suction the fat globules were separated from one another; one was drawn into the tip, whereas the other was left outside the tip. The tip was moved away slowly. Owing to the drag created by the moving tip the other fat globule followed the tip, but slowly the distance between it and the tip opening increased. Observe that, at the beginning of the clip, one could mistakenly have thought that three fat globules were already inside the capillary. However, as the tip was moved away, these fat globules were shown not to be inside, but outside of the capillary.

"15umTip 2 Glob 5 expel"

This clip starts with showing the capillary tip in a fat globule-free medium (milli-Q water). First, a single, previously harvested, fat globule is expelled, which is followed by a group of four fat globules.

7.2.4 Discussion

Practical aspects and limitations

Harvesting selected washed fat globules was shown to be possible using a micromanipulator. At the present stage of development, harvesting is a slow process mainly because the operator needs to handle five things manually at the same time (i.e., CellTram® vario to control the suction, the joystick for manoeuvring the capillary, the microscope stage, the microscope focus, and the video recorder). In particular, the manoeuvring of the capillary in three dimensions to target a particular fat globule, and the need for simultaneous adjustment of the microscope focus knob to keep either the capillary and/or the target fat globule in focus, resulted in a slow and fiddly harvesting process. Furthermore, the movement of the capillary in the sample resulted in significant fluid drag in the vicinity of the capillary tip. After a fat globule had been harvested, the capillary tip first needed to be moved away from unwanted fat globules in the vicinity of the tip before another fat globule could be targeted. This contributed to the time required for the harvesting of multiple fat globules.

Another limitation was that the inverted microscope, to which the micromanipulator was fitted, did not have a fluorescence capability. Hence, the study of fat globules beyond mere harvesting

was not pursued. Nevertheless, the potential of the technique was evident and should be explored in future work (see section below, "*Potential for future research*").

Potential for future research

Micromanipulators have proven to be useful instruments in the study of interfacial properties of emulsions. Phipps & Temple (1982) used a micromanipulator to measure the interfacial tension of individual fat globules, whereas Yeung *et al.* (2000) used a micromanipulator to study interfacial properties of micron-sized[18] water droplets in water-in-oil emulsions. The current study demonstrated that micromanipulators can also be used to harvest milk fat globules and transfer them into a different medium. This opens up new possibilities for systematic study of individual fat globules as they are affected by certain factors in their environment.

Capillaries with customised specifications can be purchased commercially. Examples of differently shaped capillary tips manufactured by Eppendorf are shown in Figure 7.10. The minimum inner diameter of the tips is 3.5 µm. The minimum length of the bevelled end is 200 µm, and the angle of the capillary can vary from 0–90°. Type 1 or Type 3 tips should be suitable for harvesting and transfer of fat globules. Type 2 tips should be suitable for holding a single fat globule in place at the end of the tip, whereas Type 4 tips should be suitable for controlled perturbation or damaging of the MFGM.

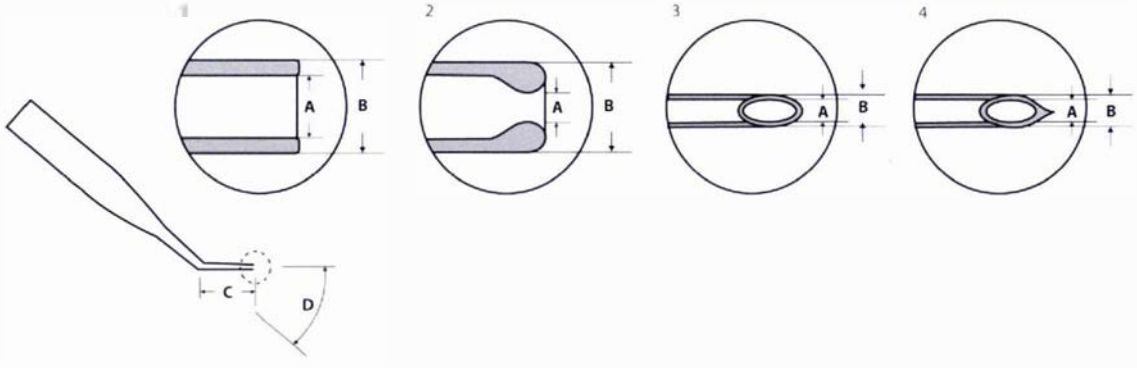


Figure 7.10 CustomTips® produced by Eppendorf. Dashed circle denotes inserts showing the different types of tip available. (1) Blunt end. (2) Holding pipette type. (3) Bevelled end. (4) Bevelled and heat-formed spike. A = inner diameter of the tip. B = outer diameter of the tip. C = Length of bevelled end. D = Angle of capillary. Illustrations courtesy of Eppendorf International, reproduced with permission.

Considering that movement of the capillary tip can be controlled at the micron scale, transfer of fat globules to a defined location on, for example, a grid should be possible. This opens up exciting possibilities for using micromanipulators in the study of properties of the MFGM of individual fat globules. Here, two experimental examples are presented that, although conceptual at the present time, should be achievable.

18 That is, similar in size to fat globules.

Scenario 1 — Precise depositing of fat globules

Using a capillary having an appropriate diameter for the intended experiment, individual fat globules could be harvested and deposited onto some retaining medium, e.g. a filter with pores of a certain size, or alternatively the fat globule could be pinioned on the tip of the capillary. In both cases, subsequent analysis by AFM might be possible in a manner similar to that for cells (Morris *et al.*, 1999a; Figure 7.11.)

Scenario 2 — Transfer of fat globules to a different medium

The harvesting technique shown in Figure 7.11A could also be used to transfer a fat globule, of which the MFGM is stained with a specific fluorescent membrane probe, to a different medium (e.g., containing a particular enzyme). Using CLSM, the distribution of the fluorescent probe in the membrane could then be studied as a function of time, temperature or other variables. Comparisons between fat globules of the same cow, or from milk of different species could be made, and thus an understanding of the differences in MFGM properties between different fat globules in the same milk or in different milks could be obtained.

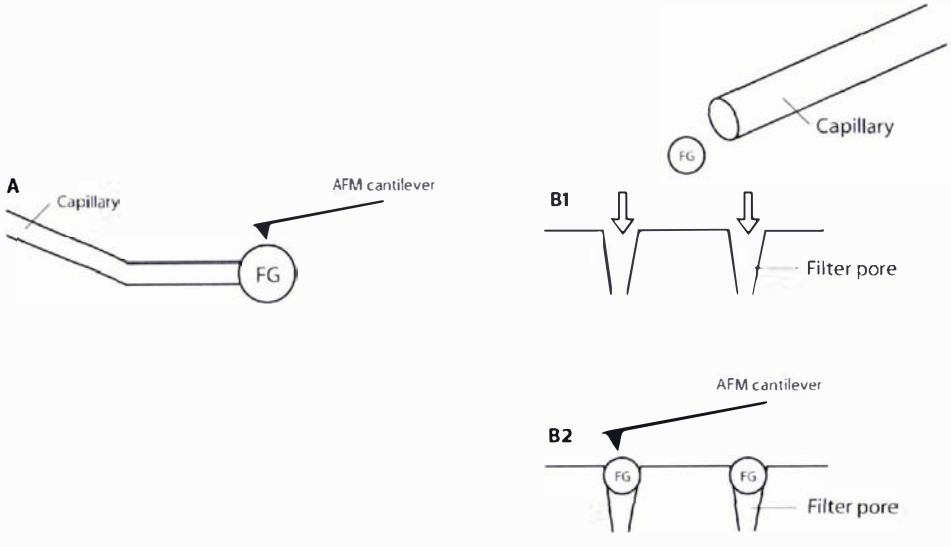


Figure 7.11 Use of the micromanipulator for precise location of individual fat globules. FG = fat globule. (A) Transfer of a fat globule pinioned on the capillary tip for subsequent assessment by e.g. AFM. (B) Depositing of fat globules onto a porous surface[19], such as a membrane shown schematically in B1 (hollow arrows denote suction), for subsequent assessment by e.g. AFM (shown in B2). Both (A) and (B) adapted from Morris *et al.* (1999a).

19 Real examples are shown in Touhami *et al.* (2003a) and Dufrêne (2004).

7.2.5 Conclusions

Washed fat globules can be harvested and transferred successfully to another medium using a micromanipulator. The development of this technique to manipulate individual fat globules opens up exciting new possibilities for studying the milk fat globule (membrane) that should be explored in future work.

7.3 Flow cytometry

7.3.1 Introduction

Flow cytometry is a well-established technique in the biological and medical fields, and is increasingly applied in food-related fields. Flow cytometry involves the counting, physical and/or chemical characterisation, and, in some instances, sorting of microscopic particles suspended in a stream of fluid. In the dairy sector, the best-known examples of the use of flow cytometry are those of rapid automated counting of somatic cells and bacteria in milk (Shapiro, 2003). The use of flow cytometry to count fat globules in milk has not been reported in the literature. In fact, analysis of cells in milk involves the destruction of components in the milk, including the fat globules. However, flow cytometry has been used successfully to study water-in-oil-in-water emulsions (Hai *et al.*, 2004).The aim of the current study was to conduct a pioneering investigation to determine whether in principle flow cytometry could be used for quantitative analysis of fat globules (essentially an oil-in-water emulsion). As existing flow cytometry protocols for milk could not be used as a basis to work from, a fat globule-specific protocol needed to be developed. This work was conducted in collaboration with, and in the laboratory of, an overseas manufacturer of flow cytometry instruments[20].

7.3.2 Principle of flow cytometry

Figure 7.12 shows a schematic diagram of the flow cytometer. The ability to count the number of particles in combination with information derived from the degree of forward scatter and side scatter of light from a laser source, as well as from the fluorescence (each particle can be stained with multiple probes), makes flow cytometry a powerful technique for quantitatively measuring distinct populations of particles within a sample.

In the current study, the fluorescence was collected in two channels, D3:FL1 (green) and D4:FL2 (red), and the relative intensities for each particle in each channel were plotted in a combined plot (Figure 7.13 and Figure 7.14). (The axes are divided into 1024 channels, which correspond to the fluorescence intensity of the measure particles).

20 This work was carried out under a confidentiality agreement between Fonterra and the manufacturer of the flow cytometer. This limits the description of some details of the work done.

7.3.3 Experimental and results

Selection of fluorescent probe

The available flow cytometer possessed a single laser only (488 nm). A fluorescent probe was required that was compatible with this flow cytometer. At the time this work was conducted, development work on fluorescent probes had not progressed to the point where WGA488 had been tested as a MFGM probe nor where $DiIC_{18}(3)$-DS had been identified as a suitable MFGM probe. Hence, DiA (ethanolic stock solution; 1 mg/mL) was selected as this probe had been shown to stain the MFGM (albeit of a small proportion of fat globules only).

Staining of milk involved the addition of DiA stock solution (10 µL) to milk, diluted milk or a suspension of washed fat globules (1 mL) and gently mixing the sample by inverting the sample tube.

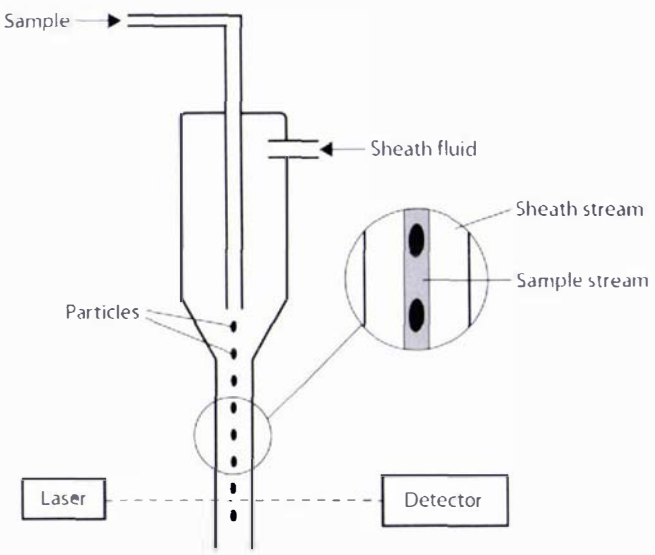


Figure 7.12 Schematic of a flow cytometer. The particles in the sample are hydrodynamically focussed into a core stream within a sheath fluid and pass a laser beam one by one. The laser beam excites fluorescent probes associated with the particles. The fluorescence and scattered light (forward scatter and side scatter) can be collected and quantified by appropriately placed detectors.

Development of an analysis protocol

Initial experiments, in which stained and unstained undiluted milk samples were presented to the flow cytometer, showed an increase in the number of particles being counted in the stained sample. However, the responses for the stained particles were located very close to those of the background particles of the milk (results not shown), and it proved to be difficult to separate the two groups of particle counts. A control experiment, in which a probe working solution (DiA stock solution diluted 100x with water) was analysed yielded a large signal[21]. This suggested

21 This was contrary to expectation as DiA is supposed to be non-fluorescent in an aqueous solution.

that the analysis of undiluted milk resulted in the contamination of the instrument, which in turn resulted in cross-contamination of successive analyses. To rule out the possibility that the DiA probe interacted with the surfactant in the sheath solution, thus yielding fluorescent particles, the probe stock was diluted with either water or the sheath solution. No significant difference in the signal between these two types of probe working solution was observed. Thus, DiA did not interact with the surfactant in the sheath and the conclusion that cross-contamination was caused by analysing undiluted milk was verified. Furthermore, the subsequent analysis of 10x diluted (aq) milk samples generally, but not always, avoided cross-contamination as evidenced by alternating the analysis of these diluted samples with dummy samples (cleaning fluid). For the latter, low counts were generally obtained.

To verify that the objects counted indeed represented fat globules, washed[22] fat globules were stained using Nile Blue. This yielded a distinct population of particles at a higher offset than that of the background (Figure 7.13) and confirmed that the instrument response represented stained fat globules that remained intact during the analysis.

Analysis of DiA-stained washed fat globules showed that the DiA signal was close to, but distinct from the background signal. A DiA "window" was constructed to count the signal from the DiA-stained fat globules (Figure 7.14, white area extending from the middle of the y-axis). However, the analysis of 10x diluted (aq) stained milk samples showed that the window parameters were not optimal as a portion of the particles fell outside the window boundaries (Figure 7.14). This approach needs to be optimised in future work.

7.3.4 Discussion

Cross-contamination

An investigative study was conducted to determine whether fat globules could be identified and counted using flow cytometry. Fat globules could not be measured in their native environment, because the use of undiluted milk samples resulted in cross-contamination of sequential sample runs. This was evident by alternating undiluted milk samples with dummy samples containing cleaning solution only; the dummy samples showed high background counts (i.e., unlabelled fat globules). The cross-contamination was presumably caused through fouling of the internal tubing of the flow cytometer. Although alternating samples with dummy samples consisting of cleaning solution might be considered a practical solution for the cross-contamination problem, the long term effects of extensive repeated fouling on the performance of the flow cytometer would be a concern. Hence, such a practice is not recommended.

22 The washing procedure used was very similar to that described in Appendix 6.

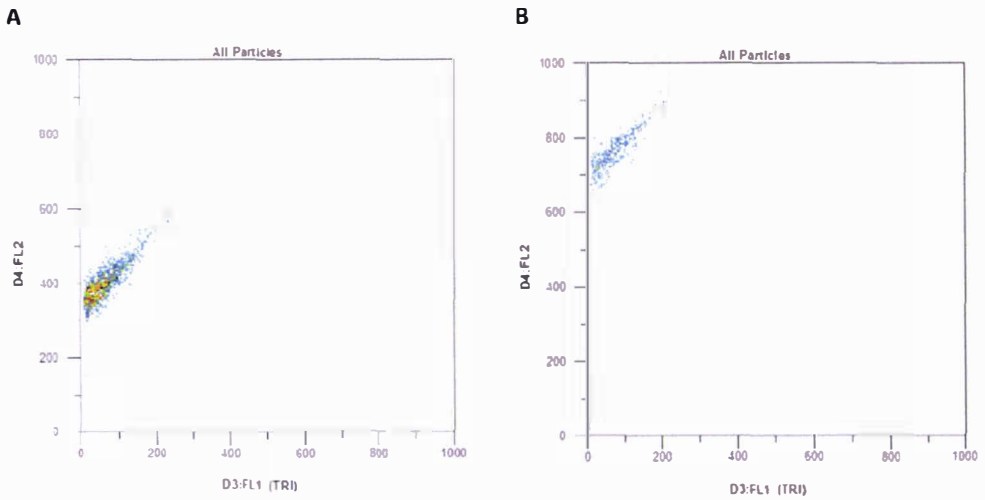


Figure 7.13 Flow cytometer plot of fluorescence as measured by the two detectors (D3:FL1 (green) and D4:FL2 (red)). (A) Unstained washed fat globules. (B) Washed fat globules stained with Nile Blue. Note the offset of the counts of the Nile Blue-stained fat globules relative to the counts for the unstained sample. This clearly shows that the counts for stained fat globules can be distinguished from background counts when suitable fluorescent probes are used.

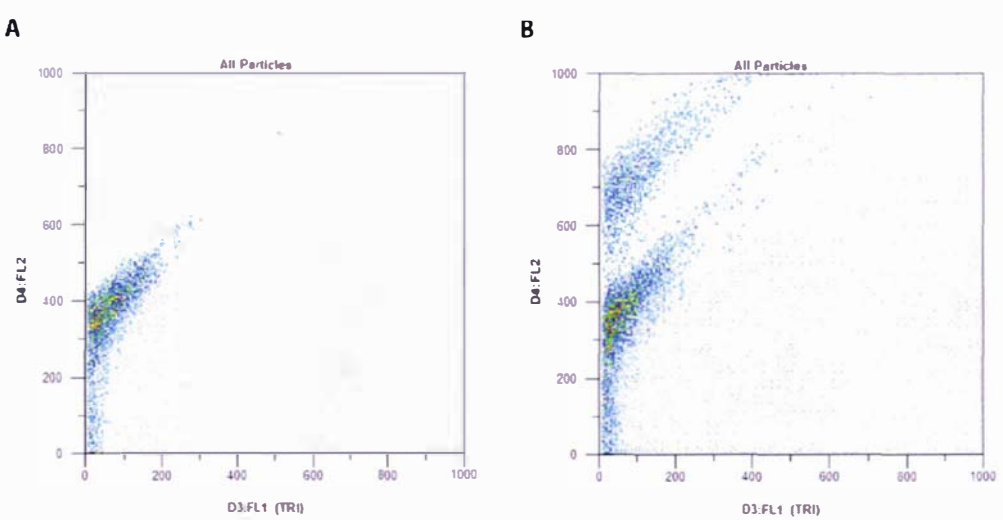


Figure 7.14 Flow cytometer plots of fluorescence as measured by the two detectors (D3:FL1 (green) and D4:FL2 (red)). (A) Background signal for unstained washed fat globules. An "inclusion" window, indicated by the light grey area, was constructed just above the background signal to count the stained particles. (B) 10x diluted milk stained with DiA. The lower boundary of the "inclusion" window proved to be correct as it successfully eliminated background particles from being counted. The other boundaries of the inclusion window were not optimal as a large proportion of stained fat globule counts were outside the upper boundary.

Diluting the milk 10x (aq) reduced, but did not completely eliminate, the cross-contamination problems. However, neither the use of greater than 10x dilutions, nor the effects of different dilution media (pH, ionic strength, etc) were tested. Future work should systematically study dilution conditions as it is envisaged that for the measurement of properties of fat globules more favourable dilution conditions might be found than a simple 10x (aq) dilution of the milk sample.

Flow cytometry can be used to analyse milk fat globules

Using the neutral fat dye Nile Blue as a fluorescent probe, it was shown that fat globules remain intact upon pumping of the sample through the flow cytometer internal tubing and flow cell. Thus fat globules can be counted by the flow cytometer as distinct particles.

Future work

Using DiA as a MFGM-specific probe showed that DiA-stained fat globules could be identified and counted using flow cytometry. As explained in Chapter 4, DiA stains only a small proportion of fat globules in the milk. Although not experimentally tested, it may be expected that other probes, such as $DiIC_{18}(3)$-DS and WGA, would be more effective than DiA for studying the MFGM by flow cytometry.

Milk contains many particles that potentially can interfere with the light emission of stained fat globules. Ideally, the background signal should be zero. However, in the current work, a significant background signal was observed (Figure 7.14). Future work should aim at reducing the background signal.

7.3.5 Conclusions

This study has shown for the first time that flow cytometry can be used to count milk fat globules. The potential of flow cytometry as a powerful *quantitative* technique for studying selected properties of fat globules and their membranes is evident. Further validation work is required to optimise staining and analysis protocols.

Acknowledgement

I thank "Company X" for allowing me the use of their laboratory facilities and for providing technical assistance during this study.

8 General discussion, conclusions and future work

Our understanding of the fat globule and its membrane has progressed significantly over the last 5 decades (for recent reviews refer to Heid & Keenan, 2005; Aoki, 2006; Bauman *et al.*, 2006; Keenan & Mather, 2006). Yet, significant gaps in our knowledge remain, particularly regarding the mechanisms controlling fat globule secretion by the mammary epithelial cell (Keenan & Mather, 2006). Furthermore, little is known about the mechanism and degree of change in MFGM composition and structure post secretion of the fat globule by the secretory cell (Chapter 1).

The ability to measure changes in the MFGM structure and composition is crucial, because of increasing interest in the MFGM for its nutritional significance (Ward *et al.*, 2006) and also its attributes as a functional ingredient in food applications (Rombaut & Dewettinck, 2006; Singh, 2006). In this context, the interaction of the MFGM with other components, whether native to milk or not, will increasingly become a subject for further investigation. Furthermore, as changes in the MFGM are implicated in practical problems encountered in the dairy industry (e.g., fouling of manufacturing equipment and off-flavour development in certain products), obtaining a fundamental understanding of the MFGM structure and how it changes on-farm and post farm upon milk harvesting, handling and processing (see Chapter 2) is paramount for the dairy industry. For the above-mentioned reasons, a range of different analytical techniques is required for studying the MFGM and its interactions with its environment in milk and milk products.

Over the years, several analytical techniques have been developed for studying some aspect of MFG(M) damage either directly or indirectly (Chapter 3). Only one of these techniques (light microscopy) allows studying the milk fat globule in its native environment. However, to date no light microscopy studies specifically dealing with the MFGM in its native environment have been published in the literature. It was postulated in this thesis that the use of fundamentally understood analytical methods is a necessary pre-requisite to advancing our knowledge of MFGM structure and the molecular arrangement of its components (Chapter 3). Ideally, these methods should allow the study of the MFGM in its native environment and in real time (Chapter 2). Hence, the focus of the current thesis was to develop, and test the potential of, a range of novel analytical techniques for studying the MFGM qualitatively and quantitatively. Future work is expected to build on the work described in this thesis to further develop and utilise these analytical techniques to advance our understanding of the MFGM. This final chapter reviews the developed techniques and makes recommendations for future work. In the previous chapters several interesting pieces of work were recommended for follow-up in future work. It is not intended to repeat these recommendations here (a summary is presented in Appendix 9), but rather to focus on additional possibilities, and particularly to outline the *directions* future work should take (i.e., the "big picture").

8.1 Fluorescence microscopy

8.1.1 Location of fluorescent probes in the MFGM

In the current study, both conventional fluorescence microscopy and CLSM techniques were developed for studying the MFGM using fluorescent probes that specifically associate with the MFGM (Chapter 4).

A range of fluorescent probes was demonstrated to reveal structural/compositional features of the MFGM (Chapter 4). The majority of these probes dissolved only in non-aqueous solvents, and were therefore not further investigated in the present study. However, some of these probes ($DiIC_{18}(3)$ and $DiOC_{18}(3)$) were found to locate partially or fully in the fat globule core. The distribution patterns of the probes in the core fat differed for the different probes; future work should try to elucidate why the probes distribute in their own peculiar patterns. This knowledge might then be applied to study certain properties of the core fat of individual fat globules as a function of certain fat globule characteristics (e.g., size).

Work in this thesis focused on the study of probes that were either somewhat soluble, or very soluble, in water and that stained the MFGM specifically. Hence, the lipophilic probes $DiIC_{18}(3)$-DS and FM4-64 and the lectin WGA were selected for further study (Chapters 5 and 6). As far is known, the use of lipophilic probes to study the structure of the MFGM has not been reported in the literature. Although WGA has been used before to study aspects of the MFGM[1], the current work describes novel applications of the lectin by applying Alexa Fluor® conjugates thereof either as a single probe or in combination with lipophilic MFGM-specific probes. Figure 8.1 illustrates the expected association of these probes with the MFGM.

$DiIC_{18}(3)$-DS is believed to orient the long axis of the fluorophore parallel to the membrane surface, whereas the two alkyl chains protrude perpendicularly into the lipid interior of phospholipid bilayers (Molecular Probes). Because $DiIC_{18}(3)$-DS is sulfonated it is more water-soluble than $DiIC_{18}(3)$, but also is known to distribute in phospholipid bilayers differently from $DiIC_{18}$ (Molecular Probes). In the current study, it was shown that $DiIC_{18}(3)$ indeed has different distribution characteristics to $DiIC_{18}$ as the former located solely in the MFGM, whereas the latter located partly in the fat globule core (Chapter 4).

FM4-64 is believed to insert only into the outer leaflet of surface membranes (Betz *et al.*, 1992; Fischer-Parton *et al.*, 2000). However, in goldfish retinal bipolar cells FM4-64 apparently slowly crossed the membrane (Rouze & Schwartz, 1998). It remains yet to be confirmed whether in MFGM FM4-64 stains the outer leaflet of the MFGM only.

WGA binds specifically to GlcNAc and NANA residues (Sharon & Lis, 2003) and thus is expected to locate in the glycocalyx by binding to relevant glycoproteins and glycolipids.

1 Either by electron microscopy or fluorescence microscopy.

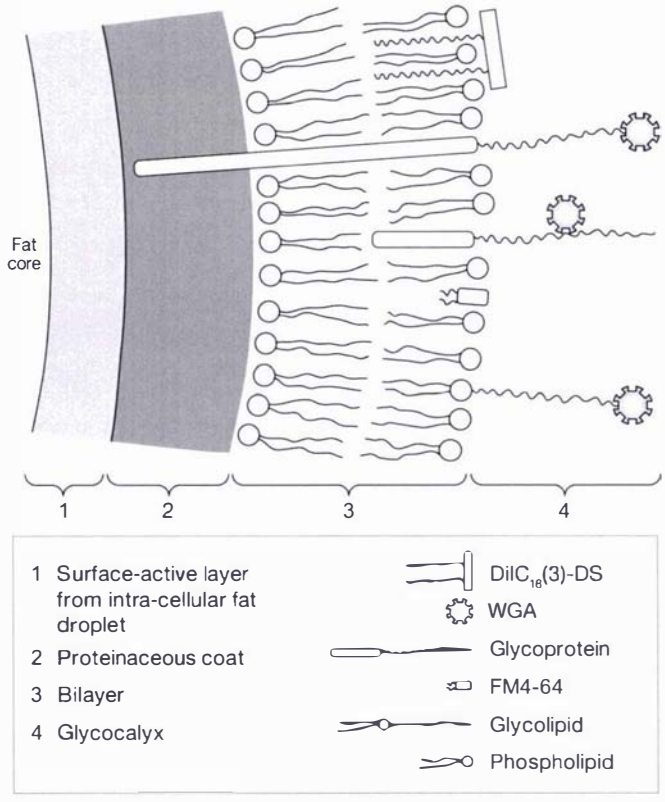


Figure 8.1 Orientation of $DiIC_{18}(3)$-DS, FM4-64 (adapted from, and used with permission by, Molecular Probes), and Alexa Fluor® conjugates of WGA on the MFGM. Highly schematic. Not to scale. Currently, there is no evidence for or against location of the lipophilic probes in the inner leaflet of the bilayer.

8.1.2 Potential of fluorescent probes

Untested probes

The theoretical assessment of the suitability of commercially available probes identified a range of fluorescent compounds that were considered to be potentially useful MFGM stains (Appendix 1). This list should not be considered exhaustive by any means as only the products of one manufacturer were considered. Other companies, such as Sigma-Aldrich[2] and Anaspec[3], may also market fluorescent probes that are suitable for studying the MFGM. In addition, as a greater understanding is obtained about the relationship between the molecular structure of the probe and its location in the fat globule (membrane), novel probes, combining the desired properties, could be synthesised and tested.

Furthermore, besides using generic fluorescent compounds, fluorescent antibodies that bind to selected MFGM compounds can reveal structural details of the MFGM (McManaman *et al.*, 2002; Robenek *et al.*, 2006). Although the latter type of probe was outside the scope of the

2 http://www.sigmaaldrich.com

3 http://www.anaspec.com

present study, evidently the use of a combination of generic and component-specific probes would constitute a very powerful technique for studying structural details of the MFGM. Hence, such an approach should be explored in future work.

DiIC$_{18}$(3)-DS

In contrast to its analogue DiIC$_{18}$(3), only a few literature reports on DiIC$_{18}$(3)-DS are available. DiIC$_{18}$(3)-DS has been used to stain bacterial membranes (Fuller *et al.*, 2000), vesicle membranes (Miller *et al.*, 2000; Saito *et al.*, 2004), ovine lymph cells (Seabrook *et al.*, 2000) and rat cells (Nilsson *et al.*, 2005). In none of these studies was the local distribution of DiIC$_{18}$(3)-DS in the membrane examined. As far as is known, the current study is the first in which DiIC$_{18}$(3)-DS has been applied to a food (i.e., milk) and in which the location of the probe has been studied in the membrane of a single object. DiIC$_{18}$(3)-DS has been shown to stain the MFGM specifically, the rate of uptake by the MFGM differing among different species. Hence, DiIC$_{18}$(3)-DS is considered to have high potential for use in future studies of the MFGM. Future work should:

1. Focus on improving staining protocols for DiIC$_{18}$(3)-DS for the MFGM of different species;

2. Investigate the suitability of DiIC$_{18}$(3)-DS analogues for staining the MFGM of different species. Possibly, the use of selected analogues of DiIC$_{18}$(3)-DS in which the length of the alkyl chains is less than C_{18} could be used advantageously to study particular properties of the MFGM (e.g., location of fluid or gel phases). Furthermore, the use of DiIC$_{18}$(3)-DS analogues which possess a longer double bond bridge between the aromatic rings, e.g. DiIC$_{18}$(5)-DS which fluoresces in the red region of the spectrum (Meers *et al.*, 2000), might overcome the current problem that DiIC$_{18}$(3)-DS cannot be combined with several other probes because of overlap of the fluorescence emission spectra.

FM4-64

FM4-64 belongs to a group of about 20 FM-analogues[4] modelled on the membrane-potential probe DASPMI, which was used to study mitochondria (Bolte *et al.*, 2004), and on the dicationic styryl probe RH414[5] (Betz *et al.*, 1992). The structures of these probes can be described as consisting of three parts (Betz *et al.*, 1996):

(1) The head (pyridinium group).
Can be neutral, monocationic or dicationic, and affects membrane penetration properties.

4 "FM" stands for Fei Mao, the name of the person who developed the FM probes from the related probe dimethylaminostyrylmethylpyridiniumiodine (DASPMI) (Bolte *et al.*, 2004).

5 The only difference between RH414 and FM4-64 is that RH414 has two double bonds in the bridge, whereas FM4-64 has three double bonds.

(2) The nucleus (the two aromatic rings and double-bond bridge).
Determines the spectral properties of the dye (increasing the number of double bonds results in longer excitation and emission wavelengths).

(3) The tail (dialkylaminophenyl group).
The length of the tail determines the lipophilic properties of the dye.

Evidently, there is plenty of scope for obtaining "tailor-made" styryl probes. Small differences in polarity of styryl probes can have a large effect on the rate of uptake and retention properties, and it is speculated that styryl probes other than FM4-64 might be useful MFGM markers. For example, in the current study, fluorescence of FM4-64-stained membranes tended to be relatively weak and prone to photobleaching (Table 4.3). However, the fluorescence intensity of styryl dyes in membranes increases with increasing length of the dialkyl tail of the molecule (Betz *et al.*, 1996; Cochilla *et al.*, 1999). The dialkyl chains in FM4-64 are very short (i.e., CH_2CH_3) and analogues having longer tails (up to C_{18}) have been synthesized (Betz *et al.*, 1996). Hence, the suitability of such analogues for staining the membranes of fat globules in milk of different species should be investigated in future work. Presumably, a compromise may have to be made between fluorescence intensity[6] and water solubility, as increasing the length of the dialkyl chains makes the probe more lipophilic (Betz *et al.*, 1996), i.e. less water-soluble. However, the property of water-solubility that some FM probes possess (Parton *et al.*, 2001) should not be sacrificed too readily in exchange for an increased fluorescence intensity in MFGM, as probe water-solubility is a major advantage for studying fat globules in their native environment.

Whether more than one FM probe can be tested in the same sample at the same time needs to be experimentally tested. In one study on frog nerve cells, FM4-64 and FM2-10[7] could be used effectively together (Richards *et al.*, 2000). However, in another study, the combined addition of FM4-64 and FM1-43[8] resulted in the quenching of FM1-43 by FM4-64 in the membrane of goldfish retinal bipolar cells (Rouze & Schwartz, 1998). The quenching observed in the latter study may be related to the use of a relatively high dye concentration (Richards *et al.*, 2000).

The distribution patterns in different types of membrane are not well understood. However, an interesting aspect of FM4-64 is that it appears to have higher affinity for membranes rich in basic phospholipids (Fishov & Woldringh, 1999). Hence, differences in fluorescence intensity across the MFGM of single fat globules, as observed in the current study (Figure 4.24-2B), may

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6 That is, fluorescence intensity when the probe is embedded in the membrane (in water, FM4-64 is only weakly fluorescent, which means that low background interference occurs).

7 The only difference between FM2-10 and FM4-64 is that FM2-10 has a one double-bond bridge, whereas FM4-64 has three double bonds.

8 FM1-43 has a one double-bond bridge between the aromatic rings and C_4H_9 dialkyl chains in the tail of the molecule, whereas FM4-64 has three double bonds and C_2H_5 dialkyl chains, respectively.

indicate the presence of MFGM domains differing in phospholipid composition. This speculation deserves further study in future work.

Finally, cellular membrane trafficking studies using FM probes have shown that these probes bind reversibly to the outer membrane; removing extracellular dye resulted in the disappearance of membrane fluorescence (Cochilla *et al.*, 1999; Parton *et al.*, 2001). In the current study, fat globules were studied while the milk plasma contained FM4-64. It is assumed that this ensured that FM4-64 stained the membranes of the fat globules present at least for the duration of the experiment. In terms of improving contrast between the stained MFGM and its background, there is little point in washing out the dye from the milk plasma, because the fluorescence quantum yield of FM dyes in water is very low compared with that in a membrane (ratio about 1:350; Brumback *et al.*, 2004). Provided that MFGMs are stained adequately, a good contrast between the stained fat globule and the surrounding milk plasma is obtained. However, it is recommended that the staining of the MFGM as a function of the concentration of FM4-64 in the milk plasma be studied systematically in future work.

WGA

WGA has previously been reported to bind to bovine MFGM (Horisberger *et al.*, 1977; Farrar *et al.*, 1980), human MFGM (Horisberger *et al.*, 1977; Farrar *et al.*, 1980; Buchheim *et al.*, 1988b; Welsch *et al.*, 1990), ovine MFGM (Welsch *et al.*, 1990) and equine MFGM (Welsch *et al.*, 1988; Welsch *et al.*, 1990). However, no investigation involved studying the fat globules in their native environment, or made use of CLSM. The results of the present study, in which WGA was shown to reveal glycosylation profiles of ovine, human and bovine MFGM[9], demonstrate that WGA conjugated with a suitable Alexa Fluor® fluorophore has a high potential as a fluorescent probe for studying the structure and composition of the MFGM. Moreover, the agglutination properties of WGA can be utilised to extract additional information in heat treatment studies. For example, when milk was stained with WGA, strands that disappeared upon heating were discovered. These structures are unreported in the literature. The significance of the strands is not known, but at least their presence in unheated milk indicates that certain glycosylated structural elements interact with the MFGM.

Future studies should expand the study of MFGM glycosylation profiles by using lectins other than WGA. In particular, the combined use of different lectins is expected to be a powerful morphological tool for studying the MFGM structure and composition.

Imaging techniques, instruments and preparation techniques

From the above it is clear that the study of the MFGM using fluorescent probes is only at an embryonic stage. There is plenty of scope for development and improvement, and it is expected

9 It may also be speculated that WGA also binds to a significant degree to MFGM of other species.

that major advances will be made in the years ahead. This section explores a range of possible developments relating to imaging techniques, instruments and preparation techniques.

In the present study, CLSM was used only in fluorescence mode. However, CLSM can also be used in reflectance mode (Figure 8.2). Although this option was not further explored in the current study, the combination of reflectance microscopy and fluorescence microscopy may offer further insights into MFGM characteristics.

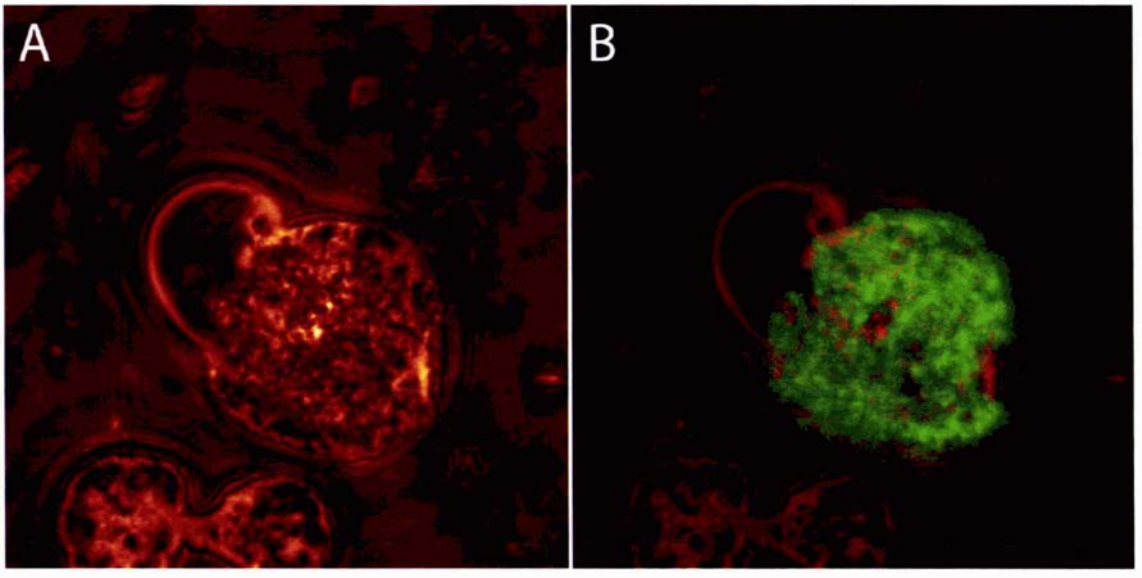

Figure 8.2 CLSM images showing two partially coalesced fat globules. (A) Reflectance image. (B) Combined reflectance and fluorescence image; green colour denotes staining by $DiOC_{18}(3)$. Comparison of the two images shows that $DiOC_{18}(3)$ is located only in that part of the coalesced fat globule that appears grainy in the reflectance image. Probably, the grainy appearance denotes the presence of fat crystals, which would indicate that $DiOC_{18}(3)$ preferentially associates with crystalline fat.

In the present study, good quality 3-D CLSM images could be obtained only of fat globules that were highly fluorescent. This limited the usefulness of the developed technique considerably, as interpretation of the 3-D results necessarily has to be restricted to the population of "highly fluorescent" fat globules in a sample. The ability to assess all stained objects in 3-D mode will require significant further development. On the other hand, 2-D CLSM images were easier to obtain as this mode of imaging did not suffer as much from adverse effects such as photobleaching of the specimen.

We can be optimistic about overcoming the limitations mentioned in the above paragraph. First, only basic sample preparation techniques were employed in the current study (i.e., simply adding the probe stock solution to the milk sample and incubating the stained sample under certain conditions). The use of anti-fades and fixatives, as well as the optimisation of probe concentration and sample incubation conditions, might result in improved sample preparation

techniques. Additionally, it may be anticipated that the use of quantum dots[10] may improve the fluorescence brightness of stained fat globules and solve the photobleaching problems encountered with 3-D imaging. Secondly, the CLSM used in the current study was an early model with limited capabilities. Newer CLSM instruments will allow a greater choice of probes, because emission spectra that differ by only a few nm, as opposed to e.g. > 50 nm, can be separated. Practically this means that limitations in combining certain probes, resulting from overlap of emission spectra as encountered in the present study, would be significantly reduced. Furthermore, other features of modern CLSM instruments should permit the study of the MFGM by using fluorescent probes to advance rapidly. Examples of such features include rapid image capturing, which means that fat globules might not have to be absolutely still before imaging can occur, automated time series experiments and 3-D multi-angle movie capability. To see an example of the latter feature open the file "*ShM 3% 6 WGA cav 63x zoom6_chan00.avi*" on the accompanying CD to see WGA488-stained ovine fat globules[11] (3-D stereo glasses are not required).

The advance of such fluorescent microscopy techniques may serve as intermediate steps for future developments. For example, there is still uncertainty about the contribution of secretory vesicle membrane or apical plasma membrane to the bilayer part of the MFGM (Keenan & Mather, 2006). Mather & Keenan (1998) suggested that in addition to developing lipid-excreting secretory cells in culture, the development of fluorescence video microscopy techniques could help resolve this issue. The fluorescence microscopy techniques described in this thesis may prove to be a crucial step on the way to achieving this resolution.

Dairy products other than liquid milk

The current work investigated fat globules in liquid milk only. However, there is much interest in using fluorescence microscopy for studying the microstructure of dairy products other than liquid milk, particularly whole milk powder and cheese (McKenna, 1997; Sutheerawattananonda *et al.*, 1997; Gunasekaran & Ding, 1999; Auty *et al.*, 2001; Guinee *et al.*, 2002; Everett & Olson, 2003; Rowney *et al.*, 2003; Michalski *et al.*, 2004a; Rowney *et al.*, 2003; Lopez, 2005; O'Mahony *et al.*, 2005; Lopez *et al.*, 2006; Ko & Gunasekaran, 2007; Lopez *et al.*, 2007; Michalski *et al.*, 2007; Ye *et al.*, 2007). Nevertheless, to date little, if any, morphological information has been forthcoming with regard to whether MFGM is still present in these products. Yet the importance of this information is evident from current discussions in the literature (e.g., refer to Michalski *et al.* (2007) for speculations on the organisation of the interface between the fat and surrounding cheese matrix, and to Ye *et al.* (2007) for speculations on the fat surface in whole milk powder).

10 Quantum dots are somewhat spherical nanocrystals of inorganic semiconductors with a typical diameter of 2–8 nm. Quantum dots can be synthesised to emit at any specific wavelength between 400–2000 nm, have very large molecular extinction coefficients, and are highly resistant to photobleaching (Smith & Nie, 2004).

11 Ovine milk was stained by adding WGA488 stock (3% v/v final concentration in the milk) and was imaged using a Leica TCS SPE DM2500 CLSM. Imaging and movie construction performed by Elizabeth Nickless.

Clearly, the application of fluorescence microscopy, using MFGM probes such as those identified in the present study, is just "waiting to be done".

8.1.3 Key conclusions

For the first time, 2-D and 3-D images have been made of the MFGM as stained by fluorescent probes. Fat globules have been morphologically studied in their native environment and in model systems by fluorescence microscopy using single probes or combinations of different probes (e.g., lipophilic and WGA). Variable distribution patterns of the probes in the MFGM were observed, which suggests that the MFGM of fat globules in harvested milk is structurally and chemically heterogeneous both within and among globules from the same species and between species. Furthermore, the use of WGA in bovine milk and model systems implicated β-lactoglobulin, α-lactalbumin, IgM and/or the glycosylated proteins PAS 6/7 in the disappearance of fat globule aggregation upon elevated heat treatment of milk.

Despite the significant developments described in this thesis, these new fluorescence microscopy techniques must be considered as being in an "embryonic stage". Hence, further development of these techniques promises huge potential for advancing the study of MFGM of different species either in their native environment, in dairy products or in model systems.

8.2 Other techniques

It was concluded in the literature review of methodology used for measuring changes to milk fat globules and/or their membranes (Chapter 3) that, "The fact that knowledge of the MFGM structure, and particularly the molecular arrangement of the components in the MFGM, is still in its infancy is a major inhibiting factor in the development of fundamentally understood methods that can be used in a routine fashion in practice. Hence, increasing knowledge of the molecular arrangement of MFGM constituents and the membrane's general structure would appear to be key objectives of future research". The work reported in this thesis is a step on the way towards that aim. First, fluorescence microscopy methods have been developed that have advanced, and in the future will further advance, our knowledge of the composition and structure of the MFGM. Secondly, the potential of a range of other techniques (AFM, micromanipulation, flow cytometry and NMR) was investigated. The first three techniques are believed to have potential in MFGM research.

8.2.1 AFM

Although AFM development work did not progress to the point where a successful technique was established (Chapter 7), the potential and value of AFM as an analytical tool for studying the molecular arrangement of the MFGM is evident. Nanomanipulation of membrane domains, or indeed of individual molecules, and assessment of inter- and intramolecular forces are just some of the possibilities (Fotiadis *et al.*, 2002). The main problems that will have to be overcome include reducing the variations in the height that the AFM tip has to negotiate,

optimising scanning parameters (in air, but particularly under liquid), and ensuring sufficient specimen rigidity to prevent the tip from damaging the specimen[12]. The use of a porous membrane to partially lower fat globules below the scanning surface (Figure 7.13), and a controlled low room temperature would be two obvious approaches to test.

More advanced applications of the AFM technique would include determining lectin-MFGM glycoprotein binding forces using functionalised AFM tips such as those developed by Touhami *et al.* (2003a) who employed carbohydrate-terminated tips. Specific carbohydrate AFM probes could thus potentially be used to map lectin receptors on the membrane (Touhami *et al.*, 2003a).

Alternatively, AFM could be used to measure differences in physical properties of different domains on single fat globules, similarly to studies on microbial cells (the size of which were similar to the average size of fat globules (3–4µm); Touhami *et al.*, 2003b; Dufrêne, 2004).

In light of the above opportunities for studying the nanostructure of the MFGM, it is strongly recommended that future research resources are devoted to AFM method development work.

8.2.2 Micromanipulator

In the current work, a micromanipulation technique was successfully developed to the stage where individual fat globules could be harvested and transferred to a new medium (Chapter 7). However, neither of the two capillaries used (∅ 4 µm or 15 µm) were optimal for harvesting fat globules. Future work should investigate systematically the effects of different tip specifications (i.e., angle of the bevelled end, internal diameter and tip shape; modified surface to avoid fat globules sticking to the tip) and develop optimised harvesting techniques. Furthermore, the capability and usefulness of the micromanipulator should be expanded by combining its use with fluorescence microscopy and AFM. It is envisaged that this will enable the researcher to harvest selectively certain fat globules and to investigate aspects of the molecular arrangement of the components of membranes of such fat globules, as well as to study the general MFGM structure of individual fat globules of different sizes.

8.2.3 Flow cytometry

The work on fluorescent MFGM membrane probes (Chapter 4) provided the basis for developing a flow cytometry method for counting stained fat globules. A successful flow cytometry method is a key component of the analytical toolbox for measuring changes to the MFGM, because it is the only technique that has the potential to *rapidly* and *quantitatively* measure certain properties of all the individual fat globules in a sample (particles can be counted at the extremely fast rate of up to 40,000 events per second; Hai *et al.*, 2004). The current work showed that stained fat globules can be detected as discrete stained particles by

12 A technique using the fluorescent styryl dye FM1-43 may be used to determine the extent to which membrane components adhere to the AFM tip and cantilever after scanning of the specimen (Schaus & Henderson, 1997).

flow cytometry (Chapter 7). As the instrument available was only a "basic" instrument (only one laser; fixed filters; no sorting capability), and as an inferior MFGM probe was used (it stained a small proportion of fat globules only), it is evident that there is a lot of scope for improvement in the flow cytometry technique. Flow cytometers that have as many as four lasers, and that can sort and collect subpopulations of stained particles, are commercially available. Such instruments should allow the development of advanced counting and sorting protocols for fat globules, particularly when the fat globules are stained by multiple probes. It should be noted that the purchase of an advanced flow cytometer would constitute a major capital investment and would require skilled personnel. Hence, as for NMR spectroscopy (see Appendix 8), it is likely that advanced flow cytometers would be used in a research environment only.

The application of flow cytometry methods to lectin-stained cells (including WGA) (Gallagher *et al.*, 1982; Kremp & Anderson, 2004; Franz *et al.*, 2006), or FM4-64-stained cells (Wendland *et al.*, 1996), has been reported. Hence, one can envisage that flow cytometry analysis protocols will be developed that sort fat globules on the basis of the degree of staining of their membranes. In turn, this should allow the ratio of polar lipids to neutral lipids to be determined. This parameter could then be used as an indicator of fat globule damage upon milk handling and treatment.

Furthermore, lately there has been increasing interest in determining differences between fat globules of certain size classes (Briard *et al.*, 2003; Michalski, 2004; Michalski *et al.*, 2004, 2005a; Fauquant *et al.*, 2005;) and manipulating the properties of some dairy products (notably cheese) by using fat globules of such size classes (Michalski *et al.*, 2003, 2004a, 2006, 2007). In these studies, the fat globules were divided into relatively broad size classes. In contrast, flow cytometry should allow the study of the relationship, if any, between the *actual* fat globule size and the related MFGM properties (or, alternatively, core fat properties), as revealed by fluorescent probe staining. It is entirely feasible that such a study might identify different populations of milk fat globules within a milk sample of an individual animal, or between milk samples obtained from different animals. Such fractions could then be collected by using the sorting capability of the instrument and be subjected to further analysis.

The above examples illustrate the value of flow cytometry methods in the study of changes in the MFGM post secretion of the fat globules by the secretory cell, and particularly post harvesting of the milk.

8.3 Final conclusions and future work

This thesis has shown that the development of novel analytical techniques has resulted in new insights into the composition and structure of the MFGM. Further development and utilisation of these techniques as summarised in Figure 8.3 are certain to give a great impetus to MFGM research efforts and will advance our understanding of the MFGM. Undoubtedly, MFGM research will prove to be a very exciting research field in the years ahead.

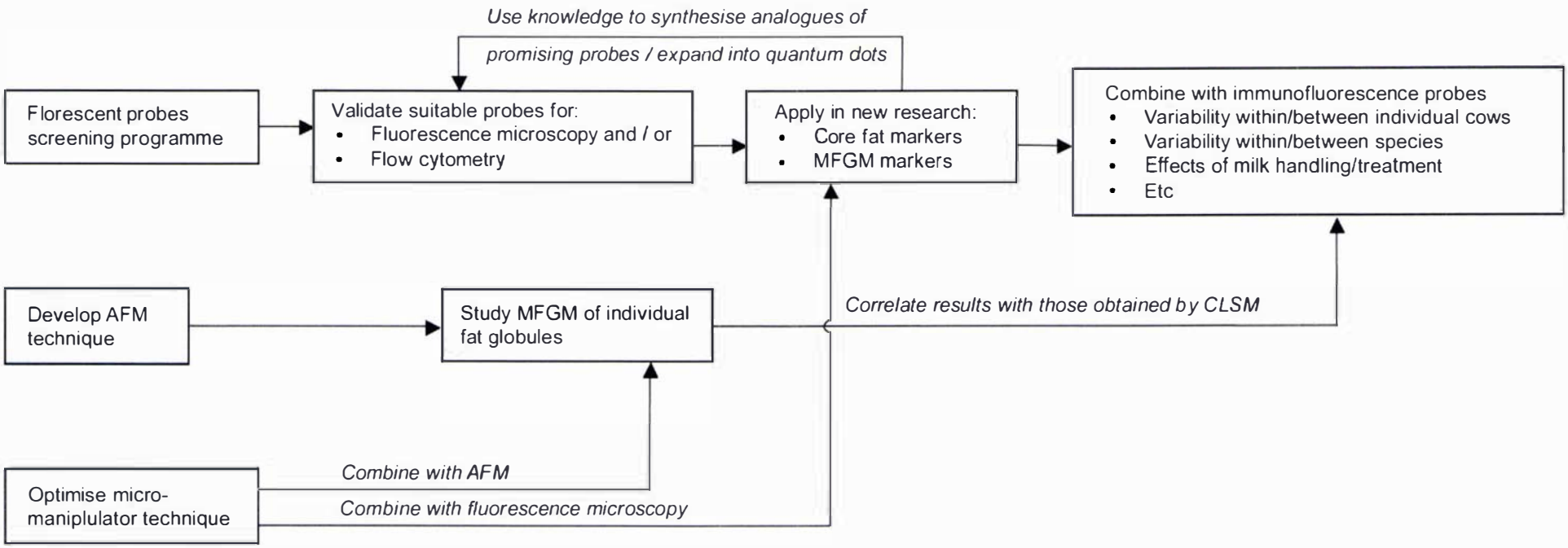

Figure 8.3 Outline of proposed future work.

Bibliography

Allen, J.C. & Humphries, C. (1977) The oxidation of lipids by components of bovine milk-fat globule membrane. *Journal of Dairy Research* 44(3), 495–507.

Anderson, M. (1983) Milk lipase and off-flavour development. *Journal of the Society of Dairy Technology* 36(1), 3–7.

Anderson, M. & Brooker, B.E. (1975) Loss of material during the isolation of milk fat globule membrane. *Journal of Dairy Science* 58(10), 1442–1448.

Anderson, M. & Cawston, T.E. (1975) Reviews of the progress of dairy science. The milk-fat globule membrane. *Journal of Dairy Research* 42(3), 459–483.

Anderson, M. & Cheeseman, G.C. (1975) Stability of the fat globule membrane. In *Proceedings of the lipolysis symposium, IDF Bulletin No. 86*, pp. 11–18. Brussels: International Dairy Federation.

Anderson, M., Cheeseman, G.C., Knight, D.J. & Shipe, W.F. (1972) The effect of ageing cooled milk on the composition of the fat globule membrane. *Journal of Dairy Research* 39, 95–105.

Andrews, A.T. (1992) Indigenous enzymes in milk - General introduction. In *Advanced dairy chemistry,Volume 1: Proteins*, pp. 286–292. Edited by P.F. Fox. London: Chapman & Hall.

Aoki, N. (2006) Regulation and functional relevance of milk fat globules and their components in the mammary gland. *Bioscience, Biotechnology, and Biochemistry* 70(9), 2019–2027.

Aristova, V.P., Serebennikova, V.A. & Radaeva, I.A. (1974) Method for separating destabilized fat from liquid milk products. *Molochnaya Promyshlennost* 6, 17–18.

Arnaudov, L.N., de Vries, R., Ippel, H. & van Mierlo, C.P.M. (2003) Multiple steps during the formation of β–lactoglobulin fibrils. *Biomacromolecules* 4(6), 1614–1622.

Asker, A.A., Hamzawi, L.F., Hagrass, A.E. & Abd-El-Hamid, L.B. (1978) Studies on buffaloe's milk fat globule membrane. II. Seasonal variations. *Egyptian Journal of Dairy Science* 6(1), 63–67.

Atkinson, S.A. & Lönnerdal, B. (1995) Nitrogenous components of milk. A. Human milk proteins. In *Handbook of milk composition*, pp. 351–387. Edited by R.G. Jensen. San Diego: Academic Press.

Aule, O. & Worstorff, H. (1975) Influence of mechanical treatment of milk on quantities of FFA and free fat in the milk, as well as on the separability of the milk. In *Proceedings of the lipolysis symposium, IDF Bulletin No. 86*, pp. 116–124. Brussels: International Dairy Federation.

Auty, M.A.E., Twomey, M., Guinee, T.P. & Mulvihill, D.M. (2001) Development and application of confocal scanning laser microscopy methods for studying the distribution of fat and protein in selected dairy products. *Journal of Dairy Research* 68, 417–427.

Bandyopadhyay, A.K. & Ganguli, N.C. (1975) Effect of heating and chilling buffalo milk on the properties of fat globule membrane proteins. *Journal of Food Science and Technology* 12, 312–315.

Banghart, L.R., Clayton, C.W., Velarde, J., Korobko, I.V., Ogg, S.L., Jack, L.J.W., Vakharia, V.N. & Mather, I.H. (1998) Butyrophilin is expressed in mammary epithelial cells from a single-sized messenger RNA as a type I membrane glycoprotein. *Journal of Biological Chemistry* 273(7), 4171–4179.

Bauer, H. (1972) Ultrastructural observations on the milk fat globule envelope of cow's milk. *Journal of Dairy Science* 55, 1375–1387.

Bauman, D.E., Mather, I.H., Wall, R.J. & Lock, A.L. (2006) Major advances associated with the biosynthesis of milk. *Journal of Dairy Science* 89(4), 1235–1243.

Baumrucker, C.R. & Keenan, T.W. (1973) Membranes of mammary gland. VII. Stability of milkfat globule membrane in secreted milk. *Journal of Dairy Science* 56(8), 1092–1094.

Becker, J.W., Cunningham, B.A., Reeke Jr., G.N., Wang, J.L. & Edelman, G.M. (1976) The molecular structure of Concanavalin A. In *Concanavalin A as a tool*, pp. 33–54. Edited by H. Bittiger & H.P. Schnebli. London: John Wiley & Sons.

Berlin, W.K. & Hanover, J.A. (1993) Characterization of nuclear and cytoplasmic glycoproteins. In *Glycobiology — A practical approach*, pp. 329–347. Edited by M. Fukuda & A. Kobata. New York: Oxford University Press.

Betz, W.J., Mao, F. & Bewick, G.S. (1992) Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *Journal of Neuroscience* 12(2), 363–375.

Betz, W.J., Mao, F. & Smith, C.B. (1996) Imaging exocytosis and endocytosis. *Current Opinion in Neurobiology* 6, 365–371.

Bhavadasan, M.K. & Ganguli, N.C. (1976) Dependance of enzyme activities associated with milk fat globule membrane on the procedure used for membrane isolation. *Indian Journal of Biochemistry and Biophysics* 13(3), 252–254.

Bhavadasan, M.K. & Ganguli, N.C. (1977) Physico-chemical properties of milk fat globule membrane proteins: dependence on the procedure of isolation. *Indian Journal of Dairy Science* 30(4), 297–303.

Bitman, J. & Wood, D.L. (1990) Changes in milk fat phospholipids during lactation. *Journal of Dairy Science* 73, 1208–1216.

Boldyrev, I.A., Zhai, X., Momsen, M.M., Brockman, H.L., Brown, R.E. & Molotkovsky, J.G. (2007) New BODIPY lipid probes for fluorescence studies of membranes. *Journal of Lipid Research* 48(7), 1518–1532.

Bolte, S., Talbot, C., Boutte, Y., Catrice, O., Read, N.D. & Satiat-Jeunemaitre, B. (2004) FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. *Journal of Microscopy* 214, 159–173.

Brew, K. & Grobler, J.A. (1992) α-Lactalbumin. In *Advanced dairy chemistry, Volume 1: Proteins*, pp. 191–229. Edited by P.F. Fox. London: Chapman & Hall.

Briard, V., Leconte, N., Michel, F. & Michalski, M-C. (2003) The fatty acid composition of small and large naturally occurring milk fat globules. *European Journal of Lipid Science and Technology* 105, 677–682.

Brumback, A.C., Lieber, J.L., Angleson, J.K. & Betz, W.J. (2004) Using FM1-43 to study neuropeptide granule dynamics and exocytosis. *Methods* 33, 287–294.

Buchheim, W. (1982) Paracrystalline arrays of milk fat globule membrane-associated proteins as revealed by freeze-fracture. *Naturwissenschaften* 69, 505–507.

Buchheim, W. (1986) Membranes of milk fat globules - ultrastructural, biochemical and technological aspects. *Kieler Milchwirtschaftliche Forschungsberichte* 38, 227–246.

Buchheim, W., Welsch, U., Huston, G.E. & Patton, S. (1988a) Glycoprotein filament removal from human milk fat globules by heat treatment. *Pediatrics* 81(1), 141–146.

Buchheim, W., Welsch, U. & Patton, S. (1988b) Electron microscopy and carbohydrate histochemistry of the human milk fat globule membrane. In *Biology of human milk. Nestle nutrition workshop series*, Vol. 15, pp. 27–44. Edited by L.Å. Hansen. New York, USA: Raven Press Ltd.

Caroll, R.J., Brower, D.P. & Thompson, M.P. (1972) Morphology of fat globule by electron microscopy. *Journal of Dairy Science* 55(5), 661–662.

Chandan, R.C., Cullen, J. & Chapman, D. (1972) Physicochemical analysis of the bovine milk fat globule membrane. III. Proton magnetic resonance spectroscopy. *Journal of Dairy Science* 55, 1232–1236.

Clausen-Schaumann, H., Grandbois, M. & Gaub, H.E. (1998) Enzyme-assisted nanoscale lithography in lipid membranes. *Advanced Materials* 10(22), 949–952.

Cochilla, A.J., Angleson, J.K. & Betz, W.J. (1999) Monitoring secretory membrane with FM1-43 fluorescence. *Annual Review of Neuroscience* 22(1), 1–10.

Collomb, M. & Spahni, M. (1995) Revue des méthodes de dosages des acides gras libres dans le lait et les produits laitiers. *Lebensmittel Wissenschaft und Technologie* 28, 355–379.

Corredig, M. & Dalgleish, D.G. (1996) Effect of different heat treatments on the strong binding interactions between whey proteins and milk fat globules in whole milk. *Journal of Dairy Research* 63, 441–449.

Corredig, M. & Dalgleish, D.G. (1998) Effect of heating of cream on the properties of milk fat globule membrane isolates. *Journal of Agricultural and Food Chemistry* 46(7), 2533–2540.

Dalgleish, D.G. & Banks, J.M. (1991) The formation of complexes between serum proteins and fat globules during heating of whole milk. *Milchwissenschaft* 46(2), 75–78.

Danthine, S., Blecker, C., Paquot, M., Innocente, N. & Deroanne, C. (2000) Évolution des connaissances sur la membrane du globule gras du lait: synthèse bibiliographique. *Lait* 80(2), 209–222.

Darling, D.F. & Butcher, D.W. (1978) Milk-fat globule membrane in homogenized cream. *Journal of Dairy Research* 45(2), 197–208.

de Boer, R. & Nooy, P.F.C. (1980) Concentration of raw milk by reverse osmosis and its influence on fat globules. *Desalination* 35, 201–211.

Debray, H., Decout, D., Strecker, G., Spik, G. & Montreuil, J. (1981) Specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins. *European Journal of Biochemistry* 117, 41–55.

Deeth, H.C. (1997) The role of phospholipids in the stability of milk fat globules. *Australian Journal of Dairy Technology* 52, 44–46.

Deeth, H.C. & Fitz-Gerald, C.H. (1975) Factors governing the susceptibility of milk to spontaneous lipolysis. In *Proceedings of the lipolysis symposium, IDF Bulletin No. 86*, pp. 24–34. Brussels: International Dairy Federation.

Deeth, H.C. & Fitz-Gerald, C.H. (1976) Lipolysis in dairy products: a review. *Australian Journal of Dairy Technology* 31, 53–64.

Deeth, H.C. & Fitz-Gerald, C.H. (1977) Some factors involved in milk lipase activation by agitation. *Journal of Dairy Research* 44, 569–583.

Deeth, H.C. & Fitz-Gerald, C.H. (1978) Effects of mechanical agitation of raw milk on the milk-fat globule in relation to the level of induced lipolysis. *Journal of Dairy Research* 45, 373–380.

Deeth, H.C. & Fitz-Gerald, C.H. (1995) Lipolytic enzymes in milk and milk products. In *Advanced dairy chemistry, Vol. 2: Lipids*, pp. 247–308. Edited by P.F. Fox. London: Chapman & Hall.

Dillier-Zulauf, A. & Wirasekara, P.R. (1971) Zur butyrometrischen Bestimmung des "freien" Fettes in Milch und Rahm. *Deutsche Molkerei-Zeitung* 92(46), 1943–1944.

Downey, M.C., Ma, C.H., Trinh, T.K., McCarthy, O.J., Fee, C., Weeks, M. & Prankerd, B. (2002) The use of particle size distribution data in the assessment of milk fat globule membrane damage. In *9th Asian Pacific Confederation of Chemical Engineering (APCChe) congress and CHEMECA 2002*, pp. Paper #367, 8 pp. Christchurch, New Zealand.

Du, H., Fuh, R.A., Li, J., Corkan, A. & Lindsey, J.S. (1998) PhotochemCAD: A computer-aided design and research tool in photochemistry. *Photochemistry and Photobiology* 68, 141–142.

Dubert-Ferrandon, A., Niranjan, K. & Grandison, A.S. (2006) A novel technique for differentiation of proteins in the development of acid gel structure from control and heat treated milk using confocal scanning laser microscopy. *Journal of Dairy Research* 73(4), 423–430.

Dufrêne, Y.F. & Lee, G.U. (2000) Advances in the characterization of supported lipid films with the atomic force microscope. *Biochimica et Biophysica Acta* 1509, 14–41.

Dufrêne, Y.F. (2004) Using nanotechniques to explore microbial surfaces. *Nature Reviews Microbiology* 2, 451–460.

Elofsson, C., Dejmek, P., Paulsson, M. & Burling, H. (1997) Atomic force microscopy studies on whey proteins. *International Dairy Journal* 7, 813–819.

Euber, J.R. & Brunner, J.R. (1984) Reexamination of fat globule clustering and creaming in cow milk. *Journal of Dairy Science* 67, 2821–2832.

Everett, D.W. & Olson, N.F. (2003) Free oil and rheology of cheddar cheese containing fat globules stabilized with different proteins. *Journal of Dairy Science* 86, 755–763.

Evers, J.M. (2003) Determination of free fatty acids in milk using the BDI method-some practical and theoretical aspects. *International Dairy Journal* 13, 111–121.

Evers, J.M. (2004a) The milkfat globule membrane--compositional and structural changes post secretion by the mammary secretory cell. *International Dairy Journal* 14(8), 661–674.

Evers, J.M. (2004b) The milkfat globule membrane—methodologies for measuring milkfat globule (membrane) damage. *International Dairy Journal* 14(9), 747–760.

Evers, J.M., Crawford, R.A., Robinson, N.P., Harding, D.R.K. & McCarthy, O.J. (2001a) "Free fat" solvent extraction methods damage milkfat globules. *Milchwissenschaft* 56(9), 504–508.

Evers, J.M. & Hughes, C.G. (2002) Analysis of Milk and Milk Products: Chemical Analysis. In *Encyclopedia of Dairy Sciences*, Vol. 1, pp. 34–40. Edited by H. Roginski, J.W. Fuquay & P.F. Fox. Amsterdam: Academic Press.

Evers, J.M. & Palfreyman, K.R. (2001) Free fatty acid levels in New Zealand raw milk. *Australian Journal of Dairy Technology* 56(3), 198–201.

Evers, J.M., Palfreyman, K.R., Russell, R.W. & Mabey, B.G. (2001b) Impact of equipment design and farm management practices on FFA levels in raw milk. *Australian Journal of Dairy Technology* 56(2), 131.

Fang, L. (1998) The effect of milk fat globule membrane damage in the absence of air on fouling in heat exchangers. MTech Thesis, Palmerston North: Massey University.

Farrar, G.H., Harrison, R. & Mohanna, N.A. (1980) Comparison of lectin receptors on the surface of human and bovine milk fat globule membranes. *Comparative Biochemistry and Physiology* 67B, 265–270.

Fauquant, C., Briard, V., Leconte, N. & Michalski, M-C. (2005) Differently sized native milk fat globules separated by microfiltration: fatty acid composition of the milk fat globule membrane and triglyceride core. *European Journal of Lipid Science and Technology* 107(2), 80–86.

Fink, A. (1986) Characterisierung technologisch bedingter Veränderungen der Emulsionstabilität von Rahm. *Deutsche Milchwirtschaft* 33, 1014–1016.

Fink, A. & Kessler, H.G. (1983) Determination of free fat in cream for the assessment of damage caused to fat globules by processing. *Milchwissenschaft* 38, 330–334.

Fink, A. & Kessler, H.G. (1985a) Changes in the fat globule membrane produced by heating. *Milchwissenschaft* 40, 261–264.

Fink, A. & Kessler, H.G. (1985b) The effect of heating on the storage stability of unhomogenized cream (30%). *Milchwissenschaft* 40, 326–328.

Fink, A. & Kessler, H.G. (1985c) The effect of heating on the state of the emulsion in homogenized cream. *Milchwissenschaft* 40, 394–397.

Fink, A. & Kessler, H.G. (1986a) Freifett-Bestimmung in Rahm zur Beurteilung technologisch bedingter Veränderungen der Fettkugelmembran. Teil 1: Zentrifugationsmethode. *Deutsche Milchwirtschaft* 10, 256–258.

Fink, A. & Kessler, H.G. (1986b) Freifett-Bestimmung in Rahm zur Beurteilung technologisch bedingter Veränderungen der Fettkugelmembran. Teil 2: Extractionsmethode. *Deutsche Milchwirtschaft* 11, 296–300.

Fink, A. & Kessler, H.G. (1986c) Veränderung der Fettkugelmembranstabilität bei der Erhitzung von unhomogenisierten Rahm. *Die Molkerei-Zeitung Welt der Milch* 40, 529–532.

Fink, A. & Kessler, H.G. (1986d) Die Stabilität der Fettkugelmembran beim Erhitzen in Abhängigkeit von unterschiedlichen Milieubedingungen. *Deutsche Molkerei-Zeitung* 38, 1246–1251.

Fink, A. & Kessler, H.G. (1986e) Auswirkungen der Erhitzung auf die Emulsionsstabilität von septisch homogenisiertem Rahm. *Deutsche Milchwirtschaft* 40, 1294–1296.

Fischer-Parton, S., Parton, R.M., Hickey, P.C., Dijksterhuis, J., Atkinson, H.A. & Read, N.D. (2000) Confocal microscopy of FM4-64 as a tool for analysing endocytosis and vesicle trafficking in living fungal hyphae. *Journal of Microscopy* 198(3), 246–259.

Fishov, I. & Woldringh, C.L. (1999) Visualization of membrane domains in Escherichia coli. *Molecular Biology* 32(6), 1166–1172.

Flückiger, E. (1987) Fettschädigung bei der Milchgewinnung. *Deutsche Milchwirtschaft* 5, 112–115.

Flückiger, E. & Hänni, H. (1980) Untersuchungen über den Gehalt der Mischmilch an freiem Fett und freien Fettsäuren in Sammelstellen mit verschiedenen Kühlsystemen. *Schweizerische Milchzeitung* 106, 66.

Flückiger, E., Hänni, H., Jegerlehner, T. & Graber, P. (1981) Einfluss der Schwarte-Milchmessanlage zur stationären Milchannahme auf den Gehalt an freien Fett und freien Fettsäuren. *Schweizerische Milchzeitung* 107(43/44), 289–290.

Fotiadis, D., Scheuring, S., Müller, S.A., Engel, A. & Müller, D.J. (2002) Imaging and manipulation of biological structures with the AFM. *Micron* 33(4), 385–397.

Fox, P.F. (2002) Fat globules in milk. In *Encyclopedia of Dairy Sciences*, pp. 1564–1568. Edited by H. Roginski, J.W. Fuquay & P.F. Fox. Amsterdam: Academic Press.

Franke, W.W., Heid, H.W., Grund, C., Winter, S., Freudenstein, C., Schmid, E., Jarasch, E.-D. & Keenan, T.W. (1981) Antibodies to the major membrane-associated protein: specific location in apical regions of lactating epithelial cells. *Journal of Cell Biology* 89(3), 485–494.

Franz, S., Frey, B., Sheriff, A., Gaipl, U.S., Beer, A., Voll, R.E., Kalden, J.R. & Herrmann, M. (2006) Lectins detect changes of the glycosylation status of plasma membrane constituents during late apoptosis. *Cytometry Part A* 69A, 230–239.

Freudenstein, C., Keenan, T.W., Eigel, W.N., Sasaki, M., Stadler, J. & Franke, W.W. (1979) Preparation and characterisation of the inner coat material associated with fat globule membranes from bovine and human milk. *Experimental Cell Research* 118, 277–294.

Fuller, M.E., Streger, S.H., Rothmel, R.K., Mailloux, B.J., Hall, J.A., Onstott, T.C., Fredrickson, J.K., Balkwill, D.L. & Deflaun, M.F. (2000) Development of a vital fluorescent staining method for monitoring bacterial transport in subsurface environments. *Applied and Environmental Microbiology* 66(10), 4486–4496.

Gallagher, J.T., Harding, M. & Dale, R.E. (1982) Sialic acid as a cell surface binding site for wheat germ agglutinin. In *Proceedings of 5th Lectin Meeting, Vol. 3*, pp. 312–318. Edited by T.C. Bøg-Hansen & G.A. Spengler. Bern: Walter de Gruyter & Co.

Gaucheron, F., Le Graet, Y. & Briard, V. (2000) Effect of NaCl addition on the mineral equilibrium of concentrated and acidified casein micelles. *Milchwissenschaft* 55(2), 82–86.

Giangiacomo, R. & Bonomi, F. (1993) A colorimetric study of fat globule structural modifications in milk handling. In *IDF special issue 9303. Protein and fat globule modification by heat treatment, homogenisation and other technological means for high quality dairy products*, pp. 107–110. Brussels: International Dairy Federation.

Gottlieb, E. (1892) Eine bequeme Methode zur Bestimmung von Fett in Milch. *Landwirtschaftlichen Versuchsstationen* 40, 1–27.

Gottschalk, A. (1960) The chemistry and biology of sialic acid and related substances. London: Cambridge University Press.

Graham, J.M. & Higgins, J.A. (1997) Membrane analysis. First edition. Oxford: BIOS Scientific Publishers Ltd.

Grappin, R. & Ribadeau-Dumas, B. (1992) Analytical methods for milk proteins. In *Advanced dairy chemistry, Volume 1: Proteins*, pp. 1–62. Edited by P.F. Fox. London: Chapman & Hall.

Greenbank, G.R. & Pallansch, M.J. (1961) Migration of phosphatides in processing dairy products. *Journal of Dairy Science* 44, 1597–1602.

Grufferty, M.B. & Fox, P.F. (1985) Effect of added NaCl on some physicochemical properties of milk. *Irish Journal of Food Science and Technology* 9, 1–9.

Guinard-Flament, J., Michalski, M-C. & Rulquin, H. (2001) Evolution of milk fat content and fat globule diameter according to milking time in dairy cows. *Rencontres autour des Recherches sur les Ruminants* 8, 92.

Guinee, T.P.F., Feeney, P., Auty, M.A.E. & Fox, P.F. (2002) Effect of pH and calcium concentration on some textural and functional properties of Mozzarella cheese. *Journal of Dairy Science* 85, 1655–1669.

Gunasekaran, S. & Ding, K. (1999) Three-dimensional characteristics of fat globules in Cheddar cheese. *Journal of Dairy Science* 82, 1890–1896.

Gunning, A.P., Wilde, P.J., Clark, D.C., Morris, V.J., Parker, M.L. & Gunning, P.A. (1996) Atomic force microscopy of interfacial protein films. *Journal of Colloid and Interface Science* 183, 600–602.

Hai, M., Barnath, K., Tawfik, D. & Magdassi, S. (2004) Flow cytometry: a new method to investigate the properties of water-in-oil-in-water emulsions. *Langmuir* 20, 2081–2085.

Halter, N., Puhan, Z. & Schmutz, M. (1978) A simple and fast method for determining fat damage in milk and cream. *Nordeuropæisk mejeri-tidsskrift* 78(7), 190–194.

Hambling, S.G., McAlpine, A.S. & Sawyer, L. (1992) β-Lactoglobulin. In *Advanced dairy chemistry, Volume 1: Proteins*, pp. 141–190. Edited by P.F. Fox. London: Chapman & Hall.

Heid, H.W. & Keenan, T.W. (2005) Intracellular origin and secretion of milk fat globules. *European Journal of Cell Biology* 84(2–3), 245–258.

Hemingway, E.B., Smith, G.H., Rook, J.A.F. & O'Flanagan, N.C. (1970) Lipase taint. *Journal of the Society of Dairy Technology* 23, 44–48.

Henson, A.F., Holdsworth, G. & Chandan, R.C. (1971) Physicochemical analyses of bovine milk fat globule membrane. II. Electron microscopy. *Journal of Dairy Science* 54(12), 1752–1763.

Herbert, S., Bouchet, B., Riaublanc, A., Dufour, E. & Gallant, D.J. (1999) Multiple fluorescence labelling of proteins, lipids and whey in dairy products using confocal microscopy. *Lait* 79(6), 567–575.

Herbst, K.D., Roiner, F. & Thormählen, H. (1984) Beeinflussung der Milch durch Tiefkühlung und mechanische Belastung. *Deutsche Milchwirtschaft* 19, 704–706.

Heywang, C., Mathe, G., D., Hess, D. & Sackmann, E. (2001) Interaction of GM1 glycolipid in phospholipid monolayers with wheat germ agglutinin: effect of phospholipidic environment and subphase. *Chemistry and Physics of Lipids* 113(1–2), 41–53.

Hinrichs, J. (1994) Grenzbelastung für Fettkügelchen. *Milchwirtschaftliche Berichte aus den Bundesanstalten Wolfpassing und Rotholz* 121, 227–231.

Hinrichs, J. & Kessler, H.G. (1995) Neue Erkenntnisse zur schonenden Förderung von Milch und Sahne - Grenzwerte für die Auslegung. *DMZ* 6, 236–242.

Hofi, A.A., Hamzawi, L.F., Mahran, G.A. & Asker, A.A. (1977) Studies on buffalo milk fat globule membrane. I. Effect of stage of lactation. *Egyptian Journal of Dairy Science* 5(2), 235–240.

Hoh, J.H. & Schoenenberger, C-A. (1994) Surface morphology and mechanical properties of MDCK monolayers by atomic force microscopy. *Journal of Cell Science* 107, 1105–1114.

Hood, L.F. & Patton, S. (1973) Isolation and characterisation of intracellular lipid droplets from bovine mammary tissue. *Journal of Dairy Science* 56, 858–863.

Horisberger, M., Rosset, J. & Vonlanthen, M. (1977) Location of glycoproteins on milk fat globule membrane by scanning and transmission electron microscopy, using lectin-labelled gold granules. *Experimental Cell Research* 109, 361–369.

Horisberger, M. & Tacchini-Vonlanthen, M. (1982) Stability and steric hindrance of lectin-labelled gold markers in transmission and scanning electron microscopy. In *5th Lectin Meeting, Vol. 3*, pp. 189–197. Edited by T.C. Bøg-Hansen & G.A. Spengler. Bern: Walter de Gruyter.

Horvath, Z. (1974) Mechanical damage of the milkfat globules during storage in bulk milk tank. In *Brief communications, Vol. 1E, 19th international dairy congress*, p. 173. New Delhi: 19th International Dairy Congress Secretariat.

Houlihan, A.V. (1992) Enzymatic activity other than lipase. In *IDF Bulletin, No. 271*, pp. 21–25. Brussels: International Dairy Federation.

Houlihan, A.V., Goddard, P.A., Nottingham, S.M., Kitchen, B.J. & Masters, C.J. (1992) Interactions between the bovine milk fat globule membrane and skim milk components on heating whole milk. *Journal of Dairy Research* 59(2), 187–195.

Hughes, R.C. (1983) Glycoproteins. First edition. London: Chapman and Hall.

Hui, S-W. & Boni, L.T. (1991) Membrane fusion induced by polyethylene glycol. In *Membrane fusion*, pp. 231–253. Edited by J. Wilschut & D. Hoekstra. New York: Marcel Dekker, Inc.

Huppertz, T. & Fox, P.F. (2006) Effect of NaCl on some physico-chemical properties of concentrated bovine milk. *International Dairy Journal* 16, 1142–1148.

Huppertz, T. & Kelley, A.L. (2006) Physical chemistry of milk fat globules. In *Advanced Dairy Chemistry, Volume 2: Lipids*, pp. 173–212. Edited by P.F. Fox & P.L.H. McSweeney. New York: Springer.

Huston, G. & Patton, S. (1990) Factors related to the formation of cytoplasmic crescents on milk fat globules. *Journal of Dairy Science* 73, 2061–2066.

Iametti, S., Versuraro, L., Tragna, S., Giangiacomo, R. & Bonomi, F. (1997) Surface properties of the fat globule in treated creams. *International Dairy Journal* 7, 375–380.

IDF (1975) Proceedings of the lipolysis symposium. IDF Bulletin No. 86. Brussels: International Dairy Federation.

IDF (1980) Flavour impairment of milk and milk products due to lipolysis. IDF Bulletin No. 118. Brussels: International Dairy Federation.

IDF (1991) Determination of free fatty acids in milk and milk products. IDF Bulletin No. 265. Brussels: International Dairy Federation.

IDF (1996) Milk. Determination of fat content. Gravimetric method (reference method). *Provisional International Standard 1D*. Brussels: International Dairy Federation.

Ikeda, S. (2003) Heat-induced gelation of whey proteins observed by rheology, atomic force microscopy, and Raman scattering spectroscopy. *Food Hydrocolloids* 17(4), 399–406.

Ikeda, S. & Morris, V.J. (2002) Fine-stranded and particulate aggregates of heat-denatured whey proteins visualized by atomic force microscopy. *Biomacromolecules* 3(2), 382–389.

Inouė, S. (1995) Foundations of confocal scanned imaging in light microscopy. In *Handbook of biological confocal microscopy*, Vol. 2, pp. 1–17. Edited by J.B. Pawley. New York: Plenum Press.

Ismail, A.A., El-ghanam, M.S. & Sirry, L. (1972a) Comparative study between some buffalo and cow cream properties I. Effect of heating, agitation, homogenization and some chemical additives. *Alexandrian Journal of Agricultural Research* 20, 69–74.

Ismail, A.A., El-ghanam, M.S. & Sirry, L. (1972b) Comparative study between some buffalo and cow cream properties II. Effect of ageing at 5°C and at room temperature. *Alexandrian Journal of Agricultural Research* 20, 75–82.

Jack, L.J.W. & Mather, I.H. (1990) Cloning and analysis of cDNA encoding bovine butyrophilin, an apical glycoprotein expressed in mammary tissue and secreted in association with the milk-fat globule membrane during lactation. *Journal of Biological Chemistry* 265(24), 14482–14486.

Janssen, M.M.T. & Walstra, P. (1982) Cytoplasmic remnants in milk of certain species. *Netherlands Milk and Dairy Journal* 36, 365–368.

Jenness, R. & Koops, J. (1962) Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Netherlands Milk and Dairy Journal* 16(3), 153–164.

Jensen, R.G., Blanc, B. & Patton, S. (1995) The structure of milk: implications for sampling and storage. B. Particulate constituents in human and bovine milks. In *Handbook of milk composition*, pp. 50–62. Edited by R.G. Jensen. San Diego: Academic Press.

Johnsen, A.R., Hausner, M., Schnell, A. & Wuertz, S. (2000) Evaluation of fluorescently labeled lectins for noninvasive localization of extracellular polymeric substances in sphingomonas biofilms. *Applied and Environmental Microbiology* 66(8), 3487–3491.

Joshi, N.S. & Thakar, P.N. (1994) Methods to evaluate deterioration of milk fat - a critical appraisal. *Journal of Food Science and Technology (Mysore)* 31, 181–196.

Kaláb, M. (1993) Practical aspects of electron microscopy in dairy research. *Food Structure* 12, 95–114.

Kammerlehner, J. & Kessler, H.G. (1980) Mechanische Einflüsse auf Rahm beim Rühren und Pumpen. *Deutsche Milchwirtschaft* 43, 1746–1748.

Kanno, C. (1986) Receptor proteins for concanavalin A and wheat germ agglutinin of bovine milk fat globule membrane probed by affinity chromatography in the presence of sodium dodecyl sulfate. *Agricultural and Biological Chemistry* 50(12), 2997–3003.

Kanno, C. (1990) Secretory membranes of the lactating mammary gland. *Protoplasma* 159, 184–208.

Keenan, T.W. (2001) Milk lipid globules and their surrounding membrane: a brief history and perspectives for future research. *Journal of Mammary Gland Biology and Neoplasia* 6(3), 365–371.

Keenan, T.W. & Dylewski, D.P. (1985) Aspects of intracellular transit of serum and lipid phases of milk. *Journal of Dairy Science* 68, 1025–1040.

Keenan, T.W. & Dylewski, D.P. (1995) Intracellular origin of milk lipid globules and the nature and structure of the milk lipid membrane. In *Advanced dairy chemistry, Volume 2: Lipids*, pp. 89–130. Edited by P.F. Fox. London: Chapman & Hall.

Keenan, T.W., Dylewski, D.P., Woodford, T.A. & Ford, R.H. (1983a) Origin of the milk fat globules and the nature of the milk fat globule membrane. In *Developments in dairy chemistry*, pp. 83–118. Edited by P.F. Fox. New York: Applied Science Publishers.

Keenan, T.W., Franke, W.W. & Kartenbeck, J. (1974) Concanavalin A binding by isolated plasma membranes and endomembranes from liver and mammary gland. *FEBS Letters* 44(3), 274–278.

Keenan, T.W., Heid, H.W., Stadler, J., Jarasch, E-D. & Franke, W.W. (1982) Tight attachment of fatty acids to proteins associated with milk lipid globule membrane. *European Journal of Cell Biology* 26, 270–276.

Keenan, T.W. & Mather, I.H. (2002) Milk fat globule membrane. In *Encyclopedia of Dairy Sciences*, pp. 1568–1576. Edited by H. Roginski, J.W. Fuquay & P.F. Fox. Amsterdam: Academic Press.

Keenan, T.W. & Mather, I.H. (2006) Intracellular origin of milk fat globules and the nature of the milk fat globule membrane. In *Advanced dairy chemistry, Volume 2: Lipids*, pp. 137–171. Edited by P.F. Fox & P.L.H. McSweeney. New York: Springer.

Keenan, T.W., Mather, I.H. & Dylewski, D.P. (1988) Physical equilibria: lipid phase. In *Fundamentals of dairy chemistry*, pp. 511–582. Edited by N.P. Wong. New York: Van Nostrand Reinhold.

Keenan, T.W., Moon, T. & Dylewski, D.P. (1983b) Lipid globules retain globule membrane material after homogenisation. *Journal of Dairy Science* 66, 196–203.

Keenan, T.W., Morre, D.J., Olsen, D.E., Yunghans, W.N. & Patton, S. (1970) Biochemical and morphological comparison of plasma membrane and milk fat globule membrane from bovine mammary gland. *Journal of Cell Biology* 44, 80–93.

Keenan, T.W. & Patton, S. (1995) The structure of milk: implications for sampling and storage. A. The milk lipid globule membrane. In *Handbook of milk composition*, pp. 5–50. Edited by R.G. Jensen. San Diego: Academic Press.

Kelly, W.G. & Hart, G.W. (1989) Glycosylation of chromosomal proteins: localization of O-linked N-acetylglucosamine in Drosophila chromatin. *Cell* 57, 243–251.

Kessler, H.G. & Fink, A. (1992) Physico-chemical effects of pasteurization on cream properties. In *IDF Bulletin, No. 271*, pp. 11–17. Brussels: International Dairy Federation.

Kim, H.H.Y. & Jimenez-Flores, R. (1995) Heat-induced interactions between the proteins of milk fat globule membrane and skim milk. *Journal of Dairy Science* 78(1), 24–35.

King, N. (1951) The behaviour of milk fat during agitation of milk and cream. *Dairy Industries* 16, 727–734.

King, N. (1958) Observations by fluorescence microscopy of casein in milk, curd, and cheese. *Journal of Dairy Research* 25, 312–319.

Kinsella, J.E. (1970) The milk fat globule membrane. A natural ready-made package. *American Dairy Review* 32(6), 46, 73–74.

Kirst, E. (1980a) Zur Lipolyse der Milch durch technologische Beeinflussungen I. Mitt. Stand der Kenntnisse und Untersuchungen zur Beeinflussung von Milch und Rahm durch Pumpen. *Die Nahrung* 24, 569–576.

Kirst, E. (1980b) Lipolytischen Vorgänge in Milch und Milchprodukten. II Mitteilung: Untersuchung zum Einfluss von Strömungsvorgängen auf das Milchfett. *Lebensmittelindustrie* 27, 314–316.

Kirst, E. (1980c) Lipolytischen Vorgänge in Milch und Milchprodukten. III Mitteilung: Untersuchung zum Einfluss der Kühlung auf die Struktur des Milchfettes. *Lebensmittelindustrie* 27, 464–468.

Kirst, E. (1981) Lipolytischen Vorgänge in Milch und Milchprodukten. IV Mitteilung: Untersuchung zum Einfluss der Schaumbildung auf die Struktur des Milchfettes. *Lebensmittelindustrie* 28(10), 461–463.

Kirst, E. (1996) Untersuchungen zu lipolytischen Veränderungen des Milchfettes und deren Auswirkungen auf die Qualität der Milch und Milcherzeugnisse. *Deutsche Molkerei Zeitung* 4, 168–181.

Klebanov, G.I., Sorokovoi, V.I. & Vladimirov, Y.A. (1972) Study of the conformational properties of biological membranes by determination of protein luminescence. *Molekulyarnaya Biologiya* 6(2), 189–195.

Ko, S. & Gunasekaran, S. (2007) Error correction of confocal microscopy images for in situ food microstructure evaluation. *Journal of Food Engineering* 79(3), 935–944.

Kobylka, D. & Carraway, K.L. (1973) Proteolytic digestion of proteins of the milk fat globule membrane. *Biochimica et Biophysica Acta* 307(1), 133–140.

Kolb, H-A., Enders, O. & Schauer, R. (1999) Morphology of native and reconstituted biological membranes and their components analyses with atomic force microscopy. *Applied Physics A* 68, 247–254.

Koops, J. & Tarassuk, N.P. (1959) The effect of various processing treatments on the partitioning of phosphatides between the fat phase and the milk plasma. *Netherlands Milk and Dairy Journal* 13(3), 180–189.

Kovbasnjuk, O.N. & Spring, K.R. (2000) The apical membrane glycocalyx of MDCK cells. *Journal of Membrane Biology* 176, 19–29.

Kremp, A. & Anderson, D.M. (2004) Lectin binding patterns of *Scrippsiella lachrymosa (Dinophyceae)* in relation to cyst formation and nutrient conditions. *Journal of Experimental Marine Biology and Ecology* 307, 165–181.

Krommenhoek, P., Gallagher, G., Jimenez-Flores, R., Hagen, J. & Gragson, D. (2006) Thermodynamic and structural analysis of milk fat globule membrane monolayers. *FASEB Journal* 20(5, Part 2), A916.

Kulozik, U., Tolkach, A., Bulca, S. & Hinrichs, J. (2003) The role of processing and matrix design in development and control of microstructures in dairy food production—a survey. *International Dairy Journal* 13(8), 621–630.

Lagoni, H. & Peters, K.H. (1959) Die "verkürzte" Röse Gottlieb-Methode zur Bestimmung des "freien" Fettes in Rahm und Milch beim Schlagen und Buttern. *Kieler Milchwirtschaftliche Forschungsberichte* 11, 291–296.

Larson, B.L. (1992) Immunoglobulins of the mammary secretions. In *Advanced dairy chemistry, Volume 1: Proteins*, pp. 231–254. Edited by P.F. Fox. London: Chapman & Hall.

Lee, S.J. & Sherbon, J.W. (2002) Chemical changes in bovine milk fat globule membrane caused by heat treatment and homogenization of whole milk. *Journal of Dairy Research* 69, 555–567.

Lefèvre, T. & Subaride, M. (2000) Interaction of beta-lactoglobulin with phospholipid bilayers: a molecular level elucidation as revealed by infrared spectroscopy. *International Journal of Biological Macromolecules* 28, 59–67.

Lehman, H. (1982) Der Einfluss der mechanischen Behandlung der Milch auf ihre Separierfähigkeit unter dem Gesichtspunkt der Gehalte an freien Fettsäuren und freiem Fett. *Deutsche Milchwirtschaft* 5, 172–174.

Lehman, H. (1988a) Rahm- und Fettkugelschädigung. Teil I: Bestimmung der freien Fettsäuren. *Deutsche Molkerei-Zeitung* 21, 634–636.

Lehman, H. (1988b) Rahm- und Fettkugelschädigung. Teil II: Bestimmung und Interpretation von freiem Fett, Vergleichsuntersuchungen mit Hilfe von Labor-Separatoren. *Deutsche Molkerei-Zeitung* 22, 671–676.

Lehman, H. (1988c) Rahm- und Fettkugelschädigung. Teil III: Korngrössen-Analyse (Fett). *Deutsche Molkerei-Zeitung* 23, 703–707.

Lent, L.E., Vanasupa, L.S. & Tong, P.S. (1998) Whey protein edible film structures determined by atomic force microscope. *Journal of Food Science* 63(5), 824–827.

Liang, I., Lee, S-L., Cheng, T-L., Lin, Y-E. & Wu, Y-C. (2000) Study on stability of the fat globule of cow's milk. *Taiwanese Journal of Agricultural Chemistry and Food Science* 36(6), 565–574.

Liener, I.E. (1976) Isolation and properties of Concanavalin A. In *Concanavalin A as a tool*, pp. 17–31. Edited by H. Bittiger & H.P. Schnebli. London: John Wiley & Sons.

Liu, C., Eickson, A.K. & Henning, D.A. (2005) Distribution and carbohydrate structures of high molecular weight glycoproteins, MUC1 and MUCX, in bovine milk. *Journal of Dairy Science* 88(12), 4288–4294.

Lopez, C. (2005) Focus on the supramolecular structure of milk fat in dairy products. *Reproduction, Nutrition, Development* 45, 497–511.

Lopez, C., Camier, B. & Gassi, J-Y. (2007) Development of the milk fat microstructure during the manufacture and ripening of Emmental cheese observed by confocal laser scanning microscopy. *International Dairy Journal* 17(3), 235–247.

Lopez, C., Maillard, M-B., Briard-Bion, V., Camier, B. & Hannon, J.A. (2006) Lipolysis during ripening of Emmental cheese considering organization of fat and preferential localization of bacteria. *Journal of Agricultural and Food Chemistry* 54, 5855–5867.

Macej, O.D., Jovanovic, S.T. & Denin Djurdjevic, J.D. (2002) The influence of high temperatures on milk proteins. *Hemijska Industrija* 56(3), 123–132.

Mackie, A.R., Gunning, A.P., Ridout, M.J., Wilde, P.J. & Morris, V.J. (2001) Orogenic displacement in mixed β-lactoglobulin/β-casein films at the air/water interface. *Langmuir* 17(21), 6593–6598.

Mackie, A., Gunning, A.P., Wilde, P. & Morris, V. (2000) Competitive displacement of β-lactoglobulin from the air/water interface by sodium dodecyl sulfate. *Langmuir* 16(21), 8176–8181.

Mallucci, L. (1976) Preparation and use of fluorescent Concanavalin A derivatives. In *Concanavalin A as a tool*, pp. 69–78. Edited by H. Bittiger & H.P. Schnebli. London: John Wiley & Sons.

Manderson, G.A., Hardman, M.J. & Creamer, L.K. (1998) Effect of heat treatment on the conformation and aggregation of ß-lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry* 46, 5051–5061.

Martin, M-J., Martin-Sosa, S. & Hueso, P. (2001) Bovine milk gangliosides: changes in ceramide moiety with stage of lactation. *Lipids* 36, 291–298.

Mather, I.H. (2000) A review and proposed nomenclature for major proteins of the milk-fat globule membrane. *Journal of Dairy Science* 83, 203–247.

Mather, I.H., Jack, L.J.W., Madara, P.J. & Johnson, V.G. (2001) The distribution of MUC1, an apical membrane glycoprotein, in mammary epithelial cells at the resolution of the electron microscope: implications for the mechanism of milk secretion. *Cell Tissue Research* 304, 91–101.

Mather, I.H. & Keenan, T.W. (1975) Studies on the structure of milk fat globule membrane. *Journal of Membrane Biology* 21(1/2), 65–85.

Mather, I.H. & Keenan, T.W. (1998) Origin and secretion of milk lipids. *Journal of Mammary Gland Biology and Neoplasia* 3(3), 259–273.

Mather, I.H., Weber, K. & Keenan, T.W. (1977) Membranes of mammary gland. XII. Loosely associated proteins and compositional heterogeneity of bovine milk fat globule membrane. *Journal of Dairy Science* 60(3), 394–402.

McCrae, C.H. & Lepoetre, A. (1996) Characterization of dairy emulsions by forward lobe laser light scattering applications to milk and cream. *International Dairy Journal* 6, 247–256.

McKenna, A.B. (1997) Examination of whole milk powder by confocal laser scanning microscopy. *Journal of Dairy Research* 64(3), 423–432.

McManaman, J.L., Palmer, C.A., Wright, R.M. & Neville, M.C. (2002) Functional regulation of xanthine oxidoreductase expression and localization in the mouse mammary gland: evidence of a role in lipid secretion. *Journal of Physiology* 545(2), 567–579.

McPherson, A.V. & Kitchen, B.J. (1983) Reviews of the progress of dairy science: the bovine milk fat globule membrane - its formation, composition, structure and behaviour in milk and dairy products. *Journal of Dairy Research* 50, 107–133.

Meers, P., Ali, S., Erukulla, R. & Janoff, A.S. (2000) Novel inner monolayer fusion assays reveal differential monolayer mixing associated with cation-dependent membrane fusion. *Biochimica et Biophysica Acta* 1467(1), 227–243.

Merel-Rausch, E., Kulozik, U. & Hinrichs, J. (2007) Influence of pressure release rate and protein concentration on the formation of pressure-induced casein structures. *Journal of Dairy Research* 74(3) 283–289.

Michalski, M-C. (2004) Milkfat globules: physico-chemical properties as a function of size. In *IDF Bulletin, No. 389*, pp. 104–107. Brussels: International Dairy Federation.

Michalski, M-C., Briard, V. & Juaneda, P. (2005a) CLA profile in native fat globules of different sizes selected from raw milk. *International Dairy Journal* 15(11), 1089–1094.

Michalski, M-C., Briard, V. & Michel, F. (2001a) Optical parameters of milk fat globules for laser light scattering measurements. *Le Lait* 81, 787–796.

Michalski, M-C., Camier, B., Briard, V., Leconte, N., Gassi, J.Y., Goudédranche, H., Michel, F. & Fauquant, J. (2004a) The size of native milk fat globules affects physico-chemical and functional properties of Emmental cheese. *Lait* 84, 343–358.

Michalski, M-C., Cariou, R., Michel, F. & Garnier, C. (2002a) Native vs damaged milk fat globules: membrane properties affect the viscoelasticity of milk gels. *Journal of Dairy Science* 85, 2451–2461.

Michalski, M-C., Camier, B., Gassi, J-Y., Briard-Bion, V., Leconte, N., Famelart, M-H. & Lopez, C. (2007) Functionality of smaller vs control native milk fat globules in Emmental cheeses manufactured with adapted technologies. *Food Research International* 40(1), 191–202.

Michalski, M-C., Gassi, J.Y., Famelart, M.H., Leconte, N., Camier, B., Michel, F. & Briard, V. (2003) The size of native milk fat globules affects physico-chemical and sensory properties of Camembert cheese. *Lait* 83(2), 131–143.

Michalski, M-C., Leconte, N., Briard-Bion, V., Fauquant, C., Maubois, J.L. & Goudédranche, H. (2006) Microfiltration of raw whole milk to select fractions with different fat globule size distributions: process optimization and analysis. *Journal of Dairy Science* 89, 3778–3790.

Michalski, M-C., Michel, F. & Geneste, C. (2002b) Appearance of submicronic particles in the milk fat globule size distribution upon mechanical treatments. *Lait* 82, 193–208.

Michalski, M-C., Michel, F., Sainmont, D. & Briard, V. (2001b) Apparent zeta-potential as a tool to assess mechanical damages to the milk fat globule membrane. *Colloids and Surfaces B: Biointerfaces* 23, 23–30.

Michalski, M-C., Ollivon, M., Briard, V., Leconte, N. & Lopez, C. (2004b) Native fat globules of different sizes selected from raw milk: thermal and structural behaviour. *Chemistry and Physics of Lipids* 132(2), 247–261.

Miller, C.R., Clapp, P.J. & O'Brien, D.F. (2000) Visible light-induced destabilization of endocytosed liposomes. *FEBS Letters* 467, 52–56.

Miller, B. & Puhan, Z. (1986a) Das "lipolysierbare" Fett als Indikator für die Fettschädigung in der Rohmilch. *Schweizerische Milchwirtschaftliche Forschung* 15, 10–17.

Miller, B. & Puhan, Z. (1986b) Fettschädigung während der Rohmilchgewinnung und -behandlung. *Schweizerische Milchwirtschaftliche Forschung* 15, 69–76.

Minana, R., Sancho-Tello, M., Climent, E., Segui, J.M., Renau-Piqueras, J. & Guerri, C. (1998) Intracellular location, temporal expression, and polysialylation of neural cell adhesion molecule in astrocytes in primary culture. *Glia* 24, 415–427.

Molecular Probes, The Handbook, www.probes.invitrogen.com.

Mondy, B.L. & Keenan, T.W. (1993) Butyrophilin and xanthine oxidase occur in constant molar proportions in milk lipid globule membrane but vary in amount with breed and stage of lactation. *Protoplasma* 177, 32–36.

Monis, B., Rovasio, R.A. & Valentich, M.A. (1975) Ultrastructural characterization by ruthenium red of the surface of the fat globule membrane of human and rat milk with data on carbohydrates of fractions of rat milk. *Cell Tissue Research* 157, 17–24.

Morris, V.J. (2004) Probing molecular interactions in foods. *Trends in Food Science & Technology* 15(6), 291–297.

Morris, V.J., Kirby, A.R. & Gunning, A.P. (1999a) Atomic force microscopy for biologists. London: Imperial College Press.

Morris, V.J., Mackie, A.R., Wilde, P., Kirby, A.R., Mills, E.C.N. & Gunning, A.P. (2001) Atomic force microscopy as a tool for interpreting the rheology of food biopolymers at the molecular level. *Lebensmittel Wissenschaft und Technologie* 34, 3–10.

Morris, V.J., Mills, E.C.N., Mackie, A.R., Wilde, P., Kirby, A.R. & Gunning, A.P. (1999b) Probing biopolymer functionality in foods with the atomic force microscope. *Leatherhead Food RA Food Industry Journal* 2(Spring 1999), 11–25.

Mulder, H. & Walstra, P. (1974) The Milk Fat Globule. Emulsion Science as Applied to Milk Products and Comparable Foods. Wageningen: Pudoc.

Murphy, D.B. (2001) Fundamentals of light microscopy and electronic imaging. New York: Wiley-Liss.

Murray, L.R., Powell, K.M., Sasaki, M., Eigel, W.N. & Keenan, T.W. (1979) Comparison of lectin receptor and membrane coat-associated glycoproteins of milk lipid globule membranes. *Comparative Biochemistry and Physiology* 63B, 137–145.

Muscatello, U., Valdrè, G. & Valdrè, U. (1996) Atomic force microscopy observations of acyl chains in phospholipids. *Journal of Microscopy* 182, 200–207.

Narine, S.S. & Marangoni, A.G. (1999) Mechanical and structural model of fractal networks of fat crystals at low deformations. *Physical Review E* 60(6), 6991.

Neeser, J-R., Golliard, M. & Del Vedovo, S. (1991) Quantitative determination of complex carboydrates in bovine milk and in milk-based infant formulas. *Journal of Dairy Science* 74, 2860–2871.

Nielsen, J.S., Friis, A. & Adler-Nissen, J. (2000) Care in processing of milk products. In *Food and drink 2000: Processing solutions for innovative products: proceedings of a conference. Institution of chemical engineers*, pp. 69–70. University of Birmingham: ICE, Rugby.

Nilsson, A., Dahlin, L., Lundborg, G. & Kanje, M. (2005) Graft repair of a peripheral nerve without the sacrifice of a healthy donor nerve by the use of acutely dissociated autologus Schwann cells. *Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery* 39, 1–6.

Ollivier-Bousquet, M. (2002) Milk lipid and protein traffic in mammary epithelial cells: joint and independent pathways. *Reproduction, Nutrition, Development* 42, 149–162.

O'Mahony, J.A., Auty, M.A.E. & McSweeney, P.L.H. (2005) The manufacture of miniature Cheddar-type cheeses from milks with different fat globule size distributions. *Journal of Dairy Research* 72, 338–348.

Oortwijn, H., Walstra, P. & Mulder, H. (1977) The membranes of recombined fat globules. 1. Electron microscopy. *Netherlands Milk and Dairy Journal* 31, 134–147.

Palfreyman, K.R. (1988) An evaluation of mixing performance and fat globule damage in cream crystallizing silos. *New Zealand Journal of Dairy Science and Technology* 23, 373–384.

Panchuk-Voloshina, N., Haugland, R.P., Bishop-Stewart, J., Bhalgat, M.K., Millard, P.J., Mao, F., Leung, W-Y. & Haugland, R.P. (1999) Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *The Journal of Histochemistry & Cytochemistry* 47(9), 1179–1188.

Parola, A.H. (1993) Membrane lipid-protein interactions. In *Biomembranes - physical aspects*, pp. 159–278. Edited by M. Shinitzky. Weinheim: VCH Verlagsgesellschaft.

Parton, R.M., Fischer-Parton, S., Watahiki, M.K. & Trewavas, A.J. (2001) Dynamics of the apical vesicle accumulation and the rate of growth are related in individual pollen tubes. *Journal of Cell Science* 114, 2685–2695.

Patton, S. (1973) Origin of the milk fat globule. *Journal of the American Oil Chemists' Society* 50, 178–185.

Patton, S., Gendler, S.J. & Spicer, A.P. (1995) The epithelial mucin, MUC1, of milk, mammary gland and other tissues. *Biochimica et Biophysica Acta* 1241, 407–424.

Patton, S. & Hubert, J. (1983) Binding of concanavalin A to milk fat globules and release of the lectin-membrane complex by Triton X-100. *Journal of Dairy Science* 66, 2312–2319.

Patton, S. & Huston, G.E. (1988) Incidence and characteristics of cell pieces on human milk fat globules. *Biochimica et Biophysica Acta* 965(2–3), 146–153.

Patton, S. & Jensen, R.G. (1976) Biomedical aspects of lactation. First edition. Oxford: Pergamon Press.

Patton, S. & Keenan, T.W. (1975) The milk fat globule membrane. *Biochimica et Biophysica Acta* 415(3), 273–309.

Patton, S., Kelley, J.J. & Keenan, T.W. (1980a) Carotene in bovine milk fat globules: observations on origin and high content in tissue mitochondria. *Lipids* 15(1), 33–38.

Patton, S., Long, C. & Sokka, T. (1980b) Effect of storing milk on cholesterol and phospholipid of skim milk. *Journal of Dairy Science* 63, 697–700.

Peixoto de Menezes, A. & Pinto da Silva, P. (1978) Freeze-fractured observations of the lactating rat mammary gland. Membrane events during milk secretion. *Journal of Cell Biology* 76, 767–778.

Phipps, L.W. & Temple, D.M. (1982) Surface properties of milk fat globules: interfacial tension studies. *Journal of Dairy Research* 49, 61–72.

Pinto da Silva, P., Peixoto de Menezes, A. & Mather, I.H. (1980) Structure and dynamics of the bovine milk fat globule membrane viewed by freeze fracture. *Experimental Cell Research* 125(1), 127–139.

Read, N.D. & Kalkman, E.R. (2003) Does endocytosis occur in fungal hyphae? *Fungal Genetics and Biology* 39, 199–203.

Reuter, H., Kurzhals, H-A., Back, W-D. & Biewendt, H-G. (1975) The influence of processing on the quality of raw milk. In *Proceedings of the 6th European Symposium of the European Federation of Chemical Engineering*, pp. 215–235. Cambridge (UK): Society of Chemical Industry.

Richards, D.A., Guatimosim, C. & Betz, W.J. (2000) Two endocytic recycling routes selectively fill two vesicle pools in frog motor nerve terminals. *Neuron* 27, 551–559.

Richardson, R.K., Evers, J.M. & Gray, I.K. (1994) Determination of cholesterol in dairy products by whole sample saponification. *International Dairy Journal* 4, 521–32.

Robenek, H., Hofnagel, O., Buers, I., Lorkowski, S., Schnoor, M., Robenek, M.J., Heid, H., Troyer, D. & Severs, N.J. (2006) Butyrophilin controls milk fat globule secretion. *Proceedings of the National Academy of Sciences of the United States of America* 103(27), 10385–10390.

Roesch, R., Cox, S., Compton, S., Happek, U. & Corredig, M. (2004) κ-Carrageenan and β-lactoglobulin interactions visualized by atomic force microscopy. *Food Hydrocolloids* 18(3), 429–439.

Rombaut, R. & Dewettinck, K. (2006) Properties, analysis and purification of milk polar lipids. *International Dairy Journal* 16, 1362–1373.

Röper, K., Corbeil, D. & Huttner, W.B. (2000) Retention of prominin in microvilli reveals distinct cholesterol-based lipid microdomains in the apical plasma membrane. *Nature Cell Biology* 2, 582–592.

Rothwell, J. (1962) Liquid cream processing. *Journal of the Society of Dairy Technology* 15, 190–195.

Rouze, N.C. & Schwartz, E.A. (1998) Continuous and transient vesicle cycling at a ribbon synapse. *The Journal of Neuroscience* 18(21), 8614–8624.

Rowney, M.K., Hickey, W., Roupas & Everett, D.W. (2003) The effect of homogenization and milk fat fractions on the functionality of Mozzarella cheese. *Journal of Dairy Science* 86, 712–718.

Rudzik, L. (1987) Nachweis von Produktschädigungen beim pumpen von Milch. *Deutsche Milchwirtschaft* 35, 1211–1221.

Russell, P., Bachelor, D. & Thornton, J. SEM and AFM: complementary techniques for high resolution surface investigations. http://www.veeco.com/pdfs.php/154. Accessed July 2007.

Saito, R., Bringas, J.R., McKnight, T.R., Wendland, M.F., Mamot, C., Drummond, D.C., Kirpotin, D.B., Park, J.W., Berger, M.S. & Bankiewicz, K.S. (2004) Distribution of liposomes into brain and rat brain tumor models by convection-enhanced delivery monitored with magnetic resonance imaging. *Cancer Research* 64, 2572–2579.

Salvatierra, S., Stannard, D.J. & Parkinson, R.D. (1978) The effect of two types of milking claws on foaming and fat globule damage in milk. *New Zealand Journal of Dairy Science and Technology* 13, 111–113.

Sasaki, M. & Keenan, T.W. (1979) Ultrastructural characterisation of carboydrate distribution on milk lipid globule membrane. *Cell Biology International Reports* 3(1), 67–74.

Schaus, S.S. & Henderson, E.R. (1997) Cell viability and probe-cell membrane interactions of XR1 glial cells imaged by atomic force microscopy. *Biophysical Journal* 73(3), 1205–1214.

Schmidt, D.G. & Buchheim, W. (1992) The application of electron microscopy in dairy research. *Journal of Microscopy* 167(1), 105–121.

Scolozzi, C., Martini, M. & Abramo, F. (2003) A method for identification and characterisation of ewe's milk fat globules. *Milchwissenschaft* 58(9/10), 490–493.

Seabrook, T.J., Hein, W.R., Dudler, L. & Young, A.J. (2000) Splenectomy selectively affects the distribution and mobility of the recirculating lymphocyte pool. *Blood* 96(3), 1180–1183.

Seok, J-S., Shimoda, M., Azuma, N. & Kanno, C. (2001) Structures of the N-linked sugar chains in PAS-7 glycoprotein sharing the same protein core with PAS-6 glycoprotein from the bovine milk fat globule membrane. *Bioscience, Biotechnology, and Biochemistry* 65(4), 901–912.

Shapiro, H.M. (2003) Practical flow cytometry. Fourth edition. Hoboken: John Wiley & Sons, Inc.

Sharon, N. & Lis, H. (2003) Lectins. Second Edition. Dordrecht: Kluwer Academic Publishers.

Shibata-Seki, T., Masai, J., Tagawa, T., Sorin, T. & Kondo, S. (1996) In-situ atomic force microscopy study of lipid vesicles adsorbed on a substrate. *Thin Solid Films* 273(1–2), 297–303.

Shimizu, M., Yamauchi, K. & Kanno, C. (1979) Proteolytic digestion of milk fat globule membrane proteins. *Milchwissenschaft* 34(11), 666–668.

Shimizu, M., Yamauchi, K. & Kanno, C. (1980) Effect of proteolic of milk fat globule membrane proteins on stability of the globules. *Milchwissenschaft* 35(1), 9–12.

Singer, S.J. & Nicolson, G.L. (1972) The fluid mosaic model of the structure of cell membranes. *Science* 175, 720–731.

Singh, H. (2006) The milk fat globule membrane – A biophysical system for food applications. *Current Opinion in Colloid & Interface Science* 11, 154–163.

Singh, H. & Havea, P. (2003) Thermal denaturation, aggregation and gelation of whey proteins. In *Advanced dairy chemistry, Volume 1, Proteins*, pp. 1261–1288. Edited by P.F. Fox & P.L.H. McSweeney. New York: Kluwer Academic/Plenum Publishers.

Singh, S. & Ganguli, N.C. (1976) Compositional and electrophoretic changes in buffalo milk-fat globule membrane proteins during lactation. *Journal of Dairy Research* 43(3), 381–388.

Singh, S., Bandyopadhyay, A.K. & Ganguli, N.C. (1977) Status of enzymes in buffalo milk fat globule membrane during lactation. *New Zealand Journal of Dairy Science and Technology* 12(3), 190–193.

Smith, A.M. & Nie, S. (2004) Chemical analysis and cellular imaging with quantum dots. *The Analyst* 129, 672–913.

Stannard, D.J. (1975) The use of marker enzymes to assay the churning of milk. *Journal of Dairy Research* 42, 241–246.

Stegemann, M., Meyer, R., Haas, H.G. & Robert-Nicoud, M. (1990) The cell surface of isolated cardiac myocytes - A light microscope study with use of fluorochrome-coupled lectins. *Journal of Molecular and Cellular Cardiology* 22, 787–803.

Stewart, D.B., Murray, J.G. & Neill, S.D. (1975) Lipolytic activity of organisms isolated from refrigerated bulk milk. In *Proceedings of the lipolysis symposium, IDF Bulletin No. 86*, pp. 38–50. Brussels: International Dairy Federation.

Sutheerawattananonda, M., Fulcher, R.G., Martin, F.B. & Bastian, E.D. (1997) Fluorescence image analysis of process cheese manufactured with trisodium citrate and sodium chloride. *Journal of Dairy Science* 80, 620–627.

Swaisgood, H.E. (1995) Chemistry of the caseins. In *Advanced dairy chemistry, Volume 1: Proteins*, pp. 63–110. Edited by P.F. Fox. London: Chapman & Hall.

Te Whaiti, I.E. & Fryer, T.F. (1973) Gelling of cream. *New Zealand Journal of Dairy Science and Technology* 8, 25.

Te Whaiti, I.E. & Fryer, T.F. (1974) Factors influencing the release of free fat from cream. *In 19th international dairy congress, brief communications, Vol 1E*. New Delhi, pp. 664–665.

Te Whaiti, I.E. & Fryer, T.F. (1975) Factors that determine the gelling of cream. *New Zealand Journal of Dairy Science and Technology* 10, 2–7.

Te Whaiti, I.E. & Fryer, T.F. (1976a) Factors influencing the release of free fat in raw milk. *New Zealand Journal of Dairy Science and Technology* 11, 91–95.

Te Whaiti, I.E. & Fryer, T.F. (1976b) The influence of free fat on lipolysis in milk. *New Zealand Journal of Dairy Science and Technology* 11, 273–274.

Tolle, A. & Heeschen, W. (1975) Free fatty acids in milk in relation to flow conditions in milking plants. In *Proceedings of the lipolysis symposium, IDF Bulletin No. 86*, pp. 134–137. Brussels: International Dairy Federation.

Torralba, S. & Heath, I.B. (2002) Analysis of three separate probes suggests the absence of endocytosis in *Neurospora crassa* hyphae. *Fungal Genetics and Biology* 37, 221–232.

Touhami, A., Hoffmann, B., Vasella, A., Denis, F.A. & Dufrêne, Y.F. (2003a) Probing specific lectin-carbohydrate interactions using atomic force microscopy imaging and force measurements. *Langmuir* 19, 1745–1751.

Touhami, A., Nysten, B. & Dufrêne, Y.F. (2003b) Nanoscale mapping of the elasticity of microbial cells by atomic force microscopy. *Langmuir* 19, 4539–4543.

Trachoo, N. (2003) Evidence of association of milk fat globule membrane with protein matrix in dairy gels as revealed by confocal microscopy. *Songklanakarin Journal of Science and Technology* 25(6), 791–797.

van Boekel, M.A.J.S. & Folkerts, T. (1991) Effect of heat treatment on the stability of milk fat globules. *Milchwissenschaft* 46, 758–765.

van Boekel, M.A.J.S. & Walstra, P. (1989) Physical changes in the fat globules in unhomogenized and homogenized milk. In *IDF Bulletin, No. 238*, pp. 13–16. Brussels: International Dairy Federation.

van Boekel, M.A.J.S. & Walstra, P. (1995) Effect of heat treatment on chemical and physical changes to milkfat globules. In *Heat-induced changes in milk*, pp. 51–65. Edited by P.F. Fox. Brussels: International Dairy Federation.

van der Meer, B.W. (1993) Fluidity, dynamics and order. In *Biomembranes - physical aspects*, pp. 97–158. Edited by M. Shinitzky. Weinheim: VCH Verlagsgesellschaft.

Walker, R.A. (1984) The binding of peroxidase-labelled lectins to human breast epithelium I – Normal, hyperplastic and lactating breast. *Journal of Pathology* 142, 279–291.

Walstra, P. (1983) Physical chemistry of milkfat globules. In *Developments in dairy chemistry*, pp. 119–157. Edited by P.F. Fox. New York: Applied Science Publishers.

Walstra, P. (1985) Some comments on the isolation of fat globule membrane material. *Journal of Dairy Research* 52, 309–312.

Walstra, P. (1995) Physical chemistry of milkfat globules. In *Developments in dairy chemistry*, pp. 131–173. Edited by P.F. Fox. London: Chapman & Hall.

Walstra, P. & de Graaf, J.J. (1962) Note on the determination of the phospholipid content of milk products. *Netherlands Milk and Dairy Journal* 16, 283–287.

Walstra, P., Geurts, T.J., Noomen, A., Jellema, A. & van Boekel, M.A.J.S. (1999) Dairy technology. Principles of milk properties and processes. First edition. New York: Marcel Dekker.

Walstra, P. & Jenness, R. (1984) Dairy chemistry and physics. New York: John Wiley and Sons.

Ward, R.E., German, J.B. & Corredig, M. (2006) Composition, applications, fractionation, technological and nutritional significance of milk fat globule membrane material. In *Advanced dairy chemistry, Volume 2: Lipids*, pp. 213–244. Edited by P.F. Fox & P.L.H. McSweeney. New York: Springer.

Warren, L. (1994) Bound carbohydrates in nature. Cambridge: Cambridge University Press.

Webb, B.H. & Hall, S.A. (1935) Some physical effects of freezing upon milk and cream. *Journal of Dairy Science* 18, 275–286.

Welsch, U., Buchheim, W., Schumacher, U., Schinko, I. & Patton, S. (1988) Structural, histochemical and biochemical observations on horse milk-fat-globule membranes and casein micelles. *Histochemistry* 88, 357–365.

Welsch, U., Schumacher, U., Buchheim, W., Schinko, I., Jenness, P. & Patton, S. (1990) Histochemical and biochemical observations on milk-fat-globule membranes from several mammalian species. *Acta Histochemica* 40, 59–64.

Welsch, U., Singh, S., Stemberger, B.H., Buchheim, W. & Patton, S. (1983) Ultrastructural changes in lactating tissue related to the suppression of milk secretion by concanavalin A. *Cell Tissue Research* 230, 527–541.

Wendland, B., McCaffery, J.M., Xiao, Q. & Emr, S.D. (1996) A novel fluorescence-activated cell sorted-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. *The Journal of Cell Biology* 135(6), 1485–1500.

Wiking, L., Björck, L. & Nielsen, J.H. (2003) Influence of food composition on stability of fat globules during pumping of raw milk. *International Dairy Journal* 13(10), 797–803.

Williams, D. (1986) Gelling in pasteurised cream. *Dairy Products - Queensland* 14(2), 25–29.

Wolf, D.E. (1988) Probing the lateral organization and dynamics of membranes. In *Spectroscopic Membrane Probes*, pp. 193–220. Edited by L.M. Loew. CRC Pr I Llc.

Wooding, F.B.P. (1971a) The mechanism of secretion of the milk fat globule. *Journal of Cell Science* 9, 805–821.

Wooding, F.B.P. (1971b) The structure of the milk fat globule membrane. *Journal of Ultrastructure Research* 37, 388–400.

Wooding, F.B.P. (1972) Milk microsomes, viruses, and the milk fat globule membrane. *Experientia* 2819, 1077–1079.

Wooding, F.B.P. (1974) Milk fat globule membrane material in skim-milk. *Journal of Dairy Research* 41, 331–337.

Wooding, F.B.P. (1975) Ultrastructural aspects of formation and fate of the milk fat globule membrane. In *Proceedings of the lipolysis symposium, IDF Bulletin No. 86*, pp. 7–10. Brussels: International Dairy Federation.

Wooding, F.B.P. (1977) Comparative mammary fine structure. In *Comparative aspects of lactation*, pp. 1–41. Edited by M. Peaker. London: Academic Press Inc.

Wooding, F.B.P. & Kemp, P. (1975) High-melting-point triglycerides and the milk-fat globule membrane. *Journal of Dairy Research* 42(3), 419–426.

Woodward, N.C., Wilde, P.J., Mackie, A.R., Gunning, A.P., Gunning, P.A. & Morris, V.J. (2004) Effect of processing on the displacement of whey proteins: applying the orogenic model to a real system. *Journal of Agriculture and Food Chemistry* 52(5), 1287–1292.

Wright, C.S. (1992) Crystal structure of a wheat germ agglutinin/glycophorin-sialoglycopeptide receptor complex. *Journal of Biological Chemistry* 20, 14345–14352.

Wu, C.C., Howell, K.E., Neville, M.C., Yates III, J.R. & McManaman, J.L. (2000) Proteomics reveal a link between the endoplasmic reticulum and lipid secretory mechanism in mammary epithelial cells. *Electrophoresis* 21, 3470–3482.

Yamauchi, K., Shimizu, M. & Kanno, C. (1978) Effect of preparation on properties of a soluble glycoprotein fraction of milk fat globule membrane. *Journal of Dairy Science* 61(6), 688–696.

Yang, H., Wang, Y., Lai, S., An, H., Li, Y. & Chen, F. (2007) Application of atomic force microscopy as a nanotechnology tool in food science. *Journal of Food Science* 72(4), R65–R75.

Yang, J., Tamm, L.K., Somlyo, A.P. & Shao, Z. (1993) Promises and problems of biological atomic force microscopy. *Journal of Microscopy* 171, 183–198.

Ye, A., Anema, S.G. & Singh, H. (2007) Behaviour of homogenized fat globules during the spray drying of whole milk. *International Dairy Journal* 17(4), 374–382.

Ye, A., Singh, H., Oldfield, D.J. & Anema, S. (2004a) Kinetics of heat-induced association of ß-lactoglobulin and α–lactalbumin with milk fat globule membrane in whole milk. *International Dairy Journal* 14(5), 389–398.

Ye, A., Singh, H., Taylor, M.W. & Anema, S. (2002) Characterization of protein components of natural and heat-treated milk fat globule membranes. *International Dairy Journal* 12(4), 393–402.

Ye, A., Singh, H., Taylor, M.W. & Anema, S. (2004b) Interactions of whey proteins with milk fat globule membrane proteins during heat treatment of whole milk. *Lait* 84, 269–283.

Ye, A., Singh, H., Taylor, M.W. & Anema, S. (2005) Disruption of fat globules during concentration of whole milk in a pilot scale multiple-effect evaporator. *International Journal of Dairy Technology* 58(3), 143–149.

Yeung, A., Dabros, T., Masliyah, J. & Czarnecki, J. (2000) Micropipette: a new technique in emulsion research. *Colloids and Surfaces. A: Physicochemical and Engineering Aspects* 174, 169–181.

Zaczek, M. & Keenan, T.W. (1990) Morphological evidence for an endoplasmic reticulum origin of milk lipid globules obtained using lipid-selective staining procedures. *Protoplasma* 159, 179–182.

Zerban, H. & Franke, W.W. (1978) Milk fat globule membranes devoid of intramembranous particles. *Cell Biology International Reports* 2(1), 87–98.

Appendix 1 List of fluorescent probes that were deemed to have potential as MFGM probes

Class	Product Code(s)	Reference to chapter in Molecular Probes Handbook	Excitation/ Emission maxima (nm)	Fluoresce in water?	Compatible with Massey University CLSM?	Unit Size	Price per unit (NZ$)	Storage & handling	Suitable solvent
Fatty acid (Bodipy)	D3834	13.2	505 / 511		Yes (weak?)	1 mg	157		
Fatty acid (Pyrene)	P1903MP	13.2	342 / 376		No	100 mg	Inquire		
Fatty acid (Dansyl)	D94	13.2	335 / 519		No	100 mg	222		
Fatty acid (NBD)	N316	13.2	467 / 539	Almost none	Yes	100 mg	168	Solid; Protect material from long-term exposure to light; may be exposed to light for short periods of time.	DMSO/MeOH
Fatty acid (NBD)	N678	13.2	467 / 539	Almost none	Yes	100 mg	440	Idem	DMSO/MeOH
Phospholipid (NBD)	N3787	13.2	465 / 534	Almost none	Yes	5 mg	168	Solid; Desiccation recommended. Store at <= -20°C. Protect material from long-term exposure to light; may be exposed to light for short periods of time.	Chloroform/EtOH
Phosphoinositides (Bodipy)	Various	13.2	refer to Table 18.3 in Molecular Probes Handbook			50 μg	658		
Phosphocholine (Bodipy)	Various	13.2	506 / 513; 530 / 550; 581 / 591		Yes (581)	100 μg	157		
Phosphocholine (DPH)	D476	13.2	354 / 428		No	1 mg	168		
Phosphocholine (Bispyrene)	B3782	13.2	340 / 473		No	1 mg	256		

Class	Product Code(s)	Reference to chapter in Molecular Probes Handbook	Excitation/ Emission maxima (nm)	Fluoresce in water?	Compatible with Massey University CLSM?	Unit Size	Price per unit (NZ$)	Storage & handling	Suitable solvent
Phosphoethanolamine (LRB/Rhodamine red)	L1392	13.2	560 / 581		Yes	5 mg	231	Solid; Desiccation recommended. Store at <= -20°C. Protect material from long-term exposure to light; may be exposed to light for short periods of time.	
Phosphatidic acid	D3805	13.2	504 / 511		Yes (weak?)	100 µg	157		
Phospholipid (Pyrene)	H361	13.2	342 / 376		No	1 mg	222		
Phospholipid (Dansyl)	D57	13.2	336 / 517		No	25 mg	303		
Phospholipid (Fluorescein and Oregon Green)	F362; O12650	13.2	496 / 519; 501 / 526		Yes	5 mg; 1 mg	231 231		
Sphingolipid (Bodipy)	D3521 / D3522	13.3	505 / 512		Yes (weak?)	250 µg	388		
Sphingolipid (NBD)	N1154 / N3524	13.3	466 / 536		?	1 mg	388		
Lipopolysaccharides (Bodipy)	L23350	13.3	503 / 515		Yes (weak?)	100 µg	249		
Lipopolysaccharides (Alexa Fluor®)	L23351 / 2 / 3	13.3	Different for the different conjugates			100 µg	249		
Oxacarbocyanines	D275	13.4	484 / 501		Yes	100 mg	413	Solid; Protect material from long-term exposure to light; may be exposed to light for short periods of time.	
Anionic sulfophenyl	D7777	13.4	556 / 573		Yes	5 mg	413	Idem	
Anionic sulfophenyl	D7778	13.4	497 / 513		Yes	5 mg	413	Idem	

Class	Product Code(s)	Reference to chapter in Molecular Probes Handbook	Excitation/ Emission maxima (nm)	Fluoresce in water?	Compatible with Massey University CLSM?	Unit Size	Price per unit (NZ$)	Storage & handling	Suitable solvent
Anionic sulfonate	D7776	13.4	555 / 570		Yes	5 mg	413	Idem	
Lipophilic Tracer Sampler Kit	L7781	13.4				1 mg each	460	Idem	
Component A; DilC18 solid	D282	13.4	549 / 565		Yes				DMSO/EtOH/ MeOH
Component B; DilC18 oil	D307	13.4	549 / 565		Yes				DMSO/EtOH/ MeOH
Component C; DiOC18	D275	13.4	484 / 501		Yes				DMSO/EtOH/ MeOH
Component D; DiA	D3883	13.4	491 / 613	None	Yes				DMSO/EtOH/ MeOH
Component E; DilC18; 5,5' disulfonic acid	D7776	13.4	See above						DMSO/EtOH/ MeOH
Component F; SP-DilC18	D7777	13.4	See above						DMSO/EtOH/ MeOH
Component G; SP-DiOC18	D7778	13.4	497 / 513		Yes				DMSO/EtOH/ MeOH
Component H; 5,5-Ph2-DilC	D7779	13.4	576 / 599		Yes				DMSO/EtOH/ MeOH
Component I; DiR (DilC18 (7))	D12731	13.4	748 / 780		No				DMSO/EtOH/ MeOH
Dil	C7000	14.4	553 / 570		Yes	20x50 μg	393		Somewhat more water soluble than D282
Styryl dye	T1111	14.4	532 / 716			5 mg	527		DMSO/EtOH/ Reasonably water soluble
Styryl dye	T3163 T35356	14.4	479 / 598			1 mg/ 10x100 μg	531/609		Water

Class	Product Code(s)	Reference to chapter in Molecular Probes Handbook	Excitation/ Emission maxima (nm)	Fluoresce in water?	Compatible with Massey University CLSM?	Unit Size	Price per unit (NZ$)	Storage & handling	Suitable solvent
Styryl dye	T3166 T13320	14.4	515 / 640			1 mg 10 x 100 µg	609		Water
Styryl dye	T3164	16.1	479 / 640			1 mg	557		
Fluorescein	D109	13.5	497 / 519		Yes	100 mg	204	Solid; Protect material from long-term exposure to light; may be exposed to light for short periods of time.	DMSO/EtOH/ MeOH
Fluorescein	H110	13.5	497 / 519		Yes	100 mg	191	Solid; Protect material from long-term exposure to light; may be exposed to light for short periods of time.	
Fluorescein	F3857	13.5	497 / 519		Yes	10 mg	204	Solid; Protect material from long-term exposure to light; may be exposed to light for short periods of time.	
Coumarin	H22730	13.5	366 / 453		No	10 mg	249		
DPH propionic acid	P459	13.5	354 / 430	Almost none	No	25 mg	440		
TMA-DPH	T204	13.5	355 / 430	Almost none	No	25 mg	460		
TMAP-DPH	P3900	13.5	354 / 429	Almost none	No	5 mg	489		
Dapoxyl derivatives	D12800	13.5	358 / 517	Weak	No	10 mg	222		
ANS	A47; A50; T53	13.5	372 / 480; 319 / 480; 318 / 443	No	No	100 mg	256 256 153		
MBDS	A11760	13.5	342 / 450	No	No		231		

Class	Product Code(s)	Reference to chapter in Molecular Probes Handbook	Excitation/ Emission maxima (nm)	Fluoresce in water?	Compatible with Massey University CLSM?	Unit Size	Price per unit (NZ$)	Storage & handling	Suitable solvent
Casein	C2990	16.1	?			25 mg	222		
LECTINS									
WGA (Alexa Fluor® 488)	W11261	7.7	495 / 519		Yes	5 mg	433	Desiccation recommended. Store at <= -20°C. Protect material from long-term exposure to light; may be exposed to light for short periods of time. This material is not considered hazardous, or is not present above 1%, or is not a carcinogen above 0.1% as defined in 29 CFR 1910.1200, the OSHA Hazard Communication Standard. Therefore, a Material Safety Data Sheet is not required.	
WGA (Alexa Fluor® 594)	W11262	7.7	590 / 617		Yes	5 mg			
Con A (Alexa Fluor®)	C11252	7.7	495 / 519		Yes	5 mg	220	Idem	Aqueous buffers
Con A (Alexa Fluor®)	C21421	7.7	650 / 668		Yes	5 mg	220	Idem	Aqueous buffers

Appendix 2 Principle of operation of the confocal laser scanning microscope (CLSM)

The major difference between a CLSM and a conventional microscope is that the CLSM excludes out-of-focus light from the image and thus allows the user to "see" optical sections below the surface of the specimen. Figure A2.1 shows the operating principle of an epifluorescence CLSM. Laser excitation light from a point source is focused onto a particular focal plane in the specimen. The beam splitter (dichroic mirror) reflects away the laser light reflected from the specimen and allows the emitted fluorescence from the specimen to pass through. The fluorescent light from the excited point in the specimen is focused at a detector confocal pinhole. A barrier filter (not shown), which is placed between the beam splitter and the detector, is used to collect the desired emission wavelength range(s). Fluorescent light from above or below the focal plane is *not* confocal with the pinhole and therefore is mostly excluded from the detector (photomultiplier tube). The excitation beam is scanned in a raster pattern across the specimen in a defined area. Emission from the focal plane from each point in the excited area is collected by the detector as photon fluxes. The changing voltage detector signal is digitised by a computer to generate pixels which are displayed on a monitor, thus generating an image. Focal planes at different depths can be scanned and the 2-D images generated can be compiled by the computer to render 3-D projections of the specimen. More detailed descriptions of the CLSM can be found in dedicated microscopy literature (e.g., Inouė, 1995; Murphy, 2001).

Bibliography

Inouė, S. (1995) Foundations of confocal scanned imaging in light microscopy. In *Handbook of biological confocal microscopy*, Vol. 2nd, pp. 1–17. Edited by J.B. Pawley. New York: Plenum Press.

Murphy, D.B. (2001) Fundamentals of light microscopy and electronic imaging. New York, Wiley-Liss.

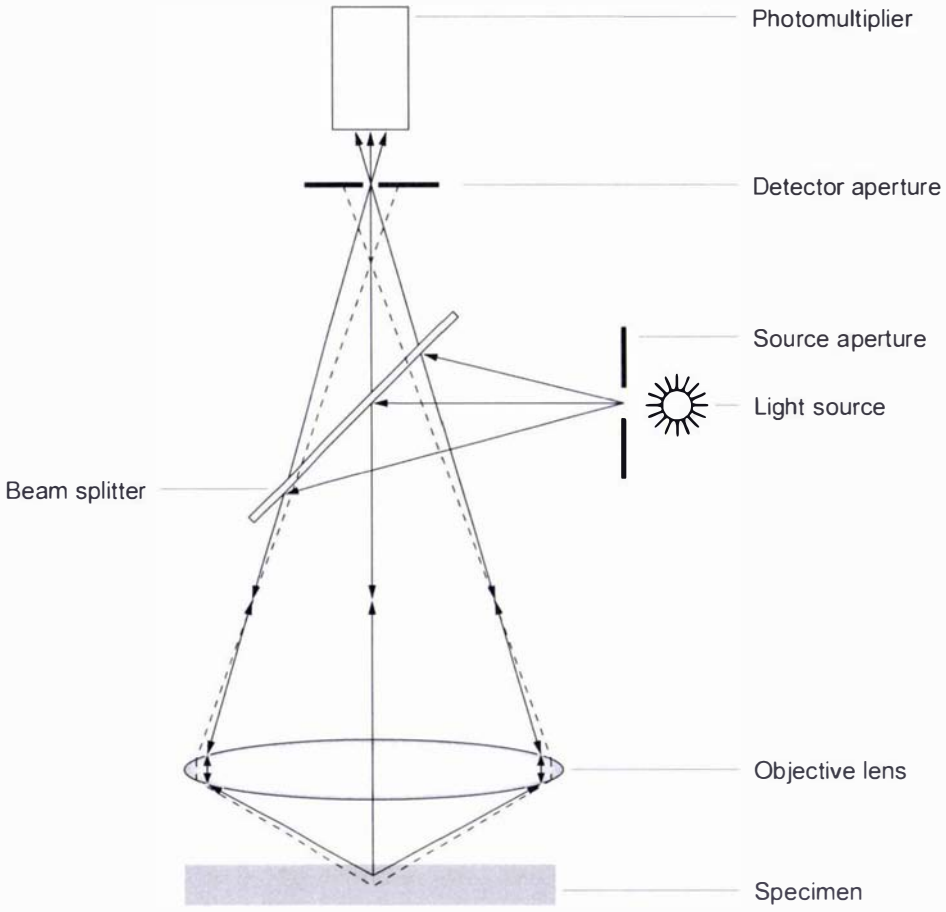

Figure A2.1 Schematic overview of the principle of the confocal laser scanning microscope. Dashed lines denote out-of-focus light which does not reach the detector.

Appendix 3 Sample staining and microscopy imaging protocol

This appendix describes the staining protocol that typically was used in all microscopy work that involved the use of fluorescent probes. Numbers within square brackets denote steps that may be omitted depending on circumstances.

[1] Prepare a probe stock solution. Wrap the vial in aluminum foil. Store the vial at the appropriate temperature when not in use.

2 Add a defined volume of the stock probe solution to a defined volume of the milk sample and mix gently by inversion of the vial.

3 Incubate the stained milk sample for a defined period of time at a defined temperature to allow proper staining of the fat globules.

4 Transfer an aliquot of the sample to a glass microscope slide. For conventional fluorescence microscopy typically a volume of 7.5 μL is suitable; for CLSM 6.5 μL is typically suitable.

[5][1] Gently place a cover slip on the sample, taking care not to cause damage to fat globules in the sample.

6 Observe the sample under the microscope.

7 Capture images that are representative of the sample.

1 This step may be omitted when using an inverted microscope.

Appendix 4 CLSM filter settings for selected fluorescent probes

Probe	Excitation laser (nm)	Beamsplitter	Channel 1		Channel 2	
			Beam splitter	Barrier filter	Beam splitter	Barrier filter
ConA488	488	RSP[1] 550	RSP580	LP[2] 515		
$DiOC_{18}(3)$	488	RSP 510	Mirror	LP 550		
$DiIC_{18}(3)$	488	RSP 510	Mirror	LP 550		
DiA	488	RSP 510	RSP 580	LP 515		
$DiIC_{18}(3)$-DS	488[3]	RSP 510	RSP 580	LP 515	Mirror	LP 530
Nile Blue	488	RSP 510	RSP 580	LP 515		
FM4-64	488	RSP 510	-	LP 590	Mirror	LP 590
FM4-64/ WGA488 combination	488	RSP 510	RSP 580	BP FITC[4]	Mirror	LP 590
FM1-84	488	RSP 510	RSP 580	BP FITC	Mirror or RSP 660	LP 530
WGA488	488	RSP 510	Mirror	LP 515		
WGA594	568	DD[5] 488/568	RSP 580	-	RSP 660	-
WGA647	647		RSP 580	-	RSP 660	LP 665

[1] Relative short pass.

[2] Long pass.

[3] Information from the manufacturer suggested that the excitation spectrum for this probe favoured excitation using a 568 nm laser. However, fat globules stained with $DiIC_{18}(3)$-DS were shown to have very poor fluorescence emission when excited at this wavelength. It appears that in MFGM the excitation wavelength range shifted from the green to the blue region of the visible spectrum.

[4] Band pass for fluoroscein isothiocyanate (515–545 nm).

[5] Double dichroic.

Appendix 5 List of lectins that have been shown to bind to intact MFGM or isolated MFGM

Lectin	Specificity[1]	BMFGM[2]	HMFGM[3]	MFGM of other species	Reference	Comments
ConA	α-D-mannose, α-D-glucose	+			Keenan *et al.* (1974)	ConA binding to washed membrane was measured using ^{3}H-acetylated ConA. This work suggested that all ConA binding sites in the membrane were externally exposed.
		<	<		Horisberger *et al.* (1977)	Electron microscopy was used to study washed fat globules, or MFGM, using lectin-labelled gold granules.
				+ (rat)	Sasaki & Keenan (1979)	Electron microscopy was used to study washed intact fat globules using ferritin-labelled ConA.
		+	+		Farrar *et al.* (1980)	Lectin binding was measured by means of an agglutination assay (1 h, 22°C) using extensively washed fat globules in PBS buffer.
				+ (caprine)	Patton & Hubert (1983)	Milk containing ^{3}H-labelled ConA was incubated at 37°C (various incubation times were tested). Fat globules were then isolated and washed and radioactivity was measured. MFGM fragments were prepared by employing the non-ionic detergent Triton X-100.
				+ (equine)	Welsch *et al.* (1988)	Fluorescent lectins conjugated with fluorescein isothiocyanate (FITC) were used. Fat globules were extensively washed and subsequently fixed in glutaraldehyde prior to assessment by conventional fluorescence microscopy.
			+		Buchheim *et al.* (1988)	It is assumed that Buchheim *et al.* (1988) used the same technique as that used by Welsch *et al.* (1988). ConA fluorescence was reported to be less intense than that of either WGA or soy bean agglutinin.
			<	< (equine); < (ovine)	Welsch *et al.* (1990)	The study of Welsch *et al.* (1990) contained only sketchy experimental details. It appears that milk was incubated (30 min) using FITC-labelled lectins. Fat globules were subsequently washed using PBS and mounted with glycerol. No microscopy details were reported.
Soy bean agglutinin	D-galactose; N-acetyl-D-galactosamine (GalNAc)	+			Horisberger *et al.* (1977)	
		+	+		Farrar *et al.* (1980)	
				+ (equine)	Welsch *et al.* (1988)	
			+		Buchheim *et al.* (1988)	

Lectin	Specificity[1]	BMFGM[2]	HMFGM[3]	MFGM of other species	Reference	Comments
			+	+ (equine); + (ovine)	Welsch *et al.* (1990)	
Wheat germ agglutinin	N-acetyl-D-glucosamine (GlcNAc); N-Acetylneuraminic acid (NANA); GalNAc;	+	+		Horisberger *et al.* (1977)	Control was performed using *N*-acetyl chitopentaose.
		+	+		Farrar *et al.* (1980)	Inhibition of agglutination was performed using GlcNAc.
				+ (equine)	Welsch *et al.* (1988)	Control was performed using GlcNAc.
			+		Buchheim *et al.* (1988)	No mention of controls.
			+	+ (equine); + (ovine)	Welsch *et al.* (1990)	Controls were performed "with corresponding inhibiting sugars". Presumably, this means GlcNAc as in Welsch *et al.* (1988).
Peanut lectin	D-galactose	–			Horisberger *et al.* (1977)	
				+ (equine)	Welsch *et al.* (1988)	
			+		Buchheim *et al.* (1988)	
			+	+ (equine); + (ovine)	Welsch *et al.* (1990)	
Griffonia simplicifolia agglutinin[4]	α-D-galactose; α-N-acetylgalactosamine	–			Horisberger *et al.* (1977)	
			<	+ (equine)	Welsch *et al.* (1988)	
				+ (equine); < (ovine)	Welsch *et al.* (1990)	
Ricinus communis Type I agglutinin	D-galactose; GalNAc	+	+		Farrar *et al.* (1980)	
				+ (equine)	Welsch *et al.* (1988)	
			+	+ (equine); < (ovine)	Welsch *et al.* (1990)	
Ricinus communis Type II agglutinin	Terminal D-galactose; GalNAc	+	+		Farrar *et al.* (1980)	
Helix pomatia agglutinin	GalNAc	+	–		Farrar *et al.* (1980)	
				+ (equine)	Welsch *et al.* (1988)	

Lectin	Specificity[1]	BMFGM[2]	HMFGM[3]	MFGM of other species	Reference	Comments
			+	+ (equine); – (ovine)	Welsch *et al.* (1990)	
Dolichos biflorus agglutinin	GalNAc	+	–		Farrar *et al.* (1980)	
				< (equine)	Welsch *et al.* (1988)	
			<	– (equine); – (ovine)	Welsch *et al.* (1990)	
Lens culinaris agglutinin	Mannose; glucose	<	+		Farrar *et al.* (1980)	
Ulex europaeus agglutinin[5]	L-fucose; di-N- acetyl-chitobiose	–			Horisberger *et al.* (1977)	
		–	–		Farrar *et al.* (1980)	
				– (equine)	Welsch *et al.* (1988)	
			+		Buchheim *et al.* (1988)	
			<	< (equine); < (ovine)	Welsch *et al.* (1990)	
Lotus tetragonolobus agglutinin	Fucose	–	–		Farrar *et al.* (1980)	
Arachis hypogaea (peanut agglutinin)	β-D-galactopyranosyl-(1-3)-N-acetyl-D-galactosamine (sometimes called Thomsen-Friedenreich antigen)	+	+		Farrar *et al.* (1980)	
Phytohaemagglutinin	GlcNAc			+ (equine)	Welsch *et al.* (1988)	
Limulus polyphemus agglutinin	Sialic acid		+		Welsch *et al.* (1988)	Pretreatment with neuraminidase was reported not to abolish the reaction with this lectin. Hence, the validity of a positive demonstration of sialic acid using this lectin was questioned.

[1] According to references cited in this table and/or Sharon & Lis (2003).

[2] Bovine MFGM; the symbols +, < and – signifiy moderate or strong binding, weak or little binding, or no binding, respectively. Absence of any symbol signifies that no published information was available.

[3] Human MFGM; symbols as for bovine MFGM.

[4] Also called *Bandeiraea simplicifolia* lectin.

[5] Also called anti-H lectin.

Appendix 6 Fat globule washing procedure

Raw bovine milk was warmed to 40°C in a water bath and weighed out into two cream Babcock tubes. These tubes were centrifuged in a Babcock centrifuge (1130 rpm, 40°C, 5 min; Super Vario N, Funke Gerber, Germany). The cream layer was transferred into preweighed empty Babcock tubes using plastic disposable pipettes (2 mL). Water (40°C) was added to fill half the tube and the cream and water were mixed by gentle swirling. The tube was then further filled with water (40°C) so that the meniscus was as high up in the neck as possible while ensuring that the weight of the two tubes agreed to within 0.5 g. The bottles were briefly (2–5 min) placed in the 40°C water bath to ensure that the temperature of the contents was about 40°C (centrifugation at this temperature ensures maximum separation of the cream phase). The bottles were centrifuged as before, and after centrifugation the removal of the cream phase, addition of water, warming of the contents and centrifugation were repeated as before. After removal of the cream phase from the original milk, three washing steps were performed in total. Assessment by bright field microscopy showed that the majority of fat globules were still spherical after three washes. Table A6.1 shows typical weights for the successive steps in this procedure.

Table A6.1 Typical weights for milk or cream and added water, and calculated dilution ratios for the cream fractions in the successive steps of the fat globule washing procedure

Sample	Milk or cream (g)	Water (g)	Dilution factor
Raw milk (a)	50.5	-	
Raw milk (b)	50.4	-	
Cream wash 1a	3.34	48.38	15
Cream wash 1b	4.31	47.84	12
Cream wash 2a	3.69	44.51	13
Cream wash 2b	3.68	44.50	13
Cream wash 3a	3.84	56.24	16
Cream wash 3b	3.73	57.20	16

Verification of absence of milk serum components was demonstrated by chemical analyses using both protein and lactose as indicators. Total nitrogen was determined according to the Kjeldahl principle[1] and protein content was calculated by employing a nitrogen conversion factor of 6.38. Lactose analyses of raw milk, skimmed milk and the serum of wash 1 were performed by the auto-analyser method[2]. However, for the serums of wash 2 and wash 3 samples, lactose

1 Fonterra Analytical Services Group method ACCA_053 version 1, *Determination of Nitrogen using Buchi.*

2 Fonterra Analytical Services Group method ACAA_01 version 7, *The Analysis of Chloride, Lactose and Phosphate by Autoanalyser.*

was determined by the phenol-sulfuric acid method[3] (which actually measures total reducing sugars, not lactose specifically) as the detection limit of auto analyser method was not sufficiently sensitive for accurate measurement of the expected low levels of lactose in these samples. Typical chemical data are shown in TableTable A6.2.

Table A6.2 Typical protein and lactose contents of the serum phases from the different fat globule washing steps

Sample	Protein (% w/w)		Lactose (% w/w)	
	Replicate 1	*Replicate 2*	*Replicate 1*	*Replicate 2*
Raw milk	3.39	–	4.94	–
Skimmed milk	3.52	3.54	5.06	5.11
Serum wash 1	0.11	0.15	0.23	0.28
Serum wash 2	0.01	0.01	0.01	0.01
Serum wash 3	0.01	0.01	< 0.01	< 0.01

The replicate results in Table A6.2 were in close agreement, which means that the washing procedure was a repeatable procedure as far as removal of serum components was concerned. The slightly higher results for both protein and !actose of skimmed milk compared with those of raw milk were due to the lowered fat content following the separation of the fat from the raw milk[4].

The total protein and lactose results of serum wash 1 were in good agreement with expected levels on a dilution calculation basis (0.3 and 0.4% w/w, respectively). The total protein results of the serums of washes 2 and 3 were at the limit of quantification for the type of samples and sample volumes used (E. Conaghan, Fonterra Analytical Services Group, personal communication, 11 September 2006). The close agreement of the level of the calculated protein content with the measured lactose content[5] and with dilution calculations[6] confirmed the absence of plasma components.

Hence, it may be concluded that after two washes of the cream phase, virtually all the milk plasma components had been removed, and that cream that had been washed three times could be considered to be completely free from milk plasma components.

3 Fonterra Analytical Services Group method ACCA_015 version 6, Determination of Lactose content in Caseins, Caseinates, WPC, MPC, Lactalbumin and Cheeses — Photometric method.

4 The fat content measured by infra-red (FT 120, Foss, Denmark) was 4.61% (w/w). Considering the low centrifugal force employed in the Babcock centrifuge, fat separation would not have been optimal and may have removed only about 4% fat from the milk sample. This estimate is in good agreement with the increases observed for both protein and lactose contents in the skimmed milk compared with those of the parent raw milk.

5 These are expected to be similar as the lactose content of the milk is of the same order of magnitude as that of the total nitrogen content.

6 For example, for serum 2 replicate 1, the expected total nitrogen result is 3.53/(16x13) = 0.02% (w/w), where the figures in the denominator are the dilution factors.

Appendix 7 Image capturing protocol for conventional fluorescence microscopy

1. View the sample under bright field using the x10 objective. This allows quick assessment of the entire slide and is particularly useful for determining whether significant fat globule clustering has occurred. Record observations.
2. Switch to the x40 objective, refocus under bright field and switch to fluorescence mode. Then quickly scan the slide (no longer than 2 s in any spot) to obtain an impression of the fluorescence of fat globules across the slide. Record observations.
3. Switch to bright field mode.
4. Switch to camera viewing mode.
5. Refocus the microscope so that in-focus images can be captured.
6. Switch to fluorescence mode and within 1 s activate the image capture command. As the camera takes several seconds to capture the image, it is important to move the stage to a different (unexposed) position to take another image of the same slide. This process will ensure that images are captured without causing any undue fading and allows for comparison of fluorescence intensity between different images.

Appendix 8 NMR spectroscopy

NMR spectroscopy is a well established analytical technique that has been in widespread use in the research community for over 50 years[1]. The work described in this Appendix was conducted in 2004 and at the time only two NMR studies relating to the MFGM had been reported in the literature (Chandan *et al.*, 1972; Murgia *et al.*, 2003). However, these studies did not involve direct NMR analysis of the milk sample[2]. The aim of the current study was to investigate whether NMR spectroscopy could be used as an analytical tool to measure the degree of damage suffered by fat globules in milk as a consequence of mechanical treatment.

A8.1 Experimental

A8.1.1 Sample treatment

Milk (1 L) from the bulk tank of Massey University dairy farm No. 1 was warmed to 37°C. About 500 mL was placed into a kitchen blender and blended at high speed for 60 s. (The intention was to test the NMR on an extreme case of mechanically agitated milk.) A subsample (~15 mL) of the blended sample and a control (i.e., untreated) sample were transferred to the Massey University NMR suite and stored at 5°C until analysis in the evening of the day of sample collection.

A8.1.2 NMR analyses

Experiments were performed using a Bruker Avance 400 NMR spectrometer (9.4 T). The NMR frequencies for ^{1}H and ^{31}P experiments were 400 MHz and 54 MHz, respectively. The sample temperature was maintained at 25°C. The spin-spin relaxation (T_2)[3] measurements were performed with the Carr-Purcell-Meiboom-Gill sequence (Callaghan, 1991) to determine the relaxation profiles for each sample.

A8.2 Results and discussion

The NMR signals were obtained from about 300 acquisitions. Although the correlation coefficients of the regression equations were not very high (0.86–0.96), the results of the experiment were sufficient for the purpose of the investigation (i.e., to determine whether differences between milk containing damaged fat globules and untreated milk can be detected using high resolution NMR).

A8.2.1 ^{1}H NMR

The relaxation constant of a molecule is related to its chemical structure and environment. The relaxation environment is a function of: (1) the level of interactions (an increased level of interaction leads to decreasing relaxation constant); (2) molecule mobility (a decreasing mobility

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1 A good general introduction to NMR can be found in Skoog & Leary (1992).

2 At a later date, a short communication was published in which buffalo milk and bovine milk were analysed directly by ^{31}P NMR (Andreotti *et al.*, 2006).

3 Also called 'traverse relaxation time". Denotes reduction of the average life time of an excited nucleus by interaction with neighbouring nuclei that have identical precession rates but different magnetic quantum states.

leads to a decreasing relaxation constant), and (3) size of the chemical environment (related to the level of interactions, the smaller the compartment the smaller the relaxation constant; Callaghan, 1991). It is assumed that the blending of the milk did not significantly change the chemical structure of the milk fat. Thus, any observed changes would only occur when the environment of the protons of the milk fat had changed. The ^{1}H relaxation curve showed bi-exponential behaviour, i.e. two relaxation zones were evident (fitted data shown in Figure A8.1).

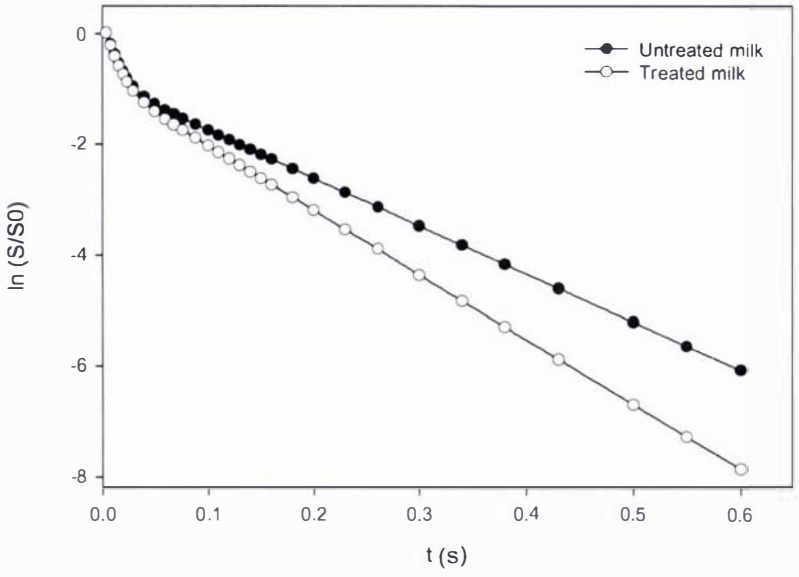

Figure A8.1 Plots of the fitted ^{1}H NMR data ($\ln[S/S_0]$) for untreated milk and treated milk, respectively.

In the fast relaxation zone (large slope; region from t = 0 s to about t = 0.04 s), no significant difference between the relaxation constants of the untreated sample and the treated sample was observed. The fast relaxation zone represents fat in a highly ordered state. This could possibly represent fat in relatively small fat globules. (The fat in small fat globules would have a more efficient interaction with the MFGM or fat globule-milk plasma interface than that in larger fat globules and thus would have fast relaxation times). Small fat globules are relatively resistant to shear forces, and they were not expected to have changed upon blending of the milk. Thus, this would explain why no significant differences were observed between the relaxation constants of the untreated sample and the treated sample. In contrast, the relaxation constants in the slower relaxation zone (smaller slope; region for which t > 0.04 s), which presumably represent the fat in larger fat globules, changed from 116 ms for the untreated milk to 86 ms for the treated milk. This indicated an increasing degree of interaction of the fat with the environment upon blending of the milk. Again, this is consistent with expectation, as the fat in larger fat globules would have undergone a change in environment upon blending of the milk, because many of the larger fat globules would have been disrupted and have generated smaller fat globules that were partially, or fully, coated with plasma protein. The combination of an

increased fat globule surface area and a proteinaceous[4] interface would be expected to result in faster relaxation times.

An alternative explanation for what the relaxation zones represent would be that the fast relaxation zone corresponds to lipid that is completely interior to all fat globules. The slow relaxation zone would then correspond to lipid that is in contact with the interface and that can move more freely than the lipid in the interior (hence slower relaxation time). Similarly to the previous explanation, the combination of an increased fat globule surface area and generation of a proteinaceous coat, through adsorption of plasma proteins onto the denuded fat upon blending of the milk, would be expected to result in faster relaxation times.

A8.2.2 ^{31}P NMR

The ^{31}P spectrum showed one major peak and some minor peaks (Figure A8.2). At the time the experiment was conducted, the major peak was assumed to represent the organic P (i.e., phospholipids). From a comparison with the chemical shift of the phosphorous peaks reported in a later study (Andreotti *et al.*, 2006), this appeared to be an erroneous assumption as the largest peak apparently represents the ^{31}P from inorganic phosphate. To amplify the organic ^{31}P signal (which was buried in the noise in the current experiment[5]), a minimum of 600 acquisitions would probably have to be made. This would require an analysis time of at least 12 h (The ^{31}P NMR analyses took 6 h for 300 acquisitions). As milk is a living medium[6], and physical changes such as creaming would occur, such a long analysis time would raise serious questions about the interpretation of the results and the practical use of the technique.

A8.3 Potential of NMR

Although ^{1}H NMR results showed differences in relaxation constants between the untreated sample and the treated sample, NMR spectroscopy was not considered to be a suitable technique for studying changes to the MFGM as a consequence of milk handling and treatment. The blending of the treated sample involved severe agitation of the milk for 60 s. Yet, only relatively small differences in NMR data between the untreated sample and the treated sample were observed. In practice, milk handling is not expected to be as extreme as that in this experiment. Therefore, NMR is unlikely to be a sensitive tool for measuring MFGM damage. Furthermore, the duration of the NMR analysis, particularly that of the ^{31}P NMR, the technically complicated environment (highly skilled NMR operators are required), as well as the high purchase price and maintenance costs preclude this technique from being used in a (semi-)routine environment for measuring changes to the MFGM. This conclusion agrees with that of

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4 Fat and protein interact via hydrogen bonding, which has the effect of changing the ^{1}H relaxation time of the fat to a value intermediate between that of its own and that of the protein. As the latter is lower than that of the fat, this would result in a reduced ^{1}H relaxation times for the fat, which agrees with the results of this study.

5 The organic phosphorous signal was not seen in the ^{31}P spectra. However, it could have been identified if the signal of the inorganic ^{31}P had been filtered out and the number of acquisitions had been increased to enhance the signal-to-noise ratio.

6 Bacterial growth may occur.

Vanhoutte *et al.* (2004) who believed that ^{31}P NMR analysis of foods would remain a research technique rather than a quality control method. Therefore, the development of an NMR technique was not further pursued in the current study.

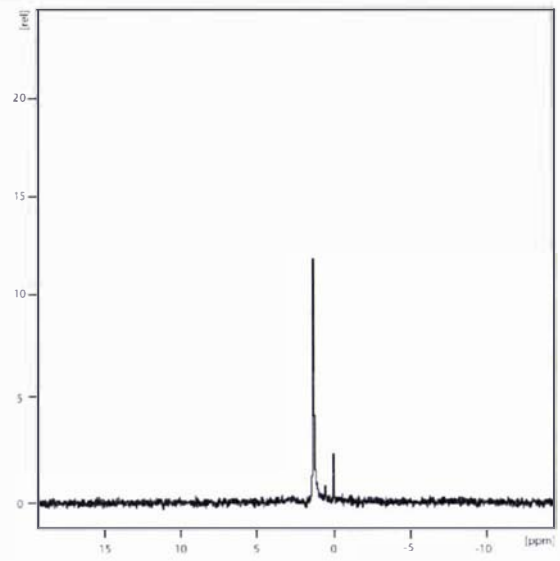

Figure A8.2 Phosphorus spectrum of untreated and treated milk samples. Phosphorus relaxation profile taken from the largest peak (inorganic P).

Acknowledgement

I thank Dr. J. P. Hindmarsh for performing the NMR analyses, analysing the data and discussions on the interpretation of the results.

Bibliography

Andreotti, G., Trivellone, E. & Motta, A. (2006) Characterization of buffalo milk by ^{31}P-nuclear magnetic resonance spectroscopy. *Journal of Food Composition and Analysis* 19, 843–849.

Callaghan, P.T. (1991) Principles of nuclear magnetic resonance microscopy. New York, Oxford University Press.

Chandan, R.C., Cullen, J. & Chapman, D. (1972) Physicochemical analysis of the bovine milk fat globule membrane. III. Proton magnetic resonance spectroscopy. *Journal of Dairy Science* 55, 1232–1236.

Murgia, S., Mele, S. & Monduzzi, M. (2003) Quantitative characterisation of phospholipids in milk fat via ^{31}P NMR using a monophasic solvent mixture. *Lipids* 38(5), 585–591.

Skoog, D.A. & Leary, J.J. (1992) Principles of instrumental analysis. Fourth edition. London, Saunders College Publishing.

Vanhoutte, B., Rombaut, R., Van der Meeren, P. & Dewettinck, K. (2004) Phospholipids. In *Handbook of food analysis*, pp. 349–382. Edited by L.M.L. Nollet. New York: Marcel Dekker.

Appendix 9 Summary of recommendations made in Chapters 2–7

Recommendation	Section
1. Confirm whether changes in the MFGM occur through membrane blebbing post secretion of the fat globule by the secretory cell.	2.2.2
2. Determine the distribution of cholesterol between the core fat, the MFGM and milk plasma upon cold ageing of milk.	2.3.1
3. Determine how cooling, ageing and pressurising milk changes the structure and composition of the MFGM.	2.3.1
4. Determine how heating affects the phospholipid content of the MFGM, and whether heating results in the release of native membrane from the fat globule.	2.3.1
5. Determine how temperature affects the fat globule size distribution of milk and cream as a function of temperature and time in the absence of mechanical agitation.	2.3.1
6. Investigate the relationship between the physical state of MFGM constituents and membrane stability.	2.3.1
7. Investigate the effect of mastitis on the MFGM.	2.3.2
8. Verify whether the quantity of membrane material is lower in mid-lactation than in either early or late lactation. Controlled experimental conditions are a pre-requisite.	2.3.3
9. Future research should increasingly focus on studying the milk fat globule in its native environment and in real time.	2.4
10. Investigate why the fluorescence intensity of $DiIC_{18}(3)$-DS-stained fat globules is greater in cold-aged milk than that in fresh milk.	4.5.2
11. Investigate the use of ethanolic stocks of fluorescent probes on the staining of fat globules or their membranes.	4.8.2

Recommendation	Section
12. Investigate whether replacing the methyl group on the pyridinium ring of DiA by a carboxylated or sulfonated alkyl chain of suitable length (or other modifications) would make the compound more soluble in water as well as promoting its location in the MFGM.	4.8.3
13. Determine what caused the differences in fluorescence intensity of FM4-64 in different parts of the MFGM.	4.8.3
14. Explore changing the length of the double bond bridge in DiIC18(3)-DS to obtain analogues that have the same, or a very similar, affinity for the MFGM as that of DiIC18(3)-DS, but that posses different excitation and emission wavelength spectra. (This is an important option to explore when developing multiple staining protocols as DiIC18(3)-DS has been found not to be compatible with some other probes because of emission spectrum overlap.)	4.8.3
15. Confirm that WGA binds to casein micelles by determining whether fluorescent particles are absent in WGA-stained milk in which the casein micelles have been dissociated or from which the casein micelles have been removed.	5.4.3
16. Confirm that β-lactoglobulin is involved in the disappearance of on-slide fat globule aggregation upon elevated heat treatment of milk by repeating the studies of Ye *et al.* (2004a, 2004b) on human milk or rodent milk, as milk from these species does not contain β-lactoglobulin.	5.8.3
17. Elucidate which MFGM proteins are lost upon using a particular washing technique, and where these were situated on, or in, the MFGM, by using a combination of a membrane-analytical approach (i.e., determining the average composition of the glycosylated compounds of the MFGM) and a microscopy approach (i.e., assessing the degree of membrane surface covered by glycosylated compounds) as a function of different washing techniques.	5.8.4
18. Further investigate the effect of WGA concentration gradient on fluorescence intensity of fat globules.	5.8.5
19. Determine the optimum concentration of WGA for different Alexa Fluor® conjugates, types of sample (e.g., washed fat globules re-suspended in a certain aqueous medium, or milk), source of fat globules (i.e., species), and type of microscope.	5.8.5

Recommendation	Section
20. The use of WGA conjugates in studying the structure and composition of the MFGM as described in this chapter should serve as a template for further studies of the MFGM using fluorescent conjugates of different lectins.	5.9
21. Extend the current studies using WGA by multiple staining studies in which both the fat globules and the milk plasma proteins (whey protein and casein) are fluorescently labelled	5.9
22. Develop a multi-staining protocol that resulted in the staining of both fat globules and other sources of membrane, including cells.	5.9
23. Determine whether the double ring phenomenon is a microscopy artifact.	6.2.7
24. Expand on the work that showed significant differences in both carbohydrate composition and distribution in MFGM of human, bovine and ovine origins, by employing different lectins that are known to bind to MFGM, and to conjugate these with selected Alexa Fluor® fluorophores to allow double or triple staining. This approach would, for example, quickly establish whether the non-fluorescent patches as shown by WGA are devoid of carbohydrate moieties altogether, or whether there is a specific grouping of other glycosylated matter at those domains.	6.4
25. Elucidate which factors affect the uptake of the tested lipophilic fluorescent probes by fat globules from milk of different species, as this will lead to a greater understanding of the differences in structure and composition of the MFGM between species.	6.4
26. Perform further screening of fluorescent probes as analogues of the fluorescent probes tested in this study might possess superior staining properties for fat globules from certain species of milk.	6.4
27. Investigate in more detail the effect of temperature-time combinations on the fluorescence intensity of fat globules stained with DiIC18(3)-DS.	6.5
28. Study the effect of heat treatment of milk by CLSM.	6.5
29. Investigate whether using an anti-fade improves the use of a range of fluorescent probes that were found to be photolabile in the current study.	6.7.1
30. Perform further experiments to evaluate whether the probes perturb the membrane or whether they reflect pre-existing membrane states.	6.7.3

Recommendation	Section
31. Develop both a wet-scanning AFM technique and a dry scanning AFM technique.	7.1.6
32. Explore the potential of using the micromanipulator technique.	7.2.4/7.2.5
33. Optimise the flow cytometry technique by optimising sample dilution conditions, reducing background signal, selecting appropriate dyes, and constructing appropriate counting windows.	7.3.3/7.3.4

Bibliography

Ye, A., Singh, H., Oldfield, D.J. & Anema, S. (2004a) Kinetics of heat-induced association of ß-lactoglobulin and α–lactalbumin with milk fat globule membrane in whole milk. *International Dairy Journal* 14(5), 389–398.

Ye, A., Singh, H., Taylor, M.W. & Anema, S. (2004b) Interactions of whey proteins with milk fat globule membrane proteins during heat treatment of whole milk. *Lait* 84, 269–283.

Appendix 10 Assessment of ethics of handling human milk

Massey University

OFFICE OF THE ASSISTANT
TO THE VICE-CHANCELLOR
(Ethics & Equity)
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 5573/350 5575
F 64 6 350 5622
humanethics@massey.ac.nz
animalethics@massey.ac.nz
gtc@massey.ac.nz
www.massey.ac.nz

20 December 2006

TO WHOM IT MAY CONCERN

Re: Mr Jacob Evers

Mr Evers submitted the following human ethics application for his doctoral research:

MUHEC: Southern A Application 06/07 – Measurement of milkfat globule (membrane) damage

The application was approved.

The Committee was subsequently advised by Mr Evers that the proposed study did not proceed for technical reasons beyond his control.

At the request of his supervisor, I discussed with Mr Evers the procedures he used in order to gather human milk samples for the conduct of his CLSM validation work and on this basis I am confident that the validation work was conducted in accordance with the requirements of Massey University's *Code of Ethical Conduct for Research, Teaching and Evaluations involving Human Participants*.

The Committee's terms of reference do not permit the consideration of applications for the retrospective approval of research that has already begun or been completed; consequently, the only ethical course of action available to Mr Evers and his supervisor is that which they have followed.

Yours sincerely

J. O'Neill

Professor John O'Neill, Chair
Massey University Human Ethics Committee: Southern A

cc Dr Owen McCarthy
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452