MASSEY UNIVERSITY

Colloidal interactions in an alternate make cheese

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Abstract

The role of emulsion structure and interactions on the material and technical functionality of an alternate make cheese (AMC) was investigated. Lab scale cheese samples (25 g comprising 23 wt.% fat and 20 wt.%) were prepared by recombining model emulsions with a separate protein phase under controlled temperature, shear speed and residence time in a rapid visco analyser (RVA). Sodium caseinate and Tween 20 were used respectively to stabilize fat globules for the model emulsions. Preliminary experiments were carried out for samples prepared using either calcium caseinate or sodium caseinate as protein phase. Structural characterisation of samples showed emulsion structure and distribution within these phases to be dependent on protein type. It was inferred that the calcium from calcium caseinate matrix modified the interfacial layer of the emulsions stabilised by sodium caseinate, as indicated by the increased fat globule size distribution after cheese making. In comparison, the size of fat globules covered with sodium caseinate appeared relatively stable in cheese produced form cheese curd. Based on these observations, caseinates were subsequently replaced by cheese curd as the protein phase for the remainder of the study.

For cheese samples prepared with low fat cheese curd, fat droplets stabilised with sodium caseinate were hypothesised as binding with the surrounding protein matrix, and thereby these fat globules could be considered as 'active fillers'. Confocal laser scanning microscopy supported this hypothesis showing homogeneously dispersed fat droplets within the protein network. This emulsion system did not show fat-protein phase separation in baking (170 °C 10 minutes) as droplets were prevented from coalescing as a consequence of entrapment within the protein phase.

Fat globules covered with Tween 20 were hypothesised as behaving as 'inactive fillers', with the adsorbed layer not anticipated to form bonds with the surrounding protein network. Confocal and scanning electron microscopy instead showed localised domains of fat droplets within the protein structure that underwent partial coalescence on cooling of the cheese after manufacture. Cheeses comprising Tween stabilised droplets exhibited phase separation on baking and visible oil-off on the surface of cheese arising

from extensive coalescence taking place within the localised regions of fat due to melting of the partially coalesced structures. Additional rheological analysis of cheeses was carried out to determine the effect of droplet-protein interactions on the material properties of the cheese samples. Notably, findings were presented in relation to a non-fat control cheese. Findings showed that, at temperatures below 30 °C when fat was crystallized, both inactive and active fillers had a higher relative modulus to the non-fat sample. However, at elevated temperature without fat crystals, inactive fillers resulted in a relative reduction in storage modulus when compared to the non-fat cheese, while active fillers increased relative storage modulus.

Model cheeses prepared with either sodium caseinate or Tween 20 stabilised emulsions were then compared to cheese samples comprising non-homogenised cream as the emulsion phase. Structural analysis of samples determined that cheeses comprising fat globules stabilized with native milk fat globule membrane behaved in a manner analogous to samples prepared with the Tween stabilised emulsion, indicating the presence of inactive droplets. However, it was also observed that increasing the residence time of cheese production within the RVA caused a transition of the interaction behaviour of the emulsion from inactive to active, as evidenced by corresponding changes to structural, material and functional properties of the cheese.

Further exploration of this transition determined that the mechanical work applied during cheese preparation was sufficient to homogenise fat droplets during extended shearing, resulting in a reduction to fat droplet size. Droplet homogenisation during shearing was also found to have disrupted the native milk fat globule membrane, allowing protein adsorption to take place. It was also determined that whey proteins were the predominant interfacial fraction adsorbed as a consequence of extended shearing, and were considered responsible for the transition of droplets from inactive to active. Combined findings have shown that the material and functional properties of an alternate make cheese composition could be strongly influenced by the interactions of the emulsion phase with the surrounding protein network. These interactions could, in turn, be manipulated through formulation and/or process design, providing greater control over product properties.

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Acronyms

AMC	Alternate make cheese
AMC_NaCas	Alternate make cheese produced from emulsified fat fully covered with sodium caseinate
AMC_Tween	Alternate make cheese produced from emulsified fat fully covered with Tween 20
AMC_NC	Alternate make cheese produced from fat globules with native milk fat globule membrane
AMC_AMF	Alternate make cheese produced from anhydrous milk fat without emulsifiers
AMF	Anhydrous milk fat
β-ME	β-Mercaptoethanol
Ca	Capillary number
CaCas	Calcium caseinate
CLSM	Confocal laser scanning microscopy
cm	Centimetre
d(0.1)	Volume-weighted diameter of 10 % smallest droplets
d(0.9)	Volume-weighted diameter of 10 % largest droplets
$d_{4,3}$	Volume-weighted average mean diameter
EDTA	Ethylene diamine tetra acetic acid
FG	Fast Green
FO	Free oil
g	Gram
G'	Storage modulus
G''	Loss modulus
G_m'	Storage modulus of non-fat cheese
Hz	Hertz
LFCC	Low fat cheese curd
m	Mass (in equations); or meter (after numbers)

min	minutes
mm	Millimetre
mg	Microgram
nm	Nanometre
NaCas	Sodium caseinate
NaOH	Sodium hydroxide
NR	Nile Red
PEG	Polyethylene glycol
Re	Reynold number
RhPe	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)
RVA	Rapid visco analyzer
rpm	Revolve per minute
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide electrophoresis
vol	volume
Tween	Tween 20
Tris	Tris(hydroxymethyl)aminomethane
µl	Microliter
WPI	Whey protein isolate
wt	Weight
w/v	Weight/ volume

Chapter 1: Introduction

Mozzarella is one of the most widely consumed cheeses across the world. Its particular material and sensory properties, especially when cooked, provide a recognisable and desirable consumer experience, which has led to its primary form of usage as a component of pizza toppings. Recently within New Zealand, the industrial production of Mozzarella cheese has been developed towards an alternate manufacturing process, where cheese structure is able to be manipulated under highly controlled conditions. Cheese structure is colloidal in nature, and thus an investigation of the colloidal properties and interactions in an alternate make cheese (AMC) prepared at laboratory scale may assist in determining how dynamic changes to cheese microstructure during processing, storage and utilisation may impact on the material, functionality and quality outputs for the product.

1.1 Objectives and research questions

The primary objective of the project has been to determine the role of emulsion structure and stability in relation to the microstructure, material properties and technical functionality of an alternative make cheese. The description of emulsion droplets as either “active fillers” or “inactive fillers” has been used throughout the thesis to define fat globule interactions in with the surrounding protein network. The particular type of interaction behavior is hypothesized as being determinant in defining the structural state of the emulsion within the cheese, such as the distribution of fat within the cheese, and the relative stability of oil droplets during manufacture, storage. Such structural changes would be expected to modify the technical functionality of the cheese during use, such as the meltability and degree of oil-off in cheese baking. Controlled modification of the interfacial layer of the cheese emulsion to be either active or inactive can be explored in the context of both formulation design and process control, thereby allowing a more iterative approach for controlling cheese colloidal microstructure, and

which in turn can potentially be applied to deliver particular technical and sensory attributes to the alternate make product.

The overarching research question can therefore be defined as:

- How do colloidal interactions in cheese manufacture, storage and baking influence cheese structure, material properties and functionality?

Within this context, the underpinning research questions can be articulated accordingly:

- What are the interactions between fat, water and protein in the cheese within active/inactive fat fillers, and can emulsions be developed that adequately represent these interaction states?
- How do the particular interactions between fat droplets and the surrounding protein network influence the structure and stability of the emulsion state within the cheese?
- What are the effects of processing conditions and formulation (manufacture temperature, shear rate and residence time) on emulsion and cheese structure dynamics during manufacture and storage?
- Can fat structure within AMC be correlated to material properties and/or functional properties of the cheese (e.g. melting behaviour)
- Will fat globules in cheese made from non-homogenised cream behave as active fillers or inactive fillers, and can this be related to the functionality of the cheese?

1.2 Thesis structure

This chapter (**chapter one**) gives an overview on thesis structure and research questions. **Chapter two** establishes the current state of the research area by reviewing prior studies on cheese structure and functionality. Aspects of the review include the derivation of active/inactive filler particles as defined in the context of model emulsion gels systems. Cheese structure and its impact on the material and functional properties of the product are discussed, along with considerations for how structure can be manipulated. Additionally, analytical methods available for determining cheese structure and

functionality are investigated to determine the most appropriate methods for meeting the objectives of this project. The details of sample preparation and analytical methods employed within the project are described in **chapter three.**

Chapter four introduces the development and characterization of model emulsion-in-protein structures, where the emulsified fat is mixed with protein powders of sodium caseinate (NaCas) or calcium caseinate (CaCas) in order to study the effect of droplet interactions in oil-in-viscous protein emulsions. Emulsion stability was investigated by comparing fat globule size distribution and emulsion structure as a consequence of processing conditions. Small strain oscillatory rheology was also used to determine the impact of droplet interfacial composition on material properties when the emulsions were mixed with the protein.

Alternate make cheese (AMC) was produced by mixing (heating and shearing) emulsified fat and fat-free cheese curd, as presented in **chapter five**. The role of the fat droplet interface in structuring the emulsion within the cheese matrix and its impact on cheese functionality was characterized. Cheese melting was analyzed by the measurement of oil-off, flowing area and moisture loss. The effect of fat structure on cheese material properties was discussed when changing the interfacial composition and/or the fat type (milk fat or canola oil). Additionally, cheeses prepared using natural, non-homogenized cream were compared to cheeses made from emulsified fat in order to better understand the colloidal interactions in commercial Mozzarella cheese.

The impact of processing on the dynamics of the milk fat globule membrane and the subsequent impact on cheese structure and functionality were studied. **Chapter six** attempts to explore this from two considerations: 1) mechanical shear work from cheese processing homogenizing the milk fat droplets; 2) damage and displacement of the milk fat globule membrane and adsorption of protein during mechanical shear. The temperature, shear speed and residence time of cheese production and protein species (casein and whey) were discussed on the modification of cheese functionality.

Chapter seven is a summary of the colloidal interactions observed within alternative make cheese and the impact of these on cheese functionality, focusing in particular on cheese melting for pizza baking. The contribution of fat interfacial properties and

emulsion structure to fat-protein phase separation and water mobility are discussed. At the end of this chapter, recommendations for future research are pointed for further development of understanding of colloidal interactions in cheese.

Chapter 2: Literature review

2.1 Colloidal interactions in traditional cheeses

2.1.1 Molecular interactions in cheese

Colloidal interactions in cheeses or cheese-like emulsion gels are primarily determined by the molecule interactions of proteins, water and fat droplets (in the form of emulsion droplets) (Dickinson 2012, Lamichhane, Kelly et al. 2017). Environmental conditions, such as production temperature, ionic strength and pH additionally play an important role in determining the nature of colloidal interactions and subsequent cheese functionality and composition (Everett and Auty 2008, Kapoor and Metzger 2008, Jana and Mandal 2011, McMahon and Oberg 2011, Ong, Dagastine et al. 2012, Ong, Dagastine et al. 2013, Lamichhane, Kelly et al. 2017). The pH of cheese, for example, can vary between 4.5 and 7.0 (Table 2.1), which affects the electrostatic interactions between protein molecules during the coagulation processes. Cheeses of low pH, such as Feta cheese, Cottage cheese and Cream cheese, are normally produced by acidifying the milk towards the isoelectric point of casein (approximate pH 4.6) (Walstra, Wouters et al. 2006), where the electric repulsions are minimized, and thereby allowing coagulation of the casein protein even prior to rennet addition. For cheeses of pH at approximate 5.5, such as Cheddar cheese, Gouda cheese, Emmental cheese and Mozzarella cheese, rennet addition and its hydrolysis and destabilization of the 'hairy' region of casein micelle (κ-casein) are considered the singular form of coagulation of the casein protein. Curd formation is further strengthened through formation of Ca^{2+} crosslinks, enabling the formation of a strong protein network, although relative pH will influence the strength of these interactions, which will be expected to decrease as pH approached the isoelectric point. In contrast, other approaches, such as the use of emulsifying salts in the manufacture of processed cheese may provide a sequestering effect, reducing the availability of Ca^{2+} with the consequence that interactions among proteins are reduced, resulting in the recognizable melting characteristics of the product.

Temperature is another important factor impacting on cheese colloidal interactions, influencing the bond energies between proteins. For example, hydrophobic interactions

become strengthened with increasing temperature. In the case of pasta-filata type cheeses, such as Mozzarella, which needs to be stretched in hot water to achieve the unique fat channeling and protein strands (McMahon, Fife et al. 1999, De Angelis and Gobbetti 2011), the amount of water associated with the protein network progressively decreases when the cheese is heated (Vogt, Smith et al. 2015). Bulk water may be either present within the fat channels as free water or entrapped within the protein matrix as entrapped water (Lamichhane, Kelly et al. 2017). In cheeses without fat channels, most of the water is present within the protein matrix; however, fat-serum channels of Mozzarella cheese are able to preserve a significant amount of water. Due to hydrophobic interactions, protein structures within the cheese contract at high temperature, with water being released from the cheese protein matrix. Conversely, unbound water tends to be reabsorbed into the cheese protein matrix during cooling and storage (McMahon and Oberg 2011). During the storage of Mozzarella cheese the amount of unbound water (which is typically located in fat-serum channels) decreases due to absorption into the protein matrix (McMahon, Fife et al. 1999, Kuo, Gunasekaran et al. 2001, McMahon and Oberg 2011). Such dynamic changes in structure can additionally impact of the functionality of the cheese.

Temperature effects, particularly under baking conditions, may also be contributory to specific chemical reactions, notably Maillard reactions, which can be influential in other aspects of cheese functionality during, cooking, such as changes in appearance (browning).

Table 2.1: Approximate composition of cheese varieties including AMC (Walstra, Wouters et al. 2006)

Cheese	Water (w/w %)	Protein (w/w %)	Fat (w/w %)	NaCl (w/w %)	Fat/ dry matter	pH	Yield (cheese/ milk)
Camembert	56	19	21	2	0.5	7.0	13%
Cheddar	39	25	31	2	0.5	5.5	10%
Emmental	38	28	29	1	0.5	5.6	8%
Mozzarella	55	23	19	1	0.4	5.2	13%
Feta	56	16	23	3	0.5	4.5	15%
Gouda	43	24	29	2	0.5	5.4	11%
Parmesan	33	36	26	2	0.4	5.5	7%
AMC	53	21	23	1.4	0.5	~5.7	N/A

Cheese can be regarded as a composite material of fat structures distributed within a viscoelastic protein matrix. Fat, and fat structures, play an important role in cheese appearance, flavour, texture and functionality (Johnson 2011). Low-fat cheeses generally display defects on sensory, texture and functionality, such as unwanted flavors, rubbery texture and poor meltability (Rodriguez 1998, Vikram 2001, Banks 2004). In the case of Mozzarella, it has been observed that a decrease in fat content from 19 % to 2 % significantly reduces the melting area and spread of the cheese (Fife, McMahon et al. 1996). During pizza baking, the limited melting induces scorching of the cheese surface with a lower incidence of oiling off (i.e. coating of cheese surface by melted fat during baking) also assisting in faster dehydration of moisture from the shred surface (Rudan and Barbano 1998).

Functional aspects, such as the relative extent of oiling off, can be related to the structure and localisation of fat phase located in anisotropic serum channels within the protein network of pasta-filata type cheeses (McMahon, Fife et al. 1999, Everett and Auty 2008, Ma, James et al. 2013). In a reduced fat Mozzarella cheese there are fewer, thinner and less connected fat channels (Ma, James et al. 2013), and consequently the cheese displays reduced oil-off in baking as it becomes more difficult for the fat to escape to the surface of the cheese during cooking (Rudan and Barbano 1998). Low fat Mozzarella is also characterised by reduced stringiness during consumption (Oberg, Oberg et al. 2015). Fat channels formed during processing assist in protein fibril formation whilst inhibiting homogeneous protein aggregation. Cheese stringiness for Mozzarella is a balance of protein-protein interactions; these should be optimised such that strong proteins bonds are prevalent *within* the fibre structures, but are limited *between* fibres. This will allow the protein to flow and stretch on melting.

For low-fat Mozzarella cheese, manipulating the structure of fat channels can provide a mechanism for improving cheese melting behaviour, and enabling an appropriate degree of oil-off to form a lubricating surface layer to allow proteins to flow easily during baking (Wadhwani, McManus et al. 2011). Changes to fat structure as a consequence of processing can also significantly impact on the functionality of the resulting cheese, and

not always in a positive way. For example, the structure of Mozzarella cheese made from homogenized cheese milk showed the emulsified fat globules to be evenly distributed within in protein matrix. Cheeses prepared in this way displayed a pronounced decrease in observed oil-off (Rowney, Hickey et al. 2003). This modification is caused by the protein adsorption at fat globule surface, which strengthens the interactions between fat globules and proteins, as well as minimising the localisation of fat into channelled structures.

2.1.2 The dynamics of cheese emulsion structure during manufacture

Traditional cheese-making converts milk to cheese, in which the casein protein component and fat are concentrated through coagulation, with much of the water and lactose expelled from the coagulum along with whey protein (Figure 2.1). Cheese manufacture includes physical changes such as gelation, syneresis, curd fusion and ingredients diffusion (Figure 2.1). Over the time-line of cheese production, there is a progressive dynamic development of the colloidal state. Gelation (coagulation) of the casein occurs after adding enzyme and/or acid, and the resultant space-filling network encloses the fat globules. Whey and some fat are lost from exposed edges after curd cutting, promoting additional curd syneresis (Guinee and McSweeney 2006). Curd fusion and ingredient diffusion during cheese ripening allow the formation of a continuous protein structure with embedded fat droplets trapped in localized domains.

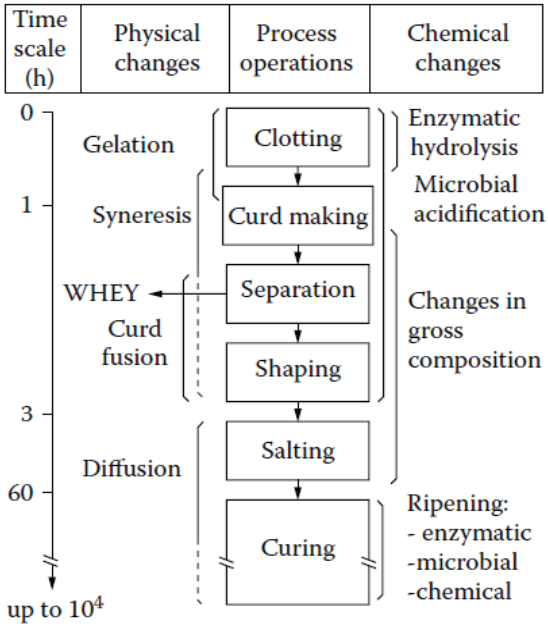


Figure 2.1: Schematic of the most essential physical and (bio)chemical changes occurring during the transformation of milk into cheese (Walstra, Wouters et al. 2006).

Cheese emulsion structure is not static, with dynamic changes taking place during manufacture and storage. These can effectively be observed using microscopic techniques. Scanning electron micrographs are able to highlight the structural connectivity of protein networks in cheese production (Figure 2.2). Taking Mozzarella cheese as an example: the renneted casein micelles are observed as aggregating to form a particle network (Figure 2.2A); draining of whey results in syneresis of water from protein network and compaction of the structure (Figure 2.2B). The protein network is then stretched into parallel strands in hot water (~ 54 °C) separated by serum channels (Figure 2.2C). After cooling and brining, scanning electron micrographs show extended domains of fat-serum channels. During cheese ripening the serum component of the channels is reabsorbed into the protein network leaving the channels predominantly occupied by fat globules (Figure 2.2D) (McMahon, Fife et al. 1999). Guo and Kindstedt (1995) also reported dramatically increased water-holding capacity of Mozzarella cheese over 14 days of ripening. The dynamic changes to cheese structure in first few weeks of cheese storage, are reflected in modification to cheese melting and water partitioning behaviour (McMahon, Fife et al. 1999).

The location and structure of individual fat droplets in cheese can be visualized using SEM, however confocal microscopy can be a more effective tool in visualizing the distribution of fat droplets within the cheese protein network. Figure 2.3, for example, shows the confocal images of Emmental cheese at different production stages (Lopez, Camier et al. 2007). Fat globules were initially seen to be entrapped in the serum pores of the casein network in the rennet-induced curd (Figure 2.3A); some fat globules subsequently aggregated and/or coalesced after curd heating (Figure 2.3B) and enlarged irregular fat domains appeared after curd pressing (Figure 2.3C). Some free fat was observed during sample preparation; serum was reduced after brining resulting in a continuous protein network (Figure 2.3D). Removing water from rennet curd seems to be essential for the formation of localized fat domains in cheese manufacture, where bulk water is lost after curd pressing and brining.

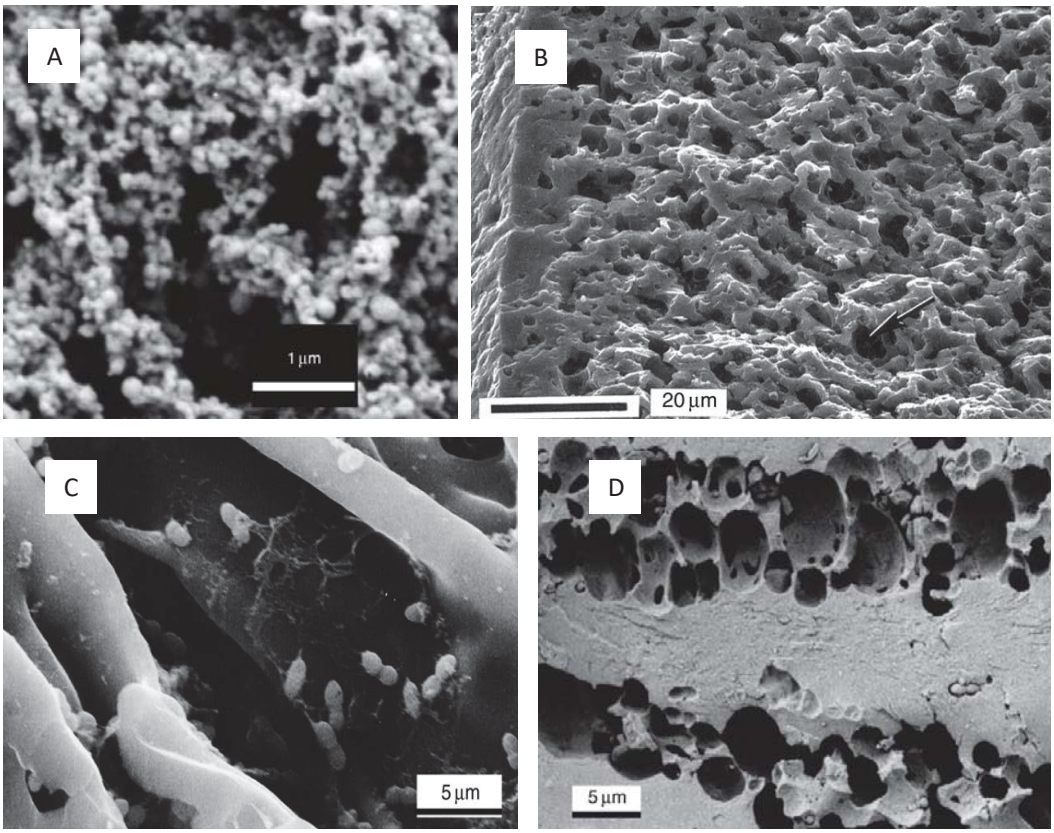


Figure 2.2: Scanning electron micrograph of Mozzarella cheese in production (McMahon and Oberg 2011). (A) milk coagulum prior to cutting, (B) curd particles after draining the whey (Oberg, McManus et al. 1993), (C) stretching (kneading) of cheese in hot water (McMahon, Fife et al. 1999), (D): cheese after four weeks in cold storage (McMahon, Fife et al. 1999).

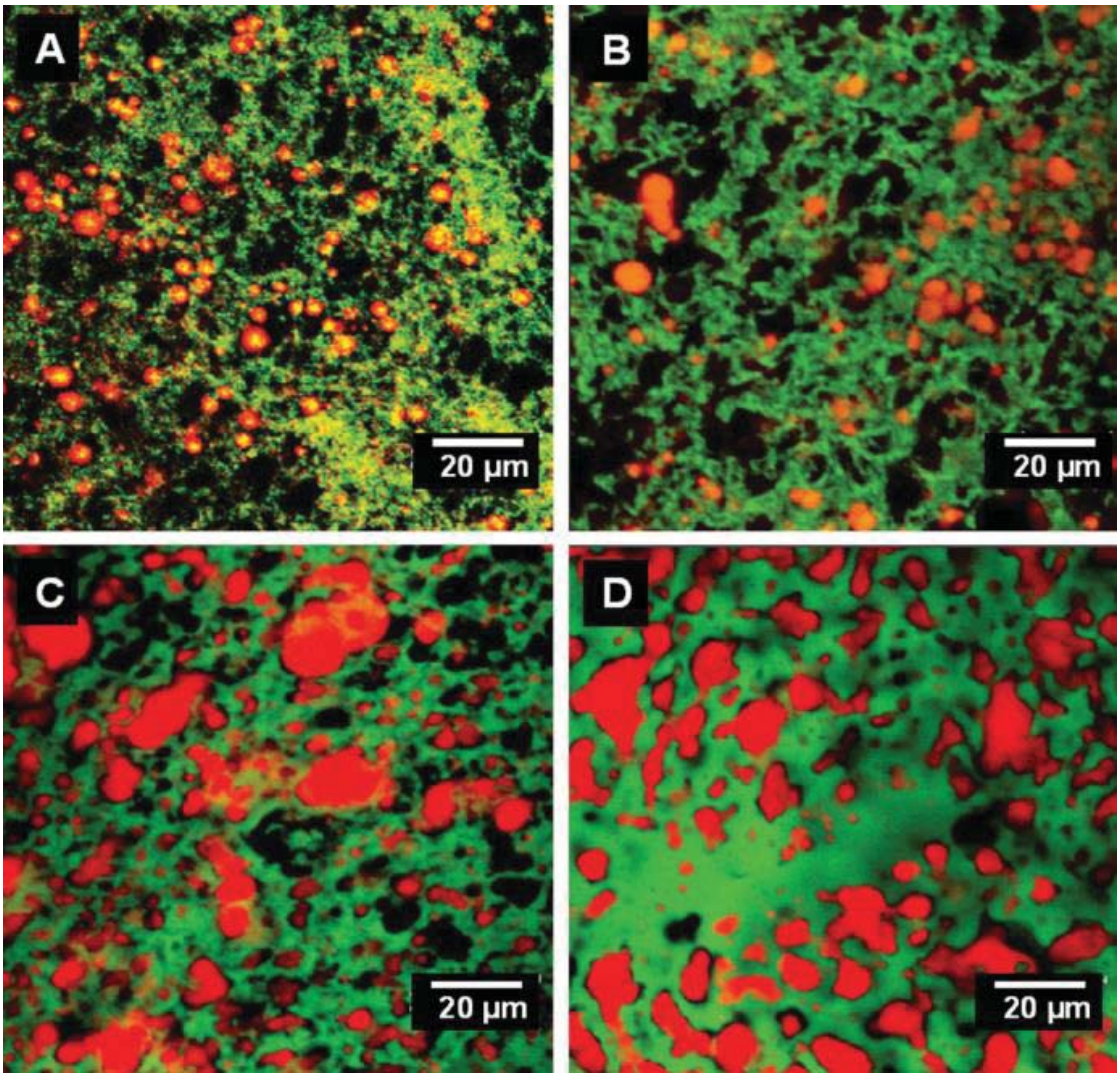


Figure 2.3: Confocal laser scanning micrographs of Emmental cheese: (A) rennet-induced curd, (B) heated curd grain before pressing, (C) Emmental cheese after pressing, (D) Emmental cheese after brining. Fat is colored red; proteins are colored green; the serum phase is black (Lopez, Briard-Bion et al. 2006).

2.2 Structural parameters influencing cheese functionality and material properties

2.2.1 Effect of fat structuring

2.2.1.1 Fat globule interface

The interfacial composition of the fat globules plays an important role in determining the colloidal interactions between the emulsion droplets and the surrounding protein matrix, which can be expected to have impact on cheese properties such as flow and melt.

Native (i.e. non-homogenised) milk fat globules are coated with membrane consisting of three layers of polar lipids and membrane proteins, and this membrane protects fat globules from coalescence in dilute emulsions such as milk (Walstra, Jenness et al. 1984, Dewettinck, Rombaut et al. 2008). However, this interfacial membrane is not mechanically very strong; notably, the application of high shear and/or the concentration of fat phase volume can promote a phenomenon called partial coalescence, which occurs as a consequence of the collision, wetting and ultimately fusion of adjacent crystalized fat globules (in which the solid fat fraction of the droplets exceeds a certain level).

Partial coalescence can cause agglomeration of droplets leading to the formation of large, irregularly shaped fat granules (Pawar, Caggioni et al. 2012). As implied, this effect occurs at temperatures where the fat droplets are crystalline. Increasing the temperature after partial coalescence, such that the fat phase of the droplets becomes fully molten (i.e. at temperatures >~40 °C in the case of milk fat), causes full coalescence of fat agglomerates into large droplets (Walstra, Wouters et al. 2006). Droplet interfacial area decreases after coalescence and some milk fat globule membrane material is released into the serum (Walstra, Wouters et al. 2006). Partial coalescence is considered an important mechanism of fat structuring in cheese manufacture and the magnitude of this effect in cheese can be dependent on milk composition (Mistry, Metzger et al. 1996, Poduval and Mistry 1999, Govindasamy-Lucey, Lin et al. 2006, Dewettinck, Rombaut et al. 2008).

Whilst cheeses are most typically produced from non-homogenised milk fat droplets, there has been some exploration of alternative interfacial materials for stabilizing the milk emulsions, particularly as a means of controlling material and functional properties. Interfacial modification has particularly focused on the use of small molecular weight surfactants, usually derived from lipids, which are able to greatly lower the interfacial tension and are frequently used to provide particular stability and structure to food emulsions. Also termed surfactants, these materials are typically comprised of a well-defined hydrophilic head group and hydrophobic tail. On emulsion interfacial surfaces, the hydrophobic tail extends into fat phase and the hydrophilic head partitions into the water phase. Surfactants can be classified as either ionic surfactants or non-ionic

surfactants, according to the charge of the head group. The most frequently used surfactants in food include phospholipids (lecithin); mono- and diglycerides (glycerol monostearate); polyethoxylated sorbitan esters (Tween); sorbitan esters (Span) and sucrose esters (Dickinson 1992). Tween 60 or Tween 80 stabilised emulsions have been previously investigated as non-interactive droplets in model protein gels (Xiong and Kinsella 1991, Cho, Lucey et al. 1999). Additionally, the influence of a Tween 80 stabilised emulsion on the properties of a model cheese has been explored. Here it was observed that the cheese exhibited increased fat loss after curd cutting when fat globules were coated with Tween 80 (Everett and Auty 2008), indicating that droplets were not only bound to the protein network, but that they were also able to be expelled as part of the whey stream during the compaction of the curd.

Biopolymers, such as milk proteins, provide an additional mechanism for stabilization and structuring of emulsions (Figure 2.4) (Walstra, Wouters et al. 2006). One such example is sodium caseinate, which adsorbs onto emulsion droplets to provide an entangled interfacial monolayer of flexible chains comprising sequences of predominantly hydrophobic domains located at the fat surface with hydrophilic 'loops' and 'tails' protruding into the aqueous phase (Dickinson 2001). Fat globules covered with proteins are generally more stable against coalescence than small molecular weight surfactants because proteins can form both a steric and electrostatic repulsive layer preventing fat droplets from approaching (whilst acknowledging that various mechanisms of interfacial cross-linking can equally result in droplet-droplet connectivity and aggregation). The enhanced mechanical properties of the adsorbed protein layer are also more effective at preventing film rupture and coalescence under circumstances where droplets are able to come into contact (e.g. under shear). The surface load of caseinates on homogenised milk fat droplets is found to be approximately 2-3 mg/m^2 (Bos and van Vliet 2001), which provides a very effective barrier layer against coalescence.

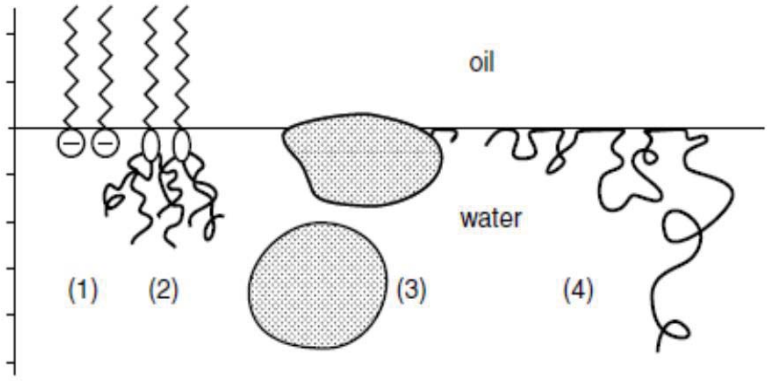


Figure 2.4: Schematic models of adsorption of emulsifiers at an oil-in-water interface. The emulsifiers from left to right are (1) soap, (2) Tween, (3) small globule proteins and (4) β-casein (Walstra, Wouters et al. 2006).

Mixed surfactant systems can provide an additional layer of interfacial complexity when incorporated into emulsion formulations. For example, there may be competitive adsorption of interfacial component at the fat surface when both proteins and lipid derived surfactants are present, which can markedly influence the stability of emulsions comprising mixtures of emulsifiers, particularly under shear. Factors influencing interfacial composition and thus stability include the surfactant/protein type, molar ratio and interactions between the two moieties, which can in turn influence interfacial mechanical behaviors, such as surface shear viscosity (Bos and van Vliet 2001, Fuller, Considine et al. 2015). This is exemplified by the addition of Tween 20 to a sodium caseinate stabilized emulsion. Displacement of the adsorbed protein layer by the Tween was found to reduce the shear stability of the emulsion, and that the extent of destabilization scaled with the concentration of added Tween (Fuller, Considine et al. 2015). With increasing surfactant concentrations, more efficient packing in the saturated monolayer leads to a lower interfacial tension for surfactants than for proteins, and then protein is competitively displaced from the interface. On the other hand, when protein concentration is high, the proteins typically predominate at the interface (Dickinson 2001). Under such conditions, low concentrations of surfactant may serve to disrupt protein-protein interactions by limited displacement of proteins from the interface and/or by the formation of a complex of protein-surfactant (Bos and van Vliet 2001). Confocal microscopy has been used to observe the competitive adsorption between

labelled proteins and various small molecular surfactants on the interface of oil in water emulsions, and the reduction in brightness indicated the displacement of proteins by the unlabeled surfactants (Heertje, Aalst et al. 1996).

As discussed, the type and concentrations of material(s) coating the surface of an emulsion droplet or a milk fat droplet invariably impacts the stability of the emulsion to mechanisms of coalescence, partial coalescence aggregation (flocculation) and ultimately phase separation. As said, for most natural cheeses, the fat phase primarily comprises milk fat droplets stabilized by milk fat globule membrane. However, modification of the milk fat emulsion interfacial layer with different surface active materials will likewise be expected to impact on the interaction of the cream emulsion with the surrounding protein network. This in turn will influence the emulsion structure during manufacture with subsequent impact on material and functional properties such as melt during baking, as discussed in section 2.3.

2.2.1.2 Fat globule size

The milk fat globule size distribution in raw milk has been found to typically range from 0.2 to 15 μm, with a maximum volume fraction around 4 μm (Mulder and Walstra 1974). Compared to fat globules, the size of milk proteins is much smaller, where whey protein radius is approximate 2 nm and casein micelle radius is 20 nm to 150 nm (Walstra, Wouters et al. 2006). In terms of imaging the structural components of milk and dairy products, figure 2.5 provides a summary of the microscopic techniques available to visualize the range of lengthscales present in such materials. The size of fat globules is in the observable range and resolution of several microscope types, including light microscope, confocal laser scanning microscope (CLSM), scanning electron microscope (SEM) and transmission electron microscope (TEM) (Everett and Auty 2008). Colloidal milk protein structures (i.e. the casein micelle and submicelle) are only suitable for imaging by SEM and/or TEM. Serum proteins (i.e. whey) and other components such as small molecular weight surfactants are too small for the microscopic techniques discussed in figure 2.5.

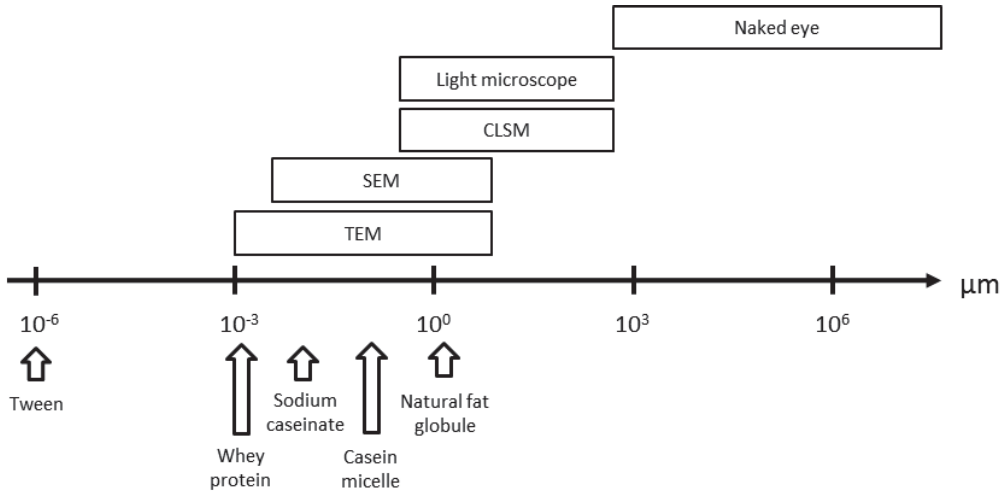


Figure 2.5: resolution of different types of microscopy and the size of milk constituents (fat and proteins) and emulsifiers. Figure is modified from the review of Everett and Auty (2008).

Homogenization is widely used to manipulate the size of fat globules, mainly from the perspective of improving emulsion stability against creaming by reducing droplet size. In the case of the native milk fat emulsion, homogenisation also influences the interfacial composition of droplets, causing the adsorption of proteins and/or surfactants at the newly formed interface. As indicated in previous sections, modification of the interfacial layer can greatly affect cheese properties, and as such, retention of the native MFGM is seen as the preferred state of the emulsion for most cheese-making processes. The polydispersity of the milk fat emulsion does, however, raise some interesting questions as to how relative droplet size influences cheese properties, particularly under circumstances where the native MFGM can be maintained. In this respect, a number of techniques are summarised here (Table 2.2) to separate fat globules on size without damaging native milk fat membrane (Bibette 1991, Goudedranche, Fauquant et al. 2000, Ma and Barbano 2000, Michalski, Ollivon et al. 2004, O'Mahony, Auty et al. 2005). Of the techniques listed, microfiltration appears most effective at fractionating the emulsion into different droplet size distributions.

Cheeses prepared from different sized fractions of fat globules that had previously been separated via microfiltration showed some interesting variations in properties (Table 2.3) (Goudedranche, Fauquant et al. 2000, Michalski, Gassi et al. 2003, Michalski, Camier et al. 2007). Moisture content was seen to be higher in Emmental cheese and Camembert

cheese produced using small milk fat globules compared to equivalent cheeses prepared from large fat globules (Michalski, Gassi et al. 2003, Michalski, Camier et al. 2007). Water could stay around the fat globules within native milk fat membrane, and small fat globules with larger interface membrane area seemed to trap more water comparing to large fat globules (Michalski, Cariou et al. 2002). Additionally, confocal microscopy showed a decrease in the size of irregular shaped fat domains in both Emmental cheese and Camembert cheese produced from native small fat globules (Michalski, Gassi et al. 2003, Michalski, Camier et al. 2007) (Figure 2.6). O'Mahony, Auty et al. (2005) also concluded the fat globule size distribution in Cheddar cheese could be manipulated by modifying the size of native milk fat globule in cheese milk, with larger non-globular fat domains observed on when cheese milk was enriched with large native milk fat globules.

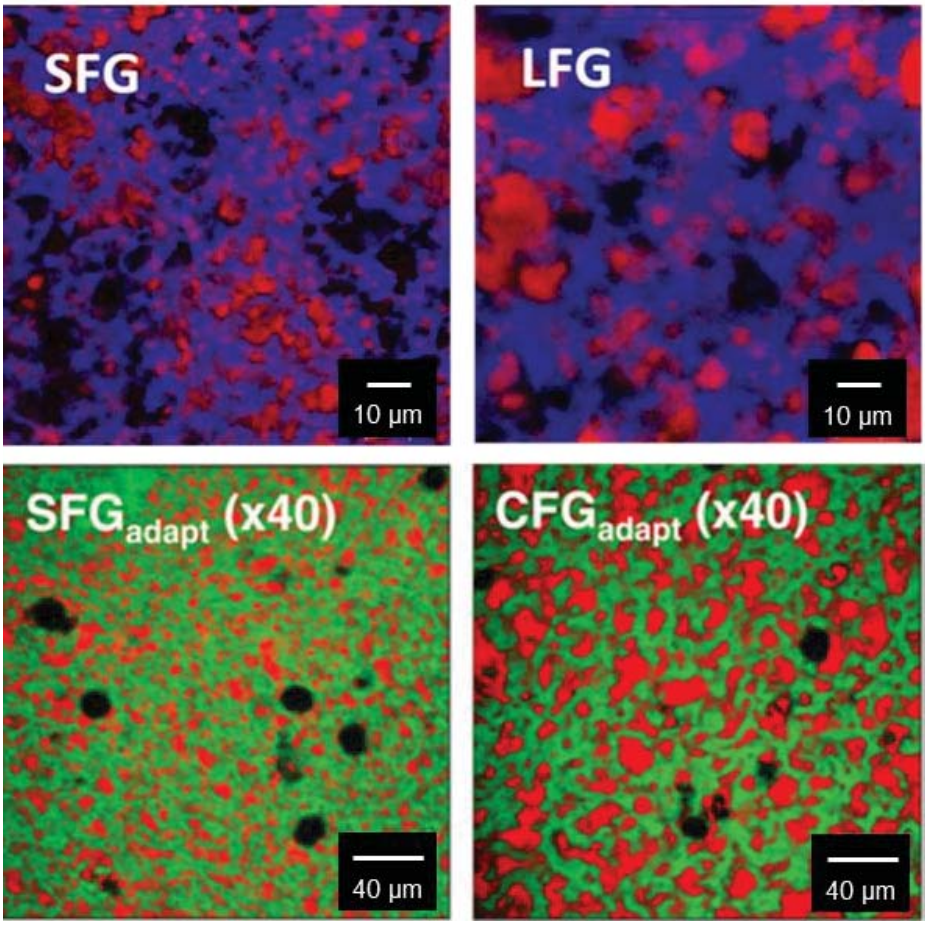


Figure 2.6: Confocal micrographs of Camembert cheese (up two photos) (Michalski, Gassi et al. 2003) and Emmental cheese (bottom two photos) (Michalski, Camier et al. 2007), which were manufactured using native small fat globule milk (SFG) and native large fat globule milk (LFG) and control raw milk (CFG). Fat is in red, and proteins is in blue (up photos) or green (bottom photos).

Table 2.2: References on different sizes of native fat globules separated from a raw whole bovine milk.

Methods to separate native fat globules by size	Fat globule size D[4,3] (μm)		Used in cheese making
	Small	Large	
Gravity separation (Ma and Barbano 2000)	< 3.2	3.64	N/A
2-stage gravity separation	3.45	4.68	Cheddar (O'Mahony, Auty et al. 2005)
Patented microfiltration (Goudédranche, Maubois et al. 1998)	1-3	6-7	Camembert (Michalski, Gassi et al. 2003) Emmental (Michalski, Camier et al. 2007)
	< 2	> 2	Camembert, mini Swiss (Goudedranche, Fauquant et al. 2000)
Depletion interactions (Bibette 1991)	0.1	2	N/A

Table 2.3: Physical, chemical and sensory properties and functionality of cheese manufactured using native small fat globule milk (SFG) or large fat globule milk (LFG).

	Emmental cheese (Michalski, Camier et al. 2007)	Camembert cheese (Michalski, Gassi et al. 2003)
Cheese texture	SFG: less firmness, greater stretching and elasticity	SFG: less firmness and rigid (greater proteolysis), higher elastic texture, smaller eyes
Cheese melting	Similar melting	SFG: higher melting
Cheese sensory	SFG: improved sensory	Different sensory properties
Cheese colour	SFG: more yellow	SFG: less yellow

2.2.1.3 Fat melting points

The material state of milk fat transitions from fully solid to fully molten across a temperature range of -40 °C to 40 °C. This broad melting point distribution is a consequence of the varied triacylglycerol species that comprise milk fat (Mulder and Walstra 1974). Fat melting point is understood to influence that fat globule size distribution of cheese both during cold storage and any subsequent heating/cooking of the cheese. Localisation of fat droplets within the curd structure during cheese manufacture typically occurs at temperatures such that the droplets are partially crystalline. This localization produces concentrated emulsion domains with high fat phase volume fractions. As discussed in section 2.2.1.1, partial coalescence can occur for

droplets comprising crystalline fat, and is more prone to occur when droplets are in a close packed state, as is the case in the fat domains within cheese (Pawar, Caggioni et al. 2012). Warming of cheese samples such that the fat phase becomes fully molten can cause partially coalesced structures to fully coalescence resulting in release of free oil during melting.

The relative solid fat content of milk fat has also been shown to impact on cheese properties. For example, cheese melting was investigated for cheeses produced from a number milk fat fractions of varied melting points (Rowney, Hickey et al. 2003, Schenkel, Samudrala et al. 2013). Schenkel et al. (2013) reported that oil-off in semi-hard cheese was significantly increased in cheese containing high melting point milk fat (it was also observed that the influence of fat melting properties on free oil was reduced in cheese made from milk of small fat globules, in which smaller fat domains were observed in figure 2.7. Curiously, research results from Rowney et al. (2003) showed Mozzarella cheese with low melting point milk fat yielded a larger amount of oil-off even though cheese microstructure did not appear to be significantly affected by the use of variable fat melting fractions. It seems that cheese oil-off is less affected by the fat melting point than the fat droplet localization in protein matrix.

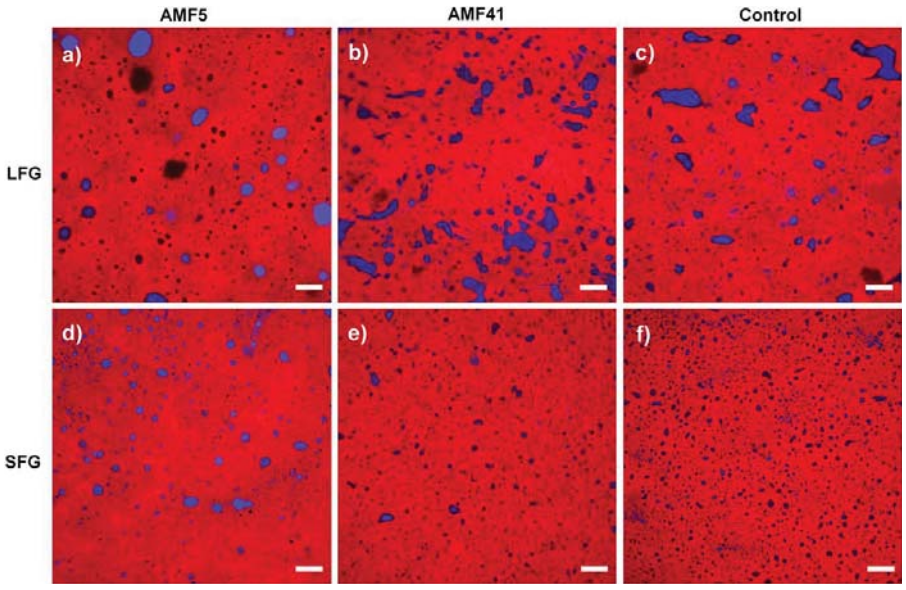


Figure 2.7: Confocal micrographs of semi-hard type cheese made of milk with different milk fat fractions (AMF5, AMF41 and butter oil as control) and large (LFG, $d_{50,3}$ ~ 2.75 µm) or small (SFG, $d_{50,3}$ ~ 1 µm) fat globules after 4 weeks of ripening at 4 °C. Protein is labeled in red, the fat is in blue and black areas reveal gas holes or the aqueous phase. Scale bar represents 20 µm (Schenkel, Samudrala et al. 2013).

2.2.2 Effect of cheese manufacture

Processing conditions, such as thermalisation (both pasteurization and sterilization) can serve to modify the native milk fat membrane interfacial layer. Heating of milk can result in complexation of surface layer components of the MFGM with milk proteins in the serum phase (Sharma and Dalgleish 1994, Kim and Jimenez-Flores 1995, Ye, Singh et al. 2004). For example, whey proteins are known to bind to the fat globule surface at temperatures above 70 ˚C (Dalgleish and Banks 1991, Sharma and Dalgleish 1993); interactions between whey proteins and milk fat membrane increased with heat time and temperature (Kim and Jimenez-Flores 1995, Corredig and Dalgleish 1996). In contrast, Dalgleish and Banks (1991) reported the amount of casein associated with milk fat membrane was independent of heat treatment, showing a decrease of β-casein and α-casein on fat membrane during heating. Dalgleish and Banks suggested denatured whey proteins might displace caseins loading on fat globule membrane. Cheese would present different functionalities when fat surface is covered with proteins, either caseins or whey proteins (see section 2.2.1.1), noting that heat treatment would also be expected to impact on casein micelle structure altering the susceptibility towards renneting and subsequent curd formation.

Other processing operations, such as homogenization, can potentially be used in cheese manufacture and may be consequential for cheese structural and functional properties. It is known that even low pressure homogenization can damage the native MFGM (Michalski, Michel et al. 2002), and as indicated earlier proteins and/or emulsifiers can adsorb onto the fat interface after homogenization when the fat membrane materials are no longer sufficient to provide saturation coverage of the surface area. Increasing homogenization pressure is known to increase milk protein concentration adhering to fat interface, but this appears to have limited effect on the stoichiometry of the milk protein species adsorbed at the interface (Cano-Ruiz and Richter 1997, Cano-Ruiz 2012). It has been demonstrated that the fat surface of homogenized cream consists of caseins and whey proteins, although whey proteins are more likely to be removed by post-homogenization washing than casein (Darling and Butcher 1978). Whey proteins denatured during heat treatment have been shown to bind to the fat interface when

homogenized (Sharma and Dalgleish 1993, Sharma and Dalgleish 1994, Lee and Sherbon 2002). Heat treatment of 65 °C up to 30 minutes do not significantly denature whey proteins, and thus after homogenization caseins in milk adsorbed preferentially over whey proteins at the fat globule surface; however, more intense heat treatment at 85 °C for 20 minutes has been shown to lead to the formation of a protein complex of whey proteins crosslinking to the casein micelle, which could then adsorb at the fat interface (Cano-Ruiz 2012).

Homogenization, particularly at high pressures, can have an effect on both the protein and fat components in cheese making. This effect has been studied through processing of cheese milk according to the following compositions: (1) homogenized full fat milk; (2) homogenized cream + non-homogenized skim milk (Metzger and Mistry 1995, Rudan, Barbano et al. 1998); (3) homogenized skim milk + non-homogenized cream (Tunick 1994, Tunick, Malin et al. 1995); (4) homogenized full fat milk + non-homogenized milk (1:1) (Rowney, Hickey et al. 2003). Cheeses prepared using homogenized milk led to curd shattering in the manufacture of fat-reduced Mozzarella cheese, resulting in poor functionalities and reduced yields (Gilles and Lawrence 1981, Tunick, Malin et al. 1993, Rudan, Barbano et al. 1998), however curd shattering was not observed when using homogenized cream (Rudan, Barbano et al. 1998). It was thought that modified curd properties were caused by restructuring of casein micelle rather than by any changes to the fat globules (Tosh and Dalgleish 1998). Oil-off in cheese baking was also observed to be greatly reduced when solely using homogenized milk. However, homogenized skim milk mixed with non-homogenized cream had no effect on oil-off of either full-fat Mozzarella cheese or low-fat Mozzarella cheese (Tunick 1994, Tunick, Malin et al. 1995). In pizza baking, the cooked cheese within homogenized fat globules showed severe browning and scorching of intact shreds with slight melting and fusion (Figure 2.8) (Rudan, Barbano et al. 1998). The implication here is that the limited oil off accelerates dehydration and causes scorched during pizza baking (Rudan and Barbano 1998). Similar oil-off behaviour was observed in fat-reduced Cheddar cheese made from homogenized cream, which exhibited a microstructure of small fat globules evenly dispersed in a protein matrix and for which the cheese was seen to display low oil-off in baking (Metzger and Mistry 1995). These findings indicated that cheese oil-off was closely

linked to the homogenisation of fat globules, with milk proteins adsorbing at the homogenised droplet interface and actively binding to the surrounding protein matrix.

Kosikowski and Silverman (1954) and (Kosikowski 1957) manufactured processed Mozzarella cheese using Mozzarella cheese curd, which resembled Mozzarella cheese physical properties and flavours but did not 'string out' well. A small scale (25-30 g) of processed cheese is successfully produced in a Rapid Visco Analyzer (RVA), which was easy and rapid for investigation on formulations and processing conditions (Kapoor and Metzger 2005). However, RVA worked in a different mechanical treatment comparing to pilot-scale equipment, and therefore manufacture profiles required a few adjustments when the processed cheese was manufactured on a pilot-scale (Kapoor, Lehtola et al. 2004, Kapoor and Metzger 2005).

Figure 2.8: Appearance of a pizza topped with reduced fat Mozzarella cheese (8-9 wt.% fat) made from non-homogenized milk (upper left), by homogenized milk treatment (bottom centre), or by homogenized cream treatment (upper right) after 30d of storage at 4 °C (Rudan, Barbano et al. 1998).

2.3 Colloidal interactions in an alternate make cheese (AMC)

2.3.1 From emulsion gels to cheese system

Gelled emulsions and protein emulsion gels have been widely used as theoretical models to investigate the structure of protein-based semi-solid and solid foods (Cho, Lucey et al. 1999, Yang, Rogers et al. 2011, Dickinson 2012, Liu, Stieger et al. 2015). According to the interactions between emulsion droplets and the surrounding protein gel network, the droplets can broadly be classified as 'active' or 'inactive' (van Vliet 1988, Dickinson and Chen 1999). Active fillers are assumed to have strong interactions with gel matrix, for example protein-coated particles forming bonds to protein gels (Langley and Green 1989, Xiong and Kinsella 1991, Dickinson and Chen 1999, Liu, Stieger et al. 2015). In contrast, inactive fillers interact weakly or not at all with the surrounding gel network, such as for the non-ionic water soluble surfactant, Tween 20, which was used to stabilise emulsion droplets which were then demonstrated as acting as inactive lipid fillers in protein gels (Chen and Dickinson 1999, Dickinson and Chen 1999, Liu, Stieger et al. 2015).

The properties of such gel systems can be influenced according to whether the droplets are active or inactive. For example, Liu, Stieger et al. (2015) observed unbound (inactive) fat droplets led to more coalescence in their emulsion-filled gels compared to bound (active) fat droplets, and decrease of solid fat content (4-48 % in total fat) caused less coalescence of the unbound (inactive) fat droplets. Liu, Stieger et al. (2015) concluded that the negligible coalescence at low solid fat content is due to low sensitivity to rupture of the droplet interface upon deformation because of the low presence of fat crystals inside the droplets.

As indicated throughout this review, cheese can be considered as a particle-filled structure composed of continuous protein gel matrix and inter-dispersed fat globules (Rogers, McMahon et al. 2010, Yang, Rogers et al. 2011). Inert (inactive) fillers or structure breakers have been used to describe behaviour of fat droplets in cheese when native milk fat globule membrane is present on the surface of fat globules (Michalski, Cariou et al. 2002, Michalski, Gassi et al. 2003). This hypothesis of inactive fat fillers was evidenced by cryo-SEM images of fat globules surrounded by serum cavities in fresh Cheddar (Hassan and Awad 2005, Everett and Auty 2008). Fat globules covered with

proteins, for example as a consequence of homogenization, are likely to interact with the cheese protein matrix and this can serve to reinforce the cheese structure (Guinee, Gorry et al. 1997, Rowney, Hickey et al. 2003). Interestingly, fat globules with limited interfacial coverage of proteins were considered as inactive fillers in the investigation of Michalski, Cariou et al. (2002) due to no significant increase in shear modulus. In this project study, the concept of active fillers and inactive fillers are used to investigate Mozzarella-type pizza cheese made using an alternate process to the traditional method of manufacture. Here, the detailed descriptions of active fillers and inactive fillers are concluded as follows:

Active fillers - filler particles form an association with the continuous phase (e.g. cheese protein matrix). The mechanical/material properties of the matrix are typically strengthened, which can be influenced further by the specific material properties of the dispersed phase particles.

Inactive fillers - filler particles lack association with the continuous phase. Shear modulus will be expected to decrease as fillers behave as structure breakers, suggesting a weakening effect on gel structure. However, this scenario can be complicated by considering different permutations for the structure and distribution of the fat phase within the gel network, as well as the specific material properties of the dispersed phase.

The interfacial components adsorbed to the fat surface of emulsion droplets are a key factor in determining the filler behaviours and their contribution to the material properties of the gel system. Figure 2.9 shows the schematic models of active fillers and inactive fillers. Active filler particle interactions with the continuous phase can include various bonding mechanisms such as covalent or electrostatic bonds, or hydrophobic interactions.

Bond formation can enable applied shear forces to be transferred from continuous phase to active fillers, resulting in filler deformation (Figure 2.9A). However, in contrast inactive fillers deform only slightly under a small strain shear (Figure 2.9B) because of 'lubricant' effect of the aqueous layer (Suchkov, Popello et al. 1997). Yang et al. (2011) used particle-filled gel models (van Vliet 1988, Luyten and Van Vliet 1990) to explain Cheddar cheese rheological properties: during small deformation the structure within

active fillers both the filler interface and matrix deform during small deformation, and overall stiffness would increase within firm fillers; the structure within inactive fillers is similar to the one filled with aqueous holes, because the mechanical forces exerted on the gel can only be passed onto the intermediate aqueous layers around the fillers, and only the intermediate aqueous layers are deformed.

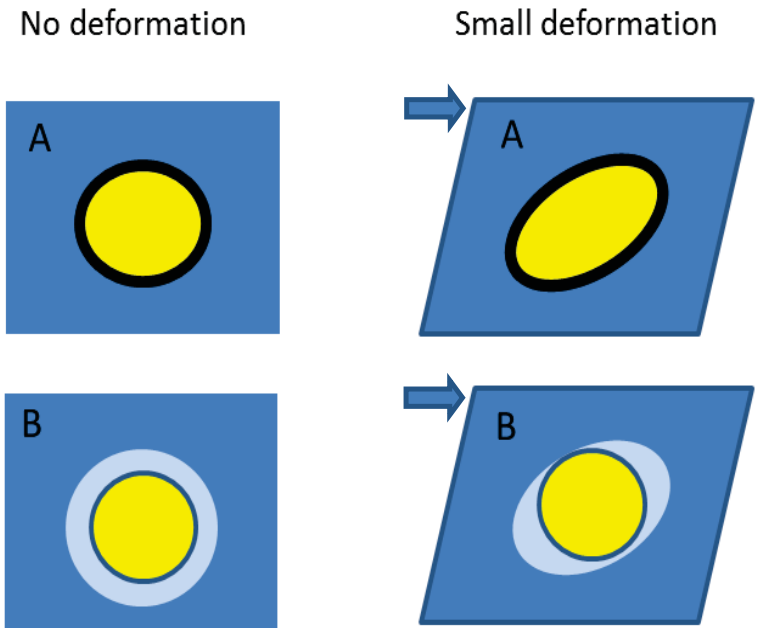


Figure 2.9: Schematic models of cheese filled with (A) active fat fillers, (B) inactive fat fillers under small deformation. Yellow droplets are the filler particles; dark blue background is the continuous phase, and light blue around filler particle is the serum. Figures are modified from models of Chen and Dickinson (1999). The arrows show the small strain force.

Such structural effects are best studied by rheological analysis, which is a widely applied tool for relating colloidal interactions to material behaviours (Dickinson 1998). For cheese systems material properties are typically investigated through the determination of shear moduli (G' and G'') under conditions of low oscillatory strain. For filled gel systems, active fillers connected to the surrounding matrix result in increased elastic modulus (G'); while inactive fillers locating in aqueous pockets of protein network would decrease sample elastic modulus (G') (van Vliet 1988, Dickinson and Chen 1999, Dickinson 2012). In the case of increased elastic modulus of (protein) gel matrix,

Dickinson and Chen (1999) observed the less effect of active fillers, but the greater effect of inactive fillers (and vice versa).

2.3.2 Fat structuring in AMC

As discussed, fat globule size and interfacial ingredients are contributing factors influencing cheese functionality and material properties, and these two factors were manipulated in AMC when preparing cream emulsions for cheese making. It has already been demonstrated that the emulsifiers sodium caseinate and Tween 20 can be used to stabilize emulsions droplets to act as either active fillers and inactive fillers within a protein matrix (Dickinson and Chen 1999, Dickinson 2012). For emulsions in which the continuous phase is fluid, steric and electrostatic repulsion from proteins loaded at the fat surface prevents droplet approach and aggregation (Figure 2.10, A1). However, where the continuous phase is solid (as for a protein gel) fat globules covered with proteins are physically trapped in the protein matrix (Figure 2.10, A2). This can also occur where droplets are stabilized by small molecular weight surfactants, for instance Tween 20, covered. Emulsion droplets globules are generally less stable against coalescence than protein covered fat globules (Figure 2.10, B1), and the inactive fat globules in cheese is more likely to coalescence in the small space of the serum pockets (Figure 2.10, B2).

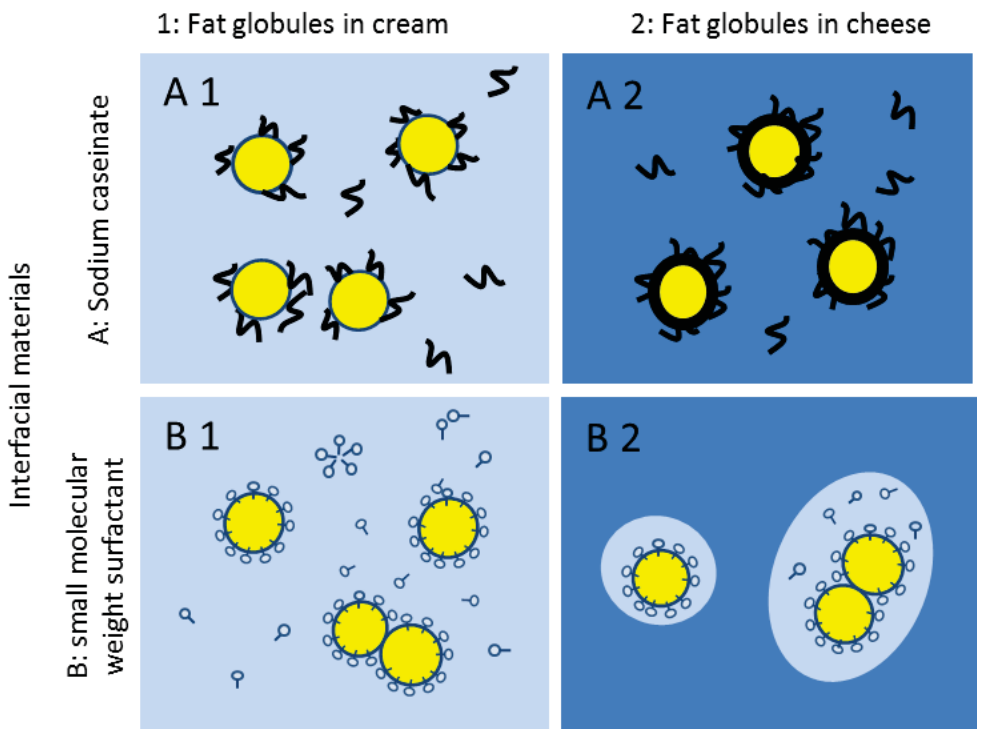


Figure 2.10: Schematic models of fat globules covered with NaCas disperse in cream emulsion (A1) and in AMC (A2), and fat globules covered with small molecular weight surfactants (e.g. Tween 20) disperse in cream emulsion (B1) and in AMC (B2). Fat globules can aggregate and/or coalescence in B1 and B2.

Determining the interfacial composition of fat droplets can be challenging in traditional cheese manufacture, because cheese milk undergoes series of time dependent physical changes in traditional cheese production (Figure 2.1). In this respect, AMC provides a more amenable model for the study of interfacial ingredients affecting cheese properties, where the interface materials can be controlled as part of the process. That said, given the nature of the AMC process, dynamic changes to the structure may occur that would not be expected to take place for conventional cheese making; for example, emulsification of fat globules may occur under large shear force from RVA, resulting in modified fat globule size and interfacial composition.

In this context, an understanding of the forces at play during processing can provide useful insights as to dynamic changes that may be taking place to emulsion droplets under shear. For example, the calculation of capillary number (2.5) can be informative in determining whether homogenization is likely to take place during mixing. The equation below indicates the factors that determine capillary number: i.e., the initial size of the

droplets, the viscosity of the continuous phase, the shear rate of blending and the interfacial tension.

η = viscosity of the surrounding continuous phase (2.1)

Ý = shear rate defined by the propeller tip speed and the gap between the tip and the wall (2.2)

Γ = interfacial tension (2.3)

R = fat globule radius (2.4)

Ca = Capillary number $= (\eta\dot{\gamma})/(\Gamma/R) = \frac{\text{stress transmitted through the matrix}}{\text{restoring stress from interfacial tention}}$ (2.5)

For active fat droplets trapped in a cheese protein matrix of high viscosity, droplet breakup is more likely to occur in the RVA during mixing than for inactive fat droplets that are localized in aqueous serum pockets. Here, the capillary number is estimated on fat globules of 4 µm in either the highly viscous protein matrix or low viscosity serum pocket phase (Appendix I). For AMC produced at 1000 rpm 60 °C, laminar flow is expected in RVA mixing because the Reynold number is below 1000 by calculation (theoretically, in laminar Re < 2300). The capillary number of fat globules in protein can be calculated as ~ 0.2, whereas the value for fat globules in serum is ~ 4.5 x 10^{-5}. Figure 2.11 indicates capillary numbers for droplet break-up (Jackson and Tucker III 2003). The viscosity ratio of the milk fat globules in AMC is ~ 2.5 x 10^{-4} at 60 °C, and thus ~4 µm fat globules should be in the stable area shown by the star points in figure 2.11. Theoretically, if active fat globules are above 10 µm, the capillary number is over 0.6 and these large droplets is probable to break-up by planar elongational flow (Figure 2.11 left) but not the shear flow (Figure 2.11 right).

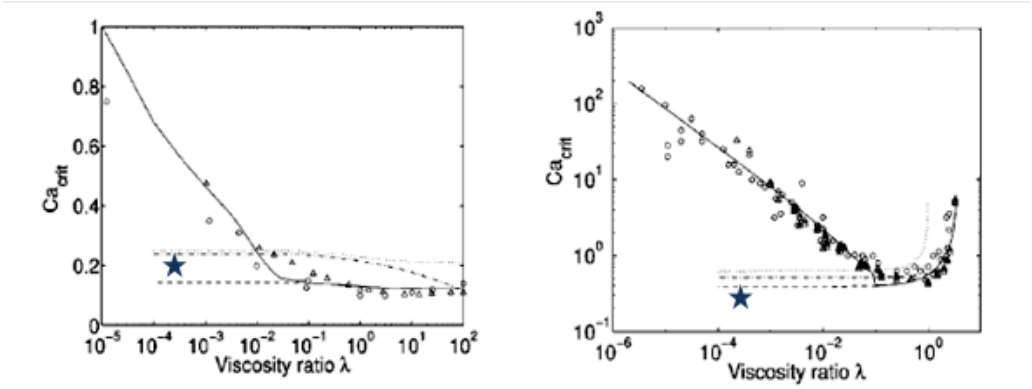


Figure 2.11: Capillary numbers for droplet break-up in planar elongation flow (left) and simple shear flow (right) (Jackson and Tucker III 2003). The star points show the 4 µm active fat globules in protein matrix of AMC blended at 1000 rpm 60 °C.

2.3.3 Analysis of AMC functionalities

2.3.3.1 Fat globule dispersion in cheese

As discussed in section 2.1.2, the dispersion state of fat globules during cheese manufacture is dynamic. The Malvern Mastersizer is a widely used tool for determining particle size distributions. However, the Mastersizer cannot directly determine whether particle represents single droplets or aggregated structures. Accordingly, microscopy is commonly used in combination with Mastersizer analysis to provide a qualitative means of determining the microstructure of cheese and the physical state of the fat. In this context, confocal laser scanning microscopy (CLSM) has been increasingly used microscopy to study fat distribution in cheese (Auty, Twomey et al. 2001). The resolution on the CLSM images is at ~ 0.2 µm (Everett and Auty 2008). Where higher resolutions are required, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) show the micro structure of cheese protein network in greater detail and with better ability to determine the aggregation state of fat droplets within the protein network (Hassan and Awad 2005, Kuo and Gunasekaran 2009). The native milk fat globules are in average size of 4 µm, and for the observation of milk fat globules dispersion in cheese proteins matrix, the resolution of CLSM is appropriate (Figure 2.5).

2.3.3.2 Cheese melting properties

Dynamic changes to cheese microstructure are most apparent when cheese is heated. These changes are also accompanied by a number of related physical and visual changes that are considered important parameters of cheese quality during cooking. These include softening, melting, oil release and surface browning.

Fat is the only ingredient in cheese that can truly melt, and milk fat completely melts above 40 °C (Mulder and Walstra 1974). The melting of Mozzarella protein matrix is essentially a relaxation of the protein network, and depends on changes in the strength of molecular interactions within the protein network. These interactions can in turn dramatically affected by manufacturing associated with the cheese, such as salt content and protein hydrolysis (Dave, McMahon et al. 2001).

From a physical point of view, cheese melting is a transform from a 'solid- like' to 'liquid-like' state. Cheese melting has been described as "the ease and extent to which cheese will melt and spread upon heating" (Gunasekaran and Ak 2003). 'Ease of melting' is influenced by a combination of heat transfer and the response of structural elements within the cheese to the application of heat (noting that not all cheeses demonstrate melting behavior on hearting); the 'extent of flow' can be defined by the rheological properties of cheese at high temperature (Gunasekaran and Ak 2003).

Varied methods have been used to investigate Mozzarella cheese melting. These include the UW-meltmeter (Wang, Muthukumarappan et al. 1998), squeeze-flow technique (Wang, Muthukumarappan et al. 1998) and helical viscometry (Kindstedt and Kiely 1992). The most widely used technique in industry is the Schreiber test (Gunasekaran and Ak 2003). The Schreiber test (a visual analysis technique) is fast and easy to conduct, as well as being low cost. A number of methodologies have been developed for the test allowing improvements in the study design to be made(Muthukumarappan, Wang et al. 1999, Altan, Turhan et al. 2005).

Small amplitude oscillatory shear characterisation is another analysis for measurement of cheese thermo-rheological properties (Guggisberg, BÜTikofer et al. 2007, Schenkel, Samudrala et al. 2013, Sharma, Munro et al. 2016). Cheese melting represents a material change in state from elastic solid-like to viscous liquid-like. Storage modulus (G') and loss

modulus (G") represent the energy storage and energy loss arising from shear deformation. The material transition in cheese melting could be indicated by the temperature where G' is equal to G" (Guggisberg, BÜTikofer et al. 2007, Schenkel, Samudrala et al. 2013). This method supplies detailed information of cheese material properties as temperature increases (Sharma, Dessev et al. 2015, Sharma, Munro et al. 2016), and has already been applied to the study of active/inactive filler properties in model protein (Dickinson and Chen 1999). Accordingly, small deformation thermos-rheology provides a useful tool in the characterization of the role of droplet interactions and fat structure within the AMC system under study.

Chapter 3: Materials and methods

3.1 Materials

3.1.1 Cheese materials

Alternate make cheeses were prepared using three fat sources. (1) Anhydrous milk fat (AMF) was obtained from Fonterra Clandeboye factory in New Zealand. A single source of AMF was used throughout this thesis, and thereby AMF was stored at -18 °C to prevent lipid oxidation, which was moved into fridge of 4 °C before using. (2) Canola oil, which was purchased from Marsanta Foods (Auckland, NZ). (3) Natural, non-homogenised cream (NC) obtained from Kapiti Fine foods (Fonterra, New Zealand), and which had been gently centrifuged to achieve ~80 wt.% fat in the pilot plant of Fonterra Research and Development Centre. The high fat cream was kept at 40 °C to avoid fat partial coalescence.

Protein powers, sodium caseinate (NaCas, which was also used as an emulsifier) and calcium caseinate 380 (CaCas), were supplied by Fonterra (92.6 wt.% proteins). Alternate make cheese (AMC) produced in subsequent chapters was produced from cheese curd, which was obtained from the pilot plant of Fonterra Research and Development Centre. The cheese curd (pH = 5.7) comprises 42 wt.% protein, 56 wt.% moisture, < 0.5 wt.% fat and 160 mmol Ca^{2+}. Cheese curd was stored at -18 °C and was thawed at 4 °C overnight prior to use.

Tween 20, sodium caseinate (supplied as above), and whey protein isolate 895 (WPI) obtained from Fonterra (69.2 wt.% β-lactoglobulin and 14.2 wt.% α-lactalbumin) were all used as emulsifiers.

All other chemicals were of Analar grade.

3.1.2 Materials for analytical methods

3.1.2.1 Walstra (dissociating) solution

Walstra solution is able to solubilise the protein component of cheese matrix allowing the release of entrapped fat globules which can then be further evaluated for size and structural attributes (Walstra 1965, Yu and Gunasekaran 2005). One Liter of Walstra solution includes 3.75 g disodium ethylenedianmine tera-acetate (i.e. Disodium-EDTA, VWR International Ltd., Poole, England) and 1.25 g Tween 20, in which pH was adjusted to 10.0 by adding 0.1 M NaOH. Walstra solution was stored in a fridge of 4 °C and would be warmed up to ambient temperature (~ 20 °C) when used.

Some cheese samples in Chapter 6 were dissociated in the EDTA solution (pH 10.0) without Tween. These dissociated cheese samples were prepared for confocal analysis.

3.1.2.2 Fluorescent staining of samples for CLSM

Confocal stains Fast Green (FG) and Nile Red (NR) were purchased from Sigma Aldrich, USA. They were dissociated in solvent (Citifluor or PEG) for 48 hours in darkness (covered with foil paper) and were stored in a fridge at 4 °C. The stains were used for up to 3 months after preparation. The stain for cheese samples is a mix of 0.4 wt.% FG and 0.5 wt.% NR in citifluor, and the stain for cream samples and dissociated cheese samples is a mix of 0.2 wt.% FG in PEG and RhPe, which should be prepared freshly. The powder of RhPe (i.e. the 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)) was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). RhPe was dissolved in chloroform (1 mg/ml) and was stored in a freezer below 0 °C. The mixing ratio of RhPe to FG is 1:3 (vol.), but the RhPe solution need to be pre-dried in a fume hood before adding the FG (0.2 wt.% in PEG), and then mixed by centrifuge and vortexed for several times. FG and NR were used to stain the dairy proteins and milk fat, respectively; and RhPe was to stain the phospholipids at milk fat globule membrane.

3.1.2.3 Buffers for protein composition analysis

The protein composition in serum was analysed using microfluidic chip SDS-PAGE (Agilent 2100 Bioalalyzer). The kits include protein chips, the gel matrix solution, the protein dye concentrate solution, the marker protein/sample buffer solution and the protein molecular mass ladder solution. However, the marker protein/sample buffer solution was modified here, mixing 1.0 ml of 0.3 mg/ml lactoferrin and 150 ml stock buffer to gain the SDS-sample buffer (working buffer). The stock buffer was prepared by adding 62.5 ml 0.5 M Tris-HCl buffer and 50 ml 10 % (w/v) SDS into 250 ml mili-Q water. The detain solution was prepared according to the protocols applied with the chips.

3.2. Equipment

Table 3.1: information of the equipments

Equipment	Detail	Comments
Ultra turrax	IKA T25 digital (Germany)	Used to prepare "pre-emulsion" before homogenisation
Two stage homogeniser	Niro-Soavi – Parma, (Italy)	Emulsions were homogenised at 80 bar – 1st stage and 20 bar 2nd stage.
Sonicator	Qsonica (USA)	
Rapid Visco Analyzer (RVA)	RVA-4, Newport Scientific (Australia)	
Malvern Mastersizer Light Scattering	Mastersizer model, flow through cell, Malvern (UK)	Diffraction technique, used for determining size distributions. Refractive index used for milk fat was 1.46 Refractive index for water was 1.33
CLSM	Zeiss LSM510 Meta, Carl Zeiss Microscopy (Germany)	
Cryo-microtome	LEICA CM 3050s	
Rheometer	Anton Paar, Physica MCR 301 Rheometer (Australia)	PP20/s plate, Peltier temperature hood of H-PTD200 (Australia)
Oven/incubator		
Agilent 2100 Bioanalyzer	Agilent Technologies,Inc., Waldbronn, Germany	

3.3. Sample preparation

3.3.1 Cream emulsion preparation

The fat component of AMC was provided in the form of model cream emulsions with varying interfacial composition. These emulsions would initially be prepared through homogenisation before blending in the RVA. Where natural cream was used in the preparation of AMC, the cream did not undergo any emulsification prior to blending with protein in the RVA. The natural cream was kepted warm in a water bath of 40°C to avoid fat partical coalescence, and would be used within 10 hours after preparation.

3.3.1.1 Emulsified cream emulsions

Varied emulsifying techniques, such as Ultra turrax, Silverson mixer and 2-stage homogenizers, were tested to produce model cream emulsions with droplet size distributions comparable to non-homogenised natural cream. The fat globules in raw milk fat are in range of 0.2 μm to 15 μm diameters with a mean particle size of ~4 μm (Mulder and Walstra 1974). Model emulsions made from the Ultra turrax and Silverson mixer obtained fat globules are much larger than 4 μm, which were unstable in the storage at 4 °C. Cream emulsions including average diameters of 1 μm fat globules were successfully produced using the 2-stage homogenizer. While the mean particle size was smaller than that of natural cream, the droplet size distribution provided a reasonable comparison.

The aqueous phase of model emulsions was prepared by dissolving the appropriate emulsifier in water (NaCas or Tween 20). Melted AMF was then mixed with the aqueous phase at temperature above 50 °C to make emulsions of 45 wt.% fat (with the exception of emulsions made using canola oil which were prepared at ambient temperatures). The emulsions were firstly prepared using Ultra turrax running at medium speed for 2-3 minutes before being passed through a 2-stage homogeniser to obtain the final emulsions. The operational homogenization pressures and passing numbers were well controlled (Table 3.2) and the resulting particle size distribution was highly reproducible. Processing conditions were adjusted according to the type of emulsifier used to try and ensure droplet size consistency across all compositions.

Table 3.2: cream emulsion ingredients and details of preparation including pressures and passing times on the two-stage homogenizer.

Emulsion ingredients	Homogenization pressure (Bar)	Passing times
AMF+ 3 wt.% or 4 wt.% NaCas	80 + 20	5
AMF+ 2 wt.% Tween 20	80 + 20	5
AMF+ 5 wt.% Tween 20	60 + 20	5
Oil + 3 wt.% NaCas	80 + 20	5
Oil + 2 wt.% Tween 20	60 + 20	6

3.3.1.2 Modified natural cream

The investigation of protein adsorption at the fat interface was studied in chapter 6. For samples used in this study in chapter 6, natural cream was modified in several ways before cheese making (Table 3.4):

(1) Preheated natural cream:

RO water was added into the high fat natural cream (~ 80 % fat) to achieve 45 % fat. They were mixed and cooked in RVA for 30 minutes at 60 °C, 15 minutes 800 rpm followed by 15 minutes 1000 rpm.

(2) Preheated natural cream within NaCas:

4 % NaCas was added into the high fat natural cream (~ 80 % fat) to achieve 45 % fat. They were mixed and cooked in RVA for 30 minutes at 60 °C, 800-1000 rpm (15 minutes 800 rpm followed by 15 minutes 1000 rpm).

(3) Natural cream loading with the serum from cheese curd:

Serum was added into the high fat natural cream (~ 80 % fat) to achieve 45 % fat. The serum was collected by squeezing the hot non-fat AMC after RVA mixing in 30 minutes at 60 °C, 15 minutes 800 rpm followed by 15 minutes 1000 rpm. The cream added within serum was sonicated in 50 % energy for 2 minutes in total, where the sonication was paused for 10 seconds in every 30 seconds running to avoid over-heating.

Another fat natural cream sample (~ 80 % fat) was sonicated within 1 % whey solution (WPI 895) to achieve cream samples of 45 % fat.

3.3.2 Cheese-making

The cheese samples were prepared at lab scale using an RVA. The process conditions, such as mixing temperature, residence time and shearing speed, could be effectively adjusted in setting up a particular RVA profile. In this respect, some of the formulated cheese samples required variation in process conditions, but the profile was kept constant whenever possible. Two types of cheese samples were produced: the model cheese and the alternate make cheese (AMC). Model cheeses in chapter 4 were produced by mixing dairy proteins (NaCas or CaCas) and emulsified cream; AMCs in chapter 5 and 6 were produced by mixing cheese curd, salt and cream. The cream could be the natural cream or the oil in water emulsions in section 3.1. The cheese-like samples after producing were shaped to cheese slice by sandwiching the hot fresh samples between two hot tile plates with smooth surface, which had been preheated in the 60 °C water-bath. Metal circles of 2.5 mm height were put between the two plates to control the thickness of cheese slices. Plastic films were placed on the tile plates to cover the cheese samples on both sides. The hot plates would be covered with dry towels and thereby cooled off slowly at ambient temperature. Temperature dropped to ~ 40 °C in 20 minutes to allow the material relaxing and then samples were stored at 4 °C. In addition, sample slices of AMF were prepared as well to analyse the material properties of milk fat (chapter 4). The AMF was firstly melted in a water bath of 60 °C, and then was poured slowly on a precooled (4 °C) tile plate, where the AMF was shaped in a metal circle of 2.5 mm height. Plastic films were placed previously on the tile plate before leaving the metal circles. The AMF sliced sample was cooling down in a fridge (4 °C) before material properties analysis.

3.3.2.1 Model cheese

Solutions of 3 wt.% NaCas and 2 wt.% or 5 wt.% Tween 20 were used, respectively, to prepare the cream emulsions for model cheese making, aiming to fully cover fat globules in cream emulsions. The hot sample of CaCas matrix model cheese made from 5 wt.% Tween 20 in cream emulsion preparation presents very good stretch-ability than the model cheese made from 2 wt.% Tween. Therefore, CaCas matrix model cheeses discussed in chapter four were using 5 wt.% Tween 20 in cream emulsion preparation.

However, NaCas matrix model cheese did not show any stretch-ability and this model cheese in chapter four was made from 2 wt.% Tween 20 in cream emulsion preparation.

30 g of model cheese was made in each trial using RVA, mixing 15.0 g cream of 45 wt.% fat, 7.5 g RO water and 7.5 g protein powders (NaCas or CaCas). The term cream applied here can be considered as being either the model oil-in-water emulsions or the diluted natural cream. The fat content of the high fat natural cream supplied from Fonterra Kapity Plant was in the range of 80 wt.% to 85 wt.%, and in this case extra RO water was added when preparing the model cheese in RVA to confirm the same fat content (45 wt.%) comparing to the cheese made from emulsified cream. The target composition of model cheese is 23 wt.% protein, 23 wt.% fat and 54 wt.% water. Table 3.3 overviews the model cheese producing temperatures, maximum shear speed and residence time discussed in chapter 4.

The sequence of adding ingredients in RVA canister is important for the success of model cheese making, especially when using the CaCas, which is not easy to dissolve in water. Water was placed at the bottom of canister, then adding protein powder, and cream was put on the top of protein powder. The ingredients were gently pre-mixed by hand using RVA impeller before starting RVA mixing.

The shear speed in RVA profile started from 20 rpm and then increased step by step to the speed of 100 rpm, 300 rpm, 500 rpm and 800 rpm in every 30 seconds (Figure 3.1). The shear speed was constant at 800 rpm after 2 min.

The processing temperature was 25 °C in the first 2 min, thereafter it went up in a linear speed before the constant temperature. Temperature was increased to a constant value over a 3 minutes ramp when producing NaCas matrix model cheese, while CaCas matrix model cheese required more gentle heating and accordingly constant temperature was reached after 6 min.

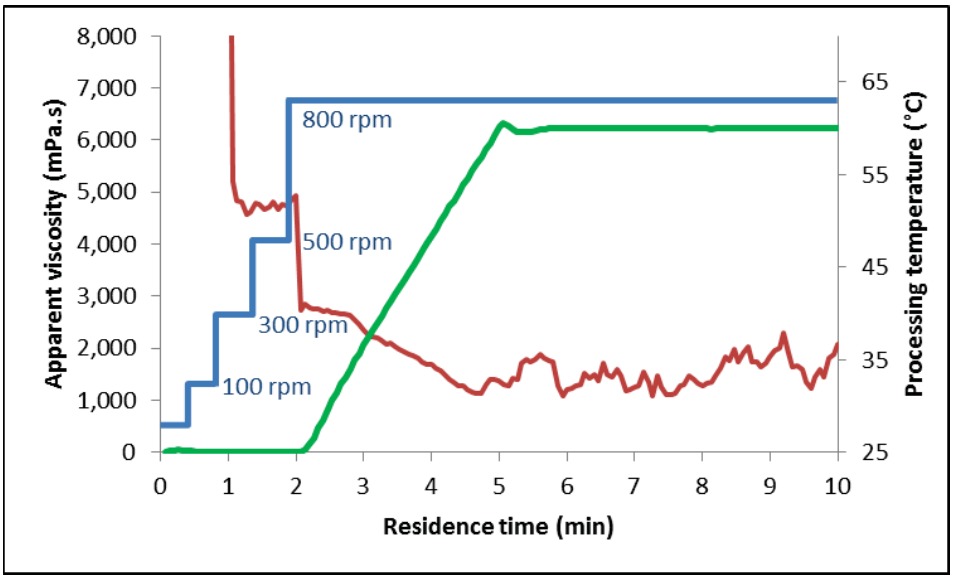


Figure 3.1: An example of AMC producing in RVA is used to explain the total shear work calculation. Green lines: processing temperature; Blue lines: shear speed; Red curves: apparent viscosity.

Table 3.3: RVA profile parameters of the model cheese production in chapter 4.

Cheese ingredients		RVA profile parameters		
Cream emulsions for cheese production	**Protein for matrix**	**Constant temperature (°C)**	**Constant shear speed (rpm)**	**Residence time * (min)**
with NaCas	NaCas	70	800	15
with Tween 20	NaCas	70	800	15
with NaCas	CaCas	70	800	10, 15, 25
with Tween 20	CaCas	70	800	10, 25, 37
		80	800	10, 25
Natural cream	CaCas	60	800	10, 25
		80	800	10, 25

*Not all the time points were discussed in result chapters.

3.3.2.2 Alternate make cheese (AMC)

The AMC was made by mixing and cooking ground cheese curd, cream emulsion, water and salt using RVA. The target composition was 21 wt.% protein, 23 wt.% fat and 53 wt.% water. Cheese curd was initially ground to the size of d < 2mm. It was kept in fridge

at 4 °C for 8 hours prior to use. Cream emulsion was the prepared oil in water emulsion or the natural cream. The natural cream would be diluted with RO water to 45 wt.% fat.

The shear speed increase over the first 2 minutes of mixing is similar as the one used in model cheese producing. The speed was 20 rpm in 30 s and then increased step by step every 30 seconds to 100 rpm, 300 rpm, 500 rpm and then the constant speed (600 rpm, 800 rpm or the 1200 rpm as required) (Figure 3.1). The shear speed was constant after 2 min.

The processing temperature was 25 °C in the first 2 min, thereafter it went up in a linear speed to the constant temperature in 3 min.

Table 3.4 gives details of AMC producing. AMCs in chapter 5 were produced to a sample size of 30 g. The ingredients were added in the order of 15.0 g grinded cheese curd, 15.0 g cream emulsions of 45 wt.% fat and 420 mg salt. The cheeses studied in chapter 6 were produced in 25 g, because the high shear speed of 1200 rpm would spread the ingredients of 30 g out of canister. The loss of cheese mass would impact the calculation of total shear work, which will be discuss in the chapter 6, and thereby, 25 g cheese was produced in all samples of chapter 6. The cheese ingredients included 12.5 g grinded cheese curd, 12.5 g cream emulsions of 45 wt.% fat and 350 mg salt.

The cream emulsions used in AMC were made from solutions of 4 wt.% NaCas or 2 wt.% Tween 20, where the concentration was able to fully cover the fat interface of 1 μm droplets. The AMC made from the cream emulsions of 2 wt.% Tween 20 presented obvious stretch on hot cheese samples.

The AMC produced with the Tween 20 emulsion was more difficult to make than the cheese comprising the NaCas stabilised emulsion, requiring adaptation of the RVA profile. This, the shear speed was increased to 800 rpm over a 2 minutes period, with a further holding period 13 minutes and followed by 15 minutes mixing of 1000 rpm; the mixing temperature was 25 °C in the first 2 minutes and then increased to the constant temperature of 60 °C in 3 min. The AMC with Tween 20 was always produced at 60 °C since demixing was observed at 70 °C. The water/oil phase causes inefficient mixing due to excessive wall-slip.

Table 3.4: RVA profile parameters in the AMC producing

Chapters	cream emulsions for cheese production	Mass of total ingredients (g)	Producing temperature (°C)	Constant shear speed (rpm)	Residence time* (min)	Comments on study area
Chapter 5	+ AMF, no emulsifier	30	60	800	10, 30	Fat interfacial ingredients
	+ NaCas, prepared cream emulsion	30	60	800	10, 30	
	+ Tween 20, prepared cream emulsion	30	60	800, 1000***	30	
	Natural cream	30	60	800, 1000	10, 20, 30	
Chapter 6	Natural cream	25	60	800	10, 20, 30	Producing temperature
		25	70	800	20, 30, 40	
		25	60	600	10, 20, 30, 40	Constant shear speed
		25	60	1200	10, 15, 20	
	Preheated natural cream	25	60	800	10	Protein adsorption on fat interface
	Preheated natural cream with NaCas	25	60	800	10	
	Natural cream in whey after sonication	25	60	800	15**	
	Natural cream in whey (WPI 895)	25	60	800	15	
	Natural cream in serum after sonication	25	60	800	15	

*Not all the time points were discussed in result chapters.

** AMC making was unsuccessful in 15 minutes using the sonicated natural cream with whey.

*** Shear speed was increased from 800 rpm to 1000 rpm after 15 min.

The target composition of the non-fat AMC was 68.8 wt.% water, but non-fat AMC produced in RVA did not contain such high moisture content probably because water was squeezed out of cheese matrix without fat. A different method to produce non-fat AMC of high moisture is required to have the same ratio of water to proteins as full-fat AMC. NaCl solution of 5.1 wt.% was added with the finely grinded non-fat cheese curd (d < 1 mm) by 1: 0.55 in mass. The mixture was kept in a sealed plastic bag of 15 cm x 23 cm and the materials fully filled the bag when leave the bag on a flat surface. The materials were sandwiched by hot solid plate (~ 60 °C) to allow the grinded curd merge together. Unabsorbed water would be reabsorbed when cooling overnight at 4 °C on a flat surface. If the ingredients were not well combined with uniform texture, the process of hot sandwiching and cooling overnight was repeated. Finally, non-fat cheese slice of 2-3 mm thickness was successfully made in this way with moisture of 68 ± 1 wt.%.

3.4. Analysis of the cream emulsion

3.4.1 Particle size distribution

Particle size distribution of the cream emulsion was measured using Malvern mastersizer 2000. Cream samples before measurement were diluted by adding a few drops in 10-15 ml RO water with gentle mixing at ambient temperature.

The refractive index of milk fat and dispersant phase (water) was taken to be 1.46 and 1.33, respectively. Measurement was repeated after 5 seconds on each sample, which was stired at speed of 1680 rpm. The sample additon will defaul in concentration of 10 - 15 wt.%. The data was analyzed using a model for general purpose, enhanced calculation sensitivity and calculation for spherial particle shape at 64 % threshhold.

3.4.2 CLSM

CLSM method was normally used to observe the cream emulsion structure, and here CLSM in Chapter 6 was to check if proteins adsorb at fat interface surface or not.

Cream emulsions of 45 % fat were diluted in the Walstra solution to a fat concentration of 0.45 %. The Walstra solution employed here did not contain Tween 20, since this could replace proteins at fat interface. The stain was a mixture of 0.2 vol. % of Fast Green in PEG and RhPe. The stain of 20 µl was mixed gently with 0.5 ml diluted cream samples. Stuck two layers of plastic tape on both left and right two edges of the slide for some space between the slide and coverslip to avoid the modification on the fat droplets when using the coverslip. The stained samples were stored for 2 hours in a dark place of 4 °C. The confocal microscope employed DPSS laser for milk fat membrane at 561 nm, and He/Ne laser for proteins at 633 nm. CLSM photos were taken when samples were magnified x10 and x40, respectively.

3.4.3 Protein composition in serum

Protein composition in serum collected from non-fat AMC was analysed by microfluidic chip SDS-PAGE, a rapid alternative method to separate and quantificate all major milk proteins (Anema 2009). Microfluidic chip SDS-PAGE just needs small volume of materials and samples, and works on the same principle as traditional SDS-PAGE, following the steps of sample loading, electrophoretic separation, staining, destaining and detection. Proteins were separated according to their size. The quantity of the protein is related to the peak area of that protein on the electropherograms.

The microfluidic chip electrophoresis used an Agilent 2100 Bioanalyzer system and the associated Protein 80 kit (Agilent Technologies, Waldbronn, Germany). The Protein 80 kit included reagents such as a gel matrix solution, a protein dye concentrate solution, a marker protein buffer solution and a protein molecular mass ladder solution.

300 µl serum squeezed from non-fat cheese just after production in an RVA mixer was mixed with 700 µl SDS-sample buffer. 20 µl β-ME (β-Mercaptoethanol) was added to 1 ml diluted serum and it was heated up for 10 minutes at 100 °C in a heating block, followed centrifugation after cooling down (3000 x g, 5 minutes, 20 °C) in a fume cupboard. Thereafter 12 µl of this serum sample was loaded onto chips for each well of S1-S9 (Figure 3.2). Skim milk was loaded to well G10 as the standard solutions to recognise milk proteins and estimate protein concentration. The lactoferrin (~80 kDa)

and the SDS in the working buffer were the upper marker and lower marker of molecular weight, respectively. Triplicate measurements of each serum samples were carried out on the chip.

The chip was primed by injecting gel matrix solution into well G1 (Figure 3.2) followed by applying air pressure (press ~ 1 ml air from syringe). Samples of destain solution (well DS, 6 µl), ladder solution (well L, 6 µl) and dye solution (wells G2-G4, 12 µl for each well) were also loaded on the same chip (Figure 3.2). The ladder solution was 1.0 ml of 0.3 mg/ml protein molecular mass ladder solution (lactoferrin) diluted in 150 ml SDS-sample buffer. The SDS-sample buffer was prepared by mixing 62.5 ml 0.5 M Tris-HCl Buffer, 50 ml 10 % (w/v) SDS and 250 ml MilliQ-water.

After preparation the chip was inserted into the Bioanalyzer, and the analysis took approximately 30 min for all ten samples. This procedure and the preliminary peak identification and integration were performed automatically by the Agilent 2100 expert software (Agilent Technologies, Waldbronn, Germany).

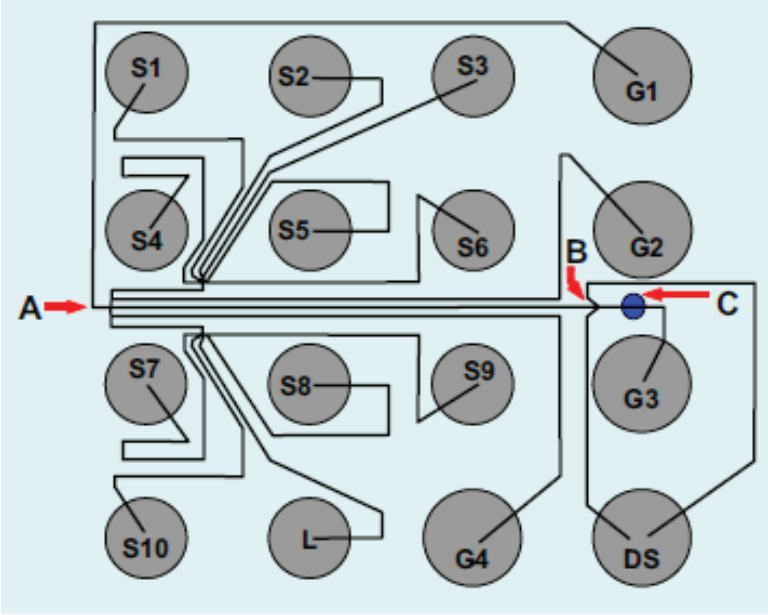


Figure 3.2: Layout of the microfluidic chip for SDS-PAGE. G1 is used for prime the channels; G2-G4 are loaded with gel/dye matrix; Wells of DS and L are designed to add destain solution and protein molecular weight standards, respectively; the measured samples are added into S1-S10. The separation channels (A), position of destaining (B) and position of the detection window (C) is marked with arrows (Anema 2009).

3.5. Analysis on cheese samples

Colloidal interactions in the cheese would not stop after cheese production. Water mobility in Mozzarella cheese was obvious in the first 7 days after production indicated by the dramatic decrease of expressible serum (McMahon, Fife et al. 1999, Kuo, Gunasekaran et al. 2001). Thereby, cheese was analysed after 7 days' storage at 4 °C. Milk fat crystallization caused partial coalescence at 4 °C would increase fat globule size when cheese was back to ambient temperature (20 °C). Particle size distribution was compared on fresh AMC without cooling and 7 days stored AMC at 4 °C.

3.5.1 Particle size distribution

The particle size distribution of cheese samples were determined using a method established by Walstra (1965). Cheese samples of 0.5 g were dissociated in 50 ml Walstra solution without stirring for overnight at 20 °C. The Walstra solution was at ambient temperature (~ 20 °C) before using. Cheese samples were taken at different places of cheese slices. The Ca^{2+} in cheese matrix would chelate with EDTA in the Walstra solution, which accelerates the dissociation of protein matrix. Fat globules were stabilized by Tween 20 in the Walstra solution. The dissociated cheese was an oil-in-water emulsion and particle size distribution was established on mastersizer. Setup was the same as that for the cream emulsions in section 3.4.1.

Cooling storage (4 °C) is able to cause partial coalescence of milk fat, and therefore fat particle size distribution was compared between the cheese without cooling (~ 20 °C) and the cheese after equilibrating for 7 days at 4 °C.

3.5.2 CLSM

The microstructure of cheese samples was observed on CLSM. CLSM images were taken from three different places across the cheese slices. They were frozen in cryo-microtome at -20 °C, cut to slices of 50 µm thickness, and stained by the mixed dye of 0.4 vol.% FG and 0.5 vol.% NR in citifluor. The model cheeses prepared with Tween 20 and discussed in chapter 4, were prepared as slices of 80 µm thickness due to the sample fragility after

freezing. Sample slices after dying were stored in a fridge of 4 °C for two days to allow the dye going inside of the samples. The confocal microscope employed Argon laser for milk fat at 488 nm and He/Ne laser for proteins at 633 nm. CLSM images were taken near the middle of the sample slices (~ 10 µm of thickness) because fat globules at the top and the bottom may be lost in the sample preparation.

The cheese samples discussed in Chapter 6 were additionally dissociated in the EDTA solution, i.e. the Walstra solution without Tween 20, aimed to observe the modified fat interface loading with proteins using CLSM. Proteins from dissociated cheese curd could adsorb at the fat interface when native MFGM rearrangements occurred upon the interface during membrane disruption from processing, shearing and/or homogenisation. Newly formed interface when fat globule size was reduced during processing created a greater surface area to which serum proteins adsorb alongside the original native membrane. The Tween 20 was not used in the Walstra solution because it would replace proteins at interface and then CLSM images would not recognize the differences. Besides, Tween 20 disturbed the signal of dyes on CLSM and could not show clearly where the fat globules were. RhPe was used to stain phospholipids on the milk fat globule membrane. The sample stain and preparation are the same to cream samples described in section 3.4.2. 3-4 CLSM photos were taken at different places when samples were magnified x10 and x40, respectively.

3.5.3 Rheology

Small deformation viscoelasticity of cheese samples was investigated by dynamic oscillatory shear rheology on an Anton Paar MCR 301 rheometer (Anton Paar, Graz, Austria) equipped with sand blasted parallel plates of 20 mm (PP20/s) and Peltier temperature control. Peltier temperature hood H-PTD 200 was used to well control the temperature of measurement. Round cheese slice of 20 mm diameter and 2.5 mm thick was placed in the centre of platform. After loading onto the parallel plate, a thin layer of canola oil was added at the edge of cheese slices to prevent water evaporation. The sample amplitude at 20 °C, in 0.1 % strain and 1 Hz was within the linear visco-elastic region in the model cheese and AMC, respectively. Therefore, storage modulus (G') and

loss modulus (G') was determined at 0.1 % strain and 1 Hz when temperature increasing from 4 °C to 80 °C with a linear heating speed of 3 °C/min. The heating speed followed the investigation from Schenkel, Samudrala et al. (2013). At a start of the measurement, the temperature should drop to 4 °C firstly before adding cheese, and the measurement started when temperature stable at 4 ± 0.3 °C for 2 minutes for cheese slices to arrive a stable temperature.

Cross temperature of G' equal to G" on the temperature sweep was compared among the AMC samples to indicate the modification on cheese melting from processing and/or interface ingredients.

To determine the particular influence of fat structuring on cheese material properties, the value of ln(G'/Gm') was calculated using the storage modulus of cheese within fat (G') and storage modulus of non-fat cheese (Gm'). The non-fat cheese included the same ratio of water to proteins as the cheese containing fat, and Gm' is assumed as the storage modulus of cheese protein matrix. The ratio of G'/G_m' should provide an indication as to how the structure and interactions of the fat component may have served to dominate, reinforce, weaken, or possibly have no influence on the material properties of the protein phase. If G' > Gm', value of ln(G'/Gm') was positive, indicating the role of fat provides a strengthening effect to the mechanical properties of the cheese; If G' < Gm', value of ln(G'/Gm') was negative, indicating the cheese material properties was weakened by the presence of the fat phase. By this calculation of ln(G'/Gm'), the impact of fat fillers on cheese texture was presented.

3.5.4 Cheese melting (Schreiber test)

Schreiber test was used to evaluate AMC melting behaviour (Muthukumarappan, Wang et al. 1999, Altan, Turhan et al. 2005). Cheese area was compared before and after baking; the amount of free oil (FO) releasing to cheese surface and the loss of water was measured as well after baking. It should be noted that not all the cheese samples have duplicate measurement on Schreiber test because sometimes samples were not enough for the second test.

Round cheese slices were prepared using a cylinder mold of 25 mm diameter. One cheese slice was placed in the center of a glass Petri-dish, and then another piece of cheese slice was put on the top the first slice to form a cheese cylinder sample of 4 mm high and 25 mm diameter. The Petri-dish was covered and then was baked at 170 °C for 10 minutes in the center of oven. The cover was removed immediately after baking to allow the water to evaporate. The cheese would cool off quickly at ambient temperature and no more water steam. The free oil on the cheese surface was dried carefully with tissues. The increased mass of tissues was the mass of free oil (m_{FO}). Free oil may sometimes spread to the dish cover, and in this case it would be dried with tissues included in the mass of free oil. The free oil was calculated using the formula:

$$FO\ (\%) = m_{FO} / (m_{cheese} * fat\ \%) * 100\ \% \quad (1)$$

Where fat % is fat content in cheese (23 wt.%).

The mass of covered dish with cheese after free oil removing (m_{rest}) was recorded. The mass of the covered Petri-dish (m_{dish}) and the mass of cheese (m_{cheese}) were recorded as well before baking. They are used for the calculation of water loss in cheese by the formula as follows:

$$Water\ loss\ (wt.\%) = (m_{cheese} + m_{dish} - m_{FO} - m_{rest})/(m_{cheese} * Cheese\ moisture\ content) * 100\ \% \quad (2)$$

The analysis of cheese moisture content is discussed in section 3.5.5.

Cheese flowability was compared by measuring the flowing area and flowing shape using the marked background circles. The Image J was used to compare the change of flowing area before and after cheese baking.

3.5.5 Moisture content in cheese-like samples

Cheese fractions (3-5 g in total) were randomly selected from different positions of the cheese slice. The edge of cheese slice was avoided. Cheese fractions were kept in aluminium cups, which had been pre-dried (105 °C, 2-3 hours) in oven and cooled to ambient temperature in desiccator before using. The cheese was dried overnight at

105 °C for 16±1 hours and cooling down to ambient temperature in desiccator. The moisture content was measured on duplicate samples calculated by formula:

Cheese *moisture content (%)* = $(m_c + m_{ch} - m_{dry\ total}) / m_{ch} * 100\%$ (3)

The m_c and m_{ch} are the mass of aluminium cup and the mass of cheese before drying, respectively. The $m_{dry\ total}$ is the total mass of aluminium cup within cheese after baking. The mass was accurate to three decimal places.

3.5.6 Total shear work calculation

Total shear work in RVA processing was discussed in chapter 6. By calculating total shear work cheese functionalities become comparable when changing processing parameters of shear speed, residence time and processing temperature.

The figure 3.1 is an example of cheese producing in RVA, describing the profile of shear speed, processing temperature and apparent viscosity in mixing. The apparent viscosity is extremely high in the first minute when shear speed is low without heating up. From 1 minutes to 5 minutes the increased shear speed and heating up lead to reduced apparent viscosity. Shear energy in the first 5 minutes is just to premix water, cream and salt, but invalid for mixing proteins because the temperature is not high enough to melt curd. Thereby, the valid total shear work for ingredients mixing was calculated from the 5^{th} minutes when temperature is above 60 °C.

Here are parameters and formulas for the calculation of total shear work in RVA.

k = mixer viscometer constant = 20.1/rev, when RVA speed > 210 rpm (Lai, Steffe et al. 2000)

N = shear speed (rps)

$\acute{Y}$ = average shear rate in RVA = kN (4)

η = apparent viscosity from RVA

σ = average stress in RVA = $\acute{Y}$ η (5)

R = radius of RVA impeller = 0.0164 m (Figure 3.3)

H = height of RVA impeller = 0.012 m

T = torque = $2\pi R^2 H\sigma$ (6)

ω = angular velocity = $2\pi N$ (7)

P = power in RVA = ωT (8)

W = total shear work = $\int P\, d(t)$ (9)

Calculate Equation (9) using Equation (8)

W = $\int \omega T\, d(t)$ (10)

Calculate Equation (10) using Equation (6) and (7)

W = $\int 4N\pi^2 R^2 H\sigma\, d(t)$ (11)

Calculate Equation (11) using Equation (5)

W = $\int 4N\pi^2 R^2 H \acute{Y}\eta\, d(t)$ (12)

Calculate Equation (12) using Equation (4)

W = $\int 4kN^2 \pi^2 R^2 H\eta\, d(t)$ (13)

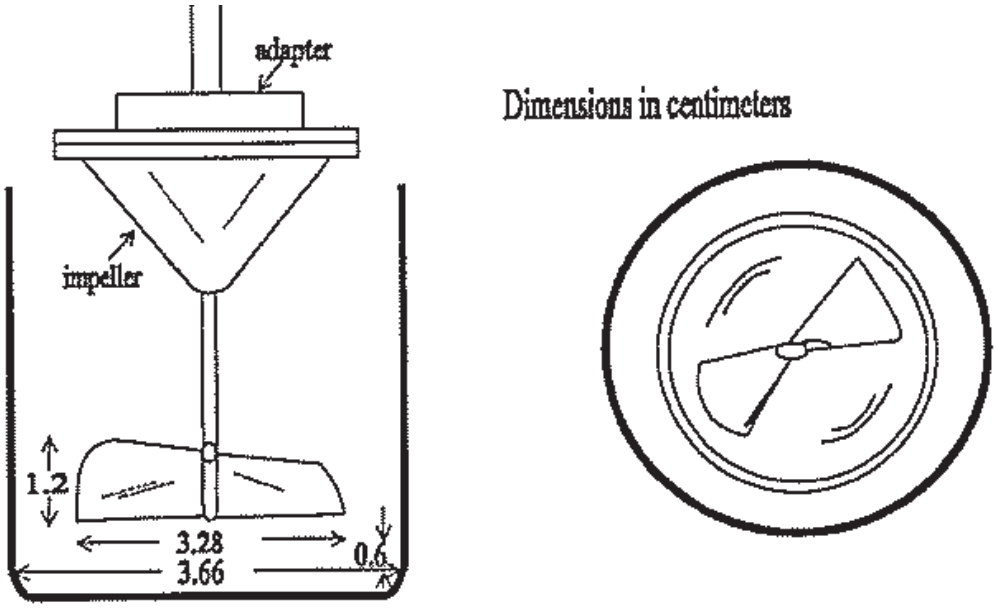


Figure 3.3: Dimensions of RVA impeller and canister. The unit is centimetre (Lai, Steffe et al. 2000).

Chapter 4: Behavior of a viscous protein matrix with emulsified lipids

4.1 Overview

Emulsifiers can stabilise oil-in-water emulsions by adding an energetic interfacial barrier to droplets that protects against mechanisms of aggregation and coalescence. Beyond interfacial stabilisation, the structuring of the continuous phase can be of particular influence on the stability and properties of emulsions. Here, structuring of the continuous protein phase in concentrated oil-in-protein emulsions is shown to greatly increase overall emulsion viscosity, such that oil-in-viscous protein matrix emulsions are formed. In these emulsions the energy barrier against aggregation arises not only from an adsorbed layer that opposes close approach of droplets, but also as a consequence of matrix phase viscous forces inhibiting the motion and interaction of droplets.

In this study the proteins comprising the matrix phase were hydrated with pre-formed emulsions, and therefore the viscoelastic properties of the protein network established during mixing would be expected to have a specific impact on the dispersion and distribution of this pre-formed emulsion during processing. Viscous emulsions were prepared in a lab scale mixer cooker with controlled shearing and temperature (the rapid visco analyser (RVA)). Under these conditions fat globules may still break up to smaller droplets in the mixing resulting in potential change to interfacial composition, interactions between droplets and surrounding matrix phase, and overall distribution of the fat. Accordingly, Capillary number of the viscous emulsions in RVA processing can be calculated to estimate this possibility, coupled with analysis of fat globule size distribution before and after RVA processing.

In this chapter, model viscous structured emulsions were made by combining and mixing two particular milk protein powders (NaCas or CaCas) with two pre-formed oil-in-water emulsions stabilised with different interfacial compositions. NaCas dissolves in water forming a homogenous matrix; however, CaCas has poor solubility in water existing as a white colloidal dispersion (Roeper 1977, Southward 1985), and a high concentration of

CaCas is expected to generate a concentrated protein phase and serum phase, which became obvious in a heat. The non-fat CaCas matrix turned to opaque and fragile at 50 °C, and some serum was able to be squeezed out. Fat globules in the pre-formed emulsion were stabilised with either NaCas or Tween 20 prior to mixing with protein powders to create different types of emulsion interface. The choice of interfacial materials would be expected to impact the behaviours of the fat globules in the emulsions, depending on the nature of their interaction with the protein matrix (van Vliet 1988, Cho, Lucey et al. 1999, Michalski, Cariou et al. 2002). Fat globules stabilised with protein are expected be dispersed and entrapped within the surrounding protein matrix, with the additional potential of forming bonds between droplet and continuous phase depending on protein type and conditions: for example, through the formation of calcium cross links between interfacial sodium caseinate and continuous phase calcium caseinate. In contrast, the droplets stabilised by the non-ionic Tween 20 would not be expected to associate with the protein. Fat globules covered with Tween 20 have little chemical affinity for the protein matrix (Dickinson and Chen 1999), and thereby tend to locate in the serum phase rather than binding to the protein matrix (Chen and Dickinson 1999, Cho, Lucey et al. 1999). Fat globules in the serum can additionally become localised during processing, creating discrete domains of high phase volume emulsion; however, fat droplets within these domains of oil-in-water may still be impervious to coalescence (at least whilst the fat is molten) due to the energy barrier provided by the surfactant layer. Aggregated fat globules would be expected to be observed in the CaCas matrix due to the existence of large serum pockets.

4.2 Sodium caseinate (NaCas) stabilised emulsion droplets

4.2.1 Oil-in-water emulsions

Milk fat emulsions of 45 wt.% fat were prepared by homogenising anhydrous milk fat in a 3 wt.% NaCas solution. Fat globule size distribution was between 0.1 µm and 3 µm and the mean particle size was 1 µm (Figure 4.1). The milk fat emulsion was found to be stable after RVA shearing (800 rpm 10 minutes 70 °C) in the absence of protein structuring of the continuous phase. There was sufficient NaCas to achieve saturation

coverage of ~ 3 mg/m^2 at fat globule surface, which is known to provide excellent stability of oil-in-water emulsions (Srinivasan, Singh et al. 1996, Bos and van Vliet 2001). Although Reynolds number by calculation (Re ≈ 2 x 10^6) indicated turbulent flow conditions in RVA processing (Appendix I), figure 4.1 showed no apparent change of fat globule size distribution.

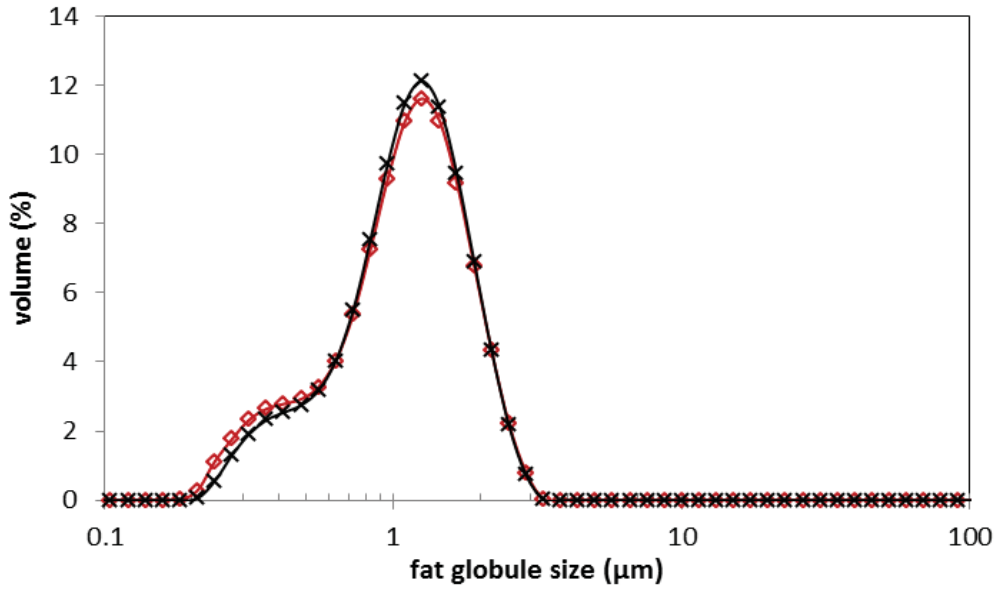


Figure 4.1: Comparison of the fat globule size distribution in oil-in-water emulsions before (◇) and after (X) RVA processing (800 rpm 10 minutes 70 °C). Fat globules were fully covered with NaCas.

4.2.2 Oil-in-viscous protein matrix emulsions

Viscous emulsions were produced by mixing 7.5 g caseinate powder, 7.5 g RO water and 15.0 g milk fat cream of 45 wt.% fat, in which fat globules were fully covered with NaCas. The RVA profile conditions were 800 rpm at 70 °C. Caseinate powder (NaCas or CaCas) created the viscous protein matrix. Findings showed that the fat globule size distribution changed as a consequence of processing, and showed considerable variation according to the caseinate type used in the assembly of the protein matrix (Figure 4.2). The high concentration of protein in the continuous phase significantly increased emulsion viscosity, however it was estimated that the 1 µm fat globules would not be expected to break up under laminar flow conditions (Re ≈ 512 in RVA) since the capillary number was determined as approximately 0.045 (Appendix I). The critical capillary number for

droplet breakup is above 10 (Jackson and Tucker III 2003), while protein-covered droplets are able to resist shear stress to a much higher degree (Erni, Fischer et al. 2005). The peak maximum of fat globule size distribution in figure 4.2 shifted to larger droplets after the creation of viscous emulsions. The D[4,3] of the NaCas stabilised emulsion system shifted from 1.195 µm (red line) to 1.523 µm (black line) upon the creation of viscous emulsion, indicating that droplets in the viscous formulation did not break up to smaller droplets but instead agglomerated to form larger fat globules. It was obvious that an increase in fat globule size had taken place in the viscous CaCas matrix with a new modal distribution of ~100 µm appearing, and with the previous modal distribution at ~1 µm dramatically decreased. The aggregation of fat globules appeared to be a consequence of the specific use of CaCas as the protein continuous phase, since only limited coalescence of droplets (as evidenced by a widening of the particle size distribution) appeared to have occurred when NaCas was used as the matrix. The larger fat globules in CaCas matrix indicated localisation and association of fat during RVA processing.

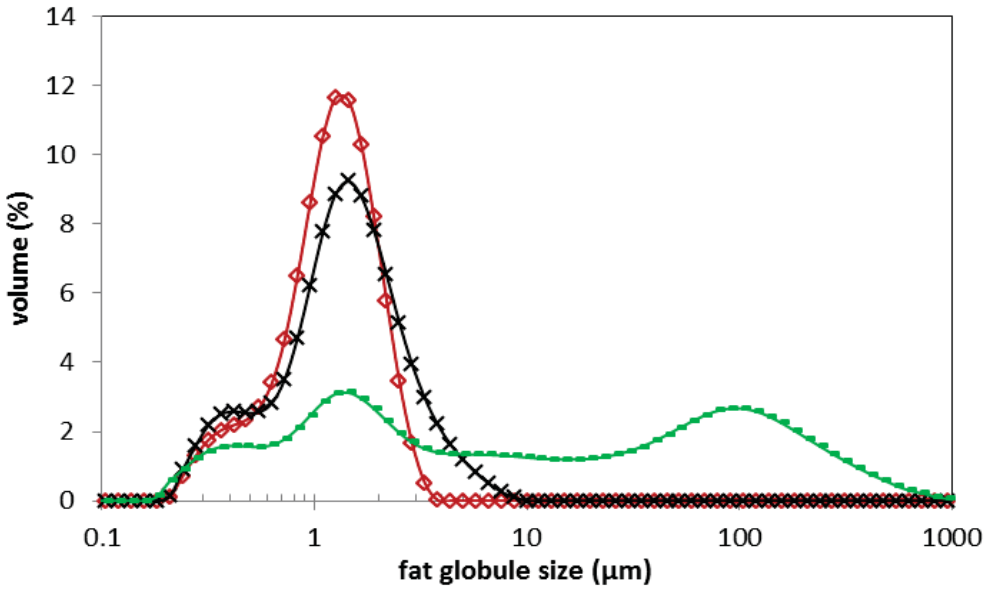


Figure 4.2: Comparison of the fat globule size distribution in the oil-in-water emulsions (◇) and the viscous emulsions of NaCas matrix (X) or CaCas matrix (–), which were manufactured from the oil-in-water emulsions (◇). Fat globules were fully covered with NaCas in the oil-in-water emulsions.

CLSM images showed how the fat globules were dispersed within the protein matrix. Small individual fat globules were homogenously dispersed within the NaCas matrix (Figure 4.3a), while a number of considerably larger fat globules were observed in CaCas matrix (Figure 4.3b). The large individual fat globules would suggest significant loss of interfacial stability leading to coalescence. Fat globules were fully covered with NaCas in the milk fat cream which is known as an effective emulsifier for providing stability against coalescence, and so the observed behaviour when combined into a CaCas protein network was somewhat unexpected.

The implication here is that the addition of CaCas in some way acted to reduce the interfacial stability of the emulsion droplets. Srinivasan et al. (2001) showed that a mixture of NaCas and CaCas in oil-in-water emulsions did not behave the same as if they were used separately. It was suggested that the protein structure was modified when using NaCas and CaCas together. The presence of Ca^{2+} ions in the NaCas solution produced the conformational change of NaCas leading to progressively less flexible structures (Alvarez, Risso et al. 2008). The aggregated proteins are less surface active than the flexible proteins (Mulvihill and Murphy 1991, Srinivasan, Singh et al. 1999), and therefore fat globules coalesced when the NaCas loading at fat surface became less flexible. To validate this hypothesis, investigation on interfacial protein is recommended.

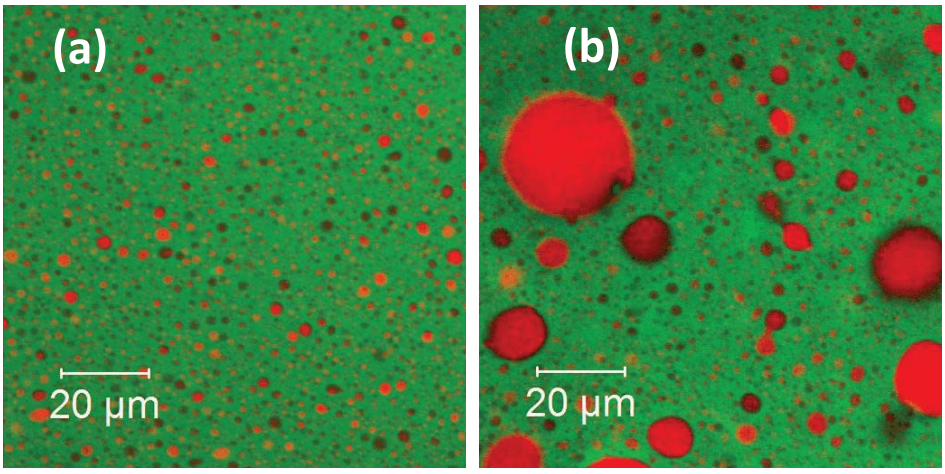


Figure 4.3: CLSM images of the viscous emulsions manufactured with NaCas matrix (a) or CaCas matrix (b). The viscous emulsions were manufactured from the oil-in-water emulsions stabled by NaCas. Red is fat and green is protein.

The small strain rheological properties were studied as a consequence of heating from 4 °C to 80 °C (Figure 4.4). The CaCas protein gels with and without the addition of the NaCas stabilised emulsion showed a reduction in storage modulus suggesting a weakening of the gel network with increasing temperature. Inclusion of the emulsion into the CaCas gel resulted in a relative increase to storage modulus compared to the fat-free sample, and which was observed across the entire temperature range suggesting that the fat component was able to reinforce the protein network. Whilst the fat phase of the structure was known to undergo a change in material state from solid to liquid (as indicated by the rheological data for AMF in figure 4.4), this change was not seen as contributing to the changes observed in the modulus for the filled emulsion gel with increasing temperature.

It can be reasonably assumed that the fat droplets with NaCas at interface were likely to increase the G' of viscous emulsion through specific interactions with surrounding CaCas matrix(van Vliet 1988), acting as active fillers (although it is not clear from confocal that this is actually the case). At high temperatures this interaction may have magnified this effect, with the formation of bonds between droplets and protein continuous phase preventing relaxation of the protein network during melting (as observed by the relative change in G' gap between the filled emulsion samples and non-fat samples at temperatures above 40 °C). This finding was consistent with the conclusion from Dickinson and Chen (1999), who considered that protein covered fat globules had more effect on material properties when protein gel matrix had lower G'.

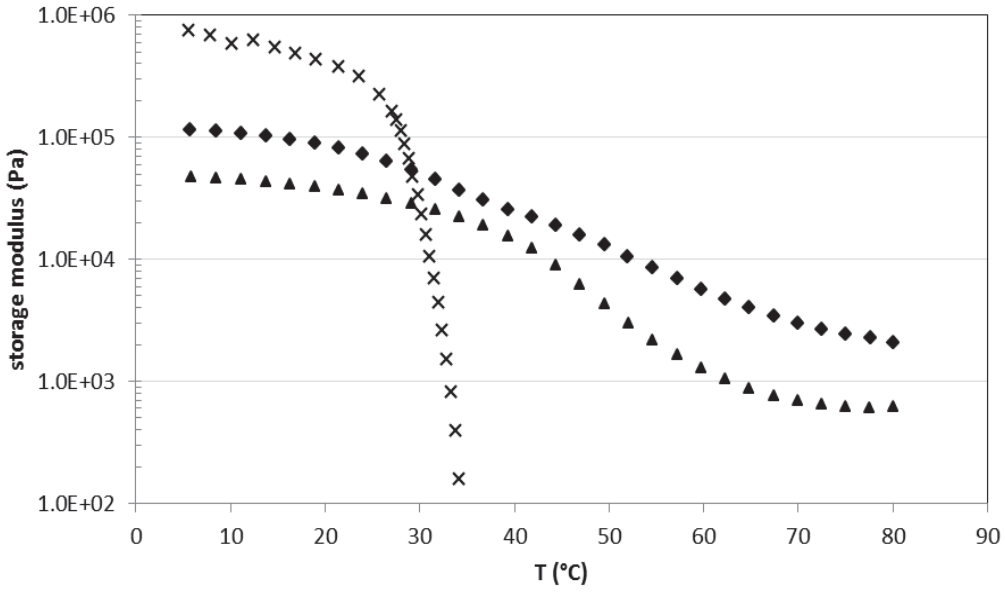


Figure 4.4: Small strain rheological properties of CaCas matrix emulsions in a heating from 4 °C to 80 °C. NaCas was used as the interfacial ingredients. The emulsion with fat (◆) was compared with the non-fat sample (▲) and anhydrous milk fat (X). The viscous emulsions were produced in RVA mixer for 25 minutes mixing.

The emulsion samples mixed with CaCas were subsequently heated in an oven at 170 ˚C for 10 minutes with little oiling off at the sample surface observed. This was perhaps unexpected give the large fat globules visualised in the CLSM images (Figure 4.3b). CaCas in high concentration forms thermally stable gels on heating (Roeper 1977), and it may be that even large domains of fat, as observed in the confocal images, could remain trapped in a strong protein network and that were able to remain isolated from each other during heating. This suggests that some critical relaxation of the protein network is required to enable sufficient structural mobility to allow fat domains to become sufficiently interconnected, thereby enabling oil release.

4.3 Tween 20 stabilized emulsion droplets

4.3.1 Oil-in-water emulsions

Emulsions (45 wt% fat) were also prepared using the non-ionic emulsifier Tween 20. Analysis of these emulsion under RVA shearing showed that the fat globule size

distribution did not change during treatment when no additional protein was present (70 °C, 10 min, 800 rpm) (Figure 4.5).

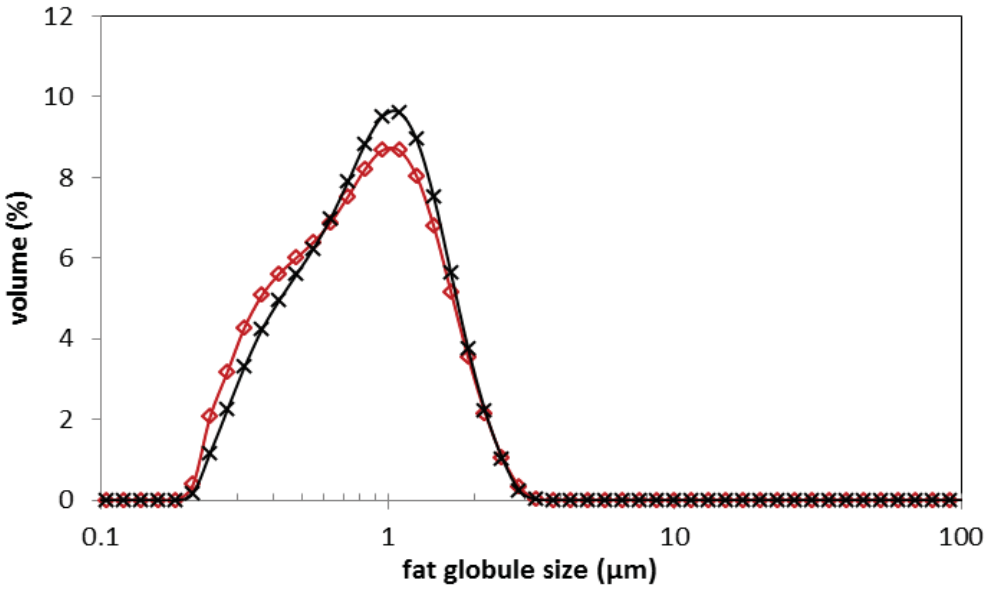


Figure 4.5: Comparison of the fat globule size distribution in oil-in-water emulsions before (◇) and after (X) RVA processing (800 rpm 10 minutes 70 °C). Fat globules were fully covered with Tween 20.

4.3.2 Oil-in-viscous protein matrix emulsions

Caseinate powders (NaCas or CaCas) were mixed and heated with the oil-in-water emulsion stabilised with Tween 20 to produce viscous protein emulsions. The fat globule size distribution of both emulsions was seen to increase after RVA mixing in the preparation of these viscous emulsions (Figure 4.6).

The mean fat globule size D[4,3] increased from 1 µm to 3 µm when NaCas was used as the matrix material, with the increase in size appearing to result from fat coalescence, as shown in the CLSM images in figure 4.7a. The presence of both Tween 20 and NaCas, two surface-active ingredients, can in some cases lead to the loss of emulsion stability due to the competitive adsorption, although it might be expected that there was sufficient Tween already adsorbed at the interface that this would not occur (Dickinson, Ritzoulis et al. 1999). It was possible that NaCas in the matrix could bind to the Tween 20 through hydrophobic bonds, with the Tween-protein composite less effective at

stabilising the interface than either species in isolation (Dickinson and Woskett 1989). In the case of fat globules in NaCas matrix, it was considered that the extent of coalescence was limited at size of 2-3 µm D[4,3] by the homogeneous distribution of droplets within the protein network, which when coupled with the increasing viscosity of the continuous phase, acted as a barrier to droplet encounters.

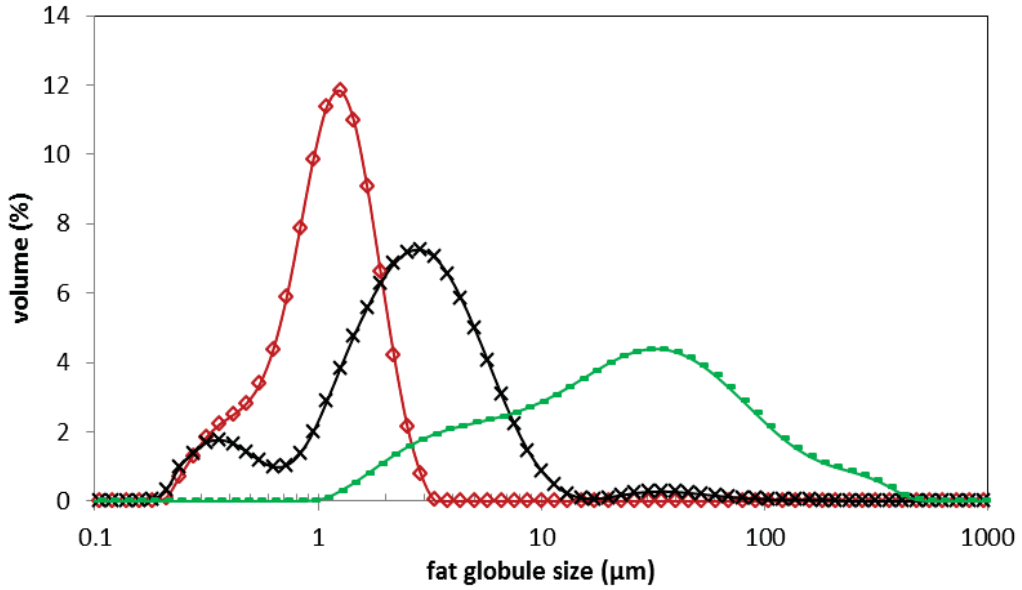


Figure 4.6: Comparison of the fat globule size distribution in the oil-in-water emulsions (◇) and the viscous emulsions of NaCas matrix (X) or CaCas matrix (–), which were manufactured from the oil-in-water emulsions (◇). Fat globules were fully covered with Tween 20 in the oil-in-water emulsions. Viscous samples have been stored at 4 °C before analysis.

In CaCas viscous emulsions the fat globule distribution could be seen to have shifted to noticeably larger values (Figure 4.6). This was possibly due to localisation of fat globules during mixing, which were then aggregated and/or coalesced within the CaCas matrix (Figure 4.7b). In the absence of fat, it was noticed that some serum was expelled from the hydrated protein network during heating and shearing. However, after cooling the serum was reabsorbed by CaCas matrix. This phenomenon suggests that fat globules covered with Tween 20 may localise in the serum pockets during heating when CaCas formed a strong network. At high temperatures, hydrophobic interactions in the CaCas strengthen, leading to the compaction of the protein network. The shrinkage of the

protein network resulted in the formation of enlarged serum pockets due to expulsion of water from the protein network, creating sufficient space for fat globules to move freely within these domains and essentially creating localized oil-in-water emulsions. When the viscous emulsion was cooled and the serum was reabsorbed by the CaCas network, the fat globules localised within the serum pockets were pushed together, increasing the effective phase volume of droplets within these domains and causing subsequent aggregation and/or coalescence.

Fat aggregates were not observed in the NaCas matrix. NaCas is a flexible soluble protein with better water binding capacity than CaCas (Roeper 1977, Southward 1985). The concentrated NaCas was uniformly dispersed in the viscous solution rather than forming protein network, and thereby large serum pockets were unlikely to be present in the NaCas matrix.

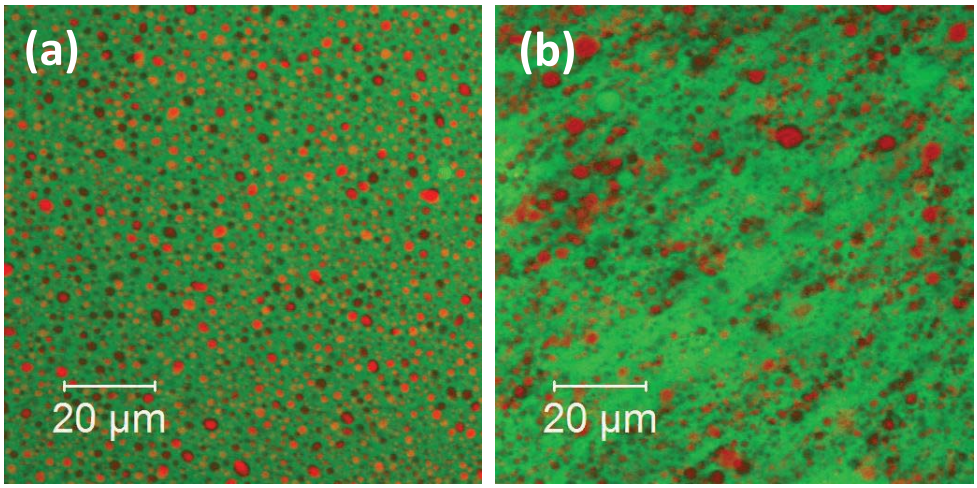


Figure 4.7: CLSM images of the viscous emulsions manufactured with NaCas matrix (a) or CaCas matrix (b). The viscous emulsions were manufactured from the oil-in-water emulsions stabilized by Tween 20. Red is fat and green is protein.

The emulsion structure of the viscous CaCas matrix stabilised by Tween 20 was seen to be unstable after extended shearing (Figure 4.8). The viscous emulsions would show little fluctuation in apparent viscosity during RVA mixing over up to ~30 minutes; however, increasing the mixing time beyond 30 minutes (at 70 °C) caused the viscosity of the system to drop to markedly lower values, wherein the emulsion lost the stretch

ability. The viscosity decline was not observed for non-fat samples even at 40 minutes mixing at 70 °C (where the ratio of water to protein was the same to the viscous emulsions); however, the material properties of the non-fat sample were seen to become firmer and less smooth textured after increased time processing at 70 °C.

The drop in viscosity of the Tween 20 viscous emulsion could be caused by a transitional change in the structure and stability of the dispersed phase. Extended shearing may restructure the protein network which in turn may influence the properties of the fat droplets. The state of the fat phase was visualised using confocal microscopy (Figure 4.8). Over short residence times of mixing, fat globules were relative evenly distributed with little aggregation observed (Figure 4.8a). Fat localisation leading to aggregation and/or coalescence occurred as residence time was increased, leading to the formation of larger fat domains (Figure 4.8b). The size of fat aggregates continued to increase until the formation of biphasic emulsion, with fat/water separated from protein matrix (Figure 4.8c). A number of cracks could also be seen in the CLSM samples of figure 4.8c (not shown in this CLSM photo) suggesting fragmentation of the protein structure. The changes in the fat dispersion within protein matrix may have occurred due to the weak association between protein and fat globules coupled with strong protein-protein interactions. Fat globules stabilised by Tween 20 were not expected to form bonds with CaCas in the continuous phase, whereas caseinate stabilised droplets may, conversely, have formed links to the surrounding protein network through Ca^{2+} cross-linkages.

It was also observed that increasing mixing temperature accelerated the changes to viscous emulsion structure. When using a higher temperature of 80 °C, the apparent viscosity dropped in 17 minutes rather than the 31 minutes at 70 °C.

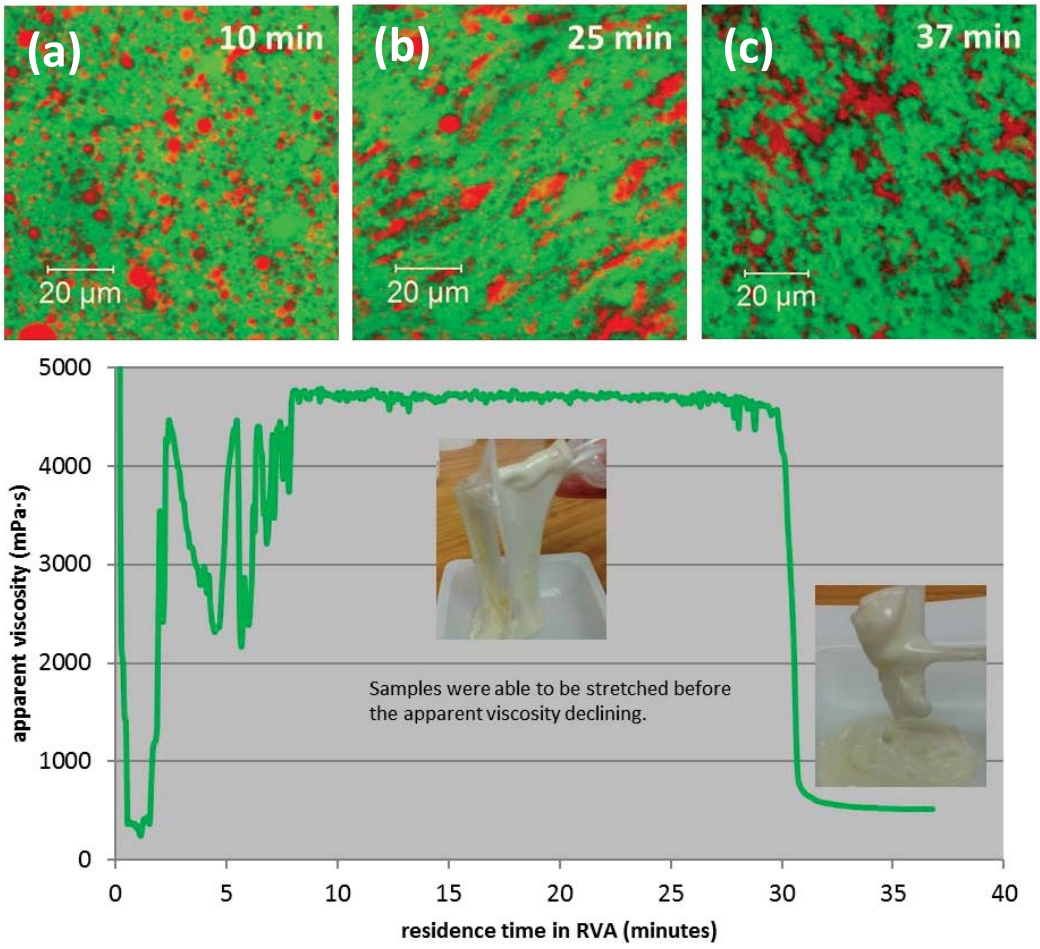


Figure 4.8: CLSM images and sample photos of the viscous CaCas matrix emulsions manufactured in 10 - 37 min (a: 10 minutes, b: 25 minutes, c: 37 minutes) and the change of apparent viscosity shown in RVA processing (800 rpm 70 °C). Fat globules were covered with Tween 20.

In the case of the non-fat sample, preparation at 70 °C for 15 minutes produced a liquid-like material during processing which became solid-like when cooled to 4 °C, behaving in a similar manner to the biophasic emulsion with Tween 20, which also turned from liquid-like to solid-like after cooling. CaCas protein could not maintain a cohesive network after extensive shearing at 70 °C. In case of using Tween 20, fat fractions increased the effect of water volume fraction because fat was locating in serum. The CLSM image in figure 4.8a indicated that fat droplets were uniformly distributed in the protein network; the decrease in viscosity observed after 30 minutes processing at 70 °C corresponds with a profound change in emulsion structure (Figure 4.8c) with fat and protein clearly separating into distinct phases (biphasic system) which the domains of

liquid fat disrupting the protein network structure and decreasing viscosity. It could also be argued that on cooling, the solidification of these separated fat domains could be of consequence in the change from liquid to solid, demonstrating the greater role of fat material properties in the overall mechanics of this particular structural state.

For fat globules covered with NaCas, fat globules were stable within the protein network, presumably due to strong interactions between interfacial ingredients and matrix, and thus the volume fraction for protein network would include fractions of protein and fat rather than just protein. A stable emulsion structure was thereby created in viscous emulsion with fat globules covered by NaCas.

To study this further, figure 4.9 compared the storage modulus of viscous emulsions with and without biphasic structuring as a consequence of thermal treatment. Where the emulsion had been prepared over short shearing times, such that the fat phase was still evenly distributed in the protein network (as for figure 4.8a) and the modulus values followed the same path as for the non-fat sample described in figure 4.4 at temperature above 30 °C when milk fat melting, suggesting limited impact from liquid fat globules within protein network. In contrast, G' of the biphasic emulsion shown in figure 4.9 was relatively higher at temperatures at which the fat phase was predominantly crystalline (<25 °C), and with an observed accelerated rate of decrease as the fat phase underwent melting to the point where the fat was entirely molten (~40°). At temperatures above 40 °C, whilst the overall modulus was lower than the viscous emulsion with the homogeneously distributed fat phase, it did appear to follow the same rate of change. Findings support the earlier observation that the phase separation of the emulsion enabled the fat phase to play a more predominant role in defining the material properties of the emulsion system, and that this was further influenced by the changes to the material properties of the fat relative to temperature.

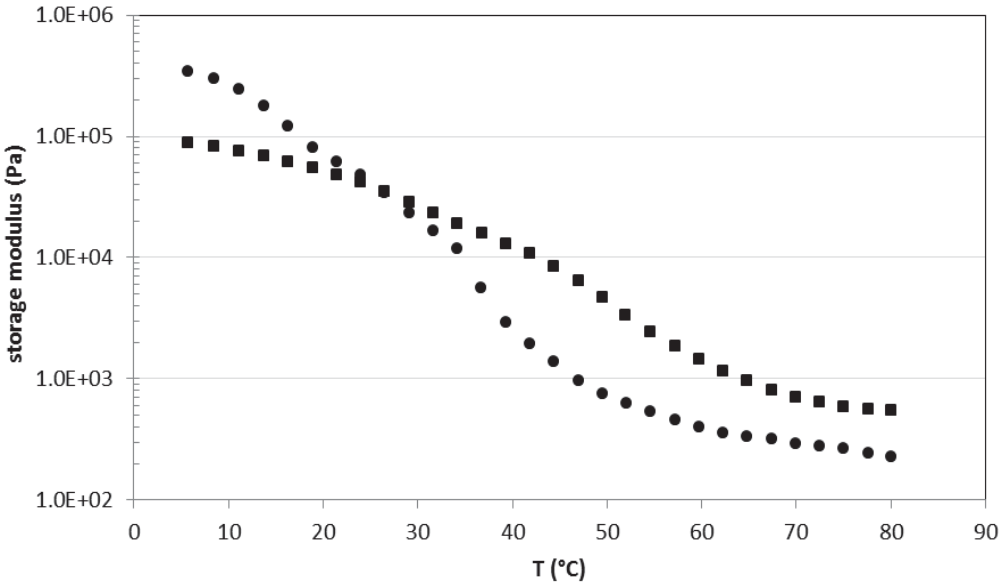


Figure 4.9: Small strain rheological properties of the viscous CaCas matrix emulsions in a heating from 4 °C to 80 °C. Tween 20 was used as the interfacial ingredients. The viscous emulsion without biphasic structure (■) was compared to over-sheared samples of biphasic structure (●).

In baking of the biphasic emulsion at 170 °C for 10 minutes in a Schreiber test, free oil was observed to be released at sample surface. The large fat domains having undergone extensive coalescence as the temperature exceeded the melting point of the fat appeared to have sufficient connectivity within the protein network to allow release of oil from within the structure. It was also noticed that the melting of the biphasic viscous emulsion was accompanied by an increase in the flow and spread of the sample, in line within expectations that free oil would enhance spreading area in cheese melting (Rudan and Barbano 1998, Everett and Auty 2008).

4.4 Viscous emulsions manufactured from natural cream

The investigation on the protein-droplet interactions for droplets stabilised with either NaCas or Tween 20 provided understanding of how interfacial interactions could influence the fat distribution in a model viscous emulsion. In order to transfer the findings into the alternate make cheese (AMC) system, an initial concept model cheese, consisting of 23 wt.% fat, 23 wt.% protein and 54 wt.% moisture, was prepared by mixing

natural cream, RO water and CaCas powder for 10 minutes (800 rpm) at 60 ˚C in the RVA mixer.

Figure 4.10 showed the change of fat globule size distribution after producing. For this measurement, the model cheese was not chilled, in order to avoid partial coalescence in the cooling storage. Milk fat in natural non-homogenised cream is covered with native milk fat globule membrane (MFGM) and this protects fat droplets from coalescence (Walstra, Jenness et al. 1984). However, large fat globules (> 100 µm) were found after processing in model cheese. The natural membrane did not appear to have stopped fat coalescence occurred during RVA processing.

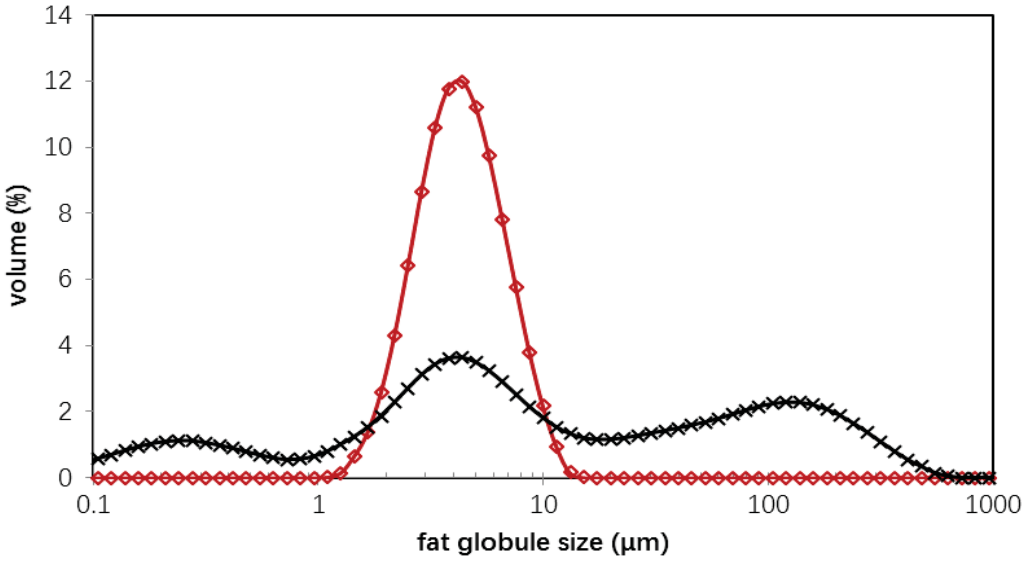


Figure 4.10: Comparison of the fat globule size distribution in the natural cream (◇) and the viscous emulsion (X) produced in 25 min at 800 rpm 60 °C by blending the natural cream, CaCas powder and water. Viscous emulsions were not chilled.

According to the investigation in section 4.2 and 4.3, it was clear that the interfacial composition at the surface of fat droplets impacted fat dispersion in the viscous emulsions. It was suggested to determine the composition at the interface during processing and any changes that may be taking place, particularly based on the consideration the droplets are being homogenised during processing. If the emulsion is

undergoing homogenisation, some CaCas may become adsorbed at the fat surface during the shearing process, which may contribute to the destabilisation of the droplets. This hypothesis was supported by the analysis on fat dispersion and melting performance: fat globules were individually dispersed in protein matrix and very few fat aggregates were observed in the CLSM images (Figure 4.11), which is similar to that using NaCas as the interfacial material (Figure 4.3b); in addition, samples presented a dry surface in heat treatment of Schreiber test suggesting fat globules were trapped in the protein matrix.

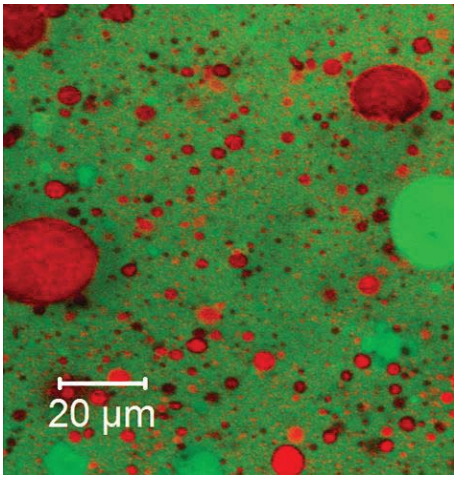


Figure 4.11: CLSM images of the viscous emulsions manufactured from natural cream and CaCas powder. The RVA processing was 800 rpm 25 minutes at 60 °C. Red is fat and green is protein.

Figure 4.12 compared the storage moduli of the model cheeses and the non-fat sample. The storage modulus of the cheese within NaCas stabilized fat globules was obviously higher than the other cheese samples, and the storage modulus decreased steadily in a heat from 4 °C to 80 °C. The fat globules covered with NaCas strengthen the cheese firmness comparing to the non-fat cheese. However, the Tween 20 stabilized fat globules and natural milk fat globules slightly impacted the cheese storage modulus which were near to the storage modulus of non-fat cheese.

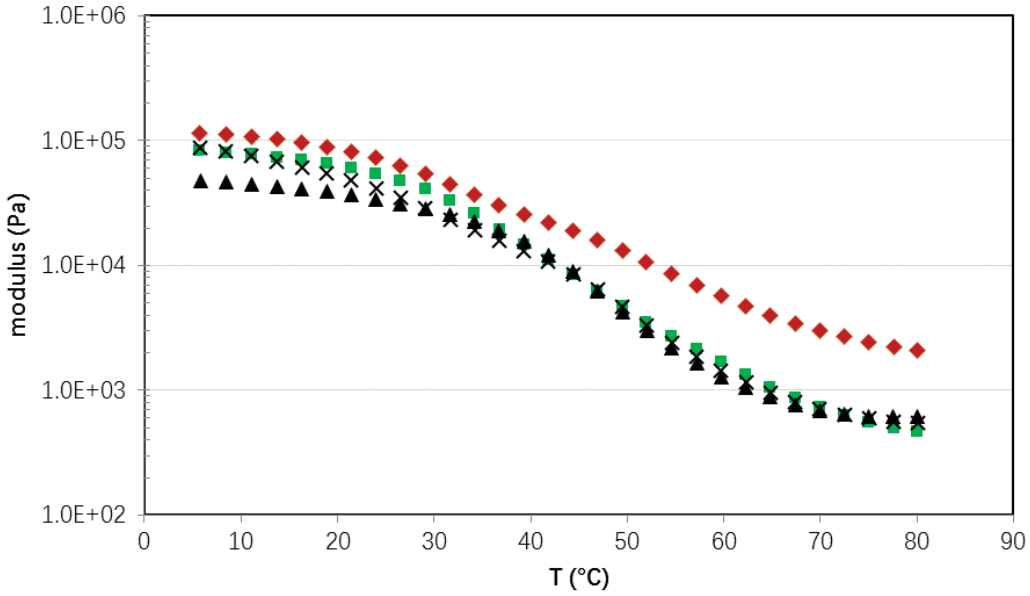


Figure 4.12: Small strain rheological properties of viscous CaCas matrix emulsions in a heating from 4 °C to 80 °C. The viscous emulsion manufactured from the natural cream (■) was compared with the non-fat samples (▲), samples manufactured from Tween 20 emulsified fat globules without biphasic structure (X) and samples within NaCas stabilized fat globules (◆). The samples were produced in RVA for 25 minutes mixing.

4.5 Conclusion

Viscous oil-in-protein emulsions of model cheeses were produced by adding cream emulsions with caseinate powder (NaCas or CaCas) in RVA mixer. Dynamic changes to fat structure were observed as a consequence of mixing, with fat globules displaying varying degrees of coalesce and/or aggregation in the preparation of these viscous emulsions. Behaviours were seen to be dependent on both the interfacial composition of the emulsion droplets and the corresponding properties of protein matrix, and the various structural outcomes are presented in Figure 4.13. When NaCas was used as both the continuous phase and as emulsifier, fat globule encounters were minimised due to the high viscosity of the protein phase, coupled with a more uniform distribution of fat droplets within the protein network (Figure 4.13a). Where the NaCas was replaced by Tween 20 as the emulsifier, competition between Tween 20 and the NaCas may be responsible for the limited fat coalescence that was observed on the increased particle size in the model cheese processing shown in figure 4.6, but the size increase was limited by high viscosity of NaCas matrix (Figure 4.13b). The use of CaCas as the continuous

phase formed a more particle gel-like network though interaction of proteins via Ca^{2+} cross-links. Compaction of the CaCas network during shearing and heat treatment caused some serum release which also allowed the formation of localised domains of fat. Where NaCas had been used to stabilize the emulsion, the fat globules were expected to cross link with the surrounding CaCas matrix through formation of calcium bridges (Figure 4.13c), although this would not necessarily account for the localisation of fat that was observed. For Tween 20 stabilized droplets, localisation was more to be expected within the serum pockets, due to the lack of interaction between Tween 20 and the surrounding CaCas network (Figure 4.13d). Structurally, this system was seen to be most sensitive to the processing conditions, ultimately forming a biphasic system appeared between protein and fat. The use of natural cream described model cheeses within evenly distributed fat globules throughout the protein network, and the milk fat membrane did not protect from coalescence in processing.

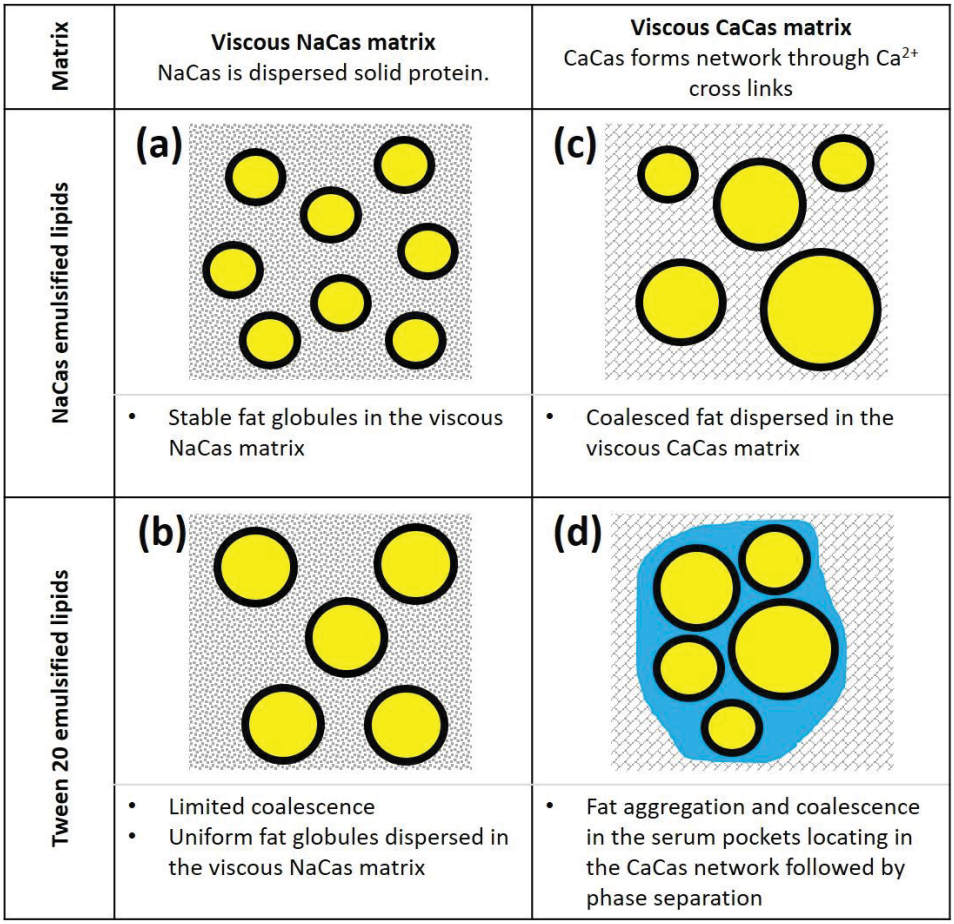


Figure 4.13: Schematic diagrams to show emulsion structure of fat globules in the viscous NaCas matrix (left) or viscous CaCas matrix (right). Yellow circles are fat globules; Blue area is the serum pocket; squared area presents protein matrix.

The aim of this chapter was to introduce the concept of 'active fillers' and 'inactive fillers' and their interactions within model viscous oil-in-protein viscous emulsions. On this understanding, active fillers can be defined as particles that interact with the surrounding continuous phase, while inactive fillers lack association with continuous phase. When using CaCas as the continuous phase, active fat fillers and inactive fat fillers showed two types of fat dispersion (Figure 4.14). NaCas stabilised fat globules strongly interacted with the protein matrix through Ca^{2+} ions cross links, and these fat globules are regarded as active fillers and they did not get close to each other (Figure 4.14a). Active fillers were more effective in building a stable viscous emulsion system, and for such structures, no oil-off occurred during a baking test. The inactive fillers, i.e. fat

globules covered with Tween 20, did not associate with protein matrix and therefore tended to localise in serum pockets (Figure 4.14b). The impact of shear on the structure of the emulsion/protein network was also evidenced with the overworking of the Tween emulsion in CaCas, which led to the formation of a biphasic fat-protein structure that displayed more pronounced oil release during heating than samples for which protein was retained as the continuous phase. This emulsion structure was unstable and biphasic emulsion appeared finally.

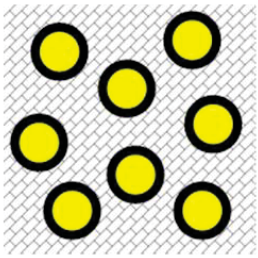
Schematic representation	(a) 	(b) 
Fat fillers	Active fillers	Inactive fillers
Interfacial ingredients	Caseinate	Tween 20
Fat dispersion	Fat globules homogenously disperse in the protein matrix.	Fat globules located in serum pockets tend to aggregate.
Emulsion stability	Stable emulsions	Phase separation may occur depending on processing
Heat stability	Fat globules are trapped in matrix.	Oil off through fat-serum channels, depending on processing

Figure 4.14: Conclusion of active fillers and inactive fillers locating in the protein matrix. Yellow circles are fat globules; Blue area is the serum pocket; squared area presents CaCas matrix.

Chapter 5: Emulsion interactions and structuring in Alternate Make Cheese (AMC)

5.1 Overview

This chapter extends the learnings of the previous chapter by investigating the role of fat globules on the colloidal interactions present in an alternate make cheese system (AMC), notably in determining how fat globules interact with each other, and the surrounding protein matrix, and as to how these interactions contribute to fat dispersion and phase separation in AMC.

In the previous chapter it was suggested that emulsion structure within a viscous protein matrix was dependent on the nature of the interfacial layers and protein matrix, with both protein-protein interactions of the viscous protein matrix impacting strongly on the state of fat dispersion, as well as interactions between the interfacial layer of emulsion droplets and the surrounding protein network. In this chapter, AMC was produced using a low fat cheese curd (LFCC), which was expected to apart different properties to the viscous protein matrix in comparison to the two previous protein types. For example, LFCC exhibits water-protein phase separation on heating due to reduced hydrophilic interactions on proteins and water, although not to the same extent as calcium caseinate (CaCas), for which calcium crosslinking plays a more predominant role in terms of protein-protein interactions.

In this respect, it can be approximated that the strength of protein-protein interactions in protein matrix is of the order: sodium caseinate (NaCas) < cheese curd < calcium caseintate (CaCas). Thereby, when using cheese curd to produce AMC, interactions between the fat droplet interface and the protein matrix may be expected to result in further variations to the emulsion structures produced as a consequence of oil-in-protein assembly.

To investigate this concept, four samples were explored with different surface compositions:

1) AMC_AMF: in which anhydrous milk fat (AMF) was directly mixed with LFCC without pre-homogenization.
2) AMC_NaCas: milk fat globules were stabilized by sodium caseinate (NaCas), prior to mixing with LFCC.
3) AMC_Tween: as for 2, but with milk fat globules stabilized by Tween 20.
4) AMC_NC: natural cream (NC), where (non-homogenised) fat droplets stabilised with native milk fat globule membrane was mixed with LFCC.

The previous chapter also introduced the concept of 'active fillers' and 'inactive fillers'. The emulsions applied in preparation of AMC within this study were hypothetically designed to conform to either of these interactive states. Thus, fat globules with NaCas at interface were expected to act as 'active' fat fillers; droplets stabilised with Tween 20 were likewise expected to act as 'inactive' fat fillers within AMC. For the AMC_NC system, fat droplets were covered with native milk fat globule membrane, comprising phospholipids and proteins (Dewettinck, Rombaut et al. 2008). The interfacial materials of the fat globules were not manipulated on the cheeses made from AMF. The fat fillers of AMC_AMF should be kind of inactive fillers due to the lack of interactions to protein matrix, and however these unmanipulated AMF fillers should behave different from the Tween covered inactive fillers.

Based on previous studies (Michalski, Cariou et al. 2002, Michalski, Gassi et al. 2003, Everett and Auty 2008) it was anticipated that AMC_NC would behave as cheese containing inactive fillers, allowing comparison to be made to the model emulsion systems used in this study. The native milk fat globule membrane is very effective at protecting fat globules from coalescence in the milk system. However, when the droplet phase volume fraction of solid fat is high enough, and droplets contain an appreciable level of solid fat, non-homogenised droplets of milk fat can be prone to partial coalescence (Pawar, Caggioni et al. 2012), particularly if the emulsion is sheared. Accordingly, the distribution of fat within the protein network (as determined by the type of interaction between interface and protein) could be expected to have a significant impact on relative stability of the emulsion droplets within the AMC, with a corresponding influence on the material and functional properties of the cheese.

For AMC prepared with active fillers (chapter 2, section 2.3), interactions with the protein matrix might be expected to lead to uniform distribution of fat within the protein network, this limiting coalescence by minimising droplet-droplet interactions. AMC prepared with inactive fillers are speculated as being more likely to coalesce in producing and/or partial coalescence in chilled storage because the emulsion can become localised within serum pockets during manufacture, with these domains behaving as concentrated oil-in-water emulsions.

To study this behaviour: fat globule size distribution, fat dispersion in matrix and cheese melting behaviour were analysed on AMCs, using the methodologies outlined in chapter 3. Fat globule size distribution and structure, as measured by laser light scattering and confocal microscopy, would be used to determine dynamic changes to fat structure and stability as a consequence of AMC processing and storage (chapter 3, section 3.5.1 and 3.5.2). Small strain rheology and Schreiber test were used to study cheese melting and flow. Rheological measurements would be able to determine how fat filler interactions would affect cheese material properties during heating. In this respect, the melting transitions of both the dispersed phase (melting of milk fat) and continuous phase (relaxation of the protein network) need to be considered as potentially contributory to cheese material properties. Therefore, the storage modulus ratio of AMC containing fat to that of an AMC composition prepared without fat ln(G'/Gm') was used to determine how fat fillers impact cheese firmness (chapter 3, section 3.5.3). Additionally, cheeses were heated up to baking temperatures (170 °C) in Schreiber test, where cheese flowing extent, oil-off and water loss in baking were analysed to ascertain the role of fat in cheese melting behaviour (chapter 3, section 3.5.4 and 3.5.5).

5.2 Microstructure of fat dispersion in protein matrix

Confocal microscope images are presented in figure 5.1 comparing the microstructure of AMC made with emulsifiers (AMC_NaCas and AMC_ Tween 20) and without emulsifiers (AMC_AMF). All three AMCs were produced using the same RVA profile (30 minutes 800 rpm 60 °C). For AMC prepared via direct incorporation of AMF, figure 5.1a showed the fat phase to comprise a relatively homogeneous distribution of spherical fat droplets.

The fat droplets were typically in the range of 10 - 50 µm. It could be seen that RVA processing was able to break up AMF into emulsified fat globules when incorporated within the cheese, but some large localised fat areas were still observed (either due to the limited application of shear in processing the AMC, or as a consequence of droplet surface area being constrained by the availability of surface active material present within the cheese materials, such as proteins).

For AMC prepared with NaCas stabilised emulsions, figure 5.1b showed many small fat droplets homogeneously dispersed within the protein matrix, with no localised domains of fat clusters. The emulsion mean droplet size D[4,3] was ~ 1 µm before making AMC_NaCas and consequently it was less easy to visualise the fat in the confocal images, where figure 5.1b shows fat droplets as tiny red/yellow points since the CLSM resolution is usually limited to particles >1 µm in size. The uniform separation of NaCas covered fat globules and their entrapment within the protein matrix minimised fat droplet-droplet contact, and thus coalescence was not observed. For Tween 20 stabilised emulsion droplets, figure 5.1c and figure 5.1d show AMC samples with irregularly structured localised domains of fat, which were not free fat pools but aggregated fat globules. The large domains of inactive fat fillers have been observed in emulsion-filled gels as well (Liu, Stieger et al. 2015) and were also visualised in the chapter 4.

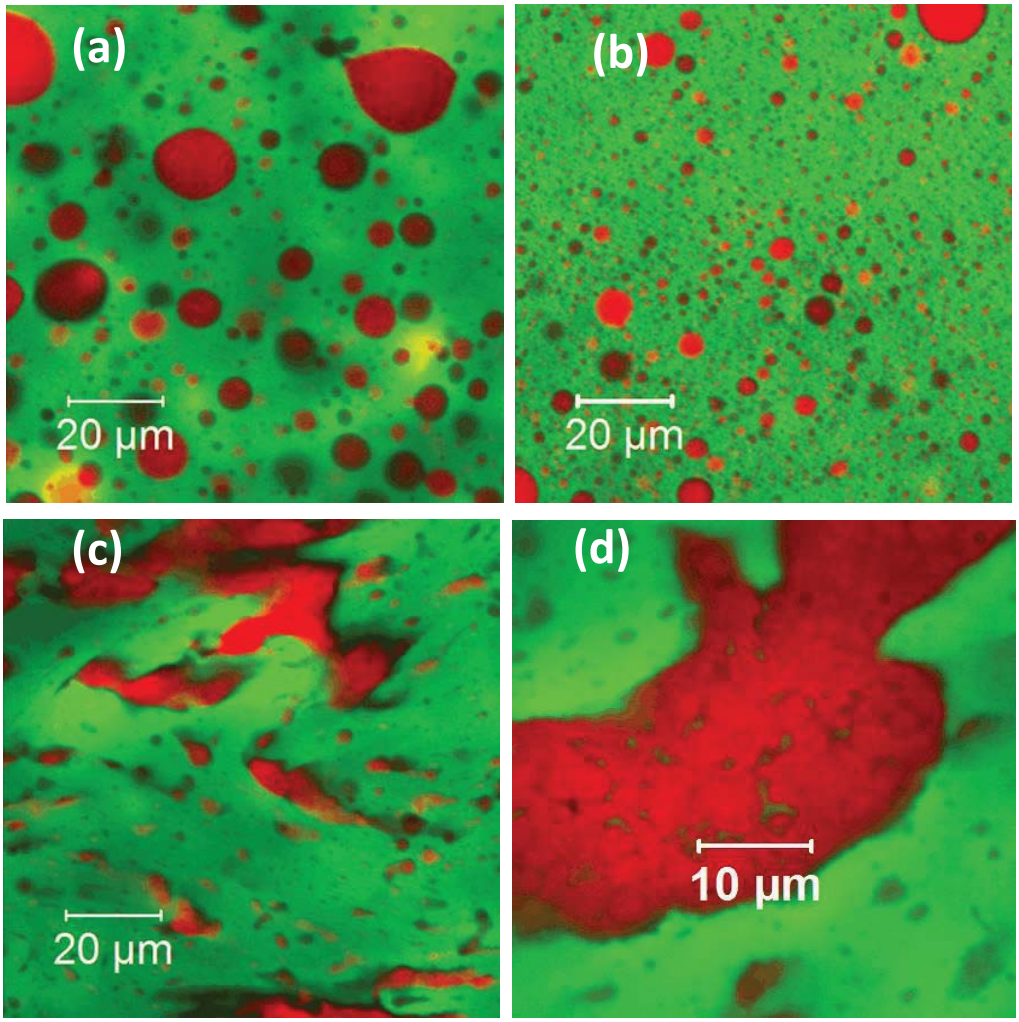


Figure 5.1: CLSM images of the AMC made from anhydrous milk fat (AMF) without emulsifiers (a), fat globules emulsified by NaCas (b) or Tween 20 (c, d) in 30 minutes RVA processing. The image of magnified fat domains is shown in (d). Red is fat, green is protein, black is serum or air.

AMC made from natural cream showed more dynamic changes in microstructure during RVA processing (Figure 5.2). In the first 10 min, when ingredients were well mixed, CLSM micrographs (Figure 5.2a) showed both localised fat domains and individual fat globules in the cheese. The size of fat domains was not that large as the regions observed in AMC_Tween, however the similarity in structure did indicate non-interactive droplets for both types of emulsion. However, it was interesting to note that the fat aggregates in AMC_NC became smaller with continuous RVA processing (Figure 5.2b). Fat channels were thinner and longer, and fat globules started to separate from each other with an increasing amount of fat being dispersed as individual droplets within the protein matrix.

After 30 minutes RVA processing, the droplet dispersion was almost entirely uniform, with the disappearance of any localised domains (Figure 5.2c). This lead to the supposition that the microstructure of AMC_NC was representative of an inactive filler emulsion at beginning of processing, with droplets displaying increasingly individual fat globules as a consequence of extended shear. The interfacial structure of natural cream could be modified during increasing residence time of AMC processing. It has been observed that serum release from molten cheese curd contained small amounts of whey and casein proteins (as reported in chapter 6). It was entirely possible that adsorption of serum proteins at the oil-water interface during extended AMC processing was likely to change fat globules from inactive fillers to active fillers. This part of study will be further explored in the chapter 6.

Hassan and Awad (2005) and Rogers, McMahon et al. (2010) both observed the fat-serum channels were disappeared in a fat-reduced Cheddar cheese compare to a full-fat Cheddar cheese. The process used in Cheddar cheese was markedly different from AMC, and the change of fat dispersion in Cheddar cheese was most likely a consequence of changes to fat volume fraction. Rogers, McMahon et al. (2010) observed coalesced fat structures in Cheddar cheese within 23.0 wt.% fat, while the study here on AMC_NC within same fat content (23 wt.%) showed the fat dispersion to be stable to subsequent coalescence, once droplets were homogeneously dispersed within the matrix.

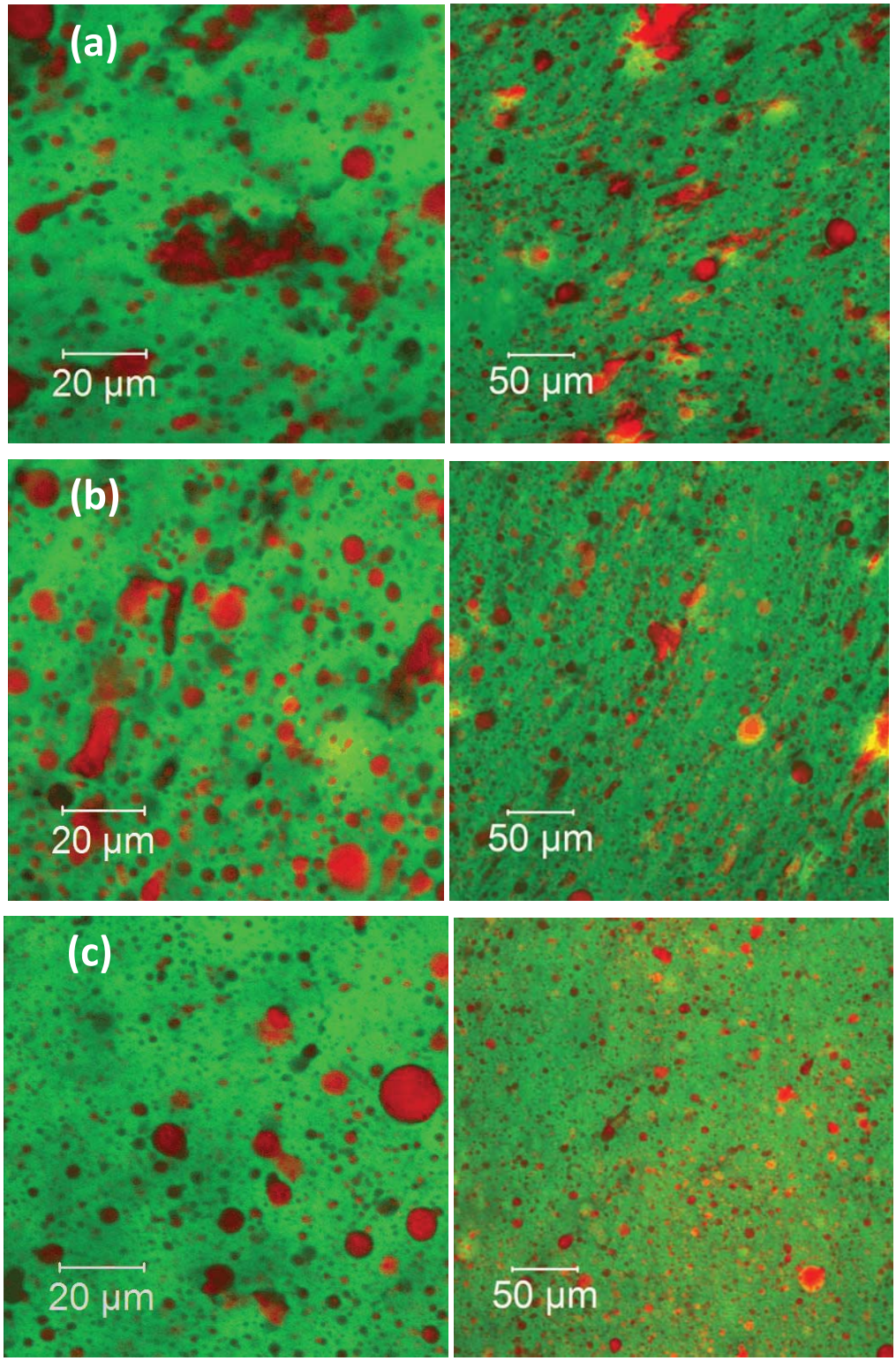


Figure 5.2: CLSM images of AMC made from natural cream in 10 minutes (a), 20 minutes (b) and 30 minutes (c). The photos were taken using the lenses of x 40 (left) and x 25 (right). Red is fat and green is protein, black is serum or air.

5.3 Fat globule size distribution

Fat globule size distribution within AMC was measured for cheese dissociated in the Walstra solution (chapter 3, section 3.5.1), which disassembles the protein network, thereby releasing the entrapped fat droplets. Analysis of Walstra treated AMC droplets can provide insights to the structural changes taking place to the emulsions, but is not without limitations. Droplets displaying no or limited change in size or structure as a consequence of AMC manufacture and storage could be readily analysed using the mastersizer. However, highly destabilised emulsions could most also produce structures outside of the measurable range of the instrument. Additionally, unstable fat tends to coalesce forming visible fat rising to the top of the Walstra solution at ambient temperature and thus they are not being effectively taken up for sizing. Accordingly, the methodology and technique can best be used qualitatively to compare structural variance between samples.

Figure 5.3 shows the fat globule size of AMC made with non-homogenised milk fat (AMC_AMF). After 10 minutes residence in the RVA, fat globule size of the AMC included a small peak of 0.1-1 µm, a primary peak of 1-30 µm and a slight peak > 100 µm. The AMC prepared after 30 minutes residence time showed a reduction in the main modal particle size distribution with an absence of droplets of > 20 µm. However, visible fat was observed in the Walstra solution for both AMC_AMF samples, and large fat domains in excess of 20 µm were observed in CLSM images of AMC_AMF samples prepared using 30 minutes RVA residence time (Figure 5.1a), indicating the likely presence of fat structures beyond the measuring range of the mastersizer. The floating fat in Walstra solution came from pockets of unstabilized AMF dispersed throughout the curd network, which were released upon the dissociation of the protein network, forming larger pools of lipids at the solution surface. The size of the floating pools of lipids would not represent in the size of droplets using mastersizer, and the CLSM images presented a better true distribution of fat within the cheese.

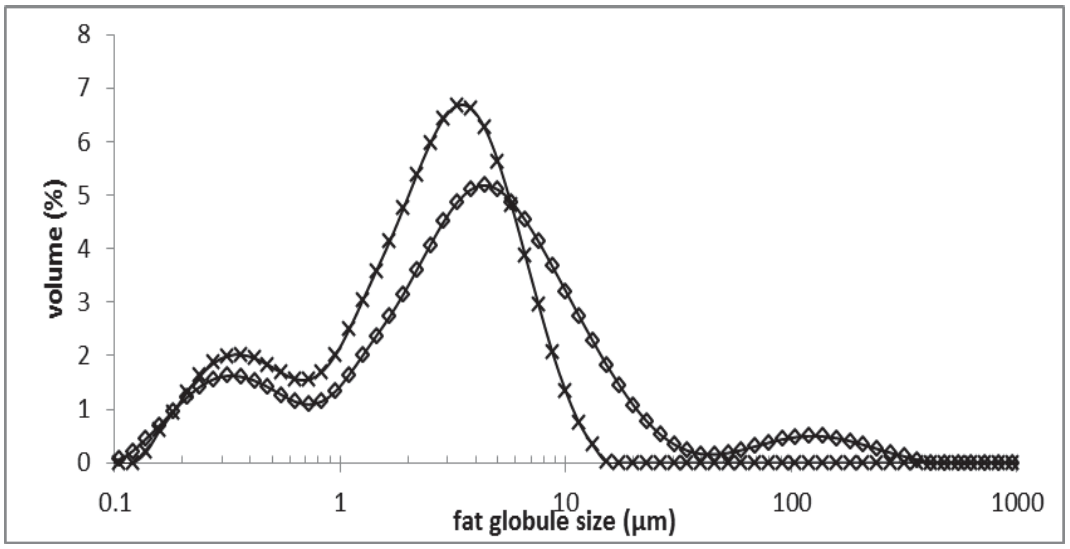


Figure 5.3: Fat globule size distribution of AMC_AMF made in RVA processing of 10 minutes (◇) and 30 minutes (x). Samples were measured after 4 °C storage.

The droplet size distribution of AMC prepared through a combination of LFCC with a sodium caseinate stabilised milk fat emulsion (AMC_NaCas) is presented in figure 5.4. Data showed that fat globule size was relatively stable in a consequence of AMC manufacture when comparing to the cream emulsion used for cheese making. Visible fat did not appear when AMC_NaCas was dissociated in Walstra solution, even for AMC that had been stored previously for 7 days at 4 °C. Fat globules trapped in matrix of AMC_NaCas (Figure 5.1b) were unlikely to encounter each other and coalesce to larger particles, and therefore particle size of fat was stable (Figure 5.4) after cooling and storage. In contrast, the fat globule size AMC prepared with the Tween 20 stabilised emulsion (AMC_Tween) appeared greatly impacted by cheese processing and storage (Figure 5.5). The mean particle size of fat droplets increased from ~ 1 µm to ~ 4 µm immediately after cheese processing. After 7 days' storage at 4 °C, figure 5.5 showed an additional modal distribution of particles in the range of 100 µm (additionally, visible fat in Walstra solution was observed when the AMC was dissociated). The large particles at ~ 100 µm and the visible fat in Walstra solution most likely resulted from partial coalescence taking place during chilled storage. The CLSM of the fat dispersion in section 5.2 helped to understand the change of particle size. The close proximity of fat droplets as a consequence of localisation of cream emulsion within the protein structure

promoted partial coalescence of fat as in oil-in-water emulsions when the cheese is chilled and the fat begins to crystallise. This would in turn lead to full coalescence of fat domains when the temperature of the cheese was increased such that the fat phase became molten. Fat coalescence in AMC_Tween has been confirmed by the increased particle size shown in figure 5.5.

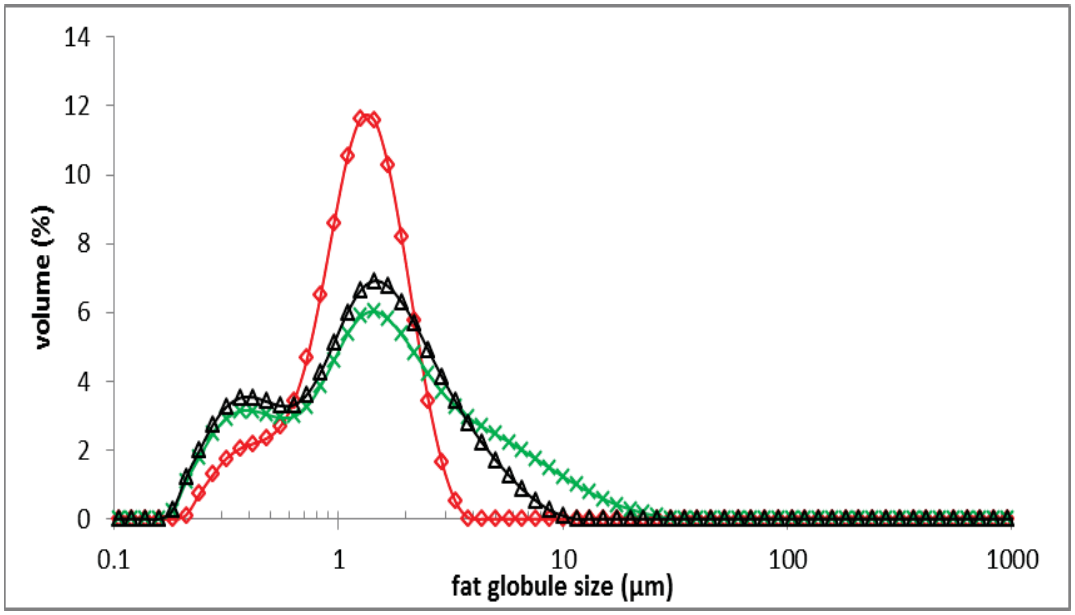


Figure 5.4: Fat globule size distribution before and after 30 minutes AMC_NaCas producing. The samples are cream emulsion used for AMC_NaCas making (◇), fresh AMC_NaCas without cooling (X) and AMC_NaCas after 7 days storage at 4 °C (△).

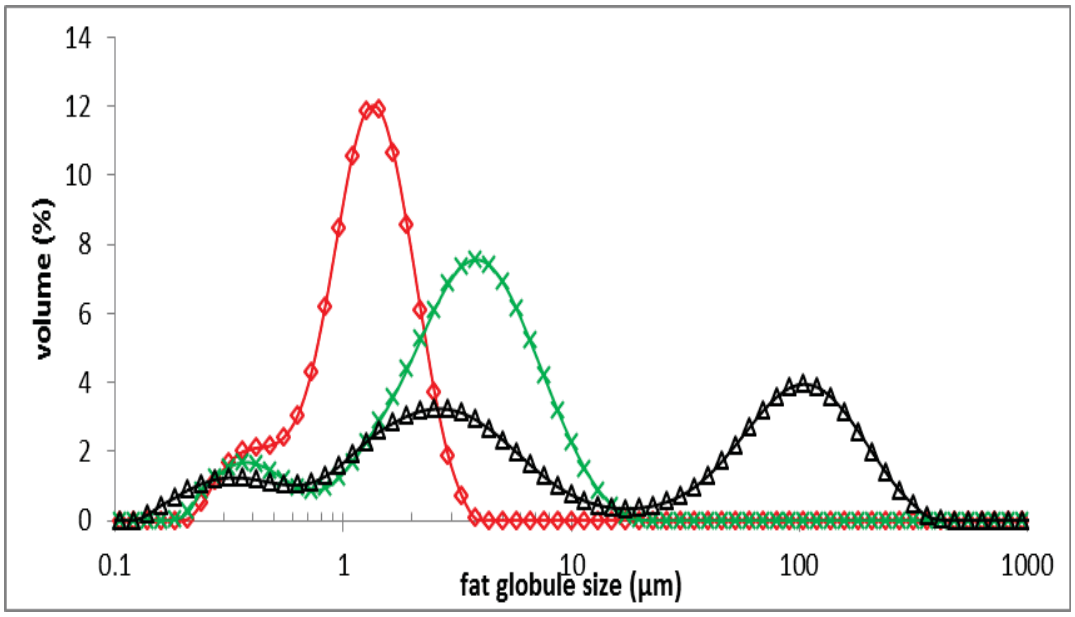


Figure 5.5: Fat globule size distribution before and after 30 minutes AMC_Tween producing. The samples are cream emulsion used for AMC_Tween making (◇), fresh AMC_Tween without cooling (X) and AMC_Tween after 5 days storage at 4 °C (△).

The results of particle size distribution for AMC prepared using non-homogenised natural cream (AMC_NC) are presented in figure 5.6. Figure 5.6a compared the particle size distribution from the cream to fresh cheese, where a similar distribution was observed, indicating relatively little change in droplet size had occurred as a consequence of preparation. However, the particle size distribution after cheese storage at 4 °C was noticeably different, being additionally influenced by the processing conditions used in preparation. AMC_NC based on 10 minutes residence time displayed a broad monomodal distribution of particles between 1 µm and 100 µm. When residence time was increased to 20 minutes the distribution showed a transition toward a bimodal distribution. When cheese was made in 30 minutes particles fewer particles in the larger size range were observed, with a more pronounced modal distribution in the range 1 – 10 µm, with little visible fat observed after cheese dissociation in the Walstra solution. Partial coalescence appeared markedly less prevalent in AMC_NC produced in 30 min. CLSM (Figure 5.2) supported the particle size distribution findings, with fat domains become progressively smaller, eventually fully disappearing over extended residence times, and were thus less likely to partially coalesce on cooling in 30 minutes

made cheese. For AMC prepared with 10 minutes residence within the RVA, particle size did increase when cooling cheese to 4 °C (Figure 5.6b). The particle size of primary peak in 10 minutes AMC_NC shifts from 4 µm to 30 µm after cooling storage, and visible fat appeared in dissociated cheese by Walstra solution.

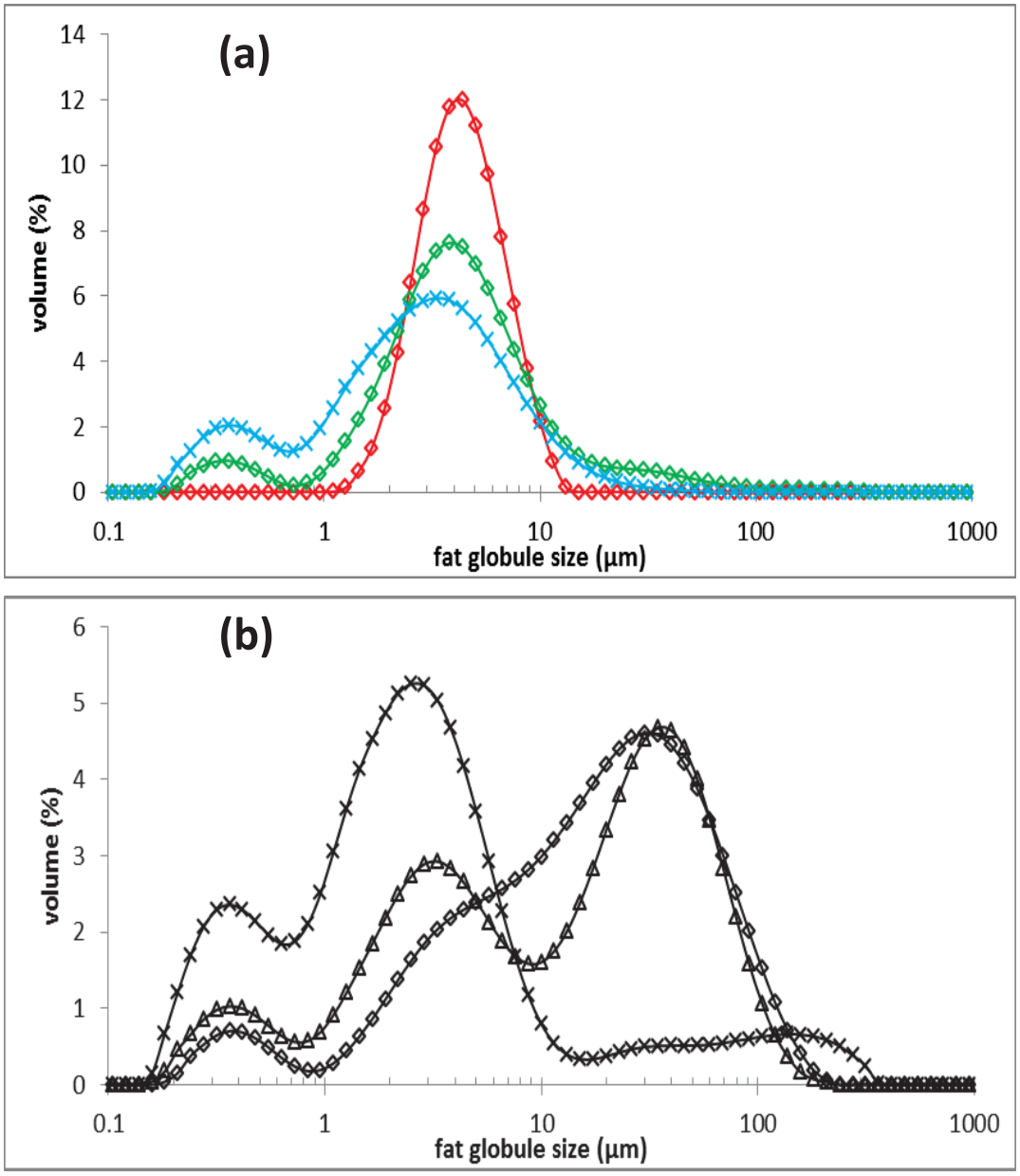


Figure 5.6: (a) Fat globule size distribution before and after AMC_NC producing. The samples are natural cream used for AMC_NC making (◇), fresh AMC_NC made in 10 minutes without cooling (◇) and fresh AMC_NC made in 30 minutes without cooling (X); (b) Fat globule size distribution of AMC_NC after 7 days storage at 4 °C. AMC were made in 10 minutes (◇), 20 minutes (Δ), and 30 minutes (x), respectively.

The impact of partial coalescence on increased particle size distribution was investigated further by manipulating the cooling temperature and cooling rate of AMC_NC after 10 minutes residence time within the RVA (Figure 5.7). Cheese was cooled from 40 °C to 8 °C in 3.5 min, at which point a sample was taken for particle size analysis. Samples were also taken after one day and seven days' storage at -18 °C. Cheeses were dissociated in Walstra solution at ambient temperatures prior to particle size analysis. The particle size fat droplets in the cheese where temperature was maintained at 40 °C cheese (the green curve in figure 5.7) did not change significantly when compared to the fat droplet size of the cream used for cheese making (the red curve in figure 5.6a). A new peak of larger particles appeared for the cheese that had been cooled to 8 °C after preparation, indicating partial coalescence had occurred at 8 °C as a consequence of solid fat crystallisation. The modal distribution of particles in the range 1 µm - 10 µm increased further during storage at -18 °C, with a modal distribution in the range 10 µm - 200 µm being predominant after one days' storage. After 7 days' storage at -18 °C the volume weighting of this distribution increased over the additional storage period. It was particularly interesting to note that the dynamics of partial coalescence were not inhibited at the temperatures where the droplets would be almost entirely crystalline (solid fat > 80 % at -18 °C (Mulder and Walstra 1974)), possibly suggesting a slow accretion process between fat crystals of neighbouring droplets located within the fat channels of the AMC. Damage to fat droplets arising from ice crystallisation at -18 °C was also considered as being a mechanism which could have been responsible for the increase in partial coalescence at these lower temperatures.

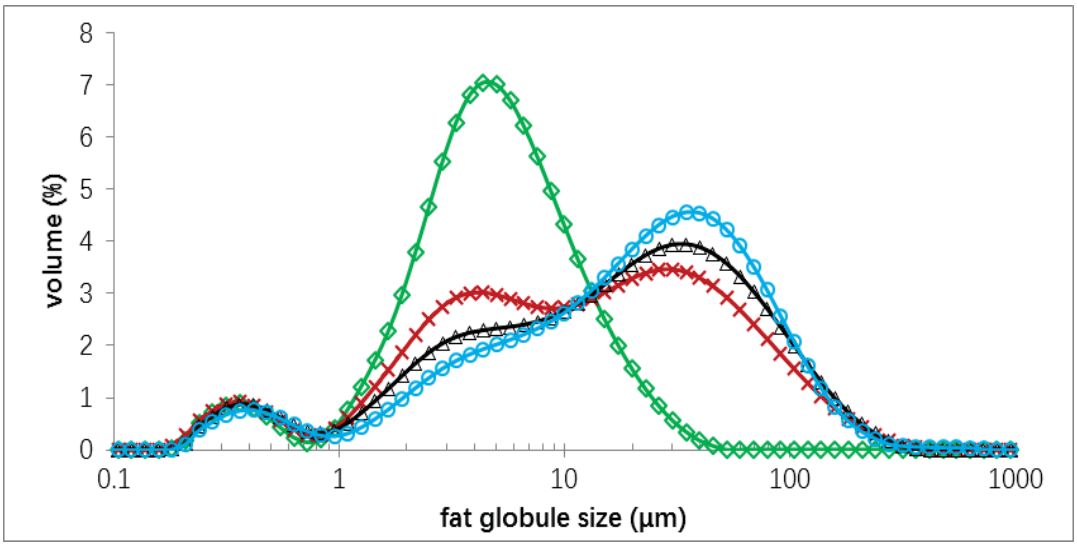


Figure 5.7: The cheeses were produced in 10 minutes using the natural cream, and the fat globule size distribution was monitored during cheese cooling down in a -18 ° C storage room. Green curve (◇) is the fresh cheese just after manufacture at 40 ° C. The cheese was cooling to 8 ° C in 3.5 min (X). The other two samples were the cheeses stored at -18 ° C for one day (△) and 7 days (○), respectively.

5.4 AMC rheological properties during heating

The small strain rheological properties (G' and G") were measured for AMC after 7 days' storage at 4 °C, and cheese samples were heated from 4 °C to 80 °C (chapter 3, section 3.5.3). For AMC samples modulus (G') was generically higher than loss modulus (G") at low temperatures, exhibiting predominantly elastic-like characteristics for all samples prepared. During heating, G' decreased faster than G", with cheeses being defined as becoming molten for G' < G". The temperature when G' crossing over G" was recorded in table 5.1 used to compare AMCs prepared with different emulsion states. AMC were prepared for both short residence time and increased residence time within the RVA. The cross-over temperature point was stable with residence time for AMC_NaCas and AMC_Tween. The moduli of AMC_NaCas crossed at ~ 62 °C, which was significantly higher than the crossover temperature of AMC_Tween (~ 53 °C).

The NaCas covered fat globules, predicted as acting as active fillers, were considered as having strong interactions with the surrounding protein matrix, serving to reinforce the protein network against relaxation and flow as the temperature rises. Conversely, Tween 20 covered fat globules, being inactive fillers, were hypothesised as not binding to the protein matrix. It could be speculated that the localised fat channels of inactive fillers

served to reduce the cohesivity of the protein network, promoting flow behaviour and releasing oil off on cheese melting, and therefore cheese was more fluid-like compared to AMC_NaCas. The drop of G' for these AMC variants during cheese heating was only slightly affected by increased residence time (Figure 5.8a) when fat globules were fully covered with NaCas or Tween 20 (i.e. AMC_NaCas and AMC_Tween). The decline of G' in AMC_NaCas was relatively flat compared to AMC_Tween, where G' decreased faster at temperatures >30 °C. As already demonstrated, the structure of AMC_Tween presented large fat domains (Figure 5.1c) which, for the reasons stated above, may be considered contributory to the lower modulus observed at elevated temperatures when compared to AMC_NaCas, where fat clusters were not observed (Figure 5.1 b)

Table 5.1: The temperature of G' and G" crossover point in small strain rheological analysis

AMC	Residence time in RVA	Crossover temperature of G' equal to G"
AMC_AMF	10 minutes	57.7 ± 3.6 °C
	30 minutes	60.7 ± 2.9 °C
AMC_NaCas	10 minutes	63.8 ± 2.2 °C
	30 minutes	64.8 ± 2.9 °C
AMC_Tween	30 minutes	53.1 ± 0.9 °C
	60 minutes	53.4 ± 2.6 °C
AMC_NC	10 minutes	56.1 ± 2.7 °C
	30 minutes	60.2 ± 0.1 °C

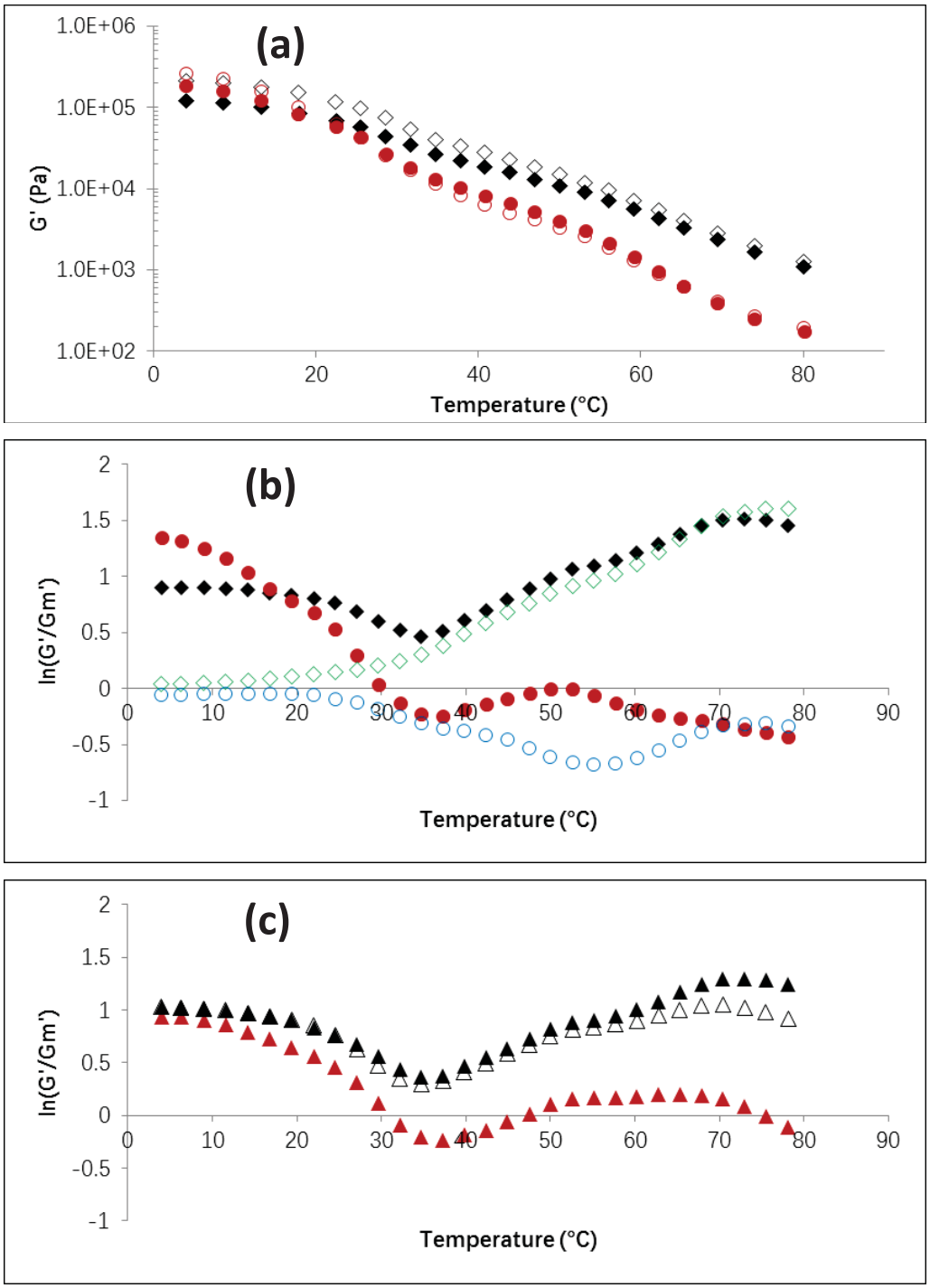


Figure 5.8: Small strain rheological properties from 4 °C to 80 °C in AMC after 7 days storage at 4 °C. (a) Storage modulus (G') of AMC made from different residence time. Samples are 10 minutes AMC_NaCas (◆), 30 minutes AMC_NaCas (◇), 30 minutes AMC_Tween (●) and 60 minutes AMC_Tween (○). (b) Storage modulus of AMC within fat fillers (G') was compared to non-fat AMC of 68 % moisture (G_m'). The non-fat AMC includes the same ratio of protein to water as AMC within fat fillers. In (G'/ G_m') indicates the impact of fat fillers on cheese texture. Samples are 10 minutes AMC_NaCas within milk fat (◆), 10 minutes AMC_NaCas within canola oil (◇), 30 minutes AMC_Tween within milk fat (●) and 30 minutes AMC_Tween within canola oil (○). (c) Comparison of AMC_NC made in 10 minutes (▲), 20 minutes (Δ) and 30 minutes (▲).

Further observations of the rheological data indicated that the crossover temperature of both AMC_AMF and AMC_NC increased as shearing residence time within the RVA was increased (Table 5.1). Notably, the crossover points of all these cheeses were below those determined for AMC_NaCas (~ 64 °C), but above AMC_Tween (~ 53 °C), possibly indicating a transitional structural state between active and inactive fillers. The change in rheological behaviour observed here could be considered a consequence of dynamic changes occurring as a consequence of mixing time. Modified microstructure of AMC_AMF and AMV_NC in the increased shearing residence time was observed in the discussion in section 5.2.

To determine which structural elements were being altered, the temperature dependent rheological profile of a non-fat AMC was measured for samples prepared with increased RVA shearing times. It was observed that the rheological profile of the non-fat AMC did not change with increased RVA residence time from 10 minutes to 30 minutes (data not shown), indicating the change on AMC_NC and AMC_AMF was most likely due to dynamic changes to fat structure and state of fat-protein interactions rather than specific changes to the protein phase.

The dynamic changes to emulsion structure of AMC_NC during shearing have been highlighted in section 5.2 and 5.3, showing a time dependent reduction in the size of the fat channels towards a more uniform distribution of smaller fat droplets. The increase in crossover temperature with increasing residence time was consistent with prior observations from particle size and microscopic analysis that the emulsion state is transitioning from inactive/localised emulsion droplets to active/homogeneously dispersed droplets.

Insight as to the effect of fat filler interactions on cheese material properties in relation to temperature could additionally be gained by determining the value of $\ln(G'/ G_m')$ across the temperature range of 4 °C - 80 °C as shown in figure 5.8b.

Figure 5.8b shows positive values of $\ln(G'/ G_m')$ for AMC_NaCas cheese for which the emulsified milk fat droplets were considered to be acting as active fillers. Thus,

interaction of the fat droplets with the protein network resulted in a strengthening role on cheese material properties. Here, protein melting caused a relaxation of the protein network, allowing flow to take place. However, this effect was to some extent inhibited due to the binding of fat droplets to the protein network, acting as anchor points that served to hold the structure together to strengthen the texture (van Vliet 1988, Xiong and Kinsella 1991, Chen and Dickinson 1999, Dickinson and Chen 1999). In contrast, inactive fat fillers, as exemplified by AMC_Tween 20, appeared to cause a weakening effect cheese material properties above 30 °C, showing relatively negative values of ln(G'/ Gm').

In the publication of Dickinson and Chen (1999), the effect from the inactive fillers was greater in the protein matrix of higher elastic modulus, but the results here indicated the weakening effect from inactive fillers becoming more pronounced as the sample was heated from 50 °C to 80 °C, and where the value of ln(G'/ G_m') becomes increasingly negative during melting and relaxation of the protein network. The dispersion of fat within the AMC structure was notably different to the heat-set whey protein emulsion gels described in the investigation from Dickinson and Chen (1999), comprising a localised distribution of fat pockets observed in AMC here, rather than a homogeneous droplet dispersion, as observed for the Dickinson's study. Increasing temperature not only caused a relaxation of the protein structure, but for the localised fat phase, lead to extensive coalescence of droplets and the formation of liquid oil channels. These fat domains created large holes which disrupted the homogeneity of the protein network resulting in a softening of AMC material properties.

Figure 5.8b also shows the greater impact of solid fat content on the material properties of AMC for the active/inactive filler emulsions. To explore the role of solid fat content, AMC samples comprising canola oil emulsions were also prepared and analysed, noting that canola oil is in a liquid state across the entire temperature range of study. For inactive fillers, at temperatures below 35 °C the value of ln(G'/ G_m') was higher in cheese made from milk fat compared to cheeses prepared with canola oil, with the gap in relative modulus increasing as temperature was lowered. The figure 4.4 in chapter 4 showed the G' of milk fat declined from ~30 °C, and was extremely low above 35 °C. The increase in cheese firmness corresponded with crystallisation and increasing solid fat

content of the milk fat as the temperature was lowered, indicating that the material properties of the fat phase provided a specific contribution to the overall material properties of the cheese where crystallisation occurred in the emulsion system.

It can also be seen that at temperatures below 17 °C, the cheese containing inactive crystallized milk fat filler droplets displayed a higher relative elastic modulus when compared to the AMC containing active milk fat filler droplets. This was attributed to the formation of large channels of partially coalesced fat clusters of inactive fillers (as observed by particle size analysis in section 5.3). The modulus of crystalline milk fat at low temperatures (e.g. <30 °C) could be higher than cheese protein matrix indicated in figure 4.4, and it was not surprising that the high modulus associated with solid fat had a pronounced firming effect on the AMC at temperatures where the fat was crystalline. The mechanical properties of the solid fat domains, observed for inactive fillers, were likely to contribute more to cheese firmness than the individual dispersed active fillers (even though the effective solid fat content is the same for both types of AMC), based on previous observations that droplet clustering and agglomeration had been found to amplify the effects of dispersed phase material properties on the stiffness of emulsion-filled gels, even in the absence of discrete bonds between adjacent emulsion droplets (Oliver, Berndsen et al. 2015, van Aken, Oliver et al. 2015). In contrast, at temperatures below 10 °C the ln(G'/ G_m') value was close to zero for model cheeses with either active or inactive droplets containing canola oil, indicating that in this temperature range cheese material properties were dominated by the protein component, and that molten fat droplets, irrespective of their structural arrangement, had little contribution to the overall material properties of the cheese.

Interestingly, at the temperatures above 35 °C, it could be seen that the rheological behaviour of the AMC prepared with canola oil active fillers matched that of the active fillers comprising milk fat (Figure 5.8b). This was perhaps to be expected, since the interfacial layer, the rheological/mechanical properties of the emulsion droplets (in that both fat types are essentially molten for temperatures >35°C), and the dispersion state of the emulsion within the AMC were equivalent for both fat types. However, for inactive fillers there was a divergence of rheological behaviour between the two fat types, even though both emulsions were fully molten, indicating that the microstructural

states of milk fat inactive fillers and canola oil inactive fillers may be different. The differences in localised fat microstructure were also expected to impact cheese functional properties, based on previous observations that, for a given fat content, the size of the inactive filler domains was influential on the material and melting characteristics of cheeses (Michalski, Cariou et al. 2002, Michalski, Camier et al. 2007).

The rheological properties of AMC made from non-homogenised natural milk fat cream are compared in figure 5.8c. For AMC_NC the residence time within the RVA was seen to significantly alter the rheological profile of the AMC produced. When compared to the profiles of both active fillers (AMC_NaCas) and inactive fillers (AMC_Tween) type AMC, as shown in figure 5.8b, AMC_NC (figure 5.8c) prepared with 10 minutes shearing residence time exhibited a rheological profile closer in behaviour to that of the AMC prepared with inactive fillers (AMC_Tween). The 10 minutes AMC_NC mostly followed the profile of the AMC_Tween, where the value of $\ln(G'/ G_m')$ dropped quickly from positive to negative at 30 °C during heating and then increasesed marginally from ~35 °C, followed by a drop again above 50 °C. However, with increased residence time the profile transitioned towards that observed for the active filler AMC. The rheological behaviour of 30 minutes AMC_NC was very close to AMC_NaCas, whilst 20 minutes AMC_NC showed somewhat intermediate behaviour between the two interaction states, particularly over the temperature range of 60 °C – 80 °C.

The impact from fat fillers in AMC_NC should come from a balance of active fillers and inactive fillers. AMC_NC made in 10 minutes exhibited both individual fat globules and fat clusters when visualised by CLSM (Figure 5.2a). Regions comprising individual fat globules were more likely to be active fillers strengthening cheese texture, resulting in firmer texture in 10 minutes AMC_NC than AMC_Tween above 50 °C. The localised fat domains in 10 minutes AMC_NC appeared smaller than the ones observed for AMC_Tween, and were presumably less influenced by fat crystallization. These localised domains gradually disappeared as cheese residence time within the RVA was increased to 20 minutes or 30 minutes (figure 5.2 b, c), and accordingly rheological behaviours showed greater similarity to that of the active filler system (AMC_NaCas). Although fat globules were seen to be homogeneously dispersed for both AMC_NaCas and 30 minutes AMC_NC, the smaller fat globule size associated with AMC_NaCas (D[4,3]=

~1µm) could have been responsible for the higher relative modulus compared to AMC_NC (D[4,3]=~4µm), possibly due to increased interfacial area as droplet size decreased. Michalski, Cariou et al. (2002) reported a similar dynamic change on the milk gels: storage modulus of the milk gels increased with the modification of milk fat globule interface materials due to mechanical treatment, where caseins and whey proteins partly or fully covered the damaged native milk fat globule surface.

5.5 Cheese melting properties

5.5.1 Flowing extent after baking

The melt profiles of AMC with varying fat structures are shown in figure 5.9. After baking in an oven at 170 °C for 10 minutes, the extent of flow was measured and compared between AMCs with various interfacial materials. For AMC prepared by direct mixing of milk fat with LFCC in the RVA (AMC_AMF), sample was seen to spread uniformly after baking and the flowing extent area (1389 mm^2) was approximately four times of the area before baking (314 mm^2) (Figure 5.9a).

For AMC prepared by mixing with the NaCas emulsion, the cheese (AMC_NaCas) was observed to be markedly more resistant to melt. Spread of flow was also seen to be non-uniform, as indicated by the arrows in figure 5.9b. The cheese did not really flow during baking, showing that one side of the slice edge did not move after baking. Oil-off was not observed for the AMC_NaCas, and the directional flow of cheese was more likely due to sudden a structural collapse due to heating, rather than uniform melting of the cheese. Findings conformed to observations made from rheological analysis, which indicated that active fillers within the cheese provided a reinforcing effect to the protein matrix as it melted. However, it was interesting to note that the spraying of a thin layer of oil on cheese sample surface had been reported as improving cheese melting (Wadhwani, McManus et al. 2011). When this approach was applied to the AMC_NaCas, it was indeed observed that the melting behaviour of this AMC was improved (Figure 5.9d) with a uniform spreading and an increased flowing area compared to AMC_NaCas without oil brushed. Here it is considered that the oil layer prevented surface dehydration and lubricated proteins to spread uniformly.

In contrast, the AMC_Tween sample comprising inactive fillers was seen to melt with a large area of flow (2778 mm^2 in figure 5.9c), approximately 9 times of the area before baking (314 mm^2). As stated previously the partially coalesced fat globules in AMC_Tween were located in serum pockets and did not bind to the surrounding protein matrix. On heating and melting, water and fat are readily released to cheese surface during baking. The surface with oil-off prevented drying and additionally the free oil assisted in the relaxation of the protein matrix, resulting in large extent of flow. Protein-protein interactions within the cheese protein phase were considered the predominant factor contributing to cheese melting behaviours, observing that cheese flow and melt area increase as protein-protein interactions were decreased (Everett and Auty 2008). However, findings within this study have consistently demonstrated that the structure and stability of the fat phase can be particular consequence in relation to the melting properties of the cheese. In this context, the particular phase separation and coalescence of localised inactive filler domains during heating can be considered as acting as structure breakers that weaken the protein-protein interactions and structures, resulting in an increased extent of flow.

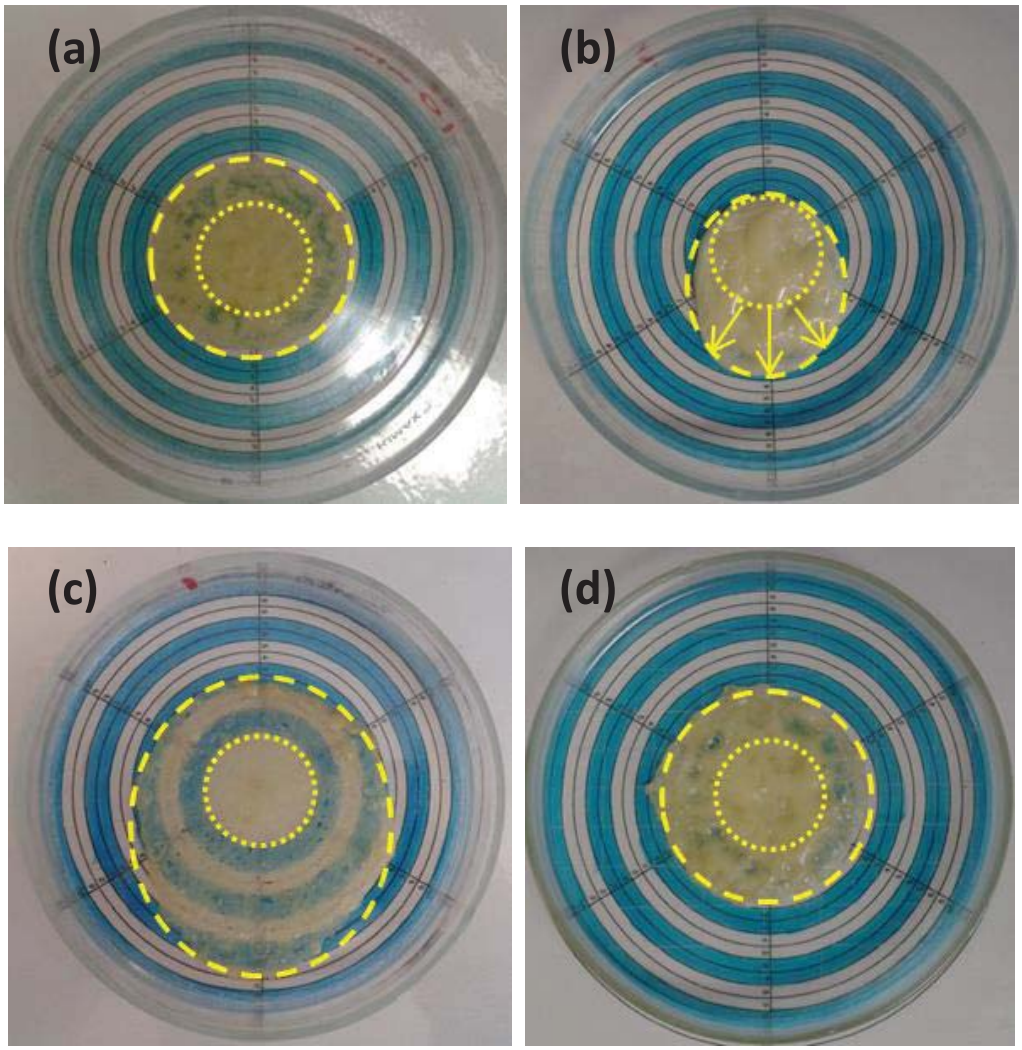


Figure 5.9: Cheese melting after 10 minutes baking at 170 °C. AMCs were made from anhydrous milk fat without emulsifier (a) and milk fat stabilised by NaCas (b) and Tween 20 (c). Photo (d) is the sample (b) with anhydrous milk fat brushed at cheese surface. The cheese area before baking was indicated by yellow dotted circles, which is the same area for all samples, and the large yellow dashed circles indicate cheese flowing in baking. The arrows indicate directional flowing by difficult melt.

Figure 5.10 is the images of baked AMC_NC produced in 10 minutes and 30 minutes, respectively. AMC_NC made with short residence time (10 minutes) was seen to melt with a flowing area of 1934 mm^2 somewhat smaller than for AMC_Tween (2778 mm^2), but clearly greater than that observed for AMC_NaCas (1389 mm^2). With increased RVA residence time the baking profile of AMC_NC samples showed decreasing extent of flow and directional spread of the melted cheese (Figure 5.10b). The extent of flow for all samples followed that order AMC_Tween > 10 minutes AMC_NC > 30 minutes

AMC_NC > AMC_NaCas. The change of flowing extent was consistent with the findings on rheological properties discussed in figure 5.8c: i.e. that fat droplets were transitioning from inactive-like to active-like when residence time was increased from 10 minutes to 30 min; fat droplets in AMC_NC were a mixture of active fillers and inactive fillers, and the performance of cheese melting depended on the balance between active and inactive droplets. The increased resistance to melt that occurred due to transition of inactive fat droplets to active fat fillers could accordingly be related to increased mechanical strength of the cheese structure due to reinforcement via formation of droplets anchoring within the protein network.

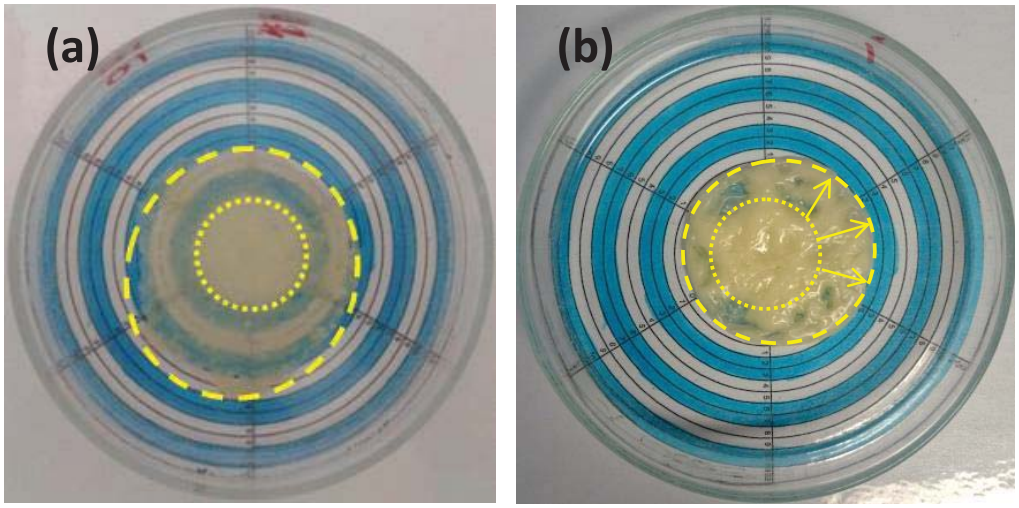


Figure 5.10: Cheese melting after 10 minutes baking at 170 °C for AMC made from natural cream in 10 minutes (a) and 30 minutes (b). The cheese area before baking was indicated by yellow dotted circles, which is the same area for both samples, and the large yellow dashed circles indicate cheese flowing in baking. The arrows indicate directional flowing by difficult melt.

5.5.2 Oil-off in baking

The amount of oil released during baking is an important quality indicator for cheese melting, as it has been shown to influence aspects of cheese melt behaviour and changes in appearance during cooking (i.e. the extent of surface browning and blistering)(Rudan and Barbano 1998, Everett and Auty 2008, Ma, Balaban et al. 2014). For these samples, processing time within the RVA was additionally varied to explore the effect of processing on AMC melt properties (Figure 5.11).

Cooking of AMC_NaCas was seen to result in no oil being released to cheese surface. The structural arrangement of the active filler particles within the AMC (as observed from confocal micrographs), in which fat droplets were relatively uniformly distributed within the protein network, limited droplet-droplet contact, and thus partial coalescence during storage was prevented. In addition, strong interactions between active fat fillers and protein matrix minimised any possible oil phase separation during cheese baking. Fat droplets therefore remained stable in cheese matrix on melting, and accordingly no oil was released.

The amount of oil released from AMC_Tween was determined as the highest of the AMC samples, as shown in figure 5.11. It was observed that 25.7 ± 1.8 % of the total fat concentration was released to the cheese surface for an AMC_Tween sample that had been prepared through 30 minutes shearing within the RVA. The oil-off increased to 34.3 ± 1.6 % when extending residence time in the RVA to 45 min; however, oil-off was seen to then decrease to a value of 17.3 ± 0.7 % for a sample that had been shear for 60 minutes during preparation in the RVA. As stated previously, fat globules covered with Tween 20 did not bind to the protein matrix but were localized in the serum pockets, where they became phase concentrated, underwent subsequent partial coalescence during storage and full coalescence during cooking.

The relaxation of the protein matrix during baking could be expected to allow localised domains of coalesced fat to become interconnected, resulting in increasing phase separation of protein and fat phases, and eventually allowing channelling of the liquid oil to the surface of the cheese during baking. The cohesion of protein network and the size of fat-serum channels were likely to both be influenced by residence time within the RVA, which in turn was seen to impact on the extent of oil-off.

This was also observed for the AMC_NC samples, where oil-off was seen to be highly dependent on RVA residence time, reducing from 11.6 ± 2.5 % to 0.5 ± 0.1 % when residence time was increased from 10 minutes to 30 min. The extent of oil-off decrease followed a log curve relative to residence time, but falling within the extremes observed below AMC_Tween and above AMC_NaCas. This intermediate oil-off behaviour again supported the findings in CLSM (Figure 5.2), rheology (Figure 5.8c) and flowing extent in

baking (Figure 5.10), in considering the that behaviour of fat fillers in AMC_NC presented a dynamic balance between inactive and active fillers, transitioning progressively towards 'active' filler properties with increased residence time during RVA preparation. The investigation of Michalski, Cariou et al. (2002) found the transition to active fat globules occurred from the membrane damage fraction above 40% when covered with caseins and whey proteins in mechanical treatment. Oil release in AMC_NC would be expected to follow the same mechanism as proposed for AMC_Tween, although it should be additionally considered that any individual fat globules in AMC_NC acting as active fillers would serve to strengthen cheese texture to stop matrix flowing/melting.

In this context, while it was interesting to observe that free oil in AMC_AMF declined with increased residence time, this trend was actually seen to be less impactful in relation to changes to the extent of flow behaviour as a consequence of processing. Instead, the magnitude of oil-off could not be related to the extent of AMC_AMF flow. From a structural perspective, oil-off was maximised when at low residence times for RVA mixing, resulting in the dispersion of fat as relatively large pockets within the protein network. As considered previously, these could become interconnected during melting of the protein network allowing release of oil to the surface. With increasing residence time, homogenisation of the fat phase resulted in a reduction in droplet size, and more uniform distribution of droplets within the protein network. On melting the greater separation of droplets would reduce the incidence of coalescence and accordingly less oil would be released. The fact that the amount of oil released had little impact on the extent of flow implied that liberation of a specific minimum concentration of oil appeared essential for flow to occur in the first place (given that AMC_NaCas for which no oiling off occurs did not flow), but that beyond this concentration further release of oil had little impact on the extent of flow. This may support the earlier observation that cheese flow was in fact governed by the formation of a surface layer of fat that promoted flow by inhibiting surface drying of the cheese.

To investigate the specific role of fat droplet partial coalescence on oil-off, AMC_Tween was made using canola oil, which would not be expected to undergo partial coalescence under AMC processing and storage conditions, since there was no solid fat present within the emulsion at these temperatures. Accordingly, oil-off measurements were

made for AMC_Tween comprising canola oil droplets with stored at 4 °C for 9 days. For canola AMC_Tween stored at 4 °C the oil-off during baking was reduced (12.4 ± 1.7 wt.%) when compared to the AMC_Tween prepared with milk fat (25.7 ± 1.8 wt.%) stored at the same temperature (4 °C). The fact that oil-off was not completely inhibited in canola AMC_Tween indicated that even though partial coalescence may not have taken place, some other form of emulsion destabilisation leading to coalescence must have occurred either during processing, storage or heating that would enable the release of free oil during baking.

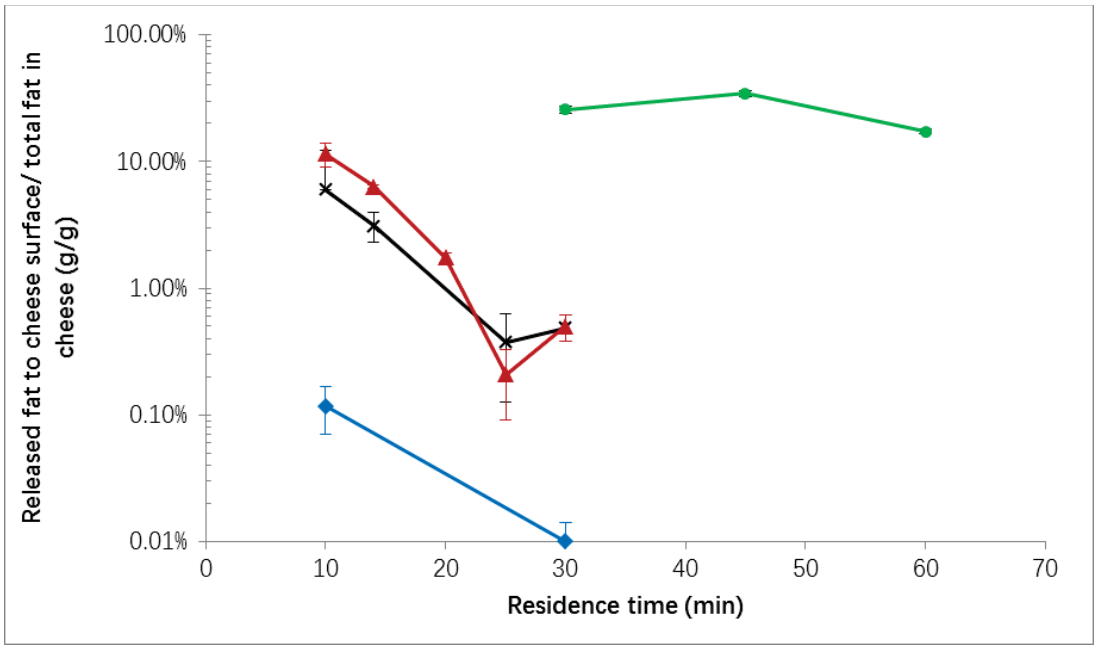


Figure 5.11: Free oil after 10 minutes baking at 170 °C was compared in AMCs within four types of fat fillers: manipulated fat globules covered with NaCas (◆) or Tween 20 (●); the anhydrous milk fat without emulsifier (x) and fat globules from natural cream (▲). X-axis is residence time of AMCs in RVA processing.

5.5.3 Water loss in baking

The loss of water in baking is considered as the change of weight of cheese samples after cooking (chapter 3, section 3.5.4), and was measured for cheeses in addition to oil-off analysis (Figure 5.12).

The water loss in AMC_NaCas (lost 29-39 wt.%) was found to be relatively low comparing to AMC_Tween (lost 63-72 wt.%) shown in figure 5.12. This indicates that the release of water from protein network may be hindered due to reinforcement of the protein network by active fat droplets. Large blisters from evaporated water were observed when baking AMC_NaCas, but the steam generated during cooking was not always successfully able to escape from the cheese matrix. The water loss in cheese baking not only comes from protein matrix but also fat-serum channels which exist in AMC_Tween. The cheese with inactive fat fillers underwent extensive flow (Figure 5.9c) and exhibited extensive oil-off (Figure 5.11). The increased spreading of the sample such as AMC_Tween under flow may provide a greater area for water evaporation from inside the cheese.

The change of water loss in AMC_NC was influenced by RVA residence time, showing behaviours consistent with other structural and functional properties. Water loss dropped from 56.2 ± 0.1 wt.% to 30.7 ± 3.4 wt.% when residence time was increased from 10 minutes to 30 minutes (Figure 5.12). The previous study found the transition of fat globules from inactive fillers to active fillers within the enhanced residence time of cheese production. The water from the fat-serum channels within the inactive fillers would evaporate with the oil-off, and the drop of water loss in baking was consistent to the decrease of oil-off shown in figure 5.11. CLSM images also indicated the disappearance of fat-serum channels in AMC made from 30 minutes (Figure 5.2).

The level of water loss in AMC_AMF likewise relatively stable with increasing RVA residence time (Figure 5.12). Fat in MC_AMF was not located in serum pockets and thereby water loss should not change with oil-off, which decreased with raised residence time (Figure 5.11). Due to the lack of fat-serum channels in MC_AMF water evaporation should come from the protein matrix, similar as the model cheese within active fat fillers (i.e. AMC_NaCas).

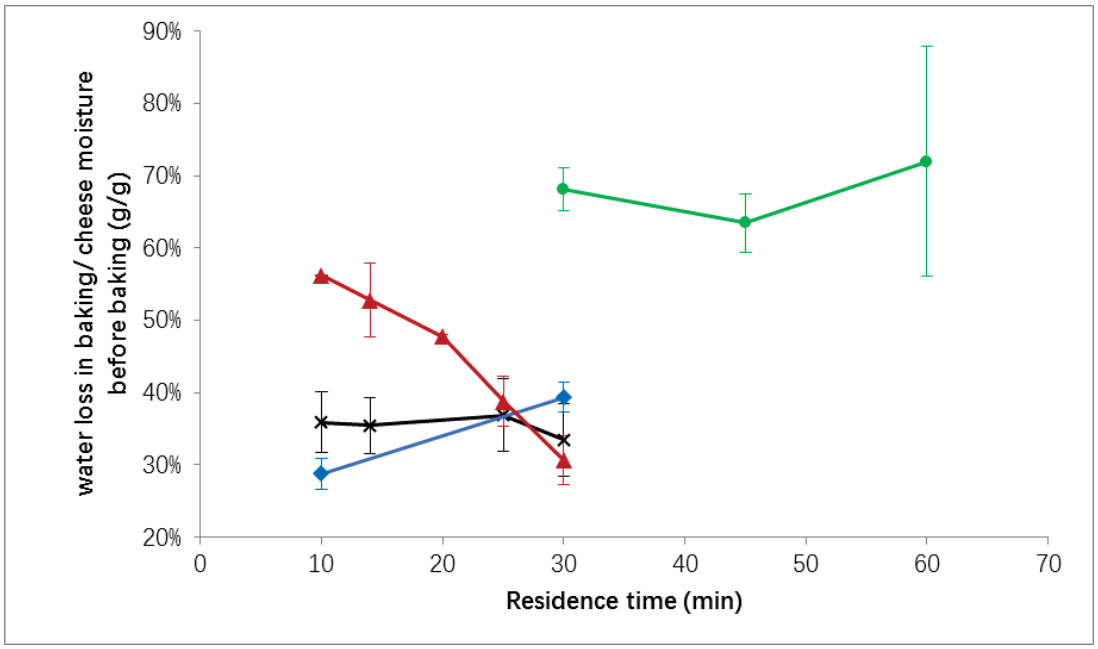


Figure 5.12: Water loss after 10 minutes baking at 170 °C was compared in AMCs within four types of fat fillers: manipulated fat globules covered with NaCas (◆) or Tween 20 (●); the anhydrous milk fat without emulsifier (x) and fat globules from natural cream (▲). X-axis is residence time of AMCs in RVA processing.

5.6 Conclusions

Figure 5.13 summarises the role of active or inactive fat fillers on cheese structural and material properties, and melting behaviours. Active fillers were seen to be homogeneously dispersed in AMC, with the interfacial layer of the emulsion droplets considered as forming bonds with the surrounding protein matrix. Cheese demonstrating this structural state did not exhibit fat-protein phase separation when heated above 100 °C, resulting in no oil-off and limited flowing extent.

Inactive fillers, which were not expected to form bonds with the protein network, were observed as forming localizing fat domains in the serum pockets, forming channelled structures of concentrated emulsion droplets within the protein structure. The serum phase surrounding within these domains was progressively reabsorbed by the protein matrix during storage at low temperature (4 °C), and the crystallization of milk fat droplets with decreasing temperature caused extensive partial coalescence within the concentrated, channelled emulsion domains of the AMC resulting in increased cheese firmness at temperatures where the fat remained crystalline. Here, the presence of solid

fat provided a greater contribution to the cheese material properties (noting that inactive filler particles comprising liquid canola oil either had no effect, or were seen to slightly weaken the protein network).

Partially coalesced domains were seen to undergo full coalescence and breaking of the emulsion as temperatures were increased above the fat melting point (> 35 °C). Inactive fat filler coalescence was able to release free oil to the cheese surface through fat-serum channels as a consequence of fat-protein phase separation during heating.

Emulsions prepared with NaCas or Tween 20 were demonstrated as acting as active and inactive fat fillers respective. In contrast. the material properties and functionalities of AMC made from natural cream, where fat globules are covered with native milk fat globule membrane (MFGM), suggested that the interaction of MFGM membrane was seen to transition from predominantly inactive filler behaviour to active filler as residence time of cheese processing within the RVA was increased. CLSM shows a combination of localised fat domains and individual dispersed fat globules for short times (10 minutes). However, the size and number of localised fat clusters were reduced with increasing residence time. The structure of the native MFGM is likely to be changed by homogenisation of the fat as a consequence of heating and shearing during RVA processing. It is hypothesised that the adsorption of soluble serum proteins at the fat droplet surface is taking place due to this process, and that it is this adsorption that subsequently renders the droplets as active fillers. This hypothesis is explored in more detail in the following chapter.

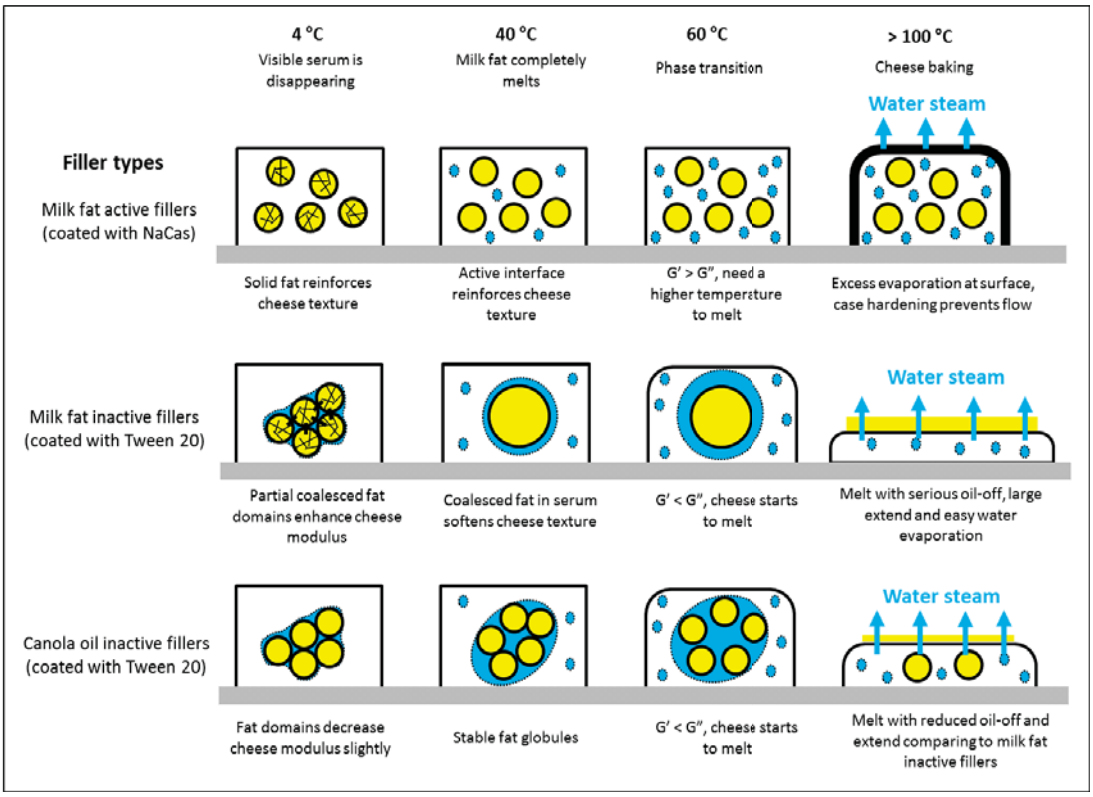


Figure 5.13: Schematic diagrams of fat and water dynamic dispersion in cheese melting. Melting behaviour in AMC within active or inactive fat fillers are described and concluded.

Chapter 6: Influence of cheese manufacture and protein adsorption on Alternate Made Cheese (AMC)

6.1 Overview

The previous chapter explored how the interfacial composition and interactions of emulsions used in the preparation of an AMC could be used to influence cheese microstructure and how this in turn was able to impact on material and functional properties of cheeses. It also began to provide evidence of how processing conditions, notably the residence time of shearing, could dynamically influence emulsion structure, leading to transitional behaviours depending on the conditions applied.

In considering the particular role of processing on microstructure and associated properties, previous research in this area has investigated the impact of mechanical treatment (screw speed) and manufacturing temperature control on Mozzarella functionality for cheeses produced on a twin-screw counter-rotating stretcher-cooker (Renda, Barbano et al. 1997, Yu and Gunasekaran 2005). Here, lower screw speeds were shown to result in greater oiling off (Renda, Barbano et al. 1997). Higher screw speeds were observed to change cheese microstructure, with SEM micrographs showing a honeycomb-like protein network rather than the network containing large holes (Yu and Gunasekaran 2005). Yu and Gunasekaran (2005) reported the modified microstructure was observed at barrel temperatures of 62 °C but not at 72 °C for the same screw speeds, indicating that manufacturing temperature played a key role in determining the cheese emulsion structure. Yu and Gunasekaran (2005) postulated that the honeycomb-like structure represented the breakdown of the protein network.

Observations of AMC processing presented within this thesis in chapter 5 have indicated that the honeycomb-like structure in previous investigation (Yu and Gunasekaran 2005) may be a modified protein matrix comprising homogeneously dispersed fat fillers. This structure arises as a consequence of overworking of the cheese resulting in modified fat globule interface ingredients. From a structural perspective, the native milk fat globules become increasingly homogenised during the mixing becoming coated with soluble

proteins present within the cheese curd. This change in structure correlates to observations made in chapter 5, in which the interactions of fat globules with the surrounding protein matrix in AMC were observed to alter from inactive-like to active-like fillers with increased residence time. The hypothesis here is the mechanical treatment in the RVA modifies fat filler behaviour, as evidenced by the changed emulsion structure and cheese material properties and functionalities.

This chapter explores how the shearing conditions and residence time contribute to the transition of inactive to active-like fat fillers in AMC. In addition, the effect of cooking temperature will be discussed. The apparent viscosity of cheese production shown in the RVA mixing in linked to the shear speed, cooking temperature and the cheese material properties. To compare the impact from shear speed, residence time and cooking temperature, the effective total shear work in cheese production has been calculated from the apparent viscosity in the RVA (as detailed in the Materials and Method chapter), and the change in cheese material properties and functionality is studied as a function of the increase in total shear work. In this way, the total shear work also takes into consideration wall-slip present during RVA treatment, which manifests itself as a significant decrease in viscosity, and thereby the analysis reflects the apparent mechanical treatment.

Shear work provides an indication as to how the stress is transmitted through cheese, but cannot necessarily predict the chemical changes taking place within the cheese. The previous chapter indicated the importance of fat interface properties to change fat filler behaviour in cheese, and how the native milk fat globule membrane is considered to be modified under shearing and heating. Critical to this behaviour is the adsorption of protein at the interface of fat droplets during processing. This chapter also seeks to further develops understanding of the role of protein adsorption at the fat interface and subsequent impact on cheese structure and functionality, through the specific incorporation of serum proteins from non-fat AMC as part of cheese manufacture, and comparison against formulations with added sodium caseinate (NaCas) or whey protein isolate (WPI). The serum from non-fat is expected to contain a mixture of proteins, including both whey proteins and caseins, although the levels of these present are likely to be influenced by processing conditions. Accordingly, the total protein concentration in

serum will be estimated at different residence time of cheese producing. The role of proteins present in the serum is investigated through the addition of this serum in AMC formation.

6.2 Impact of processing temperature

The production of AMCs prepared with non-homogenised cream is (AMC_NC) compared at 60 ˚C and 70 ˚C. Cheeses were observed as having lower viscosity during processing at 70 ˚C than 60 ˚C, resulting in lower total shear work within the same residence time (Figure 6.1). Using the data of figure 6.1, the modified cheese functionalities within raised residence time could be compared based on the total shear work.

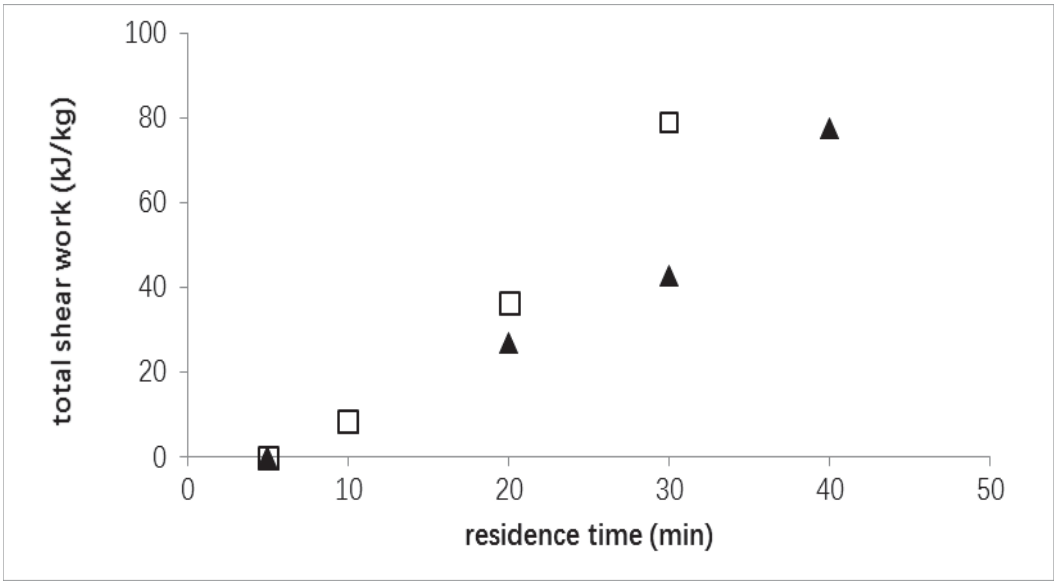


Figure 6.1: Comparison of total shear work in cheese produced at 60 °C (□) and 70 °C (▲).

6.2.1 Microstructure

Confocal microscope images of AMC produced at 70 ˚C are presented in figure 6.2. Fat channels were easily visible in cheese after 20 minutes of mixing (Figure 6.2a). These fat domains were reduced after 30 minutes and were no longer visible after 40 minutes of mixing (Figures 6.2b and 6.2c respectively). There was also an apparent, corresponding

reduction in overall droplet size and increased propensity for droplets to become uniformly distributed throughout the protein network.

Comparing the microstructures of AMCs made at 60 °C (Figure 5.2 in Chapter 5) and 70 °C (Figure 6.2), the disappearance of fat domains was can be seen to correlate to total shear work rather than the actual residence time of manufacture. The total shear work of 25-45 kJ/kg in AMCs made at 70 °C for 20 - 30 minutes was similar to AMCs made in 20 minutes at 60 °C (Figure 6.1). Confocal images showed the presence of small fat domains in these AMCs under total shear work of 25-45 kJ/kg at production temperature of 60 °C or 70 °C (Figure 6.2a and 6.2b). When total shear work was increased to ~ 80 kJ/kg (which was the value of cheese made at 70 °C for 40 minutes and for cheese made at 60 °C for 30 minutes) homogeneous fat globules appeared within the protein matrix (Figure 6.2c).

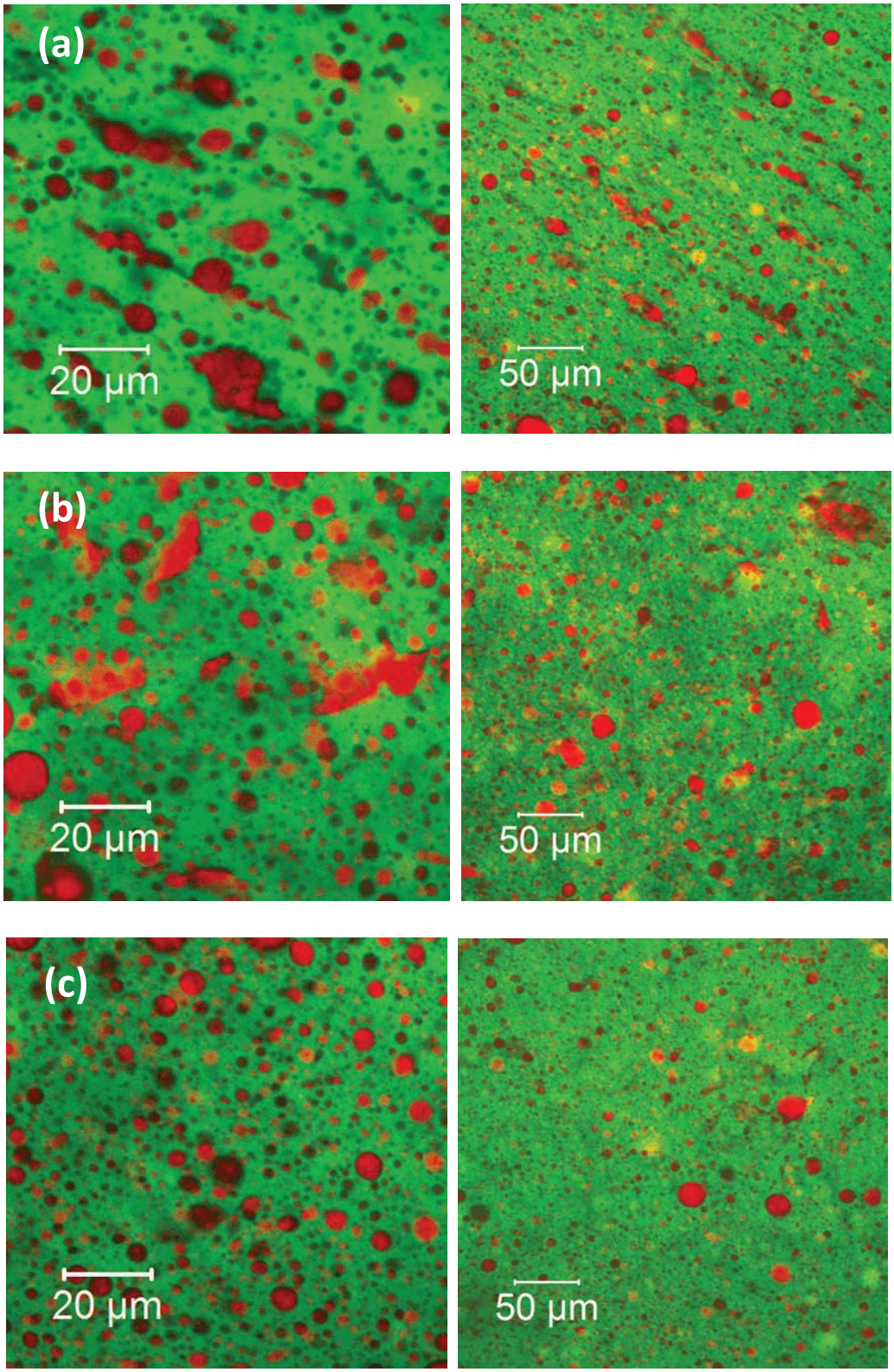


Figure 6.2: CLSM images of AMC made from natural cream in 20 minutes (a), 30 minutes (b) and 40 minutes (c) at 70 °C. The photos were taken using the lenses of x 40 (left) and x 25 (right). Red is fat and green is protein, black is serum or air.

6.2.2 Particle size distribution

The fat globule size distribution of AMC was analysed on the cheeses immediately post-processing and without cooling, and for cheeses after 7 days storage at 4 °C. Figure 6.3 shows how the volume-weighted average mean fat globule size D[4,3] changed with increasing total shear work. Prior to cooling, D[4,3] was only marginally altered by the shearing during cheese production, showing a slight decrease in size within increasing shear work. The noticeable increase in particle size observed in the chilled cheeses compared to the freshly formed cheeses experienced the same amount of shear work, which was attributed to fat partial coalescence as discussed in the chapter 5. These results confirmed that cooling was required for partial coalescence of fat droplets, and that this phenomenon predominated when the fat was localised within channelled structures as occurred for inactive filler droplets. The D[4,3] of chilled cheese was seen to decrease with increasing total shear work. This could be explained by the reduction in the relative size of the localised fat domains as shown in Figure 6.2. These localised fat domains would either contain a cluster of fully intact native partially coalesced fat globules, or be pockets of coalesced milk fat. Increased shear work would break up the fat domains and redistribute the fat globules into smaller domains in the protein matrix. The homogenised native milk fat globules could have newly formed interface from the serum proteins. Once Walstra solution was added to effectively dissociate the protein matrix, only the native globules or partially coalesced small fat domains would remain in the solution. Increased shear work reduced the number of large fat domains trapped within cheese matrix, and small fat globules may be more resistant to shear. Therefore, the reduction in size of the localised fat domains reflected the increased disruption experienced by the cheese protein matrix with shear, which could also disrupt and break the large milk fat globules, resulting in a smaller globule distribution remaining within the cheese.

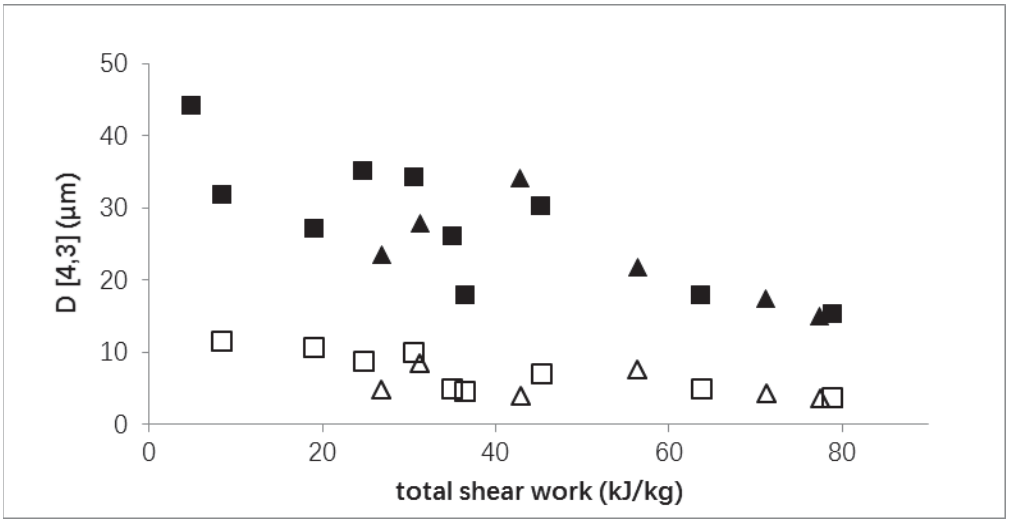


Figure 6.3: The change of average fat globule size in cheese to the total shear work made at 60 °C or 70 °C. Fresh cheeses without cooling (60 °C: □, 70 °C: Δ) are compared to the cheeses after 7 days storage at 4 °C (60 °C: ■, 70 °C: ▲).

Figure 6.4 gives details of the particle size distribution in cheese after production of varying time lengths. Cheese samples were measured after 7 days' storage at 4 °C. The particle size was distributed across three distinct modal regions: 0.1 - 1 µm, 1 - 10 µm and 10 - 200 µm. The volume weighting of particles above 10 µm decreased when total shear work increases. The fat globule size from natural cream was below 10 µm before cheese making (with D[4,3] ~ 4µm), and the large fat globules observed in the distribution above 10 µm were more likely from coalescence within fat domains. For the cheeses prepared with total shear work above ~80 kJ/kg (at either 60 °C or 70 °C), there was a noticeable decrease in the vol% of structures present in the 10 - 200 µm distribution. The relative increase in vol% of the modal distributions <10 µm in AMCs produced from 42.9 kJ/kg to 77.5 kJ/kg suggests that higher shear work resulted in a greater separation of flocculated fat droplets within the protein network, leading to a lower overall incidence of partial coalescence on cooling.

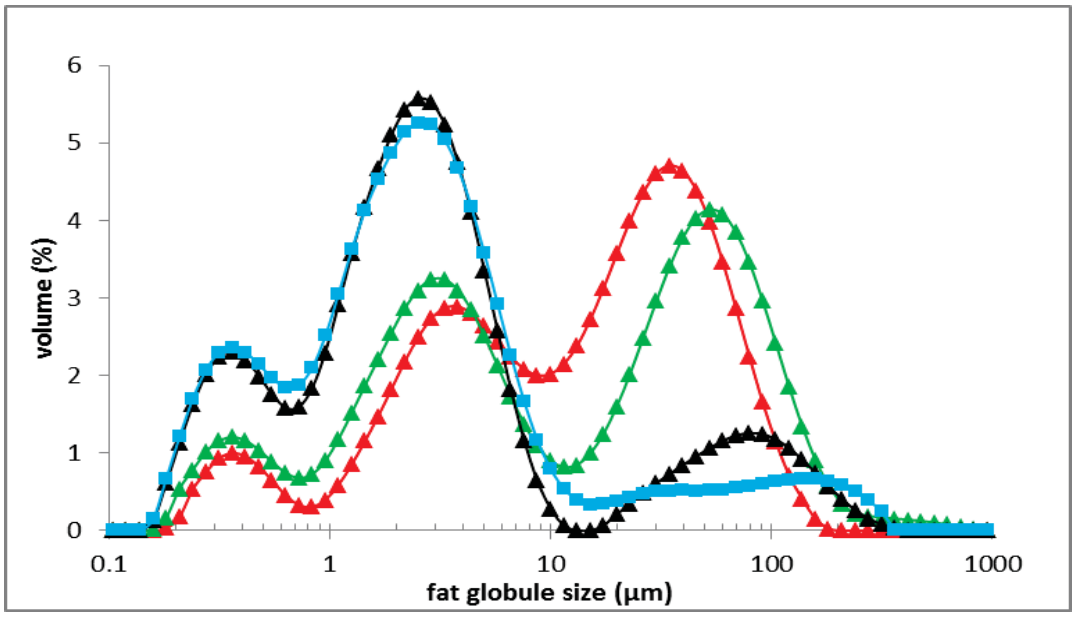


Figure 6 4: Fat globule size distribution of AMC after 7 days storage at 4 °C. Three cheese samples were made at 70 °C in 20 minutes (▲, 26.8 kJ/kg total shear work), 30 minutes (▲, 42.9 kJ/kg total shear work) and 40 minutes (▲, 77.5 kJ/kg total shear work), and another cheese sample was made at 60 °C in 30 minutes (■, 78.9 kJ/kg total shear work).

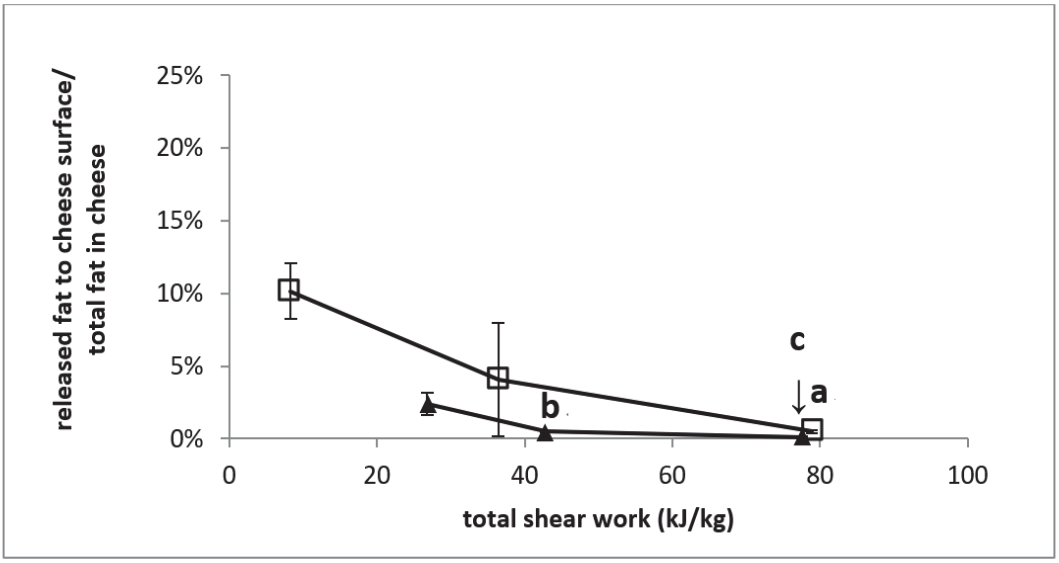
6.2.3 Cheese melting

The amount of oil-off released from the molten cheese has been used to study cheese melting behaviours as influenced by the total shear work used to process AMC (Figure 6.5). Oil-off was seen to decrease with increasing total shear work. The loss of oil-off was indicative of strong interactions between fat globules and protein matrix causing a reduction in partial coalescence on storage and full coalescence of fat droplets on heating. The size of fat domains was larger in cheeses made at 70 °C than for those made at 60 °C (Figure 5.2, and for which total shear work was ~40 kJ/kg, ~30min at 60 °C), with average particle size D[4,3] of chilled cheese noticeably larger than for the fresh cheese when made at 70 °C (Figure 6.3). However, both of the two cheeses prepared with shear work ~ 40 kJ/kg, whether made at either 70 °C or 60 °C, still exhibited little oil-off after baking (Figure 6.5). The increased temperature of 70 °C possibly strengthened the cross-linking of droplets within the protein network, because whey proteins were able to bind to fat globule surface at temperature above 70 °C (Dalgleish and Banks 1991, Sharma and Dalgleish 1993), and the interactions between whey proteins and milk fat membrane increased with the time of heat treatment (Kim

and Jimenez-Flores 1995, Corredig and Dalgleish 1996). Once fat globules were covered with whey proteins, the interactions between fat interface and protein matrix could be strengthened.

Three cheese samples displaying little oil-off were selected to compare flowing extent for a further study of cheese melting behaviour (cheese melting photos in Figure 6.5). Oil-off was minimal when cheeses were made within total shear work of ~ 80 kJ/kg at 60 °C and 70 °C (Figure 6.5 sample a and c). Both samples included homogeneously dispersed fat globules within the protein matrix and a stable particle size distribution after cooling. However, the flowing extent area was smaller in the cheese produced at 70 °C, and also displayed a less uniform direction of flow marked by the arrows in figure 6.5c. This suggested stronger interactions between fat globules and protein matrix when the cheese was produced at 70 °C comparing to the cheese made at 60 °C.

Oil-off behaviour was also measured for AMC produced at 70 °C with 43 kJ/kg total shear work (Figure 6.5 sample b). Although, from a structural perspective, it was observed that small fat domains existed in this cheese (Figure 6.2b) and fat globule size D[4,3] had increased on storage due to partial coalescence of fat domains (Figure 6.3), the baked cheese had minimal oil-off and showed non-uniform behaviour in relation to direction of flow (Figure 6.5b). The possible explanation was that reinforcement of the cheese structure through interactions of the protein network with active filler droplets inhibited relaxation of the network on melting, thus restricting the ability of the small fat domains to form channels to the cheese surface that would cause oil-off. Thus, where strong interactions of fat-protein and protein-protein occur, non-ideal cheese melting behaviour, i.e. reduced flow and oil-off, was observed.



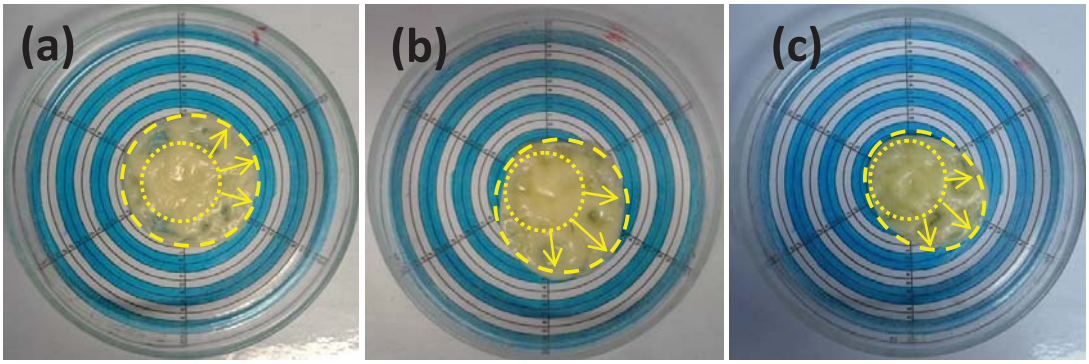


Figure 6.5: Cheeses were baked at 170 °C for 10 min. The amount of free oil released to cheese surface is decreasing when total shear work is increasing. Cheeses were made at 60 °C (□) and 70 °C (▲), respectively. Three images of molten cheese are attached: cheese (a) was 30 minutes made at 60 °C within 78.9 kJ/kg total shear work; cheese (b) was 30 minutes made at 70 °C within 42.9 kJ/kg total shear work; and cheese (c) was 40 minutes made at 70 °C within 71.3 kJ/kg. The cheese area before baking was indicated by yellow dotted circles, which is the same area for all samples, and the large yellow dashed circles indicate cheese flowing in baking. The arrows indicate directional flowing by difficult melt.

6.3 Effect of shear speed

RVA shear speed was another factor contributing to total shear work in addition to residence time. The dispersion of fat in cheese tended to become progressively more homogeneous with increased residence time, and with high shear speeds being expected to shorten the time required to achieve uniform dispersion. The shear speed for AMC production reported in section 6.2 was 800 rpm for the first 15 min, followed by

1000 rpm for the extent of residence time. The discussion of this section focuses on varied shear speed, with cheeses produced using two different shear speed levels 600 rpm (low) and 1200 rpm (high) at 60 °C. Cheese production was limited to 10 minutes at 60 °C with a constant shear speed of 600 rpm.

The role of total shear work in cheese functionalities was investigated for cheeses made from low shear speed through to high shear speeds. Cheese samples were taken at different time points (600 rpm: 10 minutes, 20 minutes, 30 minutes and 40 minutes; 1200 rpm: 10 minutes, 15 minutes and 20 minutes) and analysed for microstructure and melting characteristics. According to the calculation formula of total shear work (see section 3.5.6 in chapter 3, $W = \int 4kN^2 \pi^2R^2H\eta \, d(t)$, where N is the shear speed), theoretically the shear work at 1200 rpm should be four times of that at 600 rpm when ignoring the change on viscosity. Figure 6.6a records the total shear work of cheeses made at 600 rpm or 1200 rpm, and figure 6.6b show the viscosity recorded by RVA in cheese producing. The total shear work was not as high as expected, because the measured viscosity was lower in the processing time of 15-20 minutes for cheeses prepared at 1200 rpm compared to 600 rpm (Figure 6.6b). Cheese viscosity increased in the first a few minutes and then dropped slightly towards a constant range. The high shear rate shortened the time to reach peak high viscosity, which occurred in 12 minutes compared to the 18 minutes for low shear rate of 600 rpm (Figure 6.6b), whilst the total shear work required to reach peak viscosity could be determined as ~20 kJ/kg for both low and high shear speed samples (Figure 6.6a). Thereby, the cheese modification during preparation was more likely to be linked to the total shear work of manufacture.

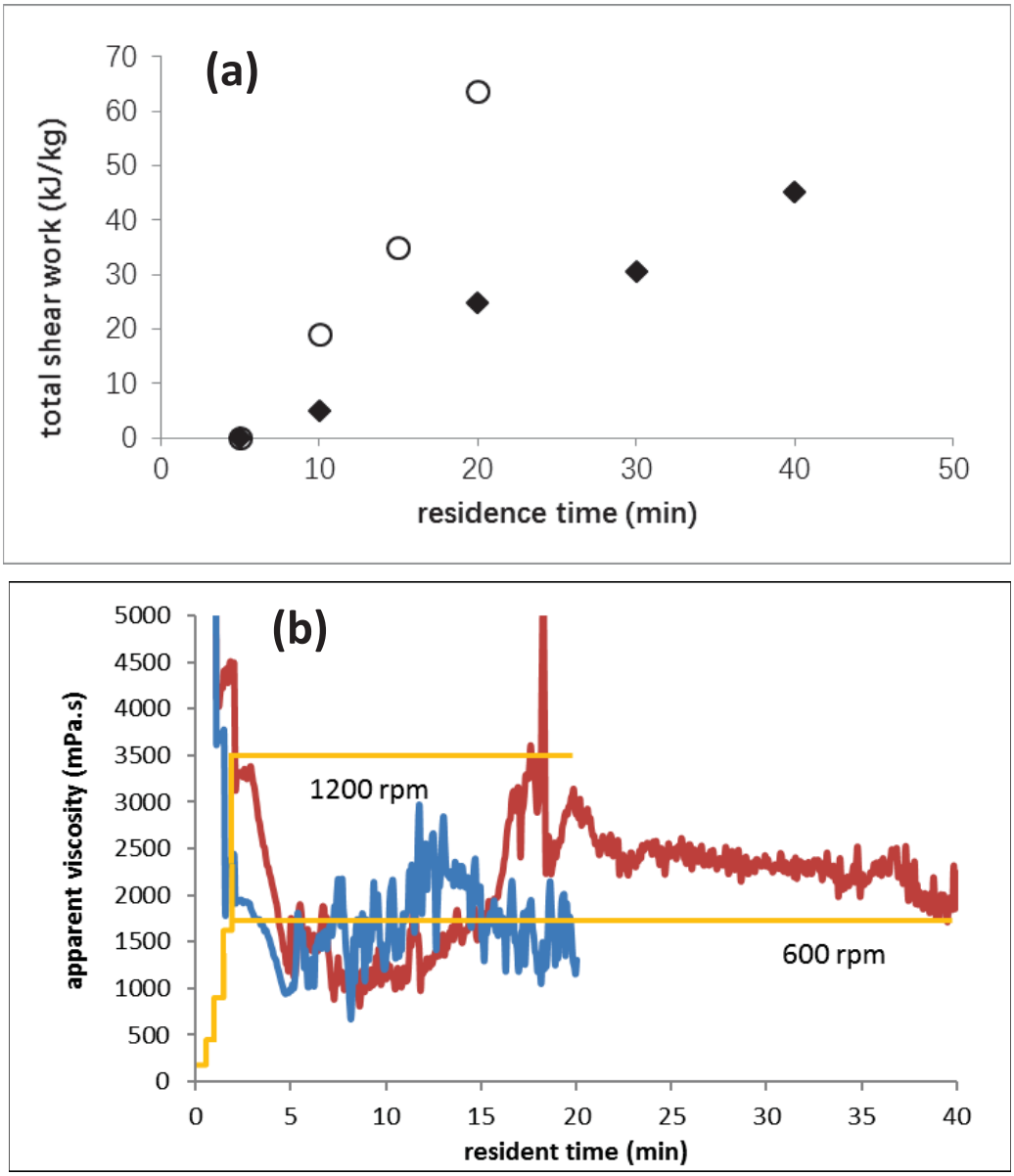


Figure 6.6: The cheeses made from the maximum shear rate of 600 rpm (◆) and 1200 rpm (○), respectively, at 60 °C. (a) Comparison of total shear work in cheese production. The total shear work was calculated after the first 5 minutes when temperature is constant at 60 °C. (b) Comparison of viscosity shown in the RVA. Yellow: shear speed profile; Red: cheese produced at 600 rpm; Blue: cheese produced at 1200 rpm.

6.3.1 Microstructure

Confocal imaging of the fat dispersion within the protein matrix was compared for cheeses produced at low shear speed (600 rpm) and high shear speed (1200 rpm). It could be seen that the initially large, localised fat domains in cheeses made at 600 rpm decreased in size as residence time increased (Figure 6.7). As residence time was

increased up to 40 min, there was a clear reduction on the size of fat domains, along with some channelling of the fat structures (Figure 6.7 b and c). In contrast, when cheeses were produced at high shear rate of 1200 rpm, most of fat globules were observed to be uniformly dispersed in matrix after only 10 minutes, and just a few droplets were close to each other forming small linear channels (Figure 6.8a). At 15 minutes mixing, these fat channels disappeared, showing a homogeneous dispersion of fat globules (Figure 6.8b). This cheese emulsion structure remained consistent, with homogeneously dispersed droplets distributed within in the protein matrix for cheeses in 20 minutes at 1200 rpm (Figure 6.8c).

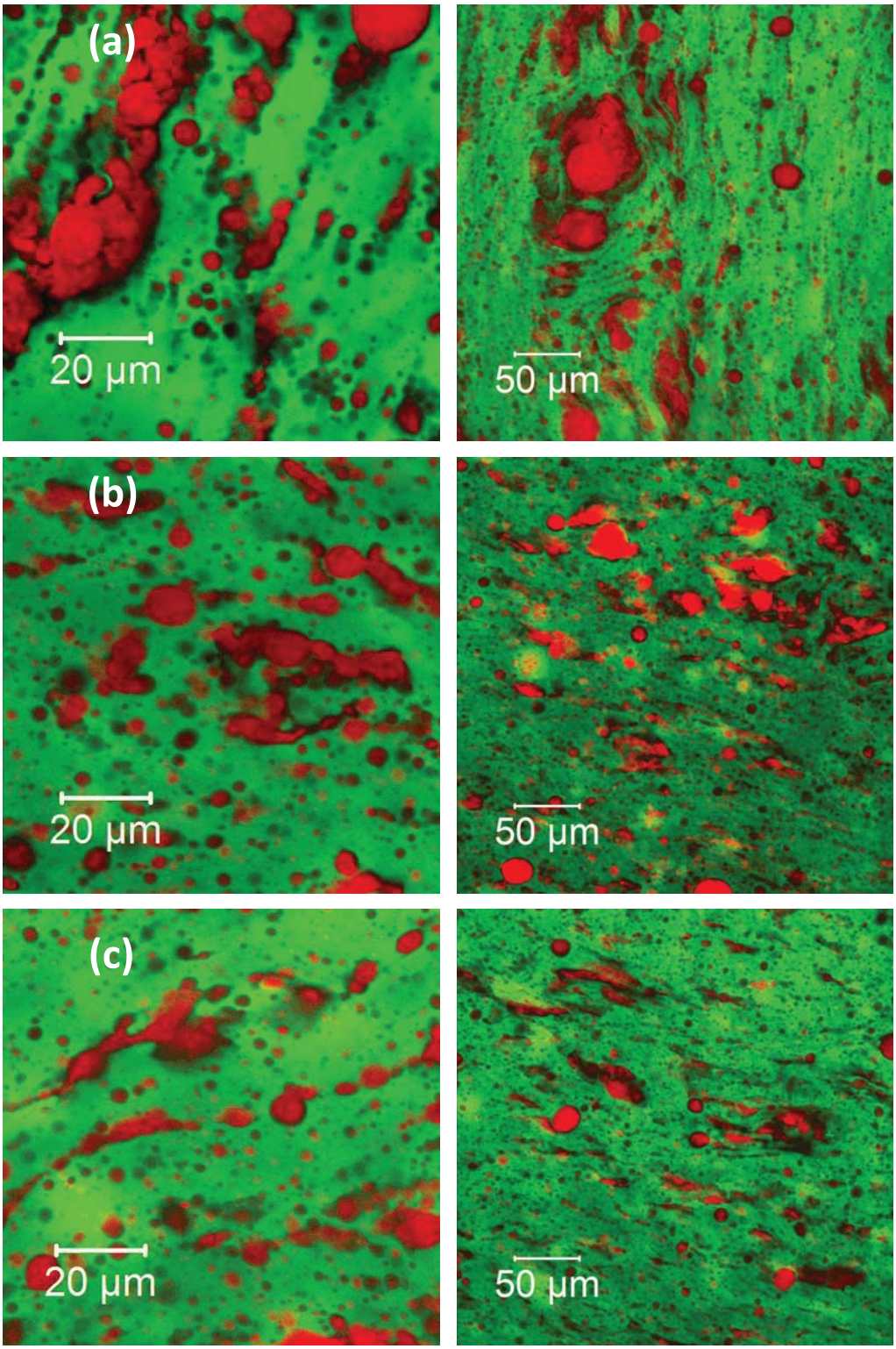


Figure 6.7: CLSM images of cheese made at 600 rpm 60 °C for 10 min (a), 20 min (b) and 40 min (c). The photos were taken using lenses of x 40 (left) and x 25 (right). Red is fat and green is protein, black is serum or air.

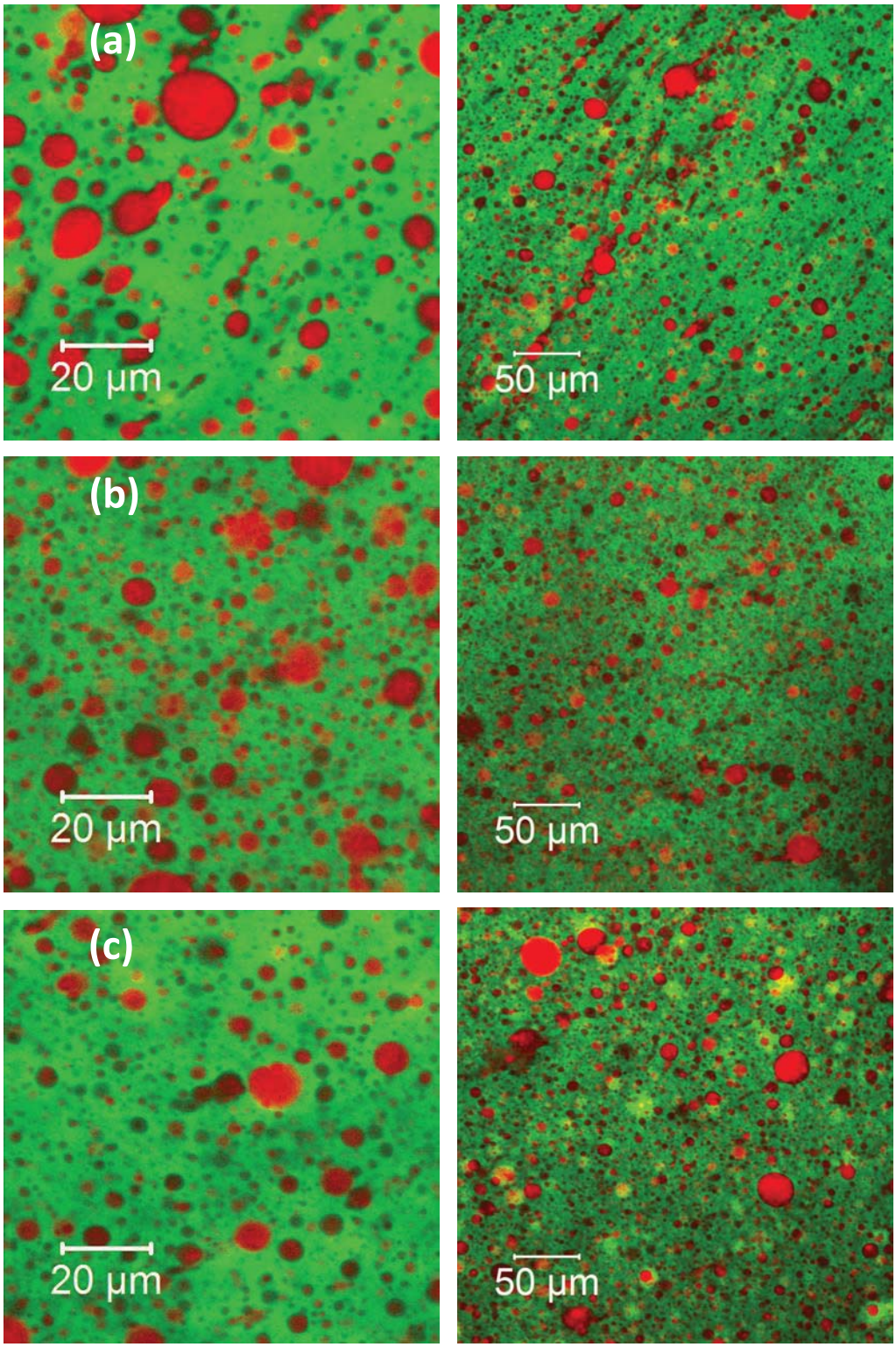


Figure 6.8: CLSM images of cheese made at 1200 rpm 60 °C for 10 min (a), 15 min (b) and 20 min (c). The photos were taken using lenses of x 40 (left) and x 25 (right). Red is fat and green is protein, black is serum or air.

Findings indicated that less total shear work was required to obtain the homogeneously dispersed fat globule structure when cheese was produced at higher shear speeds. Localised fat domains were not found in 15 minutes producing at 1200 rpm (i.e. fat distribution was essentially homogeneous at this point), which represented 35.0 kJ/kg total shear work; while the cheese made at 600 rpm in 40 minutes (45.3 kJ/kg total shear work) still displayed a number of localised fat clusters. In addition, fat domains were seen to increasingly disappear from 20 minutes to 30 minutes at 60 °C (20 min: 36.4 kJ/kg; 30 min: 78.9 kJ/kg) in confocal images (Figure 5.2 of chapter 5), where the maximum shear speed was 800 rpm for the first 15 minutes followed by 1000 rpm. These data suggested the critical total shear work to gain uniformly dispersed fat globules was closely linked to the shear speed, and that low shear speeds did not significantly impact on cheese microstructure dynamics.

6.3.2 Particle size distribution

Figure 6.9 show the fat globule size distributions for cheeses produced at low (600 rpm) and high shear speeds (1200 rpm). Figure 6.9a showed the distributions for chilled cheeses made at 600 rpm applying a total shear work of 4.9 kJ/kg, 24.7 kJ/kg and 45.3 kJ/kg respectively. The particle size distribution in these three samples showed very little change. The primary modal distribution presented a broad size range of 10 - 200 µm (which, as indicated previously was attributed to extensive partial coalescence) and the secondary modal distribution spanned droplets from approximate 1 - 10 µm, for which the vol% increased slightly with increasing shear work. However, when cheese was produced at 1200 rpm (Figure 6.9b), particle size distribution was clearly altered as shear work increased from 19.0 kJ/kg to 35.0 kJ/kg, where the primary peak shifted from 10 - 200 µm to 1 - 10 µm. The higher shear speed (1200 rpm) appeared more effective in transitioning the fat distribution in cheese, such that the modal distribution of large particles above 10 µm had dropped to very low volume fractions as a consequence of only 35.0 kJ/kg total shear work. In contrast, the lower shear speed (600 rpm) did not lead to the formation of homogeneously dispersed fat globules even when the total shear work reached 45 kJ/kg. Higher shear speeds seemed to be required to provide

sufficient mechanical force to cause homogenisation of droplets, leading to transition from inactive to active fillers.

The change average fat globule size D[4,3] as a consequence of processing is presented in figure 6.9c, where the change of particle size is described as a function of total shear work for cheeses made at 600 rpm and 1200 rpm, respectively. Particle size was measured for the freshly prepared cheeses prior to chilling (i.e. fat droplets were still predominantly molten at this point), and for cheeses after 7 days' storage at 4 °C. It could be seen that the fresh cheeses retained a relatively stable particle size distribution (5 - 10 µm) as a consequence of processing irrespective of shearing conditions, indicating that whilst fat distribution within the cheese could be influenced by shear, the individual droplets remained relatively stable up to this point.

The increase in particle size distribution observed for cheeses stored at 4 °C was therefore attributed to partial coalescence of droplets that had been concentrated within fat domains localised in the protein network. The decrease in particle size with increasing total shear work indicated an effective decrease in the relative size of these fat domains. Additionally, cheeses made at 1200 rpm showed smaller relative D[4,3] on cooling compared to cheeses made at 600 rpm, suggesting that higher shear speed had a greater homogenising and dispersing effect on fat droplets than low shear speeds (as stated above). Particle size data of the chilled cheeses was in agreement with the CLSM images (Figure 6.7 and 6.8). Fat domains were observed in all the cheese samples processed at 600 rpm (Figure 6.7), but fat domains were rarely observed in cheeses made at 1200 rpm beyond 15 minutes (Figure 6.8 b and c).

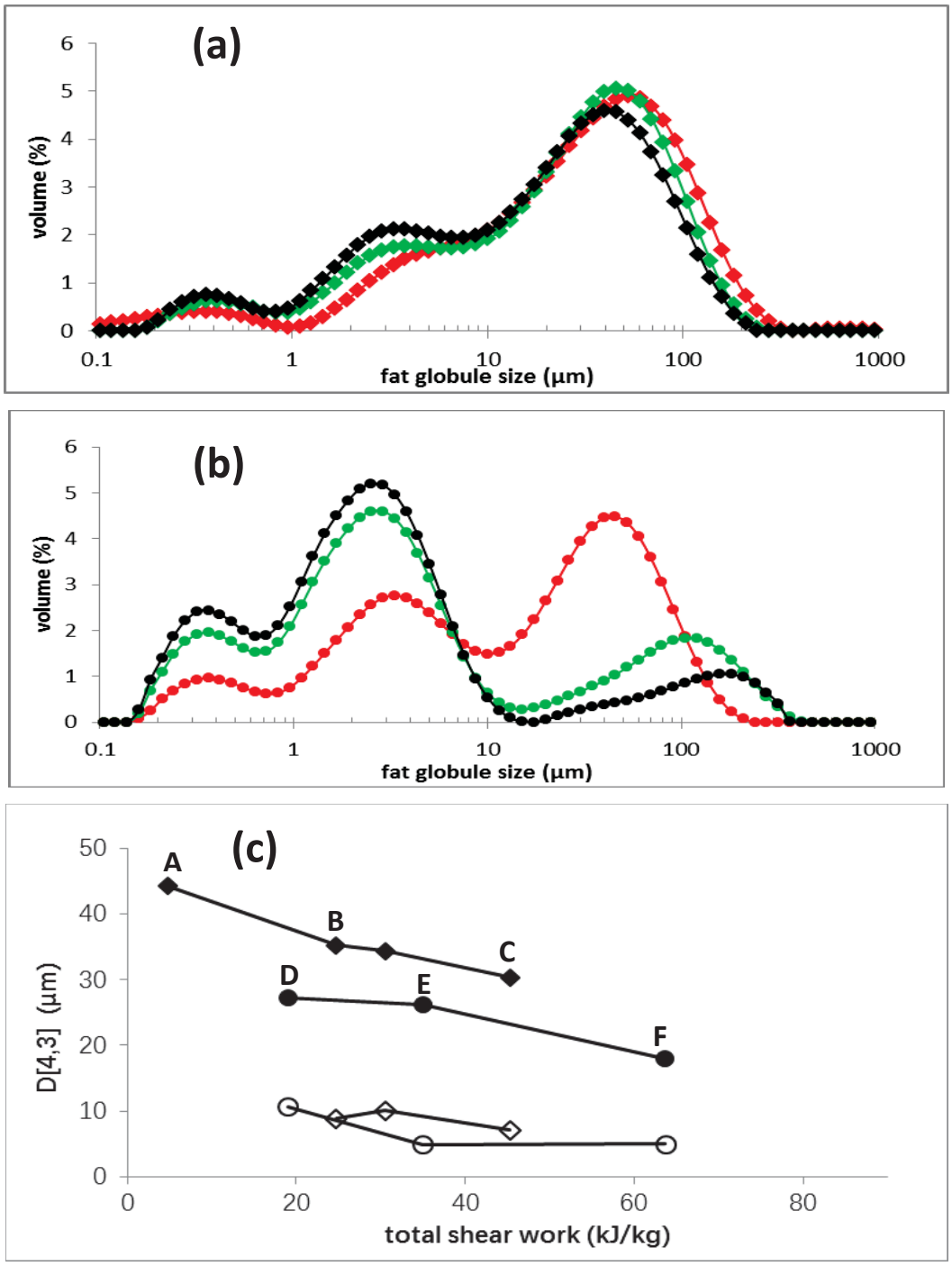
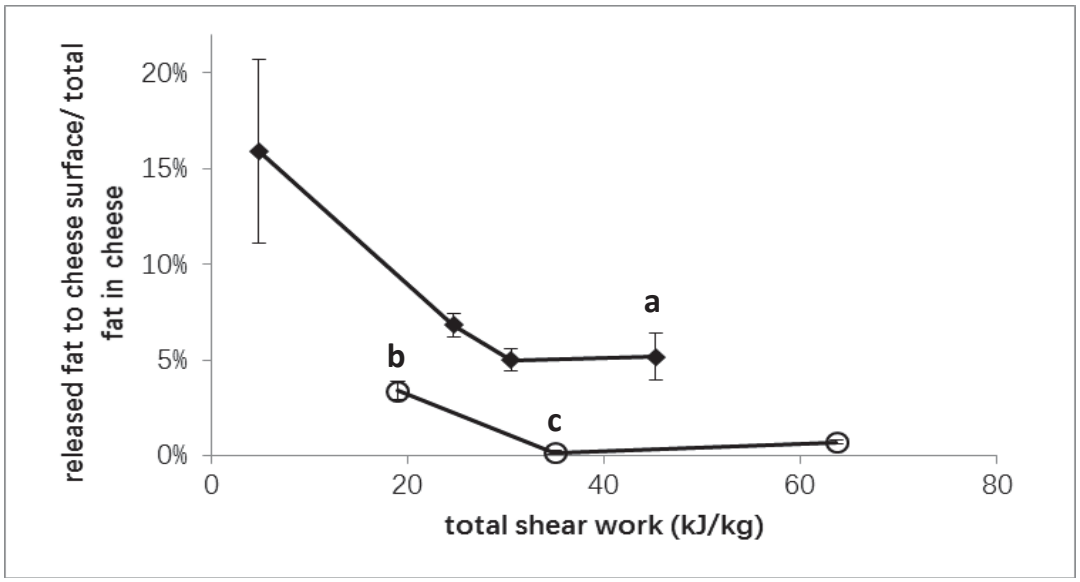


Figure 6.9: The fat globule size distribution is compared between cheeses made at low shear speed (600 rpm) and cheeses made at high shear speed (1200 rpm) at 60 °C. Figure (a) and (b) are the cheese samples in 7 days storage at 4 °C. (a) The cheeses made at low shear speed in 10 minutes (◆, 4.9 kJ/kg total shear work), 20 minutes (◆, 24.7 kJ/kg total shear work) and 40 minutes (◆, 45.3 kJ/kg total shear work), respectively. (b) The cheeses made at high shear speed in 10 minutes (●, 19.0 kJ/kg total shear work), 15 minutes (●, 35.0 kJ/kg total shear work) and 20 minutes (●, 63.7 kJ/kg total shear work), respectively. (c) Overview of the average particle size D[4,3] in fresh cheeses without cooling (600 rpm: ◇, 1200 rpm: ○) and the cheeses in 7 days storage at 4 °C (600 rpm: ◆, 1200 rpm:●). A, B and C are the three samples shown in figure (a); D, E and F are the three samples shown in figure (b).

6.3.3 Cheese melting

Oil-off during cooking (10 minutes at 170 °C) was measured to compare baking behaviour of cheese made at different shear speed (Figure 6.10). Oil-off decreased from 15.9 ± 4.8 wt.% to 6.8 ± 0.6 wt.% when total shear work was increased from 4.9 kJ/kg to 24.7 kJ/kg for cheeses made at 600 rpm, with a further reduction to approximately 5.2 ± 1.2 wt.% oil-off as shear work was increased up to 45.3 kJ/kg. At 1200 rpm, no oil-off was observed for cheese processed at total shear work of 35.0 kJ/kg. The change of oil-off supported the data of particle size distribution in figure 6.9c and confocal in figure 6.7 and 6.8: cheese was modified dramatically at high shear speed (1200 rpm) generating active-like fat droplets, while emulsion droplets in cheese made from low shear speed (600 rpm) retained inactive droplet properties even after a longer residence time and for relatively higher total shear work.

The impact of cheese processing conditions was likewise manifested in the flow properties of cheese during melting as shown in figure 6.10. Cheeses made at 600 rpm with total shear work 45.2 kJ/kg had an oil-off 5.2 ± 1.2 wt.% (Figure 6.10a). The extent of flow was similar to the cheese from 1200 rpm with a total shear work 19.0 kJ/kg, where oil-off was 3.4 ± 0.5 wt.% (Figure 6.10b). Although these two samples have low oil-off, both cheeses demonstrated uniform direction of flow on melting. However, the cheese processed at 1200 rpm, and with a total shear work of 35.0 kJ/kg, had no oil-off and accordingly presents directional flow on melting. As indicated previously, drying of the cheese surface, which can occur where no oil-off is observed, inhibits uniform flow and spread in comparison to cheeses for which the surface layer has been lubricated by the oil-off. The trapped (active) fat globules within protein matrix cannot escape to the cheese surface. The cheese melting behaviour therefore indicated that active fat fillers had been formed in the cheese from high shear speed (1200 rpm) and for which the total shear work was less than the cheese produced from low shear speed (600 rpm).



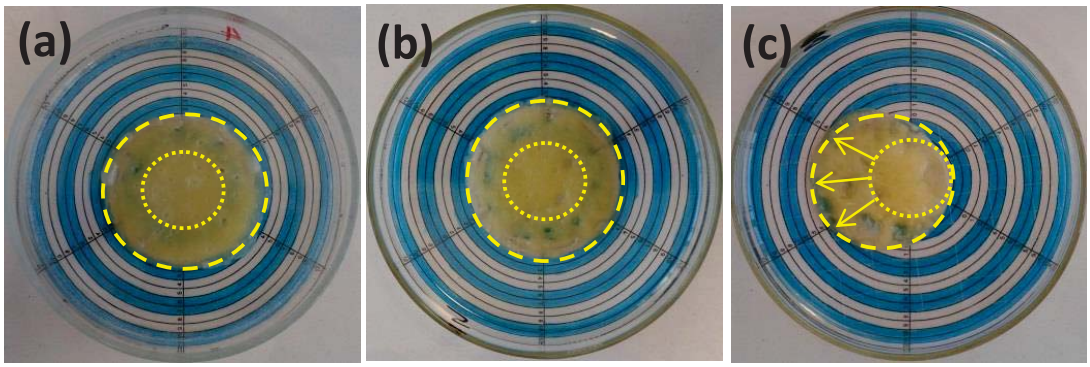


Figure 6.10: Cheeses were baked at 170 °C for 10 min. The amount of free oil released to cheese surface is decreasing when total shear work is increasing. Cheeses were made at 600 rpm (◆) and 1200 rpm (○), respectively. Three images of molten cheese are attached: Cheese (a) was 40 minutes made at 600 rpm (45.2 kJ/kg total shear work); cheese (b) was 10 minutes made at 1200 rpm (19.0 kJ/kg total shear work); and cheese (c) was 15 minutes made at 1200 rpm (35.0 kJ/kg total shear work). Small yellow circles are the size of unbaked cheeses and large yellow circles show cheese flowing extent after baking. The arrows indicate directional flowing when cheeses melt hardly.

6.4 Total shear work impacting on cheese modification

The study on temperature (section 6.2) and shear speed (section 6.3) showed how the changes to cheese microstructure and cheese melting behaviour could be linked to total shear work. Fat globules initially located in the serum pockets tended to separate from each other as RVA mixing progresses, with high shear rates causing this modification to occur even for relatively low shear work. This section compares data from temperature

and shear measurements in relation to the particle size distribution (Figure 6.11) and oil-off (Figure 6.12) of cheese samples.

The CLSM images indicated that AMC made from non-homogenised natural cream over relatively short residence times structurally comprised a mixture of individual fat globules and localised fat domains, with increased RVA shear and residence time tending to reduce the size and numbers of the fat domains. The high viscosity of protein phase generates high Capillary numbers and Weber numbers which promote droplet breakup. This comminution of fat globules to smaller droplets under conditions of high shear or prolonged residence time increased surface area, which may disrupt the native MFGM and allow adsorption of serum proteins to the newly created interface.

Figure 6.11 shows the collation of data for particle size distribution of cheese samples measured after processing without cooling, and where cheeses were produced at either 60 °C or 70 °C for varied maximum shear speed. Volume weighted mean particle size D[4,3] could be seen to decrease steadily with the increasing total shear work (Figure 6.11a). The change in size for d(0.1) in figure 6.11b, and d(0.9) in figure 6.11c also showed a relative decrease in droplet size. The cheeses made in low total shear work contain larger particles comparing to the size of fat globules d(0.9) of 10 μm in the cream for cheese production. Fat coalescence probably occurred in the early stages of RVA mixing, and then these large fat globules are likely to have broken up at increased total shear work.

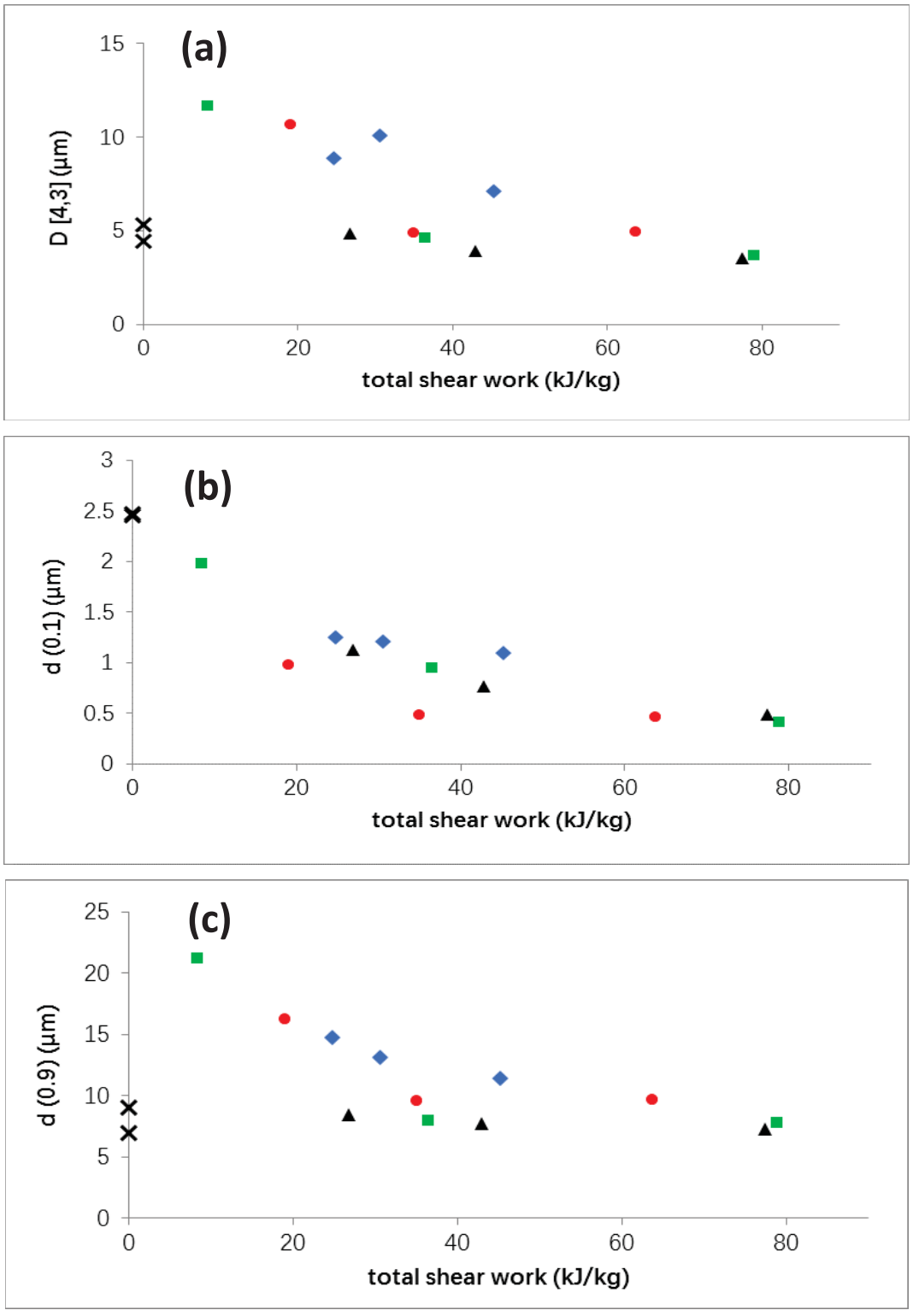


Figure 6.11: It is gathering of fat globule size distribution of average size D[4,3], the top 10 % smallest particles d(0.1) and the top 10 % largest particles d (0.9) comparing to the increased total shear work in cheese producing. Cheeses were produced at 60 °C (■, ●, ◆) and 70 °C (▲) without cooling. The constant shear speed for cheese producing is 600 rpm (◆), 800-1000 rpm (■, ▲) and 1200 rpm (●). The particle size of milk fat globules before cheese making is marked by 'x'.

The data was then replotted to probe any correlation between particle size and released free fat. The decrease in particle size with increasing total shear work in figure 6.11 (along with supporting microscopic data) suggested that a degree of homogenisation has occurred, which would presumably also alter the interfacial composition of the droplets. This was reflected when considering the extent of oil-off in baking in relation to average fat globule size D[4,3] (Figure 6.12). Oil-off could be observed to decrease with a corresponding decrease of average fat globule size D[4,3], oil-off becoming negligible for particle sizes below 4 μm.

As indicated earlier, decreasing droplet size (and thus increasing surface area) could be expected to be accompanied by disruption of the native MFGM and adsorption of soluble fractions of protein from the serum. The replacement of the native milk fat globule membrane with serum protein was considered a key mechanistic component of altering fat droplets from inactive to active particles, which were then able to bind to cheese protein matrix. This effect, in turn, was considered responsible for the transitioning of the fat structure in AMC from localised channel domains to the homogeneous distribution of fat within the protein network. The consequence of this effect was the reduction in incidence of partial coalescence on cooling (and full coalescence on heating), and the corresponding changes to cheese functionality, such as a reduction in oil-off in cheese baking.

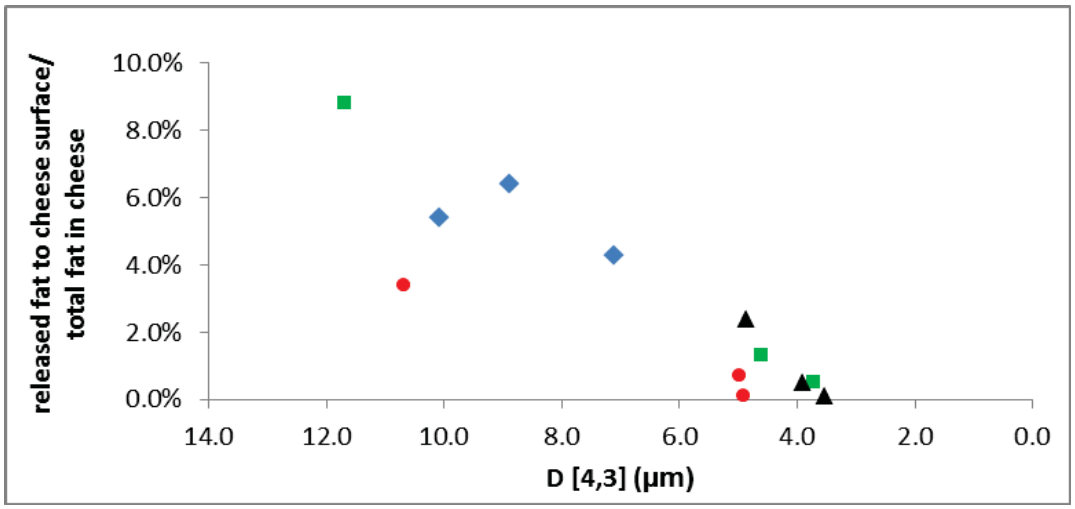


Figure 6.12: Amount of free oil release in cheeses produced in varied shear speed and temperature, in relation to average particle size D[4,3] without cooling. Cheeses were produced at 60 °C (■, ●, ◆) or 70 °C (▲) without cooling. The constant shear speed is 600 rpm (◆), 800-1000 rpm (■, ▲) and 1200 rpm (●).

Confocal microscopy was used to explore the hypothesis that protein adsorption at the fat interface was occurring during AMC producing (chapter 3, section 3.5.2). The AMC made from natural cream was dissociated in an EDTA protein dissociating solution after processing, allowing the interfacial layer of individual droplets to be visualised. Separately labelled images for fat and protein are shown in figure 6.13. The interfacial materials could then be identified as either phospholipid (in red) or protein (in green), appearing as a corona on the surface of droplets.

Cheeses made with 8.3 kJ/kg total shear work at 800 rpm showed an absence of fat globules with protein at the interface, suggesting protein adsorption had not taken place under these processing conditions. For cheeses made at higher shear speeds of 1200 rpm and with 19.0 kJ/kg total shear work, a higher incidence of protein coverage at the fat surface is clearly seen (Figure 6.13b), although a proportion of droplets were still observed as having no bound protein. The adsorption of protein at the fat surface became increasingly apparent for cheese made at 1200 rpm and with 35.0 kJ/kg total shear work. The decrease in particle size D[4,3] from 5.3 µm to 4.6 µm (Figure 6.11a) was accompanied by an observable change in interfacial characteristics where it appeared that a higher number density of droplets were now coated with protein

(Figure 6.13 c). Whilst the CLSM method gave some indication to the replacement of MFGM with protein during processing, this did not provide information on the actual protein concentration at interface.

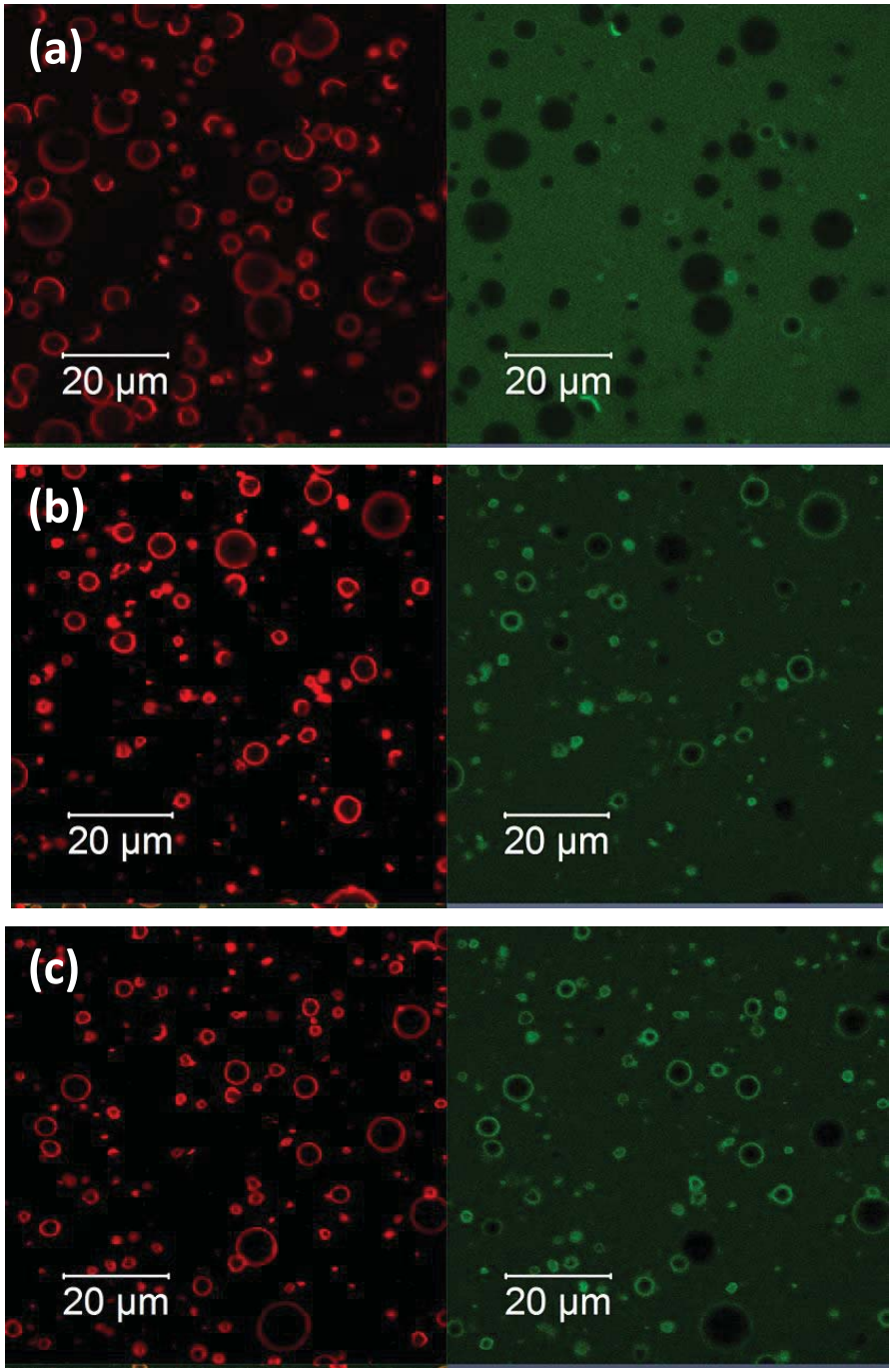


Figure 6.13: CLSM images of dissociated cheese in Walstra solution without Tween. The images are shown in two separated photos. Fat membrane is in red on left photos and proteins are in green on right photos. Image (a) is from cheese made in 10 minutes at 800 rpm (8.3 kJ/kg total shear work); Image (b) is from cheese made in 10 minutes at 1200 rpm (19.0 kJ/kg total shear work); Image (c) is from cheese made in 15 minutes at 1200 rpm (35.0 kJ/kg total shear work).

6.5 Effect of protein adsorption at fat interface on fat filler properties

As discussed previously, the native milk fat globule membrane could be altered during cheese production through a combination of heating and mixing (shear). Findings throughout the thesis have indicated that under particular processing conditions, a dynamic change in interfacial composition of fat droplets could take place that caused MFGM stabilised emulsion droplets to transition from inactive fillers to active fillers within the RVA. Active fat fillers were formed as a consequence of droplet homogenisation during processing and replacement of MFGM with an adsorbed layer of soluble protein present in the serum phase of the cheese.

In this section, the serum collected from non-fat cheese was added into natural cream and the emulsion sonicated. After sonication, proteins from the serum would be expected to adsorb at the fat surface along with any soluble protein already present in the cream. Characterisation of AMCs was carried out from cheeses produced using creams modified in three ways: (1) using preheated natural cream; (2) using preheated natural cream with added NaCas; (3) using natural cream undergoing sonication with the serum collected from non-fat AMC, and fat globules were covered with proteins from the serum (chapter 3, section 3.3.1.2). The protein composition in serum was also evaluated to estimate which proteins might contribute to the change of fat filler behaviour.

6.5.1 AMC made from preheated natural cream

Natural cream of 45 wt.% fat was preheated in the RVA at 800 – 1000 rpm 60 °C for 30 minutes mixing. A visible oil layer appeared in cream after RVA processing due to severe fat coalescence occurred prior to mixing with rennet curd. Figure 6.14a shows the increased size of particles 10 – 100 µm become the primary peak instead of the particles in natural cream at 1 – 10 µm.

AMC was then produced using this preheated cream at 800 rpm 60 °C in 10 min. In terms of fat droplet size distribution, the cheese post-processing displayed a broad modal distribution of particles in the range 1 – 40 µm. Compared to the particle size

distribution of the heated cream before cheese production, the large distribution of 10 – 100 µm clearly decreased as a consequence of AMC processing, most likely due to homogenisation of droplets in response to the shear conditions during AMC preparation. Partial coalescence was still assumed to be occurring as a consequence of cheese storage, on the basis of larger particles (~100 µm) being observed after 7-day cheese storage at 4 °C

CLSM images of the cheese (Figure 6.14b) show large, but reasonably uniformly dispersed fat droplets in protein matrix, with fat droplets in the range of 10 – 30 µm. The fat droplet size observed on confocal was consistent with the particle size distribution measured by Mastersizer on fresh cheese in figure 6.14a. Fat globules of >30 µm were seldom observed on confocal, although the particle size distribution of 7-day cheese displayed particles of 30 – 300 µm. One possible explanation for this observation was that fat globules were still relatively unstable and could readily coalesce with neighbouring droplets once the protein network was dissociated in the EDTA solution, leading to the formation of larger fat globules as measured on the Mastersizer.

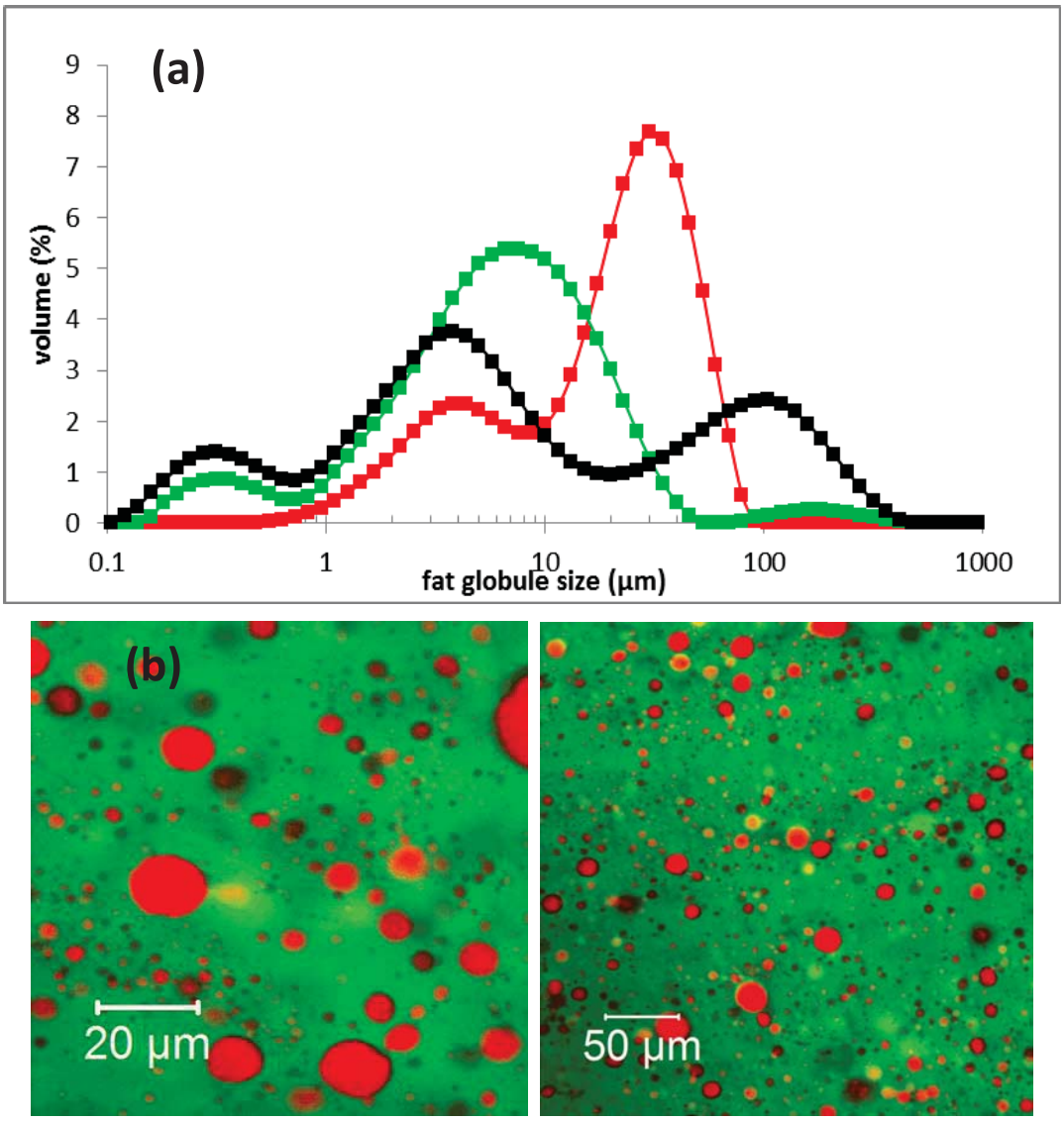


Figure 6.14: Cheeses were produced using preheated natural cream. (a) Fat globule size distribution is compared in fresh uncooled cheese (■), 7 days stored cheese at 4 °C (■) and the cream (■) used for cheese producing; (b) CLSM images were taken using lenses of x 40 (left photo) and x 25 (right photo) on cheese in 7 days storage at 4 °C. The cream was premixed with water in RVA for 30 minutes at 800 – 1000 rpm 60 °C, and then cheese was produced using this cream in 10 minutes at 800 rpm 60 °C.

6.5.2 AMC made from preheated natural cream within NaCas

A 4 wt.% NaCas solution was added into high fat cream (~80 wt.% fat) to obtain cream of 45 wt.% fat. This was then sheared for 30 minutes at 60 °C in the RVA (max. 1000 rpm) before being combined with protein to produce AMC. Here, the added NaCas in the serum was hypothesised to adsorb to the fat surface during RVA shearing of cream, which might then promote the creation of active fat fillers in the production of the AMC.

In contrast to the non-homogenised high fat cream sample with no added protein, fat globule size distribution of the cream did not change after heating and shearing (not showing in Figure 6.15), suggesting the NaCas added to the cream may have provided an inhibitory mechanism towards fat coalescence in figure 6.14a. When this cream was used in preparation of AMC, fat globule size seen to have been reduced after processing, being observed to be in the range 0.2 - 15 μm (Figure 6.15a). However, when cheese was stored in 7 days at 4 °C, three modal distributions developed: a primary distribution of large particles in the range 10 - 150 μm, and two smaller modal distributions in the range 0.2 - 0.8 μm and 0.8 - 10 μm. The increased particle size distribution suggested fat partial coalescence was still occurring within the cheese samples. Cheese confocal images showed a mixture of fat globules are individual dispersed in the protein matrix and a small fraction of these fat globules appeared to have aligned during processing to form linear small fat channels (Figure 6.15b). The shape and size of these fat channels looked different from the previous observed large fat domains of irregular shape. The fat globules were likely incompletely covered with NaCas, which is able to provide stability to the cream during heating and shearing prior to incorporation in AMC, but not necessarily at sufficient coverage to transition droplets from inactive to active filler particles during AMC processing.

This interpretation agreed with findings observed for cheese melting behaviour. For the AMC with added NaCas, the cheese was seen to spread uniformly after 10 minutes baking at 170 °C (Figure 6.15c), and 6.9 ± 1.7 wt.% fat was released to cheese surface. The oil-off was less than the cheese made in the same RVA programme without NaCas (10.1 ± 2.5 wt.% oil-off after 8.3 kJ/kg total shear work) (Figure 6.5). When considered in comparison with the particle size and oil-off data presented in figure 6.12, it might have been expected that oil-off would be negligible as the initial mean fat globule size D[4,3] was below 4 μm for the fresh cheese produced from the RVA; however, this was not the case. The reduction of fat globule size was a consequence of cream modification prior to cheese making, which had already altered the fat interfacial composition, and thereby it was perhaps understandable that the cheese melting did not follow the findings in figure 6.12. On its own, the particle size distribution of the fresh cheese could not in this case be used to predict the oil-off behaviour.

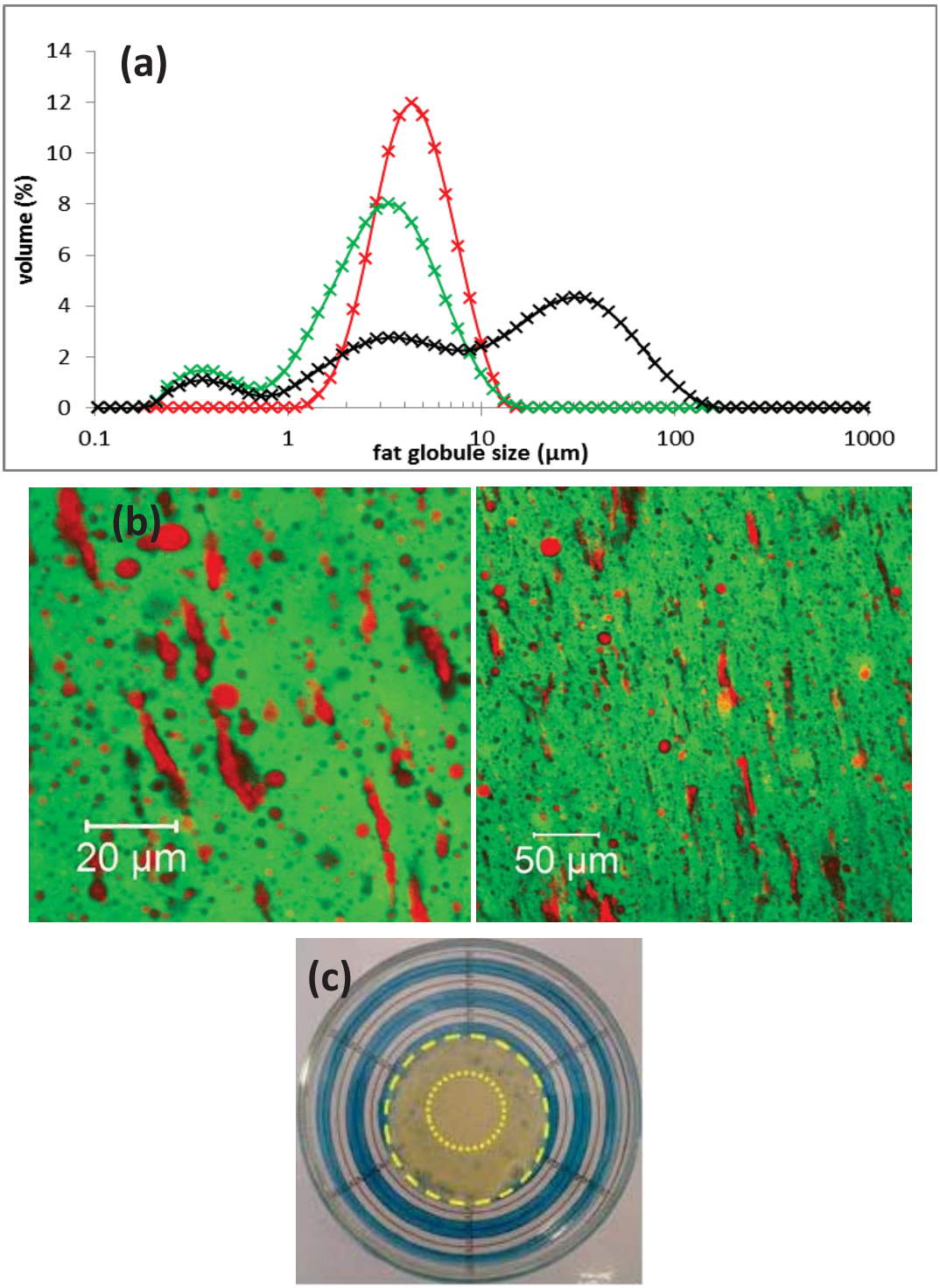


Figure 6.15: Cheeses were produced using preheated natural cream adding NaCas. The cream was premixed with 4 wt.% NaCas in RVA for 30 minutes at 800-1000 rpm 60 °C, and then cheese was produced using this cream in 10 minutes at 800 rpm 60 °C. (a) Fat globule size distribution is compared in fresh uncooled cheese (x), 7 days 4 °C stored cheese (x) and the cream (x) used for cheese producing; (b) CLSM images were taken using lenses of x40 (left photo) and x25 (right photo) on cheese in 7 days storage at 4 °C; (c) Molten cheese in 10 minutes baking at 170 °C, where cheese area is compared before and after baking which were marked by yellow circles.

6.5.3 AMC made from natural cream loading within proteins dissociated from cheese curd

As stated, where shearing conditions were sufficient to cause homogenisation of fat droplets during AMC processing, the presence of soluble proteins present within the cheese curd may have adsorbed at the fat interface during production, thereby modifying the surface properties of emulsion droplets and their interaction with the surrounding protein network.

To explore the role of soluble proteins on AMC structure and properties, the serum from non-fat AMC was collected and protein composition was analysed. Non-fat cheeses were produced by mixing cheese curd, salt and water in RVA at 60 °C using 800 rpm as the maximum shear speed in the first 15 minutes then followed by 1000 rpm. Three serum samples were collected by squeezing the hot non-fat cheese produced for 7 min, 10 minutes and 30 min, respectively. Protein composition was compared in these three serum samples (Figure 6.16).

The four primary peaks from left to right in figure 6.16 indicate α-lactalbumin, β-lactoglobulin, β-casein and α-casein. The three serums sampled at different time points all showed the presence of whey proteins and caseins. The protein peak heights were smallest in serum extracted after 7 minutes processing, and with peak height increasing with increasing mixing time. Interestingly, after 30 minutes of production time, the peak of β-lactoglobulin appeared much higher than the other three protein peaks, becoming the dominant protein in the serum, while the concentration of β-casein and α-casein obviously decreased comparing to the non-fat cheese made in 10 minutes. The production temperature of 60 °C was not high enough to denature whey proteins, and therefore whey proteins from cheese curd continued to dissolve into serum with the increase of residence time. The caseins showed a low concentration in the serum due to strong interactions in cheese protein net-work. The addition of salts in the non-fat cheese production was considered to increase the concentration of dissociated caseins.

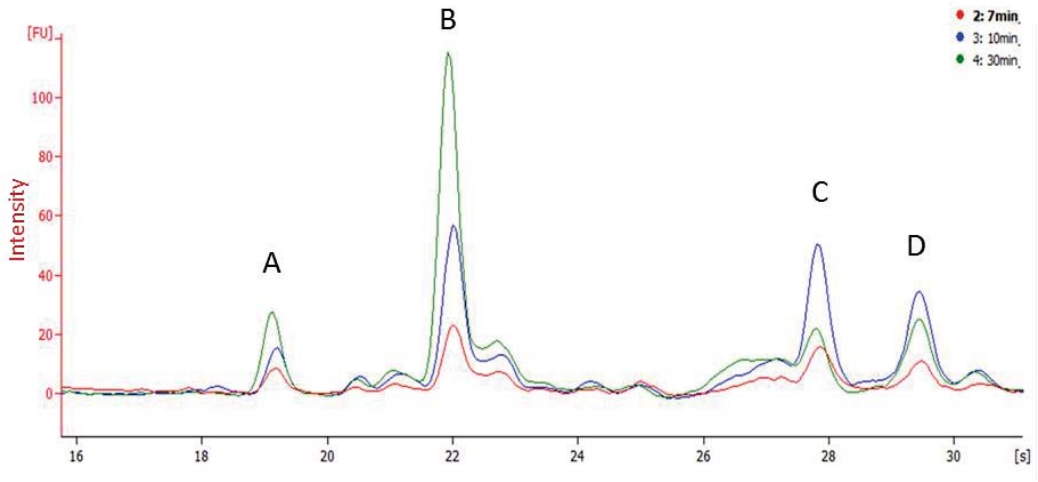


Figure 6.16: The change of protein composition in serum from non-fat cheese. The serums were collected from non-fat cheese produced in 7 minutes (red), 10 minutes (blue) and 30 minutes (green). Skim milk was used as the control sample in the measurement. A: α-lactalbumin; B: β-lactoglobulin; C: β-casein; D: α- casein.

The increase in availability of serum proteins as mixing time increases may have promoted protein adsorption at fat interface, resulting in the transition from inactive to active fat droplets. To verify this idea, high fat cream of ~80 wt.% fat was mixed with the serum phase from non-fat AMC and homogenised by using sonication. Given that active fat fillers were observed after 30 minutes processing of AMC, when produced at 800 - 1000 rpm 60 °C, serum was collected from non-fat cheese based on these parameters.

Mean fat globule size D[4,3] was seen to drop from 5.3 µm to 3.0 µm after sonication, indicating dramatically increased surface area. The fat and protein dispersion was observed on CLSM where samples were diluted 100 times in EDTA solution (no Tween). Aggregated and individual fat globules were observed in the diluted cream samples (Figure 6.17). The fat globules in aggregates appeared smaller relative to individual fat globules and the fat aggregates also showed a degree of associated protein. Although protein signals were found at surface of individual fat globules, the signals did not appear as strong as for the proteins present in the fat aggregates.

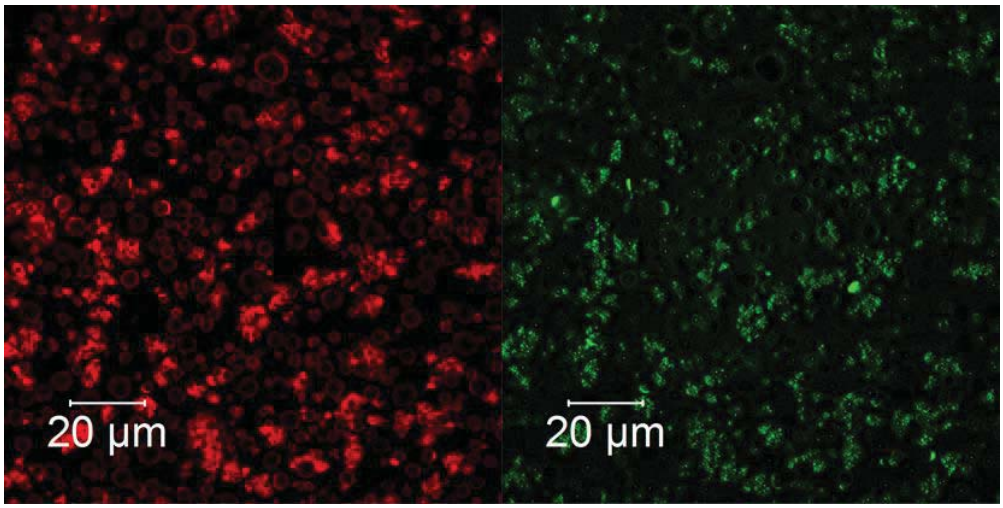


Figure 6.17: CLSM images of cream diluted by the EDTA solution without Tween. The cream was after sonication with the serum from 30 minutes non-fat cheese. The images are shown in two separated photos. Fat membrane is in red on the left photos and proteins are in green on the right photos.

AMC was made for 15 minutes at 800 rpm 60 °C using the cream under sonication with serum. Because the serum from non-fat cheese contained salt, the salt added for cheese making was reduced to match the same level as other AMCs. The small fat globules in cream after sonication were strongly linked by proteins indicated by the confocal in figure 6.17, but these aggregates of fat and protein were not observed after cheese making (Figure 6.18b).

These proteins covered fat globules would be expected to interact with the surrounding cheese protein matrix becoming homogeneously dispersed in cheese. The cheese confocal image showed a mixture of large and small, matching the findings of increased particle size distribution after cheese producing in figure 6.18a. The fat globule size distribution of cream includes the primary peak at 0.6 - 20 µm and also a small peak at 0.1 - 0.6 µm. After cheese production fat globule size distribution showed a spreading of the upper modal distribution, with droplets up to ~50 µm. The cheese cooling modified particle size distribution showed a trimodal distribution in the ranges 0.1 - 0.6 µm, 0.6 - 10 µm and 10 - 100 µm, respectively. Confocal imaging did indicate some droplets in close proximity (Figure 6.18b), and the low temperature (4 °C) of cheese storage was likely still causing partial coalescence of these fat globules, leading to the formation of the upper modal distribution (10 - 100 µm) in figure 6.18a. However, it could also be

observed that most of fat globules were homogeneously dispersed in the protein matrix, characteristic of active fat fillers and that their particle size was therefore unlikely to be affected by cheese cooling and storage. Thus, the particle size peak of 0.6 - 10 µm was observed to be the primary peak in chilled cheese. The modification from sonication did not appear to have occurred for all fat globules, with the possibility arising that a fraction of fat droplets could be present that comprised a mixed interface coated with both MFGM and protein. The fat globules in this case might therefore be expected to have acted as a combination of active and inactive fillers.

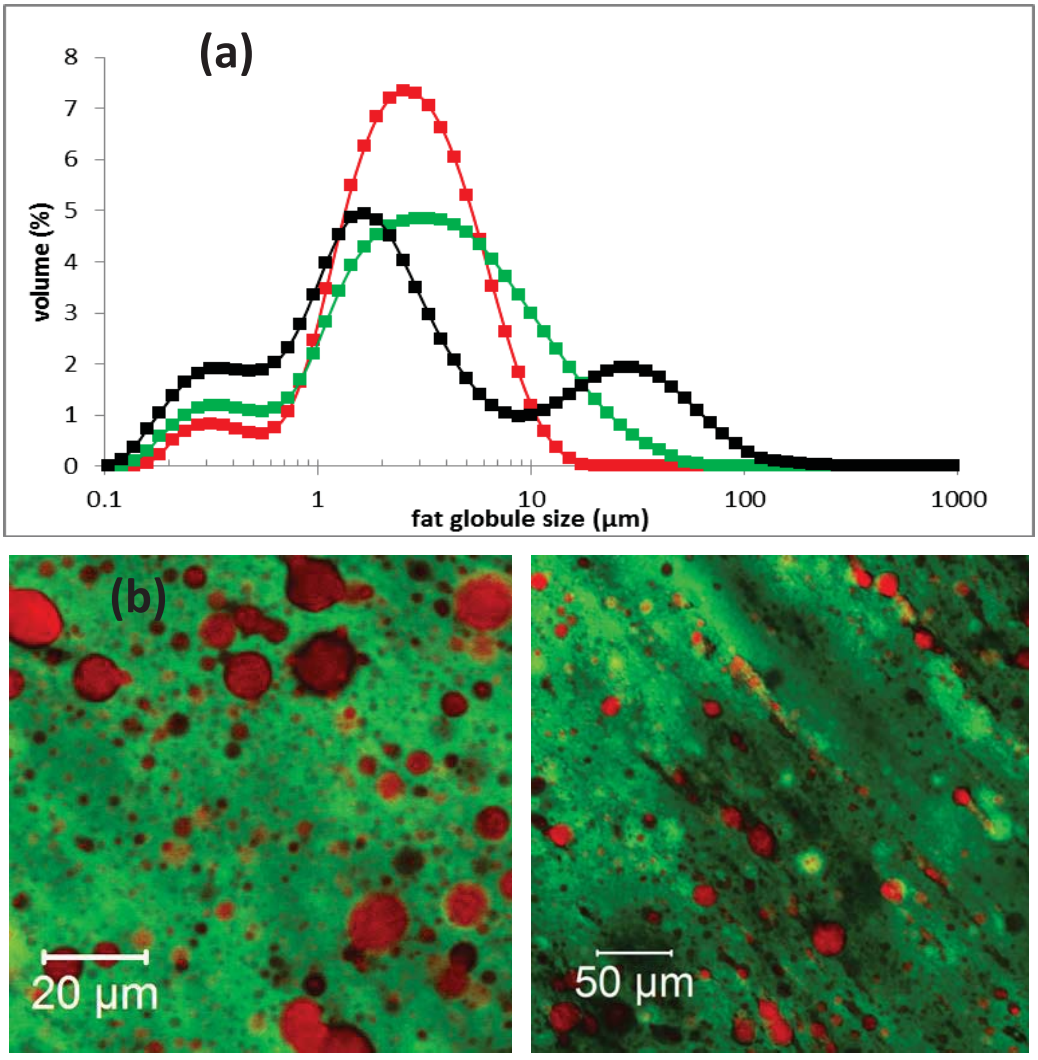


Figure 6.18: Cheeses were produced using the cream after sonication with serum collected from non-fat cheese. The cheese was produced in 15 minutes at 800 rpm 60 °C. (a) Fat globule size distribution is compared in cheese (fresh cheese, ■; 7 days 4 °C stored cheese, ■; and the cream, ■) used for cheese producing; (b) CLSM images were taken using lenses of x 40 (left photo) and x 25 (right photo) on cheese in 7 days storage at 4 °C.

β-lactoglobulin was determined as the major protein in serum after 30 minutes producing (Figure 6.16), and might accordingly be expected to be the predominant protein fraction at the fat interface after extended mixing, thus altering the interaction state of droplets from inactive to active. To validate this hypothesis, cream was sonicated with 1% whey protein isolate (WPI 895, 14.2 % α-lactalbumin and 69.2 % β-lactoglobulin indicated from product bulletin) to create whey stabilised droplets prior to AMC preparation. Fat globule size dropped to 2.9 µm after sonication with confocal imaging showing adsorption of protein at the droplet interface. Cheese production was attempted in 15 minutes up to 800 rpm at 60 ˚C using the cream sonicated with whey proteins. However, it was curious to note that not all the cream could be combined into the cheese, with 2 - 3 g cream was left in the canister for 25 g cheese making. The whey protein covered fat globules appeared less effective at being incorporated into the protein matrix, and the exclusion of cream from the cheese structure as a consequence of processing indicated that binding to the cheese protein matrix may not be taking place.

Many publications have demonstrated how fat globules covered by whey proteins behaved as active fillers in a protein matrix (Xiong and Kinsella 1991, Chen and Dickinson 1999, Liu, Stieger et al. 2015), usually as a consequence of covalent bonding between interfacial and continuous phase protein (Walstra, Wouters et al. 2006). However, whey proteins are unlikely to bind to cheese protein matrix by disulphide bonds in AMC production due to a lack of sulphydryl groups within the rennet network that would be able to form covalent bridges with the whey protein stabilised interfacial layer.

More likely, an electrostatic calcium mediated bridging mechanism was able to take place between negatively charged domains between interfacial and matrix protein. In this respect, pH may represent another important factor on molecular interactions. The pH of AMC is 5.3 which is close to the isoelectric point of β-lactoglobulin (pI 5.2) (Walstra, Wouters et al. 2006), and electrostatic interaction is weak between whey proteins and protein matrix. Although α-lactalbumin has some charge at pH 5.3, but its concentration in serum is low. Hydrophobic bonds will expose in heat, and it is probably the primary interactions between whey proteins and AMC protein matrix at 60 ˚C.

6.6 Cheese rheological properties

The impact of processing conditions and the influence of interfacial composition on cheese material properties is presented in figure 6.19. Rheological data presented in figure 6.19 showed the values of ln(G'/Gm'), for which cheese modulus is calculated in relation to a fat free analogue, thereby indicating the particular contribution of the fat phase to the material properties of the cheese over the temperature range studied. As discussed in chapter 5, samples displaying positive values for ln(G'/Gm') indicated the cheese as having a higher relative modulus in comparison to the fat free sample, with the implication that fat structures provided a mechanical strengthening to the cheese. Conversely, negative values implied that fat structures are weakening the mechanical strength of the cheese, since the modulus of these samples was relatively lower than that of the fat free sample.

All samples shown in figure 6.19 displayed decreasingly positive values for ln(G'/Gm') as the temperature was increased from 4 °C to 30 °C, indicating that fat structures provided a mechanical reinforcement when the fat phase became increasingly solid due to crystallisation at the reduced temperature.

Two of the AMC samples in figure 6.19 were shown to have reached negative values at ~35 °C, which would initially appear to have indicated the presence of inactive fillers. However, the subsequent increase in ln(G'/Gm') as temperature was increased above 45 °C could have demonstrated the presence of mixture of active and inactive droplets, rather than a dominant fraction of inactive droplets, for which negative or near-zero values for ln(G'/Gm') would have been expected for temperatures >40 °C. The similarity between the profiles of these two cheeses (x and ◆ in figure 6.19) was striking, even though their processing and formulations conditions were different. One AMC sample (◆ in figure 6.19) was produced at low shear speed (600 rpm) but with high total shear work due to the long residence time. The other AMC was produced using the preheated cream within NaCas (x in figure 6.19), in which NaCas had stabilized the fat globules in cream. The structures suggested by rheological data were found to be consistent with

observations made regarding technical functionality of the cheeses, i.e. in their relative oiling off and flow behaviours.

Figure 6.19 also includes two AMCs made in high shear speed (1200 rpm) for 10 minutes and 15 minutes respectively. The 15 minutes made AMC (1200 rpm) showed behaviour consistent with AMC made with active fillers (i.e. for emulsion droplets fully stabilised with NaCas (chapter 5, Figure 5.8b). However, the curve of AMC produced at 1200 rpm with a shorted residence of 10 minutes started to drop at temperatures above ~50 °C, indicating behaviour more consistent with inactive fillers. This cheese showed some limited oil-off (Figure 6.10) with a slightly increased particle size D[4,3] (Figure 6.11a). In contrast, the cheese prepared at 1200 rpm with the increased residence time of 15 minutes displayed a more prominent decrease in fat globule size, with subsequent changes to functionality also being observed, such as oil-off becoming negligible (Figure 6.10).

Another sample in figure 6.19 shows the rheological behaviour for AMC made at 70 °C. The particle size distribution (Figure 6.4, red) and cheese microstructure (Figure 6.2b) indicated the presence of small and finely dispersed fat droplets, suggestive of active fillers. The rheological profile was consistent with this interpretation, is was in accordance with the fact that this sample displayed poor melt and flow on heating (Figure 6.5b). Temperatures of 70 °C are able to denature whey proteins (De Wit and Swinkels 1980), and it may be that the cross-linking of whey proteins to cheese protein matrix further strengthened the interactions between fillers and matrix. Whey proteins present at the fat interface help to resist fat droplet deformation, with fat droplets behave like ‘soft capsules’ surrounded by a jammed shell (Erni, Fischer et al. 2005, Erni, Windhab et al. 2011). Thereby a more stable particle size of the larger fat globules d(0.9) was observed when compared to the increased d(0.9) in AMCs made at 60 °C (Figure 6.11c).

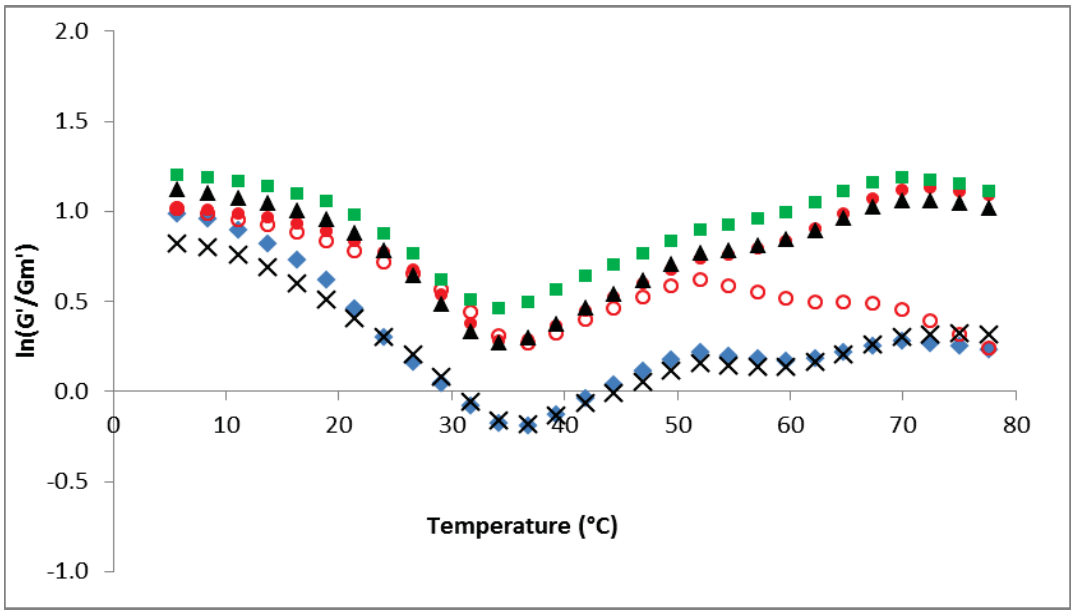


Figure 6.19: Small strain rheological properties are compared in cheese heated up from 4 °C to 80 °C. G' is cheese storage modulus and Gm' is cheese matrix storage modulus, which was measured on non-fat cheese of the same ratio of water to protein. Cheeses were made by varied constant shear speed, producing temperature and preheated natural cream. Cheese made in 40 minutes 600 rpm 60 °C (◆) within 45.3 kJ/kg total shear work; cheese made in 10 minutes 1200 rpm 60 °C (○) within 19.0 kJ/kg total shear work; cheese made in 15 minutes 1200 rpm 60 °C (●) within 35.0 kJ/kg total shear work; cheese made in 30 minutes 1000 rpm 70 °C (▲) within 42.8 kJ/kg total shear work; cheese made in 10 minutes 800 rpm 60 °C and the cream was precooked with 4 % NaCas (X); cheese made in 15 minutes 800 rpm 60 °C and the cream was after sonication with serum collected from non-fat cheese (■).

6.7 Conclusion

Modification to AMC structure as a consequence of processing could be determined in relation to the shear work applied during RVA residence. Findings indicated that fat globule size of emulsions in AMC decreased as total shear work in cheese production was increased (Figure 6.11). Structural changes manifested themselves in aspects of cheese functionality, most notably in regards to oil-off and flow during baking. Here it was observed that oil-off in cheese melting dropped to zero when total shear work was high enough to drop D[4,3] of droplets below 4 µm (the average size of natural cream) during processing without cooling (Figure 6.12). Changes to the droplet interface during processing also contributed to the structure dynamics observed under various processing conditions, enabling a transition of fat globule properties from inactive fillers to active fillers (Figure 6.20). Intensive mechanical treatment of the AMC caused both homogenisation of fat droplets and adsorption of soluble proteins at the fat globule

interface. The proteins in cheese serum included caseins and whey proteins. Whey proteins were unlikely to strongly cross-link to the protein matrix in cheese processing below whey protein denature temperature (70 °C) to form the disulphide covalent bonds (De Wit and Swinkels 1980). Other mechanisms, such as electrostatic crosslinking, may have been responsible for protein adsorption. This may also have been the case for any adsorbed casein fractions. However, the caseins in serum were found to create active-fat fillers only when milk fat globule membrane was sufficiently modified by heating and shearing during cheese producing. The addition of non-adsorbing NaCas to the fat globules covered with intact milk fat globule membrane was unlikely to create active-type fat fillers in AMC. The protein adsorption concentration and the protein cross-link strength to cheese protein matrix appeared to be the two key factors for generation of active fat fillers in AMC production.

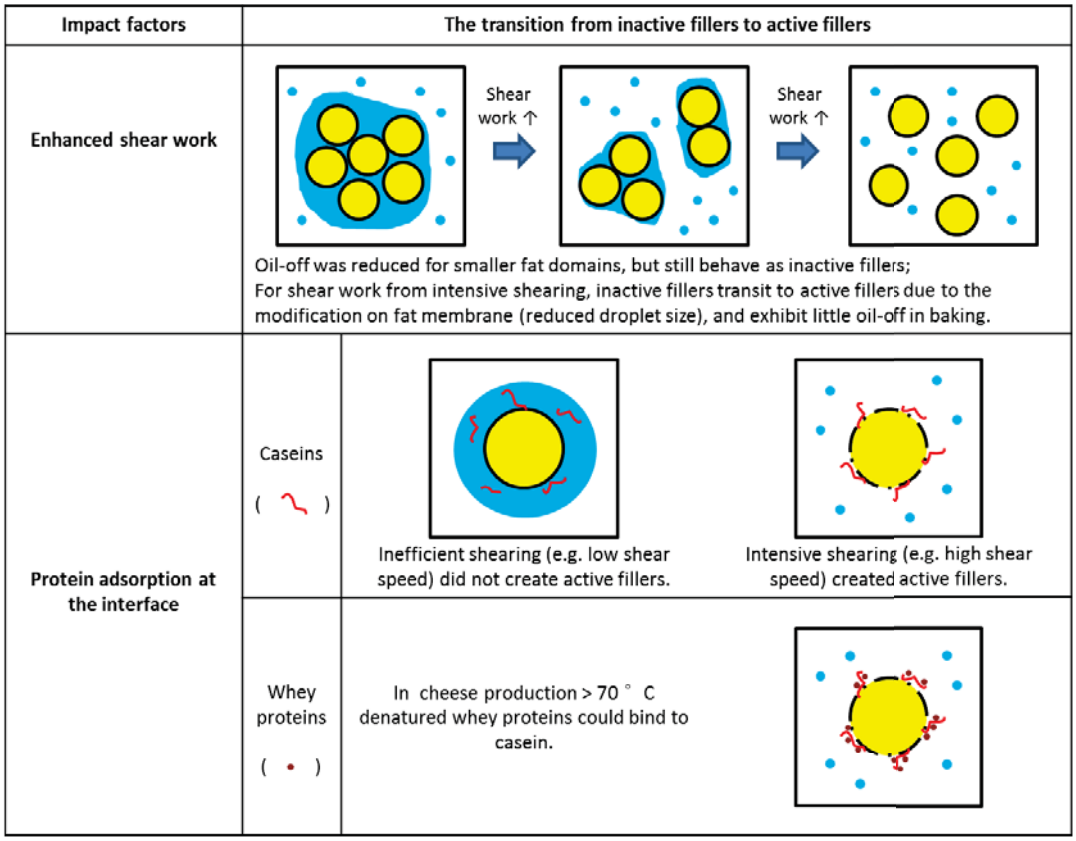


Figure 6.20: Schematic diagrams of the impact factors to transit inactive fat fillers to active fat fillers in cheese production.

Chapter 7: General conclusions

This project has sought to understand and explain the functionality of a model Mozzarella cheese as a consequence of colloidal properties of the fat, water and protein components of the cheese. The alternate make cheese (AMC) used in this study provided a good model to investigate the effect of colloidal interactions between fat and protein on cheese microstructure and related material and functional properties (such as melt/flow and oil-off). Of particular interest was how the state of the interfacial layer was able to influence the interactions, distribution and stability of emulsion droplets within the protein network, and how this could potentially be controlled through an understanding of formulation and process design. This section provides some final additional consideration to the underlying mechanisms influencing cheese properties based on the findings within the study.

7.1 Cheese melting

The release of oil during baking has been seen to be a requisite property for achieving acceptable melting behaviour, notably in regards to the flow and appearance of cheese during cooking. Findings from this study have demonstrated that the particular structural arrangement and interactions of fat droplets within the cheese can provide a significant contribution to the melting properties of the cheese. Appropriate levels of oil-off are required for pizza cheeses, since this enhances cheese melting spread ability and prevents excessive cheese browning (blistering) due to the Maillard reaction (Everett and Auty 2008). When baking a pizza with fat-free Mozzarella cheese, the dehydration of cheese surface causes scorching and limited cheese melting (Rudan and Barbano 1998) and this behavior was also observed for AMC prepared using emulsions stabilized with sodium caseinate (so-called active filler emulsion). However, the cheese displaying too much oil-off is not ideal for pizza appearance (appearing excessively oily and with no blistering). This extreme case of oil-off has been observed in the AMC produced from fat globules covered with Tween 20 (inactive filler emulsion). Less oil-off (< 12 wt.% of total fat in figure 5.11) appeared on the AMC made from nature cream, where the fat

globules are covered with milk fat globule membrane, than the AMC made from Tween 20 coated fat globules (> 16 wt.% of total fat in figure 5.11).

For AMC prepared with non-homogenised, natural cream, melting properties were found to be particularly dependent on the processing conditions within the RVA. Oil-off during baking was progressively reduced in AMC either through increasing residence time or by increasing the maximum shear speed of mixing. Characterisation of the emulsion systems within the cheese has suggested that homogenisation of fat droplets coupled with disruption of the native MFGM and subsequent adsorption of proteins in serum to the surface of fat droplets is responsible for the change in the manner in which fat droplets interact and structure within the protein network.

Protein adsorption during RVA shearing has been presented as the main mechanism by which inactive emulsion droplets can be rendered as being able to form bonds with the surrounding protein phase. Here, proteins have been found to be located at the fat interface even where fat globule size was minimally changed after short processing residence time at high shear rate. Active fat fillers were created by the coating of soluble caseins and/or whey proteins present within either the protein source or possibly within the cream. On this basis, it can be concluded that the milk fat globule membrane provides an important contribution to the inactive properties of fat droplets when non-homogenised cream is used as the primary fat source. Acknowledging that desirable melt, flow and oil-off properties area imparted by this particular fat droplet structure and its structural arrangement within AMC, a particular consideration in process design requires that the native membrane be kept intact wherever possible during cheese manufacture.

The colloidal microstructure of Mozzarella cheese comprising inactive emulsion droplets, such as provided by non-homogenised cream, is characterised by the presence of fat channels distributed throughout the protein network. These structures are considered contributory to the oil-off and protein flowing in cheese melting. The formation of fat channels allows for the localisation of inactive emulsion droplets into phase concentrated domains. Whilst (from particle size analysis) it appears that after processing (and when the fat phase is still molten) the stability of droplets in these highly

concentrated regions is retained, on cooling extensive partial coalescence occurs within these regions. This partial coalescence can enhance cheese firmness while the fat is in a crystalline state.

7.2 Phase separation

The oil-off in cheese melting occurs due to a fat-protein phase separation occurring at high temperature. This kind of phase separation is required on pizza cheese. Fat-protein phase separation observed for AMC was also evidenced as a decrease in viscosity for the model cheese prepared calcium caseinate matrix after long time mixing. Here, the phase separation within cheese structures will be briefly discussed in the context of the interactions among protein, water and fat.

1. Interactions between fat droplets and protein have been studied through manipulation of the fat interfacial composition. The active fat fillers stabilize the emulsion structure due to the strong interactions between fat interface materials and protein matrix, resulting in no oil-off in cheese baking. The interactions between inactive fillers and protein matrix are weak, and the fat is able to be released from the protein network to form the oil-off.
2. Protein-protein interactions in cheese protein matrix affect the fat-protein phase separation as well. The fat-protein phase separation was observed in the production of the model cheese of calcium caseinate matrix with inactive fat fillers. The proteins are strongly linked by the Ca^{2+} in calcium caseinate, and the high temperature of the RVA mixing accelerates protein aggregation to result in the fat-protein phase separation in cheese producing. The cheese viscosity declined after phase separation and the model cheese could not be stretched due to the breakup of protein networks, possibly rendering the protein structure as a particle gel rather than a polymer gel. According to the AMC, interactions of protein matrix are not that strong as for the calcium caseinate, and fat-protein phase separation did not occur in the cheese processing.
3. The interactions between water and protein are from the hydrophilic bonds, which are influenced by temperature. During cold storage (5 °C), most of water (serum) was adsorbed by protein matrix, depleting serum water from the fat channel domains

and further concentrating the emulsion phase in these regions. The serum pockets should appear again at temperature above 30 °C, indicated by the decreased cheese storage modulus. The water-protein interactions become weak at the cooking temperature of 60 °C resulting in visible serum in non-fat AMC. At the baking temperature, water evaporation broke through protein network, accelerating the fat-protein phase separation to release the fat from the serum-fat channels.

7.4 Recommendations

1. The effect of fat globule size should be studied on the active/inactive fillers in AMC. Smaller fat droplet sizes could be obtained by enhancing the pressure of homogenization when preparing the cream emulsions. The reduced droplet size will increase the fat interfacial area, and therefore the effect from interfacial material should become stronger on small fat globules. Cheese firmness and moisture content are probably different when changing the size of fat globules. Please see section 2.2.1.2. in literature review.
2. Whey protein denaturation needs a relatively high temperature, and thereby producing temperature should be investigated on the modification of cheese interfacial ingredients and cheese functionalities. The whey protein concentration in serum kept on increase in AMC production, and residence time should be considered when studying the effect of whey protein denaturation on fat filler properties.
3. Fat crystallization and partial coalescence in AMC have been studied in cheese samples made from milk fat and canola oil, respectively. The data have indicated fat partial coalescence would accelerate cheese melting, but not known how the partial coalescence affect oil-off and protein flowing. Study could continue at this part to manipulate the ratio of partial coalesced fat in cheese. For example, using the mixture of milk fat and canola oil in cheese production, or allowing partial coalescence to occur in cream before cheese production. The change on fat interfacial ingredients and fat globule size should be considered after partial coalescence.
4. Fat volume fraction is another important factor contributing to cheese functionalities. Inactive fat concentration is likely to affect cheese moisture, because the study shows serum-fat channels supply the space to increase cheese moisture. On this

basis, the collision on fat globules is reduced in fat-reduced cheese and fat channels of smaller size and less numbers should be found in AMC.

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Appendix I: Capillary number and Reynold number in the RVA mixing

Parameters used for calculation		Units	Values	Comments
d	diameter of particles	metre	4×10^{-6}	4 μm
ρ	density of continuous phase	kg/m^3	1000	Approximate value, water ≈ protein
n	rotational speed	1/sec	104.7	n = 2 * 3.14 * rpm/60
rpm	RVA speed	revolution/min	1000	1000 rpm
η_a	apparent viscosity in RVA	Pa*sec	2	For non-fat AMC at 60 °C
η	viscosity of water	Pa*sec	5×10^{-4}	Approximate value
R	radius of RVA canister	metre	0.019	
r	radius of RVA impeller	metre	0.016	
Ý	shear rate at the wall of canister	1/sec	446.6	Ý = n * r/ (R-r)
σ	interfacial tension	J/m^2	0.01	Approximate value

Parameters mentioned in thesis		Equations	Values (d = 4 μm)*	
Ca	Capillary number	η*Ý*d/(2*σ)	Cream: 4.5×10^{-5}	Cheese: 0.2
Re	Reynold number	$rpm*\rho*(2*r)^2/\eta$	Cream: 2.0×10^{6}	Cheese: 512
λ	viscosity ratio	$\eta_{fillers}/\eta_{dispersion}$	Cream: 1	Cheese: 2.5×10^{-4}

*: If fat droplet size is 1 μm, the Capillary number of the cream and the cheese is 1.1×10^{-5} and 0.045, respectively, and the Reynold number is little changed compared to the fat droplet of 4 μm.