Structural Aspects of the Bovine Milk Fat Globule Membrane

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"essentially, all models are wrong, but some are useful"

--- George E. P. Box (1919 - 2013)
ABSTRACT

This research provides further understanding of the native structure of the bovine milk fat globule membrane (MFGM) and the role of structure on releasing from, and binding of volatile organic compounds (VOCs) to native milk fat globules (MFGs). The MFGM is constructed from a backbone of phospholipids and proteins that encapsulate the triacylglyceride core. The MFGM is believed to play an important role in many physiological processes (as a natural vehicle for delivering nutrients from the mother to the mammalian neonate). The bio-functionalities of the MFGM are regulated, not only by individual functional molecules (e.g. phospholipids and membrane proteins), but also by their specific structural organization. To date, the native structure and organization of the phospholipids and proteins are not fully understood. The research goals of this work are to extend the knowledge on MFGM protein organization (Chapter 3) and to develop an updated version of the topology of lipid organization within the MFGM (Chapter 4). Cluster of differentiation 36 (CD 36) and fatty acid-binding protein (FABP) were more strongly bound to the MFGM under the destructive forces applied to the MFGM through washing and centrifugation. The detailed localization of phospholipids and cholesterol in the MFGM is illustrated in a newly designed schematic model system in which phosphatidylcholine (PC) and sphingomyelin (SM) are located at the outer leaflet of the membrane bilayer, and phosphatidylinositol (PI) and phosphatidylserine (PS) are relatively enriched in the inner monolayer. By using confocal laser scanning
microscopy (CLSM), detailed structural damage to the MFGM through common mechanical processing conditions (centrifugal washing processes) was observed. An artificial bilayer membrane model system, namely giant unilamellar vesicles (GUVs), was constructed for mimicking the biological surface morphology of the native MFGM (Chapter 5). By characterizing GUV membranes with controlled composition, evidence was provided that the lipid domains on the native MFGM were not necessarily formed from an association of sphingomyelin and cholesterol only, as previously reported in the literature, but also from individual high phase transition temperature (higher than room temperature) phospholipids, such as dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin. The GUV system is a powerful tool for studying the morphology and functional properties of biological membranes on a much larger scale than the native MFGM, allowing observation using CLSM of the phase separation of lipids on the GUV surface. The small size of native milk fat globules usually precludes this type of observation due to the poor resolution of CLSM. The GUV system is also an ideal tool for investigating the detailed functionalities of individual MFGM phospholipids in regulating the lateral segregated lipid domains on MFGs surfaces (Chapter 6). By manipulating the molar ratio between sphingomyelin and cholesterol in GUV bilayers, it was shown that cholesterol is a key constituent in regulating lipid domain formation in MFGM and that phosphatidylcholine may also interact with cholesterol in the MFGM, contributing to lipid domain formation. During the course of this research, an innovative technique was developed further to investigate interactions between VOC
and native milk fat globules using confocal Raman microscopy (Chapter 7). Selected VOCs with different chemical characteristics, such as hydrophobicity, were used as probes to examine the structural feature of the MFGM and to investigate physicochemical functional properties (Chapter 8).
PEER REVIEWED PUBLICATIONS AND PRESENTATIONS

Publications:


Zheng, Haotian; Jiménez-Flores, Rafael; Gragson, Derek; Everett, David W., Phospholipid Architecture of the Bovine Milk Fat Globule Membrane Using Giant Unilamellar Vesicles as a Model Journal of Agricultural and Food Chemistry. 2014, 62 (14), pp 3236–3243

Zheng, Haotian; Jiménez-Flores, Rafael; Everett, David W., Lateral Lipid Organization of the Bovine Milk Fat Globule Membrane is Revealed by Washing Processes (Journal of Dairy Science, in press).
Conference presentations:


Haotian Zheng, Rafael Jiménez-Flores, David W. Everett, From giant unilamellar vesicles (GUVs) to lipid organization of the bovine milk fat globule membrane (MFGM). ADSA annual meeting, oral presentation (Indianapolis, USA, 8-12 July, 2013).

Haotian Zheng, Rafael Jiménez-Flores, David W. Everett, Centrifugal washing processes reveal lipid organization of the bovine milk fat globule membrane (MFGM). ADSA annual meeting, oral presentation (Indianapolis, USA, 8-12 July, 2013).

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LIST OF ABBREVIATIONS

2/3D: two/three-dimensional

α-lac: α-lactalbumin

β-Lg: β-lactoglobulin

ζ-potential: zeta-potential (droplet electric potential)

Ar+: argon-ion

ADPH: adipophilin

BTN: butyrophilin

CD 36: cluster of differentiation 36

CLA: conjugated linoleic acid

CLSM: confocal laser scanning microscopy

CN: casein

CRM: confocal Raman microscopy

$d_{32}$: surface mean diameter

$d_{43}$: volume surface-weighted mean diameter

DIC: differential interference contrast

DLPC: 1,2-dilauroyl-sn-glycero-3-phosphocholine

DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine/dipalmitoylphosphatidylcholine

DR: dark region

EDTA: ethylenediaminetetraacetic acid
EFAs: essential fatty acids
ELSD: evaporative light scattering detector
ER: endoplasmic reticulum
FABP: fatty acid binding protein
GC: gas chromatography
GMV: giant multilamellar vesicle
GUV: giant unilamellar vesicle
HLB: hydrophilic-lipophilic balance
HPLC: high performance liquid chromatography
IEP: iso-electric point
ITO: indium tin oxide
kDa: kilo Dalton
Lα: liquid crystalline phase
Lβ: gel phase
Ld: liquid-disordered
L0: liquid-ordered
Log P: partition coefficient between octanol and water
M (1): washing method (1)
MFG: milk fat globule
MFGM: milk fat globule membrane
MUC: mucin
Mw: molecular weight

NBD-PC: 1-palmitoyl-2-{6-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] hexanoyl}-sn-glycero-3-phosphocholine

NMR: nuclear magnetic resonance

O/W: oil in water

PA: phosphatidic acid

PAGE: polyacrylamide gel electrophoresis

PAS: periodic acid Schiff

PC: phosphatidylcholine/ L-α phosphatidylcholine

PE: phosphatidylethanolamine/3-sn-phosphatidylethanolamine

PI: phosphatidylinositol/ L-α-phosphatidylinositol

PL: phospholipid

PS: phosphatidylserine/1,2-diacyl-sn-glycero-3-phospho-L-serine

Rd-DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine rhodamine B sulfonyl)

RP-LC-MS: reverse phase liquid chromatography-mass spectrometry

RR: relative retention (of volatile organic compounds on milk fat globules)

SDS: sodium dodecyl sulphate

SM: sphingomyelin

SMUF: simulated milk ultra-filtrate

SPE: solid phase extraction

S.S.A.: specific surface area
Stdev.: standard deviation

TG: triacylglycerol

TLC: thin layer chromatography

T_m: phase transition temperature

VOC: volatile organic compound

XDH/XO: xanthine dehydrogenase/oxidase
# TABLE OF CONTENTS

Abstract .................................................................................................................................................. i
Peer reviewed publications and presentations ................................................................. v
Acknowledgments................................................................................................................................... vii
List of Abbreviations .......................................................................................................................... x
Table of Contents ................................................................................................................................. i

Chapter 1. ............................................................................................................................................. 1
  1.1. Background .................................................................................................................................. 2
  1.2. Overview of research ................................................................................................................... 5
    1.2.1. Chapter 3 .............................................................................................................................. 5
    1.2.2. Chapter 4 .............................................................................................................................. 5
    1.2.3. Chapter 5 .............................................................................................................................. 6
    1.2.4. Chapter 6 .............................................................................................................................. 7
    1.2.5. Chapter 7 .............................................................................................................................. 8
    1.2.6. Chapter 8 .............................................................................................................................. 9

Chapter 2. ............................................................................................................................................. 11
  2.1. Bovine milk ................................................................................................................................. 12
  2.2. Bovine milk fat globule (MFG) and milk fat globule membrane (MFGM) .................. 20
    2.2.1. Composition and structure of the bovine MFG and MFGM ........................................ 20
    2.2.2. Bovine MFGM lipids ........................................................................................................ 26
    2.2.3. Bovine MFGM proteins .................................................................................................... 29
    2.2.4. MFGM isolation from raw milk ....................................................................................... 30

Chapter 3. ............................................................................................................................................. 32
  3.1. Introduction ................................................................................................................................. 33
  3.2. Materials and methods ................................................................................................................ 36
    3.2.1. Materials .......................................................................................................................... 36
    3.2.2. Isolation of Milk Fat Globules ....................................................................................... 36
    3.2.3. Fat Globule Size and Specific Surface Area ................................................................. 38
    3.2.4. Determination of $\zeta$-Potential .................................................................................... 39
    3.2.5. Total Protein and Fat Content ......................................................................................... 39
    3.2.6. Characterization and Quantitation of MFG-Associated Protein Components .......... 40
8.2. Materials and Methods .................................................................................................173
  8.2.1. Materials ......................................................................................................................173
  8.2.2. Recombined MFG emulsion system .........................................................................174
  8.2.3. MFGs + VOCs ............................................................................................................174
  8.2.4. Confocal Raman microscopy characterization .............................................................175
  8.2.5. Statistical analysis .......................................................................................................176
8.3. Results and Discussion ....................................................................................................176
  8.3.1. Spectra characterization .............................................................................................176
  8.3.2. Estimation of relative retention .....................................................................................181
8.4. Conclusions .....................................................................................................................186
Chapter 9 ................................................................................................................................187
  9.1. Conclusions ....................................................................................................................188
  9.2. Future Recommendations ...............................................................................................190
References ...............................................................................................................................193
CHAPTER 1.

Introduction
1.1. Background

Bovine milk is an important source of food for humans and has numerous applications in the food industry. The incorporation of bovine milk components into baby food formulation has resulted in significant research attention towards the nutritional needs, growth, and health of mammal neonates. Fat (31% w/w) is the second largest constituent of bovine milk dry matter having nutritional significance (Parodi, 2006), after lactose (36% w/w), and it is shaped in the form of milk fat globules (MFGs) with most of the globules having diameters in the range of 0.2 to 15 µm (Huppertz and Kelly, 2006, Walstra et al., 2006). MFGs are suspended in the aqueous phase of milk and are stabilized by a “bio-active” membrane system, namely the milk fat globule membrane (MFGM). The MFGM system as a whole or some of its associated individual components have physiological and nutritional functions (Argov et al., 2008a, Dewettinck et al., 2008); these functions are considered as the “bio-activity” or “bio-functionality” of the MFGM.

It is believed that the nutritional strategy of mammals is powered by a Darwinian engine and therefore the nutritional value of milk ingredients is maximized for infants’ survival under the evolutionary driving force. It is also implied that the reason for the synthesis of the unique structure of the MFG in the mammary gland may be related to infants’ health (Argov et al., 2008a, German and Dillard, 2006). Understanding both MFG assembly and MFGM structures is necessary for better understanding of
physiological communication between lipids and cell compartments in the GI tract. This leads to the opportunity to improve the formulation of infant formulas with high bio-accessibility and maximal mimicking of the functionality of mothers’ milk (the “bio-accessibility” may be refer as digestibility and absorption of nutrients). Although recent research has been carried out to examine the native structural organization of the MFG and MFGM in terms of lipid and protein arrangements (Lopez et al., 2011, Lopez et al., 2010, Michalski et al., 2002), the exact structure of the MFGM is not fully known, and there is still space left to improve our knowledge in this area.

MFGM-based products are currently sold on the market from such companies as Arla Foods (enriched protein powder with MFGM components “Lacprodan® MFGM-10”, Denmark) and Fonterra (MFGM-derived lipids, “dairy complex lipids”, New Zealand). Successful formulation requires fundamental knowledge of the native MFGM structure to extract the appropriate components from raw milk. Previous research has examined changes in MFGM composition, structure and functionality after thermal and mechanical treatments (Anderson et al., 1972, Bermudez-Aguirre et al., 2008, Britten et al., 2008, Cano-Ruiz and Richter, 1997, Corredig and Dalgleish, 1998, Gallier et al., 2010a, Houlihan et al., 1992a, Houlihan et al., 1992b, Rombaut et al., 2006, Rombaut et al., 2007, Ye et al., 2004a, Ye et al., 2005, Ye et al., 2002). Homogenization induces structural changes that impact upon digestion and absorption of milk components (Michalski and Januel, 2006); however, conclusions from studies such as this are controversial and the results may depend upon species used in these in
vivo experiments. Generally, homogenized milk is more easily digested than untreated milk as raw milk may result in a lower gastric emptying rate (Meisel and Hagemeister, 1984); however, in infants, native human MFGs are more favourable to gastric lipolysis than homogenised MFGs (Favé et al., 2004). These phenomena further emphasise the complexity of both structural and functional aspects of the MFGM. Nevertheless, fundamental knowledge of the MFGM physico-chemical properties enables construction of a solid database for studying more detailed structural, physical and chemical features of the native MFGM.

The complexity of the MFGM requires an innovative approach to understand the structure (Argov et al. 2008). In this current work, three ideas are proposed to provide more detailed fundamental knowledge on the structural organization of the bovine MFGM. Firstly, the relative binding strength of major membrane proteins and lipids to the membrane after washing and the location of these components within the MFGM were examined after centrifugation, a common mechanical treatment used in the dairy industry. Secondly, a model bilayer system was constructed with controlled composition to examine the morphology of the MFGM. Thirdly, an innovative approach was further developed for rapid and non-destructive characterization of MFGs and the MFGM structure and physicochemical properties using confocal Raman microscopy.
1.2. Overview of research

Six programmes were carried out to examine the structure of the MFGM. These are contained within chapters 3-8.

1.2.1. Chapter 3

A model for the topology of major bovine MFGM proteins, including relative intermolecular binding, has been developed (Keenan. and Mather., 2006, Mather, 2000, Mather, 2011). Although the general native arrangement of the major MFGM proteins has already been shown, a comparison of membrane binding strengths between these major MFGM proteins is not clear, especially after destructive forces (mechanical treatments) are applied to the surface of MFGs during dairy processing. Such detailed structural information of the rearrangement of MFGM proteins after mechanically treating the MFG surface provides further understanding of the MFGM structure, which is essential knowledge for handling raw milk, cream, and butter milk prior to MFGM isolation and fractionation. In this chapter, centrifugal washing processes were applied to the surface of MFGs, and the major MFGM proteins and structural changes to the MFG surface upon washing were characterized, along with competitive binding strengths between major MFGM proteins.

1.2.2. Chapter 4
Unlike MFGM proteins, the locations of lipids, including both phospholipids and cholesterol, in the MFGM are not definitively known. A schematic model of MFGM was proposed in which the authors hypothesised that the distribution of different types of phospholipids in the MFGM depends on the hydrophilic-lipophilic-balance (HLB) number (Michalski et al., 2002); however, experimental evidence to support this view is scarce. Moreover, understanding on the structural organization of the phospholipid monolayer located underneath the outer bilayer is still controversial. In another published model system, the phospholipids form a unitary monolayer and this is separated from a membrane protein layer (Lopez et al., 2011). In Michalski’s model system, phospholipids and membrane proteins are associated with each other and form a binary monolayer (Michalski et al., 2002). To examine this further, mechanical washing processes, as defined in Chapter 3, were applied. By characterizing the surface changes in terms of the amount of lipids and composition (the five major phospholipid species and cholesterol), and taking the phospholipid HLB into account, a native lipid structural model of the MFGM was proposed.

1.2.3. Chapter 5

The detailed structural organization of the outer bilayer of MFGM lipids is not conclusively known due to the complexity of this membrane. Lipid domain segregation on the surface of MFGs has been observed using CSLM after fluorescent staining, revealing a heterogeneous distribution of phospholipids on the outer bilayer,
and it is believed that concentration of cholesterol and SM occurs in liquid-ordered domains (L₀) (Gallier et al., 2010b, Lopez et al., 2010). Direct evidence for the composition of these lipid domains, and the mechanism of lipid domain formation in the MFGM, is still scarce due to limited methodologies and instrumental techniques to examine such small areas of lipid micro-domains and analyse the composition. A simplified artificial model membrane system where the composition is controlled and potential interactions with other components minimized would be ideal for investigating the surface morphology of MFGM and the composition of lipid micro-domains. Therefore, in this chapter, non-supported lipid bilayers, namely giant unilamellar vesicles (GUVs) containing phospholipids and/or cholesterol were constructed to mimic the surface morphology of the MFGM for revealing the nature of lipid domains in the MFGM. The phospholipid composition used for generating GUVs was controlled, along with differences in Tₘ and temperature during microscopic observation of the GUV surface by CLSM.

1.2.4. Chapter 6

Cholesterol plays an important role in inducing the formation of L₀ in lipid bilayer systems (Levental et al., 2009, McConnell and Vrljic, 2003, Simons and Vaz, 2004). Lateral segregation of lipid domains on the surfaces of native MFGs had been observed under CLSM with fluorescent staining, and these lipid domains were deduced to be L₀ phases enriched in cholesterol and SM (Gallier et al., 2010b, Lopez
et al., 2010). Further problems need to be examined, such as how cholesterol and SM regulate lipid domain formation, and which of these makes the major contribution to micro-domain formation in MFGM. In this chapter, GUV systems were constructed from a set of designed lipid formulas (made up of MFGM-derived PLs) in which the molar ratios of cholesterol and SM were manipulated and the molar volumes of PE and PC were controlled. The non-stainable areas on GUV surfaces, as observed under CLSM, were quantified. Consequently, based on the correlation between area of non-stainable region on GUVs and molar volume SM and/or cholesterol, the composition of the Lo phase on native bovine MFGs was deduced.

1.2.5. Chapter 7

The complete overall structural organization of the MFGM is not fully known due to a lack of appropriate techniques. Raman spectroscopy has been used for identifying food components for quality control purposes, and confocal Raman microscopy (CRM) has been applied to investigate microstructure of food matrixes (Li-Chan, 1996, Pudney et al., 2002). The Raman spectroscopic compositional fingerprint of bovine MFG and MFGM has been previously studied (Forrest, 1978, Gallier et al., 2011) providing fundamental information for subsequent development of structural models. The diameter of lipid micro-domains on larger MFGs (15-20 µm diameter) are around 2 µm (Gallier et al., 2010b) which is below the resolution of CRM for specifically analysing the Raman spectra of these targeted domains. More importantly,
the micro-domains are continually moving on the surface of MFGs, therefore CRM cannot easily be used to localize and map the Raman spectra of lipid domains. To realize the utilization of CRM in MFG structural studies, proper probes to label MFG with characteristic Raman spectra are needed. In this chapter, an approach was developed to examine the MFG using organic volatile compounds (VOCs) and characterizing their interactions with the membrane using CRM. It is reasonable to assume that VOCs, particularly those that are more hydrophobic, closely interact with MFGs. VOC partitioning and release from emulsion systems are often determined using headspace gas chromatography, requiring long periods of time for examination of relatively short time-scale experiments. CRM provides a method for rapid measurement of VOC adsorption to the surfaces of emulsion droplets.

1.2.6. Chapter 8

The general tri-layer structure of the MFGM had been reviewed from the perspective of the secretion pathway of MFGs from the mammary gland (Keenan. and Mather., 2006, Mather, 2011); however, experimental evidence post-milking for supporting the existence of a primary monolayer between phospholipid outer bilayer and TG inner core is scarce. As the thickness of MFGM is only around 10-20 nm (Mather, 2011, Wooding and Kemp, 1975), it is hard to distinguish layers within the MFGM by current microscopic techniques. In this chapter, VOC probes were added to MFG recombined emulsion systems for labelling MFGs. After reaching partition
equilibrium, VOC concentration at the MFG surface was measured using CRM and was used to shed insight into the trilayer arrangement of the MFGM. A hypothesis was proposed that if phospholipids and membrane proteins are homogeneously distributed in the MFGM, then the retention of VOCs at the MFG surface is simply determined from the VOC and hydrophobic TG core; otherwise, the phospholipids and membrane proteins may be assumed to be asymmetrically distributed in the MFGM with a protein-dense monolayer separate from the phospholipid bilayer. Moreover, in previous chapters, VOC retention on the MFG surface was examined using CRM and it was shown that VOCs may partition to the interior of the MFG; however, the detailed mechanism of such interaction is still not clear. The role of the MFGM in such interactions, and interactions of VOCs with the MFGM and MFG interior as separate components need to be further studied. In this chapter, the partitioning of two VOCs with differing hydrophobicity on MFG surfaces was examined using CRM, thus providing insight into the structure of the MFGM.
CHAPTER 2.

Literature Review
2.1. Bovine milk

Fundamental knowledge of the composition, structure, physicochemical properties and physiological functionalities of bovine milk has been extensively investigated (Walstra et al., 2006). Milk is secreted from mammary glands as the primary source of nutrition and energy for the neonate. The term “milk” generally includes milk from all kinds of mammals, however, in this current research the focus is on bovine milk as it widely consumed and used for producing commercial dairy products. The general composition of bovine milk is shown in Table 2.1.

Milk components are synthesised in the secretory cells of mammary glands (Keenan and Mather, 2006, Mather, 2011). Firstly, the precursors of milk components are sourced from blood and then secreted into the lumen. Milk proteins are formed in the endoplasmic reticulum then, together with other soluble components, are collected from Golgi vesicles. These vesicles eventually open and the contents are released into the lumen. Triglycerides are synthesized in the cytoplasm and packaged as small fat globules; these keep growing in size and move to the apical end of the cell, then are released out through the lumen. During this process, the cell is not destroyed. The term for such secretion is called “merocrine” (Walstra et al., 2006). Merocrine in the cow is different to apocrine in the goat in terms of milk secretion (Wooding et al., 1977, Wooding and Peaker, 1970). The mammary function may be influenced by biological active substances (Peaker and Wilde, 1996). Whey protein is considered as
feedback inhibitor of lactation (FIL) and it may regulate the rate of milk secretion. In the mammary glands where milk is accumulated, the volume of FIL is increased and milk production may be slowed; it is also reported that the fat content of milk may be increased from relatively emptier mammary glands (Jones and Spencer, 2007).

Table 2.1 General composition of bovine milk a.

<table>
<thead>
<tr>
<th>Component</th>
<th>Average content in milk (%)</th>
<th>Range</th>
<th>Average content in dry matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87.1</td>
<td>85.3-88.7</td>
<td>-</td>
</tr>
<tr>
<td>Solids-not-fat</td>
<td>8.9</td>
<td>7.9-10.0</td>
<td>-</td>
</tr>
<tr>
<td>Fat in dry matter</td>
<td>31</td>
<td>22-38</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.6</td>
<td>3.8-5.3</td>
<td>36</td>
</tr>
<tr>
<td>Fat</td>
<td>4.0</td>
<td>2.5-5.5</td>
<td>31</td>
</tr>
<tr>
<td>Protein</td>
<td>3.3</td>
<td>2.3-4.4</td>
<td>25</td>
</tr>
<tr>
<td>Caseins</td>
<td>78.3*</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Serum proteins</td>
<td>19*</td>
<td>-</td>
<td>4.9</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>2.7*</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td>Minerals</td>
<td>0.7</td>
<td>0.57-0.83</td>
<td>5.4</td>
</tr>
<tr>
<td>Organic acids</td>
<td>0.17</td>
<td>0.12-0.21</td>
<td>1.3</td>
</tr>
</tbody>
</table>

a(Walstra et al., 2006).

* Reported as the proportion of total protein (% w/w).
Milk is a whole food that meets the nutritional needs of suckling mammalian neonates. The biological significance of milk components does not depend on the proportions in milk. For instance, vitamins and minerals only account for a small portion of milk dry matter, however they have significant nutritional value (Walstra et al., 2006). Milk constituents have physiological functionalities. Immunoglobulins may ward off infection in newborns, and colostrum may provide immunity for new born mammals (Shah, 2000). Lactoferrin accounts for a minor proportion of milk, however its physiological functionality is not insignificant: *in vitro* experiments showed that bovine lactoferrin may promote the growth of infant strains of *Bifidobacterium* species (Petschow and Talbott, 1991). Lactoferrin also shows antimicrobial effects (Payne et al., 1990, Saito et al., 1991). The bacteriostatic effect of lactoferrin may be further promoted by combining with milk lysozyme and/or EDTA (Facon and Skura, 1996).

Lipids are one of the principle components of milk. There are six general classes of lipids in fresh milk, neutral glycerides, free fatty acids (FAs), phospholipids (PL), sphingolipids (SL), sterols and carotenoids + vitamin A (vitamins). The major lipid component, TGs of neutral glycerides, accounts for 98.3% (w/w) of total milk fat and is contained within the inner core of the MFG. The variety of TGs is due to the distribution of FAs. There are at least $10^5$ different molecular species of TGs present in the milk lipid fraction. TGs are non-polar and not surface active, therefore are an ideal solvent for other non-polar milk components. Small amounts (< 0.5%, w/w total
lipid) of di- and mono-glycerides and fatty acids are also present in milk (Walstra et al., 2006). The structure of TG in milk is important to be able to understand the changes that are induced by processing and the action of lipases (Jensen et al., 1991). The structure of bovine milk TG can be understood by identifying the fatty acids in each of the sn positions (Christie and Clapperton, 1982). Linear relationships between the FA content and the sn positions in TG have been found; consequently, it is possible to predict the individual TG content of milk fat from a single fatty acid analysis by using regression equations from analyses of the FA positions (Parodi, 1982, Parodi, 1983). Cholesterol is an apolar molecule that is located within the MFGM in association with PLs. Cholesterol accounts for 3.3 mg/g milk fat (in whole milk) as the major sterol (MacGibbon and Taylor, 2006) and its content is related to total fat content (Jensen et al., 1991, Jensen et al., 1990).

Polar lipids, including phospholipids and sphingolipids, account for around 1% (w/w) of total milk lipid. These constitute the backbone of the MFGM for enveloping TGs within the inner core of the MFG. Generally, phospholipids are composed of charged phosphatidic acid (PA) and various organic bases, whereas SM is built upon a sphingosine structure. Phospholipids can contain both acidic and basic groups, therefore are amphipolar molecules and are highly surface active. Thus, phospholipids are good emulsifiers to stabilize MFGs. The FA distribution in phospholipids is remarkably different to that in TGs; PLs contains more long chain FAs than TGs and glycerol-PLs have a relatively higher amount of unsaturated FAs than other milk
lipids, on average (Walstra et al., 2006). Milk polar lipids have been separated from nonpolar lipids using solid phase extraction, and the polar and nonpolar classes isolated and identified via thin layer chromatography (TLC) (Bitman and Wood, 1990). Due to the relatively high content of long-chain polyunsaturated fatty acids in milk phospholipids, they may be highly susceptible to autoxidation (Jensen et al., 1991).

From a nutritional point of view, milk fat is a primary source of energy (37 kJ/g) compared to other major milk constituents such as proteins (17 kJ/g) and sugars (16 kJ/g). This functionality is especially important for new-borns who consume milk as their only source of food (Walstra et al., 2006). The ratio between carbon and oxygen atom content determines the energy value of a fatty acid; consequently, stearic acid (C\textsubscript{18:0}) has higher energy value (9.48 kcal/g) than butyric acid (C\textsubscript{4:0}, 5.92 kcal/g). After digestion of milk fat, the relatively shorter FAs (< 12 C) may be absorbed by the intestine and eventually transferred to the liver for metabolism, whereas, the longer FAs are resynthesized into TGs and are transported into the venous circulatory system (Parodi, 2006). About one-third of FAs having low energy value in milk may be rapidly oxidized in the liver; therefore, it is considered that milk fat has less of a contribution to obesity than the equivalent amount of other dietary lipids (Parodi, 2004).
Milk fat is also a good solvent for dissolving vitamins A, D and E. Moreover, milk fat is good source of linoleic and linolenic essential fatty acids (EFAs) which are precursors for certain hormones. EFAs are also part of PLs giving long chain, low melting point FAs for maintaining the morphological surface fluidity and the structure of the MFGM (Walstra et al., 2006). Correlations between fat consumption and incidence of cancer has been studied (Kushi and Giovannucci, 2002, Kushi et al., 1995, Willett, 2001); however, it has to be pointed out that in dairy products, milk fat is consumed in a food matrix combined with anti-carcinogenic components (Parodi, 2001, Parodi, 2006). For instance, the anti-carcinogenic potential of milk fat components has been reported: conjugated linoleic acid (CLA) has the ability to inhibit mammary tumour development (Ip et al., 2003); the hydrolytes of sphingolipids, ceramide and sphingosine can restrain the development of colon cancer (Duan, 1998, Vesper et al., 1999); butyric acid in milk fat may inhibit several human cancer cells (Williams et al., 2003); and from in vivo studies, cholesterol may inhibit colon tumours via inhibition of the promotion of aberrant crypt foci (ElSohemy et al., 1996).

From a digestion point of view, the route or routes for lipid (particle) transferred from the intestine into the circulating fluid of the lymphatic system or blood is not fully known. Dietary lipids are present as TG chylomicrons in the gut, lymph and blood, and leads to a controversial discussion about whether lipids in the intestine may transport through the gut wall without alteration (Singh et al., 2009). Singh and co-
workers (2009) summarized general lipid digestion and absorption as three key steps from the perspective of emulsion structure. The first step is dispersion of dietary lipids into smaller particles in the stomach. This process creates lipid-water interfaces that are more accessible to lipases. The second step is partial hydrolysis (10-30%) of TGs by gastric lipases in the gastric juice. The hydrolysed lipids are emulsified (stabilized) and transported to the small intestinal lumen. The third step is absorption of digested lipids at the enterocyte plasma membrane. During this step, undigested phospholipid vesicles are formed and bile salt micelles play an important role in solubilisation of digested lipids, which is essential for the communication between digested lipids and enterocytes for absorption (Singh et al., 2009). The phospholipid component in the digestive processes implies that MFGM phospholipids play a role in regulating digestion rate of MFGs. It has been shown from an in vitro study that MFGM-phospholipids form liposomes that prevent the gastric degradation of lactoferrin (Liu et al., 2013).

Protein is the third major component in terms of milk dry matter content (Table 2.1). Milk protein is a term representing a mixture of different types of proteins. The types and relative proportions of milk proteins depend upon both intrinsic and extrinsic factors such as feed, environment, post-translational modifications, and genetic variants. There are two major groups of proteins in milk: caseins and serum proteins. Caseins are not soluble close to the average isoelectric point (IEP, pH 4.6). No denaturation of caseins is induced by heating (< 120°C). Caseins are present in milk in
the form of aggregates, called casein micelles, protected by a surface steric barrier of κ-casein molecules. Micelles may be clotted by rennet when κ-casein is hydrolysed through enzymatic reactions and the steric protection is lost. Clotting of caseins is an essential step for cheese-making. The α₁- and α₂-caseins account for more than 40% (w/w) of the total milk proteins. β-Casein comprises 26% (w/w) of milk proteins and may be split into γ-caseins and proteose peptone fractions by enzymatic action. Both αs- and β-caseins are phosphoproteins. Serum proteins, also called whey proteins, are globular proteins which are soluble at their respective IEPs and can be denatured during heating. α-Lactalbumin and β-lactoglobulin are major serum proteins; serum albumin, proteose peptones, lactoferrin, transferrin, enzymes and immunoglobulins comprise a minor proportion of the serum proteins. In addition to caseins and whey proteins, MFGM proteins account for 2% (w/w) of total milk proteins (Walstra et al., 2006).

Skim milk proteins, mainly whey proteins and caseins, may interact with the MFGM under specific conditions. In studies of biological layer model systems, it was found that β-casein can penetrate a 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) monolayer at specific pH values (Caro et al., 2009). Interactions between β-casein and an artificial phospholipid monolayer have been observed. During homogenization, although the whole MFGM structure is ruptured, caseins adsorb to MFGs via association with the Lₒ domains on MFGM, with PE being the key phospholipid involved in this interaction (Gallier et al., 2012). Whey proteins α-lac and β-lg can
associate with MFGM via disulphide bonds upon heating (60 – 95°C) of whole milk, and the amount of associated whey proteins increases with an increase of temperature (Ye et al., 2004b); however, the interaction behaviour of α-lac does not seem to change with temperature (Corredig and Dalgleish, 1996). Homogenization may induce adsorption of caseins but no binding of whey proteins without heating occurs to the MFGM (Lee and Sherbon, 2002).

From a structural point of view, milk is an oil-in-water (o/w) emulsion system in which the milk fat is emulsified as globules. Casein micelles are another component of the dispersion system on a smaller size scale. The aqueous phase contains a colloidal suspension in which globular protein molecules are suspended, plus a solution containing lactose and minerals (Walstra et al., 2006, Bylund, 2003).

2.2. Bovine milk fat globule (MFG) and milk fat globule membrane (MFGM)

2.2.1. Composition and structure of the bovine MFG and MFGM

The size of most MFGs in bovine milk ranges between 0.2 and 15 µm with a volume-weighted mean diameter ($d_{43}$) between 3.5 and 5.3 µm (Huppertz and Kelly, 2006). Native MFGs in milk have a negative surface potential, approximated by a zeta-potential of -12 mV (Singh, 2006). As stated earlier, the MFG is a multicomponent complex mainly composed of neutral lipids (TG) in an inner core. The TG core is
encapsulated by the MFGM containing polar lipids, cholesterol, and apical plasma membrane-derived proteins. The MFGM makes up 2 - 6% (w/w) of whole MFGs (Keenan. and Mather., 2006). Although the MFGM is only a small proportion of the MFGs in terms of weight, its structural organization is the most complicated part of the MFG and its physiological and physicochemical functionalities are significant. It is necessary to point it out that the physical properties (size and surface charge) and composition of MFG and MFGM may vary according to the breed of cow, lactation stage, and feed (Lopez et al., 2008, Ménard et al., 2010, Singh, 2006, Walstra et al., 2006).

The origin and secretion mechanism of MFGs has been studied and extensively reviewed (Keenan. and Mather., 2006, Mather, 2000, Mather, 2011, Mather and Keenan, 1975). Briefly, MFGs are generated from the secretory cells of the mammary gland. The triacylglycerol inner core is synthesized in the endoplasmic reticulum (ER) where fatty acyl chains are attached to diacylglycerols with the assistance of ER-bound terminal transferases. During secretion, it is presumed that the primary layer, containing polar lipids and proteins derived from the ER and cytoplasm, cover the surface of the nascent small lipid droplets. These lipid droplets may be transported to the apical cytoplasm and then coalesce into larger droplets. At the apical pole of cell, the newly formed larger droplets interact with the membrane material and are secreted from the epithelial cells and coated with plasma membrane (Keenan and Mather, 2006, Mather, 2011). Therefore, the general structure of the MFG is a TG droplet with
multilayer coverage of membrane material. The MFGM is considered as a trilayer system: the primary inner layer is composed of proteins and polar lipids originating from the cytoplasm and ER; the outer bilayer is mainly constructed from phospholipids and other plasma membrane materials (Keenan. and Mather., 2006, Mather, 2011, Singh, 2006).

The topology and structural organization of the MFGM in terms of both lipid and protein organization are not fully known. There are several proposed models of the native structure of the MFGM based on direct and indirect experimental evidence (Lopez et al., 2011, Lopez et al., 2010, Mather, 2011, Michalski et al., 2002). In one of the most widely accepted and updated models (Lopez et al., 2011), the backbone of the MFGM is constructed from phospholipids (including the five major species PE, PC, SM, PI, PS), and the inner layer in direct contact with the TG inner core is composed of a separate phospholipid monolayer and cytoplasm-derived protein layer; in the outer phospholipid bilayer, cholesterol is associated with SM and a distinct lipid region is formed as a liquid-ordered domain \( L_0 \) which rises above the surrounding phospholipid bilayer causing localized differences in membrane thickness. The \( L_0 \) domain is tightly packed due to the interaction between cholesterol and the FAs of SM, and as a result, fluorescently-labelled phospholipids cannot enter into this region to enable visualization of the \( L_0 \) domain by fluorescent microscopy (Lopez et al., 2010). Cholesterol molecules are asymmetrically distributed in the both layers of the outer bilayer and, therefore, \( L_0 \) domains may be formed on either side of
the phospholipid bilayer; other glycerophospholipids, including PE, PC, PI, and PS, can form a continuous fluid liquid-disordered domain (L_d); this domain is loosely packed, allowing fluorescent labelling (Lopez et al., 2011).

Three published model systems may represent the current understanding of schematic molecular organization of native MFGM in academia (Mather, 2011; Lopez et al., 2011; Michalski et al., 2002): Mather’s model describes the protein arrangement of the MFGM including the inter-molecular actions between MFGM proteins; Lopez’s and Michalski’s models are general schematic model systems of the MFGM in which the lipid and protein molecular arrangements are shown. Some controversial details may be found when comparing these models, for example, these models are deduced from direct or indirect experimental evidence and they may not represent the true molecular organization of the MFGM.

A flat supported phospholipid monolayer as a model system was constructed from bovine milk derived phospholipids (Gallier et al., 2010c) to successfully mimic lipid phase separation as observed on the MFGM; however, detailed morphological behaviour from this type of model would not necessarily be the same as for a spherical lipid bilayer system, as lipid domain formation may be affected by the surface tension and curvature of lipid bilayer (Bagatolli and Gratton, 2000, Rim et al., 2011). An artificial membrane study showed that coexistence of lipid domains within a phospholipid bilayer may be induced by immiscible phospholipids having different
phase transition temperatures ($T_m$) without including cholesterol (Bagatolli and Gratton, 2000).

Cholesterol may be bound to glycerolphospholipids in human erythrocyte membranes where SM is present, although SM has a relatively higher affinity to cholesterol (Op den Kamp, 1979, McMullen et al., 2004). Moreover, $L_0$ phases comprising PC and cholesterol have been characterized on artificial lipid bilayer model systems (Clarke et al., 2006). Consequently, it is reasonable to hypothesise that a $L_0$ phase comprised of glycerolphospholipids and cholesterol may also be found on the surface of native bovine MFGs.

A more detailed arrangement of eight major membrane proteins was illustrated by Mather (2011), and is summarised as follows. Adipophilin (ADPH) is positioned at the surface of the inner TG core. Mucin 1 (MUC 1) and MUC15 (periodic acid/Schiff III, PAS III) are trans-membrane proteins inserted into the primary inner layer of the MFGM as they lack a membrane anchor. PAS 6/7 is docked within the outer bilayer and probably bound to phospholipids in the MFGM. Butyrophilin (BTN) is considered as an integral membrane protein which probably interacts with other membrane proteins via disulphide bonds. It is believed that BTN interacts with xanthine dehydrogenase/oxidase (XDH/XO) forming a protein complex in the primary layer underneath the outer bilayer. Cluster of differentiation 36 (CD 36) is also an integral protein of MFGM. The two hydrophobic sequences, the C-terminal
and N-terminal regions of CD 36, are positioned on the cytosolic face of the plasma membrane as anchors. Fatty acid-binding protein (FABP) is enriched in the primary inner layer of MFGM and it is bound to CD 36.

The composition of MFGM had been extensively examined (Fong et al., 2007b, Gallier et al., 2011, Gallier et al., 2010a, Lu et al., 2011) using different analytical approaches including electrophoresis, chromatography, mass spectrometry, Raman spectroscopy, and microscopy. The results from compositional analyses depend upon the MFGM isolation processes employed, as the MFGM is fragile and sensitive to thermal and mechanical treatments (Walstra et al., 2006). Evers (2004) reviewed the changes occurring in the MFGM post-secretion and described methodologies for accessing damage to the MFGM (Evers, 2004a, Evers, 2004b). This knowledge is important for characterizing the integrity of MFGs and the MFGM during the MFG separation processes, and is critical for understanding the composition and structural organization of the MFGM. As MFGs and the MFGM may be disrupted during MFG isolation, the compositional results are unlikely to fully represent the native composition or the structure of the MFGM. Moreover, the stage of lactation, breed of cow, and diet may all affect the composition of the MFGM (Lopez et al., 2008, Ménard et al., 2010, Walstra et al., 2006). In raw milk, the composition of MFGM varies according to the size of the MFGs (Lopez et al., 2011).
Research has been carried out to investigate the impact of treatments on the MFGM and to characterize interactions between the MFGM and other milk components to better understand the nature of the MFGM, and to develop more efficient isolation procedures of MFGM components (Dickow et al., 2011, Le et al., 2009, Morin et al., 2007, Rombaut et al., 2006, Ye et al., 2005, Ye et al., 2002, Zheng et al., 2013b, Bermudez-Aguirre et al., 2008, Saffon et al., 2011). The processing-induced specific heterogeneous distribution of MFGM components in different phases of milk products reveals that MFGM components are constructed through a systemic design rather than through random organization (Rombaut et al., 2006, Zheng et al., 2013b).

### 2.2.2. Bovine MFGM lipids

Lipids and proteins account for about 90% (w/w) of total dry matter of the MFGM (Huppertz and Kelly, 2006). The recovery of MFGM has been reported as 3.6 ± 0.3 g/L of cream; lipid and protein made up 71.8 ± 1.7% and 22.3 ± 1.5% (w/w), respectively, of the MFGM pellet (Fong et al., 2007b). Although the MFGM is constructed from polar lipids and membrane-derived proteins, triacylglycerols account for the majority of the MFGM lipid class, with long chain FAs making up a high proportion of these membrane-associated triacylglycerols (Keenan. and Mather., 2006). It has been suggested that neutral lipids are more enriched in the inner side of the MFGM in direct contact with the TG core (Newman and Harrison, 1973). Mono- and di-acylglycerols may be found in the MFGM, but only account for a small
proportion of MFGM lipids (< 10% w/w); these are suggested as originating from MFGM constituents or products from lipolysis (Keenan. and Mather., 2006). Sterols, mainly cholesterol (accounting for > 90% w/w of the sterols in bovine milk) are found in the MFGM, originating from plasma membrane enriched in cholesterol (Blanc, 1981, Keenan. and Mather., 2006, van Meer, 1989).

Around 60% (w/w) of milk phospholipids are located in the MFGM (Huang and Kuksis, 1967, Patton and Keenan, 1971). Phospholipids make up about 26-31% (w/w) of total MFGM lipids; five major sub-species of milk phospholipids are PC (36%), PE (27%), SM (22%), PI (11%) and PS (4%) (Keenan and Mather, 2006). The composition of MFGM phospholipids and the proportion of these aforementioned sub-species depend on the breed of cow, feed, lactation stage, and the extraction and analysis methods (Avalli and Contarini, 2005, MacGibbon and Taylor, 2006). No major differences have been reported in phospholipids from both MFGM and skim milk in terms of FAs distribution; this is indirect evidence suggesting that phospholipids in the MFGM and skim phase of milk originate from the same source (Keenan and Mather, 2006, Keenan, 1995).

Relatively longer chain FA are found in milk phospholipids; no significant amount of FAs shorter than 14:0 are found in milk phospholipids. The major FA classes in glycerophospholipids are 16:0 (8-32%), 18:0 (8-26%), 18:1 (30-47%) and 18:2 (9-14%, w/w of total FAs) (Keenan and Mather, 2006, MacGibbon and Taylor, 2006).
Unlike glycerophospholipids, sphingomyelin mainly contains long chain saturated FAs: 16:0 (18-36%), 22:0 (~15%), 23:0 (17-30%) and 24:0 (11-17%, w/w) (MacGibbon and Taylor, 2006, Bitman and Wood, 1990). The major sub-species of major glycerophospholipids (PE and PC) are 18:1 containing PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DOPE, ~47% w/w, T_m = -16°C) and 16:0 containing PC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPC, ~32% w/w, T_m = 40°C) (Bitman and Wood, 1990, MacGibbon and Taylor, 2006). Phase transition temperatures (T_m) of phospholipids are sourced from Avanti® Polar Lipids, Inc. (Alabaster, AL, USA; http://avantilipids.com/).

The weight ratio between phospholipids and cholesterol in MFGM is 25:2 w/w (Huppertz and Kelly, 2006). The molecular weights of DOPE, DPPC and milk SM are 774, 734 and 785 g/mol, respectively (Avanti® Polar Lipids, Inc., http://avantilipids.com/). The content of PE, PC and SM may vary, depending on the source of the sample and the analysis method, and can be between 25-35% of total PLs (MacGibbon and Taylor, 2006), therefore, one may assume that PE, PC and SM account for the same proportion in total PLs. For simplifying the calculation of the molar ratios between PLs and cholesterol, PE, PC and SM were considered to comprise the full composition of PLs, and the major sub-species, including DOPE, DPPC represent PE and PC, respectively. The mean molecular weight (M_w) of milk PLs can be calculated as 754 (mean of M_w DOPE + M_w DPPC + M_w milkSM); assuming the
M_w of cholesterol is half of the M_w of milk PLs, then the estimated molar ratio between milk PLs and cholesterol is 25:4 (mol/mol).

### 2.2.3. Bovine MFGM proteins

To date, major MFGM-associated proteins had been identified (Keenan and Mather, 2006, Keenan, 1995). The proposed nomenclature of these major MFGM proteins have been reported (Mather, 2000) and the functionalities of the major MFGM proteins described (Dewettinck et al., 2008). MFGM proteins only account for a small proportion of total milk proteins, at around 1-2% (w/w) (Riccio, 2004). The measured composition of MFGM proteins depends upon the isolation and determination methods employed (Le et al., 2009, Zheng et al., 2013b). MFGM proteins have been separated by two-dimensional (2D) electrophoresis and identified by reverse phase liquid chromatography-mass spectrometry (RP-LC-MS/MS) (Fong et al., 2007b). In this current research, the molecular organization of the seven major membrane proteins ADPH, MUC 1, MUC 15, PAS 6/7, BTN, XDH/XO and CD 36 and the specific arrangements were studied.

Proteomic analysis of MFGM has been developed by different research groups (Fong et al., 2007b, Lu et al., 2011). Thousands of different types of MFGM proteins have been identified using the technique. The method is mostly based on gel electrophoresis coupled with tandem mass spectrometry. In the light of detailed
characterization and identification of MFGM proteins, it has been reported that MFGM proteins are not only functional in lipid metabolic or exocytosis-related biological processes, but they may also participate in signal transduction and other biological processes. A better understanding of MFGM proteins will be helpful for revealing the true secretion mechanism of MFGs and the biology of the mammary gland (Cebo, 2012). However, such identification requires standardized MFGM isolation method as the MFGM structure is fragile and can be altered by thermal and mechanical treatments, which may result in spurious conclusions about the composition and purpose of MFGM proteins.

### 2.2.4. MFGM isolation from raw milk

Methods of MFGM isolation vary between different research groups depending upon the facilities and purpose of the research (Le et al., 2009, Patton and Huston, 1986, Zheng et al., 2013b). There are general steps that are common to most isolation methods (Le et al., 2009, Mather, 2011). These include, 1) separation of MFGs from raw milk as a cream phase; 2) re-dispersing MFGs into washing buffer (such as simulated milk ultra-filtrate, SMUF) and making a recombined o/w emulsion system, 3) repeated separation and washing in buffer (non MFGM-associated proteins may be partially removed during these washing steps); 4) churning of washed cream at a cool temperature (~10ºC); 5) collection of the MFGM suspension from butter milk and butter serum after centrifuging melted butter; 6) freeze-drying or heat-drying of
Chapter 2: Literature Review

MFGM material. The critical stage of the whole process which can affect the final composition and structure of the MFGM material is initial separation of native MFGs, including processing treatments of the MFGs (Morin et al., 2007, Britten et al., 2008). During processing, MFGM-associated materials may be shed into the aqueous phase of milk, or recombined as an MFG emulsion, thus reducing the yield of MFGM during isolation (Le et al., 2009, Zheng et al., 2013b).

Gathering all the aforementioned information about the MFGM from the literature, several research questions remain to be answered: 1) How does the MFGM isolation method affect the native MFGM structure? 2) Is the tri-layer hypothesis correct? 3) Is there a protein monolayer underneath the phospholipid bilayer, and what is its purpose? 4) How does the molecular arrangement of the MFGM components impact upon the physical and physiological functions? 5) What is the composition and method of construction of “lipid rafts”?
CHAPTER 3.
Bovine Milk Fat Globule Membrane Proteins Are Affected By Centrifugal Washing Processes

This chapter has been published in Journal of Agricultural and Food Chemistry, 61(35): 8403-8411. as: Bovine Milk Fat Globule Membrane Proteins Are Affected By Centrifugal Washing Processes. by Zheng, H., R. Jiménez-Flores, and D.W. Everett, 2013 (DOI: 10.1021/jf402591f)
3.1. Introduction

Proteomics and lipidomics have been carried out to study the composition of the MFGM (Fong et al., 2007a, Gallier et al., 2010a), sourced milk from either human (Lopez and Ménard, 2011, Zou et al., 2012) or bovine milk (Gallier et al., 2010b, Lopez et al., 2010, Damodaran, 2011, Gülseren and Corredig, 2013, Zhu and Damodaran, 2011). Reviews had been published on the structure and functionality of MFGs and the MFGM (Argov et al., 2008a, Dewettinck et al., 2008, Lopez, 2011), however the structure of the native MFGM is still unclear due to its fragile and treatment-sensitive nature (Huppertz and Kelly, 2006). Different types of experimental approaches are needed to provide information to further develop current MFGM models (Lopez et al., 2011, Mather, 2011, Michalski et al., 2002).

In light of recent developments of the understanding on nutrient bioavailability, the importance of the macro-structure of food matrices in regulating the rate of digestion and absorption had been emphasized. Such work has focused on the bio-functional nature of the MFGM structure in physiological processes (Argov et al., 2008a). Several models of the MFGM have been deduced to explain experimental results, featuring a trilayer structure, trans-membrane and inner core proteins, and liquid ordered regions (or lipid rafts) rich in cholesterol and sphingomyelin (Lopez et al., 2011, Walstra et al., 1984, Lopez, 2011, Lopez et al., 2010). McPherson and Kitchen (1983) discussed the existence of a protein inner layer of the MFGM. Murray et al.
(1979) found glycoproteins in the isolated inner coat material of the MFGM, in contrast to the more widely held belief that glycoproteins are exposed at the outer MFGM surface. In a more recent review, Keenan and Mather (2006) reported on the mechanism of the development of a protein-dense layer from the perspective of MFG origin and secretion. Direct evidence for the existence of an inner protein-dense layer from studies of post-secretion milk is still scarce.

Protein structural organization in the MFGM has been reviewed by Mather (2000, 2011). It is known that key MFGM proteins are not bound to the membrane structure with equal binding force, for instance, mucin 1 (MUC 1), xanthine dehydrogenase/oxidase (XDH/XO), mucin 15 (MUC 15), periodic acid Schiff III (PAS III), and PAS 6/7 are recognized to be bound more loosely compared to cluster of differentiation (CD 36), butyrophilin (BTN) and adipophilin (ADPH). The distribution of these key MFGM proteins between the water-insoluble MFGM pellet and the MFGM supernatant during MFGM isolation has been reviewed (Bruder et al., 1982, Mather, 2000); however, there is little information on the binding strength of major MFGM proteins. It is widely accepted that both MFG and MFGM isolation processes may induce MFGM material loss, especially the loosely-bound peripheral proteins (Mather, 2000), but the relative loss of specific membrane proteins during MFG isolation from unpasteurized milk is not well known.
Fractionation of MFGM, which is essential for studying the structure, composition and bio-functionality, is achieved by three major steps: 1) isolation of washed MFGs (in physiological buffer) from unpasteurized milk; 2) release of MFGM from washed MFGs via physical and/or chemical means; and 3) fractionation of MFGM materials via physical and/or chemical methods (Mather, 2000, McPherson and Kitchen, 1983). Patton and Huston (1986) developed a washing method based on convenience of operation, less structural change to the native MFGM, and an acceptable level of protein retention. Other washing procedures employing different centrifugal forces, time of centrifugation, and washing buffers are commonly used (Le et al., 2009, Ye et al., 2002). Washing procedures may induce damage to the MFG surface resulting in contamination with milk serum proteins and will consequently alter the composition of the extracted MFGM and lead to conclusions about structure based on spurious results.

The impact of three washing procedures on the composition of the MFGM from unpasteurized bovine milk was examined to provide evidence for protein structural organization in the bovine MFGM. Different centrifugal washing forces (from mild to intensive) and duration were employed to gain insight into the competitive binding strengths of key MFGM proteins. The compositional changes of the MFGM were studied on the surface of stable MFGs without the need to destabilize MFGs and remove the MFGM from the surface.
3.2. Materials and methods

3.2.1. Materials

Fluorescent headgroup-labelled phospholipid analogue 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine rhodamine B sulfonyl) (Rd-DOPE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), dissolved in chloroform (1 mg/mL), and sealed with a Teflon lid under N₂ and kept at –20°C until used. Fast Green FCF (disodium 3-[N-ethyl-N-[4-[4-[N-ethyl-N-(3-sulfonatobenzyl) amino] phenyl] (4-hydroxy-2-sulfonatophenyl) methylene]-2, 5-cyclohexadien-1-ylidene] ammoniomethyl] benzenesulfonate) (1 mg/mL in deionized water) was kept at ambient temperature before use. Simulated milk ultra-filtrate (SMUF) at pH 6.5 was prepared (Jenness and Koops, 1962) and used as a buffer and washing solution. All other chemicals were of analytical grade (Merck, Darmstadt, Germany).

3.2.2. Isolation of Milk Fat Globules

Bovine milk was obtained from a single Jersey cow, to limit bio-variation, at mid-lactation from a local farm (Port Chalmers, New Zealand); all milk samples were collected in the morning between 8 and 9 am directly after milking. The cow was pasture-fed and milked in the same manner (in-line vacuum milking machine) for six consecutive sampling days. The milk was not pasteurized post-milking. Milk from a Jersey cow was chosen for its relatively high fat content and larger volume-based
MFG size compared to Friesian cows’ milk (Walstra, 1969). Incorporation of air through beating or stirring, temperature history, and aging during milking, transportation, and storage are known to cause compositional and structural changes to the MFGM (Walstra et al., 2006). To minimize these effects, unpasteurized milk was cooled to ambient temperature (20-22°C) immediately after milking without any mechanical treatment to minimize damage to the MFGs and ensure minimal variation in replicate samples.

Three methods were chosen to isolate MFGs from fresh unpasteurized milk namely M1 (3000 g, 5 min, cream + three washes) (Gallier et al., 2010b), M2 (3750 g, 15 min, one wash) (Patton and Huston, 1986) and M3 (15000 g, 20 min, cream + three washes) (Ye et al., 2002). Centrifugation was carried out using a high performance centrifuge (Avanti®, J-Series, Beckman-Coulter, Indianapolis, IN, USA). The M1 and M3 procedures were carried out in triplicate over the six sampling days in random order, and M2 as a control method was carried out six times over the sampling days. The M1 and M3 washes are considered to be conventional MFG isolation procedures. In all cases, cream was initially collected from milk under the M1, M2, or M3 centrifugation condition (designated as M1-cream, M2-cream, and M3-cream) and then re-suspended into ten volumes of SMUF. After 1 hour at ambient temperature with gentle agitation, the dispersion was centrifuged under the same conditions (M1, M2, or M3) again to wash off the non-MFGM proteins from the MFG surface, and the top MFG cream layer was collected. This procedure was designated as the first
washing step (M1-1, M2-1, and M3-1). The centrifugation and washing processes were repeated twice more for M1 and M3. A sucrose density gradient single wash separation (Patton and Huston, 1986), with slight modifications, was applied in M2. Briefly, in a 50 mL centrifuge tube (Nalgene Centrifuge Ware, New York, NY, USA), 15 mL of sucrose-conditioned milk (5% w/v) was layered under 30 mL of SMUF buffer using a syringe coupled with a ~100 mm length and ~1 mm diameter needle. The prepared sample was centrifuged (3750 g, 15 min), and the top layer containing washed MFGs was collected. The temperature of the SMUF buffer was kept at 30ºC. The centrifugation temperature was 25ºC for all experiments. Washing of milk took place on the same day as milking as MFGs from un-cooled milk samples were more readily dispersed into the SMUF washing solution than MFGs isolated from milk held overnight at 4ºC (results not shown). Milk and cream fractions collected after each centrifugation process were kept at -80ºC until further analysis.

3.2.3. Fat Globule Size and Specific Surface Area

Laser diffraction was used to measure particle size distribution and specific surface area on volume and surface area weighted bases (model LA-950, Horiba, Irvine, CA, USA) equipped with a red wavelength diode laser at 655 nm for larger particles (> 500 nm) and a blue light emitting diode at 405 nm for smaller particles (<500 nm). Optical parameters were taken from previous reports (Michalski et al., 2001), with modifications. Refractive index of MFGs was set to 1.460 for the diode laser and
1.470 for the light emitting diode. Samples of re-suspended MFGs were first diluted with SMUF to give the same fat content of the original milk. The MFG dispersions and milk were diluted two-fold (v/v) with 35 mM ethylenediaminetetraacetic acid (EDTA) containing 2% (w/v) sodium dodecyl sulphate (SDS) buffer solution at pH 7.0. The EDTA was used to dissociate casein micelles, and SDS to disperse aggregated MFGs. Deionized water was used as the continuous medium. Mode size (peak of frequency distribution), volume mean diameter ($d_{43}$), surface mean diameter ($d_{32}$), and specific surface area (S.S.A.) were calculated (Huppertz and Kelly, 2006) using the Horiba diffraction software. Measurements were carried out in triplicate.

3.2.4. Determination of $\zeta$-Potential

The $\zeta$-potential of milk fat globules was measured using a Zetasizer nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK) using the Smoluchowski approximation, as the thickness of MFGM is widely recognized to be less than 20 nm, which is much smaller than the diameter of MFGs (0.1-10 µm). Milk and cream dispersions were diluted $8 \times 10^{-3}$ in SMUF. Each sample was measured as five replicates.

3.2.5. Total Protein and Fat Content
The total protein content of milk and SMUF-washed MFGs was determined by a Coomassie blue-staining protein assay (Bradford, 1976). Total fat content was determined gravimetrically after a Folch total lipid extraction (Folch et al., 1957). Milk and dispersed MFG fractions were mixed in a chloroform/methanol (2:1 v/v) solvent at a ratio of 1:8 (v/v) and 1:20 (v/v) respectively. NaCl solution (0.2 volumes of 0.9% w/v) was used to wash the sample-solvent mixture to enhance the recovery of lipids from extraction. The chloroform phase, containing lipids, was dried under a gentle N₂ gas flow.

**3.2.6. Characterization and Quantitation of MFG-Associated Protein Components**

Protein characterization and relative quantification was carried out by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The pre-cast gel (bis-Tris 4-12% polyacrylamide gel), NuPAGE® LDS (lithium dodecyl sulphate) pH of 8.4 sample buffer (4x), NuPAGE® reducing agent (dithiothreitol), and SimplyBlue™ Safe Stain (Coomassie Brilliant Blue) staining agent were obtained from Life Technologies Corporation (Carlsbad, CA, USA). The dispersed MFG fractions were initially ultrasonicated at 50°C to achieve a fine and homogeneous suspension system. Milk was diluted four times in SMUF. Dispersed MFG fractions were diluted in SMUF to yield a protein content of 5 mg/mL to ensure accurate comparison of band densities in stained gels. Samples (6.5 µL) were mixed with LDS sample buffer (2.5 µL) and reducing agent (1 µL) and heated at 90°C for 10 min
before loading onto pre-cast polyacrylamide gels. Electrophoresis was run at constant voltage (160 V) for 1 h. Stained gels were scanned at 300 dots per inch (ImageScanner III, GE Healthcare, Uppsala, Sweden) and band density measured using image analysis software (ImageQuant TL, GE Healthcare). Molecular weight (MW) markers (3.5-260 kDa Novex® sharp pre-stained protein markers (Life Technologies Corporation) were used to determine the MW of MFGM proteins. Identification of protein bands was carried out by comparison with published MW data (Fong et al., 2007a, Mather, 2000, Mather, 2011, Ye et al., 2002) using standard curves for major milk proteins (Le et al., 2009). The relative density of protein bands on different gels were calibrated using the 110 and 160 kDa bands of the MW markers, allowing a comparison of protein band densities across different gels. Samples were run in duplicate on SDS-PAGE gels.

3.2.7. Microstructural Imaging by Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM; Zeiss 710 upright microscope, Jena, Germany) was used to assess protein and phospholipid changes on the MFG surface in situ. MFG-associated proteins were labelled with fluorescent Fast Green FCF and MFGM phospholipids (PLs) with fluorescent Rd-DOPE (Gallier et al., 2010b, Lopez and Ménard, 2011, Lopez et al., 2010). Prior to staining, the suspended MFGs and milk were diluted five-fold with SMUF at pH 6.5. The staining method was adopted from previous research (Gallier et al., 2010b), with slight modifications. Briefly,
prepared samples (1 mL) were mixed with Fast Green FCF at a ratio of 100:6 (v/v) in Eppendorf tubes for 10 min. Rd-DOPE solution (1 µL of 1 mg/mL) was placed on a concave microscope slide and chloroform solvent was dried in a vacuum desiccator before mixing with the protein-stained MFG sample, as chloroform may induce structure changes in the MFGM. Protein-stained MFG suspension samples (25 µL) were pipetted onto the Rd-DOPE deposited slide surface with gentle mixing using a pipette tip. An incubation time of 20 min at ambient temperature was required to allow Rd-DOPE fluorescent dye to stain the MFGM. All staining procedures were carried out in a dark room to minimize loss of fluorescence signal (photo-bleaching). Agarose (low melting point 37°C) temperature-controlled gel solution (50 µL, 0.5% w/v in deionized water) was mixed with stained samples to fix the MFGs onto the microscope slide (Gallier et al., 2010b, Lopez et al., 2010). A coverslip was gently applied to the top of the sample.

Three channels, Rd-DOPE channel 1, Fast Green FCF channel 2, and transmitted light channel 3 were used for CLSM observation. Channel 3 was used to locate the physical position of MFGs in the agarose gel (Lopez et al., 2010). Rd-DOPE-labelled MFGM PLs and Fast Green FCF-labelled MFG associated proteins were excited using a green HeNe laser at 543 nm and red HeNe laser at 633 nm, respectively. The emitted light was collected between 570 to 625 nm for Rd-DOPE and between 632 to 639 nm for Fast Green FCF. Experiments were carried out in the absence of fluorescent stains to confirm that MFGs do not display intrinsic fluorescence. The
configuration of the confocal microscope was kept the same across all samples as alteration would likely to affect the level of the fluorescent signals. This enabled relative quantitative changes of lipids and proteins on the surface of MFGs to be determined.

3.2.8. Statistical Analysis

One-way analysis of variance tests were carried out using Minitab 16 (Minitab Inc., State College, PA, USA). Significant differences ($P < 0.05$) was determined using Fisher’s test in paired comparisons between samples means.

3.3. Results & Discussion

3.3.1. Results

3.3.1.1. Milk Fat Globule Characterization

The MFG diameters, S.S.A., and $\zeta$-potential of milk and washed fractions are presented in Table 3.1. MFG modal diameters, $d_{43}$, and $d_{32}$ were larger after M1 processing with a corresponding decrease in S.S.A. M2 and M3 did not induce significant change in the particle size distribution after washing comparing with their corresponding unpasteurized cream fractions, with the exception of the third wash for M3. The $\zeta$-potential was significantly reduced after the third M1 wash compared with fresh cream (M1-cream), and was significantly increased after the second M3 wash
compared with M3-cream. M2 processing did not alter ζ-potential significantly from unpasteurized milk to the M2-1 fraction.

### 3.3.1.2. Gross Protein Profile in Washed MFG Fractions

Table 3.2 shows the percentage of initial total protein retained on the surface after washing, and amounts of non-MFGM proteins (including whey and casein proteins) in the washed MFG fractions. Results were calculated on both a fat content and S.S.A. basis. The protein content of the initial cream was considered as the reference and designated as a 100% protein load. The absolute value of non-MFGM protein content in the initial cream fractions was unable to be estimated from SDS-PAGE due to the protein load exceeding the upper limit of the standard curve. This was necessary to give clear band images of the MFGM proteins. Generally, all washing processes resulted in protein loss. M1 induced a lower ($P < 0.05$) total protein retention, 42.56% (fat basis) and 59.20% (S.S.A. basis), compared to M2, 77.22% (fat basis) and 78.28% (S.S.A. basis), and M3, 61.91% (fat basis) and 70.02% (S.S.A. basis) after the final washing steps. However, the retention of non-MFGM proteins on MFGs was higher ($P < 0.05$) in M1 (0.27 mg/g fat basis and 0.19 mg/m² S.S.A. basis) than in M3 (0.21 mg/g fat basis and 0.14 mg/m² S.S.A. basis) after the second step washing, and was not significantly different after the third washing step in M1 and M3. Although M2 showed relatively high total protein recovery after washing, it resulted in a higher ($P$
Table 3.1. Milk fat globule diameters, specific surface area (S.S.A.) and ζ-potential from different sample fractions.

<table>
<thead>
<tr>
<th></th>
<th>mode size</th>
<th>( d_{43} )</th>
<th>( d_{22} )</th>
<th>S.S.A. (^b)</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μm)</td>
<td>(μm)</td>
<td>(μm)</td>
<td>(m²/g fat)</td>
<td></td>
</tr>
<tr>
<td>raw milk</td>
<td>3.58 ± 0.03 g</td>
<td>3.57 ± 0.04 g</td>
<td>3.53 ± 0.03 e</td>
<td>1.70 ± 0.01 a</td>
<td>-12.24 ± 0.26 bcd</td>
</tr>
<tr>
<td>M1-cream</td>
<td>4.62 ± 0.26 cd</td>
<td>4.77 ± 0.07 cde</td>
<td>3.82 ± 0.17 ef</td>
<td>1.57 ± 0.07 bc</td>
<td>-12.38 ± 0.32 cd</td>
</tr>
<tr>
<td>M2-cream</td>
<td>4.79 ± 0.01 bc</td>
<td>5.11 ± 0.03 bc</td>
<td>4.47 ± 0.08 c</td>
<td>1.34 ± 0.02 e</td>
<td>-11.97 ± 0.16 b</td>
</tr>
<tr>
<td>M2-1 wash</td>
<td>5.02 ± 0.36 b</td>
<td>5.44 ± 0.25 b</td>
<td>4.65 ± 0.06 b</td>
<td>1.29 ± 0.02 e</td>
<td>-12.02 ± 0.27 bc</td>
</tr>
<tr>
<td>M2-3 wash</td>
<td>5.48 ± 0.01 a</td>
<td>7.69 ± 0.73 a</td>
<td>5.14 ± 0.03 a</td>
<td>1.17 ± 0.01 f</td>
<td>-11.38 ± 0.36 a</td>
</tr>
<tr>
<td>M3-cream</td>
<td>4.15 ± 0.01 ef</td>
<td>4.32 ± 0.09 ef</td>
<td>3.81 ± 0.07 ef</td>
<td>1.57 ± 0.03 bc</td>
<td>-12.50 ± 0.29 d</td>
</tr>
<tr>
<td>M3-1 wash</td>
<td>4.13 ± 0.01 f</td>
<td>4.18 ± 0.04 f</td>
<td>3.86 ± 0.19 e</td>
<td>1.55 ± 0.07 c</td>
<td>-12.11 ± 0.35 bc</td>
</tr>
<tr>
<td>M3-2 wash</td>
<td>4.15 ± 0.02 ef</td>
<td>4.30 ± 0.15 ef</td>
<td>3.67 ± 0.07 fg</td>
<td>1.64 ± 0.03 ab</td>
<td>-12.49 ± 0.39 d</td>
</tr>
<tr>
<td>M3-3 wash</td>
<td>4.20 ± 0.02 ef</td>
<td>4.68 ± 0.13 cde</td>
<td>3.82 ± 0.05 ef</td>
<td>1.57 ± 0.02 bc</td>
<td>-12.38 ± 0.19 bcd</td>
</tr>
</tbody>
</table>

Means within a column with different letters of significance differ \((P < 0.05)\).

\(^b\) Specific surface area.
Table 3.2. Total protein change and main non-milk fat globule membrane (MFGM) proteins on the milk fat globule surface after washing a.

<table>
<thead>
<tr>
<th></th>
<th>total protein (%) b</th>
<th>main non-MFGM proteins b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fat basis</td>
<td>S.S.A. c basis</td>
</tr>
<tr>
<td>M1-cream</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>M1-1 wash</td>
<td>66.57±3.11 d</td>
<td>78.00±3.65 b</td>
</tr>
<tr>
<td>M1-2 wash</td>
<td>50.78±1.96 f</td>
<td>61.81±2.39 d</td>
</tr>
<tr>
<td>M1-3 wash</td>
<td>42.56±3.78 g</td>
<td>59.20±2.34 d</td>
</tr>
<tr>
<td>M2-cream</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>M2-1 wash</td>
<td>77.22±3.46 b</td>
<td>78.28±2.94 b</td>
</tr>
<tr>
<td>M3-cream</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>M3-1 wash</td>
<td>84.88±0.98 a</td>
<td>87.95±0.32 a</td>
</tr>
<tr>
<td>M3-2 wash</td>
<td>72.20±3.74 c</td>
<td>71.50±1.47 c</td>
</tr>
<tr>
<td>M3-3 wash</td>
<td>61.91±1.76 e</td>
<td>70.02±1.99 c</td>
</tr>
</tbody>
</table>

a Means within a column with different letters differ (P < 0.05).
b Calculated on a total fat content and S.S.A. basis.
c Specific surface area.
d Not available; gel band density of non-MFGM proteins were too high and beyond the range of the quantification method.
3.3.1.3. Identification of Proteins

The SDS-PAGE patterns of MFG-associated proteins in different MFG fractions from the initial cream to the final washing step are shown in Figure 3.1. Non-MFGM protein bands were the most dense in the initial cream samples for M1, M2 and M3, and were largely removed after one washing step. The M2 process was least effective at removing caseins and whey proteins.

The quantification of the partial removal of MFGM proteins on MFG surfaces during the M1 and M3 washing procedures is shown in Figure 3.2. The relative changes in density from the SDS-PAGE gels of seven key MFGM protein bands (MUC 1, XO, MUC 15/PAS III, CD 36, BTN, PAS 6/7 and FABP) after three washing steps were calculated. Results are shown as a percentage change from the amount of a particular MFGM protein present in the initial cream fraction. As the total protein loading volumes were the same for all samples, the relative compositional changes of specific MFGM proteins after washing may be presented as enrichment or depletion based on a total protein basis. Enrichment, which has a positive percentage change, represents relatively stronger MFGM-binding proteins such as CD 36 and PAS 6/7 in M1, and CD 36 and FABP in M3. CD 36 was the strongest ($P < 0.05$) MFGM-binding protein and was enriched on the MFG surface by approximately 150%, even after the first washing step for both M1 and M3, and was further enriched by around 200-400% after the final M1 and M3 washing steps, respectively. PAS 6/7 together, and FABP
were the second strongest MFGM-binding proteins after M1 and M3 processing, respectively. The rest of the major MFGM proteins were more loosely bound to the MFGM.

3.3.1.4. Microstructural Imaging by CLSM

Only MFGs within the fluorescent z-stack (confocal focus) with clear and sharp boundary interfaces were used for characterizing structural and compositional changes. The microstructural comparison of unwashed initial cream and the corresponding final washed fraction for three washing methods (M1, M2, and M3) are shown in Figure 3.3. Most of the MFGs were well-covered by PLs and very little fluorescently stained protein was evident on the surface of MFGs after a single M1 centrifugation (Fig. 3.3. A-C), but there were some structural changes after the third M1 wash as shown by the evident loss of PLs (white arrows, Fig. 3.3. F). These irregular shaped MFGs (Fig. 3.3. F, white arrows) could be due to the MFGs being compressed by the agarose and coverslip, or artefacts from the microscopic imaging. For the M2 wash (Fig. 3.3. G-L), some surfaces were apparently bare without fluorescence from Fast Green FCF or Rd-DOPE (white arrows, Fig. 3.3. L), as well as rough surfaces coated with PLs (red arrows, Fig. 3.3. I). Less Rd-DOPE and Fast Green FCF fluorescent signals were observed in the M3 cream fraction (Fig. 3.3. M-O); however, the Fast Green FCF-labelled protein layer became more pronounced after three M3 washings (Fig. 3.3. P-R).
Chapter 3: Bovine Milk Fat Globule Membrane Proteins Are Affected By Centrifugal Washing Processes

Figure 3.1. SDS-PAGE gel of milk fat globule-associated proteins after different washing processes. M1-Cr.: original cream; centrifugation conditions M1: 3000 g, 5 min; M2: 3750 g, 15 min; M3: 15000 g, 20 min; the final number after the dash indicates the washing step; Mr.: molecular weight markers (kDa). MUC1: mucin 1; XDH/XO: xanthine dehydrogenase/oxidase; MUC15: mucin 15; CD 36: cluster of differentiation 36; BTN: butyrophilin; IgM: immunoglobulin M; PAS 6/7: periodic acid Schiff 6/7; FABP: fatty acid-binding protein.
Figure 3.2. Relative change to amounts of major milk fat globule proteins after different washing processes compared to the original cream. Light to dark shading refers to one to three washes. A: M1 washes; B: M3 washes. Columns for each MFGM protein within each sub-figure with different superscript letters do not differ significantly ($P<0.05$). Abbreviations for proteins given in Figure 1.
Chapter 3: Bovine Milk Fat Globule Membrane Proteins Are Affected By Centrifugal Washing Processes

Figure 3.3. Confocal micrographs of double-stained milk fat globules after washing using three different methods. A-C: M1-cream; D-F: M1 after three washes. Conditions for M1, M2, and M3 are given in the text. Column 1: PLs channel (Rd-DOPE staining), column 2: protein channel (Fast Green FCF staining); and column 3: merged channels of PLs, protein and T-MPT (transmitted light channel). White arrows: surfaces without evident protein or phospholipids; red arrows: rough Rd-DOPE-labelled phospholipid layer. Scale bar is 5 µm.
Chapter 3: Bovine Milk Fat Globule Membrane Proteins Are Affected By Centrifugal Washing Processes

Figure 3.3: Confocal micrographs of double-stained milk fat globules after washing using three different methods. Row G-I: M2-cream; J-L: M2 after one wash.

I: M2 after one wash.

L: M2 after one wash.
Figure 3.3. Confocal micrographs of double-stained milk fat globules after washing using three different methods. Row M-O: M3-cream; P-R: M3 after three washes.
3.3.2. **General Discussion**

Unpasteurized milk was sourced from a single Jersey cow to minimize variables such as breed and age of the cow, feed, and stage of lactation, which could affect the composition and structure of the MFGM and the mean size of MFGs. A single source of milk was also deemed necessary to avoid the problem of structural changes that might occur in pooled milk that is refrigerated and agitated on the farm.

The ζ-potential at the surface of shear of MFGs was used as one of the criteria to estimate the stringency of the washing processes and damage to the MFGM (Evers, 2004a). The mean ζ-potential of MFGs in unpasteurized milk was -12.24 mV, in agreement with published data in the range of -10 to -14 mV (Huppertz and Kelly, 2006, Michalski et al., 2002). The ζ-potential of MFGs increased for the M3 procedure (in absolute values) after the second and third washing steps. This increase could be considered to be due to exposure of an electron-dense proteinaceous coat (inner protein monolayer of the MFGM) (Mather, 2011) during mechanical treatments. This exposure can be attributed to damage to MFG surfaces (the outer phospholipid bilayer), as the total protein and non-MFGM protein load onto MFG surfaces did not increase with the number of wash steps in M3 (Table 3.2).

The increase in ζ-potential and greater protein content after the intensive M3 washing procedure, compared to M1 and M2, could be related to the existence of a protein coat
under the outer leaflet phospholipid bilayer in the MFGM, which has been reported in other model systems (Lopez et al., 2011, Mather, 2011, Michalski et al., 2002). This is supported by the observed increase in protein content after M3 washing compared to M1 and M2 (Fig. 3.3), which may be facilitated by damage to the phospholipid outer bilayer. Although M3 is the most intensive washing procedure used in this study, the retention of total protein on MFG surfaces in each of the isolated fractions was higher than that in the corresponding fractions in M1 and M2 (Table 3.2). Previous results showed the absence of a detectable triglyceride inner core, and a greater proportion of MFGM in vesicle-like structures smaller than 2 μm (Gallier et al., 2011). MFGM-vesicles and milk-microsomes have been found in the skimmed phase of milk, and these small “MFGM-liposomes” are thought to be formed from MFGM material released from native MFGs during centrifugation (McMullen et al., 2004). However, other research groups found that these “MFGM-liposomes” did not originate from shedding of the MFGM during centrifugation processes (Admyre et al., 2007, Argov-Argaman et al., 2010). The fat contents of M3 samples were significantly higher (P < 0.05) than the specific paired samples in M1 and M2 (i.e. M3-cream is compared with M1-cream and M2-cream; results not shown). It can therefore be assumed that a larger number of smaller MFGs collected during the more stringent centrifugation process of M3 will result in more recovered MFGM, and specifically, more MFGM proteins.
Non-MFGM proteins, bovine serum albumin and β-casein (CN), were largely removed after the first washing step in M1, M2 and M3 (Fig. 3.1). For the caseins, α-CN (combined αs1-CN and αs2-CN) were largely removed in the first washing for M1 and M3 but this combined band was still evident in the M2-1 fraction, whereas κ-CN could not be washed off by M2 and M3 but was still found after the third M1 wash (Fig. 3.1). This suggests that smaller casein micelles have a larger total summative surface area, therefore a higher amount of κ-CN that is harder to be removed from the MFGs. Bands representing β-lactoglobulin and α-lactalbumin were observed after the final washing in all three washing procedures, suggesting that whey proteins were relatively more resistant to the washing processes in all three methods, especially for M2. In practice, it is almost impossible to remove all of the non-MFGM proteins from the MFG suspensions as evident by residual non-membrane proteins remaining on the surface of MFGs (Le et al., 2009, Ye et al., 2002) and after the final washing in the current results (Fig. 3.1). It should also be noted that non-membrane proteins (such as whey proteins) in the cream fractions could be present on both the surface of MFGs and in the aqueous phase surrounding the milk fat globules.

Results of changes in key MFGM proteins (Fig. 3.2 A, B) suggest that CD 36 is the strongest bound protein to the MFGM in both M1 and M3, in agreement with a recent review of the MFGM (Mather, 2011). The CD 36 protein contains contiguous stretches of hydrophobic amino acid residues close to both the N- and C-termini which face the cytoplasm (thus are cytoplasmic-orientated) and function as trans-
membrane anchors; the hydrophobic pockets in folded polypeptides are also formed by exoplasmic domains (Mather, 2000, Sefton and Buss, 1987). Therefore, the two hydrophobic anchors and the hydrophobic exoplasmic pocket serve as unique structural elements of CD 36 that may contribute to the strong affinity to MFGM.

PAS 6/7 (co-migrating with ADPH) was shown to be less easily removed after M1 washing (Fig. 3.2 A), however it has been reported that PAS 6/7 can be displaced from washed fat globules into the skim milk phase using a concentrated salt washing solution (Butler et al., 1980, Mather et al., 1984). The similar molecular weight of PAS 6/7 to ADPH, the latter of which is associated with the lipid droplet in the protein inner layer underneath the phospholipid bilayer in the MFGM (Mather, 2011) and considered as a stable membrane protein on MFG, should be taken into account. The quantification of PAS 6/7 from the SDS-PAGE gel may have been overestimated by inclusion of ADPH. Therefore the apparent enrichment of the PAS 6/7 and ADPH band (Fig. 3.2 A) could be due to the presence of the more stable ADPH. Unlike other key MFGM proteins, PAS 6/7 are not trans-membrane proteins but instead are exoplasmic peripheral proteins bound to anionic phospholipids in the membrane via the C-terminus (Mather, 2000, Mather and Keenan, 1975). Therefore, it is reasonable to consider that the affinity of PAS 6/7 to MFGM depends on the amount of retained phospholipids rather than the internal structure and morphology of the phospholipid bilayer backbone of MFGM. M1 is considered as a mild washing treatment and did not induce much structural or physical damage to the MFGM phospholipid backbone.
on the surface of MFGs (Fig. 3.3. A-F), and the ζ-potential did not increase with the number of washing steps. However, re-arrangement of the lipid components in the MFGM during washing might allow the release of trans-membrane proteins into the washing buffer, resulting in enrichment of the PAS 6/7 and ADPH band.

The MUC 1, MUC 15, XDH/XO and BTN proteins were depleted with washing in both the M1 and M3 procedures, and are therefore considered to be relatively weakly bound proteins to the MFGM. The structural feature of MUC 15, which lacks a membrane anchor and contains an extensive exoplasmic domain (Mather, 2011), may explain the lower binding to the MFGM. It is speculated that during the washing procedures the structural organization of MFGM is altered and the soluble XDH/XO, which is located in the protein coat (underneath the phospholipid bilayer) in the MFGM (Mather, 2011) is released into the washing buffer (SMUF in this case). The BTN protein is released with XDH/XO into the washing buffer as XDH/XO is bound to the cytoplasmic tail of BTN (Ishii et al., 1995). The formation of a supra-molecular complex between XDH/XO and BTN is thought to be an essential step in the assembly of the MFGM, and XDH/XO is considered as a linker between proteins in the MFGM bilayer and proteins on the surface of the lipid inner core of MFGs (Mather, 2000, Mather and Keenan, 1998). The majority of MFGM-associated XDH/XO can be washed off using a concentrated salt solution (Bruder et al., 1982). Although the washing procedures used in these studies were different to the current study, it is still implied that XDH/XO is not a firmly bound membrane protein.
Surprisingly, BTN has been found to be firmly bound, resisting extraction with chaotropic agents and detergents (Freudenstein et al., 1979, Mather et al., 1977); however, the MFGM was removed from the surface of destabilized MFGs in the aforementioned research whereas this current study focused on the effects occurring during the washing processes on the surface of native MFGs.

In the M3 washing procedure when the MFGM was damaged (as assessed by the ζ-potential), CD 36 and FABP showed enrichment and all other key membrane proteins were depleted after the M3 process. The co-enrichment of CD 36 and FABP is probably due to CD 36 being a strong membrane-binding protein, with inter-protein binding between CD 36 and FABP (Mather, 2011). Although protein-protein interactions (CD 36 with FABP, and XDH/OX with BTN) in the MFGM are known (Mather, 2011), the depletion of FABP after the M1 washing process (Fig. 3.2 A) suggests that CD 36 and FABP proteins are partially present in an uncomplexed form due to FABP being depleted as CD 36 was enriched.

The CLSM images (Fig. 3.3) may present quantitative information based on colour (fluorescent intensities) changes, as the parameters of the CLSM were strictly controlled for all measurements. In CLSM images, Figure 3.3 O for example, it must be noted that the apparently bare MFG surfaces in the fluorescent focus plane of the confocal z-stack do not mean that membrane phospholipids and proteins were fully washed off from the surfaces of these MFGs. This phenomenon was due to the CLSM
being set at a power level that was not sufficient to excite the lesser amount of fluorescent probes on the surfaces of these MFGs. However, these apparently bare MFG surfaces indicate that some depletion of phospholipids has occurred \textit{in-situ}, therefore some structural changes to the MFGM have taken place. The fluorescently-labelled phospholipid layer on the apparently bare surfaces can be made visible by increasing the power of the CLSM (results not shown).

The increased extent of protein stained surfaces after three M3 washings (Fig. 3.3 Q, R) could indicate that MFGM proteins have been exposed, rather than being due to adsorption of milk serum proteins which are largely absent after more than one washing (Fig. 3.1). The observation of a protein-based membrane after three M3 washings is supported by the higher percentage of total proteins remaining on the surface, and these are mainly MFGM proteins due to the lower amounts of non-MFGM proteins (Table 3.2). Evidently the M1 washing procedure was not sufficiently stringent to expose membrane proteins to such a large extent, or cause the adsorption of milk serum proteins (where the outer phospholipid layer was not damaged). The observed increase in the protein stained surface after one M2 washing (Fig. 3.3 K, L) is likely due to a larger amount of adsorbed non-MGFM serum proteins (Table 3.2). From the ζ-potential, confocal, and protein measurement results, there is evidence to suggest that intensive washing procedures damaged the outer leaflet phospholipid bilayer of MFGM. The exposure of membrane proteins after the more stringent washing processes supports the notion of a protein monolayer
underneath the phospholipid bilayer, as deduced in previous models (Lopez et al., 2011, Mather, 2011).

3.4. Conclusions

This chapter describes the changes that occur in MFGs and the MFGM during the application of centrifugal washing procedures with different degrees of stringency. The type of washing processes employed will have implications for flavour and functional structure development in recombined dairy products manufactured from washed cream, particularly if specific MFGM components facilitate flavour and texture reactions. More stringent washing procedures damaged the outer leaflet bilayer of the MFGM, as assessed by the ζ-potential and changes to the amounts of adsorbed proteins. SDS-PAGE results showed the degree of integration of major MFGM proteins in the MFGM, supported by the molecular arrangement of these proteins. Evidence was provided to show the existence of a protein coat under the phospholipid bilayer in the MFGM. The results from this chapter provide further evidence for the structural arrangement of MFGM proteins and important information that can be used for fractionation of specific MFGM proteins.
CHAPTER 4.

Lateral Lipid Organization of the Bovine Milk Fat Globule Membrane is Revealed by Washing Processes

Results of this chapter had been published in the Journal of Dairy Science, as "Lateral Lipid Organization of the Bovine Milk Fat Globule Membrane is Revealed by Washing Processes" by Haotian Zheng, Rafael Jimenez-Flores, & David W. Everett, 2014 (DOI: 10.3168/jds.2014-7951)
4.1. Introduction

Food is a complex arrangement of multiple bio-components that are digested and absorbed through a dynamic and inter-related process, rather than as individual components, highlighting the importance of food microstructure in delivering health benefits. The bio-functionality of the MFGM is not simply a sum of those properties from individual molecules, but is impacted by the specific organization and structure of the PLs, cholesterol, and membrane proteins. Although current models show differences in the organization of lipids and proteins (Lopez et al., 2011, Michalski et al., 2002, Mather, 2011), there is general agreement that the MFG inner core (triacylglycerides) is coated with a trilayer MFGM system consisting of a primary monolayer of proteins and lipids and an outer PL bilayer with associated glycocalyx. Mather (2011) presented a topology of the major bovine MFGM proteins where the location and specific interactions within the MFGM were shown. Lopez and co-workers (2011) examined MFGM lipid organization and suggested co-existence and phase separation between tightly-packed liquid ordered domains (Lo, also known as lipid rafts in biological membranes) enriched in SM and cholesterol, and liquid disordered domains (Ld) enriched in other glycerolphospholipids such as PC, PE, PI, and PS, with an asymmetric distribution of Lo in the outer leaflet of the outer PL bilayer. Michalski and co-authors (2002) deduced an asymmetric distribution of PLs in the MFGM in which PLs with a low hydrophilic-lipophilic balance (HLB) are enriched in the inner monolayer in direct contact with the triacylglyceride inner core, and high HLB PLs are enriched in the outer bilayer.
Fractionation of MFGs coupled with washing to remove non-membrane associated proteins is the first step in extracting MFGM materials, and for characterizing properties of native MFGs (Mather, 2000). Many different methods have been reported utilizing different centrifugal forces, centrifugation times, and washing buffers (Huang and Kuksis, 1967, Le et al., 2009, Patton and Huston, 1986, Ye et al., 2002). It is widely accepted that mechanical treatments such as centrifugation induce depletion of MFGM materials from MFGs (Walstra et al., 2006), therefore it is reasonable to question if the MFGM compositional data generated from different isolation methods can be compared.

All of the aforementioned research and washing procedures were carried out for MFGM composition characterization only; structural information as a consequence of washing is scarce. Previous work revealed that protein structural changes in the MFGM may be induced by centrifugal washing processes (Zheng et al., 2013b) which can induce non-destructive changes and irrecoverable destructive damage to the outer phospholipid layer of the MFGM. This can be considered as a “polishing force” that can be applied to the surfaces of MFGs, thereby inducing competitive depletion of MFGM components. In this study, the relative compositional changes in lipids was measured after centrifugal washing processes. From these measurements, an updated MFGM structural model of lipid organization is proposed.
4.2. Materials and Methods

4.2.1. Materials

Bovine raw milk samples were collected from the same Jersey cow in good health and in mid-lactation stage (autumn) at a local farm (Port Chalmers, New Zealand). Milk was sampled from one cow to avoid the problem of mixing milk from cows at different stages of lactation, to avoid the need for stirring and refrigerated storage of pooled milk which would alter the MFG surface, and to avoid possible inconsistent pooling of milk from several cows. The cow was pasture-fed and vacuum milked over six consecutive sampling days. No extra mechanical or thermal treatments were applied to the fresh raw milk prior to experiments. Simulated milk ultrafiltrate (SMUF) at pH 6.5 (Jenness and Koops, 1962) was prepared and used as buffer and washing solution. The following phospholipid standards were obtained: 3-\textit{sn}-phosphatidylethanolamine (PE) and L-\textalpha- phosphatidylcholine (PC), both from bovine brain, L-\textalpha-phosphatidylinositol (PI) from bovine liver, 1,2-diacyl-\textit{sn}-glycerol-3-phospho-L-serine (PS) from bovine brain (Sigma-Aldrich, St. Louis, MO, USA) and SM from bovine milk (Avanti Polar Lipids, Alabaster, AL, USA). All other chemicals used were analytical grade and obtained from Merck (Darmstadt, Germany).
4.2.2. Isolation of milk fat globules

The same washing procedures employed in Chapter 3 were applied in this chapter. Briefly, three centrifugal isolation methods, namely M1 (3000 g, 5 min, three washes), M2 (3750 g, 15 min, one wash) and M3 (15000 g, 20 min, three washes) were used for collecting and washing MFGs according to previously published work (Gallier et al., 2010b, Patton and Huston, 1986, Ye et al., 2002) respectively, with minor modifications. M1 and M3 were carried out by washing centrifuged raw milk (cream) three times in SMUF using either the M1 or M3 centrifugation process. The cream obtained from this initial centrifugation was re-suspended into ten volumes of SMUF and left at ambient temperature for 1 h, then centrifuged again (using either the M1 or M3 process, as appropriate) to remove non-MFGM proteins from the MFG surface. This is referred to as the first wash, and was repeated a further two times using M1 or M3 to obtain the second and third washed MFG suspensions. The M2 process (Patton and Huston, 1986) used a sucrose density gradient separation as a single wash process which was applied as an intermediate stringency method. Briefly, 15 mL of sucrose-treated raw milk (containing 5% w/v sucrose) was layered under 30 mL of SMUF buffer in centrifugation tubes, and washed MFGs were collected after M2 centrifugation. A control MFG suspension sample from M2 was collected by direct centrifugation of sucrose-treated raw milk. The temperature of the SMUF buffer was 30°C and centrifugation temperature was 25°C for all experiments. Raw milk and
MFG fraction samples, which were collected after each centrifugation process, were stored at -80°C before further analysis.

4.2.3. Size distribution and assessment of specific surface area of MFGs

Volume-weighted mean diameter \((d_{43})\) and surface area-weighted mean diameter \((d_{32})\) were determined by laser diffraction using a Horiba particle analyzer LA-950 (Horiba, Irvine, CA, USA). The refractive index of MFGs was taken as 1.460 at 655 nm and 1.470 at 405 nm (Lopez et al., 2011, Michalski et al., 2001). Re-suspended MFGs were diluted by SMUF at pH 6.5 to yield a suspension with a similar fat content to that of the original milk. The diluted MFG solutions or milk were mixed with 35 mM EDTA/NaOH containing 2% (w/v) SDS buffer solution (final pH 7.0) in a 1:1 v/v ratio before loading into the particle size analyzer flow cell. The transparency of the sample cell for both light sources (655 and 405 nm) was around 90%. Measurements were carried out in triplicate. Specific surface area (S.S.A.) of MFGs was calculated from the surface area-weighted mean diameter \((d_{32})\):

\[
S.S.A. = \frac{6\varphi}{d_{32}} \quad (4.1)
\]

where \(\varphi\) is the volume fraction of milk fat (Huppertz and Kelly, 2006).
4.2.4. Total lipid extraction and quantification

A Folch extraction (Folch et al., 1957, Hundrieser et al., 1984) was carried out to extract total lipids from milk and MFG fraction samples for subsequent PL extraction and total fat content determination. Briefly, samples were mixed with chloroform-methanol (2:1, v/v) solvent in a 1:8 (v/v) ratio for milk or 1:20 (w/v) for MFG samples, respectively. The mixtures were washed with 0.2 volumes of 0.9% (w/v) NaCl solution to increase total lipid recovery. The chloroform-enriched lipid fraction phase was collected and the solvents were dried by rotary evaporation, or by a gentle nitrogen stream when the volume of solvent was not more than 3 mL. All extractions were carried out in triplicate.

4.2.5. Characterization and quantification of phospholipids

The method used for PL extraction and determination was adopted from Avalli and Contarini (2005), with slight modifications. Briefly, PLs of MFG were extracted by solid phase extraction (SPE) from the whole lipid fraction obtained from the Folch extraction. The PLs were separated using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) and detected with an evaporative light scattering detector (ELSD, Agilent Technologies). Extracted total lipids were dissolved in chloroform-methanol (2:1, v/v) to obtain 400 mg/mL of lipids for SPE extraction. A fraction of this total lipid solution (0.5 mL) was applied to silica gel-
bonded SPE cartridges (Supelclean LC-SI, 6 mL volume, 1 g sorbent, Supelco, Bellefonte, PA, USA) which was pre-conditioned with 3 mL of hexane. Non-polar lipids were first eluted with 3 mL of hexane : diethyl ether (8:2, v/v) and 3 mL of hexane : diethyl ether (1:1, v/v) followed by washing the PLs from the SPE cartridge with 2 mL of methanol and 2 mL of chloroform : methanol : water (3:5:2, v/v/v) and collected in SPE vacuum manifolds (12 ports, Sigma-Aldrich). Recovered PLs were dried under nitrogen gas and stored at –80°C before analysis by HPLC-ELSD. The PL extraction was carried out in triplicate.

Five major PLs, PE, PI, PS, PC, and SM, were separated using an analytical Zorbax Rx-SIL, 4.6 mm ID x 250 mm, 5 µm column (Agilent Technologies) on an HPLC system with a linear binary gradient program [(t0 min: 0% B, t14: 100% B, t23: 100% B, t35: 0% B, t40: 0% B; eluent A: chloroform : methanol : ammonium hydroxide (80:19.5:0.5, v/v/v), eluent B: chloroform : methanol : ammonium hydroxide : water (60:34:0.5:5.5, v/v/v/v)] at a flow rate of 1.0 mL/min. Column efficiency before and after PL analysis was tested with meta-xylene and nitrobenzene as analytes and hexane : acetonitrile (99:1, v/v) as the mobile phase to ascertain the performance for PL separation. For PLs detected by ELSD, drift tube temperature was controlled at 50°C, the pressure of nebulizer gas (nitrogen) was controlled at 3.1±0.1 bar and the signal gain was set to 6 in the Agilent chemostation software. Identification of PLs was done by comparing with the elution times of specific PL standards. The quantitation of PLs was calculated from chromatographic peak areas. Standard curves
of PLs were obtained by injecting a series with known amounts of standards (5 mg/mL for PE, PC and SM; 1 mg/mL for PI and PS) in gradient injection volumes (from 1 to 10 µL, five levels) in the HPLC. A linear regression was calculated for each standard.

4.2.6. Saponification, extraction and determination of cholesterol

The total cholesterol contents of milk and MFG fractions were determined by an enzymatic colorimetric assay on a Roche/Hitachi analysis system using “cholesterol mode” (CHOL kit, cobas®, Roche/Hitachi, Indianapolis, IN, USA) after cholesterol saponification and extraction. The extraction procedure was carried out based on previous work (Grossmann et al., 1976, Ramalho et al., 2011, Viturro et al., 2009), with modifications. Briefly, 1.0 mL of milk sample or 0.1 g of MFG fraction sample was mixed with 2.4 mL of 95% (v/v) ethanol and 1.6 mL of 50% (w/v) KOH solution. Subsequently, the whole mixture was kept at 70°C for 30 min with agitation for saponification to take place. De-ionized water (2 mL) was added into the mixture after saponification for cooling; 2 mL of n-hexane was then added and vortexed for 1 min for cholesterol extraction. The mixed solution was centrifuged at 2500 g for 10 min for phase separation. The upper cholesterol and n-hexane phase was collected and the solvent was evaporated under a gentle nitrogen stream at ambient temperature. The nitrogen-dried cholesterol sample was re-dissolved in 1 mL isopropanol and centrifuged at 21000 g for 3 min at ambient temperature. The obtained supernatant was stored at -80°C within 24 h before the final cholesterol determination by
enzymatic colorimetric assay. The system was pre-calibrated with standard blood serum sample (Roche, Basel, Switzerland) containing a known amount of cholesterol. The loading volumes of cholesterol in samples for colorimetric analysis were all within the assay capacity (0.03–8 mg/mL). The extraction was done in triplicate and analysis was done in duplicate.

4.2.7. Estimation of values of hydrophilic–lipophilic balance of phospholipids

The HLB of MFG phospholipids were calculated using Griffin’s method (Griffin, 1954; Ishii and Nii, 2005; Fukuhira et al., 2009), in which the HLB was determined from the ratio of the hydrophilic portion mass to the whole molecular mass. Each type of MFG phospholipid has several sub-species with different fatty acid composition, therefore, only the major sub-species of each PL [PE (16:0/18:0/18:1/18:2); PI (18:0/18:1); PS (18:0/18:1); PC (16:0/18:1); SM (16:0/23:0/24:0)] (MacGibbon and Taylor, 2006) were considered to simplify the calculation.

4.2.8. Statistical analysis

Significant differences ($P < 0.001$) between samples were determined by one-way analysis of variance (Fisher’s test) using Minitab 16 (Minitab Inc., State College, PA, USA).
4.3. Results and Discussion

4.3.1. Quantification of total phospholipids and cholesterol

Approximately 60–65% of PLs are associated with the MFGM, and the rest are desorbed into the aqueous phase of raw milk (MacGibbon and Taylor, 2006). This may occur during the mechanical treatment of milk during transportation and processing. It is assumed that all of PLs and cholesterol were associated with the MFGM (regardless of the size of MFGs) in the unprocessed milk samples. This does not impact upon the subsequent calculations of relative changes in these components during processing.
Table 4.1. Quantitation of total polar lipids and cholesterol in raw milk and milk fat globule washed fractions on both a total fat weight and specific surface area basis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total polar lipids</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g of fat</td>
<td>mg/m² of MFGs</td>
</tr>
<tr>
<td>M1-raw milk</td>
<td>4.69 ± 0.34^b</td>
<td>2.76 ± 0.20^b</td>
</tr>
<tr>
<td>M1-cream</td>
<td>2.49 ± 0.07^ef</td>
<td>1.59 ± 0.04^fg</td>
</tr>
<tr>
<td>M1-1 wash</td>
<td>2.22 ± 0.06^h</td>
<td>1.65 ± 0.05^cdef</td>
</tr>
<tr>
<td>M1-2 wash</td>
<td>2.14 ± 0.05^hi</td>
<td>1.66 ± 0.04^cdef</td>
</tr>
<tr>
<td>M1-3 wash</td>
<td>1.99 ± 0.11^i</td>
<td>1.70 ± 0.07^cde</td>
</tr>
<tr>
<td>M2-raw milk</td>
<td>4.78 ± 0.35^b</td>
<td>2.81 ± 0.20^b</td>
</tr>
<tr>
<td>M2-cream</td>
<td>2.54 ± 0.15^e</td>
<td>1.62 ± 0.09^efg</td>
</tr>
<tr>
<td>M2-1 wash</td>
<td>2.41 ± 0.20^fg</td>
<td>1.55 ± 0.13^g</td>
</tr>
<tr>
<td>M3-raw milk</td>
<td>5.00 ± 0.45^a</td>
<td>2.94 ± 0.27^a</td>
</tr>
<tr>
<td>M3-cream</td>
<td>2.89 ± 0.11^c</td>
<td>1.76 ± 0.07^c</td>
</tr>
<tr>
<td>M3-1 wash</td>
<td>2.72 ± 0.16^cd</td>
<td>1.73 ± 0.10^cd</td>
</tr>
<tr>
<td>M3-2 wash</td>
<td>2.62 ± 0.08^de</td>
<td>1.62 ± 0.05^defg</td>
</tr>
<tr>
<td>M3-3 wash</td>
<td>2.26 ± 0.10^gh</td>
<td>1.56 ± 0.07^fg</td>
</tr>
</tbody>
</table>

^1Results are means ± Stdev; n=9 for raw milk and washed fractions used in M1 & M3; n=18 for raw milk and washed fraction used in M2.

^2Milk fat globules.

^aData that do not share a letter within the same column are different (P < 0.001).
Table 4.2. Percentage composition of specific phospholipids on milk fat globule surfaces calculated on a specific surface area basis.  

<table>
<thead>
<tr>
<th>% S.S.A.² basis</th>
<th>PE</th>
<th>PI</th>
<th>PS</th>
<th>PC</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1-raw milk</td>
<td>27.15±0.35fg</td>
<td>5.84±0.27g</td>
<td>14.12±1.00efg</td>
<td>26.94±0.64a</td>
<td>25.62±0.62a</td>
</tr>
<tr>
<td>M1-cream</td>
<td>31.28±0.97ab</td>
<td>8.55±0.60ab</td>
<td>12.19±0.42ef</td>
<td>24.25±0.99cd</td>
<td>24.06±0.89c</td>
</tr>
<tr>
<td>M1-1</td>
<td>31.20±0.86ab</td>
<td>8.85±0.27a</td>
<td>11.65±0.49de</td>
<td>23.64±0.76de</td>
<td>24.61±0.52bc</td>
</tr>
<tr>
<td>M1-2</td>
<td>29.97±0.96cde</td>
<td>8.33±0.33bcd</td>
<td>13.60±0.83g</td>
<td>23.48±1.04de</td>
<td>24.56±0.53bc</td>
</tr>
<tr>
<td>M1-3</td>
<td>29.91±1.04de</td>
<td>8.43±0.28bc</td>
<td>13.81±0.79fg</td>
<td>22.82±0.78ef</td>
<td>25.16±0.49ab</td>
</tr>
<tr>
<td>M2-raw milk</td>
<td>27.30±0.64f</td>
<td>5.77±0.28g</td>
<td>14.55±1.00ef</td>
<td>27.35±0.89a</td>
<td>25.59±0.79a</td>
</tr>
<tr>
<td>M2-cream</td>
<td>26.50±0.71g</td>
<td>8.16±0.48cd</td>
<td>16.15±1.18bc</td>
<td>25.22±1.41b</td>
<td>24.21±0.88c</td>
</tr>
<tr>
<td>M2-1</td>
<td>26.45±0.80g</td>
<td>8.25±0.45cd</td>
<td>15.67±1.47cd</td>
<td>25.19±1.42b</td>
<td>24.23±1.07c</td>
</tr>
<tr>
<td>M3-raw milk</td>
<td>27.35±0.58f</td>
<td>5.73±0.29g</td>
<td>15.02±0.99de</td>
<td>27.40±1.15a</td>
<td>25.45±1.43a</td>
</tr>
<tr>
<td>M3-cream</td>
<td>32.02±2.95a</td>
<td>7.34±0.92f</td>
<td>16.75±1.36ab</td>
<td>25.87±1.80b</td>
<td>20.93±0.91ef</td>
</tr>
<tr>
<td>M3-1</td>
<td>30.73±1.25bc</td>
<td>7.73±0.64ef</td>
<td>16.98±0.62ab</td>
<td>25.00±1.15bc</td>
<td>20.65±0.87f</td>
</tr>
<tr>
<td>M3-2</td>
<td>29.31±0.62e</td>
<td>7.46±1.06f</td>
<td>16.33±1.61abc</td>
<td>23.91±0.46d</td>
<td>21.46±0.61e</td>
</tr>
<tr>
<td>M3-3</td>
<td>30.52±0.87bcd</td>
<td>8.00±0.28de</td>
<td>16.78±0.48ab</td>
<td>22.46±1.01f</td>
<td>22.89±1.82d</td>
</tr>
</tbody>
</table>

¹Mean ± standard deviation; n = 9 for washed fractions M1 & M3; n = 18 for washed fraction M2.
²S.S.A. = specific surface area.
³Data that do not share a letter within the same column are different (P < 0.001).
Chapter 4: Lateral Lipid Organization of the Bovine Milk Fat Globule Membrane is Revealed by Washing Processes

The total amount of PLs and cholesterol in the MFGM were calculated as a proportion of total fat (mg/g) to quantify relative changes of each compound, and also calculated on a MFG surface area basis from S.S.A. (mg/m²) values to characterize the surface changes (Table 4.1). The results of PL content in milk are comparable with reported results, 5.1 ± 1.7 mg/g of lipid (Patton and Huston, 1986), 2.5 ± 1.4 mg/g of lipid (Lopez et al., 2008), 6.3 ± 0.5 mg/g of lipid (Lopez et al., 2011), and 9.8 ± 0.0 mg/g of lipid (Rombaut et al., 2006) although different MFG isolation methods were applied in each study. In the current study, the PL content of milk samples used for the M3 process (~5.00 mg/g fat) was different (P < 0.001) than milk for the M1 process (~4.69 mg/g fat), although the samples were sourced from a same cow (mid-lactation stage) and the feed of the cow was not changed over the six-day milking period. Despite this, there was no significant difference (P < 0.001) in cholesterol content between milk samples prior to the M1 and M3 processes. A diet enriched in polyunsaturated fatty acids induces a higher yield of PLs (Lopez et al., 2008); however, the feed in the current study was controlled, suggesting that other environmental factors may affect the yield of PL. The yield of cholesterol was more consistent across raw milk samples (Table 4.1) compared to PLs, suggesting that the yield/secretion of cholesterol is less environmentally dependent than the PLs.

Total PL content of MFGs decreased significantly (P < 0.001) after the initial centrifugation of milk for all three processes, and the most intensive process (M3) yielded the highest amount of PLs on a weight and surface basis (Table 4.1).
Although depletion of MFGM material will occur during centrifugation (Walstra et al., 2006), a more intensive initial centrifugation of milk can collect a higher volume of smaller MFGs into the cream phase. Smaller lipid structures, including fat globules, have more PL content (based on both fat weight and S.S.A.) due to a smaller proportion (and in some cases, absence) of the triacylglyceride inner core of small vesicle-like structures less than 2 μm, and with a higher total surface area for smaller sized particles (Gallier et al., 2011, Lopez et al., 2011). Consequently, a higher recovery of PLs is expected when more of the smaller lipid structures are collected by centrifugation. Generally, a loss of total PLs after the three different washing processes was found when PL content was calculated on a total fat basis (Table 4.1). Surprisingly, when the S.S.A of MFGs was used as the basis for the calculation of PL content in the M1 process, a relative enrichment rather than loss of PL content was found from the cream to the third wash, but a significant loss of PL was found from the cream to the third wash in the M3 process (Table 4.1). The apparent relative enrichment of PL on a surface area-weighted basis is due to less depletion after the mild washing process with concomitant increase in the surface area-weighted mean diameter of MFGs ($d_{32}$), therefore a decrease in S.S.A. These results are in good agreement with Zheng, Jiménez and Everett (2013b and Chapter 3) where it was reported that the milder M1 process did not induce destructive damage to the PL outer bilayer of the MFGM, however the M3 process exposed the inner protein layer of the MFGM after the second wash, which was deemed to be destructive damage to the outer bilayer. During M3 washing, the PL bilayer may be destroyed by the high shear
force on the surface of MFGs during centrifugation. The M2 and M3 washing processes are recommended when a high total yield of PL is required, however the M1 process is recommended if damage to the MFG surface should be minimized.

For validation of the cholesterol assay, a standard addition method was carried out by spiking the MFG fractions with pure cholesterol; the calculated recovery of cholesterol from the fractions was 99.8±4.6%. The cholesterol values (Table 4.1) compare well with the reported value of 3.3 mg/g fat in raw milk (MacGibbon and Taylor, 2006). There was no significant difference ($P < 0.001$) in cholesterol content in raw milk samples after the first washing step for all three methods on both a weight and surface area basis, with the exception of the third wash in the M3 process (Table 4.1). The small degree of cholesterol depletion from the raw milk samples was not as much as the depletion of PL, suggesting that cholesterol is a relatively tightly bound component within the MFGM. This supports the structural model systems of the MFGM in which cholesterol is reported to interact with SM to form rigid $L_n$ domains (lipid rafts) in the MFGM bilayer (Lopez et al., 2011, Lopez et al., 2010). Surprisingly, an apparent relative enrichment of cholesterol on a weight-basis was found in the M3-3 wash sample in contrast to the depletion of total PL. This suggests that cholesterol is not desorbed together with PLs as integral MFGM fragments after the intensive centrifugation treatment. Lee and co-workers (1999) also reported that increased centrifugal force may decrease the depletion of cholesterol from MFG surfaces.
These observations suggest the following MFGM structural changes during washing. The PLs in the L_d domain of the outer leaflet of the MFGM PL bilayer (Gallier et al., 2010b, Lopez et al., 2011, Lopez et al., 2010) that are not associated with cholesterol are released from the surface of MFGs. The MFGM bilayer may rupture and the rigid L_o domains containing cholesterol then become exposed to the aqueous phase and repartition from the outer bilayer to the primary inner monolayer of the MFGM. The cholesterol content was enriched when the outer bilayers of MFGs were damaged; a process of cholesterol partitioning from MFGM to the core of the MFG was discussed by Evers (2004a).

The relative increase in cholesterol content on a surface area-weighted basis on the MFG surfaces can be considered as another indicator for assessing damage to the MFGM. If apparent relative enrichment of cholesterol on MFG surfaces is observed during mechanical treatment, then destructive damage to the outer bilayer of MFGM is likely. As a consequence, this suggests that the M1 and M2 washing processes do not induce destructive damage to the outer bilayer of the MFGM whereas the M3 process damages the bilayer during the third washing step. This is in good agreement with confocal laser scanning microscopy images where damage to the PL bilayer and exposure of the inner monolayer containing membrane proteins after the M3 washing process was observed (Zheng, Jiménez & Everett, 2013b).
4.3.2. Relative depletion/enrichment of individual phospholipids

The quantification of individual PLs by linear regression gave a high correlation coefficient squared ($R^2 > 0.99$). A standard addition procedure was carried out to measure PE and SM recovery, yielding $99.0\pm2.3\%$ for PE and $96.7\pm0.2\%$ for SM. The use of PL standards derived from milk gave a higher recovery compared to previous work (Avalli and Contarini, 2005), where standards derived from bovine brain were used. Typical chromatograms from samples of M3-cream and M3-3 washed fractions show the region where the five major PLs were eluted between 10 and 20 min (Figure 4.1). Large amounts of neutral lipids were eluted from the analytical HPLC column within the first 5 min and no noticeable peaks were shown after 20 min (results not shown). The phospholipids PE, PC, and SM accounted for most of the total PL content, and PI, PS were minor components. Four distinguishable sub-peaks of PC ($PC_2 > PC_1 > PC_3 > PC_4$) and three sub-peaks of SM ($SM_2 > SM_3 > SM_1$), both in terms of peak area, were found (Figure 4.1), as also observed by others (Avalli and Contarini, 2005, Lopez et al., 2011, Rombaut et al., 2005). The peak area ratios between sub-peaks of PC changed after three washes in the M3 process, showing a relative depletion of $PC_1$ and $PC_2$ but almost no change in $PC_3$ and $PC_4$ (Figure 4.1 A, B). Although the detailed structural organization of these four species of PC is unknown, this phenomenon implies an asymmetric distribution and tightly held structural organization of $PC_3$ and $PC_4$ in MFGM; $PC_3$ and $PC_4$ can be hypothesized
to be the tightly packed high phase transition temperature (> 30°C) phospholipids in the outer leaflet bilayer of MFGM.

Figure 4.1.
HPLC chromatograms of lipids on the milk fat globule membrane for M3-cream (A) and M3-3 (B). Marked numbers are sub-peaks for PC and SM.
Figure 4.2.
Schematic model of lipid organization of the bovine milk fat globule membrane showing the outer bilayer and inner monolayer. On the outer leaflet of the bilayer, liquid-ordered regions are shown as phospholipids with headgroups of a darker shade; the grey headgroups show the liquid-disordered regions.
The proportions of five major PLs in the MFGM were calculated on a surface area-weighted basis (Table 4.2). Generally, the initial centrifugation step for producing cream changed the proportions of individual PLs on the surface for all three washing processes, likely due to either 1) desorption of MFGM material under centrifugal forces, 2) the removal of free non-adsorbed PLs (or MFGM fragments) from the aqueous phase of milk and cream, or 3) changes in the S.S.A. of MFGs as a consequence of a shift in the size distribution. Of these three mechanisms, the first is the one more likely to damage the MFG surface and thus show potential to reveal the structural changes that occur during processing.

Comparisons of apparent relative depletion and enrichment of individual PLs on MFG surfaces was carried out with the initial isolated creams and the washed MFG suspensions in the M1 and M3 processes. The data from the original milk were excluded from the comparison to eliminate the confounding factor of free non-adsorbed PLs, MFGM fragments, and MFGM vesicles. During the M1 washing process when damage to the MFGM did not occur, relative desorption of PE and PC from the surfaces of MFGs were found from the cream to the M1-3 wash (Table 4.2) whereas PI and PS did not change, and the amount of SM increased after the M1-3 wash from the cream. A similar trend was found for the M3 method, with the exception of an increase in PI from cream to the third wash. The proportion of PE increased from M3-2 to M3-3, therefore it is reasonable to deduce that some of the PE are repartitioning from the outer bilayer into the inner monolayer (similar to the
repartitioning of cholesterol) during the third wash of the M3 process when the outer bilayer was damaged due to its peripheral location in the MFGM. Both PC and PE can be considered as loose PLs as they were significantly washed off the surfaces of MFGs in the M1 and M3 processes. These phenomena must relate to the structural properties and specific locations of PLs in MFGM.

Although the total PL content of milk samples varied across different milking days (data not shown), the proportions of individual PLs were not significantly different ($P < 0.001$; Table 4.2). The proportion of each major PL in milk (Table 4.2) is comparable with previously published work, PE ~23.2%, PI ~8.1%, PS ~16.1%, PC ~26.6% and SM ~26.0% (w/w) (Lopez et al., 2011), but different to that reported by (Rombaut et al., 2005) where the PE content is higher than 40%, and PC and SM lower than 20%. MacGibbon and Taylor (2006) noted that the measured proportions of individual PLs depend upon the analytical methods used, the breed of the cow, the diet, and the stage of lactation. The extraction method of PLs from milk is also a crucial factor (Avalli and Contarini, 2005, Gallier et al., 2010a).
Table 4.3. Estimation of HLB value of phospholipids

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>PE</th>
<th>PI</th>
<th>PS</th>
<th>PC</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0/18:0/18:1/18:2</td>
<td>18:0/18:1</td>
<td>18:0/18:1</td>
<td>16:0/18:1</td>
<td>16:0/23:0/24:0</td>
<td></td>
</tr>
<tr>
<td>HLB</td>
<td>2.6</td>
<td>8.4</td>
<td>3.9</td>
<td>3.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>

The average length of phospholipid fatty acid chains were calculated from the distribution of fatty acids in bovine milk.
4.3.3. Updated model of phospholipid organization in the milk fat globule membrane

An updated model system of lipid organization in the MFGM is proposed from observations of competitive depletion of PLs from MFG surfaces, the calculated HLB values (Table 4.3), and integrating features from previous models (Lopez et al., 2011, Michalski et al., 2002). In the current schematic model (Figure 4.2), the outer bilayer is separated into ordered domains (gel phase: black filled head groups of SM without cholesterol; L_o: black filled PC and SM with cholesterol) and liquid-disordered domains (L_d, grey filled). Lissamine rhodamine B-labelled phospholipid (DPPE) fluorescent dye was found to be segregated from the DPPE-induced gel domains and is located in the DPPC liquid crystalline domains at intermediate temperature on a non-supported artificial phospholipid bilayer system (Bagatolli and Gratton, 2000). This suggests that lipid segregation in the MFGM, as previously characterized using CLSM after fluorescent staining with lissamine rhodamine B-labelled DOPE (Gallier et al., 2010b, Lopez et al., 2010), is not only represented by L_o domains as shown in previous schematic model systems (Lopez et al., 2011, Lopez et al., 2010), but also represented by gel domains (Figure 4.2). It has been reported that SM displays a gel-liquid transition around physiological temperatures (Barenholz and Thompson, 1980, Contreras et al., 2004) which rationalizes the co-existence of gel and liquid states of SM (Figure 4.2).
The glycerolphospholipids, phosphosphingolipid and cholesterol are asymmetrically distributed in the trilayer system (as shown in Figure 4.2). There are nineteen PLs and three cholesterol molecules in the schematic model system simulating the lipid composition of the native MFGM. In the model, PE, PC and SM account for 26% each, and PS, PI, and cholesterol account for 16%, 11%, and 16% (mol/mol), respectively, of total phospholipid and cholesterol molecules. This lipid composition in the model is comparable with the estimated lipid composition of the native MFGM from previously published results (Huppertz and Kelly, 2006, Lopez et al., 2011) in which PE ~23%, PC ~26%, SM ~26%, PS ~16%, PI ~11%, and cholesterol ~16% (mol/mol).

The hydrophobicity of individual PLs and HLB values decreased in the order PI > PS > SM > PC > PE (Table 4.3). From this additional information, the proposed model (Figure 4.2) is further refined to propose the location of individual PLs as follows: PI and PS form part of the inner monolayer as they were relatively resistant to being removed after washing the MFG suspensions (from raw cream to final wash; Table 4.2). These two PLs (particularly PI) have relatively higher HLB values and are therefore more likely to stabilize the inner triacylglyceride core than PC, PE or SM to form oil-in-water (o/w) emulsions. The PS may be evenly distributed in both the outer bilayer and inner primary monolayer as there was no significant relative enrichment during the M3 washing processes (Table 4.2) when the outer bilayer was (partially) washed away, as shown in CLSM images (Zheng et al., 2013b).
It is possible that PC, SM and PS are present in the inner leaflet of the outer bilayer (Figure 4.2) as they have similar HLB values (Table 4.3). Previous reports have deduced that PE, PI, and PS are largely located within the inner layer of the MFGM bilayer whereas PC and SM are located on the outer surface (Deeth, 1997, Rombaut et al., 2006); however, there is uncertainty about the detailed distribution of PLs in the inner layers of the MFGM (the primary PL monolayer covering the triacylglyceride core and the inner half leaflet of the outer bilayer). It is proposed that PE is located in the inner half leaflet of the outer PL bilayer (Figure 4.2) as the lower HLB value is more likely to result in PE as low HLB emulsifier stabilizing a water-in-oil (w/o) emulsion system, namely the interface between the external half leaflet of the bilayer and the primary monolayer. Deducing the functionality of emulsifiers with different HLB values in terms of the preference for stabilizing either o/w or w/o emulsion systems is based on Griffin’s theory (Griffin, 1949).

The critical packing parameter (which determines the preferred association structure of molecular shape dependent on the effective volume of fatty acids, length of fatty acids, and head-group area) of PE is higher than 1, and it has an inverted truncated cone critical packing shape which makes it suitable to have an inverted packing structure (Israelachvili, 2010) in the inner leaflet of bilayer of the interface region between the PL outer leaflet of the bilayer and the PL inner monolayer (Figure 4.2).
This specific location of PE is supported by the relative enrichment of PE in the MFGM from the second to the third wash in the M3 process (Table 4.2).

More stringent washing processes (M3) damage the external PL bilayer leading to depletion of PE from the inner leaflet from cream to the third wash (M3-cream to M3-3). During the final wash of the M3 process, cholesterol was relatively enriched in the MFGs, possibly due to the partitioning of cholesterol from the outer bilayer to the primary monolayer (or the triacylglyceride inner core). A similar hypothesis could be made for the repartitioning of PE when the bilayer was damaged after the final wash in the M3 process to explain the small relative enrichment from M3-2 to M3-3 wash (Table 4.2). This small degree of relative enrichment of PE may also be due to its specific location in the native MFGM as shown in Figure 4.2.

The majority of PC is likely present in the external part of the PL bilayer as PC was partially desorbed in the M1 and M3 washing processes (Table 4.2), which is characterized by a loose arrangement of PLs in the MFGM (Rombaut et al., 2006). The proportions of PC and SM decreased after initial centrifugation for cream isolation after all three processes (more so for SM in the M3 process) whereas other PLs increased in relative proportion, with the exception of PS for the M1 process where it remained the same (Table 4.2). This suggests that PC and SM are both present in the outer layer of the MFGM (Danthine et al., 2000, Deeth, 1997, Rombaut et al., 2006). Moreover, the cholesterol content did not change significantly from M1-
raw milk to M1-cream (S.S.A. basis, Table 4.1), which together with the significant desorption of SM in the paired samples (Table 4.2), suggests that not all SM molecules are interacting with cholesterol (Figure 4.2). The outer leaflet of PL bilayers of biological membranes, such as the plasma membranes of human and animal cells, are enriched with zwitterionic PC and SM (McMullen et al., 2004, Op den Kamp, 1979). A similar observation was discussed by Rombaut and co-workers (2006) who reported PE, PI, and PS were negatively correlated with PC and SM during process-induced depletion, and who also noted a high SM content in skimmed milk. Therefore in the current results, the increase in the proportion of SM from cream to three-times washed MFGs for the M1 and M3 processes (Table 4.2) suggests that some of the SM is held rigidly in the MFGM structure and some held more loosely as shown in Figure 4.2. The rigidly held SM in the MFGM can be considered as indirect evidence for the existence of SM-enriched lipid liquid-ordered (L_0) domains which have been characterized by confocal laser scanning microscopy after fluorescent staining (Gallier et al., 2010b, Lopez et al., 2010).

It is reported that the formation of these L_0 domains is regulated by cholesterol, and are specifically formed by the interaction with SM (de Almeida et al., 2009, Lopez et al., 2010). Cholesterol not only interacts with SM but also with other glycerol-phospholipids in biological membranes, although SM has a higher affinity to cholesterol than other PLs, such as PC (McMullen et al., 2004). Therefore, some of the cholesterol in MFGM may possibly interact with other glycerol-phospholipids
(such as PC) and locate within the $L_o$ domain, and also protrude from the $L_d$ domain as shown in the proposed model system of the MFGM (Figure 4.2). Indeed, Vist and Davis (1990) characterized the existence of an ordered domain from binary mixtures of cholesterol and DPPC (one of the major species of milk PC) using differential scanning calorimetry and nuclear magnetic resonance spectroscopy. However, more direct evidence from MFGM condition and environment is still scarce. The laterally protruded $L_o$ domain, containing specific sub-species of PC (longer and saturated acyl chains) and SM in the MFGM (Figure 4.2), can be explained by the higher $trans/gauche$ ratio in the $L_o$ domains causing a thicker structure than for the $L_d$ domain (Simons and Vaz, 2004). Results from atomic force microscopy has shown a thicker structure of the $L_o$ domain in monolayer model systems formed from milk-derived phospholipids (Gallier et al., 2010c). It has also been found that the gel phase is thicker than the fluid phase on a supported PL bilayer (Giocondi et al., 2004), which supports the notion of protrusion of ordered domains (Figure 4.2).

4.4. Conclusions

The present study measured the changes in relative proportions of the major MFGM polar lipid components and cholesterol on the surfaces of native MFGs after centrifugal washing processes with different degrees of stringency. Consequently, the competitive binding strengths of five major MFGM polar lipids on native MFG surfaces were revealed. The proposed mechanism of repartitioning of cholesterol
between the MFGM outer bilayer and primary inner monolayer was described based on the relative enrichment of cholesterol after an intensive washing procedure that caused destructive damage to the outer bilayer. The general location of major polar lipids in the trilayer structure was deduced from relative depletion and enrichment of components after washing, as well as the physical properties of MFGM polar lipids. Evidence was provided that sphingomyelin is located in both the ordered (gel and liquid-ordered) and liquid-disordered regions of the MFGM, and not necessarily in association with cholesterol. This study updates the current understanding of lipid organization in the MFGM which will have important implications for MFGM extraction and purification as a dairy ingredient, and greater understanding on its physiological functions.
CHAPTER 5.
Phospholipid Architecture of the Bovine Milk Fat Globule Membrane Using Giant Unilamellar Vesicles as a Model

The results of this chapter had been published in Journal of Agricultural and Food Chemistry 62 (14), pp 3236–3243, as "Phospholipid Architecture of the Bovine Milk Fat Globule Membrane Using Giant Unilamellar Vesicles as a Model" by Haotian Zheng, Rafael Jiménez-Flores, Derek Gragson, & David W. Everett, 2014 (DOI: 10.1021/jf500093p)
5.1. Introduction

Bio-membranes act as barriers between different biological compartments that govern communication between living cells, and are involved with the passage of signals (Kahya, 2010). As a consequence, the MFGM in its native and integral form plays an important role in physiological processes including nutrient absorption, digestion and signal exchange, especially for neonates who consume milk as an essential food. Michalski and Januel (2006) reviewed the controversial and largely positive effects of homogenization of bovine milk on human health in which the macrostructure of MFGs were altered and the MFGM was broken. Coverage of MFGs with the native MFGM in human milk results in more efficient gastric lipolysis than for homogenized lipid droplets in infant formula (Favé et al., 2004). Argov et al. (2008a) extended the discussion on the importance of MFG structure and pointed out that the effects associated with MFGs are only expected to retain nutritional and structural functionality without altering the unique macrostructure of MFGs.

As cellular membranes, the MFGM has a highly intricate architecture and it is difficult to reveal the native structure in terms of lipid and protein localization and lipid-protein interactions. It is also difficult to trace the specific functions in space and time of both membrane lipid-protein network and specific membrane domains, which have been observed by confocal laser scanning microscopy (CLSM) (Gallier et al., 2010b, Lopez et al., 2010). In recent studies, a fluorescent headgroup-labelled
phospholipid analogue 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rd-DOPE) was used to label MFGs in which lateral segregation and lipid domains were characterized. These studies discussed the possibility that the non-fluorescent dark domains are lipid rafts (liquid-ordered phase, \(L_o\)) enriched in SM and cholesterol (Gallier et al., 2010b, Lopez et al., 2010) as Rd-DOPE is preferably portioned into liquid-disordered phase (\(L_d\)) (Sengupta et al., 2011). This proposal remains controversial as it has been demonstrated that the co-existence of phase regions [for instance, liquid crystalline phase (\(L_\alpha\)) + gel phase (\(L_\beta\)) and \(L_\alpha + L_\beta\)] may not always distinguish between \(L_o\) and gel regions, thus giving misleading information about lipid surfaces when using fluorescent imaging techniques (Feigenson, 2009). MFGM contains a relatively high \(L_\beta-L_\alpha\) phospholipid phase transition temperature (\(T_m\)) [for instance, 16:0-PC (DPPC) and SM] which remains in a gel-phase state at ambient temperature, and the \(L_\beta\) may contribute to the dark regions on MFG surfaces by CLSM. Although the \(L_\beta\) phase may be transformed to the \(L_\alpha\) phase by the inclusion of cholesterol (Feigenson, 2009, Zhao et al., 2007, Feigenson, 2007), the molar amount of cholesterol is less than the molar amounts of the high \(T_m\) phospholipids in the MFGM (Huppertz and Kelly, 2006), consequently, the existence of an \(L_\beta\) phase in the MFGM at ambient temperature is expected.

A supported mono-layer model system was prepared from milk-derived phospholipids to examine lipid-lipid interactions and phase co-existence (Gallier et al., 2010c); however, non-supported phospholipid bilayer systems are rarely used to model the
MFGM. Giant unilamellar vesicles as an artificial bilayer membrane system constructed from phospholipids, with or without cholesterol, have been used to mimic the morphology of native bio-membranes and to investigate interactions between proteins and lipids, as well as specific functions of lipid domains (Kahya, 2010, Menger and Keiper, 1998). Techniques such as fluorescent imaging (Morales-Penningston et al., 2010, Politano et al., 2010) have provided fundamental knowledge of GUV formation. In the current study, GUVs from milk-derived phospholipids were constructed and used as a model to examine the lipid morphology of the MFGM.

5.2. Materials and Methods

5.2.1. Materials

Indium tin oxide (ITO)-coated glass slides (surface resistivity 30-60 Ω/cm²) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The fluorescent fatty acid-labelled phospholipid analogue 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoazadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (16:0-12:0, NBD-PC, 1 mg/mL in chloroform), the fluorescent headgroup-labelled phospholipid analogue 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (18:1, Rd-DOPE, 1 mg/mL in chloroform) were chosen as fluorescent probes; 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0-PC, DPPC in powder form, Lβ/Lα phase transition temperature > 22 ± 1°C, ambient temperature), 1,2-dioleoyl-sn-
glycero-3-phosphoethanolamine (18:1-PE, DOPE in powder form, \(L_\beta/L_\alpha\) phase transition temperature < 0ºC), sphingomyelin (fractionated from bovine milk, SM, \(L_\beta/L_\alpha\) phase transition temperature > 22 ± 1ºC, ambient temperature) were all provided by Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Sucrose and glucose (both anhydrous), and chloroform, acetone, methanol and water (HPLC grade) were all sourced from Fisher Scientific (Fair Lawn, NJ, USA). A function generator (model 72-7710) was purchased from TENMA test equipment (Springboro, OH, USA) and digital phosphor oscilloscope (model TDS 3012, 100 MHz, 1.25 GS/s, 10K point) was obtained from Tektronix (Beaverton, OR, USA).

5.2.2. Electroformation of Giant Unilamellar Vesicles (GUVs)

GUVs were generated from combinations of phospholipids under an alternating current (AC) electrical field (Angelova and Dimitrov, 1986, Bagatolli and Gratton, 2000, Dimitrov and Angelova, 1987, Herold et al., 2012, Morales-Penningston et al., 2010, Veatch, 2007) with modifications. A typical apparatus setup for GUV electroformation and GUV chamber assembly are shown in Images 5.1-5.3. The detailed sample plan and operation are explained as follows.
5.2.3. Sample Plan and Preparation

GUVs were formed from unitary, binary (milk SM is considered as unitary) and quaternary systems including DPPC, DOPE, and SM, with or without cholesterol. Two sets of sample groups were prepared for studying the effects of DPPC and SM on regulating the lipid domain formation. Set 1: DPPC, DPPC + DOPE (7:3, 1:1, 3:7, mol/mol) and DOPE; set 2: SM, SM + DOPE (7:3, 1:1, 3:7, mol/mol) and DOPE. In simulating the MFGM composition, an extra sample containing DPPC, SM, DOPE and cholesterol (8:8:8:4, mol/mol) was prepared to mimic the morphology of the native MFGM. It has been reported that the weight ratio between phospholipid and cholesterol in the MFGM is 25:2, w/w (~25:4, mol/mol, calculated based on the mean molecular weight) in PE, PC and SM are the major phospholipid constituents (> 90% w/w of total phospholipid) (Huppertz and Kelly, 2006); moreover, DOPE (47.0% PE, w/w) and DPPC (32.2% PC, w/w) are the major sub-species. DPPC, DOPE and SM were chosen in this study as they are the major phospholipid species in bovine milk (MacGibbon and Taylor, 2006), and they have a broad range of L\(_{\beta}/L_{\alpha}\) phase transition temperatures (Shalaev and Steponkus, 1999, Leonenko et al., 2004, Barenholz and Thompson, 1980, Contreras et al., 2004). Phospholipids were dissolved in chloroform to yield 10 mg/mL sample solutions which contained Rd-DOPE or NBD-PC (0.25 mol%) as a fluorescent dye during the electroformation of GUVs.
5.2.4. Electroformation of GUVs

Briefly, lipid formula sample solutions (2.5 μL) were placed on the surface of the ITO slides. After completely evaporating any residual moisture in a desiccator (room temperature, 1 h), the samples were re-hydrated in 100 mM sucrose solution. GUVs were formed under AC field conditions (3 V, 10 Hz, ~55°C) in a custom designed chamber for 45 min (Morales-Penningston et al., 2010; Veatch, 2007). The prepared GUVs were taken from the electroformation chamber and placed into 100 mM glucose solutions (1/4, v/v) using a wide-mouth pipette (> 1 mm diameter), at which point the newly formed GUVs were ready for CLSM observation. It was reported (supported by observations in this current research) that ageing or insufficient cleaning of ITO slides may affect the GUV electroformation process resulting in a low yield of GUVs, therefore the ITO slides were handled by a standard cleaning procedure following a mild annealing process (Herold et al., 2012). Electroformation was carried out in duplicate for each lipid combination.

5.2.5. CLSM observation of GUVs

GUV preparations were pipetted onto observation slides containing a small well (~3 mm depth) for holding GUVs without extrinsic pressure from the coverslip. An Olympus FV1000 inverted confocal laser scanning microscope (Olympus America Inc., Center Valley, PA, USA) with oil-immersion objective lenses (40× or 60×) was
used to observe the structural properties and morphology of GUVs. Fluorescent signal collection (for rhodamine B and NBD) and the CLSM configuration were adopted from previous work (Gallier et al., 2010b), with modifications. Rd-DOPE and NBD-DLPC were excited by a diode laser (559 nm) and Ar⁺ laser (488 nm), respectively, and the emitted spectrum was measured between 570-670 nm and 500-600 nm, respectively. 3D images of GUVs were constructed from a group of individual 2D images taken across a defined z-axis section. Differential interference contrast illumination was used in the transmitted light channel for determination of the positioning of GUVs. All of the image processing was carried out at 22 ± 1°C. The operating parameters of the CLSM were not necessarily kept constant for each image as the parameters were optimized to obtain the clearest images of GUVs. This was deemed to be acceptable as relative quantification of phospholipids was not the focus of the study.
Image 5.1: Experimental setup for GUV electroformation.
Chapter 5: Phospholipid Architecture of the Bovine Milk Fat Globule Membrane Using Giant Unilamellar Vesicles as a Model

Image 5.2. Custom designed chamber compartments used for GUV electroformation.
Image 5.3. Assembled custom designed chamber ready for GUV electroformation.
5.3. Results and Discussion

5.3.1. Fluorescent Staining and Electroformation of GUVs

To test the staining ability of the headgroup dye (Rd-DOPE) and fatty acid-group dye (NBD-DLPC) for fluorescent labelling of different phospholipids and sphingolipids in the model membrane system, the two dyes were applied to two unitary GUV systems (DPPC and SM) respectively. The zwitterionic glycerophospholipid PC and the zwitterionic phosphosphingolipid SM are more enriched in the outer leaflet monolayer in the membranes of human and animal cells; moreover, in the case of MFGM, it has also been reported that PC and SM are located on the outside layer of the MFGM (Danthine et al., 2000, Deeth, 1997). Therefore PC and SM unitary systems were used in the preliminary experiments. As shown in Figure 5.1, the head group-labelled DOPE phospholipid analogue appears to stain on the surfaces of DPPC and SM induced GUVs systems well; the fatty acid group-labelled DLPC phospholipid analogue stains the DPPC induced GUVs system well whereas GUVs could not be successfully constructed from the NBD-DLPC labelled SM system in the same GUV generation condition. The reason for this unsuccessful construction is uncertain, it is probably due to the current electroformation condition is not capable to generate GUVs from this specific lipid combination (NBD-DLPC + SM). Consequently, headgroup labelled Rd-DOPE as the widely used fluorescent probe in MFG studies (Lopez et al., 2010, Gallier et al., 2010b) was used as primary dye for
probing the surface morphology of the constructed GUV systems containing milk phospholipids. No noticeable differences are apparent between head group-labelling and fatty acid-labelling when comparing between DPPC bilayer systems.

In previous work (Gallier et al., 2010b), Rd-DOPE was used for labelling the surface layer of native MFGs. The dark regions, which were not stained by Rd-DOPE, were designated as $L_o$ (lipid rafts) domains thought to be enriched in SM and cholesterol. A reasonable criticism of this characterization is that the PE-coupled headgroup fluorescent dye (Rd-DOPE) probably may not stain the outer monolayer components properly, which mainly consist of PC and SM. This would create unstained or heterogeneously stained regions on the surface of MFGs where dark regions occur through the absence of Rd-DOPE, and these regions may not necessarily be liquid disordered ($L_o$) regions. Consequently, a GUV system in which the membrane composition can be controlled is an ideal tool for testing staining capability of fluorescent label in different bilayer systems (such as Rd-DOPE). Figure 5.1 shows that the probes were distributed homogeneously on the surfaces of GUVs. Electroformation of GUVs was carried out successfully (Figure 5.2 A) where Rd-DOPE probe was located at the periphery of the GUV, and the yield of GUVs was sufficient for further analysis (Figure 5.2 B).
Figure 5.1. Comparison of GUVs (formed from different species of phospholipids) labelled by different fluorescent probes (headgroup/fatty acid labelled). A: GUV formed from DPPC, labelled by Rh-DOPC (headgroup probe); B: GUV formed from SM, labelled by Rh-DOPC; C: GUV formed from DPPC, labelled by NBD-PC (fatty acid probe). Scale bar: 10 μm.
Figure 5.2. Electroformation of GUVs (DPPC), A: 3D image of GUV formed on the surface of ITO-coated slide under electroformation conditions (AC field); B: DIC image of the yield of GUVs.
5.3.2. GUVs Constructed From DPPC and/or DOPE

The combinations of DPPC ($T_m > 22 \pm 1^\circ C$) and DOPE ($T_m < 0^\circ C$) in the formulation of GUVs was based on a range of $L_\beta/L_\alpha$ phase transition temperatures. Dark regions (non-fluorescent regions) were difficult to find in the DPPC unitary and two DPPC-DOPE binary (7/3, 1/1, mol/mol) GUV systems (Figure 5.3). There were no characteristic differences in surface morphology of these samples. The 3D images (Figure 5.3 A), built from 2D cross-sectional images, show that the Rd-DOPE fluorescent probe was homogenously spread on the surfaces of GUVs. Non-fluorescent regions were found on the surfaces of GUVs made from a DPPC-DOPE (3/7, mol/mol) binary system (Figure 5.4).

Lissamine rhodamine B is a well-characterized fluorescent probe used for studying phase coexistence on GUV surfaces (Bagatolli and Gratton, 2000, Sun et al., 2011). Bagatolli and Gratton (2000) reported that Rd-DPPE molecules are segregated from the gel domains and locate in the liquid (crystalline) disordered domains in a DPPE-DPPC GUV system, which supports the current results where the liquid disordered domains are characterized as the location of Lissamine rhodamine B-labelled PE. Rd-DOPE, as a primary fluorescent dye, has been also used to probe the lateral segregation and morphology of the native MFGM (Gallier et al., 2010b, Lopez et al., 2011, Lopez et al., 2010). Lopez and co-workers (2010) applied Rd-DOPE as a probe to determine the location of milk phospholipids in the MFGM, and found that
Figure 5.3. Comparison of GUVs formed from DPPC and/or DOPE (unitary and binary systems). A: DPPC, 3D image; B: DPPC/DOPE, 7/3 (mol/mol), 2D image; C: DPPC/DOPE, 1/1 (mol/mol), 2D image. Scale bar: 20 μm. (Dark regions on the surface of GUVs were not commonly observed).
Figure 5.4. GUVs formed from DPPC:DOPE (3:7, mol/mol). A: 2D image taken at the top/bottom section of GUV, regular circular shape of dark regions (DRs) are shown by white arrows; B: 2D image taken in the middle section of a GUV, DR is shown by white arrow. Scale bar: 5 μm.
Rd-DOPC did not partition into the triacylglycerols interior of MFGs, but was distributed on the surfaces of MFGs depending on the existence of Lo domain on the surface of MFGs (Rd-DOPC cannot stain Lo domain) (Lopez et al., 2010). Consequently, the black interior regions of GUVs with no fluorescent emission are not considered in this research.

In previous work (Lopez et al., 2010), the sizes of liquid-ordered or gel domains ranged from 0.6 to 2.7 μm depending on the size of the MFGs, and this is comparable with the current results as shown in Figure 5.4 A, B from an artificial phospholipid membrane system containing only DPPC-DOPC (3/7, mol/mol). Also, the apparent morphology of the non-fluorescent emission domains, which correspond to the circular shapes on the GUV surface (Figure 5.4 A) is in close agreement with that reported for MFGM by Lopez and co-workers (2010).

Evers et al. (2008) observed heterogeneity on the MFGM using different fluorescent probes and concluded that the lack of MFGM fluorescent emission was due to the absence of an outer bilayer. Lopez et al. (2010) challenged this hypothesis and suggested that the triacylglycerol inner core of the MFGs are unlikely to be directly in contact with the aqueous phase without surface coverage. Nevertheless, considering the current and widely accepted trilayer topology of the MFGM (Evers, 2004b, Lopez et al., 2011, Lopez et al., 2010, Mather, 2011, Michalski et al., 2002), it is reasonable
to expect that the primary monolayer containing glycerolphospholipids and membrane proteins located underneath the outer bilayer may still maintain the stability of MFGs.

An exposed protein inner monolayer on the surface of MFGs has been observed after centrifugal mechanical treatments (Zheng et al., 2013a), therefore, the explanation of the bare surfaces on MFGs, as reported by Evers et al. (2008), can be rationalized. However, this theory cannot explain the appearance of dark domains in the current GUV system containing only phospholipids, as a trilayer system is not present. Several CLSM studies (Gallier et al., 2010b, Lopez et al., 2011, Lopez et al., 2010) suggest that the dark domains on Rd-DOPE fluorescently-labelled MFGM are lipid rafts (Bacia et al., 2005, Baumgart et al., 2003, Veatch and Keller, 2003) (analogous to L_o), comprising of saturated phospho/sphingo-lipids (mainly SM) and cholesterol. Dark domains, however, were generated in the artificial non-supported model membrane system (Figures 5.4 and 5.6), containing only DPPC and DOPE (3/7, mol/mol). This phenomenon, to some extent, supports the hypothesis that the non-fluorescent dark domains on the MFGM not only represent the L_o phases (lipid rafts) which are mainly formed from SM and cholesterol (Simons and Ikonen, 1997) but also represent the gel phases formed by the high T_m phospholipids (DPPC, in this case).

In previous research (Gallier et al., 2010b, Lopez et al., 2011, Lopez et al., 2010) and in the current work, CLSM characterization was carried out at ambient temperature
(19-22°C), which is lower than the Tm of DPPC (42–60°C) required to induce liquid crystalline structure formation (Leonenko et al., 2004). Bagatolli et al. (2000) characterized the phase state of the lipid domain by using an excitation generalized polarization (GP) function at different temperatures. The CSLM GP images clearly show gel-liquid crystalline phase separation (DPPE/DPPC, 7/3, mol/mol) at the solid and fluid phase coexistence temperature region, 58–42°C. This is in close agreement with the current results where ambient temperature is within the range of the phase coexistence temperatures (Figures 5.4 and 5.6).

The 3D image (DPPC/DOPE, 3/7, mol/mol, Figure 5.6) shows more details about the gel-phase (DPPC) segregation from the liquid-phase (DOPE) in the GUV model, where irregular dendritic, flower shapes, and regular circular shapes were found. Whether the irregular shapes were formed spontaneously and individually, or were formed due to the aggregation of regular circular gel-phases induced by the fluidity of liquid-phases, is not known. The dark regions on GUVs (Figure 5.6) are similar to the dark regions that were found on the surface of native MFGs (Gallier et al., 2010b). It was difficult to observe gel-liquid crystalline phase separation in the GUVs systems as presented in Figure 5.3. This may be related to the preferred molecular arrangement of different phospholipids (DPPC and DOPE) in the non-supported model membrane systems, as shown in Figure 5.5. The diagrams shown in Figure 5.5 are based on hypotheses for predicting the homogeneity of the surface of GUVs. CLSM can be used to provide further evidence for these hypotheses.
Figure 5.5. Highly schematic illustration of proposed molecular arrangement of GUV (bilayer) systems formed from different species of phospholipids. A: DPPC/DOPE (7/3, mol/mol); B: DPPC/DOPE (1/1, mol/mol); C: DPPC/DOPE (3/7, mol/mol). Red (dashed): DPPC; green (solid): DOPE.
Figure 5.6. Apparent morphology of GUV (DPPC/DOPE, 3/7, mol/mol) in 3D image mimicking the morphology of native MFG. Scale bar: 5 μm.
According to the critical packing parameter and critical packing shape of phospholipid molecules (Figure 5.10) it may be hypothesized that PE is more favourably packed in the inner leaflet, with the bilayer system containing PC and/or SM, as the fatty acid tails of PE form cylindrical or inverted truncated cone shapes which favour planar bilayers or inverted micelles structures (Israelachvili, 2011). Consequently, the outer leaflets of GUV bilayers made from DPPC and specific DPPC+DOPE mixtures (7/3 and 1/1, mol/mol) were mainly constructed from DPPC (Figure 5.5).

It is necessary to point out that CLSM is a proper technique for characterizing the homogeneity of the surface of fluorescent labelled phospholipid bilayer and the characterization is instrumental configuration dependent [by setting up a proper configuration on CLSM, Rd-DOPE labelled DPPC concentrated GUVs (Figure 5.3. and Figure 5.5 A. & B.) may be visible]. Therefore, in the current work, the instrument is applied for characterizing the “immiscible phases” on the surfaces of GUVs rather than characterizing the individual absolute ordered or disordered phases.
5.3.3. GUVs Constructed From SM and/or DOPE

In GUV systems containing only bovine milk-derived SM, the non-fluorescent regions were observed with long elongated shapes (Figure 5.7 A, B) where the images were taken at the top and bottom section of selected GUVs. The 2D GUV image taken from the middle section (Figure 5.7 C) is similar to the MFGM images from previous reports (Gallier et al., 2010b, Lopez et al., 2011, Lopez et al., 2010); the tiny dark dots on the fluorescent GUV surface may contribute to the linear shaped micro-domains in constructed 3D images.

Unlike DPPC, SM (Avanti Polar Lipids) derived from bovine milk is a mixture of lipid molecules with different lengths of fatty acids and headgroups which gave three major peaks in HPLC-ELSD analysis. The presence of other minor SM species are also expected. This is the same three-peak profile as in the extracted bovine milk
Figure 5.7. Dark regions (DRs, shown by white arrows) presented on GUVs formed from SM. A: 2D image, top/bottom and middle section of two GUVs, DRs were observed as elongated and dot shapes; B: 2D image, top/bottom section (in white circle), DRs were observed as irregular shapes; C: 2D image, middle section. Scale bar: 5 μm.
phospholipid mixture (as shown in Chapter 4), therefore, the proposed surface arrangement shown in Figure 5.5, which only represents two types of pure lipid molecules does not apply in this case, and dark domains can occur in all SM containing samples (Figures 5.7, 5.8 and 5.9). These dark domains were probably induced by heterogeneous distribution of Rd-DOPE on the surfaces of GUVs which contained different types of milk SM. The composition of milk SM in terms of fatty acid and sphingolipid population varies considerably (MacGibbon and Taylor, 2006) and it was proposed that physical behaviour (melting) of SM depends on both the sphingosine chain and the fatty acid distribution (Shipley et al., 1974), therefore different physical behaviours and molecular packing of milk SM molecules in GUVs may be expected. The hosting capacity of fluorescent probes in phospholipid membranes depends on the phospholipid molecular packing (Gallier et al., 2010b), consequently, the observation of phase separation, which could be induced by heterogeneous distribution of fluorescent probes in the GUV system containing different types of SM, is rationalized.
Figure 5.8. DRs (shown by white arrows) were found in GUVs formed from binary systems containing SM and DOPE. A: SM/DOPE, 7/3 (mol/mol); B: SM/DOPE, 1/1 (mol/mol); C: SM/DOPE, 3/7 (mol/mol). Scale bar: 5 μm.
Figure 5.9. Apparent morphology of a GUV (SM/DOPE, 1/1, mol/mol) in a 3D image modelling part of the morphology of a native MFG. White arrow: irregular dendritic shape; green arrow: linear shape. Scale bar: 10 μm.
Figure 5.10. Highly schematic illustration of molecular packing shapes of specific phospholipids adapted from literature (Israelachvili 1991). A: PC & SM, B & C: PE. Red: critical packing shape, A: truncated cone; B: cylinder; C: inverted truncated cone.
Shipley et al. (1974) characterized a single lamellar bilayer structure in a SM (bovine brain) bilayer system, and found phase separation for a higher degree of hydration in a SM bilayer system (> 42% water, v/v) occurred at a lower phase transition temperature (25°C). This is in close agreement with the current study where bovine milk SM is highly hydrated and characterized at ambient temperature; pulsed-field gradient NMR has shown that the lateral diffusional behaviour is the same in a bilayer comprised of SM derived from either brain or milk (Filippov et al., 2006). The dark regions only account for a small proportion of GUV (SM unitary system) surfaces as discrete linear/dot shapes and larger regular shapes are not seen (Figure 5.7). This unique structure and morphology is probably due to the specific individual molecular profile of SMs derived from bovine milk and their packing structure in the bilayer. Filippov et al. (2006) found that SM derived from brain and egg may induce larger phase co-existence areas in phospholipid bilayers than milk SM, probably due to the heterogeneous hydrocarbon chain in SM which gives a less tightly packed molecular structure. Therefore, the small dark regions could be formed by a small amount of tightly packed homogeneous hydrocarbon chains of specific SM molecules.

Bagatolli et al. (2000) found similar elongated micro-domains in GUV systems (DLPC/DPPC, 1/1, mol/mol) which were stained by different probes (including Rd-DPPE). Based on a previously proposed model (Parasassi et al., 1993), the elongated domains were formed due to a particular topology (unlike a pure gel phase) of specific phospholipid molecules (DPPC in this case), presented as filaments of gel in a
particular liquid crystalline phase. Moreover, in a good agreement with the current results shown in Figure 5.9, elongated and polygonal domains have also been found in other GUV systems containing different pure phospholipids, where it has been pointed out the GUV radius of curvature and fluid line defects formed by liquid crystalline domains are important for forming and shaping lipid domains (Bagatolli and Gratton, 1999, Bagatolli and Gratton, 2000). Therefore, it can be concluded that both intrinsic factors (composition, chemical and physico-chemical nature of phospholipids, including hydrocarbon chain, $T_m$ and molecular packing shape) and extrinsic factors (size of the bilayer, environmental temperature, and physical property of liquid-domains) are important for forming lipid domains with specific morphology.

5.3.4. GUVs Mimicking the Morphology of Native MFGs

Lateral mobility of lipid domains (dark regions) with regular circular shapes was observed (Figure 5.11), in good agreement with previous work where mobility of the MFGM constituents was found (Lopez et al., 2010). The composition of the mobile non-fluorescent regions is difficult to ascertain due to the complexity of the composition and structure of the MFGM; however, in artificial membrane systems with controlled composition (Figure 5.11), it can be concluded that the mobile micro-domains were formed from a gel phase and flowed into the liquid phase. The shape and the size of non-fluorescent micro-domains did not change as a function of time, as reported before (Lopez et al., 2010). The specific shapes of non-fluorescent micro-
domains on the surface of GUVs, as shown in Figures 5.6 and 5.9. (without SM, and with a combination of SM and cholesterol), shows good agreement with the previously published surface morphology of native MFG (Gallier et al., 2010b). Based on the current results, it can be concluded that the non-fluorescent micro-domains on the surface of MFGs are not necessarily represented by the $L_0$ phase, enriched in SM and cholesterol, but may also be formed from the gel phase containing the major MFGM phospholipid species DPPC and SM.

A lipid formulation of DOPE/DPPC/SM/cholesterol (8/8/8/4, mol/mol) was used for mimicking the composition of the native MFGM. A large integrated dark region is shown in the 3D micrograph (Figure 5.12). The surface morphology is in good agreement with previous GUV systems containing cholesterol where the existence of micro-domains and domain patterns were regulated by the amount of cholesterol (Scherfeld et al., 2003). Morales-Penningston et al. (2010) showed a similar pattern of micro-domains on the surface of GUVs (composed from 18:0-SM/DOPC/cholesterol in the molar ratio of 0.56/0.24/0.20), where the larger and regular circular micro-domains were formed by the merging of smaller separated micro-domains, as induced by osmotic swelling. This osmotic swelling mechanism may explain the apparent morphology on the GUV that contains cholesterol (Figure 5.12), as an osmotic pressure gradient is formed between the external (100 mM glucose) and internal
Figure 5.11. Monitoring mobility of DRs (pointed by arrows) on the surface of specific GUVs (DPPC/DOPE, 3/7, mol/mol). 2D images were taken at the top/bottom section of GUVs. A–I were obtained individually along a fixed time interval (20 seconds). Scale bar, 5 μm.
Chapter 5: Phospholipid Architecture of the Bovine Milk Fat Globule Membrane Using Giant Unilamellar Vesicles as a Model

Figure 5.12. 3D image of a GUV formed from a lipid formula (DOPE/DPPC/SM/cholesterol, 8/8/8/4, mol/mol) mimicking the lipid composition of the native MFGM. DR is shown by the arrow. Scale bar: 5 μm.
solutions (100 mM sucrose). Cholesterol has role in inducing domain formation (Bacia et al., 2004, Wesolowska et al., 2009), and is dependent on the amount present (Scherfeld et al., 2003). Scherfeld et al. (2003) reported that phase separation may be reduced by adding 10 mol% cholesterol into the phospholipid binary system (DOPC/DPPC, 0.5/0.5, mol/mol) and can increase by adding 20 mol%. Cholesterol accounts for ~14 mol% in the current formulation for mimicking the lipid molar composition of MFGM, and phase separation can readily be observed with CLSM (Figure 5.12).

5.4. Conclusions

Phospholipids from bovine milk were successfully used to construct GUVs under a temperature-controlled AC field as a model to examine the lipid structure of the native MFGM. The DOPE analogue headgroup-labelled fluorescent probe was suitable to stain the DPPC and SM bilayers. The fatty acid-labelled probe in a non-supported phospholipid bilayer system was stable during CLSM imaging. The presence or absence of non-fluorescent micro-domains in GUVs formed from DPPC and/or DOPE binary/unitary systems provided evidence for a preferred molecular packing model which revealed the location of specific phospholipids in the bilayer (PC/SM-outer leaflet, PE-inner leaflet in the outer bilayer in MFGM). The observed non-fluorescent micro-domains were formed by the gel phase (containing DPPC and SM) in which a SM-cholesterol interaction was not involved. Cholesterol regulated
the shape of micro-domain on the GUV surface. Both circular and elongated/polygonal domain shapes, constructed of phospholipids, were found on the GUV surfaces. A model of lateral topology of MFGM lipid organization can be deduced, in which PC and SM are located in the outer leaflet of the bilayer, and specific phospholipids having high gel-liquid crystalline phase transition temperatures contribute to lateral segregation in the MFGM, suggesting that association of SM and cholesterol is not the only contributing factor to ordered domain formation.
CHAPTER 6.

Understanding How Sphingomyelin and Cholesterol Regulate Lipid Domain Formation in the Milk Fat Globule Membrane (MFGM) Using an Artificial Membrane System - Giant Unilamellar Vesicles (GUVs)
6.1. Introduction

A number of MFGM architecture model systems had been proposed (Lopez et al., 2011, Mather, 2011), nevertheless the native structure of the MFGM in terms of both lipid and protein localization, organization and surface morphology is not fully understood. Gallier and co-workers (2010b) observed lipid domains on the surface of native MFGs using CLSM in which the phospholipid head group-labelled (rhodamine B) fluorescent dye was not able to intercalate into the more tightly packed domains. Lopez and co-workers (2011, 2010) observed a similar heterogeneous distribution of phospholipids on MFG surfaces by using this same fluorescent dye, and characterized the lateral segregation of lipid domains as “lipid rafts” (liquid-ordered domains, L_o) in which milk sphingomyelin (milkSM) and cholesterol are concentrated, surrounded by a fluorescently-labelled liquid-disordered domain (L_d). Despite recent advances in knowledge about the MFGM structure, more direct evidence is still required to reveal the nature of segregated lipid domains on MFG surfaces, and understand how milkSM and cholesterol regulate lipid domain formation.

The complex native structure of the MFGM is sensitive to mechanical treatments (Zheng et al., 2013b), thereby creating challenges when designing methods to examine MFGM structure. Giant unilamellar vesicles (GUVs), featuring artificial lipid bilayers, have been built as model systems for studying lipid organization in bio-membranes (Levental et al., 2009). The specific interactions and organization of polar lipids can be studied by controlling the GUV lipid composition (Bagatolli and Gratton, 2000, Scherfeld et al., 2003); the detailed
surface morphology is able to be characterized using CLSM (Morales-Penningston et al., 2010). A GUVs system is an ideal model for mimicking the lipid morphology of the outer bilayer of the MFGM and for revealing lipid organization into domains. In this work, GUVs were constructed from different lipid formulas containing phospholipids derived from milk fat to study the nature of lipid domains within the MFGM and to examine the effect of milkSM and cholesterol on lateral lipid segregation.

6.2. Materials and Methods

6.2.1. Materials

Indium tin oxide (ITO)-coated glass slides containing a thin SiO\textsubscript{2} passivation layer between the substrate and ITO [surface resistivity \( \leq 10 \) ohms/cm\textsuperscript{2}, dimensions of 25 (±0.5) × 75 (±0.5) × 1.1 (±0.1) mm, unbevelled] were purchased from Präzisions Glas & Optik GmbH (Iserlohn, Germany). The fluorescent headgroup-labelled phospholipid analogue 1,2-dioleoyl-\textit{sn}-glycerol-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (18:1, Rd-DOPE, 1mg/mL in chloroform) as a fluorescent probe, 1,2-dipalmitoyl-\textit{sn}-glycerol-3-phosphocholine (16:0-PC, DPPC in powder form), 1,2-dioleoyl-\textit{sn}-glycerol-3-phosphoethanolamine (18:1-PE, DOPE in powder form), sphingomyelin (derived from bovine milk, in powder form, milkSM) were all purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Sucrose and glucose (both anhydrous) and solvents including chloroform, acetone, methanol and water (HPLC grade) were all sourced from Fisher
Scientific (Fair Lawn, NJ, USA). A function generator (model: 72-7710) was purchased from TENMA® test equipment (Springboro, OH, USA) and a digital phosphor oscilloscope (model: TDS 3012, 100 MHz, 1.25 GS/s, 10K point) was obtained from Tektronix (Beaverton, OR, USA).

6.2.2. Electroformation of Giant Unilamellar Vesicles (GUVs)

The general procedures for electroformation of GUVs were outlined in Chapter 5. GUVs were constructed from designed lipid formulas by adopting the electroformation method from previous work, with modifications (Angelova et al., 1992, Angelova and Dimitrov, 1986, Herold et al., 2012). GUVs were formed in a 100 mM sucrose solution in a custom designed sample chamber, and AC (alternative current) electro-field (3 V peak-peak, 10 Hz) was applied at 55±1°C for 45 min on the surface of ITO slides for creating the swollen lipid bilayers (Morales-Penningston et al., 2010, Veatch, 2007). The layout of sample formulas is explained in the following sections.

6.2.3. Sample Layout

GUVs were formed from ternary or quaternary systems with DPPC, DOPE, with and without cholesterol or milkSM. For simulating MFGM composition, a formula containing DPPC, DOPE, milkSM and cholesterol (referred to as F-MFGM, 8:8:8:4 mol/mol) was designed to mimic the surface morphology of the native MFGM. The weight ratio of polar lipids to
cholesterol in the MFGM was 25:2, thus, the calculated molar ratio was ~25:4 (mol/mol) based on the mean molecular weights of MFGM polar lipids. Phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM) were considered as the major phospholipid constituents accounting for > 90% (w/w) of total MFGM polar lipids, with DOPE (47.0% PE w/w) and DPPC (32.2% PC w/w) as the major sub-species. To simplify the formula composition, the molar ratio of polar lipids to cholesterol was set as 24:4 (mol/mol).

Based on the design of simulated MFGM formulas, two sets of formulations were made in which the added amounts of milkSM and cholesterol were varied to study lipid domain formation as a function of the amount of milkSM and cholesterol. The experimental lipid formulas were as follows. Formula set A (F.A) where the relative molar volume of milkSM was varied: 8 (F.A-8), 4 (F.A-4), 2 (F.A-2) and 0 (F.A-0) and the molar volumes of DPPC, DOPE and cholesterol were kept at 8:8:4 (mol/mol). Formula set B (F.B) where the relative molar volume of cholesterol was varied: 8 (F.B-8), 4 (F.B-4), 2 (F.B-2) and 0 (F.B-0) and the molar volumes of DPPC, DOPE and milkSM were kept at 8:8:8 (mol/mol). Lipids in different formulas were dissolved in chloroform to yield 10 mg/mL sample solutions which were pre-labelled with fluorescent Rd-DOPE (0.25 mol%). For each formula, the electroformation processes were carried out in duplicate.
6.2.4. CLSM Characterization of GUVs

GUVs were placed into the microscope slide well (~1 mm depth) which prevented extra pressure on GUVs surfaces after placement of the coverslip. An inverted confocal laser scanning microscope (Olympus FV1000, Olympus America Inc., Center Valley, PA, USA) with a 40× oil-immersion objective lens was used to observe GUVs. Rd-DOPC-labelled bilayers were excited by a diode laser (559 nm) and the emitted fluorescent signals were measured between 570 and 670 nm (Gallier et al., 2010b). The GUVs from each electroformation experiment and the physical positions of the GUVs were also observed by differential interference contrast (DIC) illumination/channel. The CLSM laser power, pinhole size, scanning speed and imaging threshold were fixed for each measurement to obtain relative quantitative information. Each 3D image was assembled from a group of 2D images taken from different sections in the z-direction. CLSM observations were carried out at ambient temperature (22 ± 1°C).

In 2D images, the ideal GUVs were characterized as fluorescent circles (as shown in Figure 6.3 A and as illustrated in Figure 6.1). The relative quantity of non-fluorescent dark region (DR) on individual GUV surfaces was correlated with the proportion of the sum of DR corresponding angles (Σα) on GUV surfaces (in 2D form in CLSM micrographs) over 360° (the full angle of circumference), as illustrated in the schematic model in Figure 6.1. Therefore, the relative quantity of DR can be represented and estimated as follows:
Relative Quantity of DR (%) = \left( \frac{\sum \alpha}{360^\circ} \right) \times 100\% \quad (6.1)

Three groups of GUVs (five ideal circular GUVs in each group) were randomly collected from duplicated electroformation runs and used for the relative quantification of the proportion of DR (n=15). DR corresponding angles (\alpha) (Figure 6.1) were measured using ImageJ software (National Institute of Mental Health, MD, USA) in which the region of interest (ROI) may be specified and quantified. The fluorescent peak intensity of ROI which crosses the GUV boundary were measured for distinguishing GUVs from GMVs (giant multi-lamellar vesicles).

6.2.5. Statistical Analysis

One-way ANOVA tests were carried out using Minitab 16 (Minitab Inc., State College, PA, USA). Significant differences (P < 0.001) was determined using Fisher’s test in paired comparisons between samples means.
Figure 6.1. Highly schematic illustration of relative quantification of “dark region” on the surfaces of GUVs and/or MFGs. Using the proportion of dark region corresponding angle “α” in angle of circumference (360°) in percentage to represent the gel/Lo phases. Black (solid): fluorescent labelled GUV/MFG; grey (dashed): dark region. n=15
6.3. Results and Discussion

6.3.1. Electroformation and Initial Characterization of Lipid Bilayers

Lipid bilayers from formulas for simulating MFGM lipid composition (F-MFGM) resulted in circular shapes under the electroformation conditions. A sufficient yield of GUVs from electroformation was checked by using DIC illumination from CLSM in which the physical location of individual GUVs could be traced. Bilayers were generated as circular shapes representing a typical GUV structure, and irregular shapes (and/or bilayer complexes) as reported previously (Estes and Mayer, 2005, Politano et al., 2010). Only regular shaped GUVs were used for image analysis. The accumulated fluorescent intensities on the edge of targeted GUVs were not significantly different, ensuring that the characterized bilayers were GUVs rather than multilayers (Figure 6.3). The efficiency of electroformation of giant liposomes depends upon the thickness of the dry lipid layer on the ITO coated slides, therefore, a spin-coating technique which can control the thickness of the lipid dry layer reaching to the optimal range (25-50 nm) for GUV formation was applied for efficient GUV electroformation (Estes and Mayer, 2005). In the current study, the classic lipid droplet dripping method, rather than the aforementioned spin-coating method, was employed for preparing lipid dry layer on ITO slide, and the GUV yield was sufficient for further characterization. A DIC image from CLSM system showed a typical yield of GUVs from the electroformation method (Figure 6.2). Irregular shaped GUVs and/or disordered coalescence of GUVs were observed by CLSM, as shown by the circles in Figure 6.2. The mechanism of
the formation of these irregular GUVs is unknown; they were possibly formed during or post-electroformation.

6.3.2. Relative Quantitative Lipid Domains are Correlated with Proportion of DRs on the MFGs and GUVs

When quantifying DRs on GUV surfaces, only true GUV bilayers were considered. The fluorescent intensity of the cross boundary of a GUV bilayer was measured and compared with the intensity obtained from the reference of a true GUV bilayer to ensure that the characterized GUVs were actual bilayers. For instance, as shown in Figure 6.3., the ideal reference GUVs are shown as No. 1 and No. 2 in Figure 6.3 A; the bilayers in Figure 6.3 B mixed together are considered to be giant multi-lamellar vesicles (GMVs), and even more complicated bilayers structures are shown in Figure 6.3 C and are considered to be GMV-complexes. Therefore, in this work, only GUVs that are similar to the bilayers in Figure 6.3 A in terms of boundary intensity were used for quantification.

DRs shown on MFGs and GUVs in CLSM micrographs are characterized as lipid domains (Lopez et al., 2010, Morales-Penningston et al., 2010, Scherfeld et al., 2003, Zheng et al., 2013b). The areas of lipid domains on MFG and GUV surfaces are quantified as a proportion of DRs on individual MFGs and GUVs. The characterized relative area of DRs from the GUV sample, which was formed from F-MFGM formulation, is not significantly different to
Figure 6.2. Representative overview of yield of GUVs (DOPE:DPPC:milkSM:cholesterol, 8:8:8:4 mol/mol) under controlled electroformation condition taken under DIC mode in a CLSM system. White circles: irregular shaped bilayers and bilayer complex. Scale bar: 30 µm.
Figure 6.3. Three major types of phospholipid layer systems are generated from electroformation condition. A: GUVs; B: GMVs (multilayers); C: GMVs-complex (cluster of multilayers). In relative quantification of “dark regions”, only GUVs (A) were taken as research objective. Scale bar: 10 μm.
that on native MFGs (Figure 6.4). The current results suggest that the artificial MFGM system (F-MFGM-derived GUVs) was successful in mimicking lipid domain formation on the surface of native MFGs.

6.3.3. Impact of Volume of milkSM or Cholesterol on Lipid Domain Formation on GUVs

In the ternary formula systems F.A and F.B, the relative ratios of DRs on GUVs were measured as a function of the relative depletion of milkSM or cholesterol as shown in Figure 6.5. The results reveal that cholesterol, rather than milkSM, plays a more important role in regulating the formation of lipid domains. Cholesterol and/or DPPC/milkSM are believed to induce lipid phase separation (as either a gel phase or a liquid ordered phase) from the liquid-disordered phase on the phospholipid bilayers (Brown and London, 2000, Levental et al., 2009, Scherfeld et al., 2003, Silvius et al., 1996), whereas the GUV containing no characterized lipid phase separation, which was made from the F.B-8 formulation (DPPC:DOPE:milkSM:cholesterol 8:8:8:8) was observed in CLSM (Figure 6.6 A) and it is considered as a rare case. This rare phenomena (shown in Figure 6.6 A) may be rationalized by considering that lipid components probably did not homogenously occur on each F.B-8-derived GUV. This suggests a possibility that a small number of GUVs contained no cholesterol, or cholesterol and DPPC/SM in combination. This explanation is supported by previous experimental evidence; although the deposited lipids for GUV formation were pre-mixed uniformly, different miscibility transition temperatures and brightness between vesicles may still be found (Veatch and Keller, 2003). Moreover, the lipid domains can be
still found in GUVs which were made from the F.B-0 formulation having no cholesterol present (Figure 6.6 B) which suggests that although cholesterol is responsible for inducing lipid domain formation in a MFGM-derived lipid bilayer environment, it is not the requisite component for phase separation as discussed in Chapter 5. It has been shown that Rd-DOPE is preferably partitioned into the L_d phase (Baumgart et al., 2007), consequently, the DRs shown on the surfaces of MFGs and GUVs can be considered as either a gel phase or a L_o phase. These results may extend the current understanding of lipid domain formation as observed on native MFGM under CLSM (stained by Rd-DOPE at ambient temperature) which previously had been deduced as milkSM and cholesterol enriched L_o phases (Gallier et al., 2010b, Lopez et al., 2010). From CLSM images in Chapter 5, gel phase formation was characterized on GUVs made from a DPPC/DOPE binary system where both milkSM and cholesterol were not present. MilkSM is not necessarily required, or may not be the only polar lipid associated with cholesterol for lipid domain formation on artificial MFGM (GUVs made from F-MFGM) as shown in Figure 6.5 A. The competitive affinity of cholesterol between glycerolphospholipids and sphingophospholipids for lipid domain formation has been reviewed, and the possibility of interactions between glycerolphospholipids (such as DPPC) and cholesterol in the presence of SM presented was highlighted (McMullen et al., 2004). Therefore, in GUVs systems made from the F.A-series formulations (whether DPPC is originally associated with cholesterol or not), with the depletion of milkSM (Figure 6.5 A), DPPC may replace milkSM to interact with cholesterol and be responsible for phase separation to create lipid domains.
Figure 6.4. Relative quantification of dark regions on GUVs/MFGs. Hollow filled bar: estimated proportion of dark regions on GUVs (DOPE:DPPC:milkSM:cholesterol, 8:8:8:4 mol/mol) surfaces; grey filled bar: estimated proportion of dark regions on native MFGs surfaces. 2D images represent the corresponding samples (pointed by patterned filled arrows). Scale bar: 10µm. *, results of the analysis of variance, means between bars with different letters differ (fisher test: P < 0.05), n=15.
Figure 6.5. The proportions of dark region (DR) as function of milkSM (A) and/or cholesterol (B). A: the molar contents/ratio of DOPE, DPPC and cholesterol were fixed (8/8/4, mol/mol) and milkSM was controlled as variable (from 8 to 0, mol; results in mean: 26.6, 25.7, 23.5, 22.6, % respectively); B: the molar contents/ratio of DOPE, DPPC and milkSM were fixed (8/8/8, mol/mol) and cholesterol was controlled as variable (from 8 to 0, mol; results in mean: 31.6, 26.6, 19.7, 10.7, % respectively). *, results of the analysis of variance, means between bars with different letter differ (fisher test: $P < 0.05$), $n=15$. 
Chapter 6: Understanding How Sphingomyelin and Cholesterol Regulate Lipid Domain Formation in Bovine Milk Fat Globule Membrane (MFGM) via artificial membrane system-Giant Unilamellar Vesicles (GUVs)

Figure 6.6. GUVs images (specific exception of interest) where cholesterol was controlled as variable. A: DOPE/DPPC/milkSM/Cholesterol, 8/8/8/8, mol/mol, 3D image of GUV (pointed by filled arrow) showing no presence of "dark region" on the surface; B: DOPE/DPPC/milkSM/Cholesterol, 8/8/8/0, mol/mol, 2D image of GUV showing presence of "dark region" (pointed by white arrows) on the surface.
Figure 6.7. Morphology comparison of GUVs containing different composition.
A: F1-8, DOPE:DPPC:milkSM:cholesterol, 8:8:8:4, mol/mol;
B: F1-0, DOPE:DPPC:milkSM:cholesterol, 8:8:0:4, mol/mol;
C: F2-8, DOPE:DPPC:milkSM:cholesterol, 8:8:8:8, mol/mol;
D: F2-0, DOPE:DPPC:milkSM:cholesterol, 8:8:8:0, mol/mol.
Scale bar: 20 µm.
The general overview of surface morphological features of four representative samples is shown in Figure 6.7 (each sub-micrograph was randomly captured). The area of the lipid domains were not significantly altered by SM, as seen by comparing Figures 6.7 A and B. The apparent lipid domain in micrographs may be determined by the molecular proportion of cholesterol by comparing Figures 6.7 C and D.

### 6.4. Conclusions

In this chapter, GUV model systems were constructed from a series of designed lipid formulas containing DPPC, DOPE, milkSM and cholesterol. In these formulas, the molar volumes of milkSM and cholesterol were altered to reveal the specific role in regulating lipid domain formation in the MFGM. The correlations between lipid domain formation and specific lipid components, SM and cholesterol were characterized. Cholesterol is a key lipid component for generating lipid domain formation in the MFGM, and the PC-cholesterol network may also possibly contribute to generation of the L<sub>o</sub> phase in the MFGM. This <em>in vitro</em> study supports the deduced lipid molecular arrangement of the outer leaflet of the PL bilayer as shown in Figure 4.2 (Chapter 4). This chapter successfully achieved the development of an innovative application of a GUV system for the detailed structural study of the native MFGM, and provided information that updates the current understanding of lipid organization in the native MFGM.
CHAPTER 7.

Using Confocal Raman Microscopy to Investigate the Interaction between Volatile Organic Compound (VOC) and Bovine Milk Fat Globules in Recombined Emulsion Systems

The results of this chapter has been published in International Dairy Journal 32(2), 68-70. As “Innovative application of confocal Raman microscopy to investigate the interaction between trans-2-hexenal and bovine milk fat globules” by Zheng, H., Gordon, K. C., & Everett, D. W., 2013, (DOI: 10.1016/j.idairyj.2013.04.011)
7.1. Introduction

Raman scattering, also known as the Raman Effect, was discovered by the Indian physicists Chandrasekhara Venkata Raman (1888–1970) and Kariamanikam Srinivasa Krishnan (1898–1961) in 1928 when the authors found a change in wavelength of scattered monochromatic visible light (Raman, 1928). This phenomena, together with the Compton Effect (Compton, 1923), were considered as convincing proof in the 1920s of the quantum theory of light, which was hypothesized by Albert Einstein (1879-1955) in 1905 (Singh, 2002). C.V. Raman was awarded the Nobel Prize in Physics in 1930 for his discovery of Raman scattering. A brief and classical picture of Raman scattering can be explained as follows: a dipole moment is induced by an incident electromagnetic wave which results in light scattering; the majority of scattered light is emitted at the same frequency of incident light and it is considered as elastic scattered light. Nevertheless, some light can be scattered at different frequencies than the original incident light and this type of scattering is referred as inelastic scattering; Raman scattering is therefore inelastic scattering. The dipole moment, $P$, is determined by the external electric field, $E$ and polarizability of the molecule, $\alpha$. These are related according to:

$$P = \alpha E$$  \hspace{1cm} (7.1)

Raman-active vibration induces a change in polarizability of the molecule; this polarizability change is represented by the polarizability derivative which determines the scattering intensity. The vibration-induced change in scattered light frequency depends on the nature of molecular system. The chemical sensitivity and specificity of Raman scattering provides a unique advantage for the application in analytical chemistry. The aforementioned
fundamental knowledge supports the development of instrumentation for Raman spectroscopy. This type of spectroscopy has been widely used in scientific research for identifying chemicals, revealing the molecular structure of target materials (Carron et al., 1992, Cherney and Harris, 2010, Dietzek et al., 2011, Forrest, 1978, Gallier et al., 2011, Li-Chan, 1996, McGoverin et al., 2009, McGoverin et al., 2010, Moros et al., 2007, Mosier-Boss and Lieberman, 2003, Okazaki et al., 2009). A modern development of instrumentation has been the coupling a confocal laser scanning microscope (CLSM) with Raman spectroscopy, called a confocal Raman microscope (CRM). CRM allows one to study the chemical and structural profile of the surface of a target material.

CRM has been applied to investigate chemical fingerprints of components of the bovine milk fat globule (Gallier et al., 2011). In this study, the authors characterized the chemical composition of individual milk fat globules (MFGs) as a function of size and breed of cow. This research enabled the study of chemical and physico-chemical interactions in recombined MFG emulsion systems. Consequently, in this current research, interactions between volatile organic components (VOCs) and individual MFGs were investigated by the innovative CRM technique. The purpose of this work was to develop a technique using CRM to investigate structural information of the MFGM using VOCs as probes, rather than study the VOC formation or retention in/on MFGs. This work provides fundamental knowledge about the efficacy of CRM to determine interactions between MFGs and VOCs, as well as to determine the correct configuration of the CRM instrument to minimise artefacts.
7.2. Materials and Methods

7.2.1. Materials

Bovine raw milk samples were sourced from Jersey cows at a local dairy farm (Port Chalmers, New Zealand) and stored at 4°C within 24 hours before use. An Olympus BX confocal microscope (Olympus Europa GmbH, Hamburg, Germany) was used for MFG in-situ observation and obtaining Nomarski differential interference contrast images of the MFGs. Raman spectra were collected by a Senterra dispersive Raman microscope (Bruker Optics, Ettlingen, Germany). The capacity of the confocal Raman microscope allowed 3-D image profiling with 2 µm depths and 1 µm high spatial resolution; the smallest spectra collection space was a column shape with 1 µm diameter and 8 µm depth.

7.2.2. MFG isolation and specimen preparation

The MFG isolation method and microscopic slide preparation were taken from previous work, with slight modifications (Gallier et al., 2011). Briefly, the raw milk was centrifuged for 5 min at 3000 × g at 4°C. The top cream layer was collected and re-dissolved into simulated milk ultra-filtrate (SMUF) buffer (Jenness and Koops, 1962) forming a 20% fat content dispersion system at pH 6.5, and then diluted ten-fold as a stock MFG emulsion (MFGs were not washed).
7.2.3. VOCs + MFGs

Six different types of VOCs (1-propanol, ethyl butyrate, octanol, 2-decanone, ethyl hexanoate and trans-2-hexenal) were selected based on the variation in hydrophobicity and vapour pressure to investigate interactions between VOCs and MFGs. Spectra of pure VOCs were individually measured by confocal Raman microscopy. To prepare samples of MFG+VOC recombined emulsions, VOCs (50 µL) were mixed with 950 µL of stock MFG emulsions in brown coloured GC vials, which were sealed with inert Teflon caps. The concentration of VOCs in the final system was 5% (v/v). The samples were prepared in triplicate. All of the sample vials were stored at 4°C overnight to allow the VOCs to interact with MFGs sufficiently to reach equilibrium.

7.2.4. Raman spectra collection

During confocal Raman microscopic observation and spectra collection, diluted stock MFGs emulsion samples were considered as the control, and MFG+VOC mixture samples were used for investigation of the interaction between MFGs and VOCs. The Raman spectra were collected and compared at three sampling positions: 1) by randomly taking any point in the membrane region (MFGM region) of specific individual MFGs; 2) by drawing an extension line which linked together the first measuring point and geometrical central point of the same MFG; the second measuring point was then taken on the extension line 2 µm away from the
membrane edge (first measuring point) on MFGs; and 3) a third measuring point was taken on the extension line 2 µm away from the membrane edge (first measuring point) in the aqueous phase (away from the MFGs).

The microscope and spectra collection were controlled by OPUS software (version 6.5, Bruker Optics). An Olympus 100 × objective (numerical aperture 0.9) with a 50 µm confocal pinhole was used to observe MFGs. Raman spectra were collected by using a diode laser (532 nm excitation wavelengths, Sentinel, Bruker Optics) with 5 mW power. The Raman spectra were collected at the aforementioned three positions. The results were taken from spectral region from 500 to 3500 cm$^{-1}$ using OPUS software. Baseline correction (additional concave rubber-band correction, 100 iterations) and normalization (offset correction) were done using the OPUS software for all result spectra for better comparison between samples. Each spectrum was the co-addition of ten 1 sec exposures. All measurements for characterizing interactions between MFG and VOC were carried out at ambient temperature and in triplicate.

7.3. Results and Discussion

7.3.1. Band assignment

Research has been previously carried out to study the natural structure of MFGs, MFGM, and other dairy constituents by Raman spectroscopy (Faiman and Larsson, 1976, Gallier et al.,
2011, McGoverin et al., 2009, McGoverin et al., 2010, Ozaki et al., 2006). From this, the band assignments of Raman spectra that were acquired are listed in Table 7.1.
Table 7.1 Band assignments of appeared peaks in Raman spectra of MFG and VOC + MFG mixture systems.

<table>
<thead>
<tr>
<th>Raman shift (cm⁻¹)</th>
<th>Reference Peaks</th>
<th>Observed Peaks</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>860-920</td>
<td>876, 910</td>
<td>v (C-C), v (C-O), CH₃rock, various amino acid specific modes</td>
<td></td>
</tr>
<tr>
<td>1063-1083</td>
<td>1060, 1075, 1086</td>
<td>v (C-C) stretching, symmetric phosphoryl stretching, gauche C-C</td>
<td></td>
</tr>
<tr>
<td>1145</td>
<td>1145</td>
<td>v (C-C) stretching</td>
<td></td>
</tr>
<tr>
<td>1158</td>
<td>1153</td>
<td>v (C-C)</td>
<td></td>
</tr>
<tr>
<td>1189</td>
<td>1189</td>
<td>v (C-C)</td>
<td></td>
</tr>
<tr>
<td>1222</td>
<td>1222</td>
<td>v (C-C)</td>
<td></td>
</tr>
<tr>
<td>1303</td>
<td>1297, 1300</td>
<td>δ (CH₂) twisting</td>
<td></td>
</tr>
<tr>
<td>1331</td>
<td>1331</td>
<td>v (C-H) bending</td>
<td></td>
</tr>
<tr>
<td>1377</td>
<td>1377</td>
<td>CH₃ symmetric deformation</td>
<td></td>
</tr>
<tr>
<td>1438</td>
<td>1438</td>
<td>δ (CH₂) scissoring</td>
<td></td>
</tr>
<tr>
<td>1519</td>
<td>1519/1515</td>
<td>v (C=C) MFGs</td>
<td></td>
</tr>
<tr>
<td>1564</td>
<td>1564</td>
<td>v (C=C)</td>
<td></td>
</tr>
<tr>
<td>1625</td>
<td>1625</td>
<td>v (C=C) stretching</td>
<td></td>
</tr>
<tr>
<td>1654</td>
<td>1652</td>
<td>v (C-C) cis unsaturation</td>
<td></td>
</tr>
<tr>
<td>1685</td>
<td>1685</td>
<td>v (C=O)</td>
<td></td>
</tr>
<tr>
<td>1744</td>
<td>1744</td>
<td>Ester v (C=O)</td>
<td></td>
</tr>
<tr>
<td>2715-2726</td>
<td>2728</td>
<td>v (C-H)</td>
<td></td>
</tr>
<tr>
<td>2853</td>
<td>2853</td>
<td>v (C-H)</td>
<td></td>
</tr>
<tr>
<td>2885±5</td>
<td>2886</td>
<td>Fermi resonance CH₂ stretching, CH₂ anti-symmetric stretching</td>
<td></td>
</tr>
</tbody>
</table>

*The observed peaks in the Raman spectra obtained from this current research were based on the Raman spectra from the following reference results and assignments (Gallier et al., 2011, McGoverin et al., 2010, Nishimura et al., 1992, Silva et al., 2007, Wallnöfer et al., 1989, Koenig and Sutton, 1969, Sebastian et al., 2009, Vijaya Chamundeeswari et al., 2011, Barry et al., 1992, Brunner and Sussner, 1973, Mizutani et al., 1994, Sajan et al., 2008, Beattie et al., 2004, Kirk et al., 2007, Li-Chan, 1996, Argov et al., 2008b, Bresson et al., 2005, Faiman and Larsson, 1976, Forrest, 1978, Krafft et al., 2005, McGoverin et al., 2009)*
For analysis of the results and interpretation, a baseline correction was carried out to give more precise comparisons between different Raman spectra. Spectra of native MFG and six VOCs with baseline correction are shown in Figures 7.1-7.7. Only the spectra of trans-2-hexenal were distinguishable and showed characteristic spectral peaks in the 1500-1700 cm\(^{-1}\) region (Figure 7.2) when comparing the spectra of native MFGs. Therefore, trans-2-hexenal was used to interpret the results of VOC+MFG mixture systems. The Raman spectra of raw MFG with a size around 10 µm (Figure 7.8) are shown as a control. The main peaks were selected individually by using the single peak pick function in the OPUS software; these were 910, 1060, 1075, 1153, 1297, 1438, 1519, 1652, 1744, 2853 and 2886 cm\(^{-1}\), respectively (Figure 7.8). The main peaks among Raman spectra of MFG are generated by vibrations of lipid molecules. Raman spectra of the minor membrane binding proteins components which associate with MFGs and the MFGM overlapped with the VOC spectra. The Raman spectra of a pure VOC, trans-2-hexenal showed notable peaks at 1564, 1625 and 1685 cm\(^{-1}\) (spectra not shown). These three bands are in the range of C=C and C=O regions, although in forms of different inter- and intra- molecular configurations. As both C=C and C=O are involved in the molecular structure of trans-2-hexenal and show characteristic peaks, the Raman intensities of the aforementioned peaks in the C=C and C=O regions were used for characterizing this VOC.

7.3.2. Trans-2-hexenal + MFGs

To investigate the interactions between MFG and VOC, trans-2-hexenal was added to the MFG emulsion system overnight to reach equilibrium. The VOC was detected on individual
MFGs and on the MFGM (Figures 7.9 and 7.10) after overnight incubation, the characteristic peaks of VOC at 1564, 1625 and 1685 cm\(^{-1}\) show a large increase from the MFGM to the interior of the MFG. The intensities of overall spectra at the first measuring point in the MFGM region (in red, Figure 7.9) are lower than that at the second measuring point on the MFG body (in blue, Figure 7.9) and no notable chemical bond can be assigned to the peak at the third measuring point (in green, Figure 7.9) in the aqueous phase. As the second measuring point (blue spectra, Figure 7.9) was taken on MFGs, and the instrument performs an in-depth (8 µm) spectral collection, more lipid molecules were included under the Raman spectra measurement at this measuring point compared to the first measuring point in the MFGM region.

Studies have shown that in food material matrices, volatile release is determined by the oil/water partition coefficient and the mass transfer coefficient of VOCs; moreover, the fat content is a crucial factor that has a noticeable effect on VOC retention/release from food matrices (Benjamin et al., 2011, Giroux et al., 2007, McClements, 2005). Consequently, this may explain why the spectra of VOC+MFGM from the 1\(^{st}\) measuring point show weaker signals than spectra of VOC+MFG from the 2\(^{nd}\) measuring point, especially at 1564, 1625 and 1685 cm\(^{-1}\) which represent VOC Raman shift regions. The results (Figure 7.9) reveal that the hydrophobic VOCs closely interact with MFGs rather than interact with the whole emulsion system, as no noticeable peaks can be observed in spectra of the aqueous phase and the VOC retention is highly dependent on fat content. These results are in good agreement with the previous work of Giroux and co-workers (2007) where they report that the VOC in the oil
Chapter 7: Using Confocal Raman Microscopy to Investigate the Interaction between Volatile Organic Compound (VOC) and Bovine Milk Fat Globules in Recombined Emulsion System

Figure 7.1: Raman spectra of native MFG (~10 μm), spectra was taken at the geometrical centre.
Chapter 7: Using Confocal Raman Microscopy to Investigate the Interaction between Volatile Organic Compound (VOC) and Bovine Milk Fat Globules in Recombined Emulsion System

Figure 7.2: Raman spectra of pure trans-2-hexenal in quartz tube
Chapter 7: Using Confocal Raman Microscopy to Investigate the Interaction between Volatile Organic Compound (VOC) and Bovine Milk Fat Globules in Recombined Emulsion System

Figure 7.3: Raman spectra of pure 2-decanol in quartz tube.
Figure 7.4: Raman spectra of pure Ethyl hexanoate in quartz tube.
Figure 7.5. Raman spectra of pure Octanol in quartz tube.
Chapter 7: Using Confocal Raman Microscopy to Investigate the Interaction between Volatile Organic Compound (VOC) and Bovine Milk Fat Globules in Recombined Emulsion System

Figure 7.6. Raman spectra of pure Propanol in quartz tube.
Chapter 7: Using Confocal Raman Microscopy to Investigate the Interaction between Volatile Organic Compound (VOC) and Bovine Milk Fat Globules in Recombined Emulsion System

Figure 7. Raman spectra of pure Ethyl Butyrate in quartz tube.
Chapter 7: Using Confocal Raman Microscopy to Investigate the Interaction between Volatile Organic Compound (VOC) and Bovine Milk Fat Globules in Recombined Emulsion System

Figure 7.9. Comparison of Raman spectra which were obtained by three measurement points/locations in VOC+MFG emulsion systems; Red: spectra obtained from MFGM (1st measuring point); Blue: spectra obtained from MFG (2nd measuring point); Green: spectra obtained from the aqueous environment outside of the MFG (3rd measuring point); the size of the chosen MFG was 10±1 µm.
Chapter 7: Using Confocal Raman Microscopy to Investigate the Interaction between Volatile Organic Compound (VOC) and Bovine Milk Fat Globules in Recombined Emulsion System

Figure 7.10. Comparison of Raman spectra of VOC + MFG and native MFG. Red: spectra obtained from MFGM (1st measuring point); Blue: spectra obtained from native MFG (control); the size of the chosen MFG was 10±1 µm.
phase, rather than in the emulsion system as a whole, determines the kinetics of hydrophobic flavour release (Giroux et al., 2007).

Methods for quantifying food components and calculating relative ratios between two related molecules of food materials based on Raman spectra had been developed in many studies (Gallier et al., 2011, McGoverin et al., 2009, McGoverin et al., 2010). Moros and co-workers (2007) used Raman spectroscopy with chemometric analysis (partial least squares method) to quantify the fat content of dairy products from two chemical groups: 1) carbonyl groups on glycerol (C=O), and 2) alkyl chain (C-H) of fatty acids. In the light of previous published information, this may support the possibility of quantification of detectable VOCs bound to MFGs and quantification of the fat content of the specific MFGs by confocal Raman microscopy. On-going work is in developing a systematic approach to quantify the relative amount of different VOCs on emulsion particles based on the intensities of measured Raman spectra. In this way, the quantification information may provide more and direct characteristic picture of the interactions between VOCs and emulsion particles.

7.4. Conclusions

Interactions between VOC and MFGs in recombined emulsion systems can be observed by confocal Raman microscopy, *in-situ*. The differences in absorption and adsorption of hydrophobic VOC into the body of MFGs and onto the MFGM surface region were observed
in real time. The hydrophobic VOC, *trans*-2-hexenal, was found to preferentially adsorb to the hydrophobic MFGs in an oil-water emulsion system rather than partition into the aqueous phase.
CHAPTER 8.

Volatile Organic Compounds as Probes to Examine the Milk Fat Globule Membrane
8.1. Introduction

Raman spectroscopy is an innovative tool based on Raman-active molecular vibration which is specific and sensitive to the chemical environment, and which has been applied in food research for compositional analyses (Li-Chan, 1996, Li-Chan, 2007, McGoverin et al., 2010). Studies had been carried out for extending its applications in quality control, compositional characterization and quantification (Cheng et al., 2010, Moros et al., 2007). Li-Chan (1996) outlined the advantages of using Raman microscopy in selective and spatial analysis of complex food matrices. The unique advantages of confocal Raman spectroscopy in food structural research by coupling this instrumentation with chemometric analysis to reveal quantitative maps of the microstructure of polymer gels, has been described (Pudney et al., 2002).

Although a number of MFGM structural models have been reported (Lopez et al., 2011, Mather, 2011, Michalski et al., 2002), knowledge about the structurally related bio-physical functionalities of MFGM is still scarce due to its complexity and the lack of direct investigation techniques. In light of previous fundamental characterization of MFGs and milk components using confocal Raman microscopy (CRM) and Raman spectroscopy (RS) (Forrest, 1978, Gallier et al., 2011, McGoverin et al., 2010), interactions between individual MFGs and a volatile organic compound (VOC, trans-2-hexenal) using CRM were successfully characterized (Zheng et al., 2013a). Preliminary work showed that CRM is capable of determining VOC retention in/on MFGs and the configuration of the instrument.
had been optimized (Chapter 7). Therefore, based on this knowledge, a technique for quantifying the relative retention of two individual VOCs and MFGs after reaching partition equilibrium was developed. The aim of this work was to study the structural feature of the MFGM by using VOCs as probes. Two VOCs were selected with a difference in partition coefficients and applied as probes. The volatile relative retention (RR) for both probes, as determined from CRM, was plotted as a function of VOC concentration. The structural features of the MFGM and bio-physical functionality were deduced by comparing the retention of the VOCs.

8.2. Materials and Methods

8.2.1. Materials

Bovine raw milk samples were sourced from Jersey cows at a local dairy farm (Port Chalmers, New Zealand) and stored at 4°C for up to 24 hours before use. An Olympus BX confocal microscope (Olympus Europa GmbH, Hamburg, Germany) was used for MFG observation in situ and for obtaining Nomarski differential interference contrast images of the MFGs. Raman spectra were collected using a Senterra dispersive Raman microscope (Bruker Optics, Ettlingen, Germany). The confocal Raman microscope allows 3-D profiling with 2 μm depths and 1 μm lateral spatial resolution; the smallest spectra collection volume is a column shape with 1 μm diameter and 8 μm depth.
8.2.2. Recombined MFG emulsion system

The MFG isolation method and microscopic slide preparation were used as previously described (Zheng et al., 2013a, Gallier et al., 2010b). Briefly, raw milk was centrifuged for 5 min at 3000 × g at 4°C. The top cream layer was collected and re-dissolved into simulated milk ultra-filtrate (SMUF) buffer (Jenness and Koops, 1962) obtaining a 20% fat content dispersion system at pH 6.5, and then diluted ten-fold as a stock MFG emulsion.

8.2.3. MFGs + VOCs

Two different VOCs, α-ionone (CAS No.: 127-41-3, from Sigma-Aldrich, St. Louis, MO, USA) and trans-2-hexenal (CAS No.: 6728-26-3, from McCormick, Sparks, MD, USA) were selected based on the differences in hydrophobicity and vapour pressure. Log P (the log of the partition coefficient between octanol and water) for α-ionone and trans-2-hexenal was 3.85 and 1.58 (0.02 and 6.60 for vapour pressure (mmHg) at 25°C), respectively as mentioned in previous research (Benjamin et al., 2012). To prepare samples of MFG + VOC recombined emulsions, two pure VOCs were separately dissolved in propylene glycol to yield 2% (v/v) VOCs solutions. Each of the 2% (v/v) VOC solutions were mixed with recombined MFGs emulsions to obtain four different concentrations of individual VOCs, 0.004, 0.04, 0.4, 1% (v/v). The yield VOC + MFGs mixtures were kept in brown coloured liquid chromatography vials (1.5 mL), which were sealed with inert Teflon lids. All of the sample vials were stored at 4°C overnight allowing the VOCs to sufficiently interact with MFGs.
8.2.4. Confocal Raman microscopy characterization

MFG + VOC mixture samples were used for characterization of interactions between MFGs and VOCs. The blank control was a diluted stock MFG emulsion sample. Samples were placed on concave microscope slides and MFGs were stabilized against movement by setting in a low-melting point agarose gel during spectral measurements. Quartz cover slips were used to cover microscopic slides. For confocal Raman microscopic observation and spectral collection, larger MFGs (10 ± 0.5 µm in diameter) were selected. The Raman spectra were collected and compared at a fixed position, the geometric centre point of each selected MFG.

The microscope and spectral collection were controlled by OPUS software (version 6.5, Bruker Optics). An Olympus 100 × objective (numerical aperture 0.9) with a 50 µm confocal pinhole was used to observe MFGs. Samples were excited using a diode laser (532 nm excitation wavelength, Sentinel, Bruker Optics) with 5 mW power. The OPUS software was used for interpretation of results. The full range of Raman wavenumbers in OPUS for collecting Raman spectra was from 15 to 4441 cm\(^{-1}\). The results were taken from spectral regions from 600 to 1900 cm\(^{-1}\) and from 2500 to 3100 cm\(^{-1}\) using OPUS software. Baseline correction (additional concave rubber-band correction, 100 iterations) and normalization (offset correction) were carried out for all spectral data to optimize the Raman spectra for good inter-sample comparisons. All measurements were carried out at ambient temperature and in triplicate.
8.2.5. Statistical analysis

Significant differences (P < 0.001) of estimated VOC relative retentions were determined by Fisher’s one-way ANOVA test (Minitab 16, Minitab Inc., State College, PA, USA) in paired comparisons between samples means.

8.3. Results and Discussion

8.3.1. Spectra characterization

Raman spectral peaks were located in two Raman shift regions: 800-1800 and 2700-3000 cm\(^{-1}\) for all samples. Raman spectra of native MFG and VOCs labelled MFGs are shown in Figures 8.1.-8.4. Generally, by adding VOCs to MFG suspensions, some peaks became more pronounced after excitation, for example, at 866 and 2800-3000 (C-C/C-O/C-H), 1076 (C-C), 1296/1298 (CH\(_2\)), 1435/1437 (CH\(_2\)), and 1742/1743 (C=O) cm\(^{-1}\) for both \(\alpha\)-ionone and trans-2-hexenal. The Raman peaks representing C=C (1623, 1651 and 1670 cm\(^{-1}\) for \(\alpha\)-ionone, and 1564, 1625 and 1627 cm\(^{-1}\) for trans-2-hexenal) on MFGs dramatically increased in size after adding VOCs. Raman peak characterization was based on the information sourced from previous research (Mizutani et al., 1994, Barry et al., 1992, Weiss et al., 2010, Brunner and Sussner, 1973, Gallier et al., 2011, McGoverin et al., 2010).
Chapter 8: Volatile Organic Compounds as Probes to Examine the Milk Fat Globule Membrane

Figure 8.1. Raman spectra of MFG and MFG + VOC (α-ionone), spectra from bottom to up: MFG, MFG + VOC (0.004, 0.04, 0.4, 1% v/v).
Chapter 8: Volatile Organic Compounds as Probes to Examine the Milk Fat Globule Membrane

Figure 8.2. Raman spectra of MFG and MFG + VOC (α-ionone), spectra from bottom to up: MFG, MFG + VOC (0.004, 0.04, 0.4, 1% v/v).
Figure 8.3. Raman spectra of MFG and MFG + VOC (trans-2-hexenal), spectra from bottom to up: MFG, MFG + VOC (0.004, 0.04, 0.4, 1 % v/v).
Chapter 8: Volatile Organic Compounds as Probes to Examine the Milk Fat Globule Membrane

Figure 8.4. Raman spectra of MFG and MFG + VOC (trans-2-hexenal), spectra from bottom to up: MFG, MFG + VOC (0.004, 0.04, 0.4, 1% v/v).
8.3.2. Estimation of relative retention

The Raman spectral peaks between 800 to 900 cm\(^{-1}\) have been characterized as originating from phospholipids, which are a signature component of the MFGM, therefore the intensities of peaks in a sub-spectral region (825–841 cm\(^{-1}\)) were used to represent MFG as the Raman spectra of headgroups of phospholipids which are sensitive to hydration (Bicknell-Brown et al., 1981). Little changes in the size of Raman peaks in this region (825–841 cm\(^{-1}\)) were found by adding VOCs compared with the more obvious changes in the size of peaks in the Raman shift region between 1600 and 1700 cm\(^{-1}\), as shown in Figures 8.1 and 8.3.

Differences in the Raman spectral profiles were observed when comparing a blank MFG with pure VOCs (Figure 8.1 – 8.4); accordingly, the vibration of C=C (\(\nu\) C=C) and the vibration of C=O (\(\nu\) C=O) were used for characterizing VOCs in the treated MFG samples. As shown in previous research, the C=O and C=C peaks are present in the following Raman shift regions: 1650-1652, 1626-1630, 1670 cm\(^{-1}\) and 1651-1685, 1625-1626 cm\(^{-1}\) (Sajan et al., 2008, Mizutani et al., 1994, Weiss et al., 2010, McGoverin et al., 2010) and therefore the peaks coming from these regions were used for evaluating the presence of \(\alpha\)-ionone and trans-2-hexenal.

The intensities of Raman spectra peaks were used for quantifying the relative amount of VOCs on the MFGs. The relative retention (RR) is calculated as the ratio between the Raman
spectral intensity of VOC to the Raman spectral intensity of MFGs. Therefore, the RR of VOC on MFGs was calculated as follows:

\[
RR = \frac{I_{VOC}}{I_{MFG}}
\]  

(8.1)

where \(I_{VOC}\) is the sum of the spectral intensities of peaks corresponding to the VOCs, and \(I_{MFG}\) is spectra intensities of peaks corresponding to the MFG.

As shown in Figure 8.5, the estimated RR of VOCs on MFGs did not significantly increase \((P < 0.001)\) by increasing concentrations from 0.004 to 0.4\% (v/v); moreover, no significant difference was found between MFGs containing α-ionone and trans-2-hexenal in terms of VOC surface retention within this concentration range. This result suggests that, unlike conventionally emulsified suspension systems, the VOC retention in recombined MFGs emulsion system is not simply correlated with their hydrophobicity and vapour pressures at relatively low concentrations (0.004 - 0.4\% v/v). A significant enrichment of VOC retention (for both VOCs) was found at 0.04\% compared with 0\% (Figure 8.5). This means that the CRM was capable of characterizing and quantifying VOC retention on/in MFGs when VOC concentration level was 0.04\%. When the concentration was further increased to 0.4\%, no significant enrichment of VOC retention was found. This suggests that there is a maximum VOC holding capacity for MFGs.
Figure 8.5. Relative retention (RR) of VOCs on MFGs. The sub-bars ranging from left to right in each section represent the “VOC + MFG” sample systems containing different VOCs concentrations [0 (unlabelled MFG as blank control), 0.004, 0.04, 0.4, 1% (v/v)]. Different letters above bars indicate significant difference for each sample (P < 0.001).
Figure 8.6. Highly schematic illustration of partitioning profile of VOC in MFG and interactions between MFG and VOC (including both α-ionone and trans-2-hexenal) when containing different VOC concentrations. Green: Phospholipid bilayer; Blue: protein-dense primary monolayer; Purple: disordered MFGM containing phospholipids and membrane proteins; Orange: Triacylglycerol inner core; Red: VOCs molecules.
The same VOCs at the concentration of 0.004% (v/v) were used in previous study where the authors characterized the release of these selected VOCs from primary o/w and multilayer o/w emulsions via headspace-gas chromatography (Benjamin et al., 2012). The aforementioned study showed that trans-2-hexenal was more readily released to the headspace than α-ionone at the concentration of 0.004% (v/v) for both primary emulsion and multilayer O/W emulsion systems. In the current study, CRM was sensitive enough for characterizing the increases of RR of VOCs on MFGs ($P < 0.001$) (Figure 8.5) at relatively low concentration (0.04%, v/v).

Giroux and co-workers (2007) observed that aroma concentration in the oil phase, rather than in the emulsion, determines the kinetics of hydrophobic volatile release. Consequently, it is reasonable to deduce that in both the conventional primary emulsion and multilayer O/W emulsion systems, the retention of trans-2-hexenal onto globules should be lower than that for α-ionone. The current results, however, showed no differences in RR on MFGs between two VOCs (Figure 8.5) at relatively low concentrations (0.004 – 0.4 %, v/v). The retention could not be altered by increasing the VOC concentration (up to 0.4%, v/v). From this result, a mechanism for VOCs absorption on native MFG is proposed: at lower concentrations (0.04 – 0.4%, v/v), the two VOCs interact only with the MFGM rather than the whole MFG assembly after reaching partitioning equilibrium. MFGM has the capability to hold a certain amount of VOCs. An interfacial layer within or underneath the MFGM (phospholipid bilayer) that seals the inner part of the MFG, thus avoiding interactions between VOCs and the triacylglycerol core of the MFG, is illustrated schematically in Figure 8.6). These results are
indirect evidence for the existence of a protein-dense monolayer separated from the phospholipid bilayer in the native MFGM, as proposed in other model systems (Lopez et al., 2011, Lopez et al., 2010, Mather, 2011). Additional evidence using CLSM has shown the presence of a protein layer after washing off the outer phospholipid bilayer (Zheng et al., 2013b). When the concentrations of both VOCs reached as high as 1% (v/v), the estimated RRs of both VOCs on the MFGs were consistent with their hydrophobicity values (Figure 8.5), suggesting that VOC absorption into MFGs is mainly determined by the hydrophobic triacylglycerol inner core (Giroux et al., 2007) rather than the MFGM. This phenomenon may be rationalized by the following proposed mechanism: at relatively higher concentrations (≥ 1%, v/v) the VOCs may rupture the protein-dense membrane monolayer barrier, or disturb the native structure of the MFGM (becoming a disordered MFGM as shown in Figure 8.6) to enable interaction of VOCs with the triacylglycerol inner core of the MFGs.

8.4. Conclusions

This work, using an innovative CRM approach, reveals that the MFGM has the capacity for hosting a small amount of VOCs, preventing interactions with the inner core of the MFGs without consideration of the VOC hydrophobicity. This capacity, however, can be saturated by increasing the volume of VOCs to a relatively higher level (≥ 1%, v/v). The current results support the idea that the MFGM is a useful vehicle for carrying and delivering hydrophobic organic compounds in food products. A natural extension to this work is to develop a formulation to contain bio-active compounds within the MFGM layer in a dairy food matrix.

186
CHAPTER 9.

General Conclusions
&
Future Recommendations
9.1. Conclusions

In the course of this research, the structural organization and properties of the native bovine MFG were investigated. Experimental evidence was provided to extend the current fundamental knowledge about the nature of the native MFGM structure. An update was provided to the current model of the MFGM. An artificial bilayer was developed and constructed as a model for mimicking the native structure of the MFGM, thus providing a new and innovative approach to examining the surface of MFGs.

By applying specifically designed washing procedures (considered as “polishing” processes) to the surfaces of MFGs, structural features of MFGM proteins in terms of competitive membrane binding strengths were shown in Chapter 3, thus providing some information about relative locations within the membrane. Washing procedures may alter the structure of MFGM differently: using CLSM, it was observed that intensive washing (centrifugation at 15000 g, 20 min, three washes, M3 in Chapters 3 and 4) may destroy the original structure of the MFGM where the phospholipid outer bilayer was partially removed and the primary protein-dense monolayer was revealed on the surfaces of washed MFGs. This provides experimental evidence to support the existence of a primary protein-dense layer located underneath the outer phospholipid bilayer in the MFGM. Although a mild washing procedure (centrifugation at 3000 g, 5 min, three washes, M1 in Chapters 3 and 4) induced compositional changes in terms of competitive enrichment of CD 36, PAS 6/7 & ADPH and SM, no significant structural changes were found by CLSM characterization. A CD 36 and FABP protein complex is a relatively stronger membrane binding protein network and may be relatively enriched on the surface when the outer phospholipid bilayer is ruptured. PAS 6/7 is enriched on MFG surfaces during mild M1 washing, but is relatively depleted after more stringent M3 washing, suggesting that the relative changes of PAS 6/7 would be a good indicator for assessing the destructive damage of the MFGM.
outer bilayer. These results provide essential fundamental information for understanding protein arrangement in MFGM during mechanical treatments and for fractionation of MFGM components from raw milk.

Washing induces competitive enrichment or depletion of the five major MFGM phospholipids on the surfaces of MFGs (Chapter 4). By characterizing the relative proportional changes of these PLs, PE, PC and SM can be considered as outer bilayer components; PI and PS are believed to preferentially locate near the inner protein-dense primary monolayer. This newly deduced MFGM PL arrangement is supported by HLB values and molecular critical packing shapes of individual major MFGM PLs. Consequently, an updated MFGM lipid topology was developed in Chapter 4 in which PC and SM form the outer leaflet of the bilayer and PE is packed in the inner leaflet of the bilayer. PI and part of PS are infused in the primary protein layer and their total molar volume is not necessarily proportional to the PL molar volume in the outer bilayer. This suggests that the primary monolayer is a fusion of PLs and membrane proteins rather than existing as a separated structure.

A non-supported GUV bilayer system, as a tool for mimicking the surface morphology of MFGs and for studying the detailed structural organization of MFGM lipids, was successfully constructed and reported in Chapters 5 and 6. In Chapter 5, by altering the composition of the model bilayer, lipid domain formation was observed by CLSM, and may be formed from relatively high T_m milk phospholipids (DPPC and SM) without the need for cholesterol. The artificially constructed lipid domains presented similar morphological features as shown on MFGs in terms of shape and size; the similar mobility of lipid domains on GUV had been observed as well. Moreover, in Chapter 6, by altering the concentration of SM and cholesterol in GUV formulas, it was found that lipid domain formation was dictated more by the presence of cholesterol than SM, and that cholesterol may interact
with glycerolphospholipids (PC) inducing lipid domains on GUV surfaces. The results from Chapters 5 and 6 provided experimental evidence supporting the deduced lipid topology as shown in Chapter 4, in which lipid domains may be formed by either SM alone, or through a PC-cholesterol network.

An innovative approach using confocal Raman microscopy to probe the structural properties of the MFGM and investigate interactions between MFG and VOC was developed in Chapters 7 and 8. The estimated relative retention of VOCs on MFG in Chapter 8 showed no significant changes as a response to the increased VOC concentrations in recombined MFG dispersion systems where VOC concentrations were in the relatively low range between 0.004 to 0.4% (v/v). The relative retention of VOC was significantly increased on MFGs when the concentration of VOC reached a relatively high level 1% (v/v), suggesting that the whole MFG assembly is not a homogenous system and there is a barrier between the surface and interior of MFG to further interactions between hydrophobic VOCs and the TG core. This barrier can be explained by a protein-dense monolayer between outer lipid bilayer and inner TG core, and is further evidence for the existence of a protein layer. These results in Chapter 8 also revealed the structural functionality of MFGM, and that hydrophobic VOCs may interact with the MFGM as a structure separate from the entire MFG assembly.

9.2. Future Recommendations

The compositional and structural properties of isolated MFGs from raw milk may be altered by manipulating the parameters of centrifugation. There is no best method for fractionating MFGs from raw milk as it depends upon the purpose of the procedure. Stringent washing procedures may partially destroy the outer phospholipid bilayer thereby inducing a significant structural change, therefore this procedure is not recommended if the native structure of MFGs is of key interest. Nevertheless, the total
recovery of MFGM PLs (w/w fat) from a stringent washing method was significantly higher ($P < 0.001$) than the recovery from the mild washing method, consequently, if the eventual yield of MFGM PLs is of key importance, then a stringent washing procedure is recommended.

Exploring the detailed compositional and structural changes of MFGs induced by other mechanical treatments, such as homogenization, would be a first step for future research on this topic. Homogenization is a common process in the dairy industry employing a destructive mechanical treatment to rupture native MFGs to produce smaller sized globules. The retention of residual MFGM components on homogenized fat globules may reflect the physicochemical nature of these components, and provide fundamental knowledge to better understand the native structure of the MFGM. Moreover, it may assist the dairy industry to provide insights into how homogenization alters the composition and structure of MFGM and MFG assembly to produce new functional food ingredients. MFGM glycoproteins are reported to be more resistant to gastrointestinal digestion than other serum milk proteins (Le, et al., 2012). Moreover, from a structural point of view, the natural digestion process of MFGs is MFGM-structure dependent (Gallier, Ye, & Singh, 2012), therefore further studies should focus on the detailed impact current models of the MFGM structure on the natural digestion of MFGs. Studies should also focus on the innovative formulation of dairy emulsion matrices containing fat droplets that mimic the native structure of the MFGM, such as in recombined milk.

The artificial GUV bilayer system for mimicking MFGM lipid arrangement in the native MFGM was a useful model for examining lipid topology. In future, the native MFGM-associated proteins, including trans-membrane and bilayer docking proteins, should be incorporated into a GUV model to show the partitioning of specific MFGM proteins between ordered and disordered lipid domains. Interactions between lactic acid bacteria (LAB) and MFG had been observed and characterized (Jimenez-Flores &
Brisson, 2008). By determining interactions between LAB and a composition-controlled GUV system, detailed mechanisms of such interactions with MFGs may be explored, thus providing information about how LAB structural location impacts upon flavour development in products such as cheese. The MFGM is involved in signal transduction processes, however, the protein structural arrangement in the MFGM is uncertain. A GUV system may be used as a platform for investigating detailed interactions between signalling proteins and specific MFGM lipids. Confocal Raman microscopy may allow for mapping the surface of MFGs, thus revealing the composition of lipid domains based on the Raman spectra of the artificial bilayer without the need for fluorescent staining that may potentially alter the surface structure.
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