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D. Kalnin
P. Quennesson
F. Artzner
O. Schafer
T. Narayanan
M. Ollivon

Monitoring both fat crystallization and self-assembly of sodium caseinate in model emulsions using synchrotron X-ray diffraction

D. Kalnin (✉) · M. Ollivon · F. Artzner
UMR CNRS 8612 Equipe Physico-Chimie des Systèmes Polyphasés, Châtenay-Malabry, France
e-mail: daniel.kalnin@web.de
Tel.: +33-1-46835465;
Fax: +33-1-46835312

D. Kalnin · P. Quennesson
Nestlé Product Technology Center Beauvais, Z.I. no. 2, 60000 Beauvais, France

O. Schafer
Nestlé Research Center, Vers-chez-les Blanc, 1000 Lausanne 26, Switzerland

T. Narayanan
European Synchrotron Radiation Facility, ESRF, BP 220, 38043 Grenoble Cedex, France

Abstract X-Ray diffraction analysis at small angles is proposed as a nondestructive method for characterization of emulsions. The paper focuses on determinations of both, the concentration of sodium caseinate (SC) used as a stabilizer and the organization of triglyceride crystals, located outside and within emulsion droplets, respectively. Measurements performed at very small angles using ID 02a at a sample-to-detector distance of 6.5 m made it possible to monitor the SC submicelle size variations at $q \sim 0.03$ Å^{-1} while exploring a SC/water binary system. Comparison with the scattering found in model emulsion shows congruent results. The characterization of the crystalline organizations of triacylglycerols was also performed simultaneously at $q \sim 0.1$ Å^{-1}. Phase transitions were directly observed within the emulsion droplets. Both characterizations allowed direct quantitative determinations of the submicellar SC concentration in the presence of emulsion and of the respective proportions of the various polymorphic forms of the fat within emulsion droplets. These experiments have shown that, knowing the primary concentrations of all ingredients, X-ray scattering at very small angles can be used, to monitor submicellar casein aggregation and fat polymorphism in a single analysis.

Keywords Triglyceride · Miceller formation · Sodium caseinate · SR · Polymorphism · Small-angle X-ray scattering · Emulsion · Interface

Introduction

Characterization of thermal and structural behavior of fat in emulsion droplets or more generally of any substance in finely dispersed systems represents a main challenge in many fields. In this respect, techniques based on scattering of light whatever its wavelength are well suited to study colloids of biological, pharmaceutical, medical or industrial interest. However, for instance, until recently, droplet size, concentration of fat in the sample and complexity of the systems prevented any direct identification of the crystalline varieties formed by triacylglycerols inside emulsion droplets [1]. Crystallization within droplets of a mean diameter of around 0.5 μm can now be monitored easily using synchrotron X-ray diffraction [1, 2, 3]. As the crystallization kinetics depend not only on the origins of the triglycerides (TAGs) and their composition but also on the size of the fat globules and the interface composition [3] it is important to handle the influence of both factors. Conversely, crystallization can be used as a reporter of the interface composition and organization [3], presuming the partition coefficients of the amphiphilic molecules between interface, bulk and dispersed phases are known.

Most dispersed multiphase systems are thermodynamically unstable per se and thus require stabilization. In the food industry, actually stabilization of emulsions is obtained by the addition of proteins such as sodium

caseinate (SC). The addition of this protein has a double stabilizing effect since it provokes together with other added surfactants an increase of both viscosity of the continuous phase and stability of the interface [4, 5].

Crystallization behavior not only depends on the time/temperature history of the samples but also on the interface of the fat globule. Then, multicomponent systems – with any component being susceptible to influence the physical chemistry of the others – render investigation of emulsions very difficult. Numerous phases coexist in emulsions. Moreover, a supplementary phase is added to the system each time one of the components crystallizes or is at least partly crystalline. In this respect, the crystallization of the interfacial film or of the oily phase frequently with melting and/or crystallization points close to room temperature even further increases the complexity of the study of thermal properties – especially when TAGs are involved [1].

The use of destructive methods for the determination of the interfacial concentration of proteins or other lipid surfactants is really time-consuming, costly and not in any case realizable. Moreover, the dependence on temperature of the interface composition is difficult to assess with these techniques.

Differential scanning calorimetry (DSC) is mainly used to elucidate thermal and structural behavior of TAGs – the main constituents of fats – which exhibit complex monotropic polymorphism. The numerous DSC peaks observed whilst heating and cooling need further identification with the help of techniques that yield information about structures (e.g. X-ray or neutron diffraction, IR spectroscopy). The coupling of both small-angle X-ray scattering (SAXS) and wide-angle X-ray diffraction (WAXS) with DSC represents a powerful technique for the characterization of crystallization in emulsion and of the influence of system parameters such as droplet size and interface composition.

This paper complements a series of studies on TAG crystallization in emulsions by providing a nondestructive and convenient method using SAXS for both the characterization of SC in the aqueous phase together with the monitoring of the polymorphic transitions of the fat within the globules.

Material and methods

Binary SC/water system

SC was industrial food grade(Armor Protéines). The same SC was used for binary water systems and emulsion stabilization. Samples for the study of the binary SC/water system were prepared by dispersing weighed amounts of SC solubilized in Millipore water and afterwards weighing for concentration determination. Each sample, about 3 cm^3, was prepared without sodium azide. An X-ray diffraction study was carried out about 24 h after preparation to ensure equilibration of protein solutions.

Model oil-in-water emulsions

Ingredients of industrial food grade were weighed before emulsion preparation. The lipid emulsifier used was GMS mainly composed of glycerol monostearate provided by Danisco Coulter (Denmark). SC was first dissolved in preheated water and was so hydrated. Then, under mechanical mixing conditions, preheated fat was added at 60° for 20 min. Model emulsions with 40% fully hydroginated vegetal fat content were prepared using a pilot plant homogenizer at about 350 bars. Different emulsifier concentrations ranging from 0.5 to 1.5% were used. Emulsifier was added to the aqueous phase prior to homogenization. All emulsions were controlled for emulsion droplet mean size using a laser light diffraction apparatus (Malvern Mastersizer, Malvern, UK). One has to note that those emulsions are thermodynamical then stable for months. They display the same fat globule size distribution providing they are lightly agitated by hand shaking before the actual measurement.

X-ray diffraction

X-ray diffraction was performed using the high flux of the synchrotron radiation beam at beamline IDE02a of ESRF (Grenoble, France) using a wavelength of 1 Å (flux through sample 8×10^{12}ph s^{-1}/100 mA at 12.5 keV). An image-intensified FReLoN charged-coupled-device SAXS detector was used for data collection using sample-to-detector distances of 1.5 and 6.5 m. All X-ray diffraction patterns were recorded by transmission using glass capillaries (GLAS W. Müller, Berlin, Germany) (diameter 1.5 mm, wallthickness 0.01 mm). Samples were prepared by filling these glass capillaries with about 20 μl sample using a laboratory-made special syringe. The sample was tempered in a multiple sample holder (20 holes) designed in our laboratory for a temperature range from 10 to 100 °C. X-ray scattering was monitored at 20 °C for equilibrium and during temperature ramps. Temperature ramps were from 20 to 60 °C, then from 60 to 10 °C for SC solutions and from 60 to 10 °C with an isothermal regime at 60 °C prior to the actual measurement in order to eliminate all temperature/time history of the sample for emulsions, respectively.

Results and discussion

Scattering patterns of emulsions stabilized by SC present an intense central scattering [1]. This scattering is already detectable at a sample-to-detector distance of approximately 1.2–1.5 m, but the diffracted signal is not quantitatively exploitable in this region owing to the vicinity of the beamstop cut at about $q = 0.025$ $Å^{-1}$ [1]. Such observation led us to the investigation of this scattering at a sample-to-detector distance of 6.5 m. Increasing the distance leads to a higher resolution and the possibility to observe scattering at lower q values. In order to determine the origin of these scattering peaks, samples containing SC solutions were examined first, then the results were compared to those of the emulsions.

Scattering from SC solutions

At a sample-to-detector distance of 6.5m, SC solutions scatter X-ray as a function of protein concentration. At first glance and from rapid analysis, the scattered

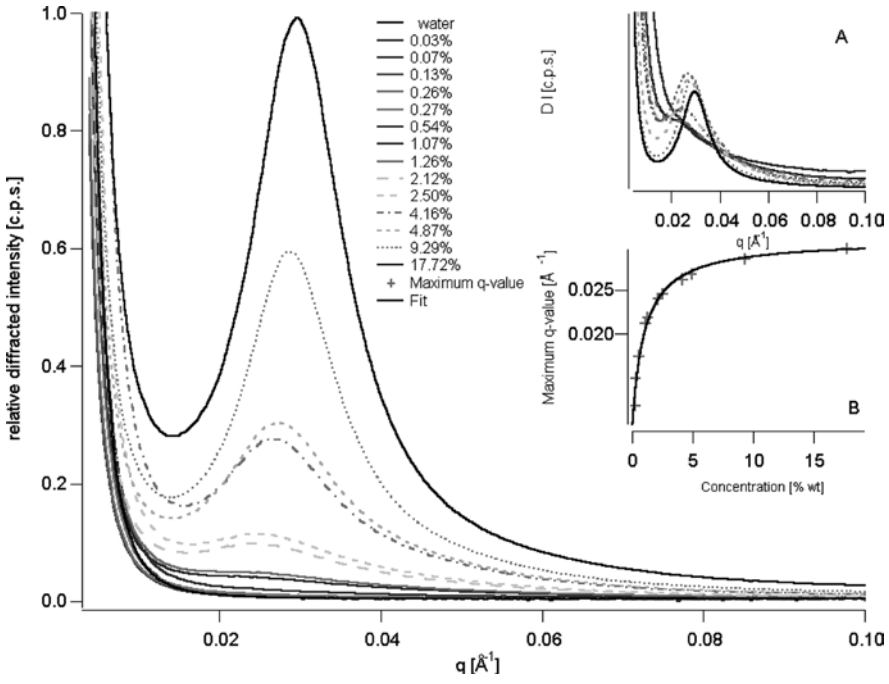

Fig. 1 X-ray scattering at very small angles (*SAXS*) patterns at a sample-to-detector distance of 6.5 m as a function of sodium caseinate (*SC*) concentration in aqueous solutions shows an increase of the scattering due to "micelle" formation. The SC concentrations examined are listed and attributed to the different line shapes *Insert A*: SAXS pattern of normalized intensity of aqueous SC solutions. Normalization is carried out by subtraction of pure water scattering as a function of the water content and by division through the SC concentration. *Insert B:* Evolution of the maximum-scattering peak position as a function of SC concentration determined from the normalized patterns using Gaussian fits

intensity is relative to the amount of SC engaged in the micelle structures. Conversely, solutions can be identified following their SC concentration very easily from scattering curves (Fig. 1).

The normalization of the scattering curves taking in account the scattering of pure water and the concentration of SC is shown Fig. 1, insert a. The plot was only normalized for SC concentration since the setup (multiple position sample holder) design did not allow rigorous scattering normalization and measurements of absolute intensities. Consequently, this plot cannot be directly compared for intensities since they were obtained from different capillaries (scattered intensities are not corrected for the different capillary sizes and sample absorption of X-ray; this mostly explains the intensity dispersion of the curves Fig. 1, insert a). However, the shift of the scattering maximum observed in this normalized plot shows that the aggregation state of the protein changes as a function of its concentration. (Fig. 1, insert a). Figure 1, insert b further evidences the variation of the scattering peak maximum as a function of SC concentration. Practically, scattering can be detected and well fitted on normalized diffraction patterns for an SC concentration of 0.26 wt% or greater. The variation of this scattering maximum, q_{max}, is not linear and beyond a certain SC concentration the shift observed becomes weaker and weaker. Two regimes are observed, up to about 3 wt% and beyond 3 wt%. The latter regime is asymptotic, with an asymptote at about $q = 0.033$ Å^{-1} which correlates to a distance of about 18 nm. What are the exact origins of this scattering and of its dependence upon SC concentration?

Taking into account the domain of the scattering vector, q, in which scattering is observed, the direct scattering from the whole casein micelles was ruled out [7, 8, 9, 10]. Casein micelles which are aggregates known to exhibit a mean diameter in the range 0.2–0.3 μm [9] are too large for the scattering lengths at which scattering is observed. If not related to casein micelles, then the scattering patterns observed result from internal organization into casein submicelles [11]. Considering the complexity of the internal organization of the casein submicelles with possibly different types of casein submicelles, the distances corresponding to the minimum and the maximum of the scattering patterns are not necessarily directly correlated to some specific length within the casein micelle or submicelle. The submicelle shape factor and the organization of the SC molecules within this aggregate should be considered to find a model of the internal organization of casein micelles into subunits. This is out of the scope of this study. While the existence of the casein submicelle has been discussed for many years [7]. Kumosinski et al. [12] proposed recently from SAXS analysis a model for the organization of these particles. According to them, the submicellar particles consist of an inner, spherically symmetrical, hydrophobic and relatively electron-dense core surrounded by a hydrophilic and less electron-dense regions. The latter loose regions of adjacent micelles interact with each other [12]. This model was refined from the comparison with electron microscopy studies of casein submicelles [13]. As this submicelle model is in agreement with the data presented earlier for scattering lengths, we concluded that the scattering signal observed in caseinate

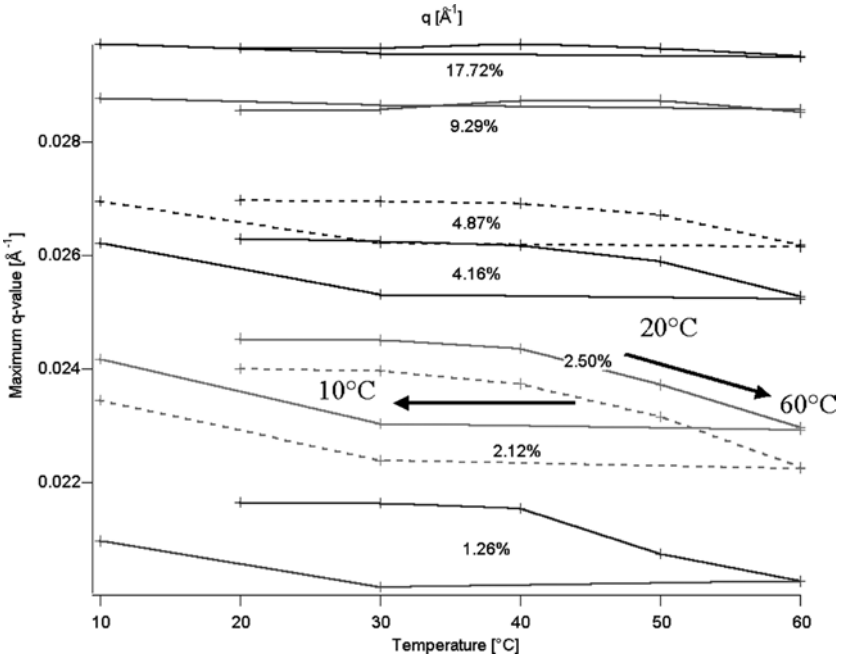

Fig. 2 Evolutions of maximum positions of SAXS peaks, determined from the normalized patterns in conditions as in Fig. 1, as a function of SC concentration and temperature. Each sample was first heated from 20 to 60 °C and then cooled to 10 °C, as indicated by *arrows*

solutions originated from a submicellar organization of SC.

Temperature dependence of the SC scattering

Solubility of SC in aqueous solutions is influenced by many factors, including temperature. The changes of the scattering maximum, q_{max}, as a function of temperature as recorded during heatings and subsequent coolings of SC solutions of various concentrations are shown in Fig. 2. As seen from Fig. 2, we were able to monitor even slight decreases of the q_{max} value thanks to peak-fitting. The q_{max} shifts indicate for all concentrations that a lower concentration of submicellar SC is observed at low temperature and this is consistent with the known higher solubility of casein at lower temperatures [9]. Such a solubility dependence is also reflected more directly from the variation of scattering intensity as a function of temperature from the same capillary, whatever SC concentration considered, a systematic decrease is observed at lower temperature (scattering intensity variations are not shown since, again, the scattering plots from different concentrations cannot be directly compared for intensity). The scattering intensity changes by about 8% at maximum, while the half height width does not compensate this temperature-induced variation. However, both intensity and q_{max} shifts indicate that fewer submicelles are formed at high temperature probably because of the increased solubility of the unaggregated casein [9].

Moreover, from the q_{max} variation versus temperature we deduced that these temperature-induced solubility changes are reversible (Fig. 2). The reversibility of this effect can also be monitored by X-ray diffraction. As shown in Fig. 2, hysteresis is observed whatever the concentration considered. This hysteresis has been attributed to slow equilibration kinetics within the submicelles. The exact time dependence of this equilibrium has not been measured. Further studies would be necessary to determine the rate of equilibration.

Scattering from SC solutions of oil-in-water fat emulsions

Emulsions stabilized with different concentrations of SC also show changes in the central scattering (Fig. 3). For SAXS, a q range from 0.03 to 0.4 Å is sufficient in order to monitor fat crystallization in these emulsions (data not shown) [2, 3]. However, X-ray recording at a sample-to-detector distance of 6.5 m (SAXS at very small angles) shows better evidence of the scattering phenomena owing to both dispersion of the fat in fat globules with a mean size of around 0.5 μm and self assembly of SC. The first-mentioned effect should be about the same for all emulsions measured because the fat concentration in the fat globule and the sizes are the same in the different emulsions. The only factor changing between the different emulsions is the SC concentration. The variations of the SAXS patterns as a function of surfactant and emulsifier content are shown in Fig. 3. In spite of the superimposed strong scattering due to the emulsion, the SC scattering of the solution is observed. This scattering can be compared to that obtained at different SC concentrations in the absence of oil droplets. Can we

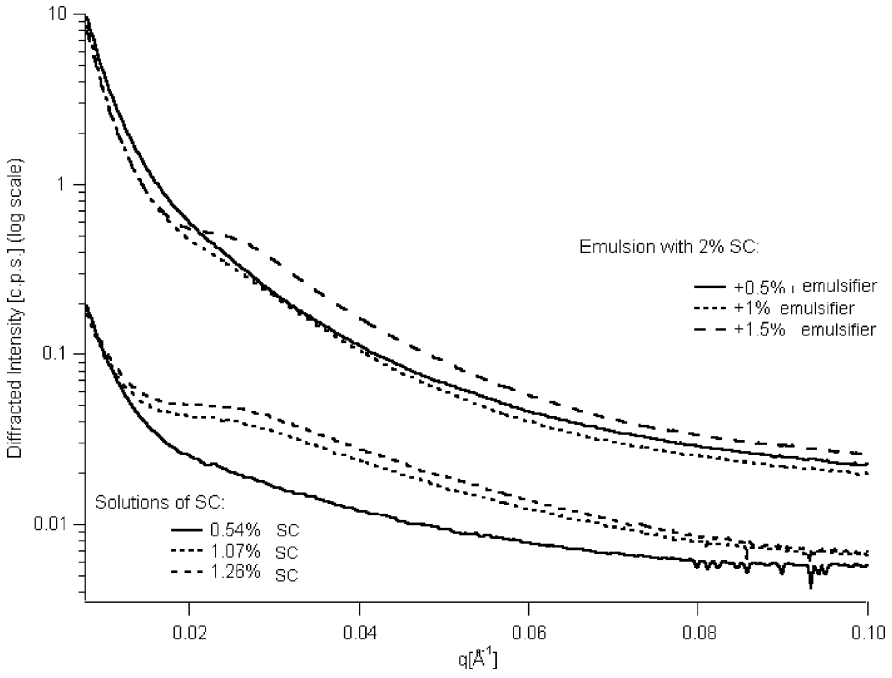

Fig. 3 SAXS patterns due to caseinate self-assembly in model emulsions (2% SC) at the different lipid emulsifier concentrations (*top*) compared for convenience to the scattering patterns (*bottom*) of three solutions of the binary SC/water system shown in Fig. 1. Both series of measurements were carried out at 20 °C

use this scattering to directly evaluate the submicellar SC content?

In an emulsified system, the "solubilization" of SC results in fact from the distribution of SC molecules at different locations and with different organizations. According to the literature, solubilization of SC is achieved by (1) SC molecules really solubilized as monomeric or oligomeric entities, and forming a true protein solution, (2) SC molecules organized in micelles and submicelles from which the scattering at very small angles originates (see earlier) and (3) that adsorbed at the fat globule interface. In this last location, depending on their nature, SC molecules can be either lying at the interface as a polymer with loop and train segments or in the form of submicelles [5, 6, 11, 12, 13]. In homogenized emulsions, the presence of casein submicelles at the globule interface can probably be ruled out. Although emulsions cannot be considered as systems at equilibrium, it can be assumed that the distribution of the SC molecules between the different locations obeys some kind of equilibrium. This can be deduced of the SC mobility and the existence of different locations without strong interactions. Then, molecules which are not in the aqueous phase can be supposed to be either at the fat globule interface or "solubilized" within the micelles and submicelles.

The comparison of SC scattering curves obtained before and after heating the emulsion from 20 to 60 °C allowed us to rule out any influence of the presence of lipid on the scattering. In this respect, it has been confirmed that a single heating did not affect the size distribution of the emulsions. Determining the maxima of the scattering peak thus makes it possible to evaluate the concentration of SC solubilized in the form of submicelles.

Diffraction from TAG crystallization within the fat globule of oil-in-water emulsions

The TAG crystallization within droplets of a SC model emulsion containing lipid surfactants has been monitored by time-resolved SAXS during sample cooling at 2 K min^{-1} from 60 to 10 °C [1, 2, 3]. Only the last frames of the time/temperature evolution are shown Fig. 4. The model emulsion (Fig. 4A) contains a high concentration of SC (5%) and 0.5% added monoglycerides. On the SAXS recordings, the large bump of scattering encountered at $q \sim 0.18$ $Å^{-1}$ is due to the fact that at this temperature liquid fat is partially liquid-crystalline. During the cooling, crystallization in the emulsion which starts by the formation of an α (rotator) chain packing of TAG precedes to an α-to-β' transformation (WAXS data not available). The longitudinal stacking change from untilted to tilted chain position visible from SAXS which accompanies this lateral chain packing transition is shown Fig. 4B. The evolution of the SAXS during the same time temperature/history at the same surfactant concentration as emulsion concentration but at a lower concentration of SC (2%) is also shown in Fig. 4B. The rate of α-to-β' transformation is strongly affected by the change in the concentration of SC.

These results demonstrate that TAG crystallization can be quantitatively monitored within the emulsion

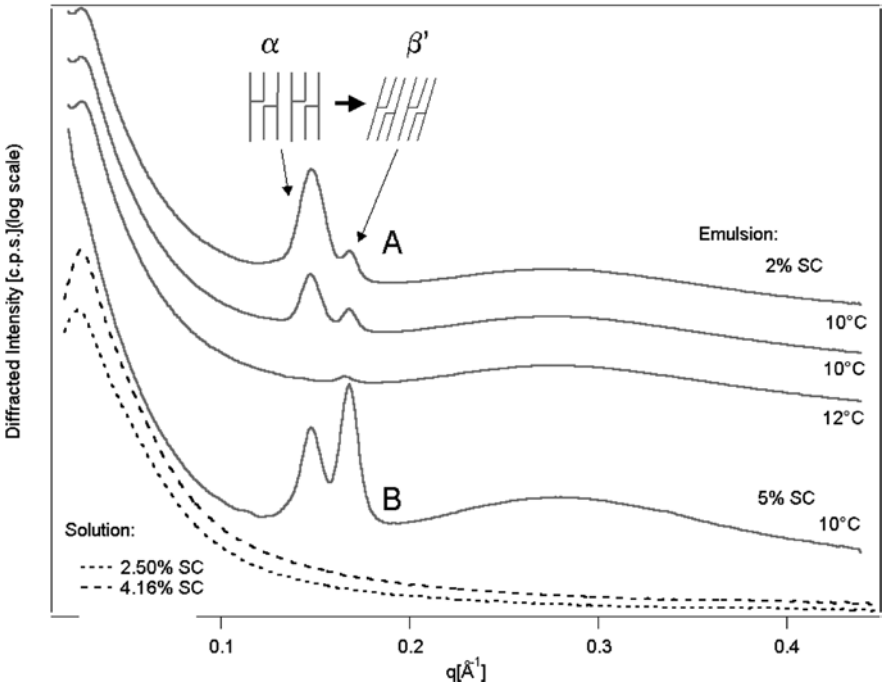

Fig. 4 SAXS monitoring at a sample-to-detector distance of 1.5 m of triglyceride crystallization within droplets of a SC model emulsion containing lipid surfactants. A Time/temperature evolution of an emulsion with a high concentration of SC and 0.5% added monoglycerides (only last frames of the time-resolved X-ray diffraction experiment are shown). *b* Same time temperature/history, same surfactant concentration as for the emulsion shown in *a* but at a lower concentration of SC (x %) (only the last pattern is drawn). Drawn *below* for comparison in *dashed lines* are the binary SC/water solutions, the concentrations of which are indicated

droplets, is affected by the concentration of SC and that SC is located partially at the interface. The data also show that the interface composition influences the TAG transition and crystallization. This point will be analyzed in more in detail elsewhere.

Competition between SC and other emulsifiers for the globule interface

The changes in the scattering patterns induced by the addition of between 0.5–1.5% monoglycerides as emulsifiers to the model emulsion are shown in Fig. 3. An increase of the scattered intensity observed at q_{max} is found upon addition of monoglycerides. How does the addition of monoglyceride affect the SC aggregation? Classical analysis is that the addition of monoglycerides has an effect on the fat globule interface, meaning that owing to its higher affinity for lipids it partially depletes the fat globules from their initial SC coating. The displacement of the protein from the interface to the solution should increase the number of submicelles and the scattering. Both influences of the addition of emulsifier on SC aggregation and on lipid crystallization will be examined in a further study.

The presence of monoglycerides in the emulsion also influences the crystallization behavior of the emulsified fats as seen Fig. 4. This means that the presence of monoglycerides in the aqueous phase has possibly a double impact on the fat globule interface: through the logical presence at the interface as a polar lipid that directly influences the crystallization behavior of the emulsified fats; through the modification of the partition coefficient of SC between the globule interface and the aqueous phase. However, it cannot be determined from this study if one of the two mechanism is preponderant.

Conclusion

SAXS permit the quantitative characterization of oil-in-water emulsions at two different levels: in the aqueous phase by the characterization of the protein self-assembly and within the fat globules by the determination of the TAG organization and polymorphism. Emulsions are complex systems in which the interface plays a critical role; however, this interface cannot be studied directly. We demonstrated here that the behavior or the structure of both phases studied is influenced by the composition and organization thanks to the location between the two phases examined. It is expected that most of the change in the interface is reflected by changes at least in one of them if not in both as observed with SC. In the latter case, then, a comparison of the influence of the two phases is very promising. However, taking into account the difficulties in the discrimination of the diverse scattering phenomenona, the influence on TAG diffraction looks simpler to analyze and to be more sensitive to the changes.

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References

1. Lopez C, Lesieur P, Bourgaux C, Ollivon M (2000) J Colloid Interface Sci 229:62
2. Kalnin D, Arnaud G, Amenitsch H, Ollivon (2002) Food Res Int (in press)
3. Lopez C et al (2001) J Am Oil Chem Soc 78:1233
4. Cornec M, Wilde PJ, Gunning PA (1998) J Food Sci 63:39
5. Dickinson E (1999) Int Dairy J 9:305
6. Farrer D, Lips A (1999) Int Dairy J 9:281
7. Walstra P (1979) J Dairy Res 46:317
8. Berry GP, Creamer LK (1975) Biochemistry 14:3542
9. Gayot P, Lorient D (1998) Structures et technofonctions des protéins du lait. Tec et Doc Lavoisier
10. Storthart DG, Cebula DJ (1982) J Mol Biol 227:391
11. Walstra P (1999) Int Dairy J 9:189
12. Kumosinski TF, Pessen H, Farrell HM Jr, Brumberger H (1988) Arch Biochem Biophys 266:548
13. Kumosinski TF, Uknalis J, Cooke PH, Farrell HM Jr (1996) Lebensm Wiss Technol 29:326