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Crystallisation of triacylglycerols in nanoparticles

Effect of dispersion and polar lipids

C. Lopez · M. Ollivon

Special Chapter dedicated to the memory of dr. Michel Ollivon

Abstract The crystallisation properties of a mixture of triacylglycerols (TG), cocoa butter (CB) 75%/miglyol 25%, were investigated on cooling at 0.5 °C/min using differential scanning calorimetry (DSC) and X-ray diffraction (XRD). The influence of (i) the dispersion of TG within nanoparticles stabilised by proteins, and of (ii) the presence of polar lipids were characterised. In bulk, crystallisation of TG successively occurred with a α 2L (49.3 Å) structure, then the formation of longitudinal stackings of 44.5 and 34.5 Å of β' form was interpreted as co-crystallisation of TG from CB and miglyol. The dispersion of TG in nanoparticles of about 400 nm induced a higher supercooling and changed their crystallisation properties. The formation of α 49.2 Å and β' 45 Å structures corresponded to the segregation of TG from CB in solid phases while TG from miglyol remained liquid. Phospholipids with saturated fatty acid chains affected the thermal properties of TG, which demonstrated their localisation at the surface of the nanoparticles. DSC and XRD revealed to be very sensitive and adapted methods to increase the knowledge about the mechanisms of crystallisation in emulsion.

Keywords Droplet emulsion · Interface · Phospholipid · Solid fat phase · DSC · X-ray diffraction

Introduction

The mechanisms of crystallization in emulsion are of primary interest to physical chemists and both pharmacy and food scientists, as highlighted by recent reviews [1–3].

Encapsulation of active substances in solid lipid nanoparticles (SLN) for drug delivery, sustained release or medical diagnostic using emulsion made from triacylglycerols (TG; esters of fatty acids and glycerol) has drawn attention recently [4–7]. However, the mechanisms of crystallization in emulsion which affect the drug release of pharmaceutical compounds through re-crystallisation and complex molecule partitioning in multiple phases are not well known [7]. Encapsulation in partly liquid SLN should be able to protect the encapsulated hydrophobic substances (vitamins, anti-oxidants, bioactive compounds…) from any unwanted biological degradation, for instance during the gastro-intestinal tract and/or in blood stream [7].

Considering dietary applications, many foods can be considered as oil in water emulsions since the fat phase is dispersed in the form of droplets (e.g. cream, mayonnaise). Moreover, the fat phase of food products is often partially crystallised as it is the case for chocolate (cocoa butter), margarine (mixture of partially hydrogenated vegetable oils) and dairy products (milk fat). Recently, Lopez et al. [8] calculated that more than 50% of fat is solid in an Emmental cheese stored at 4 °C in the fridge. The crystallisation properties of TG, which are the main constituents of natural fats, result from their fatty acid composition and the position of the fatty acids on the glycerol backbone. Such crystallisation plays an important role in the

This paper is dedicated to Michel Ollivon, who passed away on June 16th 2007.

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manufacture, storage, transport and consumption of emulsified food products. Thus, it is of great interest to understand the factors that influence the crystallisation behaviour of TG in dispersed systems.

Crystallization in emulsion droplets and in nanoparticles makes even more complex the process of nucleation and crystal growth well-described for bulk phase, due to the dispersion of TG molecules in individual particles. To study the mechanisms of crystallisation in emulsion droplets, authors use hydrocarbons, such as *n*-hexadecane [9, 10], pure TG [11] or simple mixture of synthetic TG [12] rather than natural fats because they can be obtained with a high purity and display sharp melting and crystallisation temperatures. However, these simplified systems are far from the complex composition of natural fats found in foods and pharmaceutical compounds, and then far from direct applications.

The group of Michel Ollivon investigated the crystallisation properties and polymorphism of TG in a natural emulsion, i.e. milk [13–15]. They showed that the crystallisation properties of TG in milk fat globules depend on (i) the cooling rate [13, 16, 17] and thermal history [18], (ii) the size of fat droplets [13, 19] and (iii) the composition of their surface [20]. Lopez et al. [21] showed that the presence of polar lipids formed after lipolytic hydrolylis of TG affects the crystallisation properties of fat in cheese. However, milk fat has a complex TG composition, a wide melting point range and a complex polymorphism which prevent from a full understanding of the mechanisms of crystallisation in emulsion.

Lopez et al. [22] previously defined and characterised a model fat permitting crystallisation in emulsion studies. This model fat is a mixture of TG with long-chain saturated and monounsaturated fatty acids (from cocoa butter, a natural vegetable oil) and short-chain saturated fatty acids (from miglyol, a synthetic oil). This mixture of TG leads to the coexistence of several solid phases and a liquid phase at room temperature and below thanks to the limited miscibility of the two types of TG. Moreover, the pertinence of this model fat for crystallisation in emulsion studies is enhanced by the polymorphism of cocoa butter [23–25].

The techniques most frequently used for the study of the thermal and structural properties of TG are differential scanning calorimetry (DSC) and X-ray diffraction (XRD).

DSC studies have given an insight into the thermodynamics of TG phase transition in bulk and in emulsions. DSC allows the characterization of the physical state changes if a change of energy is involved. However, DSC recordings are often difficult to interpret because of the polymorphism of TG. TG polymorphism relates to the ability of molecules to arrange themselves within a crystal lattice in a number of different ways of lateral packing of the fatty acid chains and of longitudinal stacking of molecules in lamellar structures [26]. The three main polymorphic forms frequently observed for the lateral packing of fatty acid chains correspond to different subcells that have been described in detail [26]: hexagonal (α form), orthorhombic perpendicular (β' form) and triclinic parallel (β form). TG crystals are made by the stacking of TG molecules layers, the thickness of which depends on the length and unsaturation of the fatty acid chains and their angle of tilt with respect to the basal planes formed by the methyl end groups of the TG [26]. Since DSC does not permit the identification of the polymorphs, the experiments require to be coupled with XRD, to identify the structure existing before and after the phase transition. The two levels of organization of crystallized TG, e.g. the lateral packing of the fatty acid chains and the longitudinal stacking of TG molecules in lamellae are easily identifiable from the short and long spacings observed by X-ray scattering at wide and small angles, respectively [27]. A new instrument, called MICROCALIX (CNRS, Châtenay-Malabry, France), combining time-resolved synchrotron XRD and high-sensitive DSC on a single sample has been developed by the group of Michel Ollivon to characterize simultaneously the structural and thermal properties of biomaterials as a function of temperature [28].

The objective of this study was to investigate the thermal and structural behaviors of the CB 75%/miglyol 25% mixture in bulk and after its dispersion within nanoparticles stabilized with proteins. The influence of polar lipids at the surface of the nanoparticles was also investigated. Experiments were conducted on cooling at 0.5 °C/min, using DSC and XRDT.

Materials and methods

Materials

Cocoa butter (CB) was a standard factory product originating from Ivory Coast (Barry Callebaut, France). Miglyol was purchased from Stearinerie Dubois fils (Ciron, France). The composition of CB and miglyol was previously reported in Lopez et al. [22]. Briefly, CB is mainly (>80%) composed of a mixture of three monounsaturated long-chain TG (POP, POSt, StOSt; P: palmitic acid, O: oleic acid, St: stearic acid), with oleic acid (C18:1) esterified at the central position of glycerol. Miglyol is a standard pharmaceutical excipient mainly composed of octanoic (C8:0) and decanoic (C10:0) acids with quasi-random esterification of glycerol positions. A mixture of amphiphilic proteins originating from milk was used in this study. Sodium caseinate obtained from Armor protéines SAS (St. Brice en Cogles, France) corresponded to industrial food grade powder. Polar lipids used corresponded to phospholipids and monoacylglycerols with various fatty acid chains which are liquid (oleic acid) or solid

(palmitic acid) at the temperature of the experiments. Phospholipids, dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylcholine (DOPC) and monoacylglycerols, monopalmitoylglycerol (MPG) and monooleylglycerol (MOG) were obtained pure at 99% from Sigma France (L'isle d'abeau, France).

Emulsion preparation

The aqueous phase consisted of distilled water, NaCl 80 mM and 0.04 wt% sodium azide as a preservative. Sodium caseinate solution was prepared by adding 4 wt% powder to the aqueous phase, and then stirred overnight at room temperature to ensure complete dispersion of the proteins. The pH of protein solution was adjusted to 6.7 with 1 M NaOH. The mixture of CB and miglyol (CB 75%/miglyol 25%; wt/wt), was performed in the liquid state at 70 °C. Fat mixture was added and mixed to the protein solution at 50 °C to give 45% (v/v) oil in the final emulsion. Emulsion premix was prepared using the rotor stator system Polytron PT 3000 (Kinematica, Littau, Switzerland) equipped with a 12 mm head working at 20,000 rpm for 30 s. Homogenisations of the coarse emulsions were then achieved at a pressure of 50 bars with a high pressure valve homogeniser (Stansted Fluid Power, Stansted, UK). The four lipidic emulsifiers, phospholipids and monoacylglycerols, were added during emulsion preparations at a molar ratio $r = [\text{lipid}]/[\text{protein}] = 1$.

Particle-size measurement

The oil droplet-size distribution was measured by laser light scattering using a Malvern Mastersizer (Malvern Instruments, Malvern, UK). The system was equipped with a lens of 45 mm focal length and the manufacturer's presentation code 0505 was selected to take into account the refractive index of oil. Standard parameters were calculated by the software: the volume-surface average diameter d_{32} defined as $\Sigma n_i d_i^3/\Sigma n_i d_i^2$, (where n_i is the number of fat globules of diameter d_i), and the specific surface area $S = 6 \cdot \rho/d_{32}$ (where ρ is the volume fraction of milk fat).

Thermal analysis

Thermal behaviors of CB 75%/miglyol 25% mixture in bulk and dispersed in emulsion droplets were monitored by differential scanning calorimetry (DSC) using a DSC-7 Perkin Elmer (St. Quentin en Yvelines, France). Samples were loaded in aluminum pans of 50 µL (pan, part #B014—3021 and cover, part #B014—3004) hermetically sealed. An empty, hermetically sealed aluminum pan was used as reference. Calibration was made with lauric acid (m.p. 43.7 °C, $\Delta Hm = 35.7$ KJ/mole, purity >99.9%). Crystal memory was destroyed by monitored with the temperature scanning program set from 60 to −7 °C at 0.5 °C/min.

Organisation of triacylglycerols: structural analysis

Characterizations of anhydrous CB 75%/miglyol 25% mixture and O/W emulsions were conducted with MICROCALIX, an instrument allowing simultaneously time-resolved X-ray diffraction (XRD) with high-sensitivity differential scanning calorimetry (DSC) on the same apparatus from the same sample [28]. X-ray diffraction as a function of temperature (XRDT) was monitored with the high-energy synchrotron beam at LURE (Laboratoire pour l'Utilisation du Rayonnement Electromagnétique, Orsay, France) on D22 bench. Two linear detectors allowed XRD data collection at small and wide angles with sample to detector distances of 177.4 and 30 cm, respectively (Fig. 1). All XRD patterns were recorded by transmission using thin glass capillaries (GLAS, Berlin, Germany) especially designed for XRD since they allow minimum attenuation of the beam and parasitic scattering. Samples were loaded by filling these glass capillaries with about 25 µL of melted fats or O/W emulsion. Channel to scattering vector q ($q = 4\pi \cdot \sin(\theta)/\lambda = 2\pi/d$; q in Å^{-1}, θ in degrees is the angle of incidence of X-ray relative to the crystalline plane, λ is the X-ray wavelength, d in Å is the repeat distance between two reticular plans) calibration of the detectors was carried out at wide angles with the β form of high purity tristearin, characterized by short spacings of 4.59, 3.85 and 3.70 ± 0.01 Å and at small angles with silver behenate characterized by a long spacing of 58.380 ± 0.001 Å, as previously reported [27].

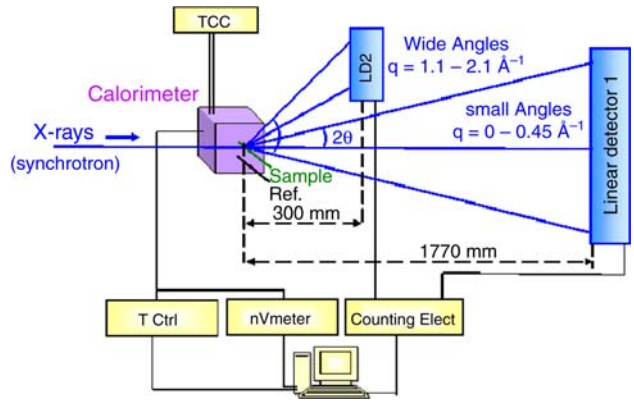

Fig. 1 Experimental set-up of MICROCALIX in the time-resolved synchrotron X-ray diffraction environment. The cell of the microcalorimeter is positioned with the capillary containing the sample perpendicular to the beam in such a way that the diffraction patterns are recorded in the vertical plane by one or two-one-dimensional proportional detectors (LD) at small and wide angles. Counting electronic (Counting Elect.), nanovoltmeter (nVmeter) and temperature controller (T Ctrl) are monitored by a single computer. The temperature-controlled cryostat (TCC) is kept at constant temperature (e.g. 6 °C)

Results

Experiments were conducted on slow cooling at $|dT/dt| = 0.5$ °C/min in order (i) to be able to separate the different solid fat phases formed by TG molecules as a function of the decrease in temperature, and (ii) to have a good signal/noise ratio permitting the study of the crystallisation properties of TG dispersed in nanoparticles with a full characterisation of the lamellar structures by XRD.

Crystallisation of triacylglycerols in bulk

The crystallization properties of CB 75%/miglyol 25% mixture in bulk were studied on cooling between 50 and −10 °C at 0.5 °C/min by coupled XRDT and DSC experiments. The final temperature (e.g. −10 °C) was chosen to allow a comparison with the crystallisation behaviour of these TG dispersed in nanoparticles (see below).

XRD patterns recorded simultaneously at small and wide angles as a function of time during cooling are presented Fig. 2. For $T \geq 20$ °C, all the TG were in their liquid state characterized by the recording at wide angles of a bump of X-ray scattering centered at 1.38 $Å^{-1}$ and no diffraction peak at small angles. For $20 > T \geq 18$ °C, the formation and increase in intensity of diffraction lines corresponded to the progressive crystallization of TG in a lamellar structure. This structure was characterized by a double chain length organization (2L) with a thickness of 49.7 Å and an hexagonal chain packing (α polymorphic form) characterized at wide angles by a single peak at 4.19 Å (Fig. 2). This structure may correspond to crystallisation of TG molecules with trisaturated fatty acids from CB (PStSt, StStSt), which have the highest melting point [25, 29]. For $17 > T \geq 13$ °C, the analysis of XRD patterns showed the growth of a α 2L (49.3 Å) structure, which corresponds to form II of CB previously identified by [23]. For $13 > T > -10$ °C, the simultaneous formation and concomitant development of two diffraction lines at 44.5 and 34.5 Å at small angles and of peaks corresponding to two β' polymorphic forms at wide angles, was interpreted as co-crystallisation of TG molecules into two structures (Fig. 3). The β' 2L (44.5 Å) structure corresponds to the form IV identified for CB [23]. This structure was formed after a $\alpha \rightarrow \beta'$ transition which lead to the tilt of the TG chains explaining the decrease in the thickness of the 2L structure from 49.3 to 44.5 Å. The β' 2L (44.5 Å) structure may be formed by monounsaturated TG from CB (saturated–unsaturated–saturated TG; Fig. 3). The β' 34.5 Å structure was interpreted as a "mixed structure" between monounsaturated TG from CB and short-chain TG from miglyol, as proposed in Fig. 3. The average difference of chain length between CB (16–18 carbons per chain) and miglyol (8–10 carbons per chain) is about eight

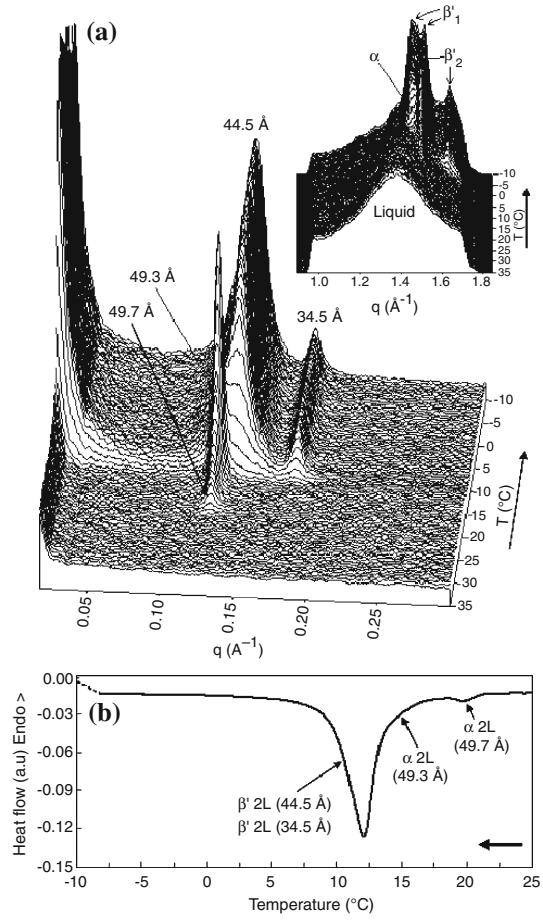

Fig. 2 Evolution of the structural and thermal properties of triacylglycerols in anhydrous state, recorded during cooling at 0.5 °C/min. (**a**) Three dimensional plots showing the evolution of the long and short (insert) spacings as determined by small and wide-angle X-ray diffraction respectively, (**b**) Differential scanning calorimetry curve recorded simultaneously on cooling

carbons and largely exceed the chain-miscibility in the solid state [26]. Then, the different chain lengths of the fatty acids from CB and miglyol TG lead to their segregation in monolayers, even if they co-crystallise with CB/miglyol = 1/1 in a bilayered structure: β' 2L (34.5 Å) (Fig. 3). Previous studies performed using DSC showed that, as a result of their different fatty acid composition, CB and miglyol TG molecules crystallize and melt in well-separated temperature domains [22]. On cooling at 0.5 °C/min, crystallisation of miglyol alone in bulk occurs in a single exotherm characterized by a temperature onset of −10.1 °C and an ending temperature of −19.2 °C. The melting behaviour of miglyol on successive heating at 2 °C/min showed three endothermic peaks until the final melting of TG at 2.25 °C [22]. Although the main proportion of TG molecules from miglyol remains liquid at −10 °C, a limited intersolubility in the solid state of TG from CB and miglyol was evidenced (Figs. 2, 3). The

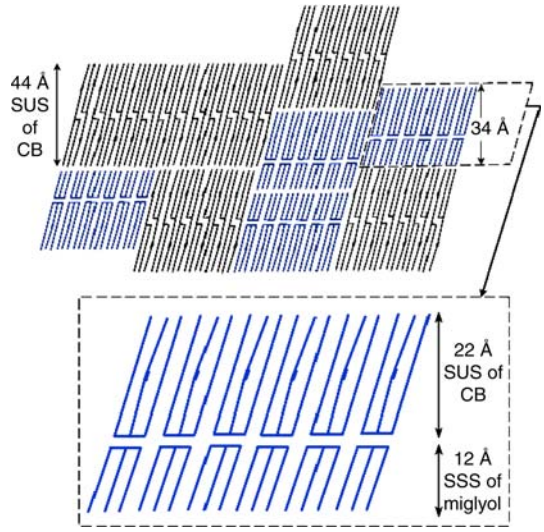

Fig. 3 Proposed structure of the solid phase corresponding to the β' packing of trisaturated (SSS) triacylglycerols (TG) from miglyol and monounsaturated (SUS) TG from cocoa butter (CB). Such a possible organisation of mixed crystals in which both short and long chain fatty acids of TG segregate from fatty acid layer to fatty acid layer corresponds to the 34.5 Å long spacing characterised by small-angle X-ray diffraction

increase in the X-ray scattering intensity recorded at very small angles ($q < 0.05$ Å^{-1}) was attributed to the small size of the mixed crystals formed on cooling (Fig. 2).

The simultaneous recording of the XRDT data and DSC signal allowed to relate the exothermic events recorded on cooling to the structural information (Fig. 2b).

Crystallisation of triacylglycerols dispersed in nanoparticles

The CB 75%/miglyol 25% mixture was dispersed in nanoparticles using the high-pressure homogenisation process. Sodium caseinate was used to stabilise the surface of the nanoparticles. Polar lipids were added in order to investigate their influence on the crystallisation properties of TG dispersed in the nanoparticles.

The structural and thermal properties of TG dispersed in the nanoparticles were characterized by synchrotron-radiation XRDT and DSC on cooling from 50 to −10 °C at 0.5 °C/min. The experiments were not conducted below −10 °C in order to avoid ice formation in the emulsions.

Dispersion of triacylglycerols in nanoparticles

The high-pressure homogenisation process created small lipid droplets with an interface between TG (hydrophobic molecules) and the aqueous phase, which was stabilized by amphiphilic proteins originating from milk, i.e. sodium caseinate. Table 1 shows the mean diameter of the droplets

Table 1 Parameters extracted from the particle size distributions of the emulsions, as a function of the composition of their interface

Interface composition[a]	Mean diameter/nm	Specific surface area/m^2 mL^{-1}
Na caseinate	470 ± 10	12.84 ± 0.03
Na caseinate + MOG	430 ± 10	14.09 ± 0.04
Na caseinate + MPG	400 ± 7	15.16 ± 0.04
Na caseinate + DOPC	400 ± 20	15.13 ± 0.57
Na caseinate + DPPC	400 ± 5	15.05 ± 0.08

[a] *Na caseinate* sodium caseinate, *MOG* monooleylglycerol, *MPG* monopalmitoylglycerol, *DOPC* dioleoylphosphatidylcholine, *DPPC* dipalmitoylphosphatidylcholine

formed using different molecules, e.g. sodium caseinate alone or with polar lipids. The small size of the emulsion droplets stabilized by sodium caseinate, i.e. 400–470 nm, can be assimilated to the formation of nanoparticles.

Figure 4a shows the XRD patterns recorded as a function of temperature at small and wide (insert) angles, which correspond to the longitudinal organization of TG molecules and the lateral packing of fatty acids, respectively. For $T \geq 13$ °C, no diffraction peaks were recorded, showing that TG molecules were in their liquid state. From about 12 °C, the simultaneous recordings of a diffraction peak at 49.2 Å (at small angles) and at about 4.16 Å (at wide angles, Fig. 4A, insert) correspond to the crystallization of TG molecules in the nanoparticles with the formation of a lamellar structure with a two-chain length organization (2L) of α form: α 2L (49.2 Å). The typical pattern recorded at wide angles indicates that the fatty acid chains were vertically packed with carbon planes randomly oriented at summits and center of a hexagonal sub-cell (α form). The α 2L (49.2 Å) structure corresponds to form II of CB [23]. Then, XRDT patterns recorded at small angles show the formation of a 2L (45 Å) structure. At wide angles (Fig. 4A, insert), 3D patterns evolution showed the progressive vanishing of the α form leading to the coexistence of β'_2 and β'_1 forms into a polymorphic transition $\alpha \rightarrow \beta'_2 + \beta'_1$. The β'_2 2L (49.2 Å) corresponds to form III of CB while the β'_1 2L (45 Å) structure corresponds to form IV of CB [23].

The thermal behavior of the emulsion, which has been recorded simultaneously to XRDT experiments is reported Fig. 4b. The DSC curve shows the successive formation of several exothermic events corresponding to the crystallization of TG molecules within the nanoparticles. The first main exothermic peak, with Tonset = 12 °C, was related to crystallization of the α 2L (49.2 Å) structure from the melt, while the second main exothermic peak with a minimum at about 4 °C was attributed to the formation of the β'_1 2L (45 Å) structure. The $\alpha \rightarrow \beta'_2$ polymorphic transition is also an exothermic process.

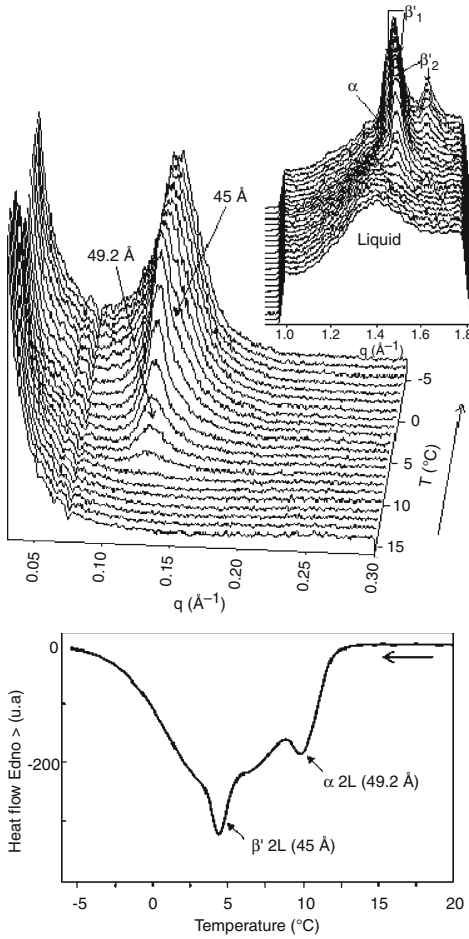

Fig. 4 Evolution of the structural and thermal properties of triacylglycerols dispersed in nanoparticules of 400 nm stabilised by sodium caseinate, recorded during cooling at 0.5 °C/min. (**a**) Three dimensional plots showing the evolution of the long and short (insert) spacings as determined by small and wide-angle X-ray diffraction respectively. (**b**) Differential scanning calorimetry curve recorded simultaneously on cooling

Influence of polar lipids at the interface

The influence of the composition of the surface of nanoparticles, mainly the lipid composition, on the crystallization behavior of TG molecules was investigated. Different polar lipids, monoacylglycerols and phospholipids, were added to the mix before homogenisation. These polar lipids were characterized by different saturations of the fatty acid chains, e.g. saturated (palmitic acid) or unsaturated (oleic acid).

The sizes of the nanoparticules formed by high-pressure homogenization in the presence of sodium caseinate and polar lipids are presented Table 1. It appears that the presence of the polar lipids in the mix decreased the size of the nanoparticules compared to the sodium caseinate stabilized emulsion, and then increased the surface area. This was attributed to the tension-active properties of the polar lipids.

The thermal properties of the emulsions were analyzed on cooling from 60 to −7 °C, using two different DSC apparatus, i.e. a DSC-7 (Perkin Elmer) and the DSC coupled with XRDT. As shown in Fig. 5, both series of recordings displayed the same main exotherms at the same temperature with the same enthalpies demonstrating that all thermal events recorded were reproducible and significant. On cooling, DSC experiments allowed the recording of the liquid → solid phase transition of TG in the nanoparticles. On another hand, the recordings of Fig. 5 were different showing the influence of interface composition on TG crystallization.

As for the sodium caseinate stabilized emulsion (Fig. 5a), the DSC curves recorded for the emulsions containing MOG (Fig. 5b), DOPC (Fig. 5c) and MPG (Fig. 5d) were characterized by a first main exotherm with a minimum at about 9–10 °C, and a second main exotherm with a minimum at about 3–4 °C. All the exothermic peaks were attributed to the crystallization of TG since these compounds were by far the major constituents able to crystallize in the emulsions in the temperature range

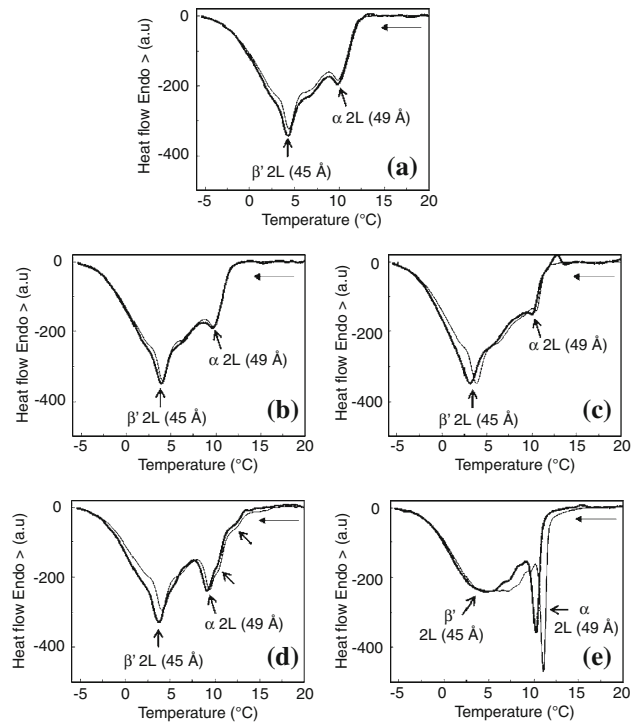

Fig. 5 Thermal properties of triacylglycerols dispersed in nanoparticles. Differential scanning calorimetry (DSC) curves recorded on cooling at 0.5 °C/min with two different calorimeters; thin line: DSC-7 (Perkin Elmer), thick line: DSC coupled to X-ray diffraction (MICROCALIX). The emulsions were stabilised by (**a**) sodium caseinate and sodium caseinate in the presence of polar lipids: (**b**) monooleylglycerol (MOG), (**c**) dioleoylphosphatidylcholine (DOPC), (**d**) monopalmitoylglycerol (MPG), (**e**) dipalmitoylphosphatidylcholine (DPPC)

considered. The formation of multiple overlapped exotherms (at least four) makes the interpretation of the DSC curves complex since only two longitudinal organisations were identified by small-angle X-ray diffraction (α 2L 49 Å and β' 2L 45 Å), and then related to the two main exotherms recorded on cooling (Fig. 5).

In contrast with the other polar lipids, a sharp exothermic peak was observed at about 11–12 °C in the presence of DPPC prior to any other crystallization of TG molecules in the nanoparticles (Fig. 5e). It should be stressed that this exothermic peak was not due to one of the transitions $L\alpha \rightarrow P_{\beta'}$ or $P_{\beta'} \rightarrow L_{\beta'}$ (corresponding to Lamellar fluid → Rippled → Lamellar gel transitions, see [30]) of this lipid since the temperatures at which they are usually observed, 41.5 and 35 °C, respectively, and the energies involved do not correspond to the transitions observed. The latter were at least one order of magnitude larger than those of pure DPPC taking its concentration into account [30]. Figure 6 shows the 3D plots of the small and wide (insert) angle XRD patterns recorded on cooling of the emulsion containing DPPC. Crystallisation of TG dispersed within the nanoparticles was initiated with the formation of a α 2L (49 Å) structure and then the formation of $\beta'_2 + \beta'_1$ 2L (45 Å) structures. The presence of DPPC in the emulsion did not change the type of crystalline structures identified by XRD as a function of the decrease in temperature. The DSC recordings show that the presence of DPPC in the emulsion shifted and triggered the crystallization of TG towards higher values (11–12 vs. 9–10 °C for the other polar lipids). Thus, DPPC catalysed the nucleation of TG dispersed in the nanoparticles. Phospholipids such as DPPC can self-organise at the surface of the nanoparticles and the fatty acid chains in the solid state may interact with the high-melting point TG of CB [11]. An alternate explanation is that DPPC exerts during the first crystallization process (formation of α 2L 49 Å structure) an indirect influence on the TG crystallization by subtracting substances needed to initiate the second crystallization process (formation of β' 2L 45 Å structure) or by changing interface local curvature. The presence of MPG in the emulsion also modified the DSC crystallization profile with two minor exotherms recorded at about 13 and 11 °C before the first main exotherm (Fig. 5d). Polar lipids containing unsaturated fatty acids such as oleic acid in MOG and DOPC, only modified moderately the DSC profile compared to that of the sodium caseinate stabilized emulsion (Fig. 5).

Figure 5 shows that the liquid → α-form phase transition of TG was sensitive to both the fatty acid chain saturation and the type of polar lipid added in the emulsion. The crystallization of TG molecules in the nanoparticles occurs via a heterogeneous nucleation induced by the molecules present at the interface.

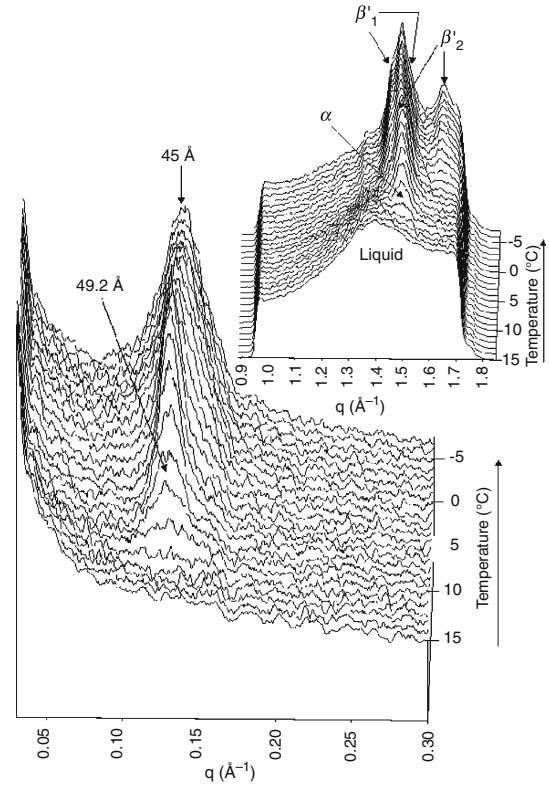

Fig. 6 Evolution of the structural properties of triacylglycerols dispersed in nanoparticules stabilised by sodium caseinate in the presence of dipalmitoylphosphatidylcholine (DPPC), recorded during cooling at 0.5 °C/min. Three dimensional plots showing the evolution of the long and short (insert) spacings as determined by small and wide-angle X-ray diffraction respectively

Discussion

Increasing the knowledge about the crystallization properties of TG and the parameters that affect their behavior when they are dispersed in emulsion is of primary importance. However, few studies focused on this subject since the investigation of the thermal and structural behavior of TG dispersed in nanoparticles is much more challenging than in bulk because of the presence of water that decrease the recorded signals by simple dilution effect.

Dispersion of TG molecules in nanoparticles of about 400 nm changed their thermal properties and their organization in the solid state. This is not surprising since compared with crystallization of TG molecules in bulk, crystallization in dispersed system is an individual event occurring in each droplet independently of the neighboring ones. A catalytic impurity has to be present in each droplet to induce the formation of a nuclei and then the growth of fat crystals. Crystallization of TG molecules dispersed in the nanoparticles (i) started with a higher supercooling $\Delta T = 19.7 - 12 = 7.7$ °C, which is due to the lack of catalytic impurities in most of the nanoparticles, (ii) span over a

larger temperature range, and (iii) exhibited a much more complex thermal behaviour, compared to bulk fat. The rapid succession of several exotherms in a small range of temperatures is the result of numerous structural reorganizations that occurred within the nanoparticles on cooling of the emulsion.

Regarding the organization of TG molecules in the solid state, the crystallization was initiated both in bulk and in the nanoparticles in the α 2L (49.2–49.3 Å) form after relaxation of supercooling from the melt. The α form is the most unstable polymorphic form of TG molecules, in which the lateral packing of the fatty acid chains is not very tight and the chains have considerable rotational freedom. Then the solid phase formed on cooling was different in bulk and in the nanoparticles. Crystallization of TG in the nanoparticles showed the successive formation of the α 2L (49.2 Å) and then the β'_2 2L (49.2 Å) and β'_1 2L (45 Å) structures which correspond to the forms III and IV identified for CB [23]. The absence of the structure characterized in bulk by a thickness of about 34 Å was interpreted as the segregation of TG molecules from CB and miglyol during cooling of the nanoparticles. While the CB 75%/miglyol 25% mixture partially co-crystallise in bulk, its crystallization behaviour shows the segregation of TG when they are dispersed in nanoparticles. Thus, only TG molecules from CB crystallized within the nanoparticles, while TG molecules from miglyol remained in their liquid state in the temperature range investigated. The analysis of the wide-angle XRD patterns showed that the β'_1 form (form IV of CB) was less important in the nanoparticles than in bulk (Figs. 2 and 4). Also, as a function of the decrease in temperature, the composition of the liquid phase may change as TG molecules migrate from the liquid to the solid phase. Thus, on cooling, we observed the following evolutions in the nanoparticles: liquid 1 → α 2L (49.2 Å) + liquid 2 → β'_2 2L (49.2 Å) + β'_1 2L (45 Å) + liquid 3. By solubilising TG from CB, the liquid fat phase may favor the polymorphic phase transitions.

The broadening of the small-angle XRD lines recorded for the solid phases of TG molecules dispersed in the nanoparticles was interpreted as a decrease of coherence length. This decrease resulted from an increase of the disorder in the crystalline organization of TG molecules and/or of a decrease of the crystal size formed within emulsion droplets [31]. The dispersion of TG molecules in nanoparticles increased the supercooling, which may lead to a fast relaxation process from metastable state. As a consequence, it is possible that fat crystals have (i) a poorer crystalline organization or (ii) a smaller size (also due to the constraint originating from the curvature of the interface) or (iii) both.

In nanoparticles, the growth of the crystals is limited by the small volume of the particles and by the interface. This study raises the question of the size and the organization of fat crystals in such small particles.

The presence in the emulsions of polar lipids containing saturated fatty acids (MPG, DPPC) influenced the crystallization behaviour of TG in the nanoparticles. We concluded that these emulsifiers (i) should be located at interface which is in agreement with their amphiphilic properties and (ii) may influence the crystallization of TG molecules by their liquid crystalline organization at interface as already observed in emulsions [32–34]. On the contrary, emulsifiers containing unsaturated fatty acids did not greatly affect the crystallization behavior of emulsified TG. The polar lipids may be localized at the surface of nanoparticles and in the aqueous phase. Particularly, phospholipids in excess were likely located in bilayered vesicles coexisting with the emulsion droplets [35, 36]. Assuming both that all phospholipid molecules are located at interface and that the surface area occupied by each molecule is about 0.6 nm^2 in excess water [26], we calculated that less than 5% (about 0.72 $m^2\ g^{-1}$ of lipid) of the surface area of an emulsion droplet stabilized by sodium caseinate in the presence of DPPC with $d_{32} = 400$ nm (15 $m^2\ g^{-1}$ of fat), was occupied by this phospholipid. This demonstrates the indirect sensitivity of the DSC and XRDT measurements to interface composition.

In this study, while it was not possible to show any role directly played by the composition of the interface on nucleation, its influence on the subsequent step of crystal growth was clearly established through the changes induced on DSC recording as well as on XRDT data.

Conclusions

XRD experiments performed using synchrotron radiation allowed (i) to study the crystallization behaviour of TG within nanoparticles (∼400 nm), (ii) to identify the longitudinal stacking of TG molecules (at small angles) and the packing of the fatty acid chains which corresponds to the polymorphic forms (at wide angles) and iii) to follow their evolution as a function of temperature. The coupling of synchrotron radiation XRD with DSC, performed thanks to the calorimeter MICROCALIX developed by Michel Ollivon [28], allowed to relate the structural information to the thermal events and then to identify the main exothermic peaks. Moreover, we showed that DSC and XRD are highly sensitive to the variations of interface composition, mainly phospholipids with saturated fatty acid chains such as DPPC. The thermal and structural properties of TG changed after their dispersion in nanoparticles. It has been shown using the CB 75%/miglyol 25% mixture previously defined as a model fat to study crystallization in emulsion [22] that (i) the dispersion of TG in nanoparticles, and (ii) the presence of polar lipids at the interface influence the crystallization behavior of TG.

It appears interesting to develop further studies investigating the influence of the size of emulsion droplets on the mechanisms of crystallization of TG molecules since the organization of fat crystals in a confined environment is still questionable.

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