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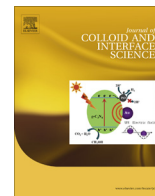
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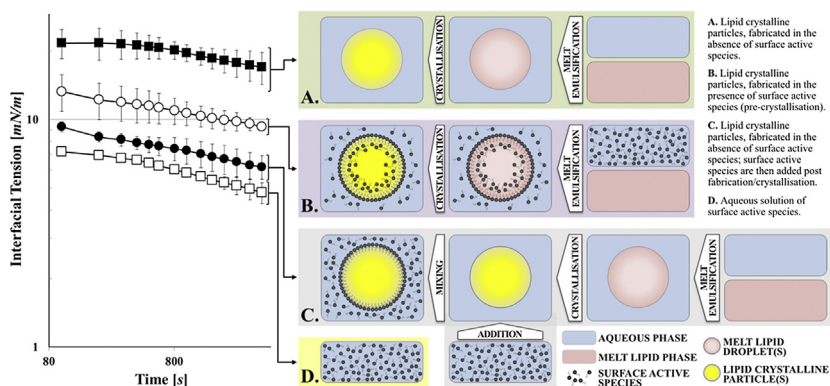


Regular Article

The role of surface active species in the fabrication and functionality of edible solid lipid particles

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ABSTRACT

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 were 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.

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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 fun-

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damental differences in regards to their stabilisation mechanisms. Particle adsorption is effectively irreversible, providing superior long-term stabilisation to the system [1]. 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 [2,3].

Despite the soar of research in the field of particle-stabilised emulsions, 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 [4,5]. To bridge the gap between Pickering emulsions and food-related applications, several natural-based materials have been investigated such as cellulose nanocrystals [6], chitin particles [7] and flavonoids [8]. The use of food-grade particulate structures also embodies lipid crystalline particles (fat crystals) [9–11], the role of which in the stabilisation of everyday foods such as butter, margarine and ice cream, is long recognised [12]. 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 [13]. 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 and 10 μm [14] and also for providing a dense surface coverage [13]. In that view, Gupta and Rousseau [15] 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. It has been shown that surfactant, and in particular its chemistry, plays a drastic role on the obtained size of lipid particles [16] as well as physical stability upon storage, and several emulsifiers have been screened towards that end [17]. Additionally, it is well-documented that surfactants influence the crystallisation profile and the kinetics of polymorphic transitions after crystallisation occurring in both bulk triglycerides [18,19] and in emulsified systems/colloidal dispersions [20–23]. 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 [24]. Moreover, surfactants have been employed as a means of tuning the hydrophobic character of lipid entities, thus their wettability characteristics [25].

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.

2. Materials & methods

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 \approx 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.

2.2. Methods

2.2.1. Solid lipid particles preparation

Solid lipid particles were prepared by a melt-emulsification method, following the procedure described elsewhere [26]. Briefly, 2.5% of lipid material relating to the total mass (wt/wt%) was heated around 5–10 $^{\circ}\text{C}$ above the melting temperature of the lipid to ensure complete melting. For instance, the triglyceride was heated up to ~ 85 $^{\circ}\text{C}$, whilst the wax was melted to around 65 $^{\circ}\text{C}$. 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 W 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 min). Droplet breakage was driven by acoustic cavitation, resulting in the formation of nanoparticles [27]. The oil-in-water emulsion formed was subsequently cooled in an ice bath, to a temperature below the crystallisation temperature of the carrier lipids. Hence, solid lipid particles (crystals) were obtained by crystallisation of the dispersed lipid. All samples were stored at refrigeration temperature (~ 4 $^{\circ}\text{C}$) until further analysis.

2.2.2. Analytical methods

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 Dyna-

san[®] 118 = 1.49, cetyl palmitate RI = 1.44, Millipore water (dispersant water) RI = 1.330. 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.

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. 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 min 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.

2.2.2.3. Thermal analysis. Thermal profiles of solid lipid particles were obtained with a high-sensitivity Setaram μ DSC7 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 °C to 85 °C at 1.2 °C/min followed by a cooling cycle from 85 °C to 5 °C at 1.2 °C/min to mimic the process that was used to obtain the crystallised particles. This ramp was used to obtain information in regards to the physico-chemical state of the micro/nanoparticles. Bulk tristearin and cetyl palmitate treated under similar conditions were used for comparison. From the differential scanning calorimetry (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. Results & discussion

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 mean diameters.

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 diameter. 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 for Pickering functionality.

Fig. 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.

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 μ m. As can be seen from Fig. 1A and B, introduction of surface active entities in the melt-emulsification step obviously affects the final particle diameter which appears to be largely dictated by their type and somewhat less by their concentration.

Upon addition of Tween 80, the mean particle diameter decreases from 35 μ m to 0.1 μ m with an increase in the surface active species' concentration (Table 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 larger diameters was still present. The transition from a bimodal to monomodal distribution at higher surface active component concentration highlights a relationship between the resulting droplet diameter 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. The large polydispersity depicted through the span values (e.g. 1.2% Tween 80) might also be impacted by potentially not well-controlled mixing during emulsification.

Similar behaviour has been reported previously in o/w emulsions formed in the presence of Tween 20 [28]. 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 diameter 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. 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

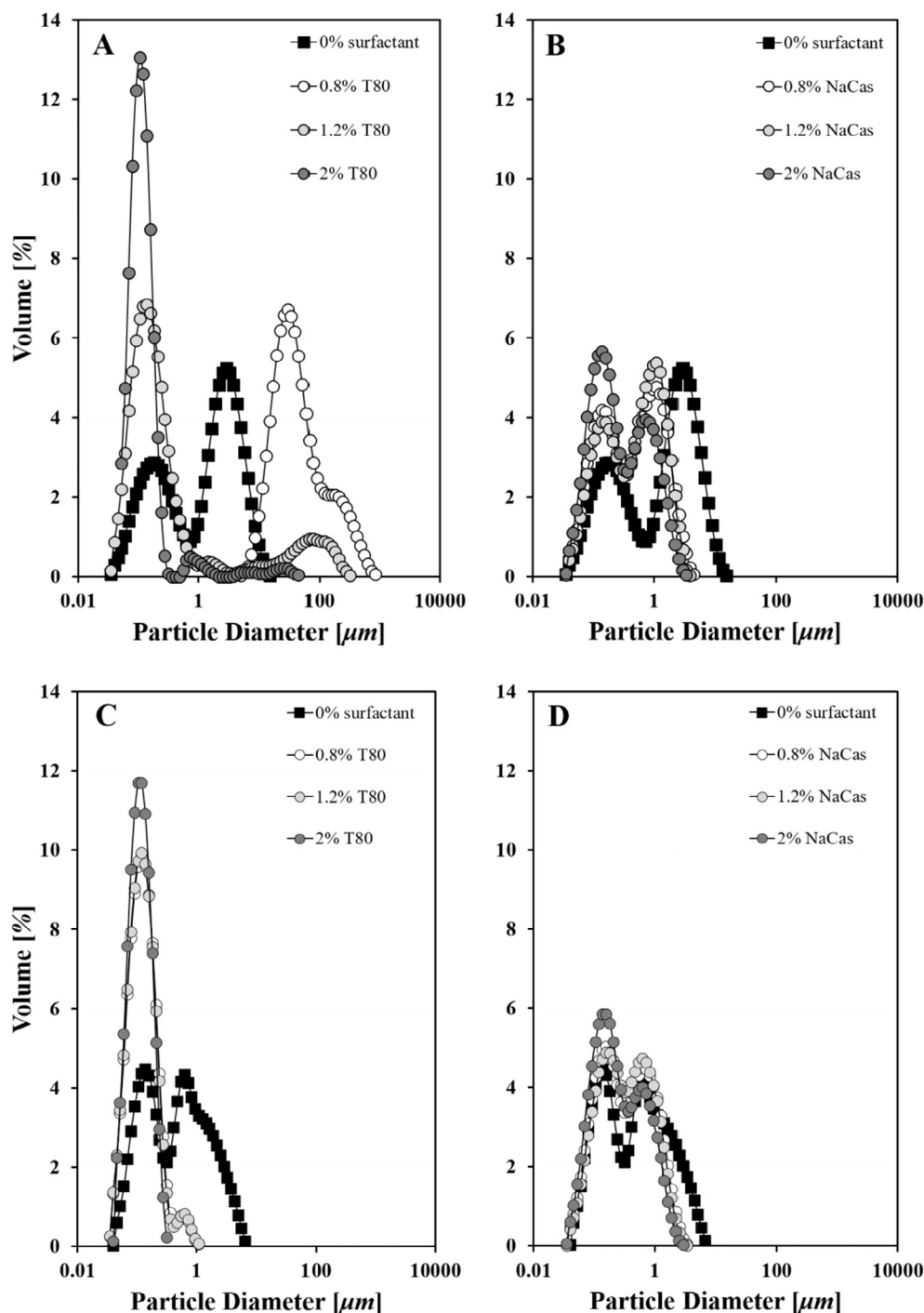


Fig. 1. Laser Diffraction measurements showing the particle size distributions of solid tristearin (top) and cetyl palmitate (bottom) particles (2.5 wt/wt%) formed in the presence of varying concentrations of Tween 80 (T80) (A and C) and sodium caseinate (NaCas) (B and D). Graphs presented are representative of three replicate samples.

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, physical and crystallographic properties, which, in turn, is expected to affect lipid particles' characteristics [29]. Cetyl palmitate crystals were produced applying the same technique and in most cases sub-micron particles (below 200 nm) were yielded. As seen in Fig. 1C and D 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, the amount of surface active component needed, and above which the particle size is no longer affected, is much lower than that of tristearin, thereby 2% Tween 80 gave similar sized wax particles to 0.8% concentration (Fig. 1C). In contrast, when sodium caseinate was used, broader sized distributions were obtained (higher span values as seen in Table 1), of which two populations were more distinct (Fig. 1D). It appears that the diameter of cetyl palmitate particles is largely dictated by the specific lipid component used and namely, by the

Table 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 °C. All data are means \pm 1 standard deviation ($\bar{x} \pm s$) for $n = 3$ batches of samples.

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	0.8	35±3.4	3.1±1.4	0.3±0.1	3.3±0.5
	1.2	0.2±0.1	200±190	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
4	0.8	19±2.6	4.3±2.4	0.2±0.1	4.4±1.7
	1.2	0.2±0.1	290±100	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	14±3.8	0.7±0.6
12	0.8	15±4.9	4.5±0.7	0.3±0.1	4.3±1.7
	1.2	0.1±0.1	150±260	0.2±0.1	3.5±0.4
	2	0.1±0.1	1.0±0.1	0.3±0.2	38±47
	CETYL PALMITATE LIPID PARTICLES				
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±6.1	0.5±0.3	12±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

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 diameter ($D_{3,2}$ values) and the span values (width of distribution) is shown in Table 1 for tristearin and cetyl palmitate particles respectively, produced in the presence of Tween 80 and NaCas.

It is evident from Table 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 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 [30].

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 were 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 [31].

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) 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 was 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 Figs. 2 and 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 [32]. 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 min (saturated IFT for Tween 80 is reported to be ~ 5 mN/m) [33]. 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 [34].

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. 2A). In this case, the much larger particle diameters obtained here ($D_{3,2} = 35$ μm , Table 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. 2A and B), where increasing Tween 80 during or post fabrication effects 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. 3A and B). 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.

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 way. 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. A representative example of the interfacial tension profile for the four investigated systems along with the physicochemical processes occurring in each one of them is shown in Fig. 4. The disparity in the location of the surface active species in relation to the crystalline lipid particles, whether these are present or absent during particles' fabrication, becomes evident from the schematic diagram. 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. 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 [21,23]. 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, differential scanning calorimetry (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. 5.

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. 5A). Cooling the melt was followed by reheating at the same rate to 85 °C (2nd heating cycle). Melting of the α -form takes place at ~ 54 °C 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 [35]. 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 [36] 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 [35]. Therefore, melting of the β -form is kinetically favoured and occurs at an onset temperature of ~ 64 °C (with a maximum at 70.5 °C). 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 -

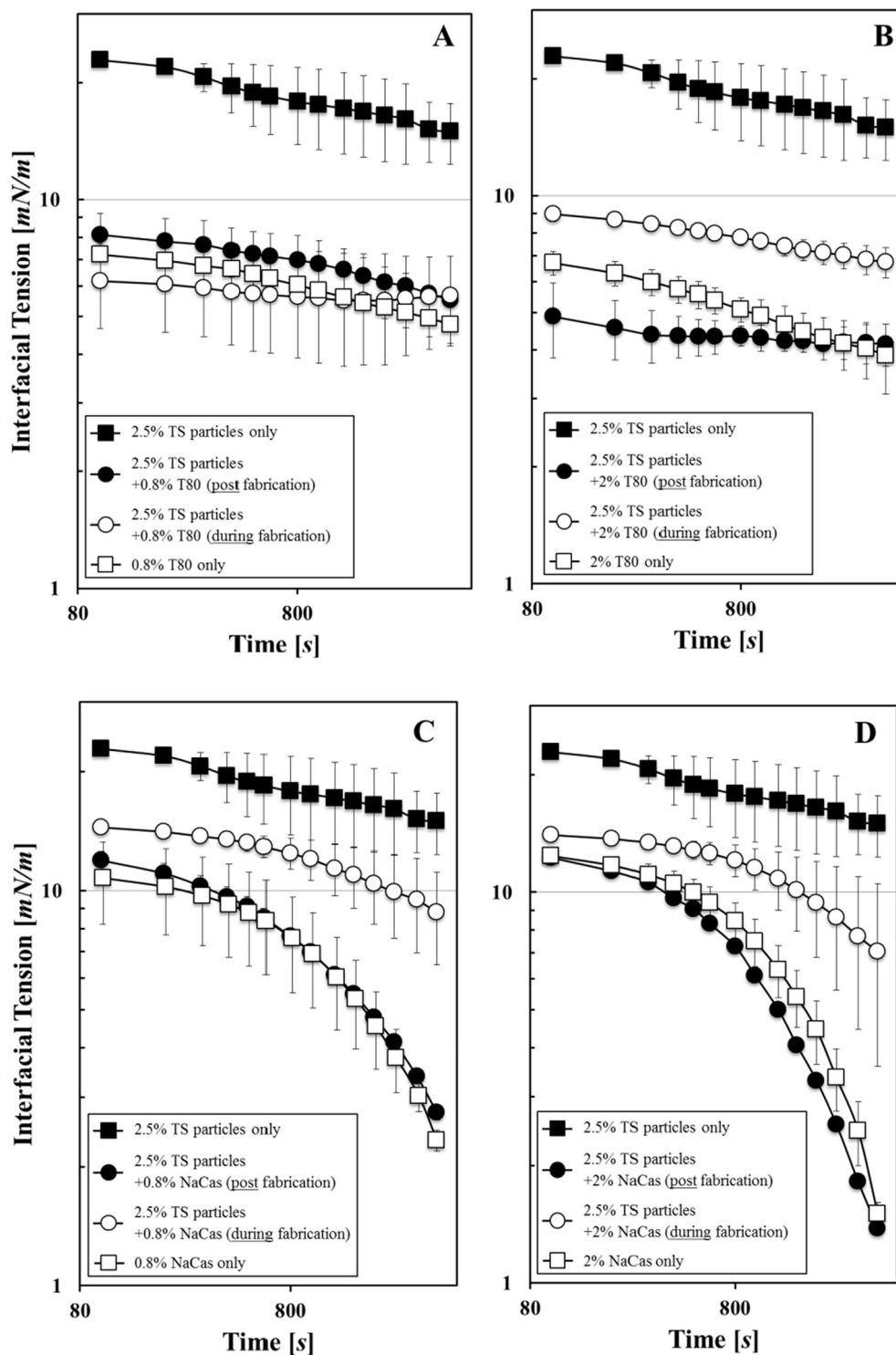


Fig. 2. Dynamic interfacial tension of aqueous dispersions of solid tristearin particles (2.5 wt/wt%) in the absence and presence of 0.8 and 2 wt/wt% Tween 80 (A and B) and sodium caseinate (C and D) added during (open circle) or after particles' fabrication (solid circle). Similar concentrations of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

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 Bun-

jes *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 [37]. 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

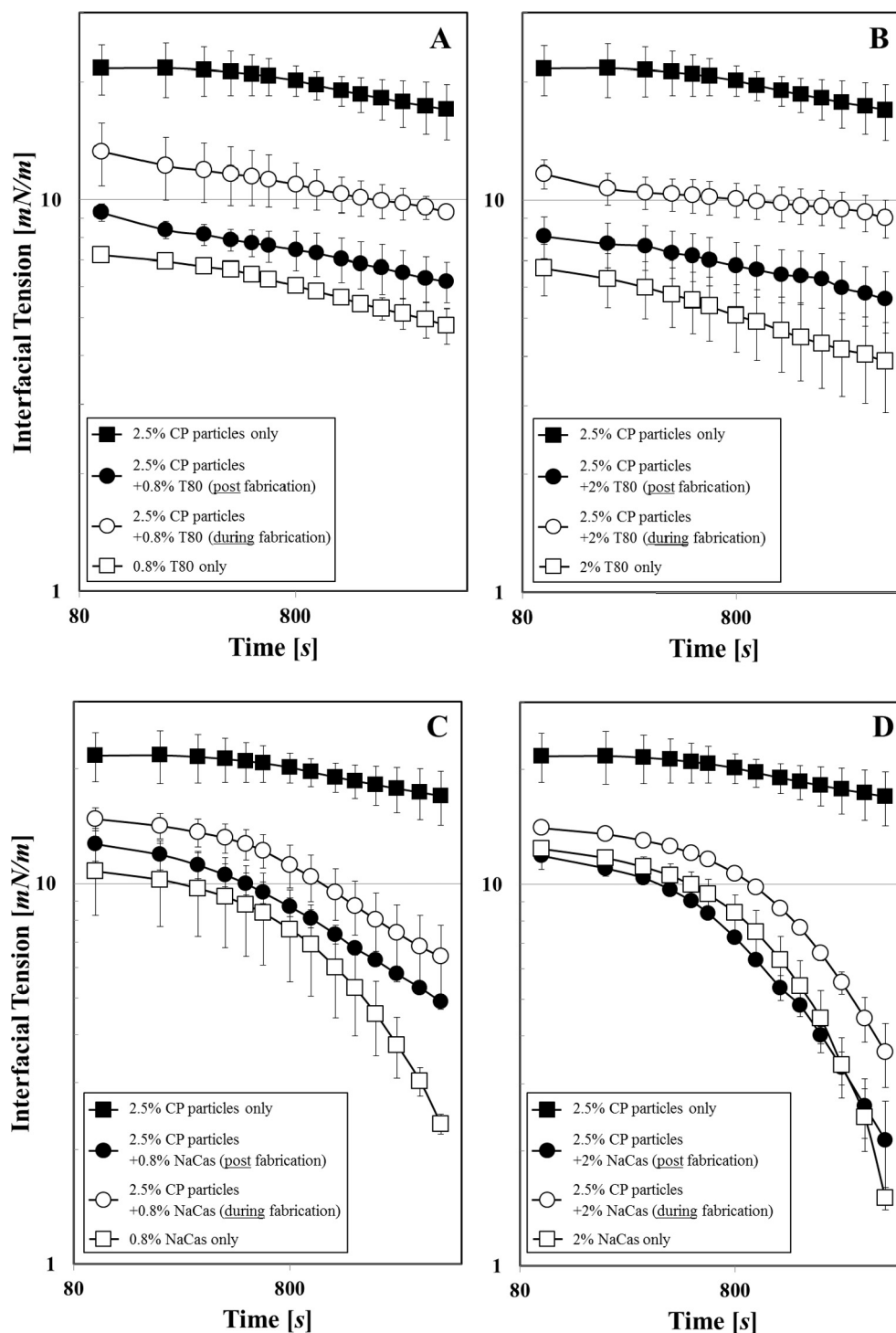


Fig. 3. Dynamic interfacial tension of aqueous dispersions of solid cetyl palmitate particles (2.5 wt/wt%) in the absence and presence of 0.8 and 2 wt/wt% Tween 80 (A and B) and sodium caseinate (C and D) added during (open circle) or after particles' fabrication (solid circle).

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 [20]. 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 pat-

terns; platelet-like particles consist of different numbered molecular layers, each one of which melts within a specific temperature range [37]. 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.

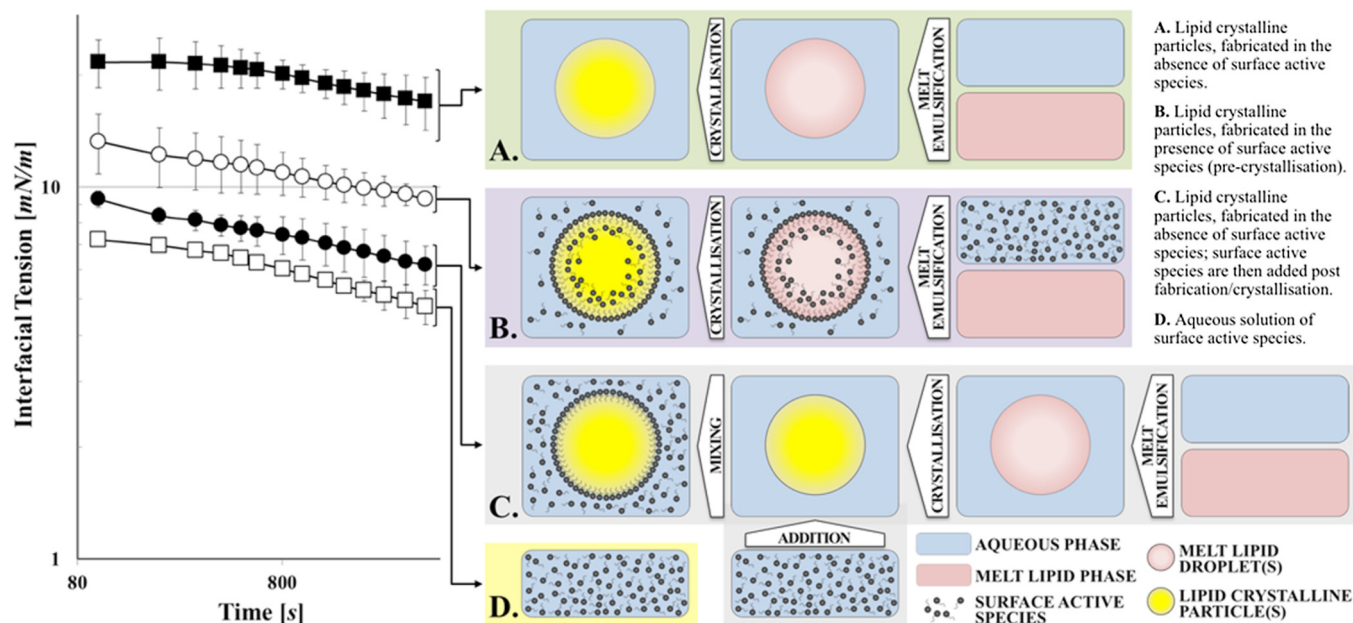


Fig. 4. A schematic diagram of the physicochemical events taking place in each of the four systems investigated for their interfacial behaviour.

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 [21,23,24], and these effects are very much related to the molecular structure of the surface active entity [21]. 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. 5A). 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 [38]. 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 [39]. 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. 6. Data from Lavigne *et al.* provide melting temperatures of bulk tristearin as obtained by DSC scans [40]. 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 top curves of Fig. 6 corresponds to a different polymorphic form of tristearin, as represented by the tabulated values (see Table S3). On the basis of the above mentioned study, the focus of this work were the three polymorphic forms as identified by Lavigne *et al.* [40]; as such, the first peak at $\sim 54^\circ\text{C}$ corresponds to the α -polymorph, the peak at $\sim 63.5^\circ\text{C}$ to the β' -form and finally the peak at $\sim 70^\circ\text{C}$ 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. 6, 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.

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 [41]. 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 Table S3). 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.* [38].

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 (2nd heating cycle) (Fig. 5A). 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 [42] where a

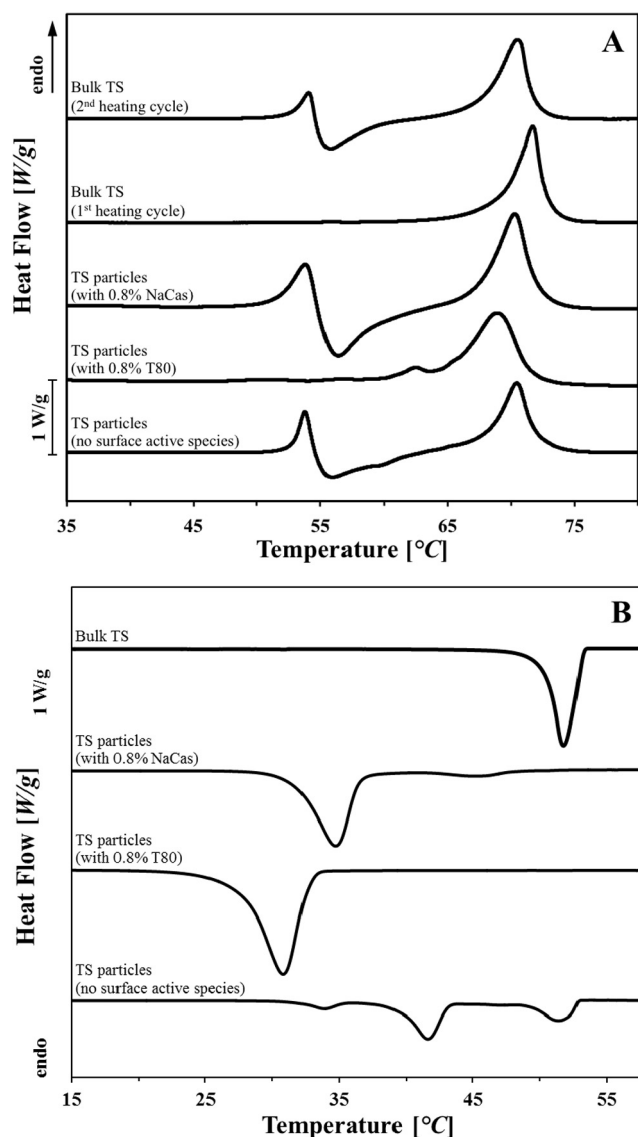


Fig. 5. DSC (A) melting and (B) crystallisation curves (scan rate 1.2 °C/min) of bulk tristearin (TS), of a 2.5% particulate tristearin dispersion without any surfactant and of 2.5% tristearin dispersion produced in the presence of 0.8% Tween 80 (T80) and 0.8% sodium caseinate (NaCas), measured after 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.

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 [25]. 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.

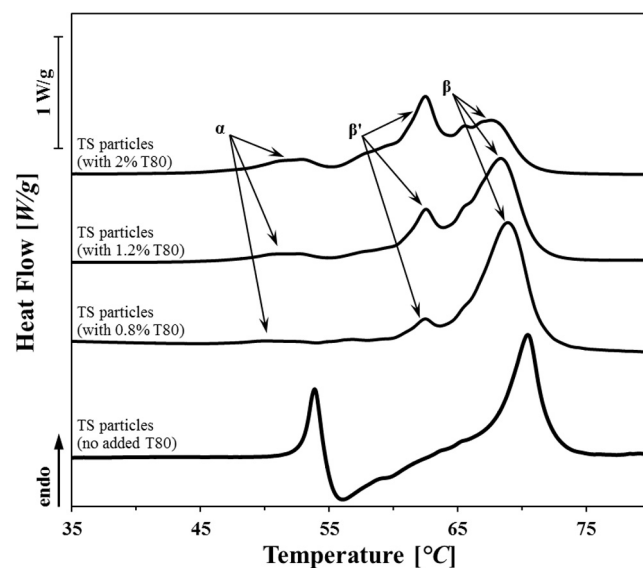


Fig. 6. DSC melting thermograms of dispersions with varying concentrations of Tween 80. The curve of the dispersion without surfactant is provided for comparison. All samples were melted from 20 to 85 °C at 1.2 °C/min. The lipid fraction is constant at 2.5 wt/wt%.

In the present study, sodium caseinate also appears to have negligible effect on the time-course of tristearin's polymorphic transitions (Fig. 5A), 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 [43–45]. 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 [45].

DSC cooling runs on the bulk tristearin revealed a single exothermic peak at ~ 52.5 °C (Fig. 5B) which corresponds to α crystals as triglycerides usually crystallise in the α -modification upon rapid cooling of the melt [46]. The enthalpy of the recrystallisation was 133 J/g which is very close to the value reported by Bunjes *et al.*, i.e. 124 J/g [46]. 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 [25]. The polymodal peaks observed in the bottom curve of Fig. 5B probably originate from the different particle size populations contained in this dispersion, rather than being distinct polymorphic forms. However, the analogous behaviour is evidenced by the same crystallisation enthalpy values (see Table S1).

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 °C) 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 °C lower than that of the bulk material is required for nucleation to occur in lipid dispersions [20,46]. Re-crystallisation for tristearin particles fabricated using Tween 80 and NaCas occurs at 31.3 and 35.3 °C 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 1), and hence would require less pronounced supercooling. The trend is the same for higher contents of Tween 80 and NaCas (see Table S2). The reason for this induced crystallisation in the case of tristearin particles produced using sodium caseinate—which takes place independent of surface active 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 while waxes only exist in the orthorhombic) [29]. Bulk cetyl palmitate revealed two melting peaks, one at 44 °C and one at 51.5 °C (Fig. 7A) which are in agreement with the values reported by Uraha *et al.* and Teeranachaidekul *et al.* [47,48]. 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 °C 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 [49].

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 Table S4). 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 Table S1), which could imply a non-significant loss of its crystallinity.

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. 7B). 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

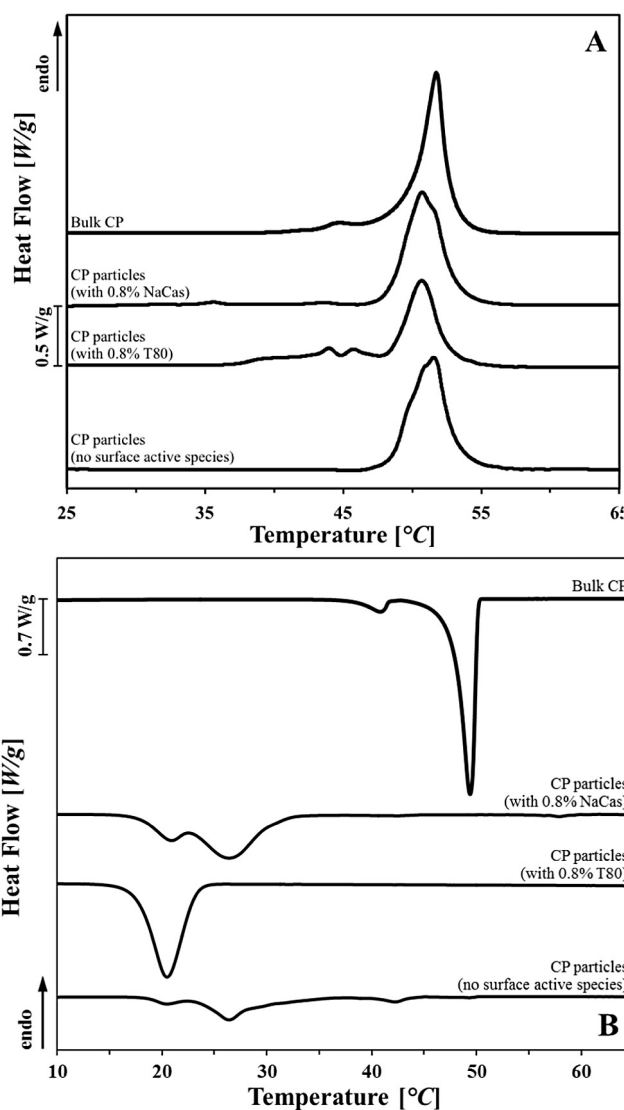


Fig. 7. DSC (A) melting and (B) crystallisation curves (scan rate 1.2 °C/min) of bulk cetyl palmitate (CP), of a 2.5% particulate CP dispersion formed in the absence of surface active species and of 2.5% CP dispersion formed using 0.8% Tween 80 (T80) and 0.8% sodium caseinate (NaCas), measured after preparation of the dispersions.

with NaCas (Table 1) required a lower degree of supercooling (onset crystallisation temperature 31.3 °C) juxtaposed to those with Tween 80 that were considerably smaller and their crystallisation event had an onset temperature of 23.1 °C. In addition to this, the bimodality of the crystallisation peak of the former particles could stem from their wider particle size distribution (Fig. 1D) as opposed to a very limited breadth of distribution for particles produced with Tween 80 (Fig. 1C).

Upon further cold storage at refrigeration temperatures (~ 4 °C) 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 Table S5). 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 [42]. 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.

4. Conclusions

Building on the previously published work on the formation and properties of solid lipid nanoparticles [17,50,51], 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 [52,53], 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 [42] 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 [25]. 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 [54]. 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 [55]) 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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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcis.2017.03.085>.

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