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Design and manufacture of lipid particles for emulsion stabilisation

By

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Abstract

Much of our everyday nutrition is based on foods that are emulsions or have been emulsified at a certain stage during their processing. Emulsions' inherent metastable state urges the introduction of emulsifiers, as a physical barrier that prevents droplets from coming together. In lieu of this approach, Pickering emulsions (i.e. droplets stabilised by solid particles) have amassed a great deal of both theoretical and commercial interest due to their scope of added functionalities. These include an exceptionally high stability and the compliance with the current demand for/appeal of formulations based on natural ingredients. Yet, their large scale adoption by the food industry has been hampered by the lack of a reservoir of edible structures that can be used as Pickering stabilisers.

This thesis suggests the use of particles made of lipids as an alternative option for the design of Pickering-type emulsion stabilisers. Colloidal crystalline structures were fabricated via a melt-emulsification and subsequent crystallisation route. Solid particle characteristics, crucial for Pickering stabilisation (e.g. size, interfacial behaviour), could be controlled by adjustments to formulation and processing parameters. Building upon the knowledge gained from this initial study, colloidal lipid particles were assessed for their effectiveness to act as emulsifiers in oil-in-water (o/w) emulsions and also, for their aptitude to undergo a dehydration and rehydration process without variation of dimension or Pickering functionality.

To my family,

teachers, and mentors

"Nothing in this world can take the place of persistence.[...]Persistence and determination alone are omnipotent."

Calvin Coolidge (1872-1933)

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Embarking on this EngD program was an amazing life journey full of challenges. It was one of the most defining times of my life and an emotional ‘rollercoaster’; exhilarating, frustrating, motivating, maddening and intellectually stimulating from start to finish. Reaching the end, I realised it was the fruit of hard personal effort and tenacity but, at the same time, a distinct contribution of so many people. It is a pleasure to thank everyone who has made this dissertation possible and this journey so unforgettable.

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I extend my gratitude to my industrial supervisor, Dr Paul Smith for providing valuable insight to my work, and also everyone at Cargill who has been involved in the ePET project over the years. Thanks goes to Dr Richard Greenwood for his support and for managing the Formulation Engineering EngD program, as well as the whole team at the School of Chemical Engineering, Kathleen Hynes, Lynn Draper and John Hooper who always dealt with our enquiries in the kindest way. A special mention to the IT Service Desk and Sailesh Pattni, in particular, who was always keen on solving my, more often than ideal, laptop issues. I gratefully acknowledge the generous financial support over the four years from the Engineering and Physical Sciences

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My parents made me the person I am today, they shaped my ethos on living, they taught me to be passionate about all endeavours, to follow my dreams and stay curious about the world around me. Σας ευχαριστώ από τα βάθη της καρδιάς μου!

Table of Contents

[1] This chapter has been published in *Colloids and Interface Science Communications*, hence the format is based on the requirements of the Journal.

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Acronyms & Abbreviations

CITREM	Citric acid esters of mono- and diglycerides of edible fatty acids
CLSM	Confocal laser light scanning microscopy
CP	Cetyl palmitate
DE	Dextrose equivalent
DSC	Differential scanning calorimetry
EHEC	Ethyl(hydroxyethyl)cellulose
ePET	Edible pickering emulsion technology
FD	Freeze-drying
FF-TEM	Freeze-fractured transmission electron microscopy
GRAS	Generally recognized as safe
GSC	Glyceryl stearyl citrate
HLB	Hydrophilic-lipophilic balance
HPH	High-pressure homogenisation
HPMC	Hydroxypropyl methylcellulose
IFT	Interfacial tension
LACTEM	Lactic acid esters of mono- and diglycerides of edible fatty acids
LbL	Layer-by-layer
LD	Laser diffraction

LMP	Low methoxyl pectin
LMW	Low molecular weight
MD	Maltodextrin
MVD	Microwave vacuum drying
NaCas	Sodium caseinate
NLC	Nanostructured lipid carriers
NMR	Nuclear magnetic resonance spectroscopy
o/w	oil-in-water
PCS	Photon correlation spectroscopy
PdI	Polydispersity index
PGPR	Polyglycerol polyricinoleate
POE	Polyoxyethylene
PVA	Poly(vinyl alcohol)
RI	Refractive index
SDS	Sodium dodecyl sulfate
SLN	Solid lipid nanoparticles
T80	Tween 80
TAG	Triacylglycerol
TS	Tristearin

w/o/w	water-in-oil-in-water
WPI	Whey protein isolate
XRD	X-ray diffraction

Nomenclature

γ	Interfacial tension	N/m
ΔE	Change in Energy	J
ΔH	Change in Enthalpy	J
ΔP_L	Change in Laplace pressure	Pa
ΔT	Change in Temperature	K
θ	Contact angle	degree
$D_{3,2}$	Surface weighted (Sauter) mean diameter	µm
$D_{4,3}$	Volume weighted (de Brouckere) mean diameter	µm
G	Gibbs free energy	J
H	Enthalpy	J
R	Radius of a particle or droplet	m
S	Entropy	J/K
T	Temperature	K

Chapter 1

General Introduction

1.1 Research Background and Motivation

In recent years, a shift towards an on-the-go lifestyle, coupled with a mounting demand for benign ingredients for diet supplementation, has evolved to become an – almost – mainstream attitude on behalf of consumers at a global level. Consumers are seeking healthier, natural, and/or minimally processed foods, while they scrutinise nutritional labels for unadulterated ingredients. This, in turn, fuels a race for food and beverage companies and formulators to develop new products, or re-formulate existing ones, to render them "clean-label". The acute awareness of sustainability together with the tilt to natural/green products, are actually some of the principal drivers of formulation efforts in the UK, as pinpointed in the findings of the Formulation Special Interest Group (SIG) pertaining to the Technology Strategy Board (TSB) (now called Innovate UK) (TSB, 2013).

A plethora of foods we consume nowadays is based on blending oil and water to form an emulsion. Butter, margarine, mayonnaise, salad dressing, ice cream and sauces are amongst the most common paradigms of food emulsions. These systems have the propensity to separate quickly after mixing. An emulsion-based food product's trek from manufacturing through to the kitchen cupboard or fridge of the consumer is full of such destabilisation pathways. It is therefore of paramount importance to maintain the integrity of a product, and emulsifiers/stabilisers have traditionally been the route towards achieving stabilisation. In addition, the emulsifiers' market has a strong potential to keep in pace with the new demands and trends in the field of food nutrition. The fact that the global emulsifier market, across a variety of sectors (including food, cosmetics and personal care, pharmaceutics), is a highly profitable one, and is expected to reach $8.44 billion by 2021 (MarketsAndMarkets, 2016), adds to its potency.

Since a significant proportion of the emulsifiers is synthetically derived, research attempts, in a bid to integrate the compelling need for natural ingredients, have been directed towards reducing or even eliminating their usage. In the quest to develop products devoid of such compounds or to identify alternative structures, food manufacturers need to face a number of challenges. The most important is ensuring efficacy and functionality without jeopardising safety and quality or any sensory characteristics (e.g. flavour, texture) of the product, as this might have adverse effects in terms of consumers' acceptability and the overall pleasurable process of eating (Kaufmann & Palzer, 2011; Norton, Wallis, Spyropoulos, Lillford, & Norton, 2014). Tackling these issues via engineering the foodstuff's microstructure at a micro- or macro- level has been proved to be an essential approach. Food structures can be designed to possess tailored properties and some highly promising showcases exist, in regards to reduction of salt (Pays, Giermanska-Kahn, Pouligny, Bibette, & Leal-Calderon, 2002), sugar (Sala, Stieger, & van de Velde, 2010), and fat (Dickinson, 2011) content, all constituting major public health burdens.

Aiming to prolong the lifespan of emulsion-based products, the most successful strategy has been the stabilisation by colloidal solid particles (Pickering emulsions). Although the concept of stabilisation by an interfacially adsorbed particulate material has long been applied in foods, e.g. casein micelles in homogenised milk or fat crystals in margarine (Binks & Horozov, 2006), the 'secret life' of Pickering emulsions has begun to be unravelled only over the past decade. Particle-stabilised emulsions benefit from an outstanding stability against coalescence originating from the mechanical barrier formed by the particles adsorbed at an interface (Dickinson, 2012). As such, the use of small molecule surfactants can be circumvented, while at the same time particle-laden interfaces hold a substantial promise of revolutionising the capabilities of emulsions.

The functionality of Pickering systems has been extensively assessed and literature is voluminous in the context of organic and inorganic materials as the building blocks of particles. Yet, these candidates seldom comply with governmental food safety standards and respective legislation. The food industry could harness the above mentioned functionality and adapt it to edible systems, as at the moment there is a limited range of food-grade species that can be produced at a commercial scale (Morris, 2011). Consequently, the need for sourcing or constructing edible Pickering particles from, preferably, readily accessible and inexpensive resources has emerged.

Designing and subsequently fabricating structures allows desired functionality to be granted, precise end-user applications to be addressed and specific materials (e.g. sustainable, risk-free) to be introduced, and all these drivers were the impetus of this project. Further to opening the gateway to improved products, the design of Pickering particles would aid to extend significantly products' shelf-lives. In the future, this approach could also be used for the formation and control of even more complex emulsion microstructures (e.g. double emulsions), particularly in relevance to their stability. Moreover, emulsion droplets with modulated interfacial characteristics could be designed in a way that novel systems, responsive to a range of external triggers, could be produced (Tang, Quinlan, & Tam, 2015). Thus, the overarching aim of this work is to establish formulation design rules as well as processing routes in order to manufacture stable edible Pickering particles for the stabilisation of oil-in-water (o/w) emulsions.

1.2 Industrial Relevance

Industrial sponsorship for this work was provided by Cargill Ltd. which is a family-owned multinational commodities firm, specialised in providing food and agricultural products, as

well as risk management and, financial and industrial services around the globe. Cargill perpetually ranks first in Forbes list of America's largest private companies, generating $107.2 billion in sales and other revenues in the fiscal year 2016 (Cargill, 2017).

This project was related to Cargill's Food Ingredients operations and, in particular, it was in close collaboration with the European Research and Development Centre in Belgium. Cargill holds a significant share of the global Food Ingredients market and supplies ingredients to several manufacturers of consumer foods. In addition, many of the products within its portfolio are emulsion-based (e.g. margarine, dressings etc.). The performance of the model systems developed in this study would need to be accurately replicated by the Applications team responsible to take the technology on board, transfer it to pilot work, test it and introduce it to their customers.

As part of the edible Pickering Emulsions Technology (ePET), it is foreseen that this technology will contribute in extending and adding value to Cargill's product portfolio. By virtue of the unique properties that they confer on emulsion systems, novel edible Pickering particles will allow the fabrication of highly functional and clean-label ingredients, and healthier/safe foods. This is well aligned with Cargill's strive to develop products that meet the shifting expectations of the modern consumer who seeks for label-friendly ingredients, devoid of artificial chemicals (E numbers) or other complicated names. The design and manufacture of particles and emulsion microstructures with reduced emulsifier levels will allow a deeper understanding of the challenges associated with this technology to be gained, and eventually the potential of more "natural", clean-label food products to reach the market. The almost irreversible adsorption of Pickering particles at an interface, would greatly favour the stability of food systems, by significantly enhancing their shelf-life.

More specifically, the knowledge emanated from the fabrication of colloidal lipid-based particles could be a great asset to Cargill's Fats & Oils product sector. It has been demonstrated (with launched products based on this technology) that, provided the crystallisation is well controlled, small amounts of hard (or saturated) fats can give rise to improved crystal structures than with conventional technology applied in the margarine processing. Consequently, up to 80% decrease in saturated fat can be achieved, while at the same time, processing is cheaper with a reduced environmental impact (Unilever, 2012).

Should the fabricated lipid Pickering particles exhibit the potential to be dried and rehydrated with a minimal loss on their properties and functionality, versatile and functional food ingredients could be yielded. This could deliver a high value for the ingredients supplied by Cargill to food and beverage manufacturers. Last but not least, the benefits accrued from this project could be spanned to cosmetics and pharmaceuticals, which are also within Cargill's activities. Generation and understanding of lipid particulate Pickering structures can be utilised to create products containing – highly desirable – natural chemicals and tailored properties, e.g. particles could be used to control the melting performance of a lipstick or the viscosity of a face cream.

1.3 Aims and Objectives

The overall objective of this work was to design and use bio-derived materials as the building blocks of Pickering particles. Emphasis was placed on the production of stable lipid-based particulates (i.e. fat crystals) and on their applicability as effective stabilisers for o/w emulsions. The specific objectives of this work are as follows:

- To fabricate and characterise lipid crystal particles with distinct chemistries and compositions via a well-controlled process
- To conceive approaches to manipulate the Pickering functionality of the manufactured particles by identifying key parameters that are linked to it (e.g. size, interfacial behaviour) and establishing stability criteria
- To explore the application of lipid particles as emulsion stabilisers of o/w emulsions and understand the stabilisation/destabilisation mechanisms
- To study the behaviour of lipid particles that undergo a drying and rehydration stage, specifically in relation to the maintenance of their functionality
- To design and produce o/w emulsions stabilised by both edible particles and surfactants (mixed emulsifier system) as separate species

1.4 Thesis Outline

The work presented in this thesis is organised into eight chapters. The four experimental chapters (Chapter 3, 4, 5 and 6) are all written in the style of peer-reviewed publications, in essence containing an introduction, an experimental section, results and discussion and concluding remarks/suggestions for future work in the end. A summary of each of the chapters is presented below:

Chapter 1: General Introduction

The chapter introduces the motivation and principal drivers of the work presented in this thesis, the relevance to the industrial sponsor, the aims and objectives of the study. It also provides an overview of the thesis organisation and details how this work has been disseminated as well as the relevant plan for the near future.

Chapter 2: Literature Review

The chapter gives an overview of the theoretical background and the current understanding of emulsions, with a special mention to emulsion stabilisation by solid particulates and the relevant work that has been conducted. It also presents a review of the up-to-date work on solid lipid nanoparticles and dehydration methods, as together these materials and processes underpin the principal research directions explored within this work.

Chapter 3: Fabrication of edible solid lipid particles in the presence of surface active species: Controlling particle microstructure attributes linked to Pickering functionality

This chapter investigates the fabrication of solid lipid particles from two lipid sources through a melt-emulsification-crystallisation method. It considers several formulation and processing parameters and their effect on particles' properties, in particular those related to Pickering functionality (e.g. size, interfacial behaviour).

Chapter 4: Oil-in-water emulsions stabilised by solid lipid particles

Work in this chapter assesses the potential of the manufactured lipid particles to act as Pickering stabilisers of o/w emulsions. The formulation and characterisation of the lipid particles-decorated o/w emulsions are presented, and new insights into the mechanisms responsible for emulsion (de)stabilisation are provided.

Chapter 5: Maintaining the Pickering functionality of solid lipid particles upon dehydration and subsequent rehydration

This chapter is concerned with the isolation of the lipid particulates from their aqueous environment via a dehydration route. The focus was placed on several formulation variables that can markedly influence the conservation or loss of the particles' original properties. Solid lipid particles can remain functional (e.g. stabilisation of emulsions) after a dehydration-

rehydration cycle, provided that certain key parameters are addressed during their initial fabrication.

Chapter 6: Emulsions co-stabilised by edible Pickering particles and surfactants: The effect of HLB value

Fabrication of particles was previously investigated on lipid crystalline structures in combination with surface active species (Chapter 3). The aim of this chapter was to explore the individual contributions of a colloidal solid structure and a surface active molecule onto such a mixed emulsifier combination/system.

A portion of the experimental work was conducted by Mr. Christopher Horridge, as part of his MEng Research project and supervised by Ioanna Zafeiri. The data analysis interpretation and discussion were carried out in their entirety by Ioanna Zafeiri.

Chapter 7: Overall concluding remarks and future recommendations

The chapter summarises the conclusions derived from the results presented in this thesis, and discusses potential application in the context of proposed future studies.

Chapter 8: Appendices

A selection of data is given in this chapter, aiming to reinforce or act complementarily to the results already presented in the experimental Chapters 3-5. It also provides a synopsis of some preliminary work conducted on the modification of lipid particles' surface properties and the induced emulsion behaviour, in terms of droplet size and stability.

1.5 Dissemination of research work

Presentations:

- Zafeiri, I., Norton, J., Smith, P., Norton, I., Spyropoulos, F. "Fabrication of Pickering particles from food-grade lipids". Poster presentation at *Nanoformulation2013* Conference, University of Manchester, June 2013
- Zafeiri, I., Norton, J., Smith, P., Norton, I., Spyropoulos, F. "Lipid particles as Pickering emulsion stabilisers". Oral presentation for the *Young Lipid Scientist Award*, SCI-University of Reading, June 2014
- Zafeiri, I., Norton, J., Smith, P., Norton, I., Spyropoulos, F. "Fabrication of food-grade lipid particles with the potential to provide Pickering stabilisation", Poster presentation at the *4th International Colloids Conference: Surface Design and Engineering*, Madrid, Spain, June 2014
- Zafeiri, I., Norton, J., Smith, P., Norton, I., Spyropoulos, F. "Lipid-based particles as novel Pickering stabilisers of O/W emulsions". Poster presentation at *2015 Rideal Meeting: Polymers in Colloid Science*, SCI, London, April 2015
- Zafeiri, I., Norton, J., Smith, P., Norton, I., Spyropoulos, F. "Lipid-based particles as novel Pickering stabilisers of O/W emulsions". Oral presentation at the *12th International Congress on Engineering and Food (ICEF12)*, Québec City, June 2015
- Zafeiri, I., Norton, J., Smith, P., Norton, I., Spyropoulos, F. "Lipid-based particles as novel Pickering stabilisers of O/W emulsions". Poster presentation at the *6th International Symposium on Delivery of Functionality in Complex Food Systems (DOF 2015)*, Paris, July 2015

Publications:

- Pichot, R., Duffus, L., Zafeiri, I., Spyropoulos, F. and Norton, I.T. (2014). Particle-stabilised food emulsions, In: Ngai, T., Bon, S.A.F. *Particle-Stabilized Emulsions and Colloids: Formation and Applications*. RSC Soft Matter No. 3. The Royal Society of Chemistry, pp. 247-277

- Zafeiri, I., Horridge, C., Tripodi, E. and Spyropoulos, F. (2017). Emulsions co-stabilised by edible Pickering particles and surfactants: The effect of HLB value. *Colloids and Interface Science Communications*, 17, pp. 5-9
- Zafeiri, I., Norton, J., Smith, P., Norton, I.T. and Spyropoulos, F. (2017). The role of surface active species in the fabrication and functionality of edible solid lipid particles. *Journal of Colloid and Interface Science*, 500, pp. 228-240
- Zafeiri, I., Smith, P., Norton, I.T. and Spyropoulos, F. (2017). Fabrication, characterisation and stability of oil-in-water emulsions stabilised by solid lipid particles: The role of particle characteristics and emulsion microstructure upon Pickering functionality. *Food and Function*; submitted
- Zafeiri, I., Smith, P., Norton, I.T. and Spyropoulos, F. (2017). Maintaining the Pickering functionality of solid lipid particles upon dehydration and subsequent rehydration. *Langmuir*; in preparation

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Dickinson, E. (2012). Use of nanoparticles and microparticles in the formation and stabilization of food emulsions. *Trends in Food Science & Technology, 24*(1), 4-12.

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Norton, J. E., Wallis, G. A., Spyropoulos, F., Lillford, P. J., & Norton, I. T. (2014). Designing food structures for nutrition and health benefits. *Annual Review of Food Science and Technology, 5*, 177-195.

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Sala, G., Stieger, M., & van de Velde, F. (2010). Serum release boosts sweetness intensity in gels. *Food Hydrocolloids, 24*(5), 494-501.

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Chapter 2

Literature Review

A proportion of the discussion contained in this chapter has been published as follows:

Pichot, R., Duffus, L., Zafeiri, I., Spyropoulos, F. and Norton, I.T. (2014) Particle-stabilized food emulsions, In: Ngai, T., Bon, S.A.F. *Particle-Stabilized Emulsions and Colloids: Formation and Applications*. RSC Soft Matter No. 3. The Royal Society of Chemistry, pp. 247-277

Synopsis

This chapter outlines the main understanding behind emulsions and colloidal systems, the mechanisms that induce alterations in emulsion properties, and also the traditional and "trending" routes to tackle it. Special mention is given to particle-stabilised emulsions and, in particular, a state-of-the-art literature survey is provided regarding food-grade particulates. The general principles and terminology related to these systems are introduced in a bid to guide the reader through the experimental Chapters 3, 4, 5 and 6. A large portion of the discussion is dedicated to solid lipid nanoparticles and the recent work pertaining to their production, characterisation, as well as the challenges associated to instability that need to be addressed to ensure the stabilising performance of the particles.

2.1 Emulsions as a class of disperse systems

Emulsions are defined as fluid systems where a liquid is interspersed into another liquid in the form of small spherical droplets (McClements, 2016). Typically, two immiscible liquids are combined via the supplementation of energy. The molecularly incompatible phases investigated in this work are oil and water, in the form of oil droplets (dispersed phase) dispersed in an aqueous phase (continuous phase), or oil-in-water (o/w) emulsions. Emulsion technology is at the forefront of a broad range of commercial applications; emulsions are the basis of several food (e.g. sauces, mayonnaise), agrochemical (e.g. crop protection products), pharmaceutical (e.g. as carriers of active agents in drug delivery systems), personal care/cosmetic (e.g. lotions and sun creams), paint (e.g. latex emulsions) and even road construction-related formulations (e.g. bitumen) and the oil industry (Chappat, 1994; Tadros, 2013).

However, the contact between oil and water molecules is not thermodynamically favourable, hence an input of energy along with the inclusion of a surface active component (also known as an emulsifier) are usually required to render the emulsion kinetically stable (McClements, 2016). These two essential criteria will de delineated in the following sections.

2.2 Emulsion formation

Having a sound understanding of the physical principles of emulsion formation and the factors that influence the size of the generated droplets is important for the selection of the right processing equipment, and also for the improvement of product quality (e.g. shelf-life, appearance, texture etc.).

Emulsification is a dynamic process which involves the simultaneous occurrence of droplet break-up (disruption) into smaller ones and re-coalescence events. The final droplet size will be a function of the balance between these two opposing physical processes. Prior to break-up, the droplet needs to be deformed and this is opposed by interfacial forces that tend to hold the droplet together. These forces are characterised by the Laplace pressure ΔP_L which is inversely proportional to the particle/droplet radius. This means that for the formation of a nanosized droplet, the resistance to deformation and disruption is such, that huge external stresses are needed. Intense disruptive forces (large pressure gradients) are provided with the aid of specially designed mechanical devices (homogenisers) and several types are used in the industry, depending on the desired emulsion properties, the energy consumption and the operating costs.

In this work, specific attention was placed on high-shear mixing, ultrasonication and microfluidisation (Fig. 2.1), since these were the methods via which emulsions were produced. Several other emulsification routes are available including colloidal mills and high-pressure homogenisers (HPH), as well as processes that build-up emulsion droplets progressively rather than disrupting them, such as membrane emulsification (Spyropoulos, Lloyd, Hancocks, & Pawlik, 2014). In certain cases, a combination of different homogenisation methods (e.g. preparation of a coarse emulsion using a high-shear mixer followed by microfluidisation) was used to further reduce the droplet size, offering at the same time an energy-efficient approach rather than using solely each technique (McClements, 2016). Energy input is supplied via these processes to break down emulsion droplets over time, thereby increasing their interfacial area.

High-shear mixers (a type of rotor-stator device) are the most widely used homogenisation instruments in the industry (Hall, Cooke, Pacek, Kowalski, & Rothman, 2011). Oil and

aqueous phase interfaces are disrupted, intermingled, and droplets are fragmented by longitudinal, rotational and radial velocity gradients resulting in the formation of droplets in the micron scale (typically r = 1-10 μm) (McClements, 2016). The rotating speed and/or processing time determine the final average size.

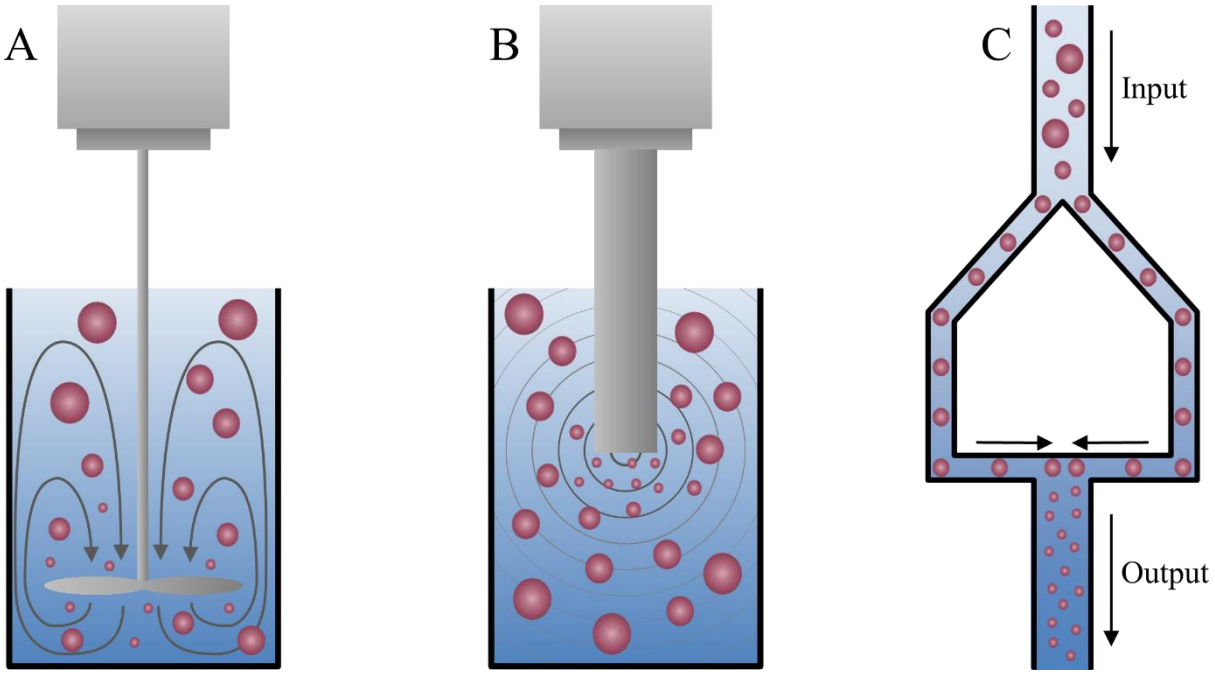

Fig. 2.1. Schematics of the used homogenisation devices' configuration indicating the mechanisms of droplet disruption; high-shear mixer (A), ultrasonic probe (B), microfluidiser (C) (adapted from (McClements, 2016)).

In contrast, power ultrasound is based on ultrasonic waves and acoustic cavitation to disperse and break down the continuous phase. Its operation is more explicitly described in section 2.5.2 as it was the principal method for lipid particles' fabrication. A high pressure device such as a microfluidiser is commonly used for the production of submicron droplets (Jafari, He, & Bhandari, 2007; Lee & Norton, 2013). Fluids are forced to collide at high velocity through microchannels and toward an impingement area, where droplet break-up is driven by inertial forces and cavitation (Maa & Hsu, 1999). Variables such as the number of passes, the

homogenisation pressure, the characteristics of the emulsifier, and the viscosity ratio between dispersed and bulk phase will dictate the obtained droplet size (McClements, 2016).

2.3 Instability mechanisms in colloidal systems

The most significant challenge that food processors need to face though, has its origins in the thermodynamically unstable character of emulsions. These systems tend to destabilise upon storage through a variety of physical breakdown mechanisms, as depicted schematically in Fig. 2.2A.

Flocculation occurs when the van der Waals attraction forces dominate over the repulsive long-range interactions. As a result, droplets/particles cluster together whilst keeping their physical integrity, and form weak (reversible) or stronger (irreversible) flocs. An increased flocculation will sequentially lead to enhanced gravitational separation, i.e. creaming or sedimentation, depending on the density differences between the dispersed and the continuous phases.

O/W emulsions are more susceptible to creaming as the oil droplets usually have a lower density than the aqueous phase. Antipodal to o/w systems, water-in-oil (w/o) emulsions or solid particle aqueous dispersions are more likely to sink and sediment. It is also possible that the sedimented or risen to the surface entities form a more compact network which ensues irreversible aggregation and/or coalescence (McClements, 2002) .

Coalescence is an irreversible process whereby a pair or more droplets merge to shape a single larger droplet. For the purposes of this study, emulsion stability against coalescence was the main mechanism investigated and no attempt to stop the emulsions from creaming was made. Ostwald ripening (disproportionation) is a process that can be found in both fat crystal dispersions and emulsions, and leads to size modifications during storage. It consists

of mass transport of dissolved matter from smaller entities (dispersed phase), diffusion through the bulk and deposition on larger entities. It is driven by the existence of pressure gradients across curved interfaces and is accentuated in samples with a high polydispersity.

Emulsions can also invert from o/w to w/o and *vice versa*, triggered by alterations in compositional or environmental variables, i.e. temperature, shear, emulsifier type and concentration, additives etc. It is a process desirable in the manufacturing of certain food products, such as butter and margarine to create a specific effect on appearance, stability and texture (McClements, 2016).

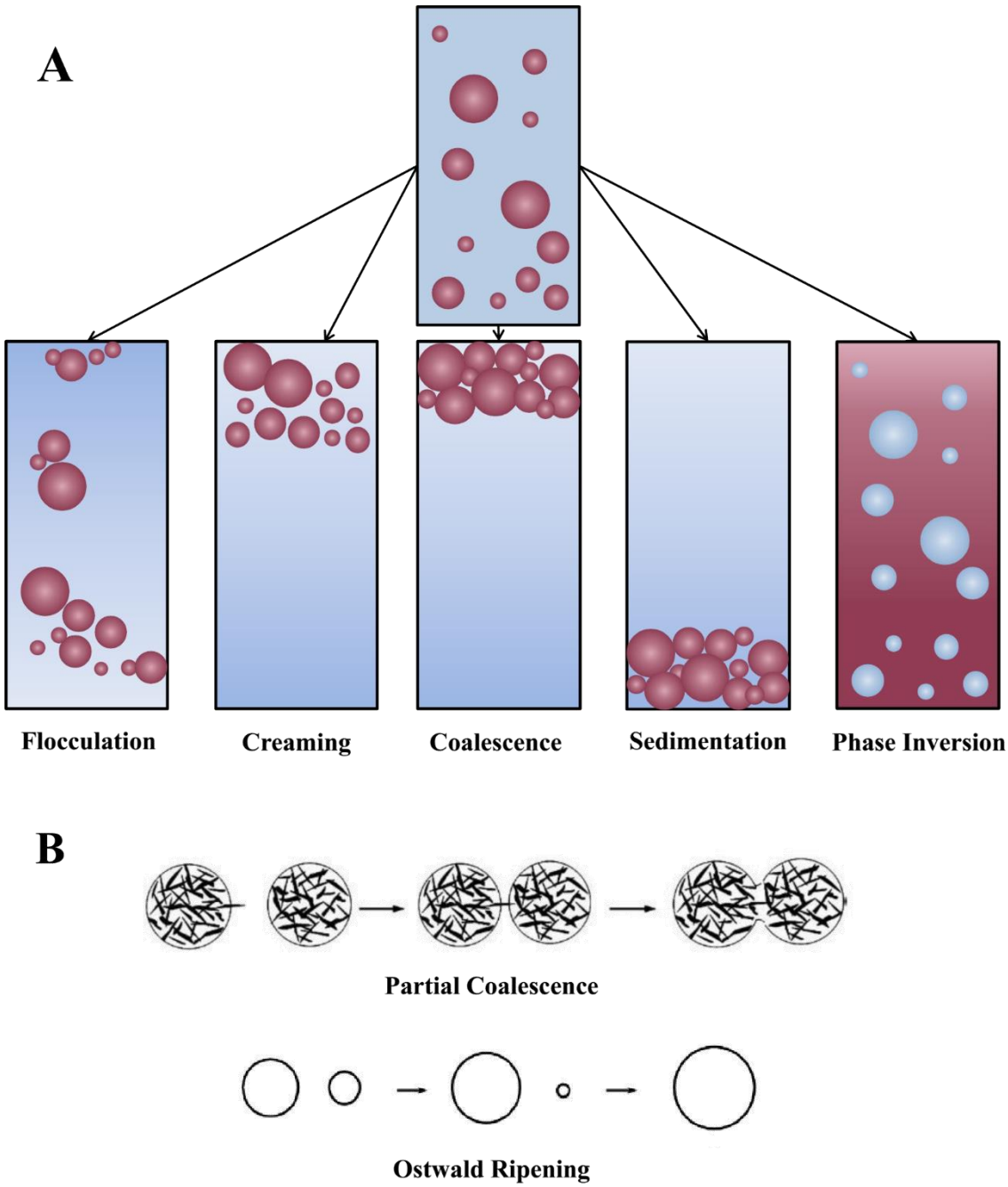

Fig. 2.2. A. The main physical mechanisms of emulsion instability (adapted from (McClements, 2002)). B. Solid dispersions become unstable also through the mechanisms of partial coalescence and Ostwald ripening (Fredrick, Walstra, & Dewettinck, 2010).

Finally, (partially) crystalline droplets (e.g. containing fat crystals) are prone to partial coalescence. This phenomenon consists of globules approaching one another (e.g. upon collision) and the fat crystals from one globule protrude the interfacial film of the other

globule, forming an irregularly-shaped aggregate (Fredrick, *et al.*, 2010). For colloidal lipid particles dispersed in a continuous aqueous phase, instability additionally relates to modifications that the lipid itself undergoes (e.g. polymorphism), as will be discussed in section 2.5.4.2. With the view to extend emulsions' lifetime and arrest/delay substantial physical emulsion destabilisation issues, emulsifiers are used as the first on hand approach.

2.4 Approaches to confer emulsion stability

2.4.1 Emulsifiers

Emulsifiers perform a dual role, during and/or post emulsification; i) lowering of interfacial tension by adsorbing to newly produced liquid-liquid interfaces and ii) formation of a – protective – interfacial film that retards or minimises all the above mentioned instability mechanisms upon emulsion's fabrication and subsequent shelf-life. Their functionality is not only limited to stabilisation as they are additionally utilised for their anti-sticking, dough strengthening, fat crystallisation inhibition, and viscosity modification properties in several food products (Hasenhuettl, 2008).

"Classical" emulsifiers used in the food industry are distinguished into low molecular weight emulsifiers (surfactants) and amphipathic biopolymers (e.g. proteins and polysaccharides), which can be synthetically or naturally derived, and they can be ionic or non-ionic. Alternatively, droplet interfaces can be fabricated and stabilised with the use of solid particles or mixed systems containing both particles and surfactants, pathways that have been attracting extensive interest within the last decade. The common feature across the conventional emulsifiers is their amphiphilicity, which enables them to adsorb at interfaces and subsequently induce a reduction of the interfacial tension. The amphiphiles owe their character to their molecular structure that comprises both polar and nonpolar regions.

The selection of an emulsifier or emulsifier blend hinges upon numerous factors which include the desired type and application of the emulsion produced, considerations relating to its composition, functional properties and manufacturing method, and also economical, legal and environmental considerations. In this work, surface active components widely used in the food industry (e.g. proteins) were employed for the fabrication of lipid particles. Subsequently, pure emulsifier-stabilised emulsions served as the basis for comparison with emulsions stabilised by solid material. Although some of these components are synthetic emulsifiers (e.g. Tween 80, PGPR), their functionality was demonstrated while present at minimal concentrations.

2.4.2 Surfactants

Low molecular weight (LMW) emulsifiers refer to small surface active entities that consist of a hydrophobic "tail" and a hydrophilic "head" group. Due to their low mass they are extremely mobile, hence particularly efficient in the reduction of interfacial tension within the rapid time scale of droplet formation. Typical molecular weights are in the range of circa 250-1200 g/mol and include monoglycerides, lecithins, fatty acids, fatty alcohols, etc.(Kralova & Sjöblom, 2009).

The proportion of the hydrophilic to hydrophobic domains of these molecules is most commonly described as the HLB (hydrophilic/lipophilic balance) value and it essentially indicates their affinity for the oil and aqueous phases. The HLB concept is usually applied to emulsifiers to classify them based on the type of emulsion that they are more suitable to stabilise; as a rule of thumb, HLB values higher than 10 represent a dominant hydrophilic character and a tendency to form water continuous emulsions (e.g. dressings, beverages). In this case, surfactants curve towards the oil phase, favouring interactions with water molecules

more than the oil ones (Binks, 2002). Conversely, surfactants with HLB lower than 10 preferentially stabilise w/o emulsions (e.g. margarine, spreads). It has been observed that very high/low HLB values do not necessarily lead to an appreciable surfactant positioning at the droplet interface (due to its low surface activity), thereby protection against coalescence is poor (McClements, 2016).

2.4.3 Protein-based emulsifiers

One of the major categories of natural food emulsifying agents is proteins. They are among the most common ingredients of several food systems, ranging from mayonnaise to ice cream, mainly present as emulsion forming and stabilising agents. Despite their extensive and conventional application, their molecular structure is complex and the "train-loop-tail" model is frequently used to describe it.

Being a macromolecular emulsifier, proteins diffuse slowly to reach and adsorb at an interface. Although the level of interfacial tension reduction is much lower than that imparted by small molecular weight surfactants, proteins enable the formation of stable emulsions via additional forces. They retard droplet coalescence by generating repulsive interactions (e.g. steric and electrostatic), and also by forming a rigid viscoelastic film around the oil droplets. Their inherent molecular structure and interactions largely dictate the properties of these protein-based interfacial coatings (e.g. thickness, viscoelasticity).

A great deal of research has been concerned with the interfacial behaviour of dairy proteins (e.g. caseins, β-lactoglobulin) (Dickinson, 1997, 2001; McClements, 2004). Sodium caseinate, extensively used in this work, is widely applied in the food industry as a stabilising agent in numerous emulsion-type products (e.g. ice-cream, whipped toppings, infant formula). It belongs to the class of flexible proteins and has a disordered structure and a relatively high

hydrophobic character. Upon adsorption at an oil/water interface during emulsification, it undergoes immediate conformational changes by positioning its train segments in such a way that a physical (interfacial) barrier against coalescence is formed (Phoon, Paul, Burgner, San Martin-Gonzalez, & Narsimhan, 2014). It is this protein chain obtained configuration that renders caseinate-stabilised emulsions more prone to depletion and bridging flocculation mechanisms (Damodaran, 2005). Apart from being an effective barrier to coalescence, the caseinate-rich interfacial layer was found to be beneficial by impeding chemical destabilisation processes too, such as lipid oxidation as underscored in a number of studies (Horn, Nielsen, Jensen, Horsewell, & Jacobsen, 2012; M. Hu, McClements, & Decker, 2003; Kiokias, Dimakou, Tsaprouni, & Oreopoulou, 2006; Phoon, *et al.*, 2014).

Last but not least, the ability of proteins to form self-assembled structures (e.g. casein micelle, globular proteins) has been exploited for the formation of (nano)particulate entities that can aptly act as stabilising agents (Dickinson, 2010) as well as wall materials and delivery vehicles for nutraceutical compounds (Chen, 2009).

2.4.4 Colloidal particles

Despite the fact that emulsifiers are relatively cheap, in most cases conveniently manufactured and presently extensively utilised at an industrial level, solid particles owing to their unmatched benefits provide a highly attractive alternative approach for emulsion stabilisation. Particle-stabilised emulsions are known to exhibit an outstanding stability against coalescence and Ostwald ripening, as opposed to emulsions stabilised by low molar mass surfactants (Tavernier, Wijaya, Van der Meeren, Dewettinck, & Patel, 2016). This feature is of utmost importance in the food industry; for instance, certain food emulsions need to be stable for a few days/months after being opened, while others might have to remain stable even years

prior to consumption (e.g. sauces, dressings, cream liqueurs). In addition, particle-laden interfaces have been shown to be more rigid and robust, enhancing stability against oxidation (Kargar, Fayazmanesh, Alavi, Spyropoulos, & Norton, 2012) and being more tolerant to processing (e.g. shear) (Niknafs, 2011).

Reports on the ability of fine solid powders to stabilise emulsions date back to the beginning of the 20th century and belong to Clayton (1898) and Ramsden (Clayton, 1923; Ramsden, 1903). A few years later this knowledge was set on a more fundamental basis via the seminal work of Pickering (1907) who also lent his name to the phenomenon nowadays widely known as Pickering stabilisation. The terms of colloidal size and wettability –currently known to be key elements of Pickering stabilisation – were also first introduced in his experimental study. Although this ability has long been recognised and several foods are based on a similar mechanism (e.g. whipped cream stabilised by fat particles or ice cream by ice crystals) (Kulozik, 2008), it has received a tremendous amount of interest only over the past decade.

The mechanism suggested is a simple one and predicts that solid particles (nano- or micro-) accumulate at a biphasic interface to form a densely packed layer that arrests droplet flocculation and coalescence. Stabilisation is imparted by a steric and/or electrostatic barrier rather than an effective reduction of interfacial tension, as is the case with conventional emulsifiers. Pickering emulsions exhibit superior stability due to the high values of the energy required to remove an adsorbed particle from an interface (detachment energy, ΔE). This energy is linked to the particle size r of a spherical particle and its wettability, best characterised by the contact angle, θ, the particle assumes at the interface (Aveyard, Binks, & Clint, 2003; Binks, 2002; Hunter, Pugh, Franks, & Jameson, 2008):

$$\Delta E = \pi r^2 \gamma_{ow} (1 - |\cos\theta|)^2, \qquad (2.1)$$

where γ_{ow} is the interfacial tension between oil and water. Based on Equation 2.1, the strength of particle attachment is largely influenced by its size; small sizes (e.g. r ~5-10 nm) will lead to enormous binding energies that are significantly larger than the thermal energy (kT) and subsequently, to almost irreversible adsorption (provided that the contact angle is not too far away from 90º). Small rather than larger particles are likely to provide full droplet coverage (Rousseau, 2000), and also particles need to be a magnitude of order smaller than the droplets they stabilise; particles in the submicron/nanometre range can stabilise emulsion droplets of 0.5-10 μm (Dickinson, 2012). Particle's location at the interface, as dictated by the multiphase contact angle θ, is often claimed to be the variable that determines emulsion stability the most (Binks, 2002). Fig. 2.3 depicts the well-established configuration of particles at planar (A) and curved (B) oil-water interfaces respectively. Particles with $\theta < 90^o$ or $\theta > 90^o$ refer to hydrophilic and hydrophobic entities respectively (Lopetinsky, Masliyah, & Xu, 2006).

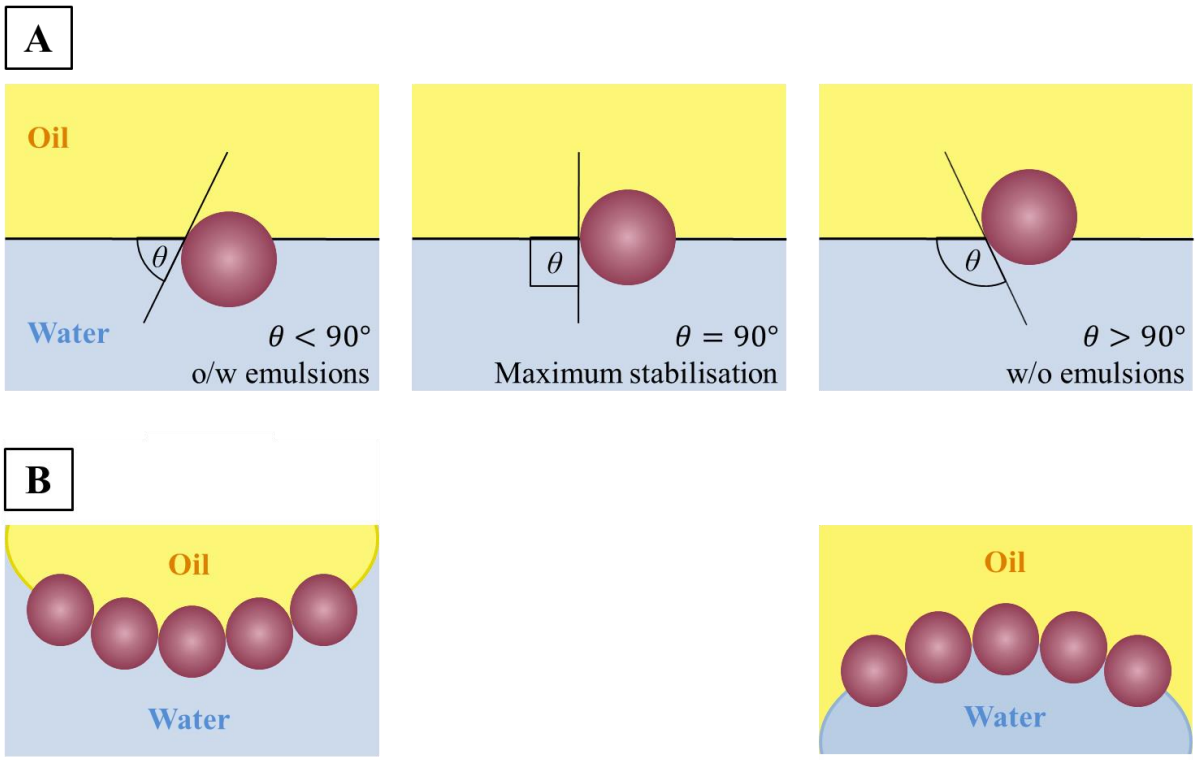

Fig. 2.3. Preferential positioning of small spherical particles at planar (A) and curved (B) oil-water interfaces and the resulting emulsion types (adapted from Aveyard, Binks, and Clint (2003)).

More specifically, a hydrophilic character means that a larger fraction of particle's surface is wetted by the aqueous phase which will be the continuous phase of the emulsion it forms. Accordingly, hydrophobic particles reside more in oil than in water and will be more suitable for forming w/o emulsions. Analogously to size, contact angle also has to be maintained within certain ranges as particles of extreme hydrophilicities/hydrophobicities will not adsorb at the interface; instead, they tend to remain dispersed in the phase they have more affinity for and eventually produce highly unstable emulsions (Binks, 2002).

Controlling particle wettability is therefore one of the principal requirements when it comes to the design or selection of solid particles with an emulsifier-like behaviour. Surfactants are most commonly utilised to aid in the modification of particle surface properties, and therefore

wettability. Various examples exist in the literature regarding inorganic (e.g. silica) (Pichot, Spyropoulos, & Norton, 2009) or bio-derived (e.g. zein protein) (Zou, *et al.*, 2016) matrices that have been rendered functional in terms of stabilisation of emulsions and foams, and this was achieved via the employment of a surfactant. This modulation of surface properties induced by the synergy between particle-surfactant mixtures is detailed in section 2.4.5.

Besides size and wettability, other factors such as particle type, shape (de Folter, *et al.*, 2014), concentration (Binks, 2002) and inter-particle interactions (Horozov & Binks, 2006) have also been identified as parameters that influence stabilisation with solid particles at fluid interfaces (Lopetinsky, *et al.*, 2006; Prestidge & Simovic, 2006). Yet, these factors were outside the focus of the present work.

Most of the aforementioned determining variables for emulsion stability have been investigated, and understanding has been gained on the basis of model inorganic and organic systems; numerous reports exist on silica with the group of Binks exploring the effects of particle's size, concentration and wettability, through to dispersed phase composition and the presence of electrolytes in the aqueous phase (Binks & Lumsdon, 1999; Binks & Lumsdon, 2000a, 2000b; Binks, Philip, & Rodrigues, 2005; Binks & Whitby, 2005). Experimental work has also included alumina, clay, polymer latex spheres and carbon nanotubes, and emulsions stabilised by them were found to be remarkably stable for up to two years in some cases (Ashby & Binks, 2000; Binks & Horozov, 2006; Shen & Resasco, 2009; Chen, *et al*., 2011). The development of particulates with well-defined and diverse microstructures and properties has resulted in gaining a deeper insight on the mechanisms involved in the stabilisation of Pickering emulsions. The mounting interest in particle-laden interfaces has extended the research beyond emulsions; it entails foams (Dickinson, 2010) as well as a wide range of applications in novel food structures that promote health and wellness (Augustin & Hemar,

2009; Dickinson, 2012). It also involves cutting-edge applications in the fabrication of nanostructures that modulate cell-membrane penetration (Verma, *et al.*, 2008), or nanocomposites that respond to external stimuli (e.g. pH or temperature) (Richtering, 2012) and can tune, for instance, their optical (Tavacoli, Thijssen, & Clegg, 2001) or electrical properties (Leunissen, van Blaaderen, Hollingsworth, Sullivan, & Chaikin, 2007). Elements derived from these technologies could be transferred and harnessed by the food industry to develop, as a paradigm, functional foods with targeted and controlled release characteristics.

2.4.4.1 Edible Pickering particles

Despite the apparent soar of research, the major challenge in food emulsion development remains the identification of edible structures that can act as Pickering particles, complying with the respective legislation on the use in food products at commercial scale. Apart from the benefits of Pickering emulsions in general, as explained before, the added-value of using specifically edible particles for emulsion stabilisation is that they are readily available and cheap, and they have the potential to undergo mass production (Xiao, Li, & Huang, 2016). They also aid in the development of emulsion systems devoid of surfactant(s) (or use of minimal surfactant concentrations) in line with the "surfactant-free" attitude adopted by both industry and consumers (Rayner, *et al.*, 2014). A relatively limited number of reports concerning edible ingredients for Pickering stabilisation have been published hitherto, although it is noteworthy that this trend is accelerating.

Particles used for stabilisation of food emulsions must be sourced from biological or biocompatible materials, and starch (Timgren, Rayner, Sjöö, & Dejmek, 2011), cellulose and cellulose derivatives (Kargar, *et al.*, 2012; Paximada, Tsouko, Kopsahelis, Koutinas, & Mandala, 2016), chitin (Tzoumaki, Moschakis, Kiosseoglou, & Biliaderis, 2011) and chitosan

(Wei, Wang, Zou, Liu, & Tong, 2012) have been found to exhibit interesting interfacial properties. A chemical treatment was a prerequisite for most of these polysaccharide-based particles to render them functional (in terms of wettability or surface activity) and they have yielded emulsions extremely stable to coalescence. Other candidates that have been screened as effective stabilisers of Pickering o/w emulsions are cocoa particles (Gould, Vieira, & Wolf, 2013), flavonoids (Luo, *et al.*, 2011) and protein-borne particles (e.g. zein extracted from corn) (de Folter, van Ruijven, & Velikov, 2012). Some recent publications provide a comprehensive inventory of the biological particulate materials, discussed briefly in this section, that are available for potential food use (Berton-Carabin & Schroën, 2015; Dickinson, 2016; Pichot, Duffus, Zafeiri, Spyropoulos, & Norton, 2014; Rayner, *et al.*, 2014; Tavernier, *et al.*, 2016; Xiao, *et al.*, 2016).

2.4.4.1.1 Lipid-based particles

Particles made of lipids have recently been added as a very promising route in the growing pace of development of edible particle-stabilised emulsions, albeit with limited research performed so far, in comparison to the other food-grade counterparts (Rousseau, 2013; Pichot, *et al.*, 2014). Due to their hydrophobic nature, lipid-based particles (e.g. fat crystals) are able to assemble at an oil-water interface assuming a contact angle larger than 90^{o}, thus being more effective in stabilising water-in-oil systems (Paunov, *et al.*, 2007); this approach has been widely investigated and reviewed by several research groups (Frasch-Melnik, Spyropoulos, & Norton, 2010; Garti, Binyamin, & Aserin, 1998; Ghosh & Rousseau, 2011; Rousseau, 2000). In particular, the wettability of fat crystals by oil and water (much like other particles) was among the principal microstructural features that were shown to largely impact on emulsion stability (Johansson & Bergenståhl, 1995; Johansson, Bergenståhl, & Lundgren, 1995). Moreover, the stated pathway has numerous applications at a commercial level, given

the number of foods that are in part or wholly stabilised by surface-active crystallised lipids that position at the oil/water interface (e.g. spreads, ice cream, whipped cream, margarine).

The most conventional mode of stabilisation (on which most of the previously mentioned studies are based) involves fat crystals' adsorption at the oil-water interface (mediated by surfactants' interfacial crystallisation) which creates a sintered "shell" enclosing water droplets (Hodge & Rousseau, 2005; Frasch-Melnik, Norton, & Spyropoulos, 2010). Crystals are formed as part of the emulsification process, in which lurks a risk of poor control over the properties directly linked to emulsion stability (e.g. size, wettability). This is in stark contrast to the stabilisation mechanism that results from the interfacial adsorption of lipid micro-/nano- particulates, a field that is still at its infancy. The work of Garti, *et al.* (1998) was the first showcase of emulsion stabilisation (water-in-oil emulsions) using nanosized tristearin particles, although agglomeration was observed within days.

Recently, few studies have reported on the potential of solid lipid nanoparticles to stabilise oil-in-water emulsions (Gupta & Rousseau, 2012; Kurukji, Pichot, Spyropoulos, & Norton, 2013; Pawlik, Kurukji, Norton, & Spyropoulos, 2016). This was achieved via the use of solely lipophilic molecules that contain one or several polar groups (surface-active), or by inducing surface activity via the synergistic combination with more hydrophilic components, such as proteins to aid in both emulsification and particle adsorption. Despite clear evidence that solid lipid nanoparticles (SLN) are able to adsorb at the oil/water interface, the interfacial properties of these nanoparticles have not been investigated so far.

Further manipulation of crystalline structures such as nanoscale fat crystals (Acevedo & Marangoni, 2010) or nanostructured lipid carriers (NLC) (Saupe, Gordon, & Rades, 2006) to display desired physicochemical properties could result in optimal emulsion stabilisation. The stated pathways could be advantageous in terms of providing a significant change in

wettability, facilitating particle size reduction and/or offering crystalline structures of controlled thermal properties. Although they don't yet offer advantages in emulsion stabilisation, due to their potential for future impact, they have been included in the discussion that follows.

2.4.5 Mixed particle-surfactant systems as emulsifiers

Aside from the sole use of surfactants or particles for emulsion stabilisation, mixed particle-surfactant systems have been historically used for emulsification (Gelot, Friesen, & Hamza, 1984; Hassander, Johansson, & Törnell, 1989), although this field has received much less attention, especially regarding food grade emulsions (Pichot, *et al.*, 2009). The underlying concept is that the benefits arising from the individual use of each of these two entities could be exploited to eliminate their drawbacks and, potentially, confer enhanced emulsion characteristics (e.g. stability). Additionally, the conjoint use of these compounds holds great promise as several emulsion-based commercial products contain particles and surfactants and many industrial processes are performed in their co-presence. In this case, the interactions developed between these species could be of a great practical interest as the possibilities, for instance, for low surfactant usage are tremendously appealing for the reduction of the economic and environmental impact (Midmore, 1998; Manousakis & Avranas, 2013).

Therefore, fundamental and applied research has dealt with the addition of surfactants to particle systems, and *vice versa*, in several occasions. The most commonly investigated candidates for particles include silica, clay, carbon and alumina while surfactants are usually cationic, anionic, non-ionic, or polymeric (Binks, Desforges, & Duff, 2007). In the majority of studies, an emulsification synergism between particles and surfactants has been reported, resulting in improved stability (Hassander, *et al*., 1989; Midmore, 1998; Binks & Whitby,

2005; Pichot, *et al*., 2009) yet, the mechanism suggested in each of these studies is different and depends on the type of the components utilised and the experimental conditions considered (e.g. pH environment, polarity of the dispersed phase etc). Pichot, *et al.* (2009) outlined a mechanism that describes the synergistic effect between two types of emulsifiers: silica nanoparticles and a low molecular weight surfactant. They proposed that the low molecular weight surfactant 'delays' the coalescence phenomena, temporarily stabilising the oil droplets. This delayed coalescence allows the less mobile Pickering particles time to diffuse and adsorb at the oil-water interface, providing long-term stability against coalescence. The study was carried out in acidic conditions where silica particles have no charge and thus, better packing at the interface was ensured. Binks and Whitby (2005) found that emulsion stability could be improved by adjustments to the oil phase polarity. In the presence of significantly polar oils, particle's wettability is modified due to adsorption of polar solvent molecules to the silica surface and emulsions exhibit remarkable stability.

The role of surfactant's addition to particle-stabilised emulsions has been described as threefold in literature; 1) to induce or limit solid particle flocculation to a level that is favourable for the particles to adsorb around the emulsion droplets (Lucassen-Reynders & Tempel, 1963), 2) to modify particle wettability, thus favouring adsorption at the liquid-liquid interface and 3) decrease the interfacial tension of the system (Midmore, 1998). It has been argued that particle flocculation can be mediated by surfactant's adsorption on particle surfaces (Binks, Desforges, *et al*., 2007). Based on some studies, the level of this agglomeration is closely related to improved emulsion stability (which depends on the adsorption of flocs at drop interfaces) against coalescence (Hassander, *et al.*, 1989). In that vein, Binks and Rodrigues (2007) and Binks, Rodrigues, & Frith (2007) showed that by means of using mixtures of particles and surfactants of opposite charge sign, coalescence and

creaming-stable o/w emulsions can be produced provided that particles are significantly flocculated. Concurrently, other studies depicted an emulsion stability where extensive particle flocculation was not a prerequisite for adsorption to take place (Midmore, 1998). Instead, the main driver affecting stability was related to surfactant's molecular structure and more specifically, the length of its polyoxyethylene (POE) chains in that case.

Early work by Schulman and Leja (1954) and Tambe and Sharma (1993) established an association between surfactant adsorption and oil-water-solid contact angle with the resulting emulsion type. Tambe, *et al.* (1993) showed that increased concentrations of an oil soluble (low HLB) surfactant (stearic acid) led to the phase inversion of emulsions from water to oil continuous. This was attributed to changes in the wetting properties of the calcium carbonate particles used for emulsion stabilisation. Although a large body of literature has examined the *in situ* modification of particle surface via adsorption of amphipathic agents (Akartuna, Studart, Tervoort, Gonzenbach, & Gauckler, 2008; Bernard P. Binks & Rodrigues, 2007, 2009; Bernard P. Binks, Rodrigues, & Frith, 2007; Gelot, *et al.*, 1984; Schulman, *et al*., 1954; J. Wang, *et al.*, 2010), only a few linked surfactant's structure with emulsion formation and stability. Binks, *et al*., (2009) showed that an increase in double-chain cationic surfactant concentration in the presence of negatively charged silica particles triggered a double emulsion inversion (from o/w to w/o and back to o/w) due to modification in their hydrophobic character, driven by the surfactant's mono- or bi-layer formation. The analogous single chain surfactant in mixtures with silica particles did not provoke any emulsion inversion (Binks, Rodrigues, *et al*., 2007). Additionally, longer chain lengths and inclusion of strong adsorbing polar groups are inherent attributes of surfactants that were found to induce an inversion of solid-stabilised emulsions from o/w to w/o type (Schulman, *et al.*, 1954).

Nevertheless, mixing particles and surfactant systems does not always result in emulsion stabilisation. The addition of silica particles to surfactant-stabilised bitumen-in-water emulsions led to droplet flocculation coupled with partial coalescence when subjected to a shear environment (Legrand, *et al.*, 2005). The possibility that both emulsifier species compete for the interface engendering, on various occasions, interfacial displacement of the particles has been described in a few recent studies (Drelich, Gomez, Clausse, & Pezron, 2010; Lan, *et al.*, 2007; Pichot, Spyropoulos, & Norton, 2010; Vashisth, Whitby, Fornasiero, & Ralston, 2010). Pichot, *et al.* (2010) highlighted the important role of surfactant's concentration which determines the behaviour at the interface. They demonstrated that the location of the particles in relation to the interface which, in turn, dictates the emulsion droplet size, is controlled by the surfactant concentration in the system; at high surfactant contents the competition for adsorption at the interface is pronounced and this leads to particles being displaced, leaving the emulsion stabilised by neat surfactant. The displacement event is also accentuated when the employed surfactant is above its critical micelle concentration, whereas at lower concentrations, particles and surfactants co-stabilise emulsion drops (Vashisth, *et al.*, 2010).

As mentioned above, another aspect of surfactant's performance in particle-stabilised emulsions is the reduction of interfacial tension. Yet, knowledge on interfacial adsorption when surfactants and particles co-exist is relatively scant. Ravera, Santini, Loglio, Ferrari, and Liggieri (2006) studied the kinetic features of particle transport to the interface via equilibrium and dynamic interfacial tension measurements supplemented by surface rheology. They found that the interfacial properties of the composite nanoparticles and surfactant are different from those of a sole surfactant, with the former being about three orders of magnitude higher in terms of equilibrium interfacial tension values. Two distinct behaviours

were observed in the dynamic surface tension profile. The first is related to the diffusion of nanosized silica particles followed by accumulation to the liquid-liquid interface, prompted by surfactant's adsorption at their surface. The second behaviour was attributed to an interfacial re-arrangement of the mixed particle/surfactant layer, where surfactants are re-distributed between the solid particles and the liquid/fluid interface.

As has been described in previous studies on systems containing both surfactants and silica nanoparticulates, particles block the diffusion of surfactant molecules to the interface by means of adsorption of the latter at particles' surfaces (Pichot, Spyropoulos, & Norton, 2012; Biswal, Rangera, & Singh, 2016). Consequently, particles' wettability characteristics are modified, i.e. particles become more hydrophobic and their attachment at the interface is highly encouraged. At the same time, surfactant molecules are depleted and the surface excess concentration of surfactant decreases, leading eventually to an increase in the interfacial tension, as has been proposed by Brian and Chen (1987).

Often the difference in charge between a surfactant and particles is claimed as a critical parameter that affects interfacial properties of the combined system. Ma, Luo, and Dai (2008) reported a slight decrease in the trichloroethylene-water interfacial tension in a system containing anionic surfactant with additional silica nanoparticles (negatively charged), counter to the absence of an effect when a non-ionic surfactant was present. This behaviour was attributed to the repulsive Coulomb forces between the similarly charged compounds that encourage surfactant diffusion to the interface. In contrast, the interactions between negatively charged particles and a non-ionic surfactant are dampened as the adsorption of surfactant is, in this case, less favourable thermodynamically (Despert & Oberdisse, 2003). In the case of oppositely charged colloidal particles and ionic surfactants a reduction of interfacial tension was found (Wang, Zhou, Nandakumar, Xu, & Masliyah, 2004). The authors explained these

recordings on the basis of the long-range electric field created by the negatively charged kaolinite particles in the vicinity of the interface, which attracted counter ions of the surfactant and thus, favoured their interfacial adsorption.

2.5 Solid lipid nanoparticles (SLN)

Solid lipid nanoparticles (SLN) were first introduced in the early 1990s. Among the first reports is the work of Speiser and co-workers who developed solid lipid microparticles (micro-pellets) by spray-drying and spray-congealing (Eldem, Speiser, & Hincal, 1991). Since then, they have become an evolving lipid formulation, as an alternative to conventional colloidal systems (e.g. liposomes, lipid emulsions, polymeric micro- and nanoparticles) in the pharmaceutical, cosmetics and, to a lesser extent, the nutraceutical and food arena. SLN are composed of lipids which are solid in room and body temperature and their size ranges between 50 - 1000 nm (Müller, Mäder, & Gohla, 2000). The solid state of the lipid matrix provides a safer 'housing' to embedded active compounds/biomolecules and guarantees a slower chemical degradation of the matrix, and also *in vivo*. Thus, there is a controlled and long-lasting release of its content (Üner & Yener, 2007). The advantages of the SLN include, *inter alia*, their tolerance within the human body (Müller, *et al*., 1995), the feasibility for encapsulation of both hydrophilic and poorly water soluble compounds and, very importantly, the ease in scaling-up (Mehnert & Mäder, 2001).

Their suitability as carrier systems (i.e. loading capacity), aspired to improve the therapeutic efficacy of the enclosed drugs, has been studied extensively for molecules with a range of hydrophobicities; lipophilic agents (Cavalli, Peira, Caputo, & Gasco, 1999; Schwarz & Mehnert, 1999), the much more challenging hydrophilic compounds (Yuan, *et al*., 2009; Becker Peres, Becker Peres, de Araújo, & Sayer, 2016) as well as other bio-macromolecules

(Almeida & Souto, 2007). In the strategy of drug targeting, several administration routes have been considered, such as parenteral, topical, oral, ophthalmic, rectal and pulmonary. Two very good reviews by Müller, *et al.* (2000) and Mehnert, *et al.* (2001) provide an overview of the most significant research work on drug incorporation within SLN vehicles and their applications in several administration pathways.

In contrast, the exploration of SLN for the encapsulation and delivery of food-related compounds has been increasingly "trending" only within the last decade (Augustin, *et al*., 2009; McClements & Li, 2010; Morris, 2011). Against the background of several contemporary dietary-related illnesses (e.g. obesity, cardiovascular disease, hypertension), the food industry has prioritised the development of health-promoting (functional) foods (Chen, Weiss, & Shahidi, 2006; Sanguansri & Augustin, 2006; Weiss, *et al*., 2008) that ensure delivery of bio-active ingredients and nutraceuticals. Exploiting attributes such as the solid nature of SLN that imparts enhanced protection and a controlled release profile, wellness-enhancing components (e.g. carotenoids, omega-3 fatty acids) have been investigated for their potential to be encapsulated and transported to the gastrointestinal tract with the aid of these carriers (Hentschel, Gramdorf, Müller, & Kurz, 2008; David Julian McClements, Decker, Park, & Weiss, 2009; Velikov & Pelan, 2008). The lipid sources and the production methods employed for food-grade SLN are inspired, in a large part, by approaches for pharmaceutical drug delivery systems (Acosta, 2009).

2.5.1 Lipid materials

The production of solid lipid nanoparticles in terms of the compounds used follows the triptych of lipid(s), emulsifier(s) and water. Lipid sources usually employed as the building blocks of SLN comprise a large group of naturally occurring organic compounds such as

triglycerides, partial glycerides (mixtures of mono-, di- and triglycerides), fatty acids, waxes, phospholipids and steroids (Mehnert, *et al.*, 2001; Rosiaux, Jannin, Hughes, & Marchaud, 2014). These materials are relatively cheap and are generally recognised as safe (GRAS) (Severino, *et al.*, 2012). The composition of physiological lipids (normal compounds of the human body such as cholesterol and some triglycerides) allows them to exhibit good tolerability once they are administered, minimising any adverse host response to the delivery system (if used within such a system), as well as any acute and chronic toxicity (Müller, *et al.*, 2000).

Triglycerides and waxes are the lipid components used throughout the study. They are the two most important classes of organic molecules that have attracted increased attention in the literature. Triacylglycerols (TAGs) of different chain lengths (e.g. trimyristin, tripalmitin, tristearin) have been preferentially investigated for pharmaceutical (Westesen, Bunjes, & Koch, 1997; Westesen & Siekmann, 1997) and food (Martin-Gonzalez, 2015; Weiss, *et al.*, 2008) applications, as well as wax formulations based on cetyl palmitate (Freitas & Müller, 1998; Martins, Tho, Souto, Ferreira, & Brandl, 2012), carnauba wax (Madureira, *et al.*, 2015) and beeswax (Kheradmandnia, Vasheghani-Farahani, Nosrati, & Atyabi, 2010).

Triacylglycerols (TAGs) are the principal components of fats and oils. Other than their biological functions, they are widely used in the food industry as the major components in cream, margarine, and confectionery fats (Pichot, *et al.*, 2014). A TAG consists of three fatty acid residues esterified to a glycerol backbone (Fig. 2.4). In contrast to TAGs, waxes are simple esters of fatty acids with alcohols, and these molecules may contain free hydroxyl groups (Fig. 2.4) (Jenning & Gohla, 2000). They are usually composed of long carbon chains, which account for their strong hydrophobic character. They are derived from various sources including animal, vegetable and microbial (Toublan, 2014).

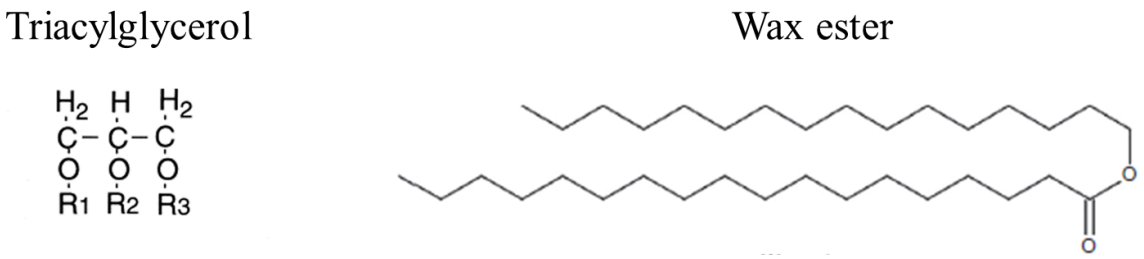

Fig. 2.4. Molecular formula of a triacylglycerol (TAG) and a wax ester.

It is understandable that these chemical composition (structural) differences will influence the lipids' physicochemical properties, e.g. thermal behaviour, as well as their features when used as carrier systems for the encapsulation of active ingredients.

2.5.2 Production methods

Solid lipid nanoparticles are obtained by emulsification of the molten lipid in a hot aqueous phase containing emulsifiers/stabilisers followed by cooling to temperatures below the crystallisation temperature of the dispersed lipid. Müller and co-workers pioneered the use of high-pressure homogenisation (HPH) for the production of particles with dimensions below 500 nm (Müller, *et al*., 1995). Since then, it has been the standard fabrication route for sub-micron sized solid lipid dispersions across all application fields. Melt-emulsification (carried out at temperatures above the melting point of the lipid) is preferentially performed via high-pressure homogenisers in a plethora of studies related to the production of SLN or the encapsulation of lipophilic bio-active agents into these crystalline matrices (Hentschel, *et al*., 2008; Helgason, *et al*., 2009a). Alternatively, melt-emulsification homogenisation can be carried out with ultrasound (Hou, Xie, Huang, & Zhu, 2003; Mei, Chen, Weng, Yang, & Yang, 2003; Silva, *et al.*, 2011; Song & Liu, 2005), which combines ease of use and low cost of the apparatus.

During emulsification by ultrasound – which was the method used in this work for particle fabrication – droplet breakage is driven by acoustic cavitation. The most prevalent mechanism is associated with the formation, growth and collapse of vapour bubbles inside the liquid medium. When the cavity collapses, it generates a shock wave that creates local pressure gradient and fluid shear that result in a reduction of the particle size because of the constant stress (Patil & Pandit, 2007). The total effective acoustic energy delivered to the insonated dispersions is a function of the instrument- and system- specific parameters. Ultrasonic-specific parameters include the frequency, the acoustic intensity and power, the insonation (residence) time and the energy density. These factors are known to affect distinctly the obtained emulsion properties (Canselier, Delmas, Wilhelm, & Abismaïl, 2002) and depending on the desired characteristics they need to be adjusted accordingly. However, this technique is also associated with major drawbacks that can be detrimental to the insonated system. Prolonged exposure to ultrasound has been reported to result in the formation of particles in the nanoscale but, concurrently, to an enhanced metal contamination from the probe (Silva, *et al.*, 2011). Also, a non-homogeneous distribution of the generated power causing a different level of droplet disruption within the same sample and thus, a broad size distribution has also been observed (Mehnert, *et al.*, 2001). In addition, Siekmann and Westesen (1994a) have shown that ultra-sonification is not very effective for lipid concentrations higher than 3% as the formulations could not be dispersed homogeneously. Therefore, instrumental factors together with formulation variables need to be considered and optimised before the employment of ultrasound emulsification.

The list of manufacturing methods for the production of SLN includes additionally microfluidisation (Helgason, Awad, Kristbergsson, McClements, & Weiss, 2008; Helgason, Salminen, Kristbergsson, McClements, & Weiss, 2015), water-in-oil-in-water (w/o/w) double

emulsion methods (Gallarate, Trotta, Battaglia, & Chirio, 2009; Becker Peres, *et al*., 2016), solvent diffusion (F.-Q. Hu, *et al.*, 2005; Quintanar-Guerrero, Tamayo-Esquivel, Ganem-Quintanar, Allémann, & Doelker, 2005), solvent emulsification/evaporation (Sjöström & Bergenståhl, 1992), microemulsion formation (Igartua, *et al.*, 2002), premix membrane emulsification (Joseph & Bunjes, 2012) and the newly reported process of microwave-assisted microemulsion (Charcosset, El-Harati, & Fessi, 2005; Shah, Malherbe, Eldridge, Palombo, & Harding, 2014).

Regarding the subsequent step of crystallisation, the cooling rate needs to be carefully selected as, apart from the size of the formed crystals, it can also induce a transition to another polymorphic form. A study by Awad, *et al*. (2008) showed that SLN formed with rapid cooling (i.e. 20 ºC/min) experienced a delay in transforming from the $\alpha \rightarrow \beta$-form which eventually made them less prone to aggregation and gelation, as evidenced by differential scanning calorimetry (DSC) and rheology experiments.

2.5.3 Influence of formulation parameters

During the development of solid lipid nanoparticulate structures, it is deemed critical to gain a complete understanding of how the interplay between fabrication methods and formulation design affects nanoparticles' characteristics and stability. With respect to formulation, several variables have been investigated for their role on SLN's physicochemical properties, e.g. the type of lipid and emulsifier or their concentrations (Martins, *et al.*, 2012). Regarding lipid composition, Boonme, Souto, Wuttisantikul, Jongjit, and Pichayakorn (2013) screened lipids of a different molecular structure and found that the number of carbon atoms contained in their fatty acid residue as well as the polarity (polar functional groups), had an influence on the resulting particle size. Microemulsion formation was not possible using lipids with no

polar groups and long hydrocarbon chains (e.g. glyceryl tripalmitate). Additionally, the smallest size was achieved with glyceryl trimyristate whose 14 carbon atoms facilitated its penetration into the lipophilic part of the surfactant as opposed to glyceryl monostearate. In a study by Grabnar, Kristl, and Smid-Korbar (1998) hard fat-based (Witepsol®W35) nanoparticles were significantly smaller sized than their triglyceride (Dynasan®118) counterparts. The authors attributed that behaviour to the high amounts of mono- and diglycerides contained in the former formulations, which are known to exhibit surface active properties.

As mentioned above, the formation of a lipid dispersion requires the presence of an emulsifier as a key design parameter. Different classes of emulsifiers have been assessed for their capacity to stabilise the lipid dispersions such as phospholipids (e.g. soybean lecithin, egg lecithin), bile salts (e.g. sodium (glyco)cholate), polymers (e.g. poloxamers), polysorbates and sucrose esters of fatty acids (e.g. polysorbate 20-80, sucrose laurate) (Mehnert, *et al.*, 2001). In general, emulsifiers are responsible for the lowering of surface tension which promotes particle (or droplet in case of hot homogenisation) fractionation. A reduction in particle size is synonymous to the creation of an enormous surface area that needs to be covered quickly by the available stabilising material. Kinetic aspects need to be considered since the final particle size will be determined by the balance between break-up and coalescence of uncovered lipid surfaces that occur simultaneously during emulsification (Niknafs, Spyropoulos, & Norton, 2011). It is understandable that a sufficient amount of emulsifier molecules needs to be present to cover the continuously formed surfaces and the speed at which this is done is a factor of both the emulsifier's concentration and molecular size (Mehnert, *et al.*, 2001).

Helgason, Awad, Kristbergsson, McClements, and Weiss (2009b) conducted a systematic investigation on the effect of surfactant concentration on the properties of solid tripalmitin

suspensions stabilised by the non-ionic surfactant Tween 20. Surfactant was added after homogenisation and given that this addition did not cause any change on particle size distribution, alterations observed in terms of SLN stability and crystal structure were related to crystallisation-induced effects. Their findings are summarised schematically in Fig. 2.5.

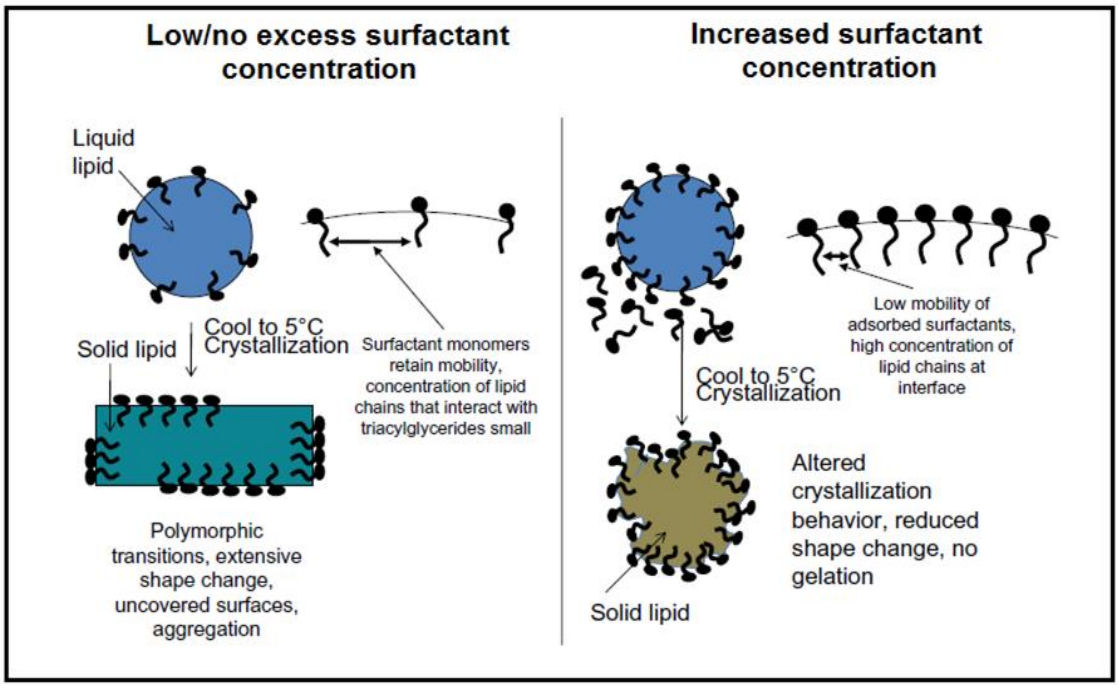

Fig. 2.5. A schematic illustrating the influence of surfactant surface coverage on interfacial crystallisation processes, as suggested by Helgason, *et al*. (2009b).

In essence, the packing of the surfactant tail groups at the oil-water interface is dictated by its concentration; at low surfactant concentration, aggregation and gelation of particles is likely to occur during the crystallisation step (Helgason, *et al*., 2008), whereas at high loads, a tighter packing of the tail groups is ensured. This, in turn, leads to the formation of a relatively rigid "shell" enclosing lipid droplets that, once crystallised, can no longer undergo any molecular motion which could lead to alteration of their shape. Martins, *et al.* (2012) also demonstrated that lipid nanoparticles were smaller and more stable for at least one year of storage when the emulsification step was conducted with high amounts of Tween 80 (2%).

The same authors correlated particle's size profile to the size of the utilised surfactant, with larger mean sizes obtained with the higher molecular weight poloxamers, and polysorbates being more effective in producing and stabilising smaller lipid nanoparticles. Using blends of emulsifiers is another approach that might be advantageous with respect to more efficiently hindering particle agglomeration. Certain studies have reported on smaller particle sizes and higher storage stability when a combination of two surfactants was used instead of a single one (Siekmann, *et al*., 1994a; Mehnert, *et al*., 2001).

Furthermore, it is well documented that emulsifiers are one of the main factors which affect, *inter alia*, lipid crystallisation and the time course of polymorphic transitions both in the bulk and in the dispersed state (Awad & Sato, 2001; Heike Bunjes, Koch, & Westesen, 2002, 2003; Garti & Yano, 2001; Rizzo, Norton, & Norton, 2015; Rosenblatt & Bunjes, 2009; Britta Siekmann & Kirsten Westesen, 1994). The majority of these studies has been concerned with triglyceride-based nanoparticles and a different thermal behaviour – with regards to melting temperature, re-crystallisation behaviour and kinetics of polymorphic transitions –, as opposed to the bulk lipid has been observed. Such discrepancies are usually attributed not only to the colloidal state of the dispersed lipid particles (hence an increase of the surface-to-volume ratio (Bunjes, Koch, & Westesen (2000)), but also to the presence of emulsifiers that, due to interactions developed with the lipid matrix, they promote stable or metastable polymorphic modifications accordingly (Bunjes, *et al*., 2003; Rosenblatt, *et al*., 2009). Further, observations by Bunjes, *et al*. (2002) revealed a correlation between surfactant's structure and its impact on crystallisation and polymorphism. It was found that stabilisation by ionic surfactants slowed down transition processes and the promotion of crystallisation was very dependent on the length of surfactant's hydrophobic chain. It can therefore be concluded that emulsifiers can alter, not only particle's properties related to colloidal state

(i.e. size, stability), but also its internal structure. This becomes more significant if lipid dispersions are used as carrier systems of bio-active agents (Helgason, *et al*., 2009a; Nik, Langmaid, & Wright, 2012) or as functional Pickering particles.

2.5.4 Characterisation of SLN's structure

Characterisation of such a system is of paramount importance to understand and evaluate its behaviour. Due to SLN's colloidal dimensions and the complexity of the system which involves dynamic phenomena, characterisation poses a serious challenge (Müller, *et al.*, 2000). The key parameters that have a direct impact on the particle's stability and overall performance are size, size distribution, and the thermal profile (polymorphic state). These factors coupled with the behaviour at an oil-water interface will, in turn, determine SLN's ability to act as Pickering particles.

2.5.4.1 Particle size and size distribution

SLN's particle size and size distribution plays a vital role during particle formation and processing, and it is the foremost parameter that is required to be evaluated for the use of these structures as Pickering stabilisers. The common practice consists of measuring particle size immediately after fabrication and also in the course of storage, to monitor changes in size. The fact that a number of techniques are employed for particle size determination highlights its importance, and possibly the difficulty in acquiring and comparing this information. However, in recent years light scattering patterns measured by laser diffraction (LD) and photon correlation spectroscopy (PCS) techniques are the preferred methods of choice within industry, due to the ease of use, fast operation and reliability (Xu, 2002). Both techniques detect light scattering effects and apply different theories to calculate particle size. PCS is routinely used in the characterisation of nanoparticles (measurement range from a few

nanometres to 10 microns), while LD is used for the detection of larger microparticles (Mehnert, *et al.*, 2001). Despite their different sensitivities, it is often suggested that these methods should be used in conjunction, especially when it comes to broad size distributions (Schubert & Müller-Goymann, 2005; Schubert, Harms, & Müller-Goymann, 2006). Results need to be interpreted with scepticism as the above mentioned analytical tools are sensitive to particle shape (they assume sphericity) and several studies have confirmed the presence of different shapes in solid lipid nanodispersions (e.g. platelet, disk-like, etc.) based on the obtained crystal modifications (Westesen, Siekmann, & Koch, 1993; Mühlen, Mühlen, Niehus, & Mehnert, 1996; Bunjes, Steiniger, & Richter, 2007). When the particle shape is of relevance, then light microscopy or other microscopic techniques (e.g. AFM) need to be used complementarily.

2.5.4.2 Crystallinity and Polymorphism in SLN

Polymorphism is defined as the ability of molecules to crystallise in multiple arrangements in the solid state (Larsson, 1994). In the case of triacylglycerols, it is well known that they have the inherent ability to pack in three main polymorphic forms, namely the α (hexagonal subcell), β' (orthorhombic-perpendicular subcell) and β (triclinic-parallel subcell) (Fig. 2.6A). Each polymorph has a different value of Gibbs free energy (G), which is the driving force for the transformation between them and their stability; α is the least stable, β' has an intermediate stability, β is the most stable polymorph and has the lowest Gibbs free energy. The thermodynamic stability of the polymorphic forms is usually mapped via a Gibbs free energy-temperature diagram (Fig. 2.6B) which follows the equation $G = H\text{-}TS$, in which H, T, S refer to enthalpy, temperature and entropy respectively. The formation and stability of these polymorphs are also largely influenced by the TAG fatty acid composition. Polymorphic crystallisation is mainly determined by the rate of nucleation, which depends on many factors

such as cooling rate, supersaturation or shear rate. For example, Sato and Kuroda (1987) showed that when subjected to cooling, α tripalmitin form appears first, followed by β' form and then the most stable form β.

These different crystalline arrangements have distinct melting temperatures and unique X-ray diffraction patterns (Himawan, Starov, & Stapley, 2006) and therefore, their identification in lipid dispersions is a necessity. On the other hand, waxes exhibit a suppressed polymorphism where an orthorhombic subcell arrangement prevails and the polymorph transition is occurring at low rates (Jenning & Gohla, 2000).

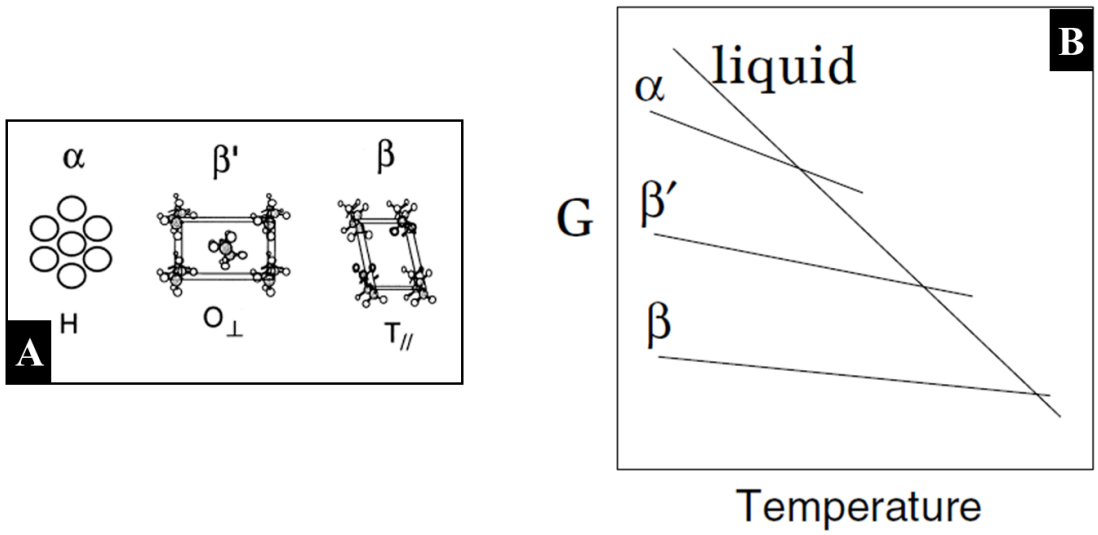

Fig. 2.6. Subcell structures of the three major polymorphic forms in TAGs (A). Schematic diagram of Gibbs energy (G) versus temperature of the three triacylglycerol polymorphs (B) (Sato & Ueno, 2005).

Since the preparation protocol involves thermal treatment and a subsequent crystallisation step, issues related to polymorphism and polymorphic transitions need to be considered during the manufacturing of these nanodispersions. It is common that particles may undergo polymorphic transformations post-crystallisation which may endure for a prolonged time after solidification (Bunjes & Siekmann, 2006). It is essential to confirm the polymorphic state

(crystalline or amorphous) of the lipids within the SLN dispersions, as this determines properties such as shelf-life stability. Differential scanning calorimetry (DSC), frequently in combination with X-ray diffraction (XRD), are the most widely used techniques for the investigation of lipid particles' physical status and the distinction between the various lipid polymorphs (Westesen, *et al*., 1993; Siekmann, *et al*., 1994b). Electron microscopy and neutron scattering have also been employed to monitor thermal transitions taking place in emulsified lipids (Bunjes & Unruh, 2007). In addition, a study by Awad, *et al*. (2008) showed that non-destructive temperature scanning ultrasonic velocity measurements could follow complex melting patterns of solidified tripalmitin SLN in accordance with results obtained by DSC, and thus, they can be used as a promising alternative to conventional DSC measurements.

Several other parameters have been identified as important variables that determine SLN's stability and functionality, e.g. surface charge and particle morphology, albeit they were out of the focus of this study.

2.5.5 Nanostructured Lipid Carriers (NLC)

Nanostructured lipid carriers (NLC) were developed at the turn of the millennium as an attempt to overcome the flaws associated with SLN's use. The improved version of SLN is composed of blends of solid and –chemically very different – liquid lipids (oils). This leads to the creation of more imperfect crystals that increase the loading capacity of particles when they are used as carrier systems. Due to the incorporation of a "foreign" molecule, the melting point suffers a depression compared to the neat solid lipid, and the water content of the dispersion is minimised (Montenegro, *et al.*, 2016). Concurrently, expulsion of any embedded active as a result of the crystallisation process itself or the polymorphic transformations

occurring upon storage is prevented (Müller, Radtke, & Wissing, 2002), rendering overall NLC more physically stable than the SLN.

Similar manufacturing methods have been used for the production of NLC, for instance high-pressure homogenisation where the lipid mixture is melted and mixed with a hot aqueous surfactant solution (Hentschel, *et al.*, 2008). Different lipids and oils have been used in the literature for the production of oil-borne particles. Among them, monostearin and soybean oil, and mixtures of triglycerides of behenic acid with medium chain triglycerides (Jores, Mehnert, & Mäder, 2003; Severino, *et al.*, 2012).

Three types of NLC have been described during the first years of their launch: i) imperfect, ii) amorphous (structure-less) and iii) multiple (oil-in-fat-in-water), as illustrated schematically in Fig. 2.7A. Several analytical techniques have been implemented since then in order to gain a deeper insight on the structure of oil-loaded SLN (Jenning, Mäder, & Gohla, 2000; Jenning, Thünemann, & Gohla, 2000). The research group of Jores has investigated in detail physicochemical characteristics of NLC structures with a range of methods (Jores, Haberland, Wartewig, Mäder, & Mehnert, 2005; Jores, *et al.*, 2004; Jores, *et al.*, 2003). They postulated that oil distribution within the lipid crystalline matrix is a function of the former's concentration. More specifically, when present at low contents the oil molecules are localised on the surface of solid lipid particles forming spots on it. Upon an increase of their amount, oil molecules form droplets that were found to stick on the lipophilic solid surface in "nano-spoon" structures, as shown in Fig. 2.7B.

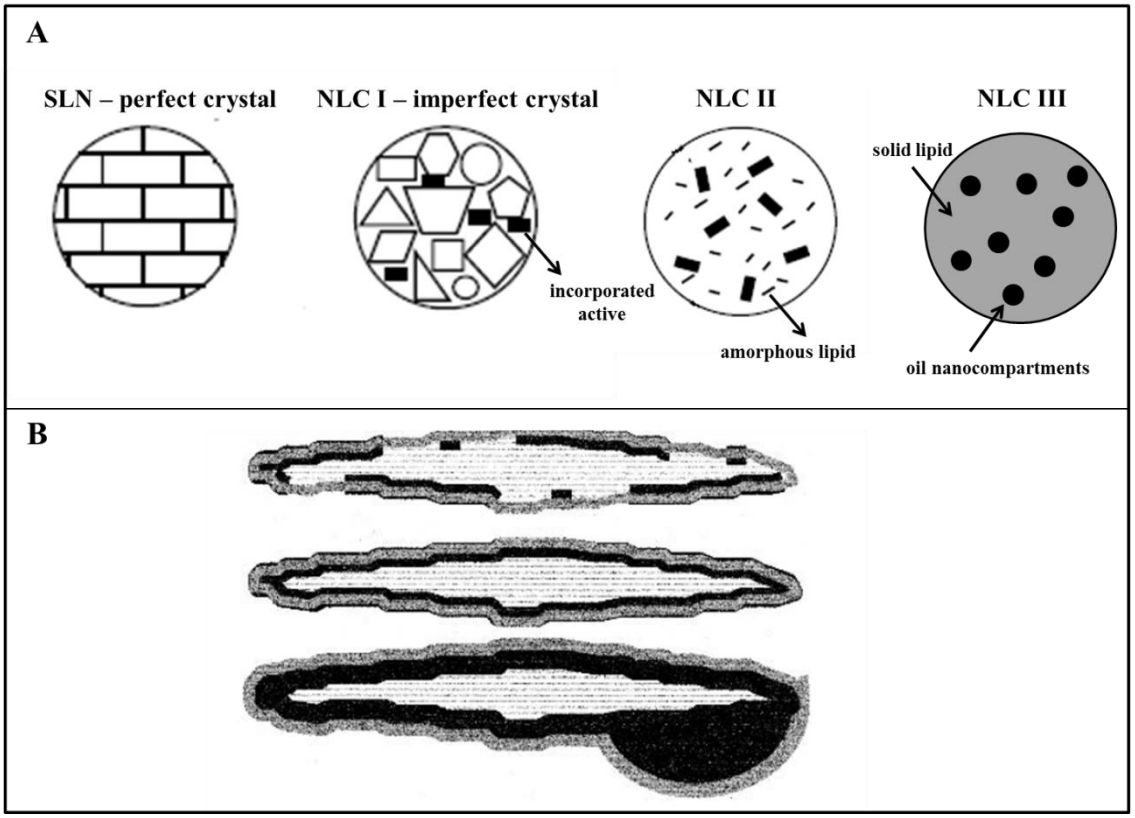

Fig. 2.7. Proposed structures of the three types of NLC (A) (adapted from Müller, *et al.* (2002)). A schematic representation of the NLC model developed by Jores, *et al.* (2003) where variations are a function of increasing oil loading in the system. White areas represent the solid lipid, black regions the liquid oil and the grey contour is the surfactant (Jores, *et al.*, 2003) (B).

In spite of the absence of an accurate model of NLC's structure, the second generation of lipid nanoparticles has been investigated for several administration routes and for their potential to accommodate, protect and release active components such as *β*-carotene, and co-enzyme Q10 (Teeranachaideekul, Souto, Junyaprasert, & Müller, 2007; Hentschel, *et al*., 2008). More relevant to stabilisation of emulsions, the use of NLC structures as potential Pickering stabilisers has not yet been reported in the literature.

2.5.6 Towards extending stability of SLN dispersions

Physicochemical stability of the developed SLN systems can be ensured via a number of composition/formulation and process-related pathways. The term physicochemical stability usually relates to the prevention of degradation reactions (e.g. hydrolysis), and the maintenance of the original size and size distribution within the desired storage time. This is particularly critical if SLN are used as Pickering particles as size is one of the key characteristics.

SLN aqueous dispersions that are prepared with a large volume of water can be stabilised by means of water removal via dehydration approaches. It is expected that the resulting solid state nanoparticles will exhibit an enhanced stability compared to aqueous lipid dispersions.

2.5.6.1 Dehydration/drying

Dehydration is defined as "the application of heat under controlled conditions to remove the majority of the water normally present in a product by evaporation" (Fellows, 2009). The underlying objective is to extend a product's shelf-life by halting microbial growth and this is achieved by reducing water activity. At the same time, the reduced weight and volume of dried products leads to a concomitant reduction in transportation/shipping and storage costs, and depending on specific food applications, they can potentially offer convenience (e.g. instant food products that can be reconstituted simply by addition of water) (Berk, 2013).

The conversion of the solid lipid nanoparticle dispersion (liquid) into a dry product (SLN granulates or powders) endows flexibility and additional possibilities of their incorporation into tablets, pellets or capsules (Mehnert, *et al.*, 2001). SLN have been converted into dry powders by spray-drying (Freitas, *et al.*, 1998), evaporative drying at low temperatures (Cavalli, Gasco, Barresi, & Rovero, 2001) and, most commonly, via freeze-drying

(lyophilisation) (Schwarz & Mehnert, 1997; Zimmermann, Müller, & Mäder, 2000). Nonetheless, such dehydration events could significantly affect the aqueous dispersions of lipid particles because they become prone to several changes as a direct result of the process itself (e.g. temperature events taking place), or it is likely that they suffer severe modifications of their structure upon rehydration (Abdelwahed, Degobert, Stainmesse, & Fessi, 2006).

2.5.6.2 Freeze-drying

During the last couple of decades, freeze-drying has evolved to a well-established drying method for the preservation of heat labile materials and the convenient production of solid forms, employed for food products, biological materials as well as drug delivery systems (Franks, 1998). It combines a number of assets that render it advantageous over conventional drying methods, such as high yield, conservation of structural/morphological as well as functional properties (e.g. biological, immunological), more favourable processing conditions (e.g. low temperature, oxygen and shear), extended shelf-life and production of lightweight products that facilitate storage, transportation and handling (Liu, Zhao, & Feng, 2008; Berk, 2013). Despite the benefits, its principal flaw is the enormous energy consumption and the high costs related to both operation and maintenance which, ultimately, make lyophilisation the most expensive drying method (Ratti, 2013). In the case of SLN, lyophilisation has been widely used as the method of choice for dehydration due to the unmatched benefits of chemical degradation and Ostwald ripening inhibition (Mehnert, *et al.*, 2001).

The basis for freeze-drying operation is removal of water by sublimation. In more detail, system temperature is lowered to temperatures below the crystallisation point of the solvent or suspending medium (typically water) so that it crystallises and thereafter sublimates under vacuum from the solid to the vapour phase directly, followed by desorption of the bound

water (Abdelwahed, *et al.*, 2006). It is therefore composed of three sub-processes; freezing, primary drying (sublimation) and secondary drying (desorption). The heat of sublimation is most commonly supplied by the combination of radiation from the hot surfaces and conduction (through contact with hot surfaces), as schematically shown in Fig. 2.8. The resulting product is (almost) anhydrous, with 1-3% moisture content being the usual target of a freeze-drying cycle (Berk, 2013). The cycle is also considered successful when reconstitution time is short, the physicochemical properties of the freeze-dried product are maintained and long-term stability is achieved (Abdelwahed, *et al.*, 2006).

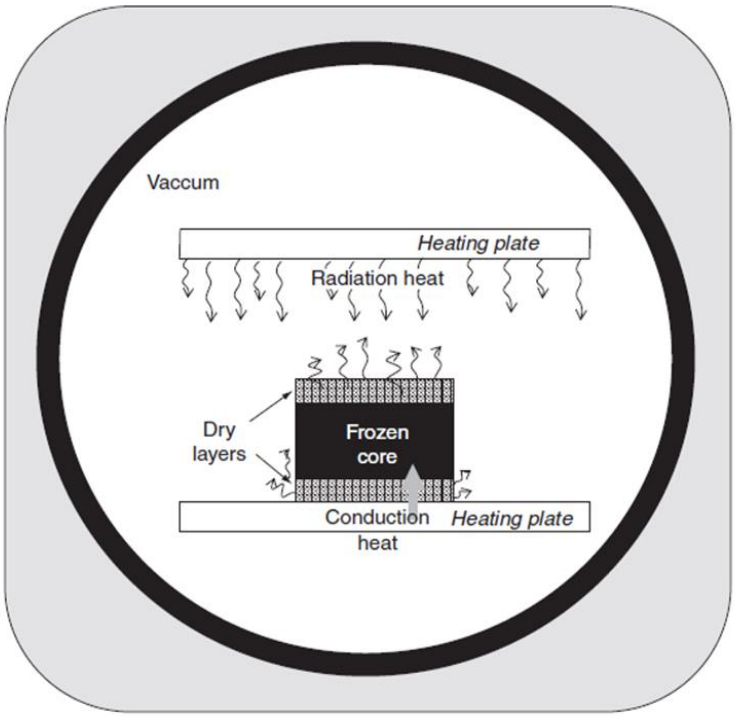

Fig. 2.8. Heat and mass transfer mechanisms occurring within the product during freeze-drying (Ratti, 2013).

Upon lyophilisation, the system is subjected to mechanical stresses (primarily linked to ice formation) that can destabilise the colloidal structure of a particulate preparation by promoting aggregation and/or irreversible fusion. In addition, upon the subsequent

rehydration of the lyophilised powders, events such as the low water and high particle content coupled with high osmotic pressure can also induce particle aggregation (Mehnert, *et al.*, 2001). Consequently, the redispersed product will contain larger particles and wider size distributions. Numerous compounds have been used in the literature in an attempt to circumvent freezing and desiccation stresses. Such components are usually added to the suspending medium prior lyophilisation and are known to endow protection and prevent damages occurring during the process (cryoprotectants). Polysaccharides, disaccharides, amino acids, polyols and honey are amongst such additives reported in the literature (Abadias, Benabarre, Teixidó, Usall, & Viñas, 2001; de Valdez, de Giori, de Ruiz Holgado, & Oliver, 1983). In the context of nanoparticles, sugars such as sucrose, glucose, trehalose and mannitol are usually the preferred protective excipients, supplemented at high concentrations (Morais, *et al.*, 2016). The yielded results will be a function of the cryoprotectant properties and concentration, system composition (e.g. type of lipid, stabiliser/surface active component), processing parameters (e.g. cooling rate) and so on (Zhang, Liu, Qian, & Chen, 2008; Morais, *et al*., 2016). A precise selection of materials has shown that freeze-drying is feasible even without the use of cryoprotective agents. Vighi, Ruozi, Montanari, Battini, and Leo (2007) demonstrated that cationic stearic acid-based SLN subjected to lyophilisation in the absence of cryoprotectants, could maintain the size and morphology of the fresh prepared samples upon resuspension and, most importantly, their functionality to complex DNA.

In emulsified systems, the presence of cryoprotectants in the formulation is not always desired, given the alterations caused in powder's composition (Adelmann, Binks, & Mezzenga, 2012). Therefore, research efforts have been directed towards alternative routes that could stabilise an oil-water interface and thereby, protect the drops from the aggressive freeze-drying conditions. Despite a dearth of published reports, the most significant approach

described in the literature is the use of a Pickering emulsion precursor. On this basis, oil-based powders and gels (depending on water evaporation rate) could be prepared from silica nanoparticles-stabilised o/w emulsion templates (Adelmann, *et al.*, 2012). More relevant to food systems, encouraging results were also obtained by using natural solid particles such as quinoa starch (Marefati, Rayner, Timgren, Dejmek, & Sjöö, 2013) and cellulose derivatives (via complexation with tannic acid) (Hu, Marway, Kasem, Pelton, & Cranston, 2016) as the stabilisers of emulsion precursors. Both studies showed that the fabricated emulsions could be dried and easily redispersed into water, with only minor levels of aggregation observed in the reconstituted emulsions. There is certainly a lot to be learnt from such microstructural strategies that allow preservation of key characteristics and that could be transferred to particulates undergoing a dehydration process.

2.6 Concluding Remarks

In lieu of sourcing particles and subjecting them to (minimal) processing treatment to ensure functionality, evidence described in this review suggests that fabrication of SLN has a great potential as a route towards emulsion stabilisation. These particulates can adsorb at oil-water interfaces (hence function as effective Pickering stabilisers of o/w or w/o emulsions) provided that they have appropriate size and wettability characteristics. Establishing formulation design rules and processing methods will ensure that these properties are addressed, as they dictate, in turn, the kinetics of adsorption and desorption from the interface and eventually, emulsion stability. The proceeding experimental chapters offer an analytical insight on that.

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Chapter 3

Fabrication of edible solid lipid particles in the presence of surface active species: Controlling particle microstructure attributes linked to Pickering functionality

Data and discussions contained within this chapter have been published within:

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Synopsis

Lipid particles are very promising candidates for utilisation as Pickering stabilisers, and fabrication of these species has been attracting considerable academic and industrial research. Nonetheless, current understanding of these systems is hindered by the fact that, as a whole, studies reporting on the fabrication and Pickering utilisation of lipid particles vary significantly in processing conditions being utilised and formulation parameters considered. The present study investigates, under well-controlled processing and formulation conditions, the fabrication of edible lipid particles from two lipid sources in the presence of two different types of amphiphilic species (surfactant or protein) via melt-emulsification and subsequent crystallisation. Fabricated solid lipid particles were assessed in terms of their particle size, interfacial and thermal behaviour, as well as stability, as these microstructure attributes have established links to Pickering functionality. Lipid particle size and stability was controlled by the type and concentration of the used amphiphilic species (affecting the melt-emulsification step) and the type of lipid source (influencing the crystallisation step). Interfacial behaviour was closely linked to the type and concentration of the surface-active component used. Finally, the types of lipid and amphiphilic agents employed were found to affect lipid particle thermal behaviour the most.

3.1 Introduction

It is well known that solid particles can adsorb at the oil-water interface in a manner similar to surfactant molecules, yet with fundamental differences in regards to their stabilisation mechanisms. Particle adsorption is effectively irreversible, providing superior long-term stabilisation to the system (Binks, 2002). Inorganic materials such as silica (Binks, *et al*., 1999; Daware & Basavaraj, 2015), clay platelets (Guillot, Bergaya, de Azevedo, Warmont, & Tranchant, 2009; Nonomura & Kobayashi, 2009), iron oxide (Ahn, Jung, & Choi, 2015), metal hydroxides (Tan, Wang, Wang, Xu, & Sun, 2011) and carbon (Godfrin, Tiwari, Bose, & Tripathi, 2014; Briggs, *et al.*, 2015) have been the mainstream for solid structures that provide Pickering stabilisation in emulsions.

Certain key particle attributes have been linked to effective Pickering stabilisation performance. Particle size, shape, wettability and level of particle-particle interactions are amongst those features (Tambe & Sharma, 1994; Binks, *et al*., 1999). For example, resultant emulsion droplets are affected by the size of the particle adsorbed to the interface; it has been suggested that the average particle size needs to be at least an order of magnitude smaller than the emulsion droplet size (Dickinson, 2012). Wettability, quantified by the contact angle θ that the particle assumes with the oil-water interface is often viewed as a factor of primary importance for emulsion stability (Binks, 2002). This is because it determines – together with the size – the particles' attachment energy as well as the emulsion type, depending on how particles reside at the interface between the two immiscible liquids (Simovic & Prestidge, 2004).

Despite the soar of research, there is a significant scope to extend the range of Pickering particles that originate from food-approved components and could be manufactured at

commercial scale, which, owing to their special properties, could be an asset for the food industry (Dickinson, 2010; Morris, 2011). To bridge the gap between Pickering emulsions and food-related applications, several natural-based materials have been investigated such as cellulose nanocrystals (Cherhal, Cousin, & Capron, 2016), chitin particles (Tzoumaki, Moschakis, Kiosseoglou, & Biliaderis, 2011) and flavonoids (Duffus, Norton, Smith, Norton, & Spyropoulos, 2016). The use of food-grade particulate structures also embodies lipid crystalline particles (fat crystals) (Hodge & Rousseau, 2005; Binks & Rocher, 2009; Rousseau, 2013), the role of which in the stabilisation of everyday foods such as butter, margarine and ice cream, is long recognised (Ghosh & Rousseau, 2011). As also mentioned above for Pickering particles, when fat species are used as emulsion stabilisers, their mean size and microstructure (e.g. morphology and polymorphism) will highly define their effectiveness (Rousseau, 2000). In turn, these properties will be dictated by processing conditions and formulation parameters. It is critical to produce particles in the sub-micron size range as this is a prerequisite for stabilising emulsion droplets ranging between 0.5-10 μm (Dickinson, 2012) and also for providing a dense surface coverage (Rousseau, 2000). In that view, Gupta and Rousseau (2012) fabricated solid lipid nanoparticles based on inherently surface-active glyceryl stearyl citrate (GSC) that retained their initial diameter (~152 nm) for 6 months and partially covered and stabilised ~459 nm oil-in-water (o/w) emulsions for 12 weeks.

The co-presence of surface active agents in a fat system is apt to bring about a number of benefits in fat crystals behaviour which would subsequently improve their performance within emulsions. It has been shown that surfactant, and in particular its chemistry plays a drastic role on the obtained size of lipid particles (Shi, Li, Yu, Jia, & Zheng, 2011) as well as physical stability upon storage, and several emulsifiers have been screened towards that end

(Mehnert & Mäder, 2001). Additionally, it is well-documented that surfactants influence the crystallisation profile and the kinetics of polymorphic transitions after crystallisation occurring in both bulk triglycerides (Garti & Yano, 2001; Bunjes & Koch, 2005) and in emulsified systems/colloidal dispersions (Awad & Sato, 2001; Bunjes, Koch, & Westesen, 2002, 2003; Siekmann & Westesen, 1994). For instance, in the field of food production (e.g. chocolate, margarine) low amounts of certain surfactants are added to the fat in order to delay undesirable polymorphic transformations (Aronhime, Sarig, & Garti, 1988). Moreover, surfactants have been employed as a means of tuning the hydrophobic character of lipid entities, thus their wettability characteristics (Pawlik, Kurukji, Norton, & Spyropoulos, 2016).

Nonetheless, current understanding of these systems, especially in the area of food, is somewhat hindered by a level of disconnection exhibited in the literature. This is largely due to the fact that, as a whole, studies reporting on the fabrication and Pickering utilisation of lipid particles vary considerably both in terms of the processing conditions used and formulation parameters considered. The present study aims to investigate, under well-controlled and uniform (processing and formulation) experimental conditions, the impact of the type of lipid source (used for particle fabrication), and type and concentration of surface-active species (used to facilitate particle fabrication) on specific particle microstructure attributes, established in literature as clear drivers of Pickering functionality. This was achieved via fabrication of solid lipid particles from lipids of different chemical structures by a hot emulsification method. To that end, a pure monoacid triglyceride (tristearin) and a model wax (cetyl palmitate) were selected as the bulk lipid materials. The structural diversity of these lipids leads to different thermal properties (e.g. melting/crystallisation temperatures), which is expected to impact differently particles generated from these precursors. Lipid particles were produced in the presence of two surface active species that are widely used in

the food industry (i.e. a low molecular weight surfactant and a protein) and that were chosen on the basis of their distinct physicochemical properties. The difference in chemistry and size of these compounds was anticipated to have a dual effect; that is, providing stability against particle-particle interactions, and controlling wettability of the created particles, hence their behaviour at an oil-water interface. Overall, it was aspired that these formulation variables would enable the control of indicators that have been shown of being important for Pickering performance. The constructed lipid-based particles were characterised in terms of their size, stability upon storage, interfacial behaviour and thermal properties.

3.2 Materials & Methods

3.2.1 Materials

In terms of the lipid components used, microcrystalline glyceryl tristearate (tristearin, TS) (Dynasan® 118) and cetyl palmitate (CP) were kindly provided from IOI Oleo (IOI Oleochemicals GmbH, Germany) and Gattefossé (France) respectively. Polyoxyethylene sorbitan monooleate (Tween 80) and casein sodium salt (NaCas) from bovine milk, were purchased from Sigma-Aldrich (Sigma-Aldrich, UK). The dairy protein NaCas was used in its native state (i.e. pH≈6.8). The lipid phase used for interfacial tension measurements was commercially available sunflower oil, which was used without further purification. Double distilled water from Milli-Q systems (Millipore, Watford, UK) was employed throughout the study.

3.2.2 Methods

3.2.2.1 Solid lipid particles preparation

Solid lipid particles were prepared by a melt-emulsification method, following the procedure described elsewhere (Silva, *et al.*, 2011). Briefly, 2.5% of lipid material relating to the total mass (wt/wt%) was heated around 5 to 10 ºC above the melting temperature of the lipid to ensure complete melting. For instance, the triglyceride was heated up to ~85 ºC, whilst the wax was melted to around 65 ºC. Temperature was monitored with a digital thermometer (TGST3, Sensor-Tech Ltd., Ireland). Tween 80 and NaCas were dissolved in the aqueous phase at different concentrations (0.8, 1.2 and 2 wt/wt%) which was subsequently heated to the same temperature as the molten lipid. The hot aqueous phase was then added to the molten lipid phase and mixed with a magnetic stirrer for a few minutes at a moderate speed. The hot pre-emulsion was homogenised using a high intensity ultrasonic vibracell processor (Sonics & Materials, Inc., CT, USA) operating in a continuous mode, at 750 Watt and 20 kHz. The sonication amplitude and thus the power output, was set at 95% of the nominal power and sonication was conducted over a controlled period of time (2 minutes). Droplet breakage was driven by acoustic cavitation, resulting in the formation of nanoparticles (Patil & Pandit, 2007). The oil-in-water emulsion formed was subsequently cooled in an ice bath (average cooling rates ~1-2 ºC/min), to a temperature below the crystallisation temperature of the carrier lipids (to 1-3 ºC). Hence, solid lipid particles (crystals) were obtained by crystallisation of the dispersed lipid. All samples were stored at refrigeration temperature (~4 ºC) until further analysis.

3.2.2.2 Analytical methods

3.2.2.2.1 Particle size analysis

Particle size and size distribution profiles for all samples were measured using laser diffraction (LD) (Mastersizer 2000, Malvern Instruments, UK) equipped with a small manual dispersion unit (Hydro SM). For measurement, the sample was dispersed in distilled water at 1200 rpm until an obscuration rate of 5-10% was obtained. Optical properties of the materials used were the following: refractive index (RI) of Dynasan® 118 = 1.49, cetyl palmitate RI = 1.44, Millipore water (dispersant water) RI = 1.330. An absorption index of 0.01 was employed across all measurements. The instrument produces a volume distribution for the analysed light energy data, independently of the number of particles in the sample (Rawle, 2011). The mean diameters $D_{3,2}$ (representing the surface average value), and the span values (a measure of the width of distribution) were obtained. Samples were prepared in at least duplicates and are reported as the average of three measurements. Particle size measurements were taken immediately after production and were repeated 1, 4 and 12 weeks after preparation.

3.2.2.2.2 Interfacial tension measurements

The sunflower oil/water static interfacial tension (IFT) was determined using the Wilhelmy plate method on a K100 Krüss Tensiometer (Krüss GmbH, Germany). The interfacial tension of the systems with lipid particles prepared in the presence or absence of surface active components was measured. It is assumed that the impurities present in the commercially available oil are not significantly influencing the adsorption behaviour of surface active agents at the oil/water interface. All the experiments were conducted at room temperature. To perform the measurement, ~50 mL of sunflower oil were carefully pipetted onto the surface of

the aqueous phase containing the different formulations of lipid particles and measurement commenced. Measurements were performed between 50 and 70 minutes although some systems did not reach equilibrium values within this time frame. All measurements were conducted at least in duplicate on each different sample and the average value as well as the standard deviation (±1) was calculated.

3.2.2.2.3 Thermal analysis

Thermal profiles of solid lipid particles were obtained with a high-sensitivity Setaram μDSC7 evo microcalorimeter (Setaram Instrumentation, France). ~600 mg of aqueous lipid dispersions and ~7.5 mg of bulk lipids were analysed as sample materials. Samples were subjected to a scan program consisting of: a heating cycle from 20 ºC to 85 ºC at 1.2 ºC/min followed by a cooling cycle from 85 ºC to 5 ºC at 1.2 ºC/min to mimic the process that is used to obtain the crystallised particles. This ramp was used to obtain information in regards to the physicochemical state of the micro/nanoparticles. Bulk tristearin and cetyl palmitate treated under similar conditions were used for comparison. From the DSC curves, peak temperature and enthalpy during melting and crystallisation transitions were determined by extrapolation via the software (Calisto Processing) or via deconvolution of the acquired peaks. All measurements were performed in at least duplicate.

3.3 Results & Discussion

3.3.1 Particle Size Analysis

Aqueous dispersions of solid lipid particles were produced via a melt-emulsification (ultrasonication) technique. Two lipid compounds (i.e. triglyceride and wax) were used as the building blocks of the particles. The dispersions also contained two different types of surface

active species (i.e. Tween 80 and sodium caseinate). These entities were added during the melt-emulsification stage at different concentrations (0.8, 1.2, 2 wt/wt%) in order to obtain lipid melt droplets (particle precursors) of small sizes.

Initially, pure lipid dispersions with no surface active species present were produced at a range of concentrations for both lipid materials, in order to establish a relationship between lipid content and resulting particle size. It was observed that above 2.5 wt/wt% lipid fraction, the generated particle size distributions were polymodal with predominantly micron-sized particles for both the triglyceride and the wax. Therefore, for the purposes of the present study the focus was on 2.5 wt/wt% in an attempt to ensure production of solid lipid particles within a size range that is desirable to promote Pickering functionality.

Fig. 3.1 shows the particle size distributions for tristearin and cetyl palmitate particles fabricated using different concentrations of Tween 80 or NaCas and measured immediately following production.

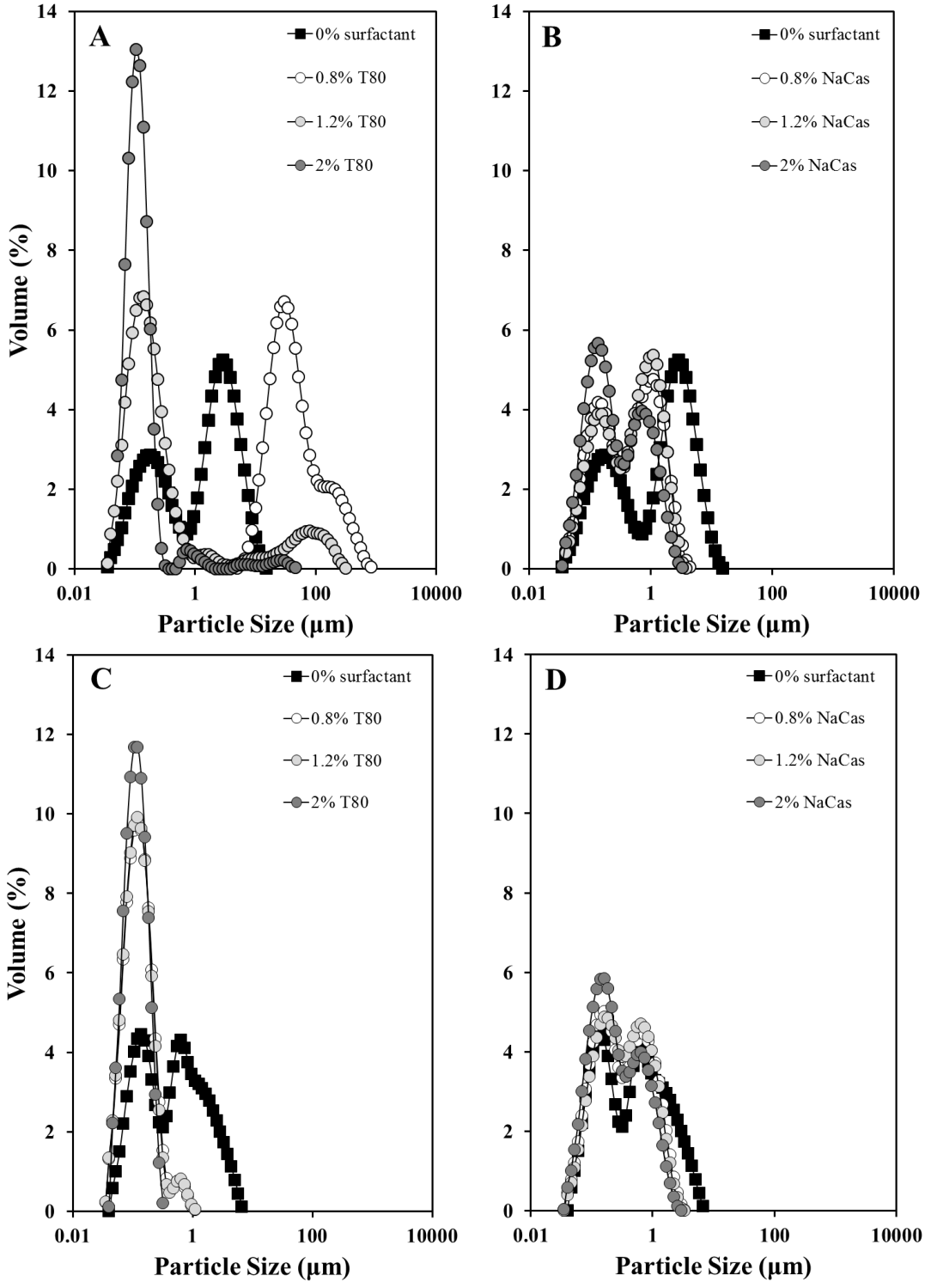

Fig. 3.1. Laser Diffraction measurements showing the particle size distributions of solid tristearin (top) and cetyl palmitate (bottom) particles (2.5 wt/wt%) formed in the presence of varying concentrations of Tween 80 (T80) (A, C) and sodium caseinate (NaCas) (B, D). Graphs presented are representative of three replicate samples.

The production of lipid particles is essentially composed of two stages, melt-emulsification followed by cooling (solidification of liquid droplets) and formulation variables were found to affect differently each one of these, as will be discussed below. Tristearin particles were initially formed in the absence of any surface active species. The resulting lipid particle suspension appeared milky white with some bigger lumps of solidified fat and a bimodal distribution with peaks at 0.2 and 2.8 μm. As can be seen from Fig. 3.1A and Fig. 3.1B, introduction of surface active entities in the melt-emulsification step obviously affects the final particle size which appears to be largely dictated by their type and somewhat less by their concentration.

Upon addition of Tween 80, the mean particle size decreases from 35.5 μm to 0.1 μm with an increase in the surface active species' concentration (Table 3.1). When a low concentration (0.8 wt/wt%) of Tween 80 was employed, micron sized particles were produced. Increasing the amount of surfactant to 1.2 wt/wt% led to a significant reduction in the particles' diameter, yet not completely as a second peak at higher sizes was still present. The transition from a bimodal to monomodal distribution at higher surface active component concentration highlights a relationship between the resulting droplet size and surface active species available to cover/stabilise droplet interfaces during the fabrication process. At 0.8 wt/wt%, the high energy provided by ultrasound together with the lowering of interfacial tension caused by Tween 80 forces the creation of a huge number of small droplets that cannot be efficiently covered by the available surfactant. Therefore, the system is more prone to coalescence. Similar behaviour has been reported previously in o/w emulsions formed in the presence of Tween 20 (Niknafs, Spyropoulos, & Norton, 2011). The authors reported an increase in droplet size at surfactant concentrations as low as 0.01 wt/wt% and attributed it to the increase of the system's interfacial area due to the enhanced break-up events. In this way,

the limited surfactant content is depleted which, in turn, encourages coalescence events. On the contrary, particle size seemed not to be influenced by the surface active agent's concentration in the case of sodium caseinate as two characteristic size populations were obtained regardless of the protein content (Fig. 3.1B). NaCas due to its high molecular weight has lower potential to reduce interfacial tension, hence droplet break-up and coalescence will not be affected as much by its concentration as was the case with the small molecule of Tween 80.

Tween 80 appears to drive a reduction in the size of the melt triglyceride droplets that are initially formed, further than NaCas does. Thus, in this case the size of the melt lipid droplets (precursors of the lipid particles, which are ultimately obtained) is mainly controlled by the ability of the used surface active component to lower the interfacial tension in the system (at a faster rate and to a lower equilibrium value). Once these melt emulsion droplets then undergo cooling and crystallisation to render the final lipid particle structures, the type and concentration of the surface active species previously used also appear to play an important role. Conversely to the melt-emulsification step, the role of the surface active agents is to limit/hinder interactions/contacts between the lipid bodies as they undergo crystallisation, to transform from almost liquid droplets to solid crystalline material. Here, it is the protective barrier that is interfacially provided by the surface active component present which is most important. Interfacially active species that can form a thicker barrier will successfully inhibit contacts between lipid bodies, even when present at relatively low concentrations (NaCas). On the other hand, a denser (as opposed to thicker) interfacial layer can have similar benefits, but this can be achieved at relatively higher concentrations of the surface active agents in question (Tween 80).

A lipid with different thermal properties, namely the wax cetyl palmitate was comparatively investigated for the fabrication of lipid particles. The two lipid components evaluated possess distinct chemical (tristearin is an ester of a fatty acid while cetyl palmitate is an ester derived from a fatty acid and a fatty alcohol), physical (tristearin is a larger molecule and requires melting at higher temperatures) and crystallographic (triglycerides usually crystallise in three different subcell arrangements whereas in waxes an orthorhombic lamellar lattice dominates) properties, which, in turn, are expected to affect lipid particles' characteristics (Jenning & Gohla, 2000). Cetyl palmitate crystals were produced applying the melt-emulsification-ultrasound technique and in most cases sub-micron particles (below 200 nm) were yielded. As seen in Fig.3.1C and Fig. 3.1D no particles bigger than 3 μm were detected in any of the cetyl palmitate-based systems fabricated using either Tween 80 or NaCas.

Specifically in the presence of Tween 80, solid lipid particles consisting of wax matrices were mono- or quasi- monodisperse (Fig.3.1C). Mean particle diameters in the range of 100-110 nm were obtained. It is interesting to observe that the amount of Tween 80 needed, and above which the particle size is no longer affected, is much lower than that of tristearin, thereby 2% (wt/wt) Tween 80 gave similar sized wax particles to 0.8 wt/wt% concentration (Fig. 3.1C). In contrast, when sodium caseinate was used, broader sized distributions were obtained (higher span values as seen in Table 3.1), of which two populations were more distinct; one population in the nanoscale and another one reaching a few microns (Fig.3.1D). It appears that the size of cetyl palmitate particles is largely dictated by the specific lipid component used and namely, by the small temperature difference (ΔT) between melting and crystallisation temperatures. This allows for the melt wax droplets that are initially formed (as compared to melt triglyceride droplets) to undergo crystallisation over much shorter timescales (note that the cooling rate in both cases is comparable).

Fabricated lipid particles were evaluated in terms of their long-term physical stability over a time period of 3 months (stored at refrigeration temperature). The evolution of the mean particle size ($D_{3,2}$ values) and the span values (width of distribution) is shown in Table 3.1 for tristearin and cetyl palmitate particles respectively, produced in the presence of Tween 80 and NaCas. All formulations refer to a fixed amount of lipid phase (2.5 wt/wt%).

Table 3.1. Mean diameters of solid tristearin and cetyl palmitate particles produced using Tween 80 and sodium caseinate. Measurements were performed by laser diffraction after production of the lipid crystals and over a span of 12 weeks, while stored at 4 ºC. All data are means ± 1 standard deviation ($\bar{x}\pm s$) for n=3 batches of samples.

TRISTEARIN LIPID PARTICLES

Period of storage (weeks)	Concentration of surface active species (wt/wt%)	Tween 80		Sodium Caseinate	
		$D_{3,2}$ (µm)	Span	$D_{3,2}$ (µm)	Span
0	0	0.4±0.1	3.3±1.1	0.4±0.1	3.3±1.1
	0.8	35.5±3.4	3.1±1.4	0.3±0.1	3.3±0.5
	1.2	0.2±0.1	205.9±194.4	0.3±0.1	3.1±0.6
	2	0.1±0.1	1.1±0.2	0.2±0.1	4.6±0.2
4	0	0.5±0.3	7.0±5.9	0.5±0.3	7.0±5.9
	0.8	19.4±2.6	4.3±2.4	0.2±0.1	4.4±1.7
	1.2	0.2±0.1	293.6±102.6	0.3±0.1	4.0±0.1
	2	0.1±0.1	1.1±0.2	0.2±0.1	4.3±0.4
12	0	0.7±0.6	13.7±3.8	0.7±0.6	13.7±3.8
	0.8	14.6±4.9	4.5±0.7	0.3±0.1	4.3±1.7
	1.2	0.1±0.1	153.2±262.8	0.2±0.1	3.5±0.4
	2	0.1±0.1	1.0±0.1	0.3±0.2	37.9±47.1

CETYL PALMITATE LIPID PARTICLES

Period of storage (weeks)					
0	0	0.3±0.1	6.1±2.4	0.3±0.1	6.1±2.4
	0.8	0.1± 0.1	1.6±0.1	0.2±0.1	4.0±0.3
	1.2	0.1±0.1	1.3±0.2	0.2±0.1	3.8±0.8
	2	0.1±0.1	1.1±0.1	0.2±0.1	4.0±0.3
4	0	0.5±0.3	12.5±6.1	0.5±0.3	12.5±6.1
	0.8	0.1±0.1	1.2±0.1	0.2±0.1	3.4±0.3
	1.2	0.1±0.1	1.1±0.1	0.2±0.1	3.7±0.5
	2	0.1±0.1	1.0±0.1	0.2±0.1	3.7±0.1
12	0	1.8±0.6	6.6±3.1	1.8±0.6	6.6±3.1
	0.8	0.1±0.1	1.2±0.1	0.2±0.1	3.9±0.9
	1.2	0.1±0.1	1.1±0.1	0.2±0.1	3.4±0.5
	2	0.1±0.1	0.9±0.1	0.2±0.1	3.5±0.3

It is evident from Table 3.1 that the stability of lipid particles in the absence of surface active components is compromised, with both $D_{3,2}$ and span values increasing along time. Furthermore, the type of the surface active species and the structure of the lipid material both seemed to have a significant impact on the stability upon storage. The highest concentration of either surfactant (i.e. 2 wt/wt%) yielded the most stable lipid particles for the investigated period of 3 months.

From Table 3.1 it can be seen that tristearin particles produced with Tween 80 were somewhat less stable as opposed to the ones produced with NaCas, and the instability was more pronounced for Tween 80 concentrations ≤ 1.2 wt/wt%. The differences detected between the storage behaviour of tristearin particles constructed using sodium caseinate and Tween 80 can be ascribed to several factors; the steric properties that impart electrostatic and/or steric stabilisation, the thickness of the interfacial film formed by the distinct surface active species and the polymorphic changes that are potentially occurring upon particles' storage (Fredrick, Walstra, & Dewettinck, 2010).

In regards to cetyl palmitate-based particles, there were no differences depicted in the mean diameter within the replicates of freshly-prepared solid wax matrices with either surface active component, as expressed by the low standard deviation values. Cetyl palmitate-based formulations showed excellent physical stability upon storage; the mean particle diameters as well as the distribution width did not change, or changed negligibly by a few nanometers during 90 days. The very low span values yielded, especially in the presence of Tween 80, was almost unchanged after 3 months. This appears to be in good agreement with the literature where cetyl palmitate formulations stabilised by polysorbates (20,40, 60 and 80) were stable during 1 year (Martins, Tho, Souto, Ferreira, & Brandl, 2012).

3.3.2 Interfacial behaviour

It is difficult to evaluate how much of the amount of the surface active species is still associated with the formed particles versus the amount that remains "free" in the aqueous phase. Measurement of a property that is sensitive to such changes could give an indication of the amount of surface active component that each time participates. For this reason, interfacial tension measurements (IFT) between sunflower oil and the continuous (aqueous) phase were conducted for four different systems; lipid particles formed in the absence of added surface active components, lipid particles fabricated with surface active entities added during the melt-emulsification stage, lipid particles where the surface active components were added once the particles have formed crystalline structures (and are dispersed in water), and solely surface active species in aqueous phase. It was anticipated that the behaviour of lipid particles with surface active species supplemented at different stages of their production would be within the margins (i.e. maximum and minimum interfacial tension recordings) corresponding to particles without any added surfactant and bare surfactant in solution respectively.

It needs to be noted that it was not central to evaluate interfacial behaviour at equilibrium. Rather, the behaviour of the manufactured systems in terms of interfacial tension reduction capacity at shorter time scales that are more representative of the action of surface active species during emulsification conditions than equilibrium IFT values, was more relevant to the purpose of this study.

The interfacial behaviour has been investigated for both fabricated tristearin and cetyl palmitate lipid particles in the presence or absence of the two surface active species. Interfacial tension data obtained for the tristearin and cetyl palmitate particles are presented in Fig. 3.2 and Fig. 3.3. The interfacial performance of lipid particles fabricated with the lower (0.8%) and higher (2%) concentrations of surface active agents are only presented here, as

these represent the behaviour observed for all lipid dispersions of all surface active component content.

Tristearin and cetyl palmitate particles produced without any emulsifier, only slightly alter the interfacial tension of water/sunflower oil (measured value 24.3 ± 0.3 mN/m). These systems have practically the same interfacial tension as sunflower oil/water and any small reduction is probably due to impurities present in the commercial sunflower oil (Gaonkar, 1989). In regards to Tween 80, its small molecular size allows it to adsorb at an oil/water interface fast, and it appears to reach a certain level of thermodynamic equilibrium within the first 15 minutes (saturated IFT for Tween 80 is reported to be ~5 mN/m) (Apenten & Zhu, 1996). Unlike Tween 80, sodium caseinate due to its larger molecular weight and its bulkier structure diffuses much slower to a liquid-liquid interface. Upon adsorption at an interface, proteins will undergo a long process of conformational changes (i.e. surface denaturation) where their hydrophobic domains are exposed to the oil and their hydrophilic to the aqueous phase of the biphasic system, thereby lowering the IFT (McClements, 2004).

Specifically for the tristearin systems, adding the interfacially active species to particles fabricated in the absence of Tween 80 or NaCas produced a response, in terms of interfacial tension, that in all cases is almost identical to that of only the specific active component itself. This clearly suggests that in both cases, the surface active components are relatively unhindered to interfacially adsorb and lower interfacial tension. Particles formed in the presence of either Tween 80 or NaCas appear to reduce interfacial tension to a lesser extent than the former two cases. The only exception is Tween 80 at the lowest concentration investigated (0.8%) which appears to coincide with both the 0.8% Tween 80 only data as well as with the 0.8% Tween 80 added post fabrication system (Fig. 3.2A). In this case, the much larger particle sizes obtained here ($D_{3,2}$ = 35.5 μm, Table 3.1) drive interfacial tension to lower

values (6.2 versus 8.9 mN/m for 0.8% and 2% T80 respectively, present during the fabrication stage) given the difference in the availability of surfactant molecules in each case.

The interfacial tension data recorded for triglyceride-based particles shows a link to surface active agent concentration (Fig. 3.2A and 3.2B), where increasing Tween 80 during or post fabrication affects the tension at the oil/water interface. However for the wax (cetyl palmitate) particles, increasing the surface active species' content appears to have little effect (Fig. 3.3A and 3.3B). The behaviour of cetyl palmitate particles when surface active entities were added following their formation resembles that of the neat entity in solution. This trend was observed for both Tween 80 and NaCas and is akin to what has been discussed earlier for tristearin systems.

As also seen in both Fig. 3.2 and 3.3 the interfacial profile of systems containing sodium caseinate reaches values that are significantly lower than the ones in the presence of Tween 80. This could indicate that a proportion of NaCas is adsorbing to the Wilhelmy plate, making the contact angle at the plate non-zero, i.e. reducing the measured value of the weight of the meniscus (equal to the force recorded by the device) which would lead to smaller interfacial tension values. Indeed, it has been observed that some surfactants can adsorb so strongly on metals, such as platinum (Wilhelmy plate) altering plate's wetting properties and eventually, introducing a possible error source by changing the measurement conditions (Drelich, Fang, & White, 2002).

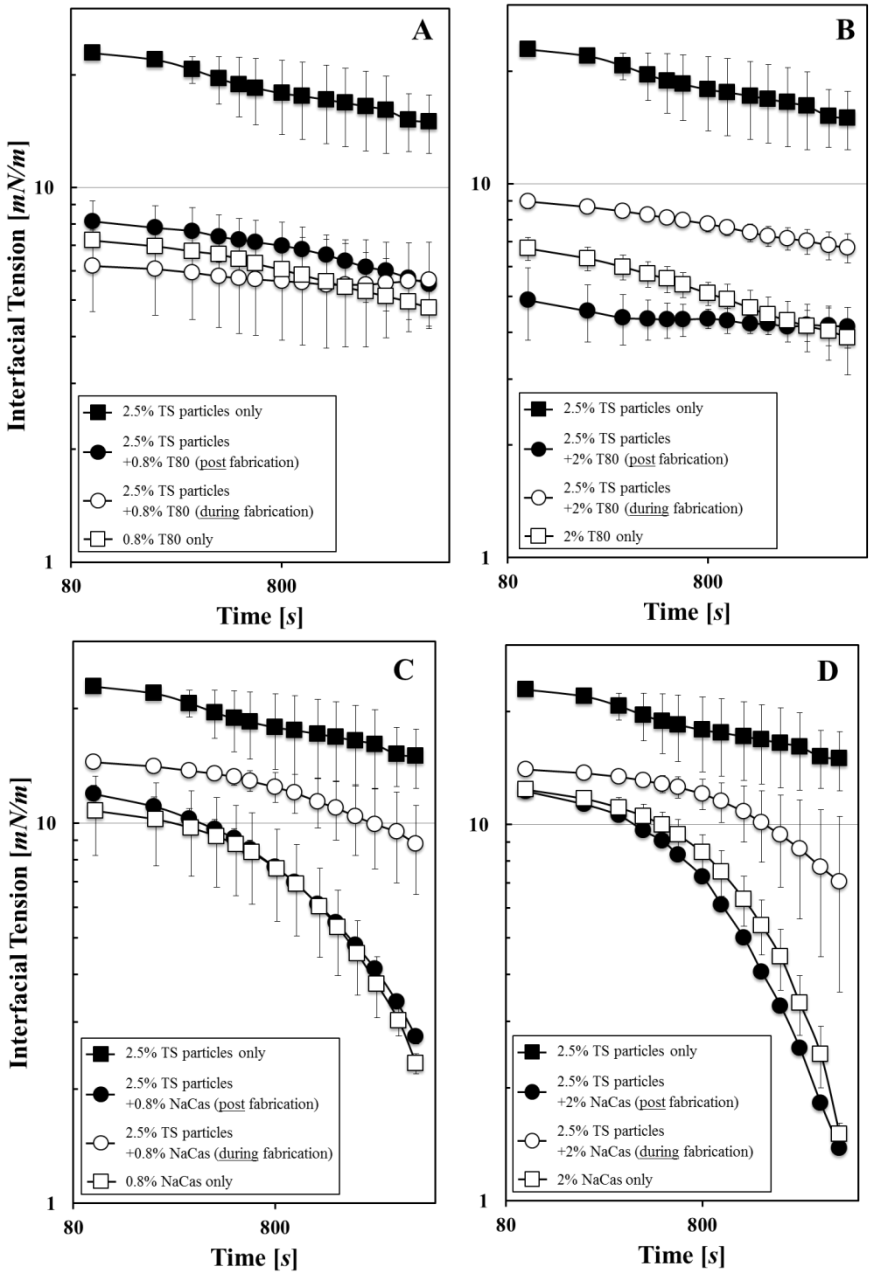

Fig. 3.2. Dynamic interfacial tension of aqueous dispersions of solid tristearin particles (2.5 wt/wt%) in the absence and presence of 0.8 and 2 wt/wt% Tween 80 (A,B) and sodium caseinate (C,D) added during (open circle) or after particles' fabrication (solid circle). Similar concentrations of pure surfactant solutions are presented on the graph as a comparison. Samples were measured at least in duplicates and error bars represent ±1 standard deviation. When not visible, error bars are smaller than symbols.

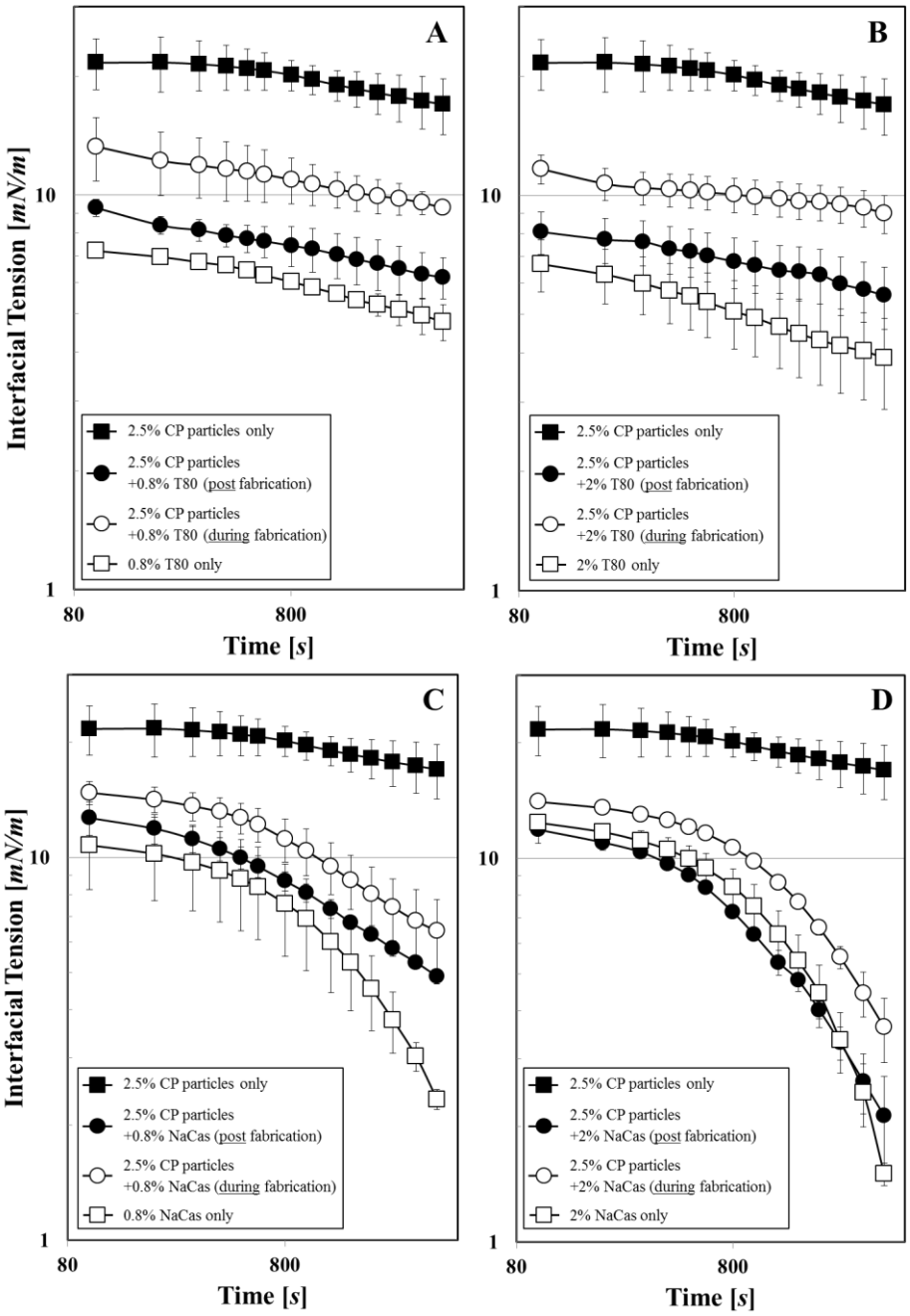

Fig. 3.3. Dynamic interfacial tension of aqueous dispersions of solid cetyl palmitate particles (2.5 wt/wt%) in the absence and presence of 0.8 and 2 wt/wt% Tween 80 (A,B) and sodium caseinate (C,D) added during (open circle) or after particles' fabrication (solid circle).

Overall, the trends generated for both lipid systems show that the composite particles and surface active species have an interfacial profile that falls within the range defined by the

individual entities, rather than in a cumulative effect. The type of the surface active component plays a significant role in the rate of interfacial tension reduction. Based on the discrepancies in the case where this component is added during or post fabrication of particles, trapping a proportion of these species during particle production course appears a strong likelihood. The possibility for the surface active species to participate in the crystal lattice or to be simply confined/trapped is further discussed in a later section.

3.3.3 Thermal behaviour

The crystallisation temperature along with the polymorphic transitions are parameters of critical importance in the production of solid lipid nanoparticles. Both of these factors are known to be influenced by surfactants, although this might be to a different level (Bunjes, *et al.*, 2002, 2003). To obtain a better insight on the influence of surface active species on the phase transitions and to investigate any potential correlations between the structure of these species and their effect on crystallisation and polymorphic transitions, DSC measurements were performed.

The melting and crystallisation behaviour of microcrystalline tristearin as bulk material and in the form of solid particles, is presented in Fig. 3.4. Comparing the melting curves (in terms of shape and enthalpies) of the lipid dispersions prepared with surface active components to the bulk lipid (Fig. 3.4, see Appendix A1, Table A1.1) allows conclusions to be drawn regarding the effect of the type of these components on the triglyceride's phase transitions.

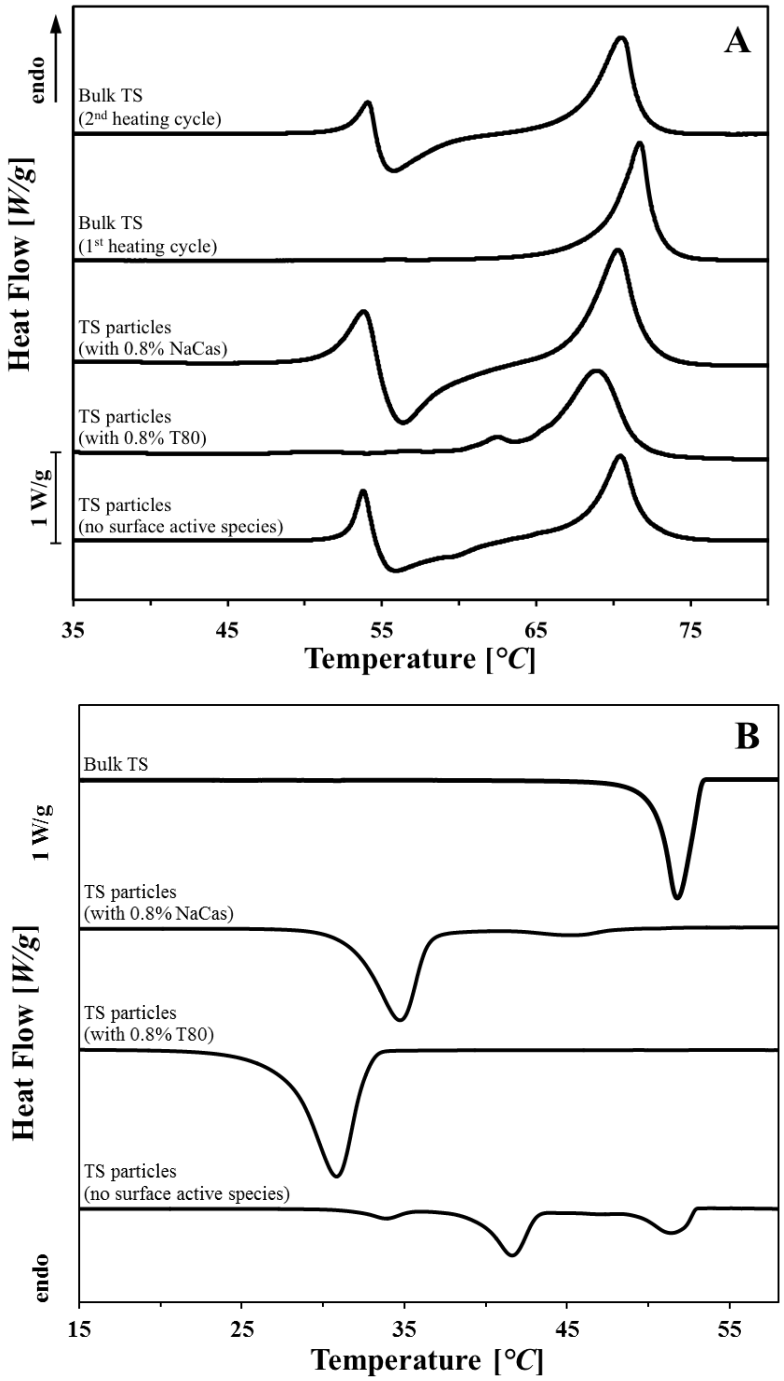

Fig. 3.4. DSC (A) melting and (B) crystallisation curves (scan rate 1.2 °C/min) of bulk tristearin (TS), of a 2.5% particulate tristearin dispersion without any surfactant and of 2.5% tristearin dispersion produced in the presence of 0.8% Tween 80 (T80) and 0.8% sodium caseinate (NaCas), measured after preparation of the dispersions. The curves are normalised for the sample weight and the amount of crystalline material present, and displaced along the ordinate for better visualisation.

The melting behaviour of the different polymorphs ascribed to monoacid saturated triglycerides is dictated not only by the triglyceride's inherent composition but also by the transformation kinetics. In the thermograph of the bulk material, heating of the crystalline tristearin (1st heating cycle) gives rise to the formation of the most stable polymorphic form (β) upon storage (Fig. 3.4A). Cooling the melt was followed by reheating at the same rate to 85 ºC (2nd heating cycle). Melting of the α-form takes place at ~54 ºC during the second heating cycle, and is followed by a broad exothermic phase transition indicating an $\alpha \rightarrow \beta$ transition process (re-crystallisation process) that extends till the onset of the β polymorph melting. The $\alpha \rightarrow \beta$ transition is essentially a complex process of nucleation and crystal growth rather than simply lipid molecules rearranging (Kellens, Meeussen, Gehrke, & Reynaers, 1991). The two phase transitions (melting of the α-form and re-crystallisation) occur almost concurrently due to their low stability (low energy state), and are represented by successive and overlapping endothermic and exothermic peaks as was also observed by Kellens and Reynaers (1992) in a study on tristearin's polymorphism. X-ray data have shown that the α-form transforms rapidly to a β-form without transformation to the intermediate β'-form, even at decreased heating rates (Kellens, *et al.*, 1991). Therefore, melting of the β-form is kinetically favoured and occurs at an onset temperature of ~64 ºC (with a maximum at 70.5 ºC). In essence, the differences between the first and the second heating cycle of bulk tristearin indicate that the lipid material was originally in the β crystal form and the small fraction of the α-modification resulted only from re-crystallisation following the melting of the β-form.

The thermal behaviour of fabricated tristearin particles within an aqueous medium was found to be almost identical to that of the bulk material. The two polymorphic α and β forms were present in the thermograph, as well as the exothermic re-crystallisation process, as shown for

the bulk tristearin. As demonstrated by Bunjes *et al.* in colloidal state triglycerides, there is a direct relationship between particle size and melting behaviour, regardless of the lipid component and the surface active agent's composition (Bunjes, Koch, & Westesen, 2000). Due to the high surface-to-volume ratio of (tri)glyceride particles, these will be expected to exhibit broader melting peaks together with lower melting temperatures (as compared to the bulk component), which eventually leads to a lower degree of crystallinity (decreased heat of fusion). The size-dependent melting behaviour of triglycerides has been investigated in previous studies based on a theoretical approach, as a basis to interpret the depression of the nanoparticles' melting temperature in comparison to the bulk phase (Siekmann, *et al.*, 1994). This dependency was also explored experimentally by Bunjes *et al.* via morphological studies (Freeze-Fractured Transmission Electron Microscopy (FF-TEM) in combination with DSC and X-Ray Diffraction (XRD)) revealing that the thickness of particles' molecular layered structure determines the exhibited melting patterns; platelet-like particles consist of different numbered molecular layers, each one of which melts within a specific temperature range (Bunjes, *et al.*, 2000). However, within the present study no such reduction was observed. This is more likely to have occurred as, unlike most of the studies in literature which are concerned with dispersions where a stabiliser is present, no surface active component participates in the current system. In conjunction with this absence, particle sizes are significantly larger (up to 7 μm) than the colloidal sizes that are usually investigated in other studies.

Regarding the influence of the surface active species on the liquid phase transitions, it is well documented that such components influence the polymorphism and crystallisation temperature of triglyceride nanoparticles (Aronhime, *et al.*, 1988; Bunjes, *et al.*, 2002, 2003), and these effects are very much related to the molecular structure of the surface active entity

(Bunjes, *et al.*, 2003). In the current study, the presence of a liquid polysorbate surfactant (Tween 80) had a significant influence on the shape of the DSC melting curve, compared to the neat tristearin. When Tween 80 was used in the fabrication of the tristearin particles, the onset and peak temperature as well as the shape of the liquid crystalline transition was altered (Fig. 3.4A). The Tween 80 molecular structure contains a fatty acyl moiety (primarily oleic acid) and as such, it can give rise to strong interactions with the triacylglycerol component in the tristearin dispersed phase. As reported by Helgason *et al.* these interactions can result to complex crystalline structures, as indicated by multiple melting events (Helgason, Awad, Kristbergsson, McClements, & Weiss, 2009b). For example, a comparison between high (i.e. Tween 60) and low-melting surfactants (i.e. Tween 80) in a study by Helgason *et al.* showed that the latter surfactants promoted the formation of β' and β (in detriment of α) stable crystals in tripalmitin solid lipid nanoparticle (SLN) suspensions (Helgason, *et al.*, 2009a). They have explained this behaviour by the ability of the tail layer of the high-melting surfactant to act as a template for nucleation and subsequent crystallisation of the lipid matrix within the droplets into the α-subcell crystal form.

The DSC melting profiles of solid tristearin particles produced in the presence of different concentrations of Tween 80 are presented in Fig. 3.5. Data from Lavigne *et al.* provide melting temperatures of bulk tristearin as obtained by DSC scans (Lavigne, Bourgaux, & Ollivon, 1993). The polymorphic transitions from that study were assigned to transitions observed in the spectra here, although slightly suppressed in the presence of Tween 80. Each of the peaks observed in the three bottom curves of Fig. 3.5 corresponds to a different polymorphic form of tristearin, as represented by the tabulated values (see Appendix A1, Table A1.3). On the basis of the above mentioned study, the focus of this work were the three polymorphic forms as identified by Lavigne *et al.* (Lavigne, *et al.*, 1993); as such, the first

peak at ~54 ºC corresponds to the α-polymorph, the peak at ~63.5 ºC to the β'-form and finally the peak at ~70 ºC can be assigned to the stable β-form. Based on this, the area under the curve for each of the distinct peaks was calculated, aiming to quantify the contribution of each of the polymorphs formed during the melting of tristearin particles produced using Tween 80. As can be clearly seen from Fig.3.5, the first and the second peak increase with increased concentration of Tween 80, at the expense of the third peak that decreases with an increase of the amount of Tween 80.

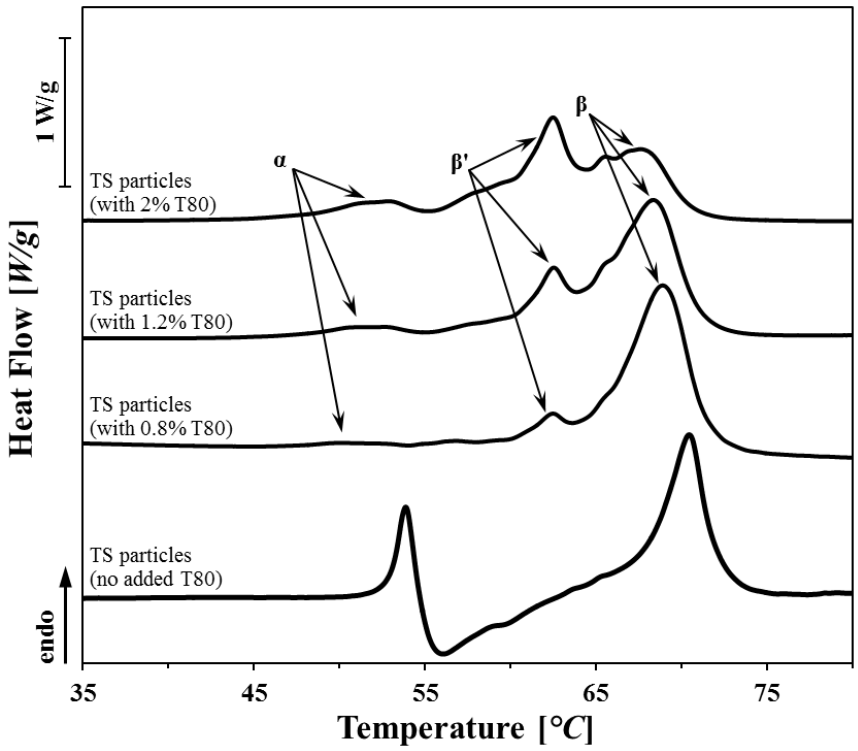

Fig. 3.5. DSC melting thermograms of dispersions with varying concentrations of Tween 80. The curve of the dispersion without surfactant is provided for comparison. All samples were melted from 20 to 85 ºC at 1.2 ºC/min. The lipid fraction is constant at 2.5 wt/wt%.

The similar chemical structure between the polysorbate and tristearin encourages molecule-molecule interactions at the interface in the liquid state. Consequently, upon crystallisation, a fraction of the surface active species' content (depending on its overall concentration) is expected to be entrapped within the crystallised lipidic matrix. The "foreign" surfactant entity

within the crystal structure restricts a tight packing of lipid molecules and thus, a higher triacylglycerols (TAG) ordering. Essentially, this results in enhanced β'-form stability to the detriment of the more thermodynamically stable β-form as the Tween 80 concentration is increased. This observation is in agreement with a study by Nik *et al.* who postulated that the structural affinity between Tween 20 and canola stearin (TAG) could lead to inclusion of the surfactant within the crystal structure at the interface (Nik, Langmaid, & Wright, 2012). This, in turn, prevents the mobility of the TAG molecules (transition to β-form), whereas it encourages the stability of the intermediate β'-form.

In the present formulations, a higher fraction of β-form crystals was detected in particles prepared with 0.8% Tween 80 when compared with the high fraction of α crystals in the 1.2% T80 formulation (see Appendix A1, Table A1.3). The 2% T80 sample evidenced the highest and the lowest fractions of the α and the β polymorphs respectively. A higher concentration of Tween 80 means that its alkyl tails are packed more tightly at the oil-water interface, giving rise to more rigid structures. The crystal structure becomes more complicated and the suspension exhibits more complex melting patterns as was previously reported by Helgason, *et al*. (2009b).

On the other hand, when molecular compatibility between the crystallised lipid matrix and the surface active component is absent, as in the case of sodium caseinate, the melting pattern resembles that of the bulk lipid material (2^{nd} heating cycle) (Fig. 3.4A). The fact that the β-polymorph corresponds to a larger area under the curve (as compared to the preceding exotherm) suggests that a fraction of crystalline matter was already in the most stable β-form prior to heating. This is in agreement with the findings in the study by Rosenblatt and Bunjes (2009) where a similar pattern was obtained from the melting of trimyristin nanoparticles stabilised with poly(vinyl alcohol) (PVA). It was postulated that PVA stabilises triglyceride

nanoparticles in the metastable α-modification due to its polymeric nature and subsequent steric hindrance effects. The increased viscosity or immobilisation of the molecules in the interfacial vicinity prompted by the presence of PVA, impedes conformational reorientation processes that are necessary for the $\alpha \rightarrow \beta$ transition. The behaviour is also consistent with the findings of Pawlik *et al.* who investigated whey protein isolate (WPI)-stabilised tripalmitin particles (Pawlik, *et al.*, 2016). The authors ascribed this performance to the interfacial positioning of WPI and proposed two mechanisms in support of it. The protein adsorbs and positions at the interface without penetrating the fat matrix and therefore, it exhibits no special effect on tripalmitin's polymorphic transformations. Alternatively, interfacially adsorbed WPI forms a viscoelastic film that arrests crystal movement, making the system behave similarly to crystalline particle dispersions constructed in the absence of surface active species.

In the present study, sodium caseinate also appears to have negligible effect on the time-course of tristearin's polymorphic transitions (Fig. 3.4A), suggesting that there is no "participation" of the protein molecule in the formed crystalline lattice. The protein might have been treated as an impurity (this is because its quantity is significantly small) which due to its large intrinsic size and non-compatibility with the fat molecules is excluded from the formed crystals. Consequently, it is likely trapped within the grain boundaries upon the cooling stage of the production process. The proposed mechanism is analogous to the phase behaviour of binary model systems, i.e. colloidal crystals, which has been studied to some extent (Nozawa, *et al.*, 2013; Yoshizawa, Okuzono, Koga, Taniji, & Yamanaka, 2011; Yoshizawa, Toyotama, Okuzono, & Yamanaka, 2014). In particular, Yoshizawa *et al.* reported that impurity particles were excluded from the crystals during grain growth and were swept away to the grain boundaries, owing to sizes and/or charge being different to the bulk (Yoshizawa, *et al.*, 2014).

DSC cooling runs on the bulk tristearin revealed a single exothermic peak at ~52.5 ºC (Fig. 3.4B) which corresponds to α crystals as triglycerides usually crystallise in the α-modification upon rapid cooling of the melt (Bunjes, Westesen, & Koch, 1996). The enthalpy of the re-crystallisation was 133 J/g which is very close to the value reported by Bunjes *et al.*, i.e. 124 J/g (Bunjes, *et al.*, 1996). The differences fall within the experimental error and they could also be a consequence of the much lower scanning rates in the current study. In addition, the fact that the values of crystallisation enthalpy are in all cases lower than respective melting values, suggests melting and crystallisation processes could be occurring on different polymorphic forms. In regards to the surface active species-free dispersion, it is expected to behave similarly to the bulk lipid since the precedent melting (in the DSC) leads to coalescence of the lipid droplets and phase separation, and hence crystallisation in a bulk fat manner (Pawlik, *et al.*, 2016). The polymodal peaks observed in the bottom curve of Fig. 3.4B probably originate from the different particle size populations contained in this dispersion, rather than distinct polymorphic forms. However, the analogous behaviour is evidenced by the same crystallisation enthalpy values (see Appendix A1, Table A1.1).

The crystallisation thermographs of the tristearin particles in the presence of surface active entities all evidence increased supercooling (retarded crystallisation/hysteresis between heating and cooling curves). The onset and peak temperatures of crystallisation for both surface active components are considerably lower (approximately 15-20 ºC) than that of the bulk tristearin. The enhanced supercooling tendency is common for triglyceride systems in the colloidal state, as has been reported previously. Pronounced supercooling that can reach temperatures even 20 ºC lower than that of the bulk material is required for nucleation to occur in lipid dispersions (Bunjes, *et al.*, 1996; Siekmann, *et al.*, 1994). Re-crystallisation for tristearin particles fabricated using Tween 80 and NaCas occurs at 31.3 and 35.3 ºC

respectively. This temperature difference cannot be explained by size differences since tristearin particles in the presence of this concentration of Tween 80 were substantially larger than the ones that were formed with NaCas (Table 3.1), and hence would require less pronounced supercooling. The trend is the same for higher contents of Tween 80 and NaCas (see Appendix A1, Table A1.2). The reason for this induced crystallisation in the case of tristearin particles produced using sodium caseinate–which takes place independent of surface actives species content – is not yet clear, or at least it cannot be explained based on particle size data. Additionally, the crystallisation enthalpy value for particles formed with Tween 80 is higher than the neat tristearin and the particles formed with NaCas because of the higher melting polymorphs (e.g. β') formed during the melting as discussed above.

The thermal behaviour of the fabricated wax-based particles was also studied, and in this case the occurrence of polymorphism was clearly suppressed in comparison to the tristearin systems due to their different intrinsic composition (triglyceride crystals exist in hexagonal, orthorhombic and triclinic arrangements (α, β′ and β forms respectively) while waxes mainly exist in the orthorhombic) (Jenning, *et al.*, 2000). Bulk cetyl palmitate revealed two melting peaks, one at 44 ºC and one at 51.5 ºC (Fig. 3.6A) which are in agreement with the values reported by Uracha *et al.* and Teeranachaideekul *et al.* (Teeranachaideekul, Souto, Junyaprasert, & Müller, 2007; Uracha, *et al.*, 2008). These peaks can be attributed to a metastable low melting α and a stable higher melting β polymorphic form. In the presence of either surface active agents, the spectra obtained are very similar to the tristearin systems; hence, congruent conclusions regarding the influence of each agent on the thermal profile of the wax colloidal particles can be drawn. Against this background, wax particles formed with the use of NaCas, behaved as a surface active species-free dispersion. However, when Tween 80 was used in the production of particles, participation of the molecule in the crystal lattice

through specific interactions slowed down polymorphic transitions. In fact, as seen before for tristearin particles and Tween 80, higher concentrations of the surface active entity led to a gradual increase of the α-polymorph fraction to the detriment of the β-form which decreases with increasing Tween 80 content (data not shown). The peak at 43.3 ºC beside the melting peak of the α crystals has previously been attributed to a thermodynamically unstable modification, though the authors did not provide further details (Saupe, Wissing, Lenk, Schmidt, & Müller, 2005).

The melting enthalpy values for particles formed with either Tween 80 or NaCas are not very far from those of the dispersion without the surface active species addition, with all of them somewhat lower than the values for the bulk lipid (see Appendix A1, Table A1.4). Assuming that crystallinity is 100% for the bulk cetyl palmitate, the decrease in crystallinity for the dispersions is within the range of 14-21% which in turn, means less ordered structures. Unlike the wax-comprised solid particles, tristearin particles in the presence of Tween 80 for example, exhibited a melting enthalpy akin to the pure lipid material (see Appendix A1, Table A1.1), which could imply a non-significant loss of its crystallinity.

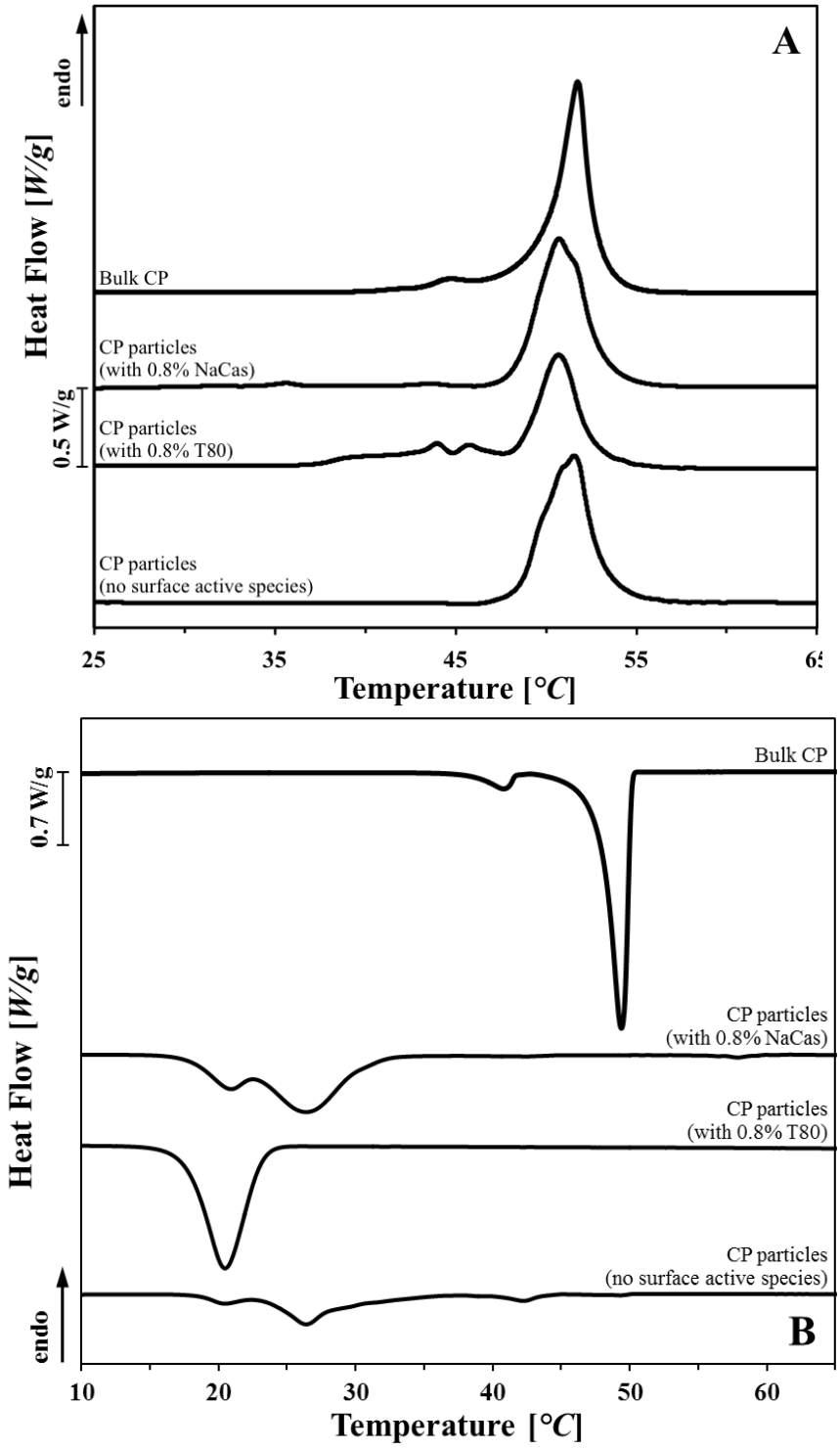

Fig. 3.6. DSC (A) melting and (B) crystallisation curves (scan rate 1.2 ºC/min) of bulk cetyl palmitate (CP), of a 2.5% particulate CP dispersion formed in the absence of surface active species and of 2.5% CP dispersion formed using 0.8% Tween 80 (T80) and 0.8% sodium caseinate (NaCas), measured after preparation of the dispersions.

During crystallisation, bulk cetyl palmitate showed two exothermic peaks, ascribed to α and β crystals. For the wax dispersions, it was anticipated that the original size range ("fresh" crystallised particles) has been preserved which is why a level of supercooling is exhibited across all dispersions (Fig. 3.6B). A level of coalescence would actually be expected in the case of particles formed in the absence of surface active components, although this was not demonstrated. The larger sizes of the particles produced with NaCas (Table 3.1) required a lower degree of supercooling (onset crystallisation temperature 31.3 ºC) juxtaposed to those with Tween 80 that were considerably smaller and their crystallisation event had an onset temperature of 23.1 ºC. In addition to this, the bimodality of the crystallisation peak of the former particles could stem from their wider particle size distribution (Fig. 3.1D) as opposed to a very limited breadth of distribution for particles produced with Tween 80 (Fig. 3.1C).

Upon further cold storage at refrigeration temperatures (~4 ºC) of the lipid particle dispersions (up to 9 months), only small changes were detected in the enthalpy values of the α-endotherm, the β-endotherm as well as the exotherm (see Appendix A1, Table A1.5). Furthermore, in tristearin particles formed with surface active species that do not participate in the crystal lattice (i.e. NaCas), the α-polymorph was stable for prolonged storage time. This enhanced stability of the α-modification by means of the presence of NaCas is akin to the behaviour observed in a previous study where PVA was used as the emulsifier of triglyceride (i.e. trimyristin and tristearin) nanoparticles (Rosenblatt, *et al.*, 2009). The systems were stored for 9 months (refrigerator temperatures) and this high stability was found to be a function of storage conditions, since storage at higher temperatures (but below the melting point of the α-form) provoked transformation into the β-modification, accompanied by an increase in particle size. The authors provided a tentative explanation, suggesting an impact of PVA on

triglyceride crystal growth in a way that the polymer favours the formation of a less ordered structure.

In the current study, the persistence of thermal behaviour over long times suggests that any potential Pickering functionality attributed to microstructural characteristics is also expected to be maintained over similarly extended periods of storage.

3.4 Conclusions

Building on the previously published work on the formation and properties of solid lipid nanoparticles (Mehnert, *et al.*, 2001; Saupe, Gordon, & Rades, 2006; Uner, 2006), this study has demonstrated that triglyceride and wax particles can be produced to exhibit tailorable microstructural attributes, established as drivers of Pickering functionality. Particulates of micron or nano- dimensions were constructed from lipid sources with distinct melting and crystallisation temperatures and in the presence of different surface active entities, following a melt-emulsification method. Previous reports on emulsion stabilisation using lipid particles have studied the formation of fat crystals during the emulsification process (Frasch-Melnik, Spyropoulos, & Norton, 2010; Garti, Binyamin, & Aserin, 1998), leading to limitations in terms of controlling particle characteristics. Nonetheless, the route employed within this study separates the two processes, emulsification followed by crystallisation, allowing well-controlled and uniform experimental conditions to be established.

It was shown that the importance of the type of surface active species selected in regards to the performance of the generated structures was twofold: a) depending on its size, an improved stability layer against inter-particle interactions and therefore aggregation, could be endowed (e.g. NaCas) and b) depending on its chemistry, interactions could be developed with the crystalline matrix resulting in a strong effect on lipid particles' phase transitions and

polymorphism not only upon production, but also after several months of storage. While the assignment of polymorphic forms in the fabricated crystals was not the aim of this study, the obtained modifications would certainly need to be correlated with XRD data. Polymorphic forms are particularly important should the manufactured structures provide housing for active compounds and control their subsequent release (Rosenblatt, *et al.*, 2009) in potential future applications.

Previous work has shown that by using a variety of different size and chemistry surfactants during tripalmitin particles' fabrication process, the particles' polarities and therefore their tendency to form o/w or w/o emulsions could be manipulated (Pawlik, *et al.*, 2016). Yet, an insight into particles' interfacial properties has not been provided. In the present study, tensiometry was proved to be an invaluable tool for studying the different interfacial dynamics caused by the presence of Tween 80 and NaCas. Interfacial behaviour was shown to be heavily dominated by the type and concentration of the surface active species used in the investigated systems. It also enabled the gaining of a better understanding of the amount of interfacially active component that remains associated with the formed particles rather than "free" in the continuous phase.

Emulsion production is under way to assess the fabricated lipid particles' potential to act as Pickering-type stabilisers. Although this study was concerned with a model wax, it can be extended to include waxes more relevant to the food industry, such as rice bran, carnauba or candelilla (Toublan, 2014). Despite demonstrating that functionality exists even at minimum concentrations of surface active entities, it would certainly be interesting from an industrial perspective to fabricate particles without any surfactant that could be used as encapsulation vehicles and other purposes such as taste masking or delivery of nutrients. Additionally, the approach that was described in this study could be used to develop emulsions with a tailored

melting profile or emulsions that are temperature responsive, and to extend the pool of components that have been investigated so far (e.g. microgels (Richtering, 2012)) to also include food-grade stimuli-responsive agents. Systems that are responsive to different triggers such as pH could also be developed following this approach, which would render it more promising for food applications.

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Chapter 4

Oil-in-water emulsions stabilised by solid lipid particles

Data and discussions contained within this chapter have been submitted for publication within:

Zafeiri, I., Smith, P., Norton, I.T. and Spyropoulos, F. (2017). Fabrication, characterisation and stability of oil-in-water emulsions stabilised by solid lipid particles: The role of particle characteristics and emulsion microstructure upon Pickering functionality. *Food and Function*.

Synopsis

The quest to identify and use bio-based particles with a Pickering stabilisation potential for food applications has lately been particularly substantial and includes, among other candidates, lipid-based particles. The present study investigates the ability of solid lipid particles to stabilise oil-in-water (o/w) emulsions against coalescence. Results obtained showed that emulsion stability could be achieved when low amounts (0.8 wt/wt%) of a surface active species (e.g. Tween 80 or NaCas) were used in particles' fabrication. Triple staining of the o/w emulsions enabled the visualisation of emulsion droplets' surface via confocal microscopy. This disclosed an interfacial location of the lipid particles, hence confirming stabilisation via a Pickering mechanism. Emulsion droplet size was controlled by varying several formulation parameters, such as the type of the lipid and surface active component, the processing route and the polarity of the dispersed phase. Differential scanning calorimetry (DSC) was employed as the analytical tool to quantify the amount of crystalline material available to stabilise the emulsion droplets at different intervals during the experimental timeframe. Dissolution of lipid particles in the oil phase was observed and evolved distinctly between a wax and a triglyceride, and in the presence of a non-ionic surfactant and a protein. Yet, this behaviour did not result in emulsion destabilisation. Moreover, emulsion's thermal stability was found to be determined by the behaviour of lipid particles under temperature effects.

4.1 Introduction

Emulsions refer to bi- or multiphasic systems that consist of two immiscible liquids (typically oil and water) where one is dispersed into the other in the form of small spherical droplets. The list of industrial applications for emulsions is extensive and includes foods, personal care products and cosmetics, pharmaceuticals, agrochemicals as well as crude oil. Given the importance of emulsions, a great deal of research has been directed towards ways to overcome the energy barrier between the state of the high (unstable) and low (stable) free energy, and impart kinetic stability (i.e. for months) to the thermodynamically unstable system. Traditional approaches for providing stabilisation for a period of time have been relying on addition of substances known as emulsifiers. These are amphipathic molecules that are able to adsorb at newly formed o/w interfaces within an emulsification process. By means of formation of a protective interfacial layer, emulsifiers assist in maintaining a sufficient distance between emulsion droplets, thereby hindering/preventing their aggregation/coalescence. Typical emulsifiers are low molecular weight surfactants, phospholipids and also macromolecular species such as proteins, and polysaccharides (McClements, 2016).

Despite their established effectiveness, there is a growing shift in the food industry towards the – partial or full – replacement of emulsifiers in emulsion-based products by –at least perceived to be as – natural, and more sustainable ingredients (Morris, 2001; French, *et al*., 2016). This is in line with the drive for "clean-label" food products fuelled by the growing consumers' demands for healthy, safe and at the same time as natural as possible foodstuffs (Odriozola-Serrano, Oms-Oliu, & Martín-Belloso, 2014). The most appealing candidates are undoubtedly colloidal particles which owing to their insurmountable features, they have

attracted a significant surge of interest among physical scientists, both academically and industrially. Emulsion droplets stabilised by colloidal particles (Pickering emulsions) combine the benefits of prolonged stability against coalescence (Dickinson, 2010; Pichot, Spyropoulos, & Norton, 2009), improvement in lipid oxidative stability (Berton-Carabin, Ropers, & Genot, 2014; Kargar, Fayazmanesh, Alavi, Spyropoulos, & Norton, 2012), increased resistance to shear (Lee, Niknafs, Hancocks, & Norton, 2013; Niknafs, 2011) and controlled release across the interface (Frasch-Melnik, Norton, & Spyropoulos, 2010). It is important to note that the aforementioned enhanced functionality can be seen in the absence to very low concentrations of synthetic surfactants (Chapter 3).

Fat crystals (e.g. triacylglycerols (TAGs), etc.) are a significant class of solid particles that confer stability in a number of everyday food products such as margarine, whipped cream and ice cream. It has been proposed that their stabilisation mechanism is based on either the interfacial adsorption of previously formed crystals and/or originate from a three-dimensional particle network formed in the continuous phase (Ghosh, Tran, & Rousseau, 2011; Rousseau, 2013). Fat crystals with tailored properties have also been investigated for their potential to stabilise emulsions, although relevant work in the literature is relatively limited. In particular, there is a dearth of studies centered on production of lipid-based particles where emulsification and crystallisation take place as separate processes. Garti, Binyamin, and Aserin (1998) prepared sub-micron α-polymorph tristearin crystals via flash-crystallisation within a liquid oil, and then used these dispersions as the continuous phase of w/o emulsions. The obtained emulsions were prone to aggregation and flocculation, and the authors showed that sufficient amounts of an emulsifier were necessary to trigger adsorption of particles to the interface. More recent work by Pawlik, Kurukji, Norton, and Spyropoulos (2016) demonstrated a preference of solid tripalmitin particles to form oil or water continuous

Pickering emulsions depending on the chemistry of the emulsifiers used during particle fabrication. The aqueous lipid dispersions were produced by melt-emulsification followed by crystallisation, and subsequent use as the continuous phase of emulsions. The emulsions were stable to coalescence for a storage period of 70 days.

An important point is that the amount of solid matter that provides stabilisation needs to be maintained over time if these structures are to be utilised commercially. Evidence from previous studies showed that within an emulsion, transfer of lipids between liquid droplets is taking place and this mechanism hinges on the type of lipid molecules (e.g. size and polarity) and the presence of surfactant micelles and co-solutes in the aqueous phase (J.N. Coupland, Brathwaite, Fairley, & McClements, 1997; J.N. Coupland, Weiss, Lovy, & McClements, 1996; Weiss, Coupland, Brathwaite, & McClements, 1997; Weiss & McClements, 2000). Specifically for solid lipid nanoparticles (SLN), the addition of liquid oil to the aqueous suspension of SLN triggers mass transfer phenomena that result in the gradual dissolution of the particles over the course of storage time (Samtlebe, Yucel, Weiss, & Coupland, 2012). The authors of that work have looked at the stability of solid lipid nanoparticles in the presence of pure liquid oils (alkanes) and suggested a dissolution mechanism which embodies solubilisation of lipid molecules from the oil droplets into the aqueous phase, diffusion and subsequent adsorption at the SLN surface, and finally SLN dissolution in the adsorbed liquid surface layer. It needs to be stressed that SLN in that case were not in contact with the emulsion droplets.

In Chapter 3, it was demonstrated that lipid particles, depending on the lipid source and the type of the surface active species used in their manufacturing, can be designed to own microstructural characteristics that are closely linked to Pickering functionality; i.e. size and interfacial behaviour. The aim of the present study is to fully assess the potential of these

solid lipid particles to act as Pickering stabilisers of oil-in-water (o/w) emulsions. Particles were prepared from a wax (cetyl palmitate) and a triglyceride (tristearin) source and were fabricated in the presence of Tween 80 or sodium caseinate (NaCas). Emulsion formation and subsequent stability were investigated as a function of the former formulation variables. Different dispersed phase mass fractions and production methods of varying shear levels were assessed. Emulsions produced in the presence of solid lipid particles were characterised by laser diffraction and confocal microscopy, both upon fabrication and following varying periods of storage. A specific attempt was made to link the level of solid matter and emulsion stability with the resulting microstructure over time, or as a result of temperature conditions, as well as type of the dispersed phase.

4.2 Materials and Methods

4.2.1 Materials

Lipid particles were prepared using microcrystalline glyceryl tristearate (tristearin) (IOI Oleochemicals GmbH, Hamburg, Germany) and cetyl palmitate (Gattefossé, France). The particles were formed in the presence of two surface active species: polyoxyethylene sorbitan monooleate (Tween 80) and casein sodium salt (NaCas) derived from bovine milk (Sigma-Aldrich, UK).

For emulsions production, commercially available sunflower oil was used without further purification. In addition, pure alkanes *n*-dodecane and *n*-hexadecane (Sigma-Aldrich, UK) were also used for comparative purposes. Double distilled water from Milli-Q systems (Millipore, Watford, UK) was used throughout.

4.2.2 Methods

4.2.2.1 Preparation of oil-in-water emulsions

Oil-in-water (o/w) emulsions were prepared with a 80% (wt/wt) aqueous phase containing solid lipid particles and a 20% (wt/wt) sunflower oil phase. Lipid particle suspensions (tristearin, cetyl palmitate) were first prepared via an emulsion route; more specifically, melt-emulsification was carried out at temperatures above the lipids' melting points followed by quench cooling to form crystalline particles (for further details see Chapter 3). Systems were then stored at 4 ºC until further analysis.

Emulsions were fabricated using two emulsification techniques at varying energy inputs. In particular, emulsions were produced using a high-shear mixer (Silverson L5M, Silverson Machines Ltd, UK) with an emulsion screen of 19 mm diameter for 2 minutes at 9000 rpm, as well as an ultrasound generator for 30 seconds at 95% ultrasonic amplitude (i.e. acoustic power) (Sonics & Materials, Inc., CT, USA). During both processes to avoid shear-induced heating of the sample, emulsification was carried out whilst the vessel was immersed in an ice bath. Phase inversion was tested by simple dilution to confirm that all emulsions remained water continuous. The resultant o/w emulsions were analysed directly post production or were stored at 4 ºC until further analysis.

4.2.2.2 Droplet size analysis

Stability against coalescence as a function of time was assessed by optical observation and droplet size measurements. Droplet size distributions for o/w emulsions were analysed by laser diffraction using a Malvern Mastersizer 2000S (Malvern Instruments, UK) equipped with a Hydro S dispersion cell. A refractive index of 1.47 (RI of the sunflower oil dispersed

phase) was used for the calculation of the droplet size distribution. Samples were analysed immediately and after 7, 14 and 30 days following emulsion preparation.

4.2.2.3 Imaging emulsions microstructure by confocal laser light scanning microscopy (CLSM)

Pickering performance (i.e. the location of the particles) was assessed visually using microscopy. Emulsion droplets were visualised at room temperature using a confocal laser light microscope, (Leica TCS SPE (Heidelberg, Germany)), equipped with laser operating at a wavelength of 532 nm. The various emulsion components were individually stained at various times throughout production. Rhodamine B (0.01 wt/wt %) was added to the aqueous phase containing surface active species before mixing with the melted lipid. The solid lipid component was stained through addition of perylene (0.01 wt/wt%) to the hot o/w emulsion following ultrasonication, prior to cooling. To image the oil phase Nile Red (0.01 wt/wt%) was added to the sunflower oil prior to emulsification. All the stains used were purchased from Sigma-Aldrich (Sigma-Aldrich, UK).

4.2.2.4 DSC measurements

The melting properties of the emulsions were monitored at regular intervals by differential scanning calorimetry using a high-sensitivity Setaram μDSC7 evo microcalorimeter (Setaram Instrumentation, France). A system equilibrium was achieved (20 ºC) for 15 min prior to a heating ramp of 1 ºC/min until a maximum temperature of 90 ºC was reached. Within this temperature range the oil phase does not undergo any transition, thus, the endothermic peaks observed correspond to the melting of lipid crystalline particles present in the o/w emulsions.

4.2.2.5 Thermal stability of emulsions

Adsorption of the lipid particles to the interface was studied by heating o/w emulsions (20 wt/wt%) stabilised using cetyl palmitate particles (2.5 wt/wt%) formed in the presence of sodium caseinate (0.8 wt/wt%). DSC thermographs for the melting cycles presented in Chapter 3, showed melting events to commence at ca. 48.2 ± 0.2 ºC with peak temperatures close to 50.7 ± 0.1 ºC. A cross comparison between emulsion droplet sizes at temperatures both significantly lower and higher than the particle's melting point provides a better insight to the stabilising properties of the wax particles. As such, emulsions were placed in an oven (Advantage Lab, Belgium) at two preconditioned temperatures; 36 and 58 ºC for 1 and 5 days. Changes in droplet size were monitored via laser diffraction measurements. Sodium azide (Sigma Aldrich, UK) was added to all systems at a concentration of 0.001% (wt/wt) to prevent microbiological growth.

4.3 Results and Discussion

Emulsification was investigated in the presence of solid lipid particles formed with different surface active species (Tween 80 and NaCas) and o/w emulsions were prepared with sunflower oil as the dispersed phase. Emulsions were formed by using an ultrasonic probe and by a conventional high-shear mixing process. Preliminary studies showed that tristearin and cetyl palmitate particles fabricated in the absence of surface active species could not produce stable o/w emulsions, on one hand due to their inherent hydrophobic character and on the other, due to their large particle sizes. As such, the emulsions assessed all contained lipid particles (2.5 wt/wt% of the aqueous phase) formed with either Tween 80 or NaCas (both at 0.8 wt/wt% of the aqueous phase). The parameters studied were the lipid type that the

particles were composed of, the type of surface active agents used in the production of the particles, as well as the mass fraction of sunflower oil.

4.3.1 Effect of formulation parameters on emulsion droplet size

The effect of the presence of particles formed with a different surface active component on the emulsion's droplet size and as a function of the oil's fraction is shown in Fig. 4.1. This was measured directly after emulsification, and also after two months of quiescent storage at refrigeration temperatures.

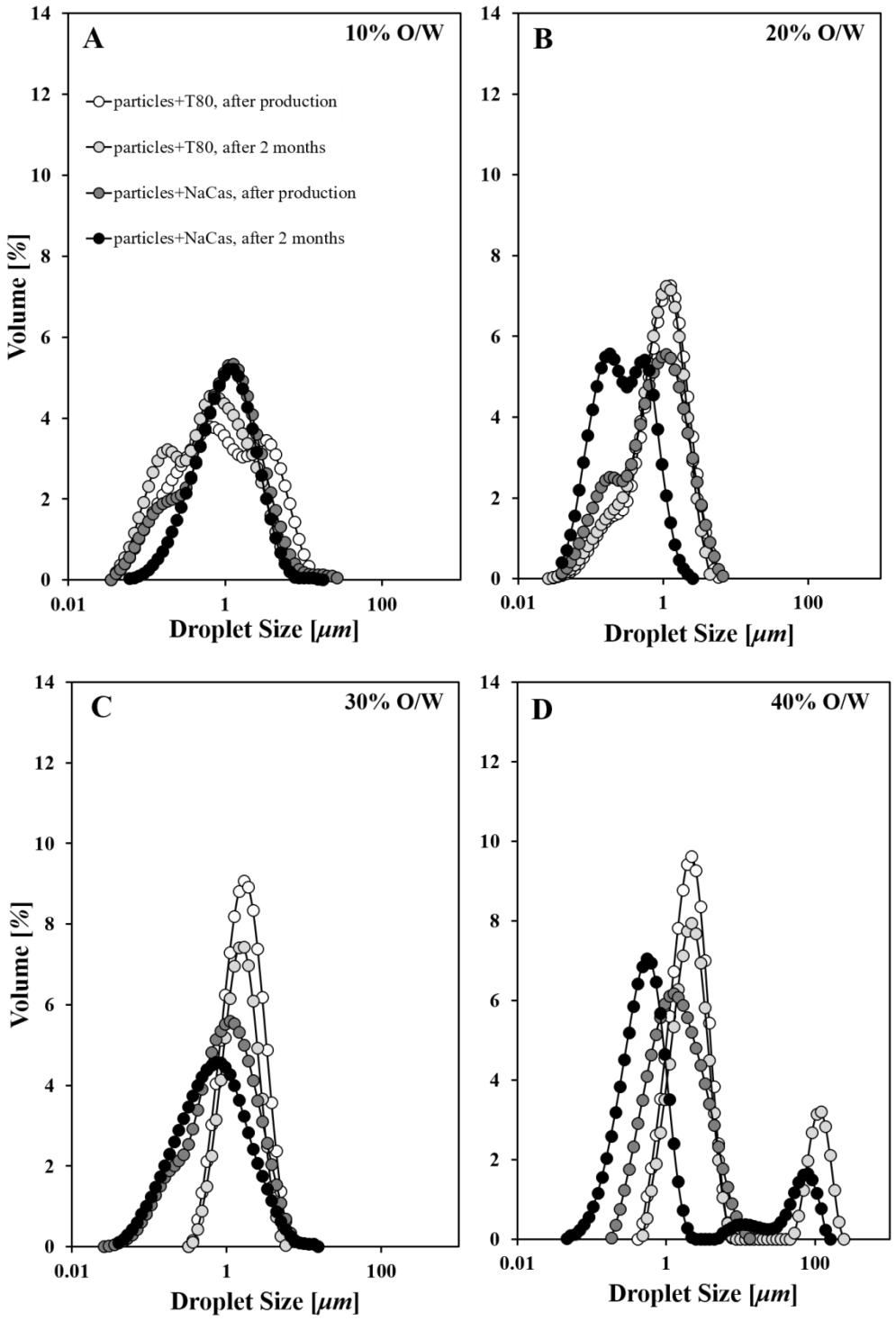

Fig. 4.1. Variations of emulsion droplet size as a function of dispersed phase mass fraction and different surface active species used in the preparation of solid wax particles. Cetyl palmitate and surface active species were used at a constant concentration of 2.5 and 0.8 wt/wt% of the aqueous phase respectively across all samples.

It can be seen that the average droplet size ($D_{4,3}$) of all emulsions after ultrasound emulsification was ~1-2 μm. Emulsion droplet size increased slightly with an increase in the internal phase mass fraction for lipid particles containing either of the two surface active species. It is anticipated that at a constant surface active component-to-oil ratio, an increase in the oil mass fraction engenders an enhanced rate of collisions and coalescence, with the coalescence rate increasing much faster than the rate of droplet break-up (Hakansson, 2015). This is common place for emulsions where Tween 80 or NaCas have been used as the sole emulsifiers and/or stabilisers (Sun & Gunasekaran, 2009).

Additionally, when particles were formed with sodium caseinate the resulting emulsion had smaller average droplet sizes than when Tween 80 was used, and were also more stable over time, particularly for intermediate oil mass fractions (i.e. 20 and 30%) (Fig. 4.1B, 4.1C). This droplet size discrepancy is likely to originate from the lower levels of interfacial tension induced by Tween 80. This reduction in interfacial tension during initial droplet formation results in an increased number of small emulsion droplets (within a fixed volume) which, in turn, increased the probability of droplet-droplet collisions (during processing) (Liu, Yang, & Yu, 2011). Further, potential differences in the thickness of the interfacial film that particles with Tween 80 or NaCas would provide, could be accounted for the droplet size disparity observed; enhanced steric properties caused by the thicker protein-stabilised interface would form a barrier to droplet coalescence (Berton-Carabin, *et al.*, 2014). Another factor that needs to be considered is the amount of free Tween 80 or NaCas (i.e. not associated with the lipid particles) in the original particle dispersions, which could also be having an impact, e.g. acting to adsorb both to the oil droplet and wax particle interface. In all cases, it has to be considered that during sonication, there's always the risk of jeopardising the integrity of solid matter due to local temperature increase.

Upon ageing, droplet coalescence was observed only for the highest dispersed phase content (i.e. 40%) with a population appearing at around 100 μm for emulsions stabilised by lipid particles containing either surface active species. It is proposed here that at an oil mass fraction of 40%, instability becomes more prominent as a result of the loss of particulate matter, the mechanism of which will be the focus of a latter section. An enhanced desorption of solid lipid particles from the interface or dissolution is probably taking place, which leads to droplets having their surfaces devoid of stabilising material, therefore prone to inter-droplet contacts and subsequent coalescence. Similar observations were made by Gupta and Rousseau (2012) that used emulsion surface charge measurements and TEM images to explain the destabilisation process occurring in SLN-decorated o/w emulsions. A significant drop of 10 mV (emulsion surface charge) was recorded by 24 weeks, and non-covered emulsion interfaces together with SLN in the continuous phase were depicted in the micrographs, providing evidence of the gradual destabilisation of the emulsion.

4.3.2 Effect of processing method on emulsion behaviour

Emulsions were also produced using a conventional processing method that provides a lower level of shear in comparison to sonication, e.g. high-shear homogenisation. A study investigating differences between emulsions formed in the presence of cetyl palmitate or tristearin particles fabricated with low amounts of sodium caseinate was conducted. There was no control over instability due to creaming and all the obtained emulsions creamed within minutes following production. However, there was no oil layer observed with time; this would have indicated the occurrence of coalescence phenomena. The mean droplet sizes of these systems are illustrated in Fig. 4.2 for different storage times (at 4 ºC).

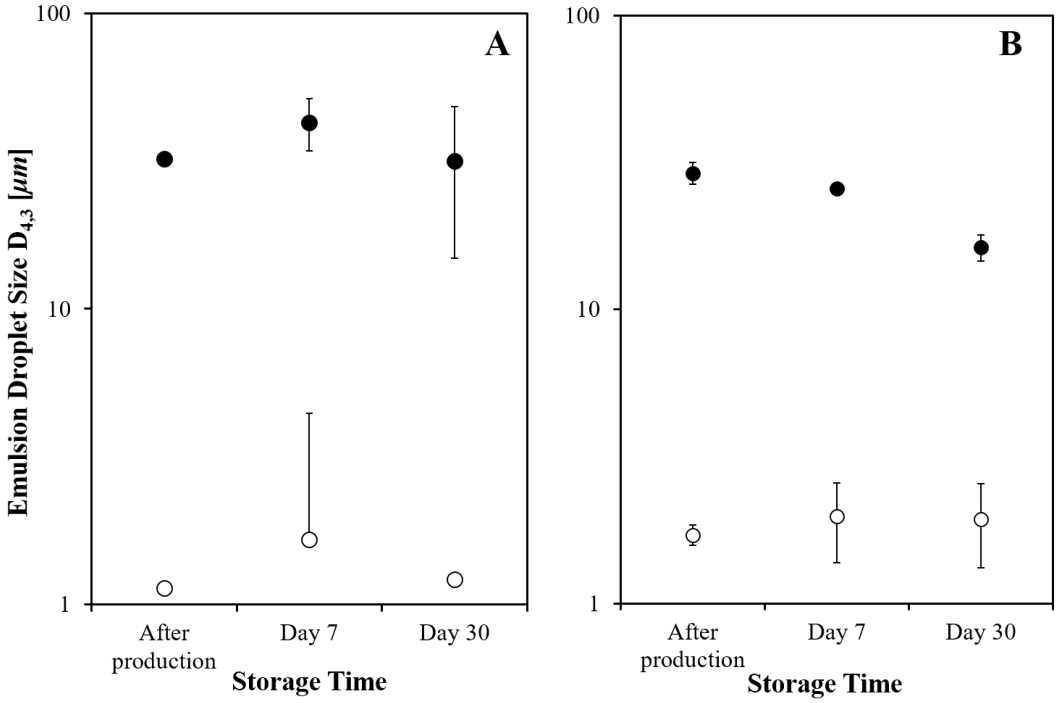

Fig. 4.2. Volume-weighted mean droplet diameters ($D_{4,3}$, μm) of 20% o/w emulsions formed with wax (A) and triglyceride (B) particles with 0.8% sodium caseinate, using different processing conditions, measured immediately after preparation and after 7 and 30 days. Closed and open circle symbols refer to emulsions prepared by high-shear mixer and ultrasound respectively. Where not visible error bars are smaller than the symbols.

It is evident from Fig. 4.2 that the use of ultrasound generates emulsions with droplets of about an order of magnitude lower than the ones produced via a high-shear device. It is known that ultrasound, due to cavitational collapse, promotes an enhanced droplet break-up, enabling the eventual formation of very fine/nano droplets (Canselier, Delmas, Wilhelm, & Abismaïl, 2002; Kentish, *et al.*, 2008; Leong, Wooster, Kentish, & Ashokkumar, 2009). Moreover, the smaller sized emulsions were also more stable over the investigated time, yet this is also true for the emulsions with cetyl palmitate particles produced with a high-shear homogeniser (Fig. 4.2A). The initial droplet size does not appear to depend on the lipid source of the particles, however the long-term emulsion stability was more sensitive to that parameter. The $D_{4,3}$ value of the emulsions with tristearin particles and NaCas manufactured

by a high-shear mixer, drops from 29.1 to 16.2 μm following one month storage. The relatively broad distribution was composed of two populations, one at ~1 μm and a larger one at ~23 μm (see Appendix A2, Fig. A2.1). The smaller peak is likely to correspond to lipid particles that are not associated with the oil droplets ("free"). Over time, this peak appears to grow at the expense of the bigger one, potentially pointing to increased desorption of particles from the interface. This shift in the distribution is what also causes the subtle drop in $D_{4,3}$ values as seen in Fig. 4.2B. In regards to emulsions produced in the presence of cetyl palmitate particles formed with Tween 80, emulsification was not possible under high-shear mixing and a system containing a cream and a foam layer was obtained. Foaming is common during the operation of high-shear mixers and protein molecules, being surface active, compete for both interfaces (oil/water and air/water) which might have affected emulsion droplet stabilisation.

In order to confirm that emulsion stabilisation was imparted by the lipid particles rather than the surface active agents used for their fabrication, emulsion's microstructure was visualised. Most commonly, cryo-SEM has been used for visualisation of emulsion microstructure and for lipid crystals residing at the interface in particular (Frasch-Melnik, Spyropoulos, & Norton, 2010; Kurukji, Pichot, Spyropoulos, & Norton, 2013; Pawlik, *et al.*, 2016). In addition, confocal laser light microscopy (Heisler, Oehlke, Greiner, & Steffen-Heins, 2013) and TEM (Gupta, *et al.*, 2012) have been employed for investigation of the ultra-structure of o/w emulsions decorated by solid lipid nanoparticles.

Fig. 4.3A compares droplet size distributions of emulsions prepared with particles and sodium caseinate and those ones stabilised by protein only. Fig. 4.3B displays a CLSM image of a 20% o/w emulsion prepared in the presence of the same species and produced with a high shear mixer, taken within two hours after its production.

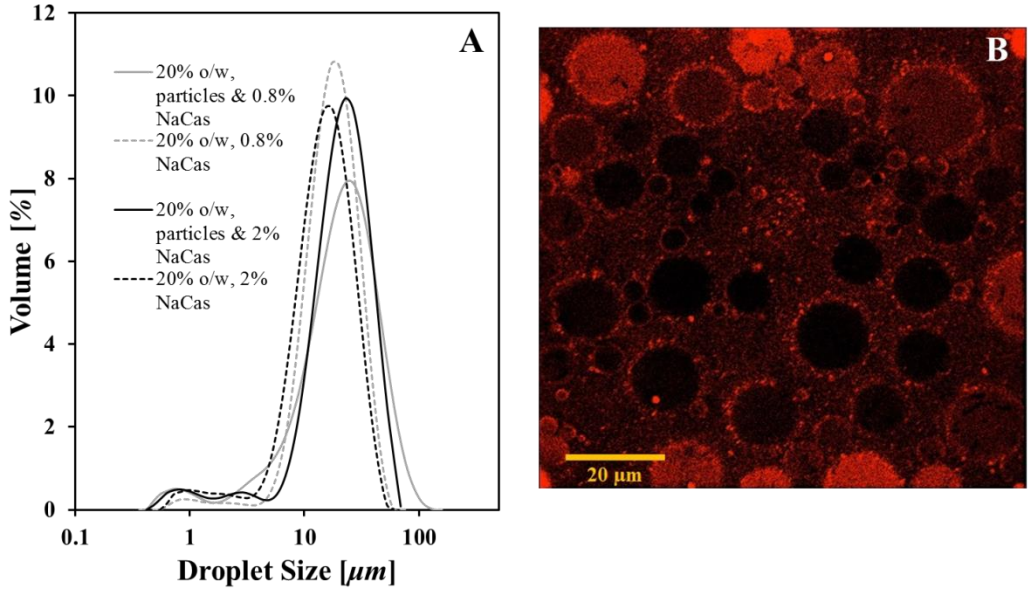

Fig. 4.3. Droplet size distribution of 20% sunflower-in-water emulsions containing solid wax nanoparticles formed in the presence of sodium caseinate (0.8, 2%) or stabilised by the same concentration of neat sodium caseinate (A). Measurements were conducted immediately after production. Representative confocal image of the emulsion formed with wax particles and NaCas (B). The micrograph was taken within 2 hours following emulsion production.

It can be seen from Fig. 4.3A that the droplet size of lipid particle-stabilised emulsions is not significantly different to protein-stabilised systems. Any shift can be attributed to the different level of interfacial tension reduction that each of the species provides. A closer view to emulsions' microstructure obtained via confocal microscopy suggests the presence of a close association of lipid particles with the interfacial area of the emulsion (Fig. 4.3B). It appears that wax particles reside at the space around the oil droplets and are adsorbing at the interface. This data provides evidence of a Pickering-type stabilisation with solid lipid particles – rather than the surface active agents utilised for their fabrication – acting as emulsion stabilisers. The demonstrated mechanism is also in agreement with a previous study by Pawlik, *et al.* (2016) who confirmed a Pickering performance of tripalmitin particles fabricated with whey protein isolate (WPI) in the stabilisation of sunflower-in-water emulsions.

4.3.3 Lipid particles' functionality under different formulation parameters

The effect of the dispersed phase polarity on the resulting emulsion droplet size and stability was studied by using pure alkanes of different molecular sizes (chain length). Fig. 4.4 presents laser diffraction measurements performed on dodecane and hexadecane-in-water emulsions where the aqueous phase contained wax particles formed with Tween 80. Emulsion stability was monitored over 10 and 75 days of storage (at 4 ºC).

It was anticipated that altering the polarity of the oil phase would impact solid particles' surface characteristics, i.e. how particles adsorb and reside at an emulsion droplet interface. In general, oil type is one of the parameters that influence interfacial tension, solids' contact angle, as well as the energy of particle anchoring to an interface (Lopetinsky, Masliyah, & Xu, 2006). The order of increased polarity of the oils investigated in the current study is hexadecane < dodecane < sunflower oil (Rampon, *et al.*, 2004). Comparing the droplet sizes of the emulsions produced with apolar oils, it is evident that larger sizes were yielded in the presence of a less polar oil (Fig. 4.4B). Previous work on the determination of interfacial tension for *n*-alkane/water systems has revealed a linear dependence between the carbon atoms in the molecule and interfacial tension (Aveyard & Haydon, 1965; Zeppieri, Rodríguez, & López de Ramos, 2001). The authors of both studies reported that as the number of carbon atoms increases, interfacial tension increases accordingly. As such, dodecane is more effective than hexadecane in lowering the oil-water interfacial tension and therefore, droplets fragmentation is enhanced which, in turn, results in smaller sizes. We therefore postulate that interfacial properties are altered due to the presence of less polar oils, and this enables the formation of o/w emulsions with droplet sizes that depend on the polarity of the dispersed phase.

These emulsions also appeared to be more prone to instability mechanisms over time, as the distribution was shifted to larger sizes following 10 days of cold storage pointing to coalescence (Fig. 4.4B). Earlier studies of Davis and Smith (1976) demonstrated a decreased emulsion stability (due to droplet coalescence) upon increase of oil phase's polarity. The emulsions in that case were stabilised solely by a surfactant (i.e. sodium dodecyl sulfate (SDS)) and different aliphatic hydrocarbons (C_6-C_{16}) as dispersed phase oils were screened. More relevant to particle-stabilised emulsions, studies on model silica systems of similar hydrophobicities demonstrated an oil-type dependence of emulsion's continuous phase; particles within non-polar oils preferentially form o/w emulsions, whereas polar oils tend to produce w/o emulsions (Binks & Lumsdon, 2000). This behaviour was explained based on the work of adhesion between water and oil and the contact angle between solid-oil-water (i.e. particles' hydrophobicity increased at polar interfaces). In the present study, it is expected that wax particles have different solubility in dodecane and hexadecane, and this is likely to largely explain the long-term stability of the produced emulsions.

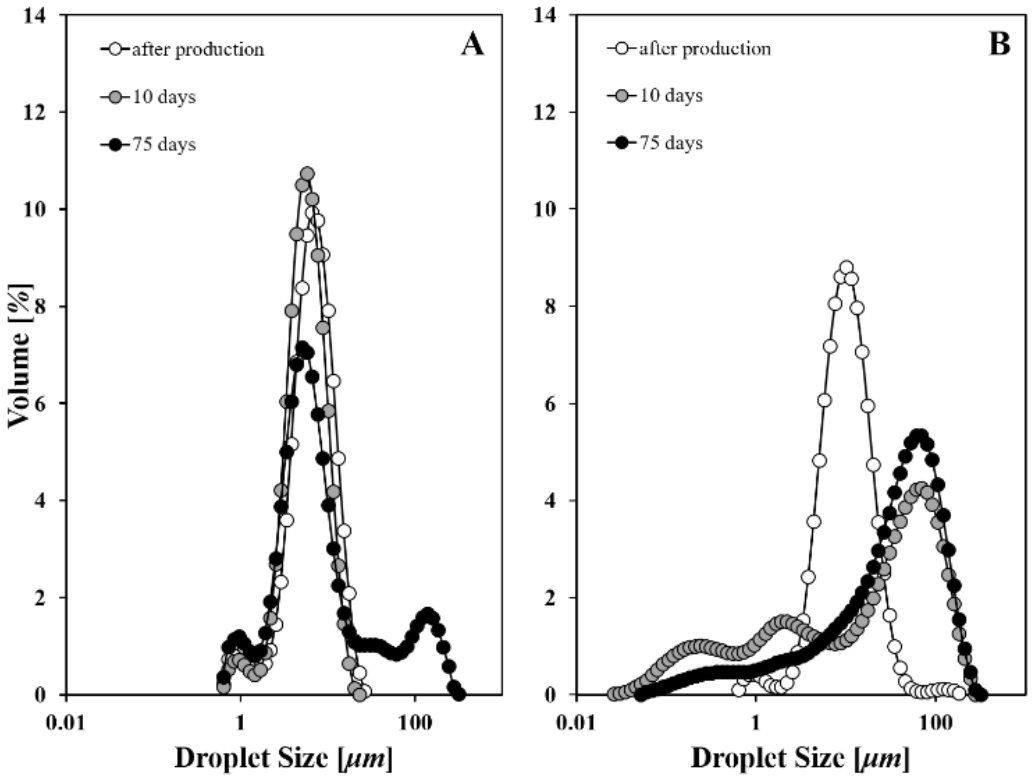

Fig. 4.4. Droplet size distributions of 20% dodecane (A) and hexadecane (B)-in-water emulsions stabilised by 2.5 wt/wt% cetyl palmitate particles formed with 0.8 wt/wt% Tween 80. Measurements were performed immediately after production and following 10 and 75 days of cold storage.

4.3.4 Stability of solid lipid particles within sunflower oil emulsions

Dissolution of solid lipid particles within the disperse phase is anticipated to occur upon introduction of the oil phase, emulsification and upon storage. The migration of lipid molecules (from the solid lipid particle to the dispersed phase) will be driven by the solubility of the two species (Samtlebe, *et al.*, 2012). Microcalorimetry was employed as the technique to track the evolution of the solid fat content in o/w emulsions formed in the presence of 2.5 wt/wt% wax particles and 0.8 wt/wt% surface active species for different oil phase fractions. The melting thermograms of these emulsions are shown in Fig. 4.5A and 4.5B. Their melting enthalpies are expressed as a ratio $\Delta H_{emulsion}/\Delta H_{dispersion}$ and are plotted versus

the disperse phase mass fraction (Fig. 4.5C, 4.5D). Each of these ΔH values (units: W/g of crystalline material) corresponds to the single thermograms presented in Fig. 4.5A and 4.5B. The above mentioned ratio is used in order to denote the fraction of the solid matter that remains in the emulsion system following introduction of a liquid oil phase and subsequent emulsification, and along different storage times (at refrigeration temperatures). As such, a value of 1 would obviously suggest that the solid content is maintained, whereas a value that approximates zero would mean that any solid matter has been lost.

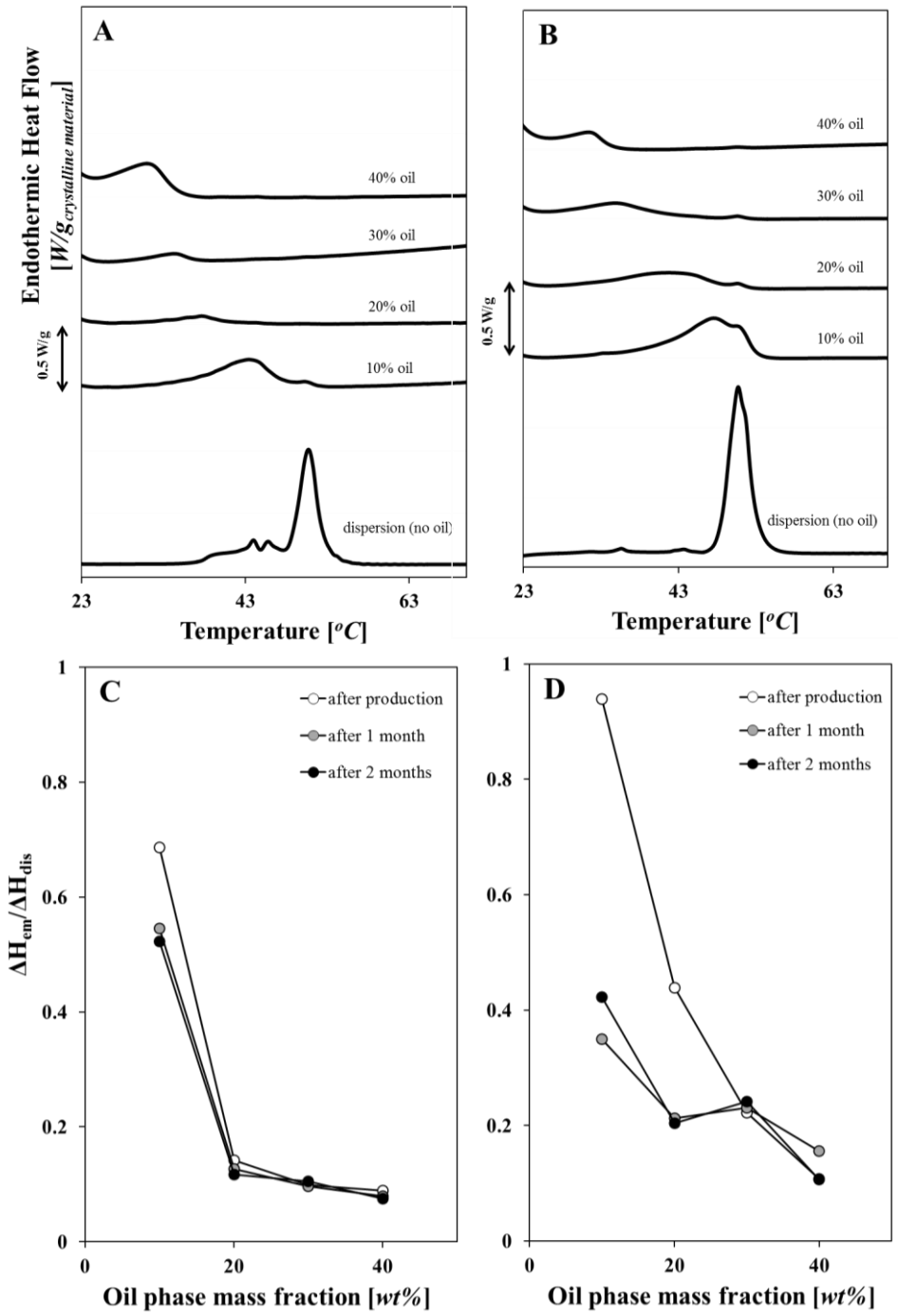

Fig. 4.5. Melting thermograms immediately after production of 10, 20, 30 and 40% o/w emulsions produced via sonication for 30 sec (A, B). Emulsions were produced using 2.5 wt/wt% wax particles and 0.8 wt/wt% Tween 80 (A) or NaCas (B). The "dispersion" sample is the aqueous dispersion of cetyl palmitate particles formed in the presence of surface active species which is the continuous phase of all the emulsions. Time evolution of the amount of crystalline material present within emulsions ($\Delta H_{em}/\Delta H_{dis}$) formed with lipid particles and Tween 80 (C) or NaCas (D) for different oil percentages. Results shown are from a representative experiment.

Dispersions of solid wax particles fabricated with Tween 80 or NaCas melted at approximately the same temperature (~50.5 ºC is the main melting peak that corresponds to *β*-form crystals of cetyl palmitate), regardless of surface active species type. When the dispersion was mixed with sunflower oil to form the emulsion, the respective melting endotherm of the resulting emulsion occurred progressively at lower temperatures, as the dispersed phase (oil) mass fraction in the emulsion was increased (Fig. 4.5A, 4.5B). The magnitude of the change in the endothermic transition, as also depicted from the enthalpy ratio values (Fig. 4.5C, 4.5D), was significantly greater when the oil mass fraction was high (i.e. 40%). This indicates that solid matter suffers a greater loss ($\Delta H_{em}/\Delta H_{dis} < 0.2$) with an increase in the dispersed phase fraction, and this appears to be independent of the type of the surface active component. The results are well aligned with a study by Samtlebe, *et al.* (2012) who investigated *n*-tetradecane emulsions in the presence of crystalline *n*-eicosane particles. The authors observed a marked difference in the melting thermograms of 9:1 and 1:9 mixtures of *n*-eicosane SLN and *n*-tetradecane emulsions after different storage times. They ascribed the distinct melting profiles to changes in solid fat content, as measured by nuclear magnetic resonance spectroscopy (NMR).

In the case of emulsions stabilised by wax particles and Tween 80 the enthalpy ratio decreased sharply from 0.68 to 0.14 with an increase from a 10 to a 20% mass fraction of sunflower oil in the system and decreased only marginally for higher oil contents (Fig. 4.5C). In earlier studies for *n*-alkane droplets in the presence of a non-ionic surfactant, it has been observed that oil exchange takes place between droplets and the rate of this exchange increases in a linear fashion with the droplet surface area (McClements & Dungan, 1993). In the current study, a high rate of oil transfer would essentially mean increased dissolution, hence, to an extent, the preceding observation is true as well. It is also worth noting that in the

emulsion containing 40% oil, the dissolution of particles is so significant that only approximately 9% of the initial lipid crystals (contained in the initial aqueous dispersion) were present in the emulsion following its production. While the emulsion with a 10% oil phase fraction suffered an almost 24% decrease in the amount of solid lipids, the rest of the fractions appeared to have negligible changes over the course of two months. This clearly suggests that loss of solid matter occurs shortly following emulsification, and prolonged storage does not substantially result in any further losses.

In contrast, the melting enthalpies ratios for the emulsions stabilised by wax particles with sodium caseinate were higher (as absolute values) (Fig. 4.5D) than the respective ones for wax particles in the presence of Tween 80. This behaviour persisted across all investigated mass fractions, except the 40% where the two systems display similar levels of solid matter losses (Fig. 4.5C, 4.5D). Counter to the system in the presence of Tween 80, sodium caseinate doesn't give a plateau behaviour at oil mass fractions ≥ 20% and an increase in the dispersed phase mass fraction produces a further drop in the remaining solid material.

A number of studies has shown that the rate of oil exchange between o/w emulsion droplets is highly dependent on the type and concentration of the surfactant present (D. J. McClements & Dungan, 1993; D. J. McClements, Dungan, German, & Kinsella, 1993a, 1993b; Weiss, *et al.*, 2000). More specifically, McClements and Dungan (1993) demonstrated that solubilisation of hydrophobic molecules and subsequent transport between oil droplets is promoted by surfactant micelles. The data presented above provide evidence of solubilisation of hydrophobic material from the SLN and potential transfer into the oil droplets. This is promoted by increases in the mass fraction of the oil phase and it is also facilitated by the surface active species present, most likely by its ability to form micelles and assist transportation from SLN to the oil phase.

Based on evidence in literature, the rate of SLN dissolution is also affected by the lipid source, e.g. its molecular weight. It has been found that the rate of oil mass transfer in emulsions decreases with an increase in lipid's molecular weight (Samtlebe, *et al.*, 2012). Therefore, the loss of solid matter in the presence of sodium caseinate was further investigated on whether it can be enhanced or maintained by changing the source of lipid particles to a lipid of a higher molecular weight (hence, reduced solubility). Oil-in-water emulsions were prepared (via ultrasound) using tristearin particles formed with sodium caseinate and their melting enthalpy ratios were juxtaposed to the ones obtained from the respective ones of emulsions with wax particles. These ratios are presented in Fig. 4.6A along with the emulsions' droplet size evolution for each investigated oil mass fraction (Fig. 4.6B, 4.6C).

As depicted from Fig.4.6A, dissolution of solid tristearin particles in sunflower emulsions was significantly suppressed. There was no evidence of substantial loss of solid matter in the time allowed and that appeared to be independent of dispersed phase concentration. This indicates that the amount of tristearin particles when fabricated using sodium caseinate is negligibly affected by the presence of liquid oil. A percentage as high as 87% of the original crystals is still available after one month storage to stabilise the oil droplets.

One would expect that the almost 52% loss of solid content in the case of wax particles (Fig. 4.6A) following one month storage (at 20% oil mass fraction) would have an impact on their functionality as emulsion stabilisers. The fact that no differences were detected in the droplet size patterns within a month (Fig. 4.6B) potentially points to a sufficient amount of particles being available in the first place to stabilise the interfaces.

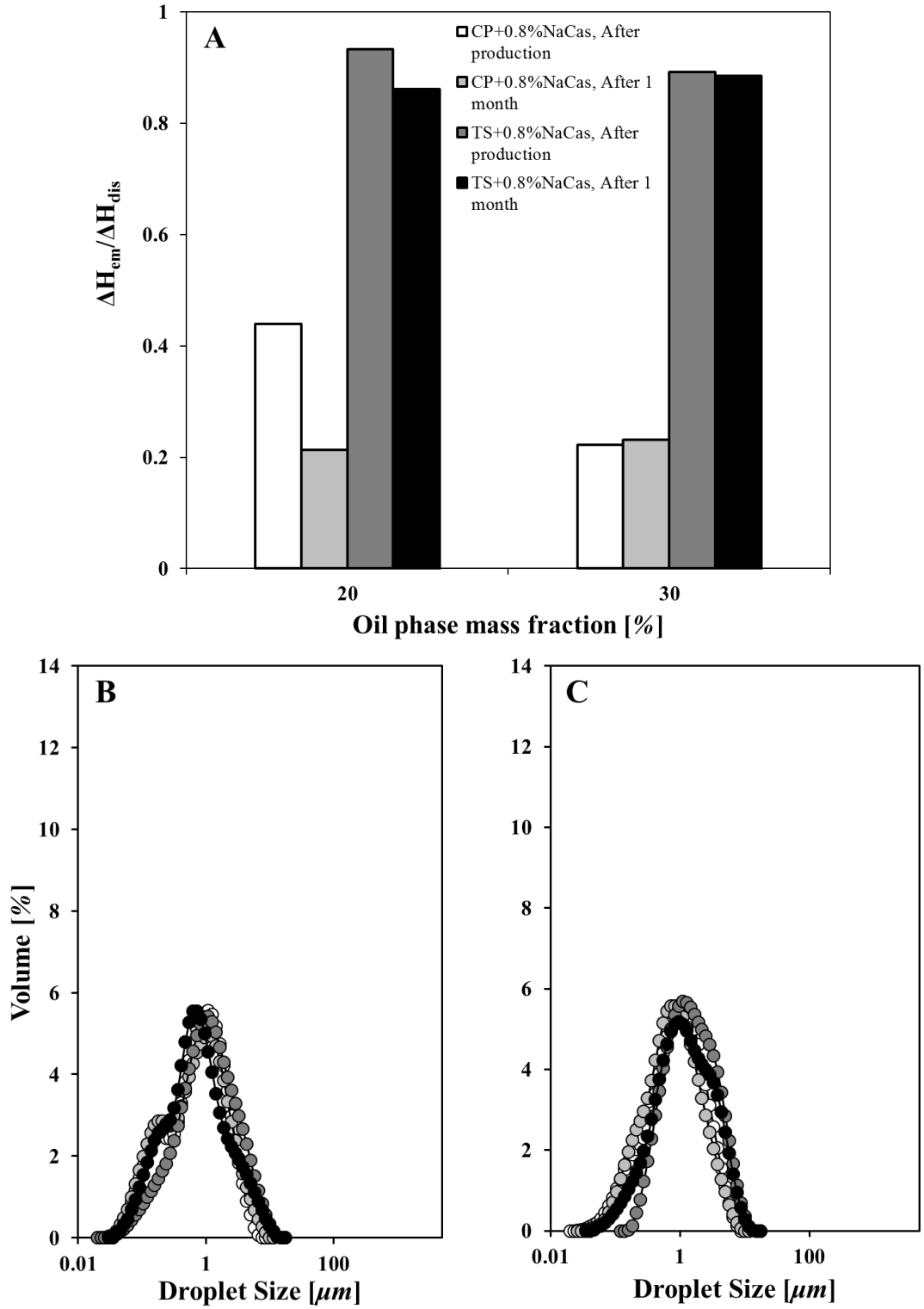

Fig. 4.6. Time evolution of the ratio $\Delta H_{em}/\Delta H_{disp}$ for emulsions stabilised by cetyl palmitate and tristearin particles, both formed in the presence of 0.8% NaCas (A). Droplet size distributions of the respective emulsions at 20% (B) and 30% (C) dispersed phase mass fraction.

4.3.5 Thermal stability of emulsions stabilised by solid lipid particles

This section is looking at the thermal stability of the emulsions' microstructure in an attempt to investigate the link to temperature effects on the lipid particles themselves. For this purpose, o/w emulsions stabilised by cetyl palmitate particles that have been formed in the presence of 0.8% NaCas were stored at elevated temperatures; at a temperature well below the melting point of the lipid particles (i.e. 36 ºC) and at a temperature that the crystalline material is already melted (i.e. 58 ºC).

Fig. 4.7 depicts the droplet size profiles obtained upon exposure of emulsions to increased temperatures and also upon ageing.

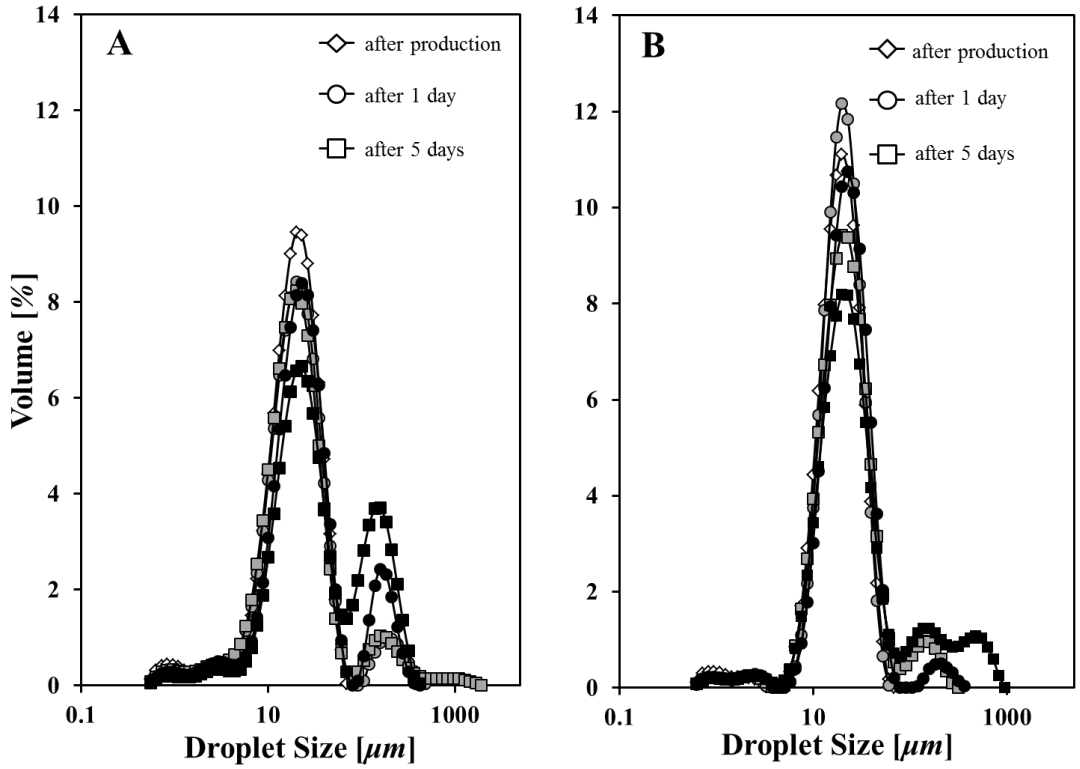

Fig. 4.7. Comparative size distributions of solid wax particles and NaCas (0.8%) stabilised emulsions with 20% (A) and 40% (B) sunflower oil. Open symbols represent emulsions after production, while coloured symbols show emulsions stored at elevated temperatures; grey and black colour symbols represent emulsions stored at 36 ºC and 58 ºC respectively.

Laser diffraction measurements showed that subjection to elevated temperatures (either 36 or 58 ºC) resulted in the formation of additional size populations and a shift towards higher measured emulsion diameters (Fig. 4.7A, 4.7B). This evidence of coalescence occurring in the systems regardless of the dispersed phase mass fraction, appears to be more accentuated at temperatures above cetyl palmitate's melting point (i.e. 55 ºC) rather than at 36 ºC. Stability of the emulsion systems clearly depends on the thermal properties and this has come about due to the presence of lipid particles themselves.

4.4 Conclusions

This study demonstrated the potential of solid lipid particles of colloidal dimensions to act as Pickering stabilisers of oil-in-water emulsions. Particles that were fabricated in the presence of minimal amounts of a surface active component could form stable to coalescence o/w emulsions for at least two months. Emulsion stability was corroborated to dispersed phase mass fraction and polarity, processing method employed, as well as inherent thermal properties of the stabilising lipid particles. Dissolution of the solid matter upon introduction of liquid sunflower oil, subsequent emulsification and over a storage period was shown to take place and was monitored via DSC. This phenomenon was more significant for the smaller lipid molecule (e.g. cetyl palmitate) as opposed to the long-chained tristearin. Dissolution was also more pronounced in the presence of a surface active species that is capable of forming micelles (i.e. Tween 80). Despite the loss of crystalline material from the interface there was no concomitant compromise in particles' Pickering functionality and emulsions remained stable for one month. This was ascribed to a sufficient amount of solid lipid particles being present, although screening of different concentrations of lipid particles would provide a

deeper insight into the interplay between particle content/interfacial coverage and emulsion stability.

Taking this work further, it would be interesting to see how the surface active components' content, irrespective of particle size, affects particles' Pickering performance with the ultimate goal being Pickering stabilisation only in the presence of colloidal lipid structures. The amount of surface active species could be removed via a range of purification/washing procedures (e.g. ultracentrifugation, diffusion through dialysis tubing, cross flow filtration, etc.) (Veeken, 2012). Last but not least, assessment of longer storage stability of emulsions would certainly be more relevant to commercial applications.

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Chapter 5

Maintaining the Pickering functionality of solid lipid particles upon dehydration and subsequent rehydration

Synopsis

The aim of this study was to investigate whether key formulation elements controlled to produce lipid particles with a Pickering functionality can also impact upon the ability of these structures to withstand drying and subsequent rehydration events without loss to their original capacity to provide stable o/w emulsions. The formulation parameters studied included the type of the lipid matrix and the type and concentration of two surface active species. Freeze-drying was used as the dehydration process for the fabricated lipid particles, while two methods for the rehydration of these structures were tested. The use of compounds with a known capacity to act as cryoprotectants (e.g. sugars) is also explored. The findings presented here clearly demonstrate that the addition of minimal amounts of surface active species at the fabrication level, contributed not only in enhancing particles' Pickering functionality but also in conserving the key features of Pickering systems (e.g. size) throughout the drying process. Results suggest that particles fabricated using sodium caseinate during the melt-emulsification step can maintain their original size characteristics after a lyophilisation stage; therefore, once reconstituted, they form o/w emulsions of the same droplet sizes as the respective particles that haven't undergone the freezing and desiccation stage. That is not the case in the presence of a typical small molecular weight surface active component or even with a traditional drying aid, such as cryoprotectants in the formulation.

By studying drying kinetics, moisture content and the effect of storage conditions of the novel dried powders, we were able to demonstrate that as long as particles are designed using a specific type of surface active species, those parameters affect imperceptibly the final behaviour. This work could pave the way for the use of lipid particles as functional food ingredients, capable of reducing the emulsifier content in foods and enhancing their shelf-life, reducing their transportation costs and, in the long run, handling by the consumer.

5.1 Introduction

Stabilisation of emulsions by particles of colloidal dimensions (Pickering stabilisation) from food grade sources has latterly been an energetic field of research, with a number of industrial applications, including those in the food and pharmaceutical sectors. Proteins, biopolymers and lipids are the most commonly investigated edible building blocks for these particulate stabilisers (Pichot, Duffus, Zafeiri, Spyropoulos, & Norton, 2014; Dickinson, 2016). With respect to solid lipid nanoparticles (SLN) in particular, considerable share of research effort has been directed towards improving the physical and chemical stability and Pickering functionality of these structures within their host (usually aqueous) environment (Gupta & Rousseau, 2012; Pawlik, Kurukji, Norton, & Spyropoulos, 2016).

Nonetheless, current literature appears to lack an equally large body of work focusing on the removal/isolation of these structures from their aqueous surroundings (dehydration) and subsequently on the recovery of their Pickering functionality upon reintroduction of water (rehydration). Understanding the dehydration/rehydration behaviour of these particulates will not only allow for greater flexibility in their use as food ingredients, but also prolong their shelf-life by halting microbial growth and ultimately assist in their storage, distribution, and handling (Marefati, Rayner, Timgren, Dejmek, & Sjöö, 2013). However, retention of particle performance following dehydration and later rehydration of these delicate structures is extremely problematic (Mehnert & Mäder, 2001). The challenges involved are a direct result of the dehydration process itself (e.g. high temperatures) and the close proximity between the (dehydrated) particles, brought up by the drastic reduction in the amount of solvent/water in the system. Unfavourable processing conditions (during dehydration) and uncontrolled particle-particle contacts (post-dehydration), can encourage/promote physical and chemical

modification(s) to the particles, which then, upon rehydration, fail to recover their original performance (Abdelwahed, Degobert, Stainmesse, & Fessi, 2006; Morais, *et al.*, 2016).

Over the last couple of decades, freeze-drying (lyophilisation) has been used by industry for the convenient production of solid forms or the stabilisation and preservation of perishable materials (Liu, Zhao, & Feng, 2008; Kasper, Winter, & Friess, 2013). Specifically in the case of SLN, lyophilisation has emerged as the preferred route for dehydration. Literature suggests that lyophilisation of SLN can improve their storage stability by inhibiting chemical degradation (e.g. hydrolysis) and Ostwald ripening. It has also been suggested to enhance the tolerance of these structures to temperature changes that may occur during transportation, as well as enhance their incorporation into tablets, pellets or capsules (Mehnert, *et al.*, 2001). Freeze-drying operation is based on the principle of lowering the system's temperature to subzero values, where the solvent or suspending medium (in this case water) crystallises, and subsequently allowing the frozen aqueous component to be removed by sublimation (direct transition from solid to vapour phase) and desorption under vacuum (Abdelwahed, Degobert, Stainmesse, *et al.*, 2006; Ratti, 2013). Lyophilisation consists of three stages, namely freezing, primary drying and secondary drying, and therefore a plethora of processing parameters need to be controlled/optimised so that the resulting dehydrated product meets specific quality standards (e.g. high yield, residual water content, structure and activity) (Liu, Zhao, & Feng, 2008).

In addition to processing elements, formulation aspects associated with the original "hydrated" structure must be tuned so that the lyophilisate can withstand the mechanical stresses involved during the freezing and desiccation steps; damage mainly associated with ice formation. A large body of literature reports on numerous compounds screened for the protective capacity (cryoprotectants) they can provide to structures undergoing lyophilisation; polysaccharides,

disaccharides, amino acids, polyols and honey are amongst such agents that have been reported (Abadias, Benabarre, Teixidó, Usall, & Viñas, 2001; Champagne, Gardner, Brochu, & Beaulieu, 1991; de Valdez, de Giori, de Ruiz Holgado, & Oliver, 1983; Soltanizadeh, *et al.*, 2014). With regards to freeze-drying of nanoparticles, sugars such as sucrose, mannitol, trehalose and glucose are the most commonly investigated cryoprotective agents, with their effect being accentuated when added in concentrations from 5-20% (in the case of emulsified systems) (Morais, *et al.*, 2016). Two main mechanisms have been suggested in order to explain the manner by which these compounds impart stabilisation upon freeze-drying. The first one proposes that cryoprotectants form a glassy matrix that encloses the lyophilisate (vitrification theory), thus maintaining a sufficient distance from its close particulate neighbours (hindering aggregation) and also abating any mechanical damage caused by ice crystal growth (Abdelwahed, Degobert, Stainmesse, *et al.*, 2006). The second mechanism is relevant to systems containing surface active agents and postulates that the hydroxyl groups contained in the cryoprotectants can substitute water molecules in the course of drying. In essence and by using the example of lecithin, Zhang, Liu, Qian, and Chen (2008) explained that this replacement compensates for the water loss upon drying and essentially acts similarly to the hydrated layer around lecithin which, prevents lecithin layers to come close enough and fuse (which would result in a size increase).

Further to process control and incorporation of cryoprotective species, there is also scope to explore the role that formulation components used in the fabrication of hydrated SLN can have in terms of supporting the recovery of particle functionality following transition to a dehydrated state and later rehydration. In contrast to formulation species with functionalities that only emerge during dehydration (e.g. as in the case of cryoprotectants), formulation components referred to here already possess very specific microstructural function within SLN

systems in the hydrated state, but could then, during dehydration and rehydration events, also provide additional (in the case of lyophilisation "cryoprotective-like") advantages enabling SLN to revive their original (pre-dehydration) performance. To our best knowledge, such dual formulation functionality has not been previously reported for SLN undergoing lyophilisation. However, comparisons can be drawn from a limited number of studies in the area of emulsions, where microstructural design is analogously employed to enable subjection of oil droplets (as opposed to particles) dispersed within an aqueous continuous phase to a lyophilisation process with minimal impact on their original microstructure upon rehydration (Hu, Marway, Kasem, Pelton, & Cranston, 2016; Marefati, *et al.*, 2013). Both of these studies focus on food-grade particle-stabilised Pickering emulsions and highlight the vital role of the nature and properties of the stabilising material in the recovery of emulsion functionality following a dehydration stage. In more detail, Marefati, *et al.* (2013) investigated the manufacture of oil-filled powders from the dehydration of quinoa starch (OSA-chemically modified) stabilised Pickering oil-in-water emulsions. The authors found that partial gelatinised starch granules were able to preserve the original emulsion template throughout a freeze-drying process, with only minor levels of aggregation observed in the reconstituted emulsions. In that vein, complexation of tannic acid (added after emulsification) with cellulose derivatives (methyl cellulose or hydroxyethyl cellulose) already stabilising oil-in-water emulsions, promoted condensation of the cellulosic "shell" around the oil droplets, allowing the generation of solid dry emulsions that were then shown to be easily redispersed in water (Hu, *et al.*, 2016).

The present study focuses on lipid-based nanoparticulate structures and investigates how formulation parameters (e.g. lipid source and type and concentration of surface active species), previously demonstrated to be crucial for particle fabrication (Chapter 3) and

subsequent Pickering functionality (Chapter 4) can impact on the ability of these particle systems to withstand a harsh dehydration process and regain their microstructure and Pickering performance upon reconstitution. Edible lipid particles from two discrete lipid sources (either a pure monoacid triglyceride (tristearin) or a model wax (cetyl palmitate)), in the presence of two different types of amphiphilic species (either Tween 80 (surfactant) or sodium caseinate (protein)), were initially subjected to lyophilisation over different timescales and then rehydrated following two reconstitution methods of varied energy input. Lipid particle microstructure characteristics such as size and size distribution, interfacial behaviour, as well as their ability to stabilise oil-in-water emulsions (Pickering functionality) were studied before and after lyophilisation/rehydration. Dried powders were reconstituted either immediately or following storage (under cold or ambient temperature conditions) of up to one month. Finally, retention of lipid particles' microstructure and Pickering performance were linked to both formulation (type of lipid and type/concentration of surface active component) and processing (drying time, storage time, reconstitution method) parameters.

5.2 Materials & Methods

5.2.1 Materials

Microcrystalline glyceryl tristearate (Dynasan® 118) (tristearin hereafter) and cetyl palmitate were gifted from IOI Oleo (IOI Oleochemicals GmbH, Hamburg, Germany) and Gattefossé (France) respectively. Surface active components polyoxyethylene sorbitan monooleate (Tween 80) and casein sodium salt from bovine milk were purchased from Sigma-Aldrich (Sigma-Aldrich, UK). High molecular weight hydrocolloids (hydroxypropyl)methyl cellulose (HMPC) (M_{HPMC}≈86 kDa) and low-methoxylated citrus pectin DE (LM-pectin, GENU® pectin type LM-104 AS) were purchased from Sigma-Aldrich (Sigma-Aldrich, UK) and CP

Kelco (Copenhagen, Denmark) respectively. The disaccharide cryoprotectants D-(+)-trehalose dihydrate ($C_{12}H_{26}O_{13}$; molecular weight:378.33 g/mol; ≥98.5% purity) and sucrose ($C_{12}H_{22}O_{11}$;molecular weight:342.30 g/mol; ≥99.5% purity) were supplied by Fisher Scientific and Sigma respectively. A range of commercially available maltodextrins were also tested as drying aids during the dehydration process. To this end, maltodextrins of increasing dextrose equivalent values, i.e. decreasing molecular weights, were screened. Maltodextrin (DE 4-7) was provided by Sigma-Aldrich (Sigma-Aldrich, UK) and corn maltodextrin (DE 17.9, C*Dry MD 01915) was gifted by Cargill (Haubourdin, France). Commercial sunflower oil was used for all emulsions. Double distilled water from Milli-Q systems (Millipore, Watford, UK) was used throughout.

5.2.2 Methods

5.2.2.1 Particles preparation

Solid lipid micro- and nano- particles were produced via a melt-emulsification method (as described in Chapter 3). Firstly, an o/w emulsion is prepared at a temperature above the melting point of the lipid in the presence of surface active agents (Tween 80 or sodium caseinate) and using ultrasonication (2 minutes at 95% amplitude). The emulsion is then cooled resulting in the formation of discrete lipid particles. Final dispersions contained 2.5% (wt/wt) lipid with varying concentrations of surface active components. The protein was used at its native state (pH ~7.01).

Solid wax particles were also fabricated using 2% (wt/wt) (hydroxypropyl)methyl cellulose (HMPC) and low methoxyl pectin (LMP) as alternative to sodium caseinate high molecular weight species. Samples were subjected to lyophilisation and then characterised for their mean size.

5.2.2.2 Lyophilisation of lipid particles

Lyophilisation was undertaken using a bench top freeze dryer, Scanvac model 110-4 (Copenhagen, Denmark). Particulate dispersions were first cooled to -20 ºC overnight before being transferred to the freeze dryer. The freeze-drying process was carried out at -110 ºC under constant vacuum (0.100 hPa) for 48 hours. Post-lyophilisation, powders were collected, weighed and rehydrated at room temperature using the same volume of water (deionised) removed by the drying process. Particles were redispersed, via agitation supplied by vortex mixer for 30 seconds, followed by probe ultrasound treatment (Sonics & Materials, Inc., CT, USA) operating at 95% amplitude for approximately 20 seconds.

5.2.2.3 Effect of cryoprotectants

Solid lipid particle dispersions (2.5 and 5% (wt/wt)) formed in the presence of Tween 80 (0.8 and 2% (wt/wt)) were mixed with 2.5 and 5% (wt/wt) aqueous cryoprotectant solutions (sucrose, trehalose) in a weight ratio of 1:1 prior to freezing (total resulting lipid content: 1.25 and 2.5% wt/wt% respectively). Mixtures were subsequently lyophilised for 72 hours and the obtained powders were rehydrated as described in section 5.2.2.2.

5.2.2.4 Lyophilised powders – Storage conditions

A one-month stability study was conducted on the powders under different storage conditions. Freeze-dried samples were kept either at room temperature in an air-tight desiccator filled using silica gel as the desiccant material, or at 4 ºC (refrigerated) for fixed storage times (t=7, 30 days). At each time point, samples were reconstituted with distilled water and analysed for their physicochemical properties.

Powder water activity (a_w) was measured at ambient conditions, using a dew point hygrometer (AquaLab Series 4TE, Decagon Devices, Inc). Measurements were performed

straight after powder collection to minimise moisture uptake. 1 g of lipid-based powder was used in each case, with means showing at least duplicates.

5.2.2.5 Emulsion preparation

Re-suspended samples were subsequently used as the aqueous phase for following oil-in-water emulsions. O/W emulsions (sunflower oil 20% and 80% aqueous dispersions containing lipid particles and Tween 80 or sodium caseinate) were produced using a high shear mixer (Silverson L5M, UK) at 9,000 rpm for 2 minutes or via ultrasonication at 95% amplitude for 30 seconds. Emulsions were additionally fabricated using stored (30 days) reconstituted lyophilised lipid particles. Both sets of emulsions were analysed for droplet size.

5.2.2.6 Characterisation of powders and emulsions

5.2.2.6.1 Particle and droplet size analysis

Particle size distributions for both fresh and reconstituted samples were determined via static light scattering (SLS) using a Mastersizer 2000 (Malvern Instruments, UK). Means show the result of at least duplicated results. For redispersed systems refractive indices of 1.49 and 1.44 was used for tristearin and cetyl palmitate respectively.

Sunflower oil-in-water emulsions were also analysed using laser diffraction immediately after production (RI=1.47). Average droplet sizes were reported as volume weighted means, $D_{4,3}$.

5.2.2.6.2 Interfacial Tension measurements

Static sunflower oil/water interfacial tensions were determined using a K100 Krüss Tensiometer (Krüss GmbH, Germany) equipped with a Wilhelmy plate. The interfacial tension of freshly prepared and dried/resuspended lipid particle systems was measured at 20 ºC. To perform the measurement, ~50 mL of sunflower oil was carefully pipetted onto the

surface of the aqueous phase (~25 mL). All measurements were conducted at least in duplicate, and average values along with standard deviations were calculated.

5.3 Results & Discussion

5.3.1 Drying and rehydration of solid lipid particles

Lipid particles were produced following the method described earlier in the presence of either Tween 80 or sodium caseinate, and from two different lipid sources; a triglyceride (glyceryl tristearate) and a wax (cetyl palmitate). As described in Chapter 3, the sizes of the fabricated particles were found to be affected by the type and the concentration of the employed surface active species, as well as the physicochemical properties of the lipid material used. Particle manufacturing was followed by lyophilisation and subsequent rehydration of the lyophilisates, by adding back the same weight of distilled water that was lost during the drying process.

5.3.1.1 Effect of formulation parameters and reconstitution method

Reconstitution of the lyophilised lipid particles was carried out via two different methods: mixing with a magnetic stirrer for approximately 2 hours or vortexing for 30 seconds followed by sonication for another 15 seconds. The applied methods were selected based on the fact that they are the most common ones described in the literature (Abdelwahed *et al.*, 2006), and also because they differ significantly in the level of shear they yield. Variation in shear involved to systems' reconstitution provides a measure of the magnitude of events that happen to particles upon drying, and shows whether they can overcome them given the different levels of shear (Ding & Pacek, 2008).

The obtained size distribution of the reconstituted cetyl palmitate particles formed in the presence of different concentrations of surface active entities in comparison to non-freeze-dried ones is shown in Fig. 5.1 for 0.8 and 2 wt/wt% surface active component's concentration.

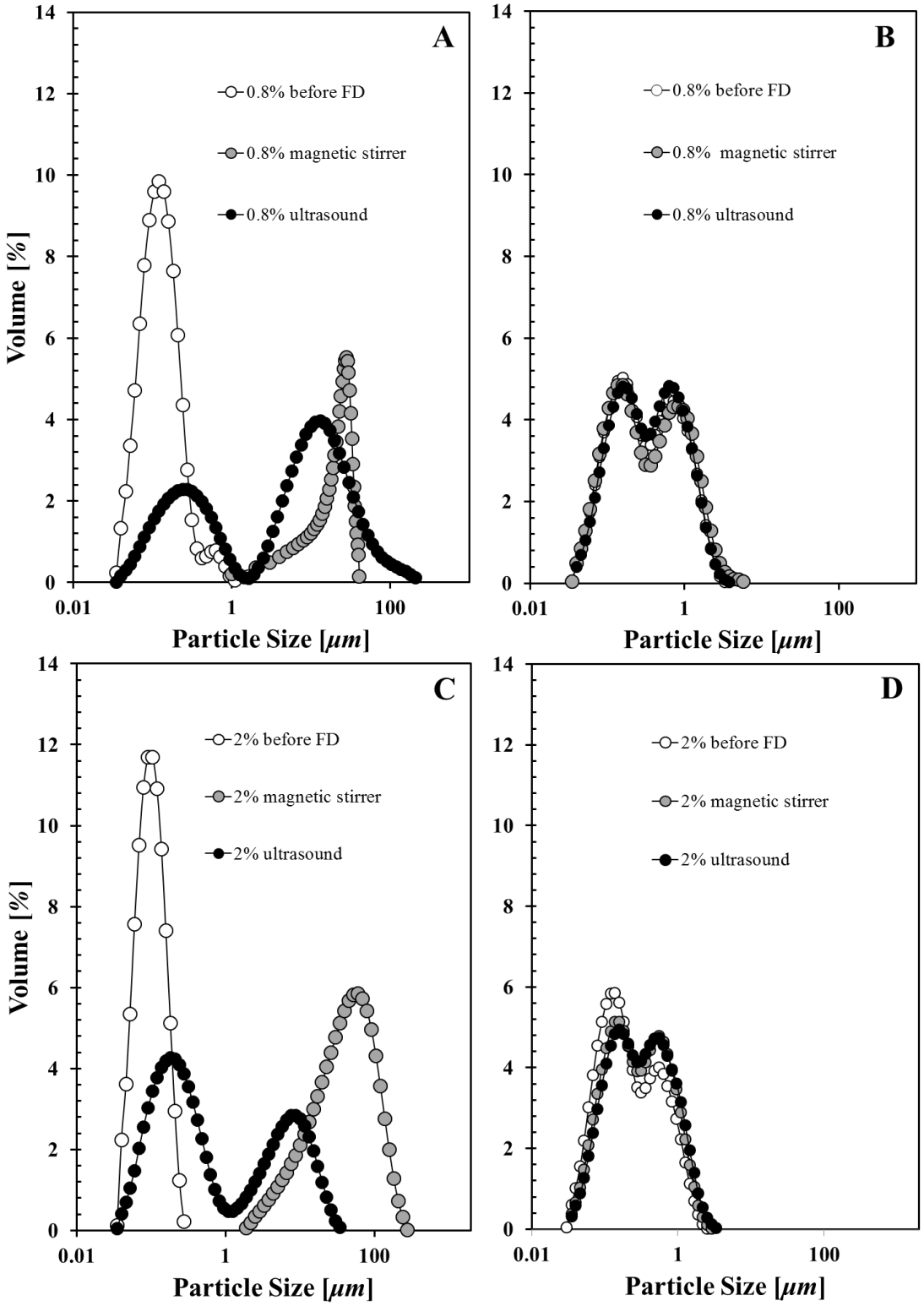

Fig. 5.1. Laser Diffraction measurements showing the particle size distributions of solid cetyl palmitate (wax) particles in the presence of 0.8 and 2 wt/wt% Tween 80 (A, C) and sodium caseinate (B, D) before and after freeze-drying (FD) and for different reconstitution methods.

As can be seen from Fig. 5.1, the type rather than the amount of the surface active component present in the original particles suspension appears to dictate the recovery of particles. In the case of wax particles produced using Tween 80 and when a low energy agitation such as the one conducted by a magnetic stirrer is applied for their resuspension, large micron-sized aggregates are formed, regardless of the amount of surface active component (Fig. 5.1A, 5.1C). Assuming that drying brings particles into closer contact and creates aggregated structures rather than individual bodies, a reconstitution method via high energy systems would revert any aggregates, if present, to discrete entities. In our study, mild reconstitution generated large bodies, while provision of more extensive energy levels had only a small effect at low Tween 80 concentrations where smaller particle populations accompanied by expanded span values (width of distribution) become evident (Fig. 5.1A). Fig. 5.1C depicts a tendency to maintain some integrity of the redispersed particles with an increase in the amount of Tween 80, however the size recovers only partially to initial ranges. The particle sizes of the redispersed wax nanoparticles lyophilisates still cover, in part, the submicron range, although they are larger than the original dispersion (~200 nm as opposed to ~130 nm). Despite being effective in producing particles at the sizes desired for Pickering functionality, Tween 80 appears to be unable to maintain those sizes after a drying stage.

On the other hand, sodium caseinate in addition to its capacity to produce small particles – although not at the same level as Tween 80 – it also demonstrated a capacity to protect them during harsh drying conditions, and conserved the original sizes, with no aggregates observed following rehydration. In fact, this behaviour seemed to be independent of the resuspension method used or the amount of surface active agent present in the original formulation (Fig.5.1B, 5.1D).

This surface active species-induced behaviour during the drying and rehydration process seemed not to be affected by the type of the lipid matrix employed, since a similar profile was acquired when solid tristearin particles were lyophilised (Fig. 5.2).

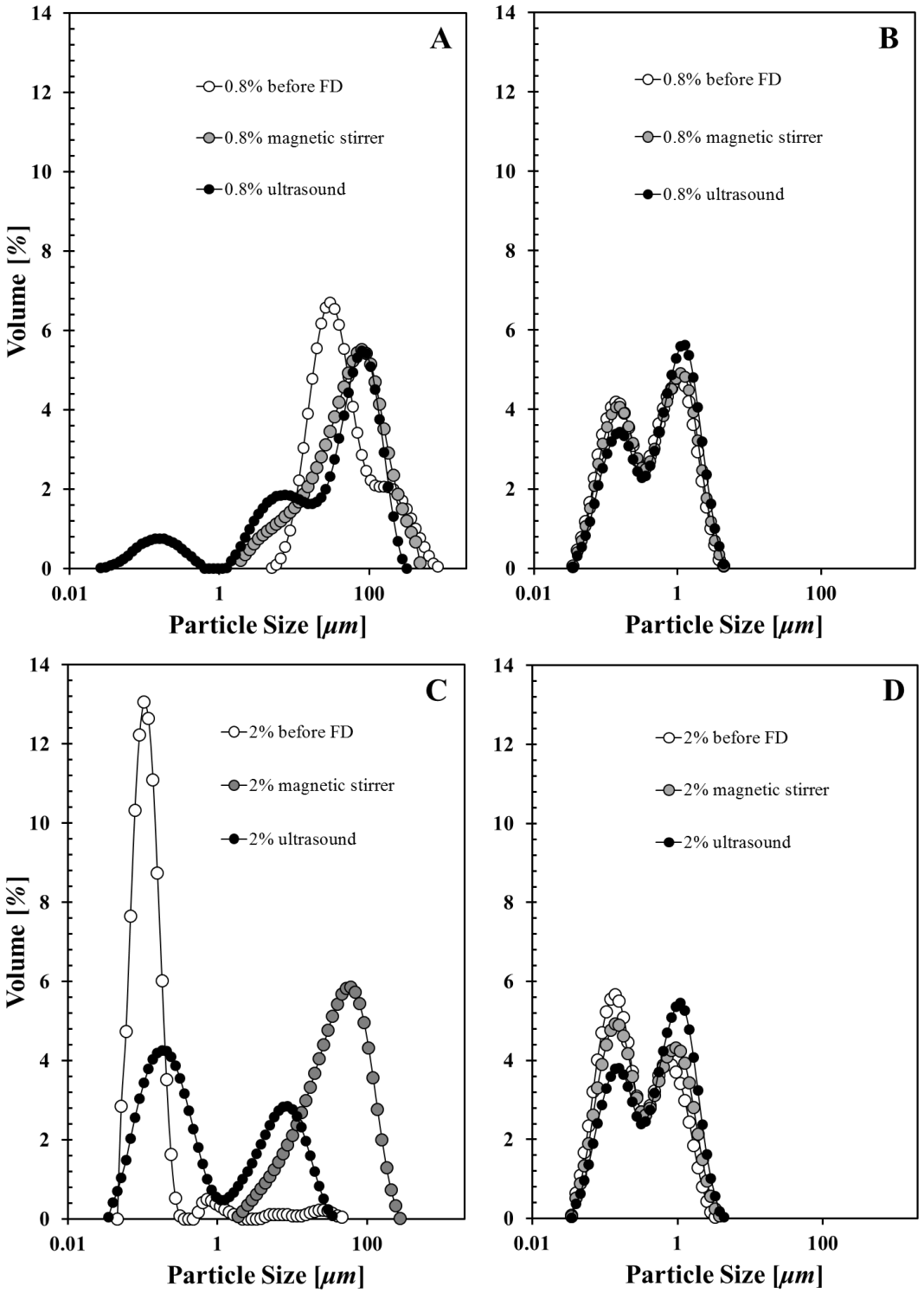

Fig. 5.2. Laser Diffraction measurements showing the particle size distributions of solid tristearin (triglyceride) particles formed with 0.8 and 2 wt/wt% Tween 80 (A,C) and sodium caseinate (B, D) before and after freeze-drying (FD) and for different reconstitution methods.

For 2 wt/wt% Tween 80, the average particle sizes ($D_{4,3}$ values) of the lyophilised samples were around 30 and 450 times higher than the initial triglyceride dispersions for the mixing with a magnetic stirrer and ultrasound as rehydration methods respectively (Fig. 5.2C). On the contrary, the presence of sodium caseinate at any concentration in the fresh prepared formulation proved to be key for maintaining an homogeneous particle dispersion with the original size (Fig. 5.2B, Fig.5.2D).

In view of this data, it is likely that the capacity of the surface active species initially used to fabricate the particles and to also provide a certain level of microstructural recovery following a drying and subsequent rehydration event is very much dependent on the type of surface active component used. In fact, our evidence suggests that this is primarily dependent on its capacity to form a significantly robust barrier against particle-particle interactions encouraged within the low moisture environment resulting from drying. This level of protective effect that a surface active component imparts to a particulate dispersion during a drying stage appears to be a function of its size. A surface active agent of a small molecular size such as Tween 80, is not able to prevent the contact between particulate entities which is favoured by the removal of water and subsequent increase of particle concentration, and finally enhanced coalescence. In contrast, particles that are coated by a high molecular weight surface active component such as a protein prior to drying, retain their original properties (in terms of size) upon addition of water. Evidence from previous work has shown that skim milk proteins, either alone or in combination with other components, have the potential to endow protection to labile systems subjected to freeze-drying. In particular, early studies have demonstrated the efficacy of the proteins contained in milk for the preservation of lactic acid bacteria (Champagne, *et al.*, 1991) and the increase in viability of freeze-dried yeast cells and fungus spores (Berny & Hennebert, 1991), attributed to the formation of a protective coat.

There are various physicochemical processes that accompany ice formation and water removal during freeze-drying of oil-in-water emulsions that can lead to the destabilisation of these systems. The amount of liquid water is lessened upon freezing, hence the emulsifier molecules adsorbed to droplet surfaces are not fully hydrated and this could favour interactions between the surfactant-coated oil droplets (Zhang, *et al.*, 2008). Additionally, the protective effect of the interfacial membranes around the oil droplets is dampened because of ice crystals that can protrude and exert a disruption on them. Depending on their innate surface activity, emulsifiers can adsorb at the surface of newly formed ice crystals reducing their total content in the system. Consequently, it is likely that there is not sufficient material to provide surface coverage to emulsion droplets (McClements, 2004). In the course of both freezing and drying, it is possible that certain emulsifiers (e.g. proteins) are denatured and eventually lose their functionality (Farshchi, Ettelaie, & Holmes, 2013).

In this study, it is proposed that sodium caseinate provides a robust interfacial layer that prevents lipid particles from coming in close proximity during the lyophilisation stage and eventually, enables them to maintain their size and size distribution profiles. Similar observations have previously been reported when poly(vinyl alcohol) (PVA) or modified PVA (containing a long alkyl chain at the end of the molecule) was used as the stabiliser of poly(ε-caprolactone) nanocapsules or liposomes (Abdelwahed, Degobert, Stainmesse, *et al.*, 2006; Takeuchi, *et al.*, 1998). These cryoprotectant-free systems were found to be able to undergo freeze-drying without any aggregation/fusion taking place during freezing. Takeuchi, *et al.* (1998) attributed the observed behaviour to the thick polymeric layer that is formed at the liposome's surface owing to the high molecular weight of the modified PVA together with its anchoring-like coating capacity.

It is also possible that advantages in terms of the recovery of particle performance could be arising from the presence of free (non-adsorbed) surface active species in the system. Abdelwahed, Degobert, Stainmesse, *et al.* (2006) highlighted the importance of the presence of free PVA in addition to the surface-adsorbed PVA, which possibly via inhibition of ice nucleation, protected the fragile nanocapsules throughout the freezing step.

This behaviour of sodium caseinate was only observed at its native state (pH ~7) as any modification of pH to values closer to its isoelectric point (e.g. pH=5) before lyophilisation resulted in considerably bigger particle sizes (See Appendix A3, Fig.A3.2). This is probably occurring because the decrease in protein's charge favours the attractive forces between the droplets in detriment of the electrostatic repulsion that keeps the droplets apart.

Although the conservation of the size properties of dried and rehydrated lipid particles originally fabricated in the presence of NaCas was so far associated with NaCas location at the interface, the possibility that the enhanced performance results also from its presence in the bulk phase, cannot be completely ruled out. This scenario was investigated by substituting sodium caseinate for two different biopolymers, namely hydroxypropyl methyl cellulose (HMPC) and low methoxyl pectin (LMP). The underlying concept was to assess a component that has a level of affinity for the interface but is larger than NaCas (i.e. HPMC), or take it even further and use a biopolymer that has no apparent emulsion stability capacity (i.e. LMP) but solely its presence in the bulk (aqueous) phase could induce a larger barrier to particle aggregation due to lyophilisation. HPMC and LMP were used in the fabrication of particles instead of Tween 80 and sodium caseinate, following exactly the same experimental protocol. These were subsequently freeze-dried and rehydrated and the yielded size and size distribution profiles of the reconstituted lipid particles are presented in Fig. 5.3.

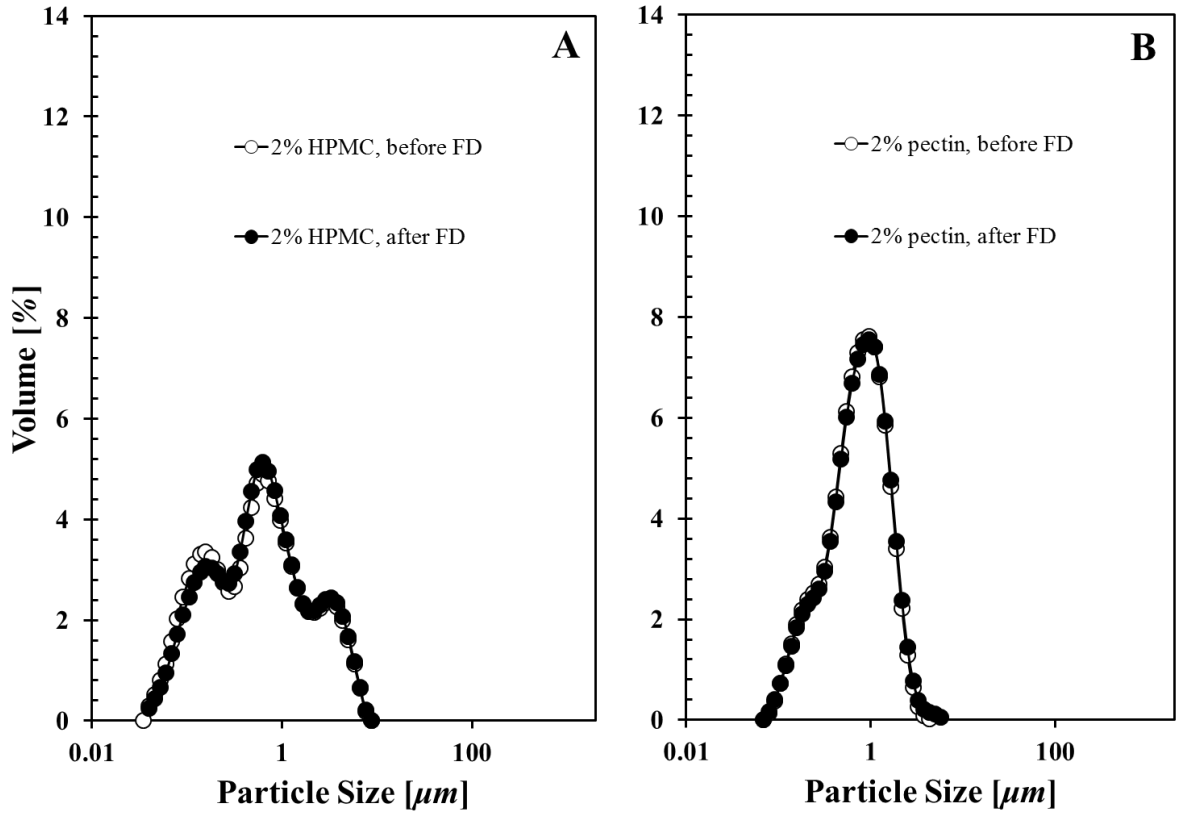

Fig. 5.3. Solid cetyl palmitate particles fabricated in the presence of 2% HPMC and 2% LMP before and after freeze-drying.

Fig. 5.3 shows that when high molecular weight entities were used in the actual preparation of particles, a size distribution with a major peak at around 1 μm was obtained. The size of these particles was also maintained for both polymers after a lyophilisation cycle. This data demonstrates that water soluble macromolecular polymeric compounds surround/co-exist with particles upon reconstitution, acting as a barrier that prevents their fusion. Although there is an impact in terms of conserving the size of dried and redispersed lipid particles, the investigated large molecular weight hydrocolloids don't seem to provide particles within the desirable size ranges for Pickering functionality (i.e. submicron sizes are essential for stabilisation of droplets of 0.5-10 μm (Dickinson, 2012)) as interfacially active species would. As a result, and given their inherent lack of surface activity, they don't impart any lowering of the interfacial tension (see Appendix A3, Fig. A3.3). Due to both pectin's and HPMC's

minimal or at least lower than NaCas ability to reduce the interfacial tension, these redispersed lipid particles dispersions were not further investigated for their potential to stabilise o/w emulsions.

5.3.1.2 Effect of the type of cryoprotective agent

In an attempt to improve the recovery performance of lipid particles originally formed in the presence of Tween 80, standard cryoprotective species used in relevant studies were additionally incorporated. Such species are special excipients and typically sugars, which are added to the colloidal suspension before freezing with a view to protect the lyophilised product from the freezing stresses and improve its stability upon storage (Abdelwahed, Degobert, Stainmesse, *et al.*, 2006). The non-reducing sugars sucrose (Abdelwahed, Degobert, & Fessi, 2006; De Chasteigner, Cavé, Fessi, Devissaguet, & Puisieux, 1996; Lim & Kim, 2002) and trehalose (Dulieu & Bazile, 2005; Schwarz & Mehnert, 1997; Zimmermann, Müller, & Mäder, 2000) are among the most common cryoprotectants used in the literature for the freeze-drying of nanoparticles.

Therefore, these compounds were added at different amounts after fabrication of the dispersions of wax particles in the presence of Tween 80. Starch derivatives such as maltodextrins of a variety of dextrose equivalent (DE) values, ranging from 5 to 18 were also used as an alternative to small molecular excipients. These carbohydrates have also been shown to possess a cryoprotective efficacy (Mun, McClements, & Surh, 2011; Reddy, Awasthi, Madhu, & Prapulla, 2009). Different DE values correspond, among others, to different glass transition temperatures (T_g'), hence it is expected to affect in a distinct way the structural parameters and physical properties of the lyophilised systems.

The samples were lyophilised and rehydrated employing a high shear reconstitution method (i.e. ultrasonication) as described earlier. The resulting size distributions of lipid particles with variation to the type and concentration of cryoprotective agents are shown in Fig. 5.4.

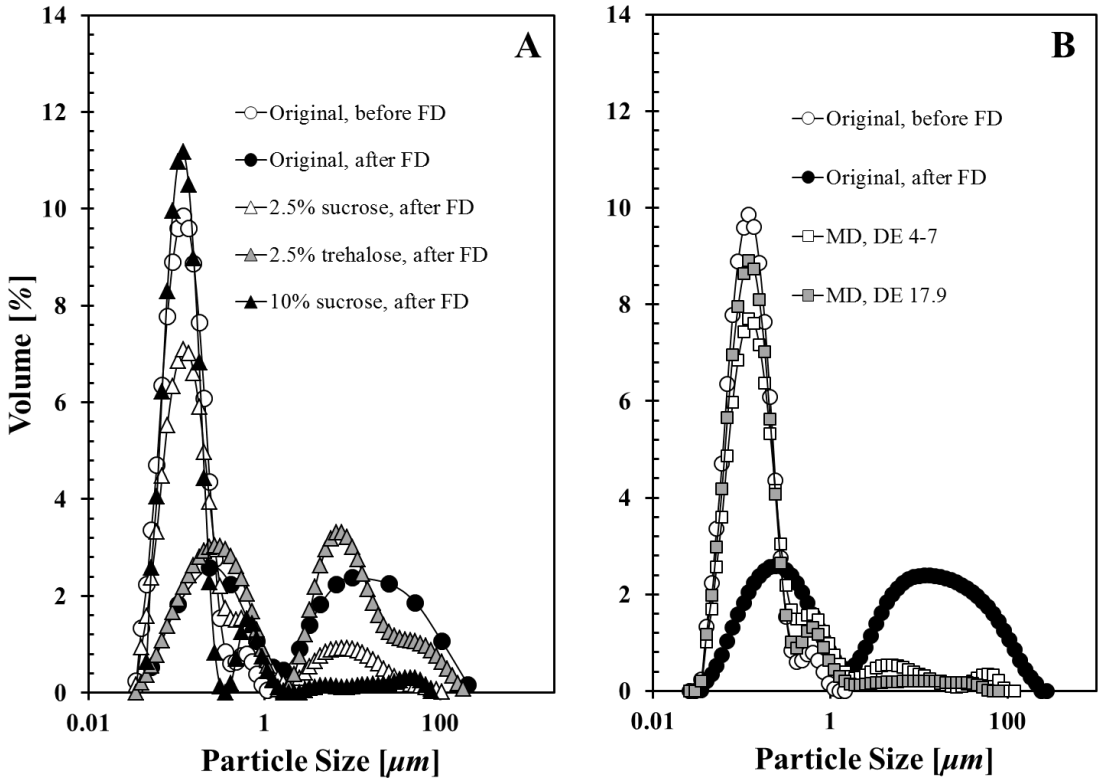

Fig. 5.4. Particle size distributions of 2.5 wt/wt% solid cetyl palmitate particles produced with 0.8% Tween 80 after reconstitution of the lyophilised samples in the presence of sucrose and trehalose as cryoprotectants at different concentrations (A), as well as different DE maltodextrins (MD) at 10 wt/wt% concentration (B).

As becomes evident from Fig. 5.4A, only high concentrations of sugars seemed to have a drastic role on the freezing resistance of cetyl palmitate nanoparticles and mediate their recovery upon rehydration to sizes close to their original ones. A high concentration of a cryoprotector was identified in several studies as a formulation parameter that enhances to an optimum level the reconstitution properties of lyophilised products (Schwarz, *et al.*, 1997). This behaviour appears analogous to the polysaccharides' effect on the stability of frozen

dairy desserts (e.g. ice cream). Stability in these o/w emulsion-based systems is affected by sugar content due to the impact of the latter upon the size and growth of ice crystals (Goff, Caldwell, & Stanley, 1993). The cryoprotective effect of the non-reducing sugars is accentuated at higher concentrations as they confer increased viscosity of the aqueous medium on one hand, and immobilisation of the droplets within a glass matrix on the other (Zhang, *et al.*, 2008).

In regards to the role of maltodextrins, they appeared to behave in a very similar way to sucrose when used at high concentrations. The size of the redispersed wax particle suspensions was only minimally altered, regardless of maltodextrins' dextrose equivalent value (Fig. 5.4B). This could be ascribed to the increase in the higher glass transition temperatures that maltodextrins trigger which results in powders with improved flow characteristics and quality properties (Mosquera, Moraga, & Martínez-Navarrete, 2010). Differences in glass transition temperature signify differences to the amount of unfrozen water and as a general principle, the lower the T_g' values, the more the lyophilisates have the tendency to collapse (De Chasteigner, *et al.*, 1996). The low glass transition temperatures of sugars is responsible for the stickiness of dried powders (Augustin & Hemar, 2009) which will influence, among others, moisture ingress upon rehydration, an effect that is probably diminished when it comes to starch or maltodextrins that possess higher T_g' values. Nonetheless, the behaviour of maltodextrins in relation to their glass transition temperatures was not the focus of this study.

5.3.1.3 Effect of drying time on moisture removal and redispersion behaviour

An optimised freeze-drying process ensures that the duration of the freezing and drying stages (primary and secondary) are such that the requirements for specific final residual moisture

contents are met and at the same time, time-consuming cycles that could lead to over-drying and unnecessary energy usage are avoided. Primary drying is usually the most extensive and multifactorial drying step; it allows ice sublimation to take place which, in turn, is dictated by several factors such as the shelf temperature and chamber pressure, the heat transfer coefficient of the containers and the geometrical characteristics of the product (e.g. thickness of frozen cake). Secondary drying involves water desorption which has normally faster kinetics at constant temperature and pressure. It has been suggested that times longer than 3-6 hours have a negligible effect on the reduction of the moisture content (Tang & Pikal, 2004).

Although the determination of the two drying steps end points were not the aim of this study, it was postulated that different drying times would result in different moisture contents and potentially affect the rehydration ability of the lyophilised powders, for instance by keeping more/less particles intact. Certainly, the moisture levels of the freeze-dried product depend on the interplay between formulation parameters, equipment characteristics as well as the cycle *per se*.

Lyophilisates were left to be dried for different time durations (i.e. 24, 40, 48 and 72 hours), powders were weighed and the amount of water that was removed was calculated via a mass balance. The drying curves for the wax systems along with the water activity at each drying period are plotted in Fig. 5.5.

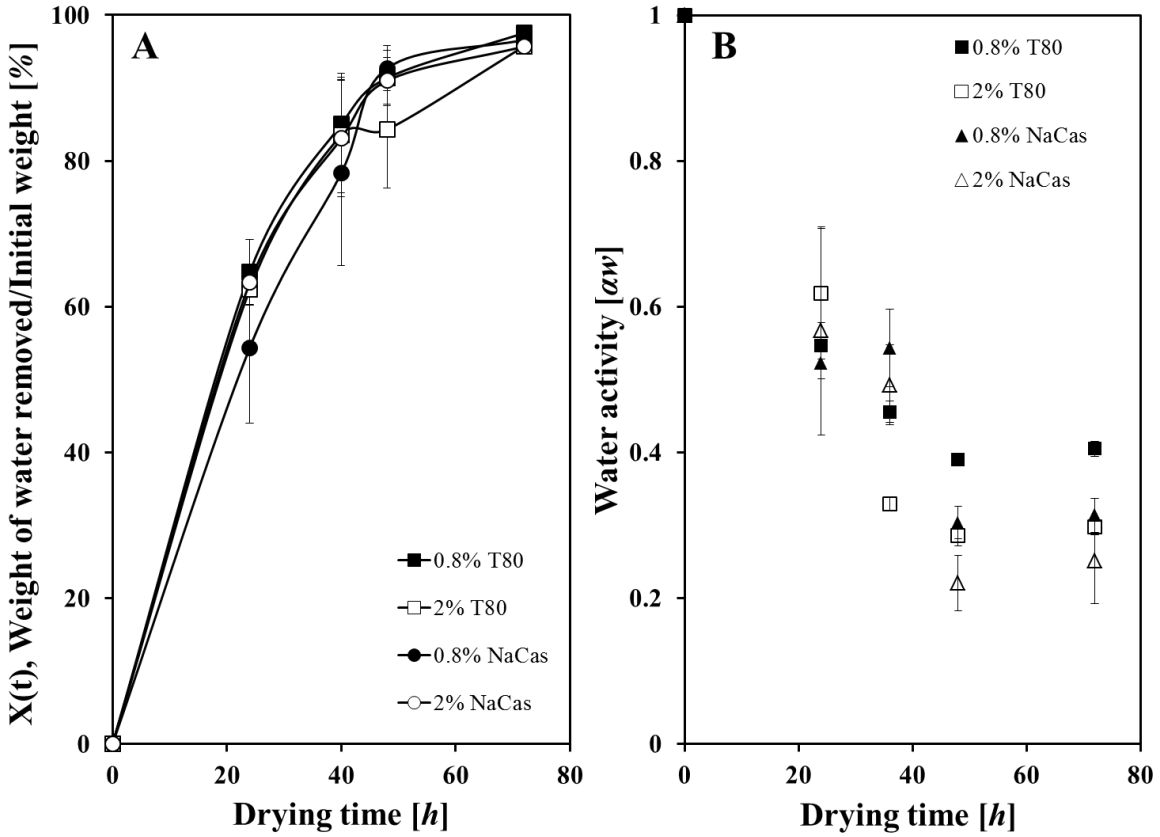

Fig. 5.5. Drying curves for different amounts of Tween 80 and NaCas for dried and resuspended cetyl palmitate particles formed in the presence of either surface active species (A). Water activity measurements of the dried powders (B). Measurements were carried out in duplicates with the mean values shown and the error bars representing ± 0.5 standard deviations.

The drying curve of cetyl palmitate-based particles presented in Fig. 5.5A exhibited two drying periods. During the initial 24 hours the amount of water that is removed increases rapidly for all four systems investigated. At the end of this 24 hours period, 54-65% of the initial water contained is lost by sublimation. This provokes a sharp decay in the water activity values (α_w) over the course of this period (Fig. 5.5B). The time length of this desiccation stage is principally controlled by the preceding freezing step and more specifically, by the number and size of ice nuclei formed which in turn define the product resistance to vapor flow and also the surface area that is available for desorption during secondary drying (Patel, Bhugra, & Pikal, 2009). The process of ice nucleation is affected by

the freezing rate, vials' features and certainly by solution properties, such as composition of the original product, water content or viscosity. For instance, a different lipid or a contained cryoprotectant is anticipated to influence ice crystal shape and size in a different way, and accordingly speeding up or slowing down the sublimation process. This increased drying rate region is followed by a less drastic increase (in the range of 23-30%) within the next 24 hours in the lost frozen water by sublimation. Between 40 and 48 hours there is only a low percentage of water that is still being removed. Accordingly, a decreasing trend was acquired from the water activity measurements, where α_w gradually decreases over time evidencing a reduction in the free water that reaches a level that is only tightly bound within the powder (e.g. at 48 hours). Variations in formulations appear to have a not very significant effect on the amount of water that is sublimed within 48 hours.

In the last stage of drying (i.e. between 48 and 72 hours) only a negligible amount of water is abstracted and also independently of the type and the amount of surface active species present, indicating that the product is dry and there is no further benefit in terms of moisture removal by extending the cycle to 72 hours. The latter observation was confirmed by very low water activity values close to 0.2 which indicate a dry powder already formed at 48 hours.

The freeze-dried powders that were collected at predefined time intervals were subsequently redispersed in order to evaluate the effect of moisture content on the size profile of the rehydrated powders. The example of cetyl palmitate particles fabricated in the presence of low amounts of surface active components is presented here (Fig. 5.6).

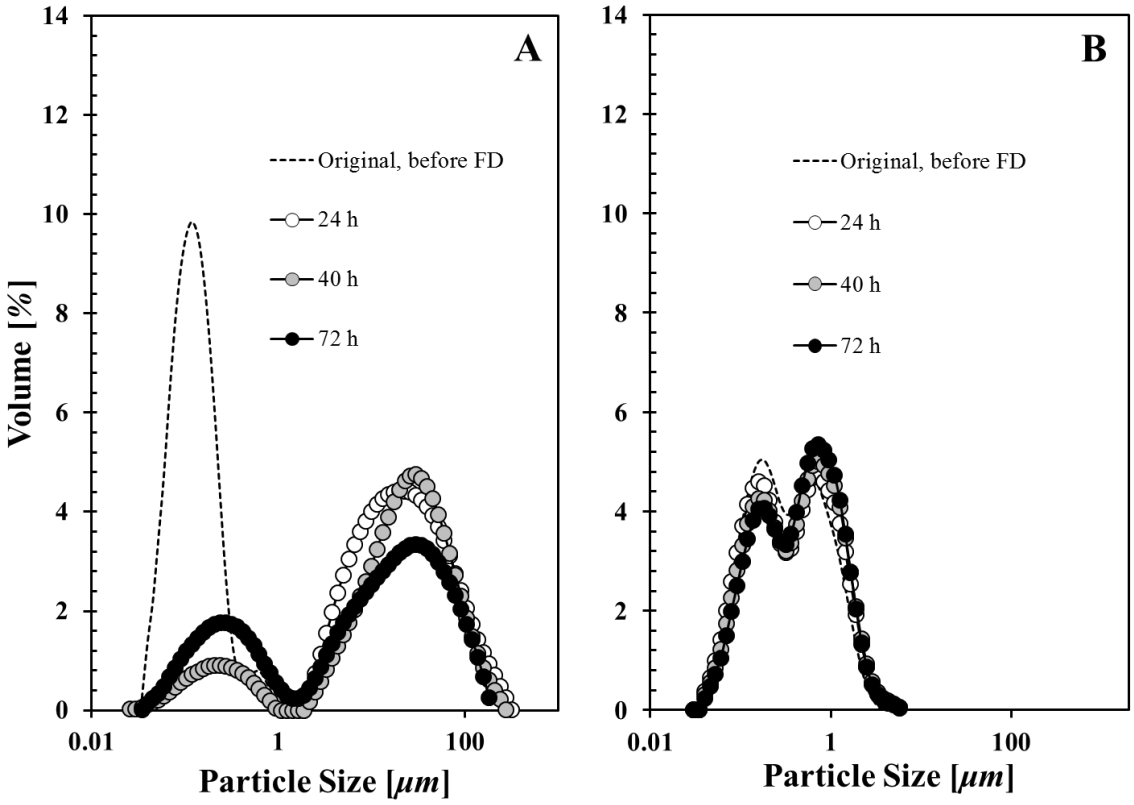

Fig. 5.6. Particle size distributions of dried and redispersed 2.5 wt/wt% solid cetyl palmitate particles produced in the presence of 0.8% Tween 80 (A) and 0.8% NaCas (B) for different drying times. Original wax particles with the same amounts of surfactants serve as a reference.

The samples that were removed after the course of 24 hours had cold vial surfaces and ice was noticeably present, indicating that the freeze-drying cycle was still at its primary drying stage. This was also confirmed by the high values of measured water activity (α_w in the vicinity of 0.6 in most cases), an effect which is only slightly more pronounced for the Tween 80 rather than the NaCas systems (Fig. 5.5B).

Increasing the drying time from 24 to 72 hours is accompanied by a gradual decrease in Tween 80-wax particles size up to 48 hours ($D_{4,3}$ = 40.65 μm, 35.64 μm, 14.96 μm) and an increase to 24.76 μm at the end of the cycle (Fig. 5.6A). It is not entirely clear whether these measurements are a true representation of what the structure is experiencing during this prolonged drying. It is highly likely that the discrepancies are an artefact of the measurement

as reflected by standard deviations in the range of ±1.4 - 2.1, mainly because of the large size of these particles. Overall, extending the run for another day seemed to be of no improvement to the performance of dried and rehydrated wax particles fabricated in the presence of Tween 80.

Antithetical to the small molecule surfactant, almost identical size distributions were obtained for sodium caseinate (at both 0.8 and 2 wt/wt% concentration), from which it can be stated that the protein acts to retain the original particle properties, independently of the duration of drying and the residual moisture contents (Fig. 5.6B). As long as the lipid droplets are sufficiently covered by protein molecules before being subjected to the freezing and drying stages, they remain protected against the stresses developed during the process and they overcome the inter-particle aggregation via the formation of a protein-rich thick interfacial film. It has been proposed that upon exposure to a convective drying environment, this film converts into a glassy matrix that grows in thickness along the drying process (Adhikari, Howes, Bhandari, & Langrish, 2009).

5.3.1.4 Effect of storage conditions

A one-month stability study was performed to evaluate the effect of different storage times and conditions (room or refrigerated temperature) on the performance of stored lyophilised powders upon their reconstitution. Water activity of the dried particulates was recorded over the course of one month. Dried formulations were rehydrated under all different conditions and the generated particle size was measured. Data obtained from these measurements are presented in Fig. 5.7.

The general trend across all stored formulations was that they suffered an increase in their mean particle size, even after one week storage at any temperature, an effect which was

significantly more pronounced for Tween 80-stabilised particles (Fig. 5.7A). Although the powders that were stored at 4 ºC were more exposed to much higher humidity levels, they appeared to maintain better the size obtained after one week. Upon storage, water activity values increase (i.e. water is less strongly bound) as the systems are extremely susceptible to moisture uptake, particularly when stored in a moisture-rich environment (i.e. refrigerator) (Fig. 5.7B). The effect of temperature was also crucial, as an increase in temperature was accompanied by a reduction in the mould-free shelf life.

In the presence of Tween 80 in the original formulation, the mean particle size of resuspended powders stored at low temperatures for one month appeared to experience no variations. However, the span value increased by almost 80% (data not reported) which clearly points to particle aggregation. Given that this aggregation tendency was not observed when cetyl palmitate particles formed in the presence of Tween 80 and not having undergone freeze-drying were stored for the same time period at 20 ºC, the behaviour must be due to the lyophilisation process and the different microstructures formed. The size of rehydrated wax particles produced with sodium caseinate remained unchanged after one month of storage at 4 ºC. Nonetheless, room temperature conditions allowed an intense particle agglomeration, as seen by a doubling in the $D_{3,2}$ values (Fig. 5.7A).

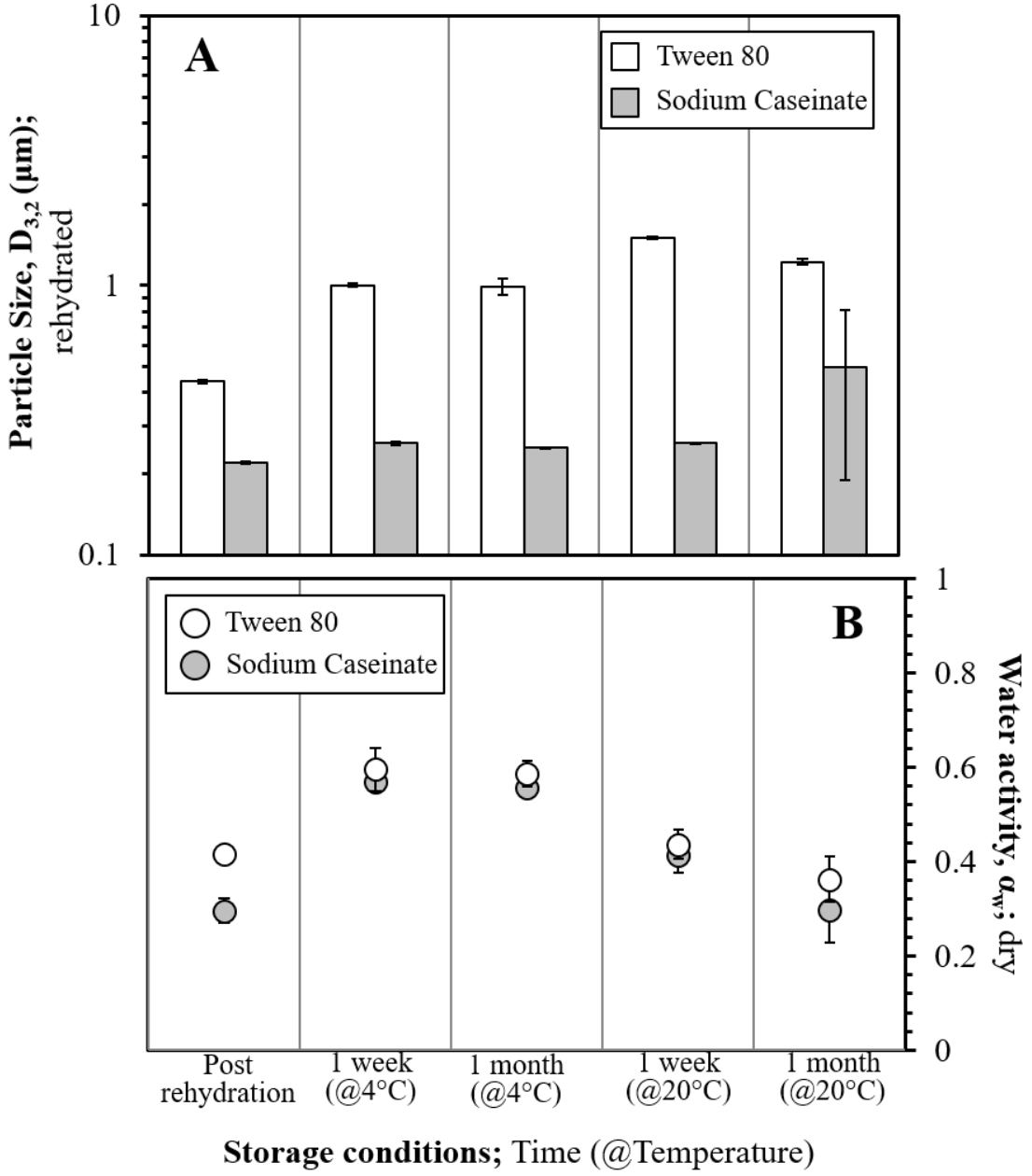

Fig. 5.7. Size properties (A) of redispersed cetyl palmitate particles formed with 0.8% Tween 80 or sodium caseinate under different storage times and conditions. Water activity of these systems measured in dry state (B).

5.3.1.5 Interfacial behaviour

The potential of particles to act as Pickering stabilisers of o/w emulsions is also dependent on their performance at an oil-water interface as has been shown in Chapter 3. Therefore, the aim was to study whether interfacial behaviour, analogously to size, can be maintained following drying and rehydration. The systems of focus here were reconstituted lipid particles that previously demonstrated a capacity to maintain their initial size post-lyophilisation (i.e. formed in the presence of sodium caseinate). The obtained profiles were compared to particles in the absence of that further treatment stage (Fig. 5.8).

As seen in Fig. 5.8 lipid particles formed in the presence of sodium caseinate (before FD) exhibit an intermediate behaviour ascribed to a level of surface active component being entrapped/contained within the crystalline structure as discussed in Chapter 3. Accordingly, interfacial tension increases but not to the level of either lipid particles formed in the absence of interfacially active entities, and not as low as neat surface active species in solution.

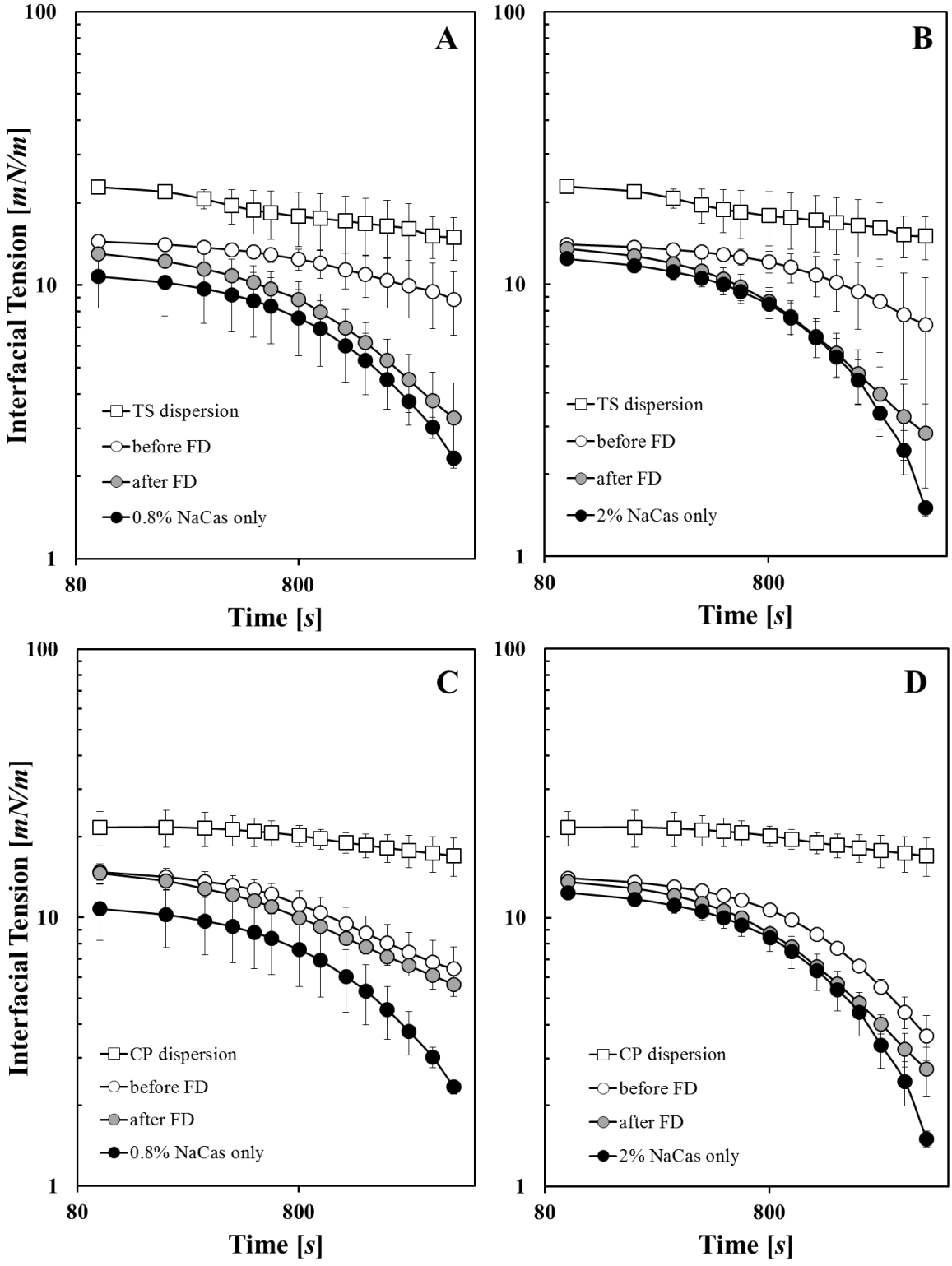

Fig. 5.8. Interfacial tension between sunflower oil and aqueous dispersions of solid tristearin (A,B) and cetyl palmitate particles (C,D) formed in the presence of 0.8 and 2 wt/wt% NaCas before and after freeze-drying (FD). Surfactants in solution are presented on the graph as a reference. All formulations contain 2.5 wt/wt% of lipid material.

However, upon freeze-drying interfacial tension data shows a behaviour close to solely NaCas systems, an effect which is slightly more accentuated in the case of resuspended tristearin particles (Fig. 5.8A, 5.8B) and for higher NaCas concentrations. An increased content of surface active species in the bulk which is responsible for IFT reduction (compared to before FD) is likely to suggest that some of the protein (NaCas) associated with particles has been removed. Clearly, more experiments or analytical methods would be needed in order to elucidate the exact composition of the interfacial layer and the effect of the freeze-drying process on it.

Preliminary interfacial tension measurements conducted with lipid particles and Tween 80 after lyophilisation, yielded a behaviour very similar to Tween only systems, or even in some cases exceptionally lower than that (see Appendix A3, Fig. A3.4). It appears that during freeze-drying part of Tween is removed from the particles and upon rehydration it is no longer associated with them, leading to irreversible aggregation as has also been discussed previously. This could be ascribed to the large size increase of those systems.

5.3.1.6 Emulsion stabilisation / Pickering functionality

It was previously shown that key features linked to Pickering functionality such as size and interfacial behaviour can be controlled via process and formulation parameters (Chapter 3). Additionally, in this Chapter we have demonstrated the maintenance of these attributes after a secondary processing step (i.e. lyophilisation). Such characteristics enabled lipid-based particles fabricated in the presence of surface active species to act as Pickering stabilisers in o/w emulsion systems (Chapter 4). It was thus the ultimate objective of this study to investigate whether particles withstand a drying and dessication environment and preserve their Pickering functionality.

To that end, lyophilised and reconstituted lipid particles formed with two different surface active species were used to produce 20% o/w emulsions. The effect of drying time on the ability of these systems to exhibit Pickering functionality was also investigated. In particular, it was explored whether duration of drying, therefore the moisture content of particles, had an effect on the capacity to stabilise emulsions. Droplet size distribution curves and mean droplet size values for o/w emulsions formed with cetyl palmitate particles are presented in Fig. 5.9 and listed in Table 5.1.

As can be seen from Fig. 5.9, the capacity to stabilise emulsions seems to recover after a freeze-drying and rehydration cycle. This data suggests that maintaining key particle attributes such as size and a level of IFT performance appears to also correspond to a recovery of Pickering functionality. This capacity seemed also not to be influenced by either short or longer drying times. Interfacial tension measurements for the NaCas-coated reconstituted lipid particles disclosed a profile that was nearly identical across the different drying times (see Appendix A3, Fig.A3.5).

Conversely, this was not the case with a lipid particles-Tween 80 mixture where all the resulting emulsions had larger average droplet size (e.g. ~4.11 μm for wax particles) compared to the non-dried particles (~1.25 μm) (see Appendix A3, Fig.A3.6) This was an expected result as bigger sized particles such as the ones acquired after the lyophilisation of Tween 80-present systems, would form and stabilise larger emulsion droplets.

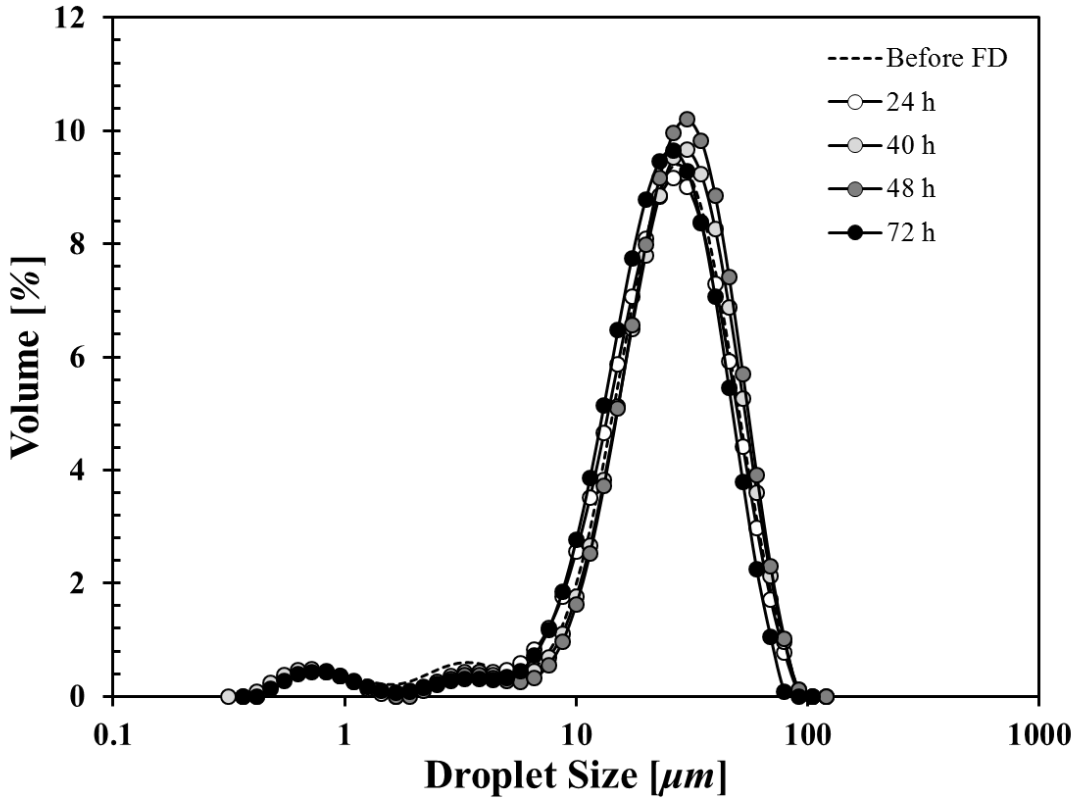

Fig. 5.9. Droplet size distribution curves for 20% sunflower oil emulsions formed with dried and reconstituted 2.5% wax particles and 0.8% NaCas for different drying times. The same emulsions produced with non-freeze-dried particles are also included for comparison.

Table 5.1. Mean droplet size and span values for 20% sunflower oil emulsions formed with dried and rehydrated 2.5% cetyl palmitate particles and different amounts of NaCas, for different drying times.

	0.8% NaCas			2% NaCas		
Drying time (h)	**$D_{3,2}$ (μm)**	**$D_{4,3}$ (μm)**	**Span**	**$D_{3,2}$ (μm)**	**$D_{4,3}$ (μm)**	**Span**
0	10.4±1.5	28.9±2.7	1.8±0.1	8.4±0.3	20.2±3.2	1.7±0.1
24	15.0±4.1	31.2±3.1	1.5±0.1	8.8±0.7	31.1±0.1	1.8±0.1
40	13.0±1.1	31.2±0.1	1.5±0.1	9.3±0.3	26.7±3.8	1.7±0.1
48	22.1±1.5	31.5±1.9	1.4±0.1	8.6±0.6	24.0±2.6	1.8±0.1
72	15.0±3.5	34.5±9.7	1.7±0.3	7.6±0.5	20.5±0.7	1.8±0.1

O/W emulsions were also produced after resuspending the powders that were stored for different time lengths. As shown previously, wax-based dried powders were more stable when stored at 4 ºC rather than at 20 ºC, hence the systems stored at the former temperature were chosen to stabilise sunflower-in-water emulsions. The resulting droplet sizes for a short and a longer duration storage time as a factor of the type of the surface active component (here NaCas) are presented in Fig. 5.10.

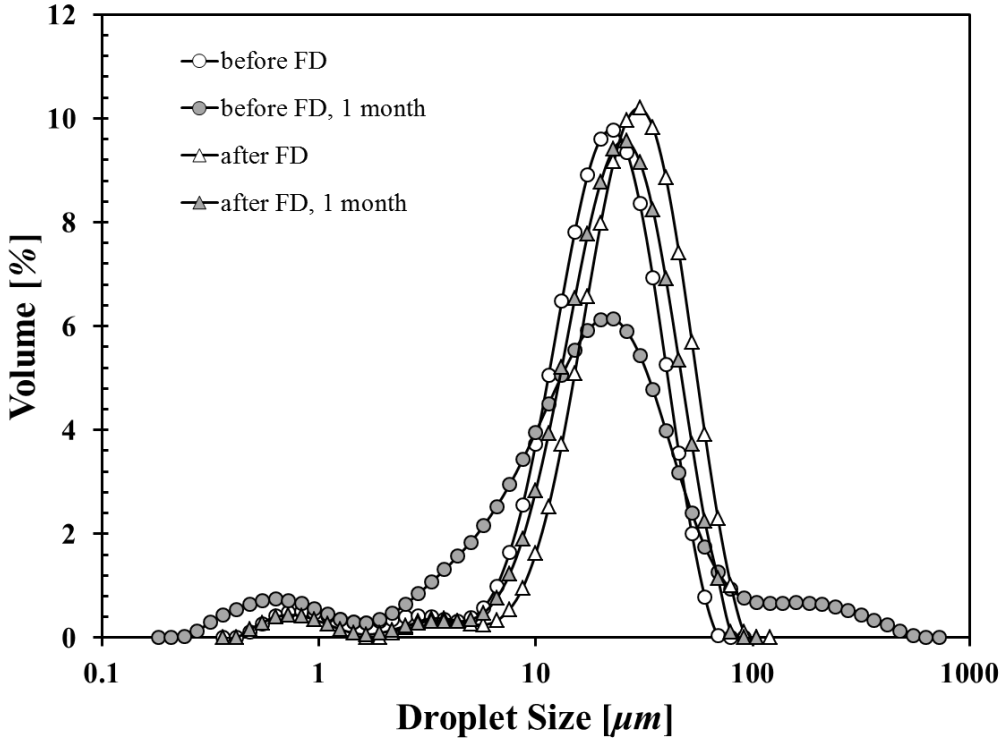

Fig. 5.10. 20% o/w emulsions formed with dried and rehydrated 2.5% wax particles and 0.8% NaCas stored for different times at refrigeration temperatures. The same emulsions produced with non-freeze-dried particles are also included for comparison.

In contrast to cetyl palmitate systems that were not subjected to a lyophilisation process, the emulsions formed with the dried and redispersed ones, appeared to be less sensitive to coalescence, with only minor changes in their droplet sizes after one month storage in the fridge. This behaviour is somewhat improved in the case of Tween 80 (see Appendix A3,

Fig. A3.6). It seems that dried lipid particles not only maintain their Pickering functionality following a freeze-drying step, but also confer enhanced stability to coalescence when used as o/w emulsion stabilisers. These observations are speculative and require a more extensive study to elucidate the impact of a lyophilisation and rehydration process on the subsequent behaviour of particles as emulsion stabilisers.

5.4 Conclusions

In conclusion, this study showed that the choice of surface active species during the initial fabrication of lipid particles could be very crucial when it comes to drying or isolating the particulates from their aqueous environment. Sodium caseinate emerged as a "smart" drying aid which enabled particles to withstand the harsh freezing and desiccation conditions and retain their original size. This is in stark contrast to a low molecular mass surface active component (e.g. Tween 80) or the conventional cryoprotective agents which did not enable the conservation of the initial particle properties. In parallel, rehydrated particles formed with NaCas could generate o/w emulsions with droplets of a size similar to emulsions formed with particles that had not undergone the additional processing step.

Lipid-based Pickering particles and emulsions stabilised by such particles could be destined not only for food but also for cosmetics, pharmaceutical or agrochemical applications and, in particular, when drying is the intention. In the long run, it is envisaged that dried particulates could be manufactured by a pool of alternative templates such as natural lipids and waxes (e.g. carnauba, rice bran) together with thick interfacial layer forming surface active components and the use of rapid drying techniques (e.g. microwave vacuum drying).

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Chapter 6

Emulsions co-stabilised by edible Pickering particles and surfactants: The effect of HLB value

Data and discussions contained within this chapter have been published within:

Zafeiri, I., Horridge, C., Tripodi, E. and Spyropoulos, F. (2017). Emulsions co-stabilised by edible Pickering particles and surfactants: The effect of HLB value. *Colloids and Interface Science Communications.* 17, pp. 5-9

Synopsis

This study focuses on the impact of surfactants' hydrophilic/lipophilic balance (HLB) value on emulsions also co-stabilised by Pickering particles. This co-stabilisation approach was investigated by using hydroxypropyl methylcellulose (HPMC) as the food-grade Pickering particle and two surfactants typically used for the stabilisation of oil-in-water or water-in-oil emulsions; Tween 80 (high HLB) and PGPR (low HLB), respectively. Mixed-emulsifier systems of HPMC together with either of the two surfactants were initially interfacially characterised and subsequently used to formulate and stabilise o/w emulsions through different processes. Co-stabilised emulsions possessed smaller droplet sizes and exhibited enhanced stability against coalescence. Results suggest that both these features were influenced by the concentrations of the two components and more importantly by surfactant's HLB value. It was further demonstrated that the reported co-stabilisation approach can provide stable o/w emulsions even in the presence of surfactants of low HLB value. These findings persisted even when emulsions were generated under intense shearing.

Over the last two decades there has been an upsurge of research interest in the study of Pickering emulsions. The benefits of having particle-laden interfaces, mainly relating to prolonged emulsion stability, have been extensively reviewed (Binks, 2002; Dickinson, 2012). Nonetheless, adoption of Pickering stabilisation strategies within the foods arena is still somewhat hampered due to the challenges associated with the development of Pickering structures from edible elements (Berton-Carabin & Schroën, 2015; Pichot, Duffus, Zafeiri, Spyropoulos, & Norton, 2014). Polysaccharides and proteins are the two most commonly investigated materials when it comes to the use of particles of biological origin as stabilisers of emulsions and foams. Amongst semi-crystalline polysaccharides, several cellulose derivatives of interesting particle shapes have been utilised; e.g. bacterial cellulose fibres, ethylcellulose, hydroxypropyl methylcellulose phthalate and starch (Lam, Velikov, & Velev, 2014).

Hydroxypropyl methylcellulose (HPMC) is a hydrophobic cellulose derivative approved for use in foodstuffs; e.g. baked goods, sauces, dressings, and whipped toppings (Coffey, Bell, & Henderson, 1995). A chemical modification that involves addition of methyl and hydroxypropyl groups to the anhydroglucose backbone induces increased polymer hydrophobicity due to these groups. This renders the polymer interfacially active; in essence being able to adsorb to liquid-liquid interfaces and lower interfacial tension (Camino & Pilosof, 2011; Camino, Sánchez, Rodríguez Patino, & Pilosof, 2011). High hydrophobicity leads to the polymer forming aggregates within an aqueous environment, hence adopting a behaviour that resembles more that of a colloidal particle. Several studies have reported on the ability of HPMC to form and stabilise emulsions, mainly evaluating the effect of the polymer's molecular weight, degree of substitution with methoxyl groups (Schulz & Daniels,

2000) or that of its blends with other biopolymers such as β-lactoglobulin (Camino, Sanchez, Rodríguez Patino, & Pilosof, 2012).

For Pickering particles acting as emulsion stabilisers, emulsion type is governed by the balance between the hydrophilic and lipophilic domains of their microstructure, in this case best described by their wettability (Pichot, Spyropoulos, & Norton, 2009). This is somewhat different to surfactants which have traditionally been used to stabilise emulsions, due to their inherent amphipathic character. The type of emulsion that a given surfactant species tends to facilitate forming (oil-in-water, o/w, or water-in-oil, w/o) can be primarily predicted based on its hydrophilic-lipophilic balance (HLB) value. When used in conjunction with colloidal particles, low molecular weight surfactants (even at relatively low concentrations) have the potential to provide emulsions with much lower droplet sizes compared to systems where each of these entities is used as the sole interfacial stabiliser (Pichot, *et al.*, 2009). It has been proposed that long-term storage stability in an o/w emulsion containing both a surfactant and colloidal particles is ensured via a two-part synergistic mechanism where each component has a well-defined task (Pichot, *et al.*, 2009; Pichot, Spyropoulos, & Norton, 2010). This behaviour was found to be dependent not only on the concentrations of both components but also on the type of surfactant employed (Pichot, *et al.*, 2010). Surfactant usage in the presence of colloidal particles can therefore be exploited as a means of adjusting particle wettability and thus tailoring the emulsion's interfacial composition. The majority of work in this area has focused on mixtures of silica nanoparticles with a range of surfactant species, with studies on colloidal particles of closer association to what would be perceived as an edible Pickering structure being scarce. For example, it has been demonstrated that when cellulose ethers were mixed with anionic or double chain cationic surfactants, a time-dependent synergy was developed between the neutral polymer aggregates and the surfactant (Dal-Bó, Laus, Felippe,

Zanette, & Minatti, 2011; Manousakis & Avranas, 2013). Nevertheless, both these studies focused on analysing the dynamic and equilibrium adsorption behaviour of these polymeric-surfactant mixtures at the air/water interface and in the presence of electrostatic effects.

The current study investigates the impact of the surfactant's HLB value on the formation and stability of mixed-emulsifier stabilised emulsions (systems stabilised by both surfactants and Pickering particles) produced through different processing routes. In this view, o/w emulsions stabilised solely by conventional surfactants of markedly different HLB characteristics (Tween 80, PGPR) or edible colloidal particles (HPMC) or mixture of both species, were formed using different emulsification methods. For all investigated systems the achieved emulsion droplet sizes and their storage stability were assessed, and the role of the HLB value of the used surfactant on emulsion behaviour was determined.

For the stabilisation of the o/w emulsions the non-ionic synthetic surfactants Tween 80 (HLB_{T80}=15, M_{T80}≈1310 g/mol) and PGPR (HLB_{PGPR}=1.5, M_{PGPR}≈353.51 g/mol), which are typical o/w and w/o stabilisers respectively, were used in the study. HMPC (M_{HPMC}≈86 kDa) was employed as the edible Pickering particle component. Both particle and surfactant concentrations in the investigated systems are calculated and provided as weight percentages of the overall mass of the relevant emulsion (wt%). Mixed-emulsifier stabilised emulsions were fabricated by initially dissolving the surfactant component (depending on its specific HLB value) in either the distilled water or sunflower oil phases; as such, Tween 80 was initially dissolved in the water phase while PGPR in the oil phase. HPMC was added to the surfactant solutions and was then mixed with either pure sunflower oil or distilled water to emulsify them using a high shear mixer (L5M, Silverson, UK). These o/w emulsions were analysed as such or were further processed using an air-driven microfluidiser device (M110-S, Microfluidics, USA). Following production, all mixed-emulsifier stabilised emulsions were

assessed in terms of their stability against coalescence via droplet size measurements conducted using laser diffraction (Mastersizer Hydro 2000SM, Malvern, UK). In addition, the interfacial behaviour of all systems was measured as a function of time through equilibrium interfacial tension measurements at room temperature. These were carried out in a tensiometer (K100 Krüss, Germany) using the Wilhelmy plate method.

The ability to stabilise emulsions stems from the ability to lower interfacial tension, hence the two systems were analysed as a pure particle/surfactant or as a mixture of particles and surfactant for their behaviour at the water-sunflower oil interface. Fig. 6.1 depicts the interfacial tension between water and oil as a function of the concentration of Tween 80 in the presence of a constant concentration of colloidal particles.

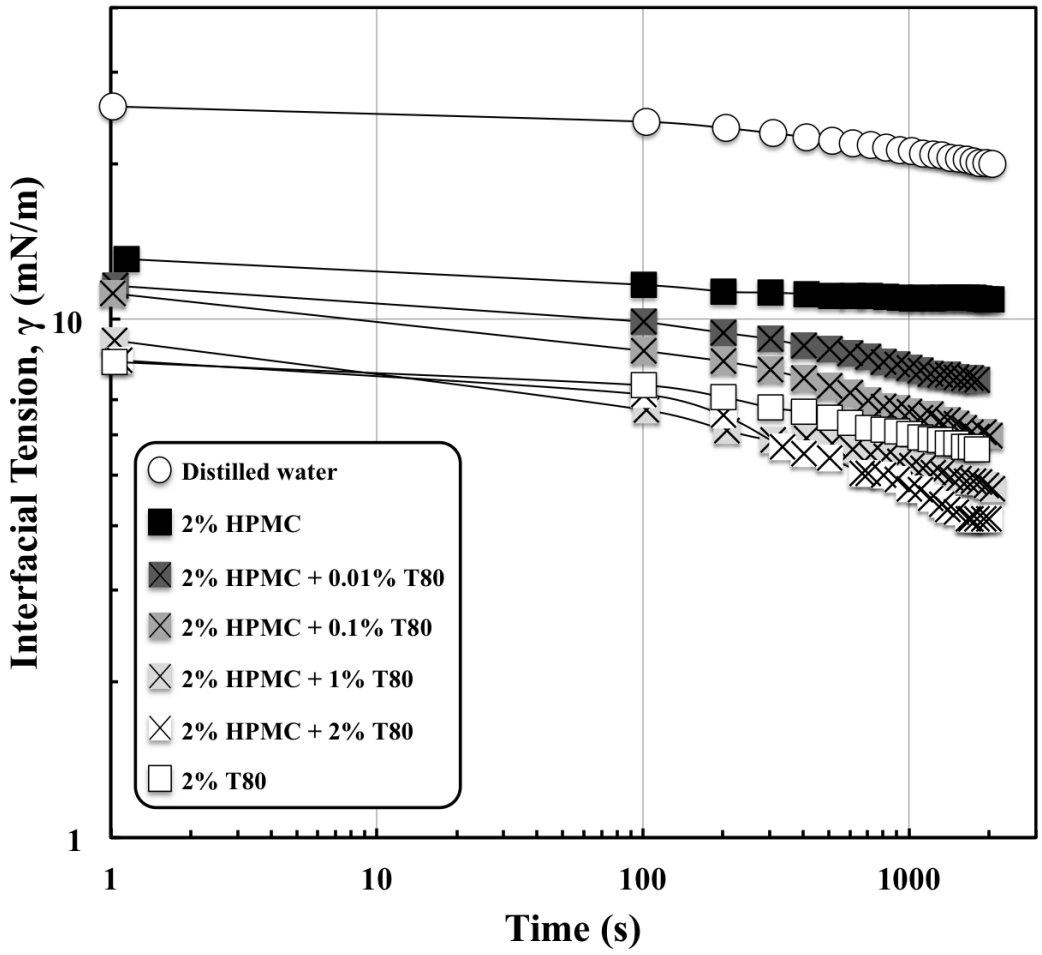

Fig. 6.1. Dynamic interfacial tension data (between sunflower oil and an aqueous phase) as a function of a pure system containing particles or surfactant and of a mixed system containing 2 wt% HPMC and different concentrations of Tween 80. A logarithmic scale in both axes gives a better view of the time-scale of the experiment.

Although it is debatable whether significant differences exist, the interfacial tension profile shows some trends that are worth discussion. Fig. 6.1 demonstrates that HPMC particles delay the adsorption at the interface in the case of mixed systems as the values obtained at t=1 sec, range between the pure HPMC and Tween 80 system (13.07 mN/m and 8.27 mN/m respectively). At the initial stages of the measurement that are more related to droplet break-up and formation, the values follow the stepwise addition of surfactant (i.e. decrease when the amount of surfactant increases). Similarly to the case of hydrophilic silica particles and

Tween 60 (Pichot, Spyropoulos, & Norton, 2012), the ability of a high HLB value surfactant to reduce interfacial tension is affected by the presence of colloidal particles and seems to be dependent on the amount of surfactant. More specifically, by increasing the concentration of Tween 80 in the mixed-emulsifier systems the interfacial tension decreases significantly for low amounts (7.6 mN/m for 0.01 wt% and 6.04 mN/m for 0.1 wt%) and only slightly for concentrations above 1 wt%.

For higher concentrations of Tween 80 (≥1 wt%) in the mixed-emulsifier systems, the interfacial tension evolves differently and becomes even lower than solely Tween 80 after 16 minutes (5.6 mN/m at equilibrium), anticipating a much more efficient droplet break-up during emulsification and a subsequent droplet size decrease. This slight reduction, compared to the pure surfactant, could be attributed to the fact that the concentration of Tween 80 in the aqueous phase when HPMC particles are present, is slightly higher than Tween 80 on its own, affecting accordingly the tension at the interface. It is also possible that this behaviour is a result of a rearrangement of the stabilising species taking place at the interface. HPMC particles are potentially displaced from the interface, driven by the increase in Tween 80's concentration in the system.

This synergism between a polymeric based particle and a surfactant system has been observed before for mixtures of cellulose ethers such as ethyl(hydroxyethyl)cellulose (EHEC) or HPMC with ionic surfactants. The interaction between these two components engenders associated structures with higher surface activity than each of the entities alone (Manousakis, *et al.*, 2013). These interactions have a great practical importance such as, among others, reducing the total amount of surfactant is tremendously appreciated from an environmental and economic perspective (Dal-Bó, *et al.*, 2011).

The effect of a low HLB surfactant on the interfacial tension in the presence or absence of HPMC particles is shown in Fig. 6.2. As opposed to Tween 80, for concentrations of PGPR as low as 0.1 wt% the mixed system behaves identically or almost identically to the pure surfactant. Similar to previous observations apropos the mixture of silica particles and a w/o surfactant (e.g. lecithin) (Pichot, *et al.*, 2012), HPMC particles appear to have a negligible effect on the interfacial tension, particularly at surfactant concentrations ≥0.1 wt%. An additional difference between Fig. 6.1 and Fig. 6.2 lies in the thermodynamic equilibrium state that in the case of Tween 80 is not reached within the time frame of 1000 sec as interfacial tension decreases continuously with time, while for PGPR it is reached immediately upon adsorption of surfactant into the interface. Caused by variations in surfactants' architectural characteristics which affect how they pack at the interface, steric hindrance is what prevents or allows impurities in the oil phase to enter the interface and affect the behaviour (Pichot, *et al.*, 2012).

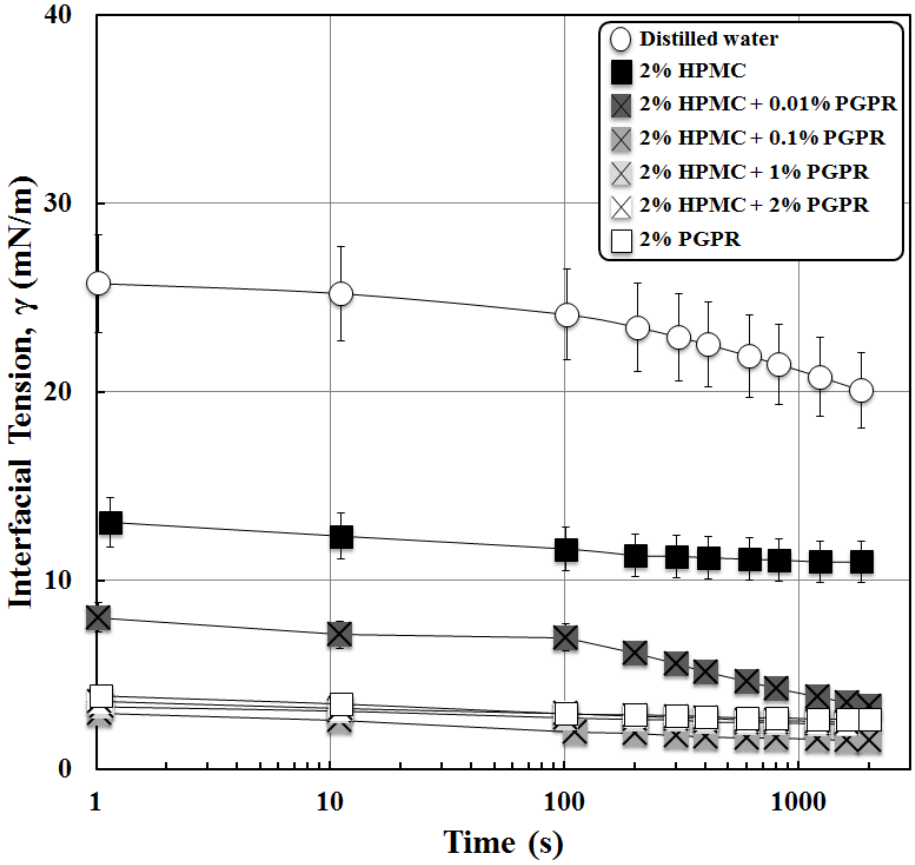

Fig. 6.2. Dynamic interfacial tension data as a function of a pure system containing particles or surfactant and of a mixed system containing 2 wt% HPMC and different concentrations of PGPR.

Having determined the interfacial performance of different HLB value surfactants in the presence of colloidal particles, the role of these systems on the droplet size and stability of emulsions was studied next. Oil-in-water emulsions (w_{oil}=0.1) were stabilised solely by either 2 wt% HPMC or 2 wt% Tween 80 and also conjointly by HPMC and various amounts of Tween 80, and were prepared by high-shear mixing (2 minutes at 10,000 rpm). Fig. 6.3 shows the generated droplet size ($D_{3,2}$) values as a function of surfactant concentration measured immediately after emulsification and after one and two weeks of storage at room temperature.

With the exception of 0.01 wt% Tween 80 in the mixed-emulsifier systems, all emulsions fabricated with the o/w surfactant in the presence or absence of Pickering particles were stable

against coalescence with only minor changes in droplet size with time. Droplet sizes of particle and surfactant-stabilised emulsions showed no notable difference between them (7.7 μm and 7.1 μm following emulsification), despite the lower interfacial tension values of Tween 80 as discussed above. The balance between the competing events of droplet break-up and re-coalescence that take place during the emulsification is the one that determines the final emulsion droplet size. Tween 80 might be able to promote droplet break-up via the reduction of the interfacial tension, but HPMC particles, as long as they breach the oil/water interface, are more efficient in reducing the coalescence rate, producing eventually similar droplet sizes.

Upon addition of Tween 80 to a 2 wt% HPMC aqueous solution, emulsions of smaller droplet sizes are produced (Fig. 6.3). The average droplet diameter is reduced as a function of the surfactant concentration for the entire concentration spectrum investigated, while in all cases it is significantly smaller than those in the presence of surfactants or particles alone. Importantly, the reduction in droplet size follows the same trend as the interfacial tension profile, i.e. a gradient reduction with increasing surfactant concentration.

The observed droplet size pattern comes into contrast with the behaviour reported previously for emulsions containing silica particles and o/w surfactant (Tween 60 or sodium caseinate) (Pichot, *et al.*, 2010). In that case, variations in surfactant concentration dictated the location of the particles in relation to the interface which, in turn, dictated the final droplet size. Specifically, Pichot, *et al.* (2012) identified a surfactant concentration above which there is no synergistic action between the surfactant and the colloidal silica particles, and the interfacial tension profile is determined only by the amount of surfactant. It was argued that this behaviour is closely linked to the positioning of the particles at the emulsion interface. In our study, 0.01 wt% Tween 80 in the mixed system fulfills its role of promoting further break-up,

generating smaller size droplets post-emulsification; yet this behaviour is only temporary as, after 7 days, the system returns back to a particle-stabilised emulsion. The fact that mixed-emulsifier stabilised emulsions of higher Tween 80 concentrations retain their initial size after 2 weeks suggests that given the increased amount of surfactant, the tendency for droplet-droplet collision is minimised.

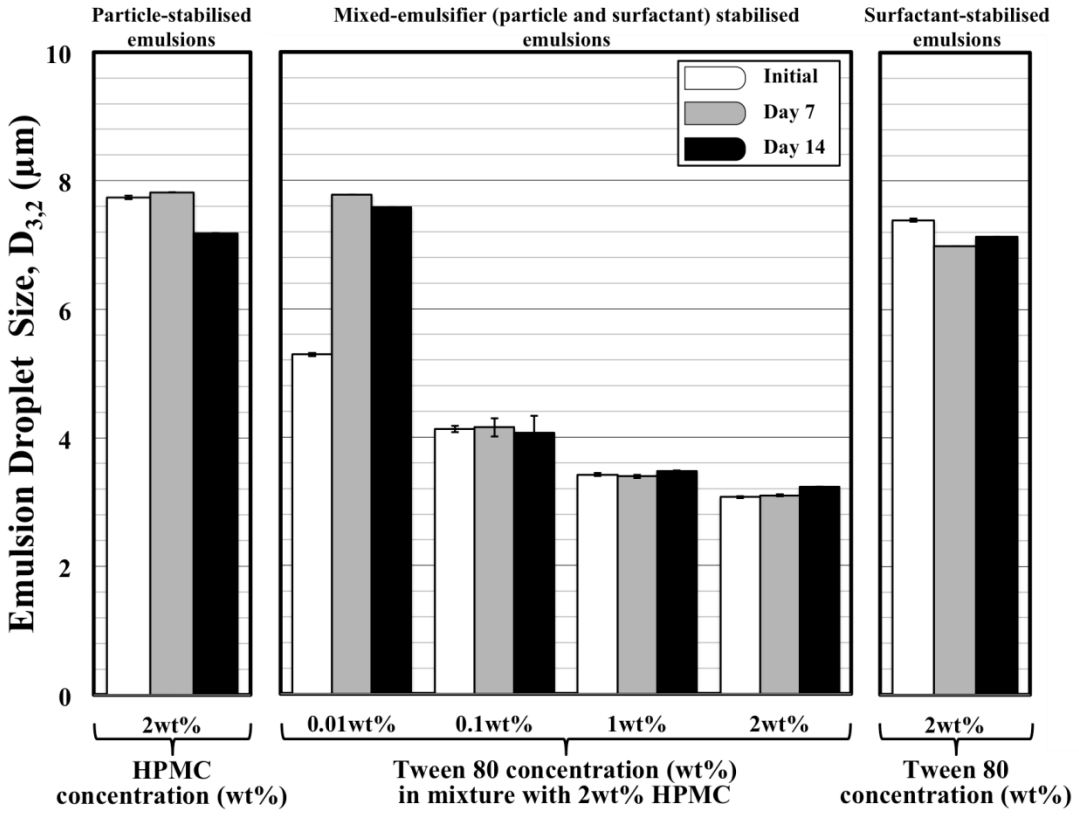

Fig. 6.3. Average droplet sizes of emulsions stabilised by solely HPMC, solely Tween 80 and different concentrations of Tween 80 in the presence of 2 wt% HPMC over time and as a function of the surfactant concentration. Where not visible, error bars are smaller than symbols.

Having demonstrated that a high HLB surfactant enhances the functionality of these mixed systems, we sought to investigate whether such synergism persists in the case of low HLB value surface active species, e.g. PGPR. The dependency of emulsions' droplet size on the

surfactant's concentration, in the absence and presence of 2 wt% HPMC particles, is shown in Fig. 6.4 along with their evolution with storage time.

The inclusion of PGPR in the formulation affects the resulting droplet sizes considerably, albeit in a different manner to Tween 80 systems. Although PGPR on its own initially seems to encourage droplet disruption to the same extent as Tween 80 (Fig. 6.1, Fig. 6.2), it eventually fails to render the droplets stable against coalescence and leads to a phase-separated system. This is usually the case as emulsifiers of low HLB values will tend to stabilise w/o rather than o/w emulsions. However, when used in combination with HPMC particles and at low concentrations of PGPR, a significant benefit arises; in fact, a 22.8% and a 35.6% decrease in droplet size is observed when small concentrations of the surfactant (0.01% and 0.1% respectively) are used together with 2% cellulose particles. As Fig. 6.4 shows, at concentrations of PGPR up to 1%, droplet sizes decrease and are overall slightly bigger than HPMC-Tween 80 stabilised ones. For high PGPR amounts (i.e. ≥1 wt%), there is no further benefit observed through subsequent surfactant addition, and o/w emulsions produced are not stable, with large droplets appearing over time (Fig. 6.4).

The mechanisms that lie behind the adsorption of particles and surfactants to the interface during the emulsification process could account for this behaviour. In emulsions stabilised by a mixture of silica particles and lecithin, SEM analysis of the emulsion microstructure revealed that there is no dependency between the surfactant concentration and the acquired droplet size (Pichot, *et al.*, 2010). Emulsions were stabilised by the particles (Pickering stabilisation) and any increase on lecithin's concentration did not lead to their desorption from the interface. Nevertheless, this is not entirely true with our system as at 2 wt% PGPR concentration, a small deposit of particles was observed at the bottom of the vessel. One could argue that this was the result of particles' displacement, which also explains the more

surfactant-dominated performance as seen in Fig. 6.4. Further experiments with different concentrations of PGPR and visualisation of the emulsions' droplet surface would shed some light on the exact mechanism(s) taking place.

A comparison between Fig. 6.3 and Fig. 6.4 suggests a far superior performance of PGPR than Tween 80, at very low concentrations. An important factor is the surfactant's HLB value and its molecular architecture. We hypothesise that a low HLB value surfactant has a stronger tendency than a one of high HLB value to shift the hydrophobicity of HPMC at lower concentrations and therefore render an o/w emulsion more stable. Essentially, PGPR having more lipophilic sites than Tween 80 has more chances to bind to the somewhat hydrophobic particles through hydrophobic interactions and increase the hydrophilic character, making them more stable in the aqueous phase. In addition, the molecular geometry of a surfactant determines factors such as the critical packing parameter, which in turn affects interfacial curvature. The highly lipophilic PGPR having an area per chain larger than the hydrophilic head group has a tendency to curve around water. This surfactant configuration does not favour long-term stabilisation of o/w emulsions. This change in curvature angle could also be responsible for the larger droplet sizes of PGPR as opposed to Tween 80 mixed stabilised emulsions.

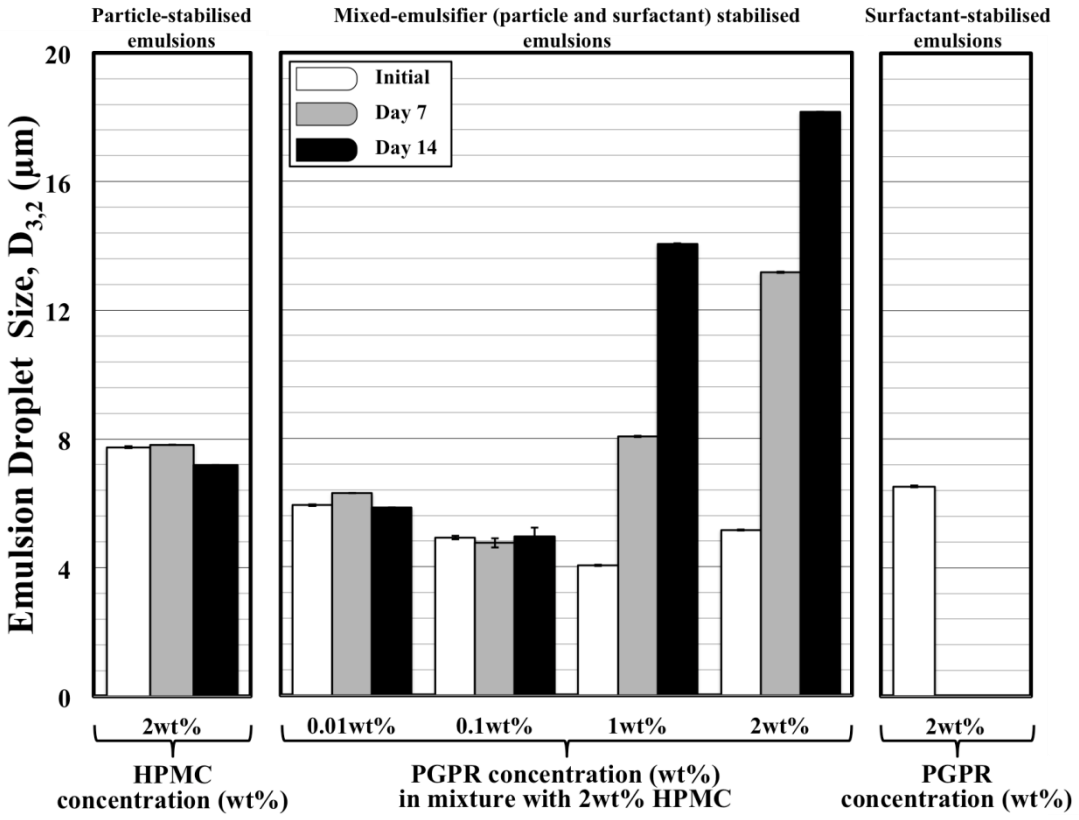

Fig. 6.4. Average droplet sizes of emulsions stabilised by solely HPMC, solely PGPR and different concentrations of PGPR in the presence of 2 wt% HPMC as a function of the surfactant concentration.

The effect of processing method, and in particular how the effect of a surfactant's HLB value would be altered by using an emulsification process of significantly higher energy input was investigated next. A series of o/w emulsions were produced with 10 wt% sunflower oil and the mixtures HPMC-Tween 80 and HPMC-PGPR at the same concentration regimes as the emulsions produced using a high-shear mixer. Droplet size data for the two types of mixed systems at 1000 bar and for one pass as measured straight after emulsification and after one week storage at room temperature, are shown in Fig. 6.5.

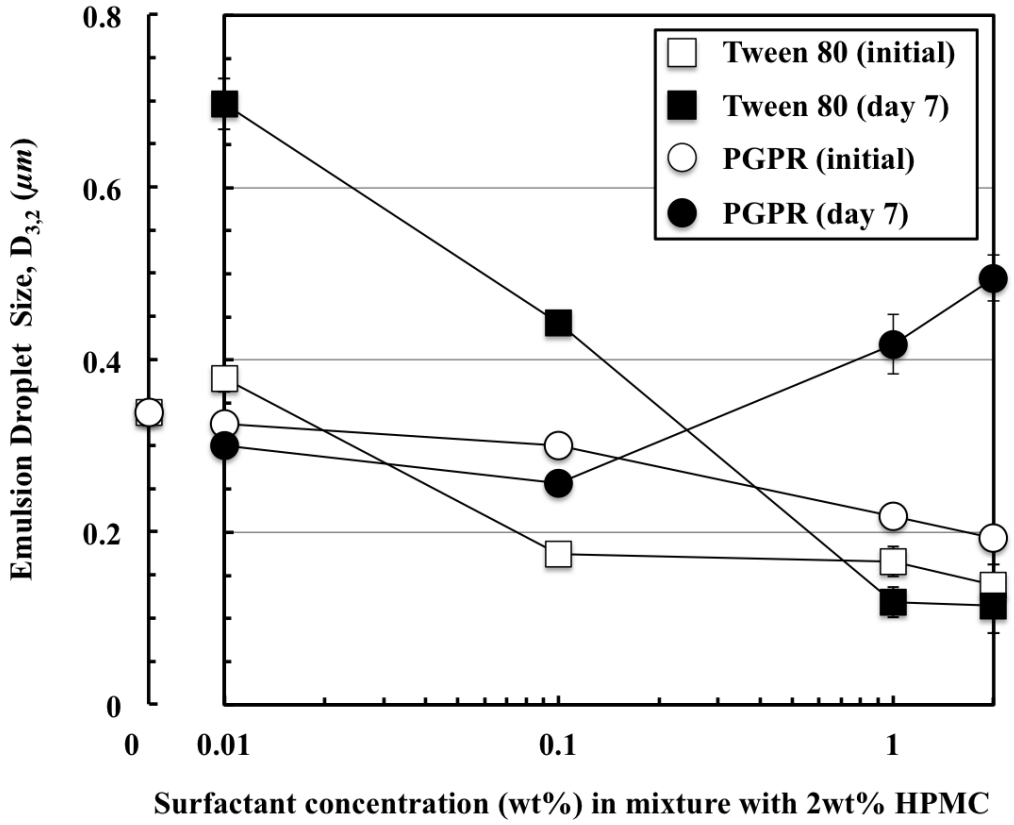

Fig. 6.5. Average droplet sizes of 10% o/w emulsions stabilised by mixed HPMC-Tween 80 and HPMC-PGPR (2 wt% particles & 2 wt% surfactant) produced at 1000 bar using the microfluidiser, as a function of the surfactant concentration.

Fig. 6.5 clearly suggests that the exertion of high pressures in both systems induced a dramatic decrease in the generated droplet sizes. A single pass through the microfluidiser was sufficient to produce submicron emulsion droplets with a size range of 200 – 400 nm in both cases. The trends observed are quite similar to the ones acquired by the high shear device, however for both surfactants a more or less constant size is reached for concentrations above a specific value. Long-term stability was also conferred to the systems in a way rather similar to the stability of emulsions produced with a high-shear device.

In terms of the mixed systems containing the high HLB value surfactant, at low concentrations of Tween 80 (0.01 wt%), the effect of the surfactant component seems to be

negligible. Indeed, the resulting emulsion has a mean droplet size of 0.378 μm which is even higher than the HPMC particles on their own (0.338 μm). A gradual increase in the amount of Tween 80 leads to a 58% decrease in the droplet size which then almost reaches a plateau up to the highest concentrations. The mixed HPMC and PGPR systems behaved analogously. Unlike the emulsions produced on the high shear mixer, over the same surfactant concentration range, droplet sizes continue to decrease with subsequent addition of PGPR, steeply for concentrations up to 1 wt% and at a less rapid rate for 2 wt%. Fig. 6.5 obviously illustrates the absence of droplet size dependency on surfactant concentration for concentrations higher than 0.1 wt% and 1 wt% for the Tween 80 and PGPR mixed systems respectively. This pattern variation probably implies a different location of particles and surfactants in the bulk and at the interface, dictated by the distinct shear regime to which the emulsions are subjected inside the microfluidiser.

The intense disruptive forces applied on the system in the microfluidiser result in an increased specific surface area. The increased interfacial area of the emulsions compared to that produced on a high shear mixer will require higher concentrations of surfactant available to stabilise and prevent coalescence. Upon increasing the surfactant concentration in a mixed-emulsifier stabilised emulsion, the surfactant begins to dominate the system at much lower concentrations compared to a high shear device, as more of it will be used to cover the increased interfacial area.

In summary, this work advances the current understanding on mixed-emulsifier stabilised emulsions, by specifically focusing on the influence of the (employed) surfactant's HLB value. This study demonstrates that addition of small amounts of surfactant can enhance the functionality of an edible Pickering particle component in terms of the emulsion droplet sizes that are generated and the stability to coalescence that is induced. The findings provided here

offer strong evidence that these emulsion features are directly influenced by the concentrations of the two components and more importantly by the surfactant's HLB value. In addition, the co-stabilisation approach reported here is shown to provide stable oil-in-water emulsions even in the presence of surfactants of low HLB value (conventionally used as stabilisers of water-in-oil emulsions). Furthermore, the impact observed persists across different processing conditions (i.e. at relatively low and high shear environments). The observed synergy between the two interfacial entities is foreseen to provide applications in a wide range of commercial settings where optimisation of emulsions structure, utilisation of sustainable interfacial species and/or reduction of surfactant content are required.

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Chapter 7

Overall concluding remarks and future recommendations

7.1 Overall Conclusions

The principal objective of this work was to deliver the design rules for particles to provide Pickering stabilisation for o/w emulsions, and also the means of their manufacture. Despite the fact that, as discussed beforehand, Pickering stabilisation technologies hold an immense potential, their application in foods is currently hindered by the limited knowledge on edible structures that can act as Pickering particles. This was confronted in this thesis, by the use of lipids as the building blocks of the particulates, deployed as templates to construct lipid crystals and subsequently, candidates for Pickering stabilisation.

The main conclusions derived from this work are summarised in the following sections:

Solid lipid particles in the micron and nanoscale were fabricated from the food-grade tristearin and the model wax cetyl palmitate. Crystalline particles were produced by the melt-emulsification method in the presence of a low (i.e. Tween 80) and a high (i.e. sodium caseinate) molecular weight surface active component. The obtained oil-in-water emulsion was cooled to form solid lipid particles, which were characterised for their mean size and size distribution, thermal behaviour, behaviour at an oil/water interface, and long-term physicochemical stability.

Carrying out emulsification and formation of crystalline structures as separate processes enabled both experimental conditions and the resulting particles' properties to be well-controlled. Same concentrations of Tween 80 led to different size behaviour patterns between triglyceride and wax particles due to the distinct liquid-crystalline transition times of the two lipid sources. The disparity in the adsorption kinetics of the two surface active species during the (hot) o/w emulsion formation, together with their size, appeared to play a determining role in the width of particle size distribution. Lipid nanoparticles (of either lipid) with a mean

particle diameter of ~110 nm (which was the bottom limit for the employed ultrasound method) and a (close-to-) monodisperse size distribution were produced in the presence of 2 wt% Tween 80 and remained stable (at 4 °C) for 12 weeks. In contrast, all the distribution profiles were bimodal when NaCas was present during particle formation. Yet, these particles exhibited good storage stability imparted by a thick interfacial film that acts as a barrier against particle-particle interactions, thus aggregation.

Interfacial tension measurements suggested differences in interfacial composition as a function of the type and the concentration of the amphiphilic agent. In particular, interfacial tension recordings for lipid particles constructed in the presence of surface active species were, in most cases, within the ranges defined by solely particulates in dispersion (highest value) and solely surface active entities in solution (lowest value). Introducing the interfacial species post-fabrication of particles generated a different interfacial profile, pointing to entrapment of these species within the crystalline structure during the particles' production step.

Assessment of lipid particle thermal and polymorphic behaviour revealed a strong dependency on the type of the surface active component. Interactions between Tween 80 and lipids due to molecular structure compatibility had a more significant influence on the melting profile and the rate of polymorphic transitions; β' and β-forms were observed for tristearin particles fabricated in the presence of Tween 80, while sodium caseinate at similar concentrations stabilised the α polymorph and slowed down β formation. Dissimilarities in the original crystal structure affects the subsequent polymorphic behaviour, therefore polymorphism was significantly supressed in the case of cetyl palmitate.

The properties of the manufactured solid lipid particles could render them ideal Pickering emulsion stabilisers. This potential was explored by forming o/w emulsions in their presence. Emulsions were stable to coalescence for 10-30% dispersed phase mass fraction for up to two months (stored under refrigeration conditions). The resulting droplet size post-emulsification was mostly related to the processing route, with yielded volume-weighted mean droplet size of ~1.5 μm and ~30 μm following sonication and high-shear mixing respectively. Droplet size was found not to be greatly dependent on the type of the lipid source for emulsions formed with lipid particles and sodium caseinate. Solid lipid particles reside at the oil-water interface and provide stabilisation via a Pickering mechanism which was confirmed through microscopic visualisation. Results taken from confocal microscopy in combination with droplet size measurements suggest that droplets are first covered by the free sodium caseinate molecules that are present in the aqueous phase and subsequently, by the lipid particles which are also expected to contain NaCas on their surface, as represented schematically in Fig. 7.1. The polarity of the oil phase was also found to affect emulsion's droplet size, possibly due to different levels of interfacial tension reduction which, in turn, governs droplet break-up in the course of emulsification. Low polarity oils (e.g. hexadecane) formed emulsions of large droplet diameters that became unstable within ten days.

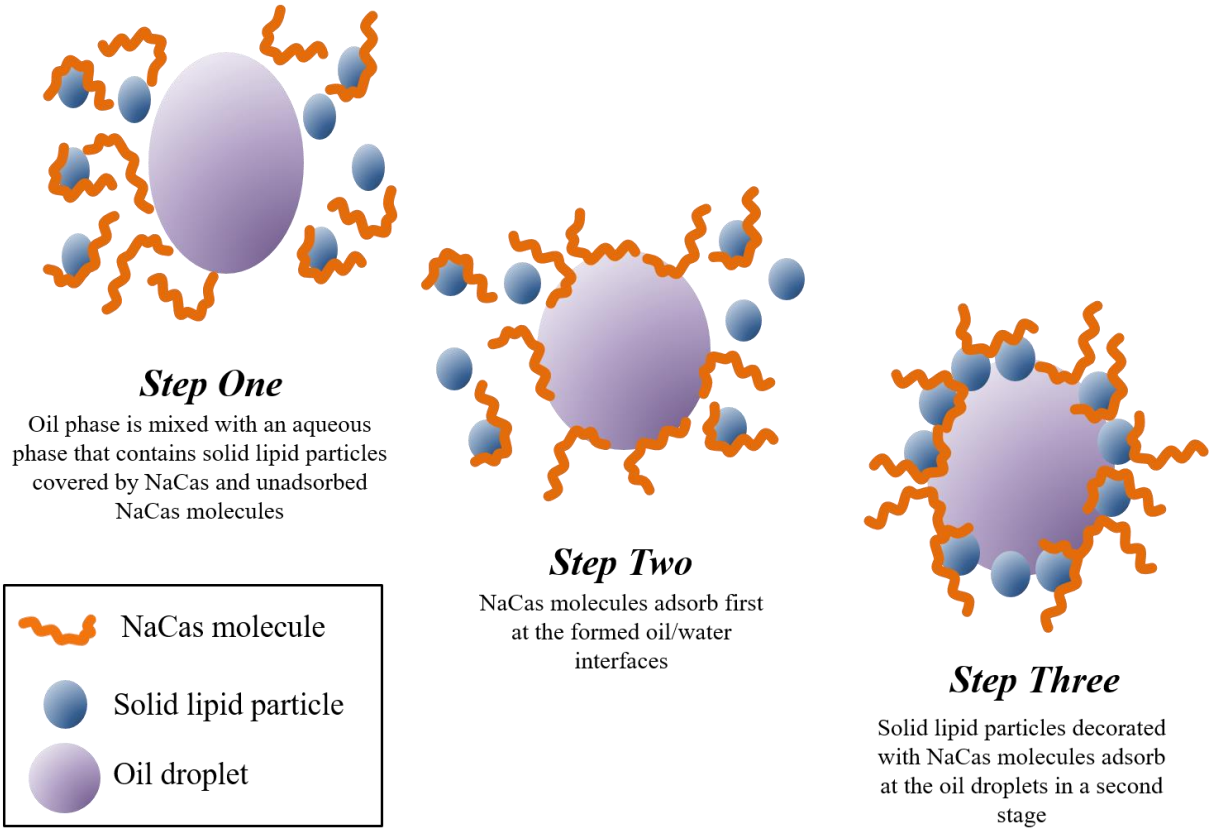

Fig. 7.1. Schematic drawing illustrating the potential sequence of events occurring at an oil droplet interface within the emulsions formed in the presence of lipid particles and sodium caseinate (NaCas).

The ratio between the melting enthalpy of emulsions produced in the presence of solid lipid particles (fabricated with surface active agents) and the respective of particles in dispersion, proved to be an invaluable tool for monitoring the evolution of the solid fat content upon introduction and emulsification with an oil phase, and subsequent storage. Dissolution of the solid matter in emulsions was more pronounced when lipid particles were formed with Tween 80 rather than NaCas and appeared to experience negligible changes with time. This behaviour was ascribed to the capacity of the surface active species to form micelles which facilitate the transfer of oil molecules. It was also shown that dissolution is a function of the molecular weight of the lipid material, as emulsions stabilised by tristearin particles suffered a

reduced loss of solid matter (or at least at a slower rate). Despite dissolution, emulsions were still stable to coalescence which was attributed to sufficient coverage provided by the existing amount of lipid particles. Last, the thermal stability of emulsions appeared to be dictated by the thermal properties of the lipid particles present; emulsion stability was markedly compromised at temperatures above the melting point of the lipid.

The formulation elements (e.g. lipid source, type and concentration of surface active species) shown to control the production of lipid particles with a Pickering functionality (Chapters 3 and 4) were further investigated with regard to how they impact upon the ability of these structures when subjected to a dehydration-rehydration route. Dehydration of solid lipid particles formed in the presence of Tween 80 or sodium caseinate was conducted via the widely used and industrially applicable lyophilisation (freeze-drying) process. The obtained lyophilisates were reconstituted via different methods and characterised for their mean size and size distribution, interfacial behaviour and also, the ability to stabilise o/w emulsions.

Results suggested that the type of the surface active component present was more significant than the resuspension technique employed, in terms of conservation of the original properties. Irrespective of the type of lipid material, dried and rehydrated particles produced in the presence of Tween 80 (both at 0.8 and 2 wt/wt% concentration) appeared to depend on the energy input of the redispersion method, however, in general, they were unable to maintain their initial size. This was ascribed to the small size of Tween 80 which does not prevent fusion of particles, favoured by water subtraction and subsequent increase of particle concentration. Even after addition of conventional cryoprotective agents such as sucrose, trehalose or maltodextrins prior to drying, lipid particles could not be recovered to their

original sizes, albeit a 10 wt/wt% cryoprotectant concentration had a more drastic effect on that.

Unlike Tween 80, particles produced in the presence of a high molecular weight surface active agent (i.e. NaCas) managed to withstand the freezing and desiccation stresses and resulted in particle sizes identical to the initial ones. This behaviour was found to occur regardless of the reconstitution method or concentration of sodium caseinate. It was postulated that NaCas provides a robust interfacial layer that protects lyophilised particulates from interactions and subsequent aggregation. A level of protection could additionally be provided by the presence of a large molecular mass entity in the bulk phase and this was demonstrated via the use of two biopolymers (i.e. hydroxypropyl methylcellulose (HPMC) and low methoxyl pectin (LMP)).

Retention of the lipid particles' microstructures was also observed upon different drying times (hence moisture contents) and storage of the dried powders under refrigeration temperatures for up to one month. This was not the case with lipid particles formed with Tween 80 as the resulting microstructures were more vulnerable to changes due to the above mentioned processing parameters. Colloidal crystalline structures fabricated in the presence of NaCas were shown, for the first time, to have the potential to undergo a drying and rehydration stage with a minimal loss on their Pickering functionality. The same emulsion droplet sizes were obtained by those particles in both hydrated and dehydrated states.

So far lipid particles were investigated in combination with a surface active component. As an extension, the contribution of each of these entities in a mixed particle-surfactant (mixed-emulsifier) system was explored with respect to its effect on o/w emulsion stabilisation. The

edible Pickering component was, in this case, cellulose-based particles (HPMC), and the surfactants assessed had a low (PGPR) and a high (Tween 80) HLB value. The mixed-emulsifier system was characterised for its behaviour at an oil/water interface and mixed-emulsifier stabilised emulsions were produced via different processing methods, and were analysed for their droplet sizes and storage stability.

The interfacial behaviour of mixtures of colloidal particles and surfactants resembled that of solid lipid particles fabricated in the presence of such surface active entities. Interfacial tension for composites of HPMC-Tween 80 and HPMC-PGPR was lower than the neat HPMC in dispersion and higher than either surfactant in solution. Regarding the behaviour within emulsions, it was demonstrated that mixtures of surface active species in combination with natural Pickering structures, can provide stable oil-in-water emulsions up to two weeks storage in quiescent room temperature conditions. These co-stabilised emulsions possessed smaller droplet sizes than systems stabilised by either of the two species in isolation, and showed enhanced stability against coalescence. Results obtained provided evidence that both of these features are directly influenced by the concentrations of the two components and more importantly by the surfactant's HLB value; at low concentrations (i.e. 0.01 wt%), the performance of PGPR was superior to Tween 80 as mixed-emulsifier stabilised emulsions formed in the presence of the latter were unstable over time. The reported co-stabilisation approach was shown to provide stable oil-in-water emulsions even in the presence of surfactants of low HLB value (conventionally used as stabilisers of water-in-oil emulsions). It was proposed that this behaviour is due to a greater shift in the particle's hydrophobicity, induced by the lower HLB value surfactant, as pictorially represented in Fig. 7.2. The observed performance was also found to persist across an emulsification method of a higher energy input (i.e. microfluidisation).

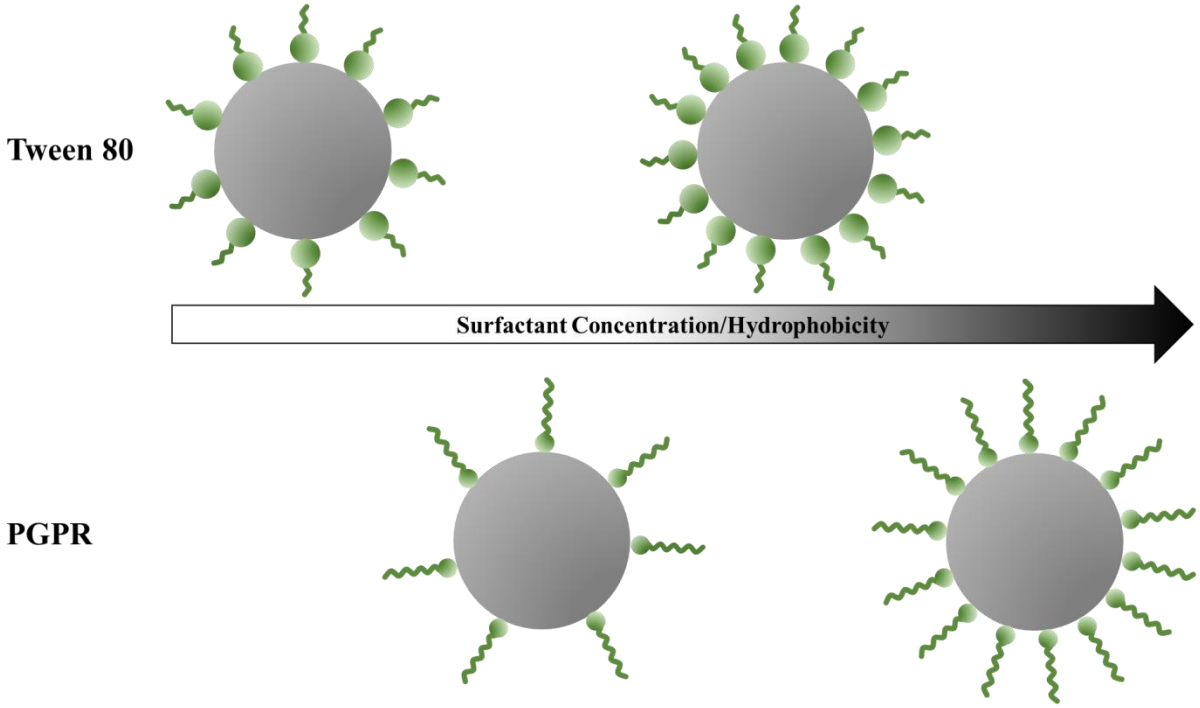

Fig. 7.2. Schematic description of the proposed effect of surfactant type (low and high HLB values) and concentration on HPMC particles' hydrophobicity.

7.2 Future Outlook

On the basis of the findings obtained from this work, a few areas that warrant further investigation were identified.

Assessment of more natural lipids as the building blocks of Pickering particles

Gaining a sound understanding of the way fats and waxes function as Pickering stabilisers would allow the extension of the work to waxes, for instance, from botanical sources that would be more relevant to the food industry. Naturally occurring waxes such as rice bran, beeswax, carnauba or sunflower wax have a GRAS (Generally Recognized as Safe) status and could offer an appealing pathway for the development of Pickering-type emulsifiers based on natural and sustainable materials. Some of these components might even have advantageous properties over the commonly used triglyceride-based nanoparticles; for example, beeswax exhibits only a single crystalline modification resulting in a physical stability that is likely to be superior to solid triglyceride particles (Souto, Doktorovova, & Boonme, 2011), hence stable Pickering crystalline structures for prolonged time could be produced.

Surface active species' content elimination, targeting for a surfactant-free system

This work demonstrated that the presence of a surface active component (even at low concentrations) is essential for the fabrication of lipid particles with the desired attributes for Pickering functionality (Chapter 3) and subsequently, for acting as a Pickering stabiliser (Chapter 4) and maintaining particle integrity during a drying-rehydration cycle (Chapter 5). Yet, Pickering stabilisation only in the presence of colloidal lipid structures still remains the 'holy grail' from both an academic and industrial perspective. Therefore, approaches for the removal of surface active agents should be implemented. This could be done either at the

initial fabrication step (i.e. investigate whether and how lipid structures can be formed in the absence or under reduced surface active species loads), or post-manufacturing, by eliminating the amount of non-adsorbed ("free") surface active components. A number of purification technologies for nanoparticles could be implemented towards that end, e.g. (ultra)centrifugation, dialysis, filtration, size-exclusion chromatography (Robertson, *et al.*, 2016; Veeken, 2012). Alongside the progressive removal of surface active entities, emulsions could be produced enabling an estimation of how close to a surfactant-free system one could get. Additionally, manufacturing nanoparticles without surfactant opens new avenues to several industrially appealing applications; they can be used as encapsulation vehicles for the delivery of nutrients, texture modification and taste masking across a variety of emulsions, including, but not limited to, food emulsions.

Alteration of lipid particles' surface properties

As discussed in the literature review and along the experimental Chapters of this thesis, surfactants can facilitate the manipulation of particle surface properties and, accordingly, modify their affinity for an interface, hence their Pickering performance. As such, a careful selection of surfactants with specific physicochemical properties could result in a more efficient control over particle emulsion stabilisation capacity. Apropos the investigated lipid particles, the use of a different type of protein that anchors itself within the crystalline structure but its hydrophilic parts, exposed to the aqueous phase, are tuned or, the use of a surface active species that matches the alkyl chain groups of the lipid used could enable, eventually, a better/prolonged anchoring of particles at an oil/water interface.

Alternatively, fabricating solid lipid particles based on mixtures of different hydrophobicity lipids (e.g. nanostructured lipid carriers (NLC) structures) could be a novel strategy towards their use as the sole emulsifiers of o/w emulsions. Some preliminary work was carried out on these systems and the most prominent findings are presented in Appendix A4.

Further exploitation of the interfacial layer

This work underscored the significance of the type of the surface active species (and therefore interfacial composition) for the control of lipid particles' characteristics that determine a Pickering performance, and for imparting an optimum redispersion capacity to lipid particulates following a dehydration process. It is thus logical that understanding and further exploiting the possibilities linked to smaller or larger interventions on the interfacial layer should be within the scope of future work.

The layer-by-layer (LbL) adsorption technique (or multilayer coatings) involves the sequential assembly of substances from oppositely charged compounds at an interface. The method allows the formation of structures with engineered attributes, including size, shape, composition and functionality (Johnston, Cortez, Angelatos, & Caruso, 2006). In parallel, it has been thoroughly explored and is versatile since it spans across physical, device, chemical and biological usages, and stands as highly promising for numerous others (Ariga, Hill, & Ji, 2007). Specifically in emulsion science, the LbL methodology has been applied to produce mixed biopolymer particles destined for stabilisation of emulsions and it has been proposed that the formation of these complexes prior to accumulation at the interface might be advantageous (Dickinson, 2008). LbL has not yet been tested as much in the field of SLN, however its introduction could inculcate novel physicochemical properties to SLN and expand their repertoire of applications.

A very recent example of LbL design was presented in literature and is concerned with the preparation of multilayered coated SLN by means of hydrophobic and electrostatic deposition, as schematically shown in Fig. 7.3.

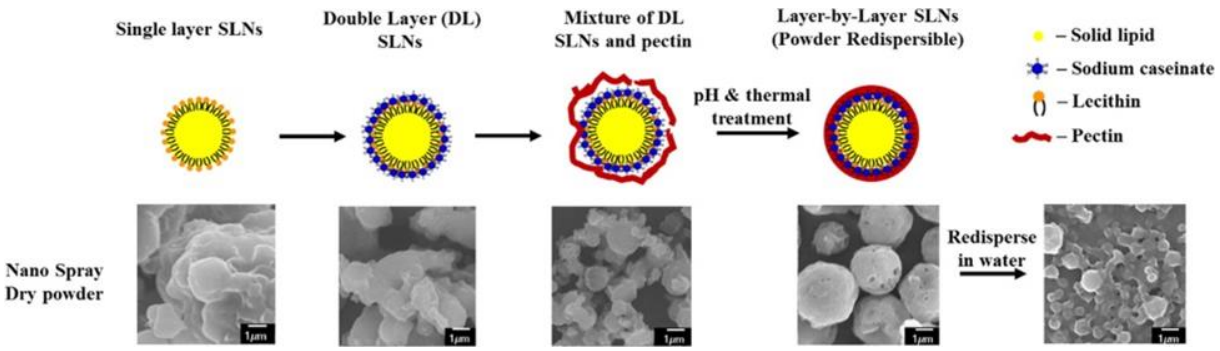

Fig. 7.3. Pictorial representation of fabrication of layer-by-layer coated SLN along with SEM images of spray-dried powders (Wang, *et al.*, 2016).

The resulting composite was found to exhibit excellent redispersion properties post-spray drying and would also be suitable for the encapsulation of heat-sensitive lipophilic compounds (Wang, *et al.*, 2016).

Unravel the fundamental mechanisms of stabilisation conferred by lipid particles as emulsion stabilisers

The stabilisation mechanism(s) of the fabricated oil-in-water emulsions are yet to be fully elucidated. For this reason, it would be worth investigating further and obtaining concrete evidence on the kinetics of the interfacial adsorption-desorption process, as well as how these are influenced by formulation parameters (i.e. type of lipid and surface active component etc.). In light of this, an accurate determination of particles' contact angle would aid in further understanding occurring interfacial phenomena. It is anticipated that interfacial species of different chemistries and sizes would distinctly modify the wetting of lipid particles at the oil/water interface. A relationship could then be established linking the free energy of

displacement from the interface and the varied lipid particle types formed with surface active species (Equation 2.1), which would eventually allow prediction of their effectiveness as emulsion stabilisers.

Deploy an alternative drying method

In this work, freeze-drying was employed as the dehydration method for the lipid particulate systems. However, as mentioned in section 2.5.6.2, energy expenditure as well as time duration are significantly greater in this process compared to other techniques. It would therefore be necessary to seek for alternative methods in order to provide a cost-effective and less time-consuming solution for industrial scales. In addition, the final moisture levels achieved by each of these methods need to be taken into consideration. Experiments on the lipid systems showed that 96% of the initial moisture could be removed within 48 hours at a drying rate of 0.0002 g/min utilising the freeze-dryer.

Two attractive examples of such techniques are the conventional spray drying (atomisation) and, the much gentler, microwave vacuum drying (MVD). Both of these routes combine several advantages and merit a more in-depth study to demonstrate whether they are equally advantageous when it comes to rehydration.

Adaptation from a concept system to a commercial food product

It is a requirement for food emulsions to remain kinetically stable for several months, even years. With reference to the o/w emulsions produced in this work, no attempts were made to stabilise them against creaming. A translation into the food industry though would demand creaming to be arrested. This is usually done by adding another component (e.g. gelling agent) to the emulsion microstructure. As a last step, formulation efforts would need to be directed

towards developing desired sensory profiles for manufactured prototypes; such profiles should match consumer acceptance, liking and trends, as this is, ultimately, the cornerstone of a commercial food emulsion.

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Chapter 8

Appendices

A1. Detailed thermal properties of the lipid-based systems investigated in Chapter 3

Table A1.1. Melting and crystallisation enthalpies for bulk tristearin and tristearin-based solid lipid particles. The absolute value of enthalpy of both particles produced without any added surfactant and particles produced with sodium caseinate should be taken with caution due to difficulties in estimating a suitable baseline. The two values in some of the melting events correspond to the *α* and *β* polymorphic forms.

		TRISTEARIN (TS)
		Enthalpy (J/g)
Bulk Lipid Phase (TS) – 1ST heating cycle	**Melting**	206.1± 7.8
	Crystallisation	-133.7± 5.3
Bulk Lipid Phase (TS) – 2ND heating cycle	**Melting**	24.5±1.1
		182.3±1.3
	Crystallisation	-137.5 ± 3.6
Aqueous Dispersion of TS Lipid Particles: 2.5% (wt/wt) TS without Surfactant	**Melting**	33.9 ± 1.0
		165.4 ± 3.9
	Crystallisation	-128.6 ± 4.1
Aqueous Dispersion of TS Lipid Particles: 2.5% (wt/wt) TS with 0.8% (wt/wt) T80	**Melting**	202.8 ± 11.4
	Crystallisation	-172.8 ± 6.0
Aqueous Dispersion of TS Lipid Particles: 2.5% (wt/wt) TS with 1.2% (wt/wt) T80	**Melting**	197.8 ± 6.4
	Crystallisation	-178.8 ± 9.1
Aqueous Dispersion of TS Lipid Particles: 2.5% (wt/wt) TS with 2% (wt/wt) T80	**Melting**	192.1 ± 5.2
	Crystallisation	-179.1 ± 2.6
Aqueous Dispersion of TS Lipid Particles: 2.5% (wt/wt) TS with 0.8% (wt/wt) NaCas	**Melting**	47.7 ± 2.1
		163.9 ± 8.5
	Crystallisation	-128.7 ± 11.9
Aqueous Dispersion of TS Lipid Particles: 2.5% (wt/wt) TS with 1.2% (wt/wt) NaCas	**Melting**	44.4 ± 1.5
		166.7 ± 1.5
	Crystallisation	-128.0 ± 2.7

Aqueous Dispersion of TS Lipid Particles: 2.5% (wt/wt) TS with 2% (wt/wt) NaCas	**Melting**	48.2 ± 1.1
		144.6 ± 0.4
	Crystallisation	-127.8 ± 1.1

Table A1.2. Crystallisation transitions for tristearin particles formed in the present of various concentrations of Tween 80 and NaCas. All data are means ± 1 standard deviation of at least two measurements.

TRISTEARIN (TS) LIPID PARTICLES

Crystallisation Parameters	**Concentration of Tween 80 (wt/wt%)**			**Concentration of NaCas (wt/wt%)**		
	0.8	**1.2**	**2**	**0.8**	**1.2**	**2**
Onset Temperature (°C)	32.7±0.1	32.2±0.1	31.9±0.1	37.2±0.1	37.2±0.2	36.3±0.6
Peak Temperature (°C)	30.8±0.1	30.4±0.1	30.1±0.2	35.2±0.1	34.9±0.2	34.1±0.3

Table A1.3. Relative percentages of the peaks ascribed to polymorphic forms, after integration applying the trapezoidal method.

Tween 80 Concentration (wt/wt%)	**Relative percentages of polymorphic forms (%)**			**Melting temperature of tristearin polymorphs (°C)** (Lavigne, Bourgaux, & Ollivon, 1993)		
	α	***β′***	***β***	***α***	***β′***	***β***
0.8	2.4± 1.0	13.0±1.8	79.1±1.7	54.5	64.5	72.5
1.2	4.5±2.0	22.2±3.5	65.9±3.8			
2	12.1±5.3	43.5±5.2	34.2±3.9			

Table A1.4. Melting and crystallisation temperatures and enthalpies for the bulk cetyl palmitate and the cetyl palmitate-based solid lipid particles.

CETYL PALMITATE (CP)

		Enthalpy (J/g)
Bulk Lipid Phase (CP)	**Melting**	224.2± 8.6
	Crystallisation	-224± 4.6
Aqueous Dispersion of CP Lipid Particles: 2.5% (wt/wt) CP without Surfactant	**Melting**	175.8 ± 14.0
	Crystallisation	-179.3 ± 16.5
Aqueous Dispersion of CP Lipid Particles: 2.5% (wt/wt) CP with 0.8% (wt/wt) T80	**Melting**	190.8 ± 1.7
	Crystallisation	-197.9 ± 0.8
Aqueous Dispersion of CP Lipid Particles: 2.5% (wt/wt) CP with 0.8% (wt/wt) NaCas	**Melting**	192.6 ± 3.6
	Crystallisation	-196.1 ± 5.8

Table A1.5. Melting and crystallisation enthalpies for tristearin and cetyl palmitate-based solid lipid particles formed using Tween 80 and NaCas, after 9 and 5 months of cold storage.

		Enthalpy (J/g)	
TRISTEARIN (TS) LIPID PARTICLES		**Following particle fabrication**	**Following prolonged storage**
2.5% (wt/wt) TS with 0.8% (wt/wt) T80	**Melting**	202.8 ± 11.4	198.7 ± 1.9[a]
	Crystallisation	-172.8 ± 6.0	-179.8 ± 1.2[a]
2.5% (wt/wt) TS with 0.8% (wt/wt) NaCas	**Melting**	47.7 ± 2.1	45.5 ± 1.3[a]
		163.9 ± 8.5	166.1 ± 2.2[a]
	Crystallisation	-128.7 ± 11.9	-134.4 ± 2.4[a]
CETYL PALMITATE (CP) LIPID PARTICLES			
2.5% (wt/wt) CP with 0.8% (wt/wt) T80	**Melting**	190.8 ± 1.7	197.9 ± 1.1[b]
	Crystallisation	-197.9 ± 0.8	-200.9 ± 1.4[b]
2.5% (wt/wt) CP with 0.8% (wt/wt) NaCas	**Melting**	192.6 ± 3.6	188.5 ± 3.1[b]
	Crystallisation	-196.1 ± 5.8	-189.1 ± 2.1[b]

[a]*DSC data (TS with T80 and NaCas) collected following 9 months of cold (4 ℃) storage.*

[b]*DSC data (CP with T80 and NaCas) collected following 5 months of cold (4 ℃) storage.*

A2. Selected droplet size distributions for o/w emulsions presented in Chapter 4

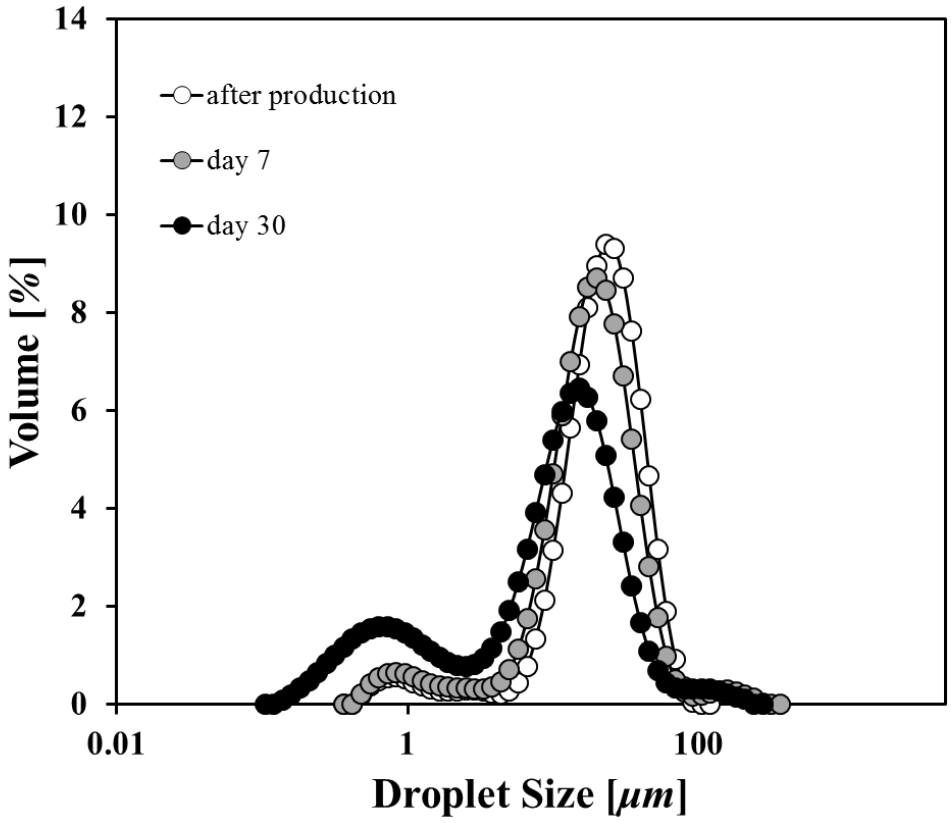

Fig. A2.1. Laser Diffraction measurements showing droplet size distributions of 20% o/w emulsions produced with tristearin particles formed with sodium caseinate, at various time points.

A3. Selected particle and droplet size distributions and interfacial tension profiles for the results presented in Chapter 5

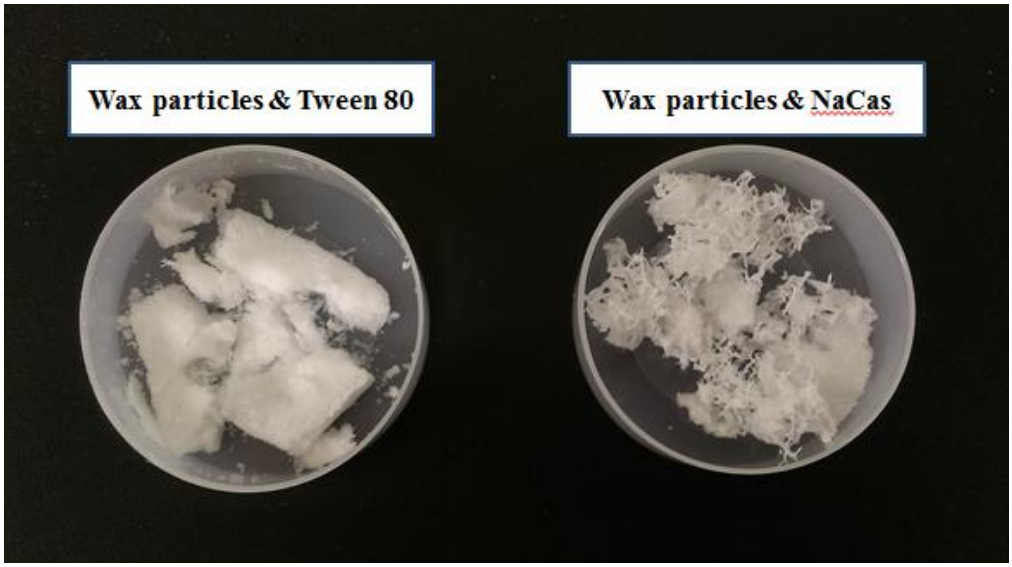

Fig. A3.1. Representative images of the lyophilised powders after their removal from the dryer.

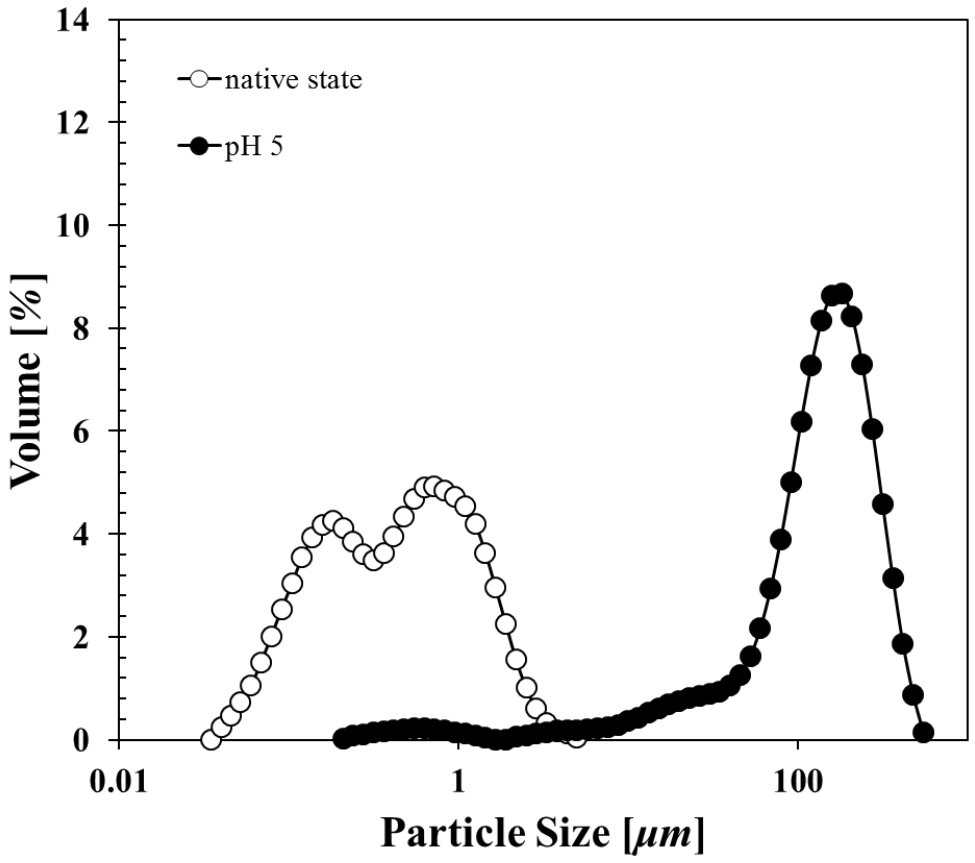

Fig. A3.2. Effect of protein's pH on lipid particle's redispersion behaviour. Size distributions refer to cetyl palmitate particles formed with 0.8% NaCas.

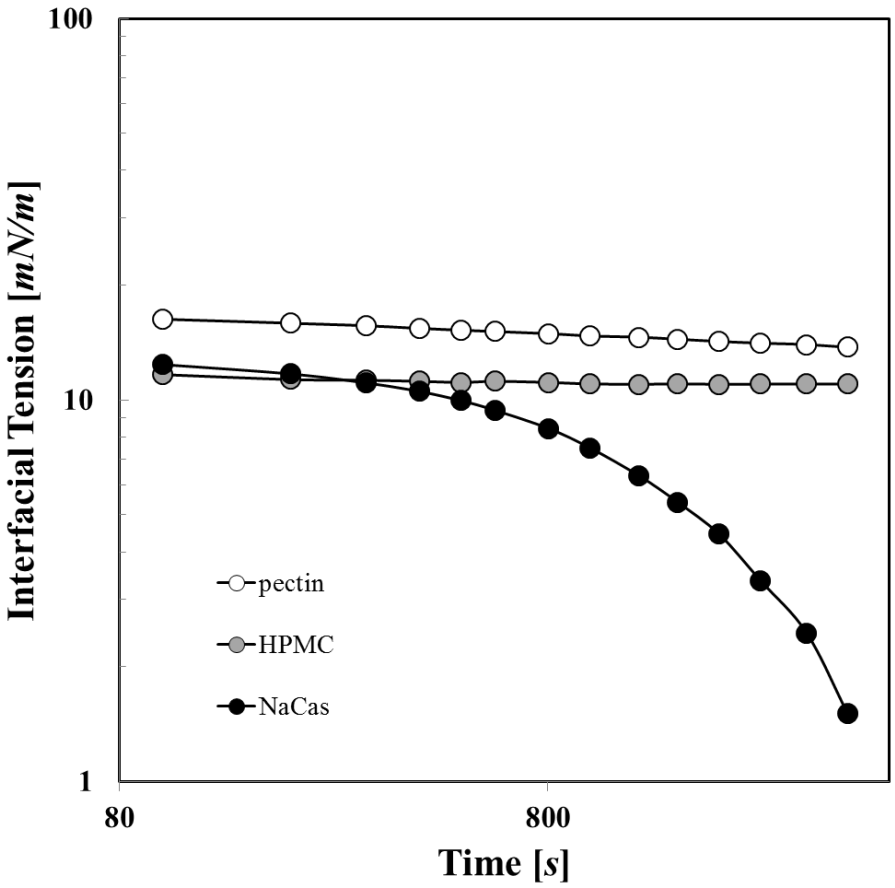

Fig. A3.3. Behaviour of 2% pectin, HPMC and NaCas (in solution) at the sunflower/water interface.

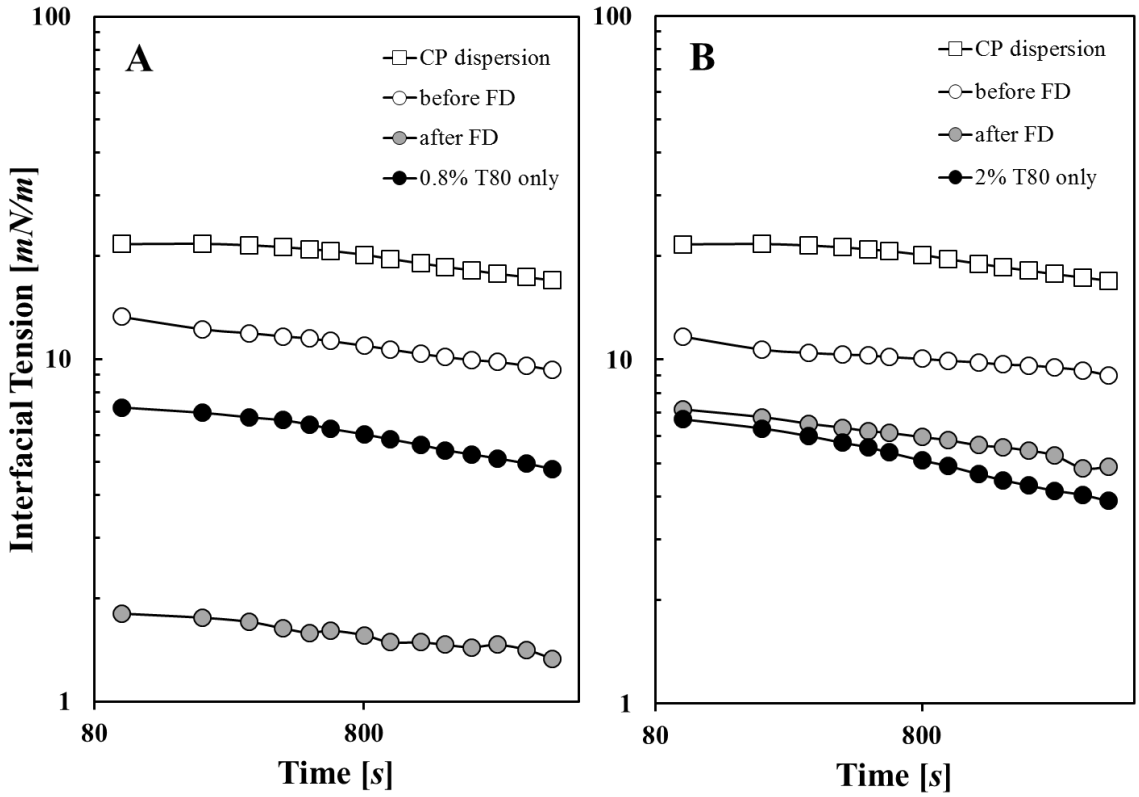

Fig. A3.4. Interfacial tension between sunflower oil and aqueous dispersions of solid cetyl palmitate particles formed in the presence of 0.8 (A) and 2 wt/wt% (B) Tween 80 before and after freeze-drying (FD).

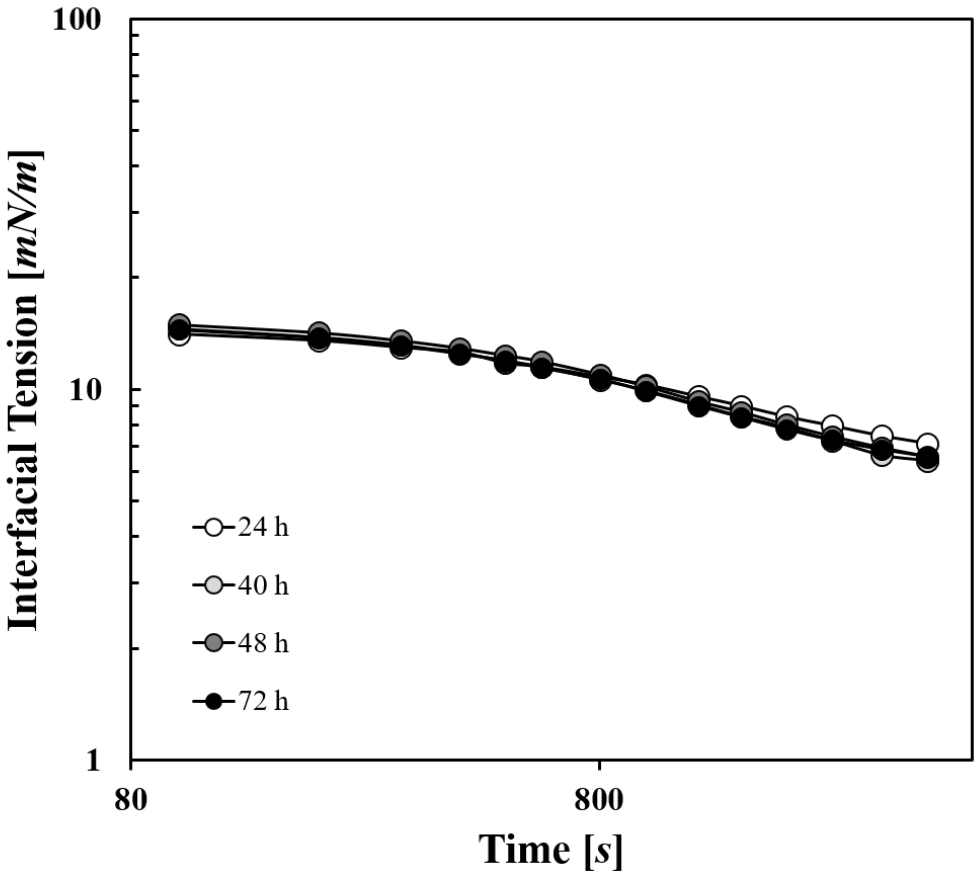

Fig. A3.5. Interfacial tension between sunflower oil and aqueous dispersions of dried and redispersed solid cetyl palmitate particles originally formed in the presence of 0.8 wt/wt% NaCas for different drying times.

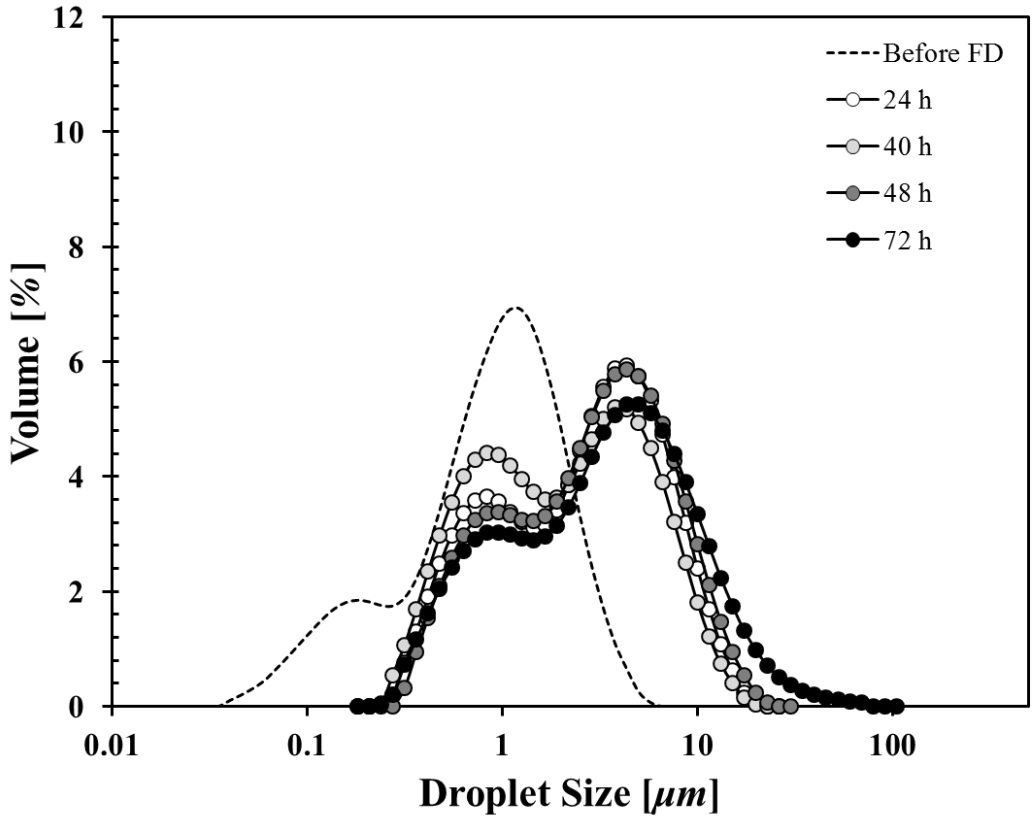

Fig. A3.6. Droplet size distribution curves for 20% sunflower oil emulsions formed with dried and reconstituted 2.5% wax particles and 0.8% Tween 80 for different drying times.

Table A3.1. Mean droplet size and span values for 20% sunflower oil emulsions produced in the presence of dried and rehydrated 2.5% cetyl palmitate particles and different amounts of Tween 80, for different drying times.

	0.8% Tween 80			2% Tween 80		
Drying time (h)	**$D_{3,2}$ (μm)**	**$D_{4,3}$ (μm)**	**Span**	**$D_{3,2}$ (μm)**	**$D_{4,3}$ (μm)**	**Span**
0	2.2±0.2	18.0±18.3	20.3±25.0	0.4±0.1	1.4±0.1	3.6±0.1
24	1.2±0.6	5.9±2.8	4.7±2.9	0.7±0.6	4.7±3.3	10.9±11.6
40	0.9±0.6	5.4±2.9	6.3±4.6	0.5±0.3	3.9±2.1	12.9±12.4
48	1.3±0.6	7.3±4.3	4.7±2.8	0.7±0.6	3.4±0.6	27.2±34.6
72	1.0±1.2	3.2±2.5	13.4±14.7	0.8±0.9	3.1±1.1	26.8±33.4

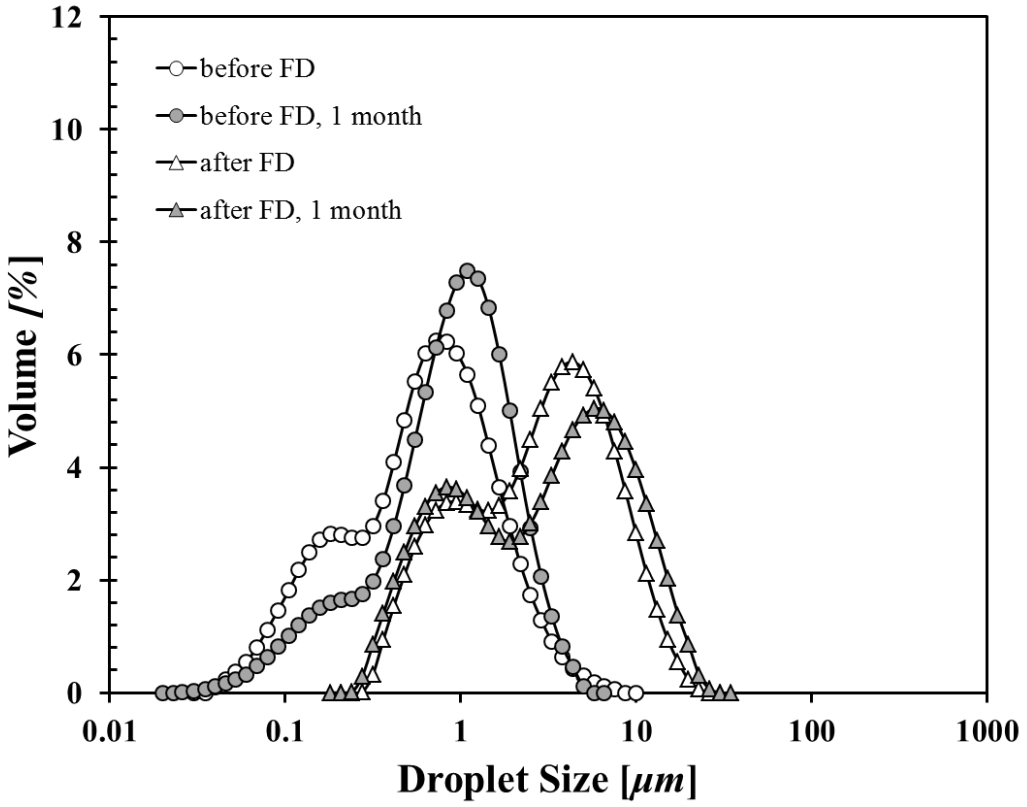

Fig. A3.7. 20% O/W emulsions formed in the presence of dried and rehydrated 2.5% wax particles and 0.8% Tween 80 stored for different times at refrigeration temperatures. The same emulsions produced with non-freeze-dried particles are also included for comparison.

A4. A structural-based approach for enhanced Pickering functionality of solid lipid particles

The aim of this preliminary study was to investigate the potential of nanostructured lipid carriers (NLC) to act as solid particle emulsifiers for the stabilisation of o/w emulsions.

Glyceryl citrate/lactate/linoleate/oleate (CITREM/LACTEM, commercial name: Imwitor 375) was used as the liquid lipid and cetyl palmitate as the solid lipid component of the NLC. This mixture of glyceryl monoesters is a sunflower based food-grade lipid (E472b, E472c compliance) (Ash & Ash, 2004) that has an HLB value of 10-12. In the food industry it is normally used as an emulsifier, stabiliser, anti-spattering agent, as well as an ingredient that improves aeration, foam stability, texture and volume (Gaupp & Adams, 2004). It was anticipated that, in this manner, solid lipid particles can be rendered surface-active and behave as the sole emulsifiers, without any surfactant present in the formulation.

Cetyl palmitate was mixed with the liquid lipid at different mass ratios (1:3, 3:1 CP:Imwitor 375) and at a total particle concentration of 2.5 wt/wt%. Particles were produced via an ultrasound-assisted melt-emulsification method as described in Chapter 3. Manufactured structures were characterised for their size and size distribution, and also their ability to form and stabilise o/w emulsions.

Results obtained together with a few comments and observations are presented below.

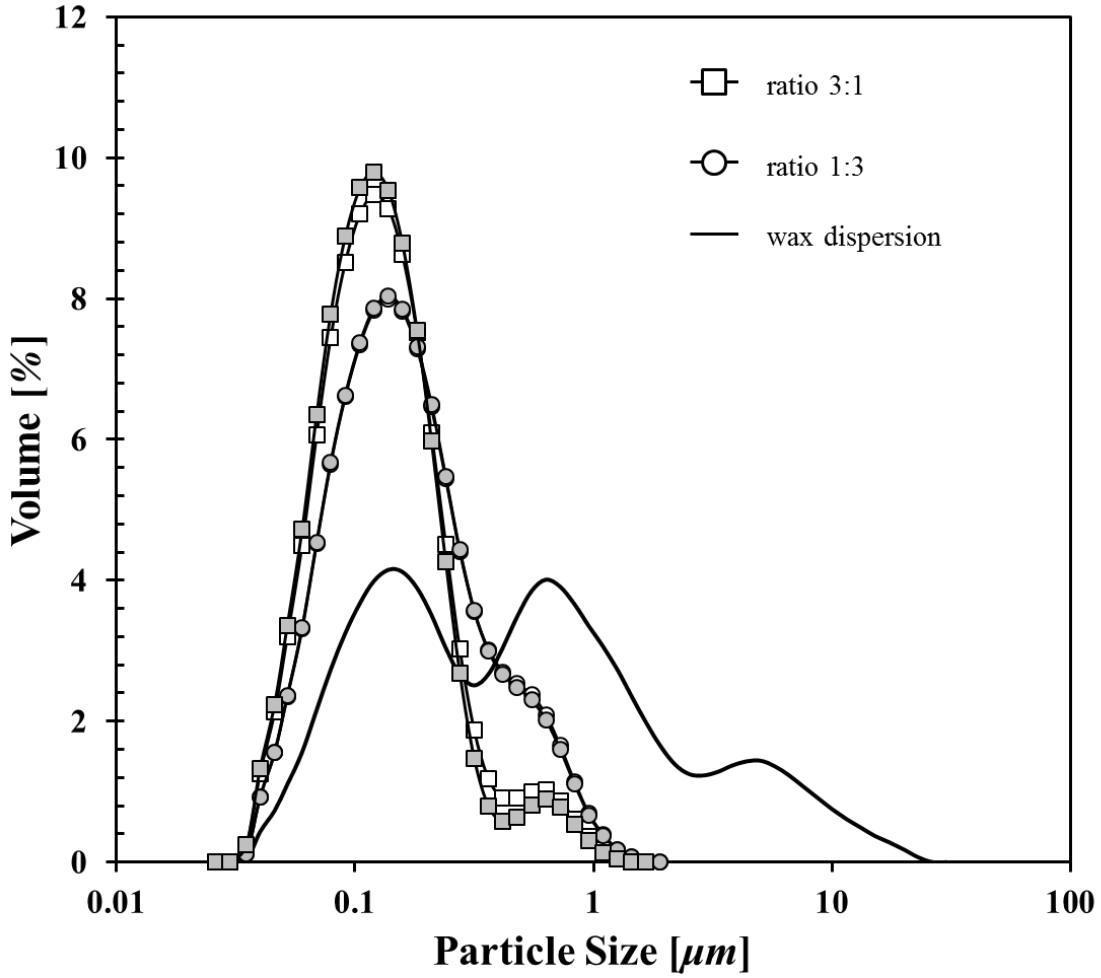

Fig. A4.1. Laser diffraction measurement of particle size distribution of a NLC system where solid and liquid lipids are mixed at different ratios. Open and coloured symbols represent particles after production and after one month storage (at 4 °C) respectively.

- Addition of liquid lipid shifts the particle size distribution to values in the nanometric range; all the lipid blends-based particles were smaller than 1 μm, having a mean particle diameter of ~0.12 μm ($D_{3,2}$) which is usually the expected sizes for NLC (i.e. below 500 nm) (Hentschel, Gramdorf, Müller, & Kurz, 2008).
- Size differences could be attributed to differences in the viscosities of the lipid components at high processing temperatures, and how these vary with increasing liquid lipid amounts in the system.

- No increase of particle size or span values was observed within the one month storage time (at 4 ºC), indicating a stable dispersion without the use of any additional surfactant in the system.

A fixed mass of 20 wt/wt% sunflower oil (dispersed phase) was added to the continuous aqueous dispersion containing 2.5 wt/wt% lipid particles at different solid:liquid lipid ratios. Oil-in-water pre-emulsions were obtained by emulsifying these mixtures at 10,000 rpm for 2 minutes using a high shear mixer. Submicron emulsions were produced by passing these coarse pre-emulsions through an air-driven microfluidiser for up to 4 passes at 1000 bar. For droplet size analysis, dynamic laser light scattering system (DLS) was used. Measurements were performed immediately after emulsification and after 3 weeks of storage at 4 °C.

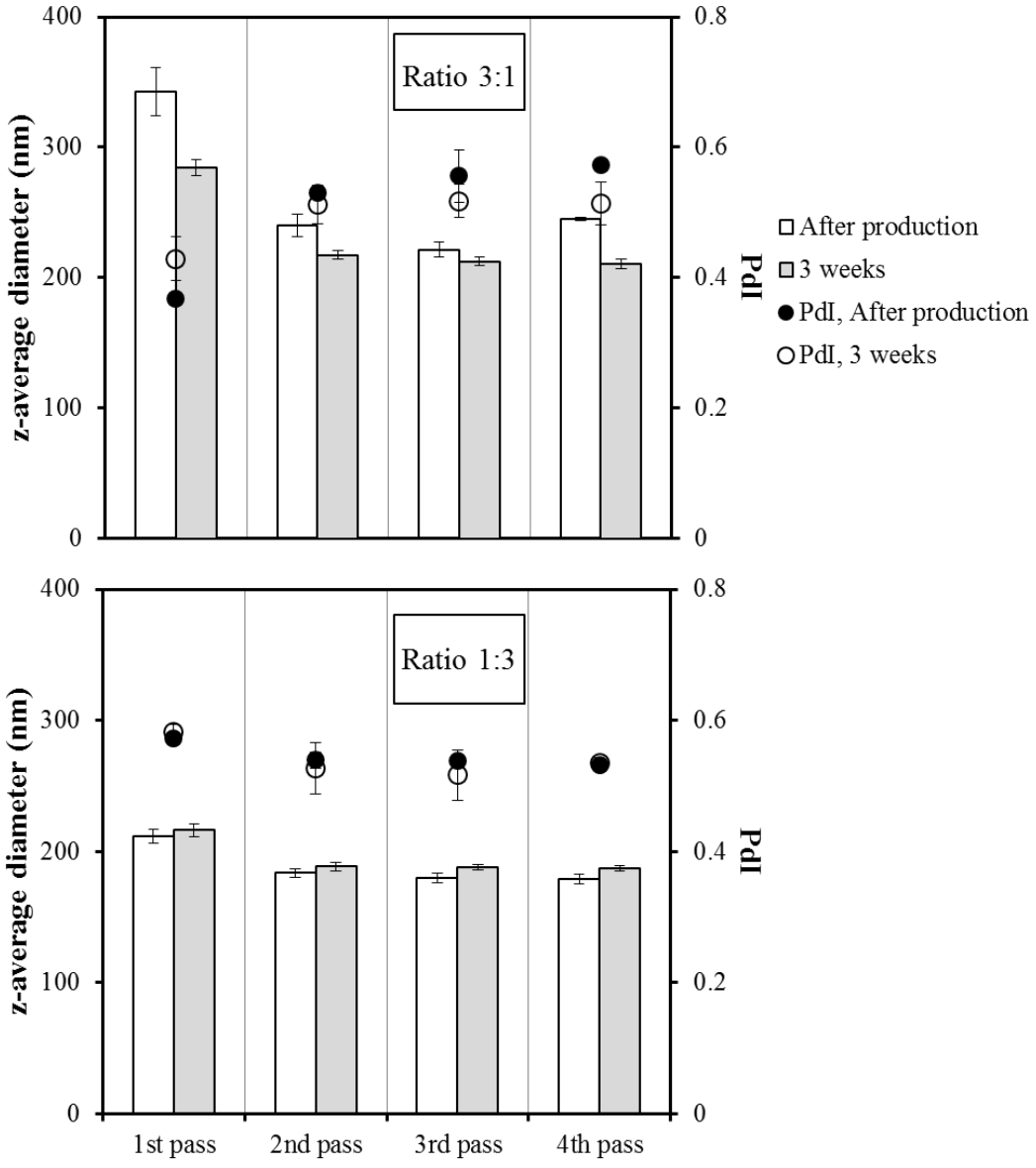

Fig. A4.2. Effect of pass number and storage stability on 20 wt/wt% o/w emulsion droplet size and polydispersity index (PdI) in a microfluidiser. Emulsions are stabilised by mixed lipid particles at a 3:1 and a 1:3 solid:liquid ratio.

- Incorporation of a more polar oil within the lipid phase of the particles, imparts a level of hydrophilicity which allows particles to be significantly more surface active. Therefore, oil-in-water emulsions with sub-micron sized droplets can be formed, albeit with a relatively broad size distribution (PdI $\geq$ 0.5).

- Emulsions prepared in the presence of high concentrations of Imwitor 375 in the NLC yielded overall smaller droplets than the ones with the lower amounts of liquid lipid. At a solid:liquid ratio of 1:3 a greater level of interfacial tension reduction is anticipated and therefore, an enhanced droplet break-up during homogenisation. If the model proposed by Jores, Mehnert, and Mäder (2003) regarding NLC's structure is adopted (for more details see section 2.5.5) , then it is expected that a cetyl palmitate matrix is enclosed in a film made of the liquid lipid which is sparsely surrounding the solid lipid at low amounts, and fully covering it at increased concentrations. Consequently, droplets might not be sufficiently covered by NLCs depending on the liquid lipid amount and this in turn, makes them more prone to coalescence events (e.g. at a 3:1 ratio).
- All the emulsions were stable against coalescence upon ageing.

This preliminary study demonstrated that stabilisation of emulsions can be achieved with the use of NLC as the sole emulsifiers. When appropriate molecules are mixed with the lipid matrix it appears that the latter's hydrophobicity is tailored, hence wettability can be manipulated. Additional work would benefit from a better understanding of the interfacial architecture generated in the case of NLC coupled with the mechanism of emulsion stabilisation.

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