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In vitro digestion of emulsions: high spatiotemporal resolution using synchrotron SAXS

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Abstract

Although the biochemical processes of lipid digestion are well-known, the biophysical ones, responsible for the assembly of molecules into functional structures, lack studies resolving both time and space scales. About 35 years ago, the seminal microscopy study of Patton and Carey constituted a major advance to reach this goal. Nowadays, new perspectives arise from the availability of large facilities scattering techniques, able to monitor the dynamics of multi-scale assemblies with unprecedented resolutions. The present small angle X-ray scattering (SAXS) study focused on the roles of the emulsifier and triglyceride in the formation of lipid assemblies during emulsion digestion in vitro. By developing several interpretations of the data in the whole space range (qualitative, shape-dependent and shape-independent models), the characteristic size of the assemblies and their transition times were obtained, which depended on the triglyceride, but not on the emulsifier. The major assembly formed was found to be a spherical mixed micelle, but vesicle was also found to coexist throughout the digestion, although in a lower proportion. The quantitative determination of the sizes and proportions of these assemblies, as well as the evolution of these characteristics during digestion are precious information for nutritional sciences, as these assemblies are the vehicles of lipophilic nutrients and micronutrients towards their absorption site.

Introduction

From the historical perspective back to the ancient Greek philosophers, there was a debate whether food digestion consists of physical or chemical processes. Though the importance of both was recognized with the scientific revolution, it remains that modern and contemporary

studies mostly investigated the chemical aspects (nutrients production) in relation to the physiology (biology, motility) of the gastrointestinal tract.¹ The emerging of works related to the physics of ingested food is more recent. We indeed reported only a few articles in the early 20th century concerning the structural aspects of the digestion of dispersed systems whereas, since the pioneering work of Patton and Carey,² much more studies were published.³

The case of lipids is an intriguing one, as they are able to form numerous molecular assemblies depending on physicochemical conditions. The control of their self-assembly was indeed identified in 2005 as one of the 25 big questions facing science.⁴ From the nutrition point of view, various lipid assemblies are known to form during digestion,⁵ but their efficiency to carry lipophilic micronutrients (or lipophilic drugs) are still poorly known.^{5,6,35} Moreover, the lipid composition and type of emulsifier often modify digestion kinetics and could in turn affect this carrying efficiency.^{3,35} Studying the dynamics of lipid assemblies in wide ranges of time and space scales is not a trivial task, and only a few studies were able to resolve both since the use of microscopy to monitor the digestion of emulsion droplets by Patton and Carey.² But, as theirs, most laboratory works only resolved the transitions between droplets, vesicles (or a lamellar phase), and micelles.^{3,7}

In this article, further to our laboratory investigations,^{7,8} we report dynamical and structural data obtained in synchrotron large scale facilities on a SAXS beamline. The study was designed to monitor emulsion intestinal digestion in undiluted conditions, with time from several seconds to several hours and space from a hundred nanometer to the nanometer. Then, our goal was to develop detailed interpretations in the whole space range, allowing the identification of the geometry of the digestion assemblies, the characterization of their sizes and relative proportions, and their evolutions during digestion.

Experimental Section

Materials

Tricaprylin (glyceryl trioctanoate T9126, 99% purity), triolein (glyceryl trioleate T7140, 99% purity), sodium oleate (O7501, 99% purity), sodium glycodeoxycholate (G9910, purity 97%) and

pancreatic lipase type II from porcine pancreas (L3126) were provided by Sigma-Aldrich. Betalactoglobulin was purified from whey protein isolate (Prolacta 90 batch no. 273 supplied by Lactalis Industrie, Laval, France) in our laboratory. Milli-Q water having an electrical resistivity of 18.2 M Ω cm was used.

Emulsion preparation and digestion

Oil-in-water emulsions were composed of 190 mg.mL⁻¹ (20 v%) oil phase of tricaprylin (TC) or triolein (TO), stabilized by beta-lactoglobulin (BLG) or sodium oleate (NaO) respectively. Emulsions were prepared by sonication as previously reported.^{7,8} The pH of the continuous aqueous phase was adjusted to 7.5 using 10 mM Phosphate buffer (NaH₂PO₄). As already reported,⁷ the surface-based mean droplet diameter was 361 ± 25 nm for all emulsions, except for TC-NaO emulsion, for which it was 242 ± 12 nm. The intestinal digestive fluid used for the *in vitro* digestion experiment was prepared by mixing 20 mg.mL⁻¹ of sodium glycodeoxycholate (NaGDC, a bile salt) and 1 mg.mL⁻¹ pancreatic lipase in 130 mM NaH₂PO₄ buffer and adjusting to pH 7.5. The emulsions analyzed during intestinal digestion were composed of 47 mg.mL⁻¹ (5 v%) triglyceride, 1.5 mg.mL⁻¹ emulsifier, 10 mg.mL⁻¹ NaGDC, and 0.5 mg.mL⁻¹ pancreatic lipase in 100 mM NaH₂PO₄ buffer at pH 7.5.

SAXS experiments

SAXS experiments were performed at the SWING beamline at the Synchrotron SOLEIL (Saint-Aubin, France). The sample-to-detector distance was fixed to 1.164 m and X-ray energy was 12 keV, corresponding to a wave vector between 0.009 Å⁻¹ < q < 0.8 Å⁻¹. The scattering from the capillary and air was subtracted from the total scattering intensity. Scattering patterns were acquired for at least 1 hour with a 20 s interval. FOXTROT, a homemade program, was used to acquire data and reduce 2D patterns to 1D curves.

In vitro emulsion digestion experiment monitored by SAXS

The setup used for the *in vitro* digestion of emulsion is shown in fig. S1. Only the small intestine part was mimicked as this is mainly where lipid digestion occurs and assemblies form. Dilution in the mouth and stomach was taken into account by mixing 1 mL of emulsion and 1 mL of 130 mM NaH₂PO₄ buffer at pH 7.5, without enzymes to avoid the macroscopic structural changes that could occur during these digestion steps. The diluted emulsion was transferred to a glass vial (reactor), which was sealed and connected to a syringe containing the digestive fluid and to the quartz capillary. The glass vial was placed on a magnetic stirrer with continuous stirring at 500 rpm and a temperature control of 37 °C. The emulsion was circulated between the 2 mm diameter quartz capillary and the glass vial using a peristaltic pump at a flow rate of 0.5 mL.min⁻ ¹. The digestion was initiated by injecting 2 mL of digestive fluid into the glass vial containing the emulsion using an operated syringe driver. The duration of injection was approximately 60 s. In order to remove material adsorbed on the surface of the capillary after each digestion, a 2 ways 6 ports valve was added in the system to connect the capillary to a cleaning robot. The cleaning process was realized in one minute and had two steps. The first consisted in the injection of a cleaning solution containing detergents and the second was a water washing. When the process was finished, the reactor was reconnected to the capillary. In this way, we ensured a perfect subtraction of the contribution of the capillary scattering to enhance the quality of the data.

Results

Time-resolved graphical interpretation

The time-resolved spectra obtained from the SAXS experiments during emulsion digestion are presented in figs. 1 and S2. Plotted in log-log scales, they provide qualitative information about the assemblies in the system, as the local log-log slopes are related to characteristic geometries. For instance, the large slope at low-q in the beginning of digestion indicates 3D objects, typically spheres, which are the emulsion droplets. As digestion proceeds, this low-q slope decreases, indicating evolution towards objects of local lower dimension, such as the bilayer of some lamellar phases. Towards the end of the digestion, a plateau at intermediate q indicates the presence of micelles. At high-q, a broad peak is present throughout the digestion. This

spatiotemporal representation allows the visualization of all the data simultaneously, revealing that the major parameter affecting the digestion assemblies is the type of triglyceride (TG).



Figure 1: Full data for the spatiotemporal SAXS profiles (background is not subtracted) of TC-NaO (left) and TO-NaO (right) emulsions during *in vitro* intestinal digestion. The first digestion profile was obtained 100 s after the digestive fluid injection, then the normal time step was 20 s (see fig. 2 for exceptions).

To be quantitative with respect to time, normalized I(q) for different characteristic regions (values of q) were plotted as a function of digestion time. The most representative variations are presented in fig. 2, plateaus and slopes revealing the stages and transitions between the digestion assemblies. At low-q, reflecting the large assemblies (TG droplets), the decrease of the intensity starts about 3 min (150-180 s) after the beginning of the digestion, except for TO-BLG (320 s, about 5 min). Then, it decreases almost exponentially until it reaches a plateau, after about half an hour (1740-1860 s) for the TC systems, and after about 2 hours and a half (8200-9000 s) for the TO systems. At high-q, reflecting the small assemblies (lamellar structures and micelles), the increase of the intensity is consistent with the low-q behavior, but starting at latter times. It was expected, as TG droplets have to be hydrolyzed in order to generate other assemblies. For the TO systems, an intermediate plateau lasting about 10 min indicates a stage in the digestion process, before a specific assembly is generated.

From these graphical interpretations, it appears that the systems under study are complex mixtures of different assemblies, coexisting as droplets, lamellar structures and micelles. This coexistence is not thermodynamically stable, evolving as TG droplets are hydrolyzed (low-q), progressively releasing the digestion products (fatty acid and monoglyceride) which form lamellar structures and micelles (high-q).³⁶



Figure 2: Time-resolved evolution of the normalized scattering intensity at two q values (0.01 and 0.2 Å^{-1}) for all emulsions. The characteristic times are indicated by the arrows, highlighting the stages and transitions during digestion.

Shape-independent model interpretation

Complementary information can be obtained by transforming the reciprocal space data (q space) into real space data (r space). This is done by calculating the pair-distance distribution function p(r) using the indirect Fourier transformation (IFT) method⁹ to solve $I(q) = 4\pi \int_0^\infty p(r) \frac{\sin(qr)}{qr} dr$. The features of the p(r) curve give information on the geometries and characteristic lengths of the assemblies in the system.

This analysis was done for the four emulsion systems, at different digestion times after the addition of lipase and bile salt, and for the reference emulsions, in the same conditions as the digested ones but with no lipase and no bile salt. Fig. 3 shows the fits obtained with the IFT

method for the TC-NaO and the TO-NaO emulsions, in the whole q range (i.e. up to a maximal r value of 700 Å). From these fits, the p(r) functions were calculated and are reported in fig. 4. The results for the TC-BLG and the TO-BLG emulsions are not reported, as they are very similar to those for the TC-NaO and the TO-NaO emulsions, respectively.



Figure 3: SAXS intensity profiles (background is not subtracted) for the NaO reference emulsions and at selected times during digestion. Successive curves are multiplied by a factor of 10 for clarity. The continuous black line represents the best fit by IFT. The local maximum at high-q is indicated by a dashed line.



Figure 4: Pair-distance distribution function p(r) calculated by IFT of the SAXS intensity profiles in fig. 3. The resolution is 0.27 Å.

The p(r) curves for the reference emulsions are similar, reflecting a spherical geometry with features at low r. Then, during digestion, the main peak decreases and some new features appear at low r. The main peak captures the smallest emulsion droplets, the intensity of the peak decreasing as the droplets get digested into new assemblies. The low r features should capture the characteristic lengths of these assemblies through their local minima and maxima. On the contrary, the oscillations at intermediate r for long digestion times in the TO-NaO case (and also the TO-BLG case) should not be used as they interfere with the droplet peak. Nevertheless, they reflect the presence of lamellar structures such as vesicles. By performing another IFT analysis in the high q range (up to a maximal r value of 70 Å), it is possible to finely resolve the characteristic lengths at low r, which are reported in table 1.

Sample	r_1 (Å)	r_2 (Å)	<i>r</i> ₃ (Å)
TO-NaO Ref	15	27	
TO-NaO 100 s	14	26	
TO-NaO 1900 s	11	29	44
TO-NaO 5820 s	12	29	43
TO-NaO 8760 s	12	30	51

TC-NaO Ref	12	19	
TC-NaO 160 s	12	19	
TC-NaO 980 s	13	22	32
TC-NaO 1680 s	14	23	32
TC-NaO 2440 s	14	24	32

Table 1: Characteristic lengths appearing in the pair-distance distribution function p(r) obtained for $r \le 70$ Å. The resolution is 0.027 Å.

As a first interpretation, these characteristic lengths were compared to the literature values. The smallest length was found to be close to the characteristic thickness of an emulsifier monolayer at the oil-water interface. It is indeed around 10-15 Å for emulsions stabilized by tween 60, an emulsifier comparable to sodium oleate.¹⁰ Similar values are found for the TC-BLG and the TO-BLG emulsions, what was expected as BLG also forms a layer of thickness around 10-15 Å.¹⁰ It is unclear why this thickness decreases or increases during the digestion of the TC-BLG and the TO-NaO emulsions, respectively. However, the same behaviors were found for the TC-BLG and the TO-BLG emulsions.

The largest length was found to be close to the diameter of mixed micelles composed of bile salts and lipid digestion products.^{7,11} This diameter is smaller for the TC-NaO system compared to the TO-NaO system. And again, similar values were found for the TC-BLG and the TO-BLG emulsions, what was expected as the nature of the emulsifier did not affect the characteristics of the assemblies when it was used in small quantity.⁷

The intermediate length was found not to be related to an assembly, but to an arrangement of the triglyceride itself. Indeed, such a length was reported as the long spacing of the arrangement of molten triglycerides,¹²⁻¹⁷ or free fatty acids,¹⁷ or even simple alcohols.¹⁸ In the q space, this arrangement is associated to a broad peak at high-q. The q values at the broad peak maximum are indicated by a dotted line in figs. 3 and 5, around 0.37 Å⁻¹ for TC-emulsions and 0.26 Å⁻¹ for TO-emulsions. These values correspond to correlation distances in the *r* space (D= $2\pi/q$) around 17 Å and 24 Å, respectively. These are indeed close to the intermediate lengths reported in table 1.

This first interpretation is quantitative but does not resolve the size and shape of the lamellar structure identified previously. To fully characterize the assemblies present at a given digestion time, a physical model accounting for the different objects (shapes) in the system is thus required.

Shape-dependent model interpretation

The background subtracted scattering intensity I(q) is related to the shape and arrangement of scattering monodisperse particles in the matrix and can be expressed as:⁹

$$I(q) = NV^2 \Delta \rho^2 P(q). S(q) \tag{1}$$

where *N* is the number density of the objects, *V* is the single particle volume, $\Delta \rho$ is the scattering length density contrast between the solvent and scattering object, *P*(*q*) is the form factor which is dependent on the shape and morphology of the particle, and *S*(*q*) is the structure factor originating from the correlations in the arrangement of scattering centers in the matrix. In this model, we first assume the coexistence of two types of scattering objects (a) micelles and (b) vesicles as the lamellar structures.

We suppose that the micelles formed during the digestion are polydisperse spheres of uniform electron density and radius R_{mic} . The form factor F(q) is given by the following expression:

$$F_{mic}(q) = Z(q, R_{mic}, \Delta \rho_{mic-sol})$$
⁽²⁾

with R_{mic} , the micelle radius, and $\Delta \rho_{mic-sol}$ the difference of scattering length density between the solvent and the micelle. The form factor of the micelles is considered as a special case of spherical core-shell form factor where the core of the structure has the same scattering length density as the matrix.

For the vesicles, with core radius R_{ves} and shell thickness of dR_{ves} (bilayer thickness), the form factor is given by:¹⁹

$$F_{ves}(q) = Z(q, (R_{ves} + dR_{ves}), \Delta\rho_{ves-sol}) - Z(q, R_{ves}, \Delta\rho_{ves-sol})$$
(3)

where the function Z is defined as:

$$Z(q, R, \Delta \rho) = \frac{4}{3}\pi R^3 \Delta \rho \left(3 \frac{\sin(qR) - (qR)\cos(qR)}{(qR)^3}\right)$$
(4)

As the size of the scattering objects changed continuously during the digestion, we assumed that they were polydisperse in size. The polydispersity in size can be described by a log-normal distribution $D(R, R_0, \sigma)$:

$$D(R, R_0, \sigma) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-\frac{1}{2\sigma^2} \left(ln\frac{R}{R_0}\right)^2\right]$$
(5)

where *R* is the radius, R_0 is the mean particle radius and σ is the standard deviation of the distribution and considered as polydispersity index (PDI) in scattering object.

Thus, taking the polydispersity into account, the form factor can be written as:

$$P(q) = N \int D(R, R_0, \sigma) |F(q)|^2 dR$$
(6)

Concerning the structure factors, we assume that during digestion, the number density of large objects at low-q (vesicles) is low enough to have a constant value of 1 for the structure factor. In contrast, the number density of objects at high-q is high, thus the structure factor arising from small objects is taken into account. In this case, it corresponds to the distance by which the objects are separated. We approximate these correlations on the basis of the structure factor of non-interacting hard spheres. The analytic expression for $S_{HS}(q)$ is as follows:²⁰

$$S_{HS}(q) = \frac{1}{1+24\varphi_{qR_{HS}}^{G(q)}}$$
(7)

where φ is the volume fraction of the particles in the dispersion and G(q) is given by:

$$G(q) = \frac{(\sin A - A\cos A)}{A^2} + \beta \frac{(2A\sin A + (2 - A^2)\cos A - 2)}{A^3} + \gamma \frac{(-A^4\cos A + 4[(3A^2 - 6)\cos A + (A^3 - 6A)\sin A + 6])}{A^5}$$
(8)

with
$$\alpha = \frac{(1+2\phi)^2}{(1-\phi)^4}$$
; $\beta = -6\phi \frac{(1+\phi/2)^2}{(1-\phi)^4}$; $\gamma = \frac{\phi\alpha}{2}$ and $A = 2R_{HS}q$

11

where R_{HS} is the hard sphere repulsion.

Neglecting cross terms, the total scattering intensity $I_{total}(q)$ can be described as a sum of contributions from the different objects and is given by:

$$I_{total}(q) = N_{mic}V_{mic}^2\Delta\rho_{mic-sol}^2P_{mic}(q) \times S_{HS}(q) + N_{ves}V_{ves}^2\Delta\rho_{ves-sol}^2P_{ves}(q)$$
(9)

All the experimental data in the whole q range were fitted using SASfit program (https://kur.web.psi.ch/sans1/SANSSoft/sasfit.html). An example of fit obtained for the TC-BLG emulsion using equation 9 is shown in fig. S3.a. At low-q, the fit did not reproduce the experimental observations. For this q-range, the q-dependence of the scattering intensity can be described by a power law. The increase of the intensity at q < 0.02 Å⁻¹ can be attributed to scattering from large objects in the dispersion. As already mentioned, this contribution is due to the undigested TG droplets. Since their mean diameter is much larger than $2\pi/q_{min}$, we expect a Porod scattering regime corresponding to the TG droplets at q < 0.02 Å⁻¹ so the low-q slope of the undigested emulsions should correspond to q⁻⁴. Any deviations from this Porod scattering regime can be attributed to the interfacial roughness of the TG droplets in the dispersion. To account for this contribution, equation 9 can be modified as:

$$I_{total}(q) = N_{mic}V_{mic}^2 \Delta \rho_{mic-sol}^2 P_{mic}(q) \times S_{HS}(q) + N_{ves}V_{ves}^2 \Delta \rho_{ves-sol}^2 P_{ves}(q) + Aq^{-\alpha}$$
(10)

where A is the constant obtained by fitting the experimental curve and α corresponds to the slope.

An example of fit obtained for the TC-BLG emulsion using equation 10 is shown in fig. S3.b. The scattering length densities were calculated for each component. Six adjustable parameters thus composed our model: R_{ves} (vesicle radius), dR_{ves} (bilayer thickness), N_{ves} (number density of vesicles), R_{mic} (micelle radius), N_{mic} (number density of micelles), S_{HS} (hard sphere structure factor taking into account the hard sphere repulsion length and the volume fraction). To minimize the error when combining the different form factors, we fixed the values of the bilayer thickness (dR_{ves}) deduced from different fits and corresponding to values found in the literature for vesicles obtained for similar systems.²¹ The bilayer thickness of vesicles generated during the digestion of the TC and TO emulsions was fixed to 16 Å and 19.5 Å, respectively.

Equation 10 was used to monitor the assemblies formed during lipid digestion. First, it was used to fit the background subtracted SAXS intensity profiles for the four reference emulsions (fig. 5).

In all cases, equation 10 captured: (i) the broad peak at high-q, and (ii) the steep decrease of the scattering intensity at low-q. In contrast, the term for the vesicle contribution tended towards zero, which was expected as no vesicles were present in the initial emulsions. So this term was removed for the fitting of the intensity profile of these reference emulsions. The parameters obtained from the best fits are reported in table 2. The hard sphere repulsion lengths are very close to the intermediate length reported in table 1, indicating that the structure factor in equation 10 captures the lipid arrangement instead of the micelle arrangement as initially projected. Moreover, the micelle radius corresponds well to the small length reported in table 1, that is the interfacial layer thickness. It confirms that the broad peak at high-q contains the characteristics of arrangements at the single molecule scale. At low-q, the scattering intensity comes from the droplet surface since the emulsion droplets are large and their form factor is not accessible in the experimental q range. The decrease in scattering intensity is captured by a power law. The classical behavior observed for smooth interfaces is a q⁻⁴ decay.²² This behavior is observed for the two emulsions stabilized by sodium oleate (TC-NaO and TO-NaO). For the emulsions stabilized by beta-lactoglobulin, the scattering intensity gives a variation as q^{-3} due to the roughness of the interface formed by the protein.



Figure 5: Background subtracted SAXS intensity profiles for the reference emulsions (not submitted to digestion). The continuous black line represents the best fit obtained by combining

Sample	R _{mic} (Å)	R _{HS} (Å)	N_{mic} (cm ⁻³)	α
TC-NaO	13	21	8.67x10 ¹⁸	4.0
TC-BLG	13	20	7.22×10^{17}	3.0

28

29

TO-NaO

TO-BLG

14

13

a spherical form factor and hard sphere structure factor with a power law. Successive curves are multiplied by a factor 10 for clarity. The local maximum at high-q is indicated by a dashed line.

Table 2: Fitting parameters of the reference emulsions data in fig. 5. See text for parameters meaning.

 1.10×10^{19}

 4.97×10^{17}

3.9

3.1

In a second step, the background subtracted scattering intensity profiles at different digestion times after the addition of lipase and bile salt were fitted by equation 10. Fig. 6 shows the resulting fits for the triolein (fig. 6.a) or the tricaprylin (fig. 6.b) emulsions stabilized by BLG. Again, very similar curves and fits were obtained for the emulsions stabilized by NaO (fig. S4). During digestion, equation 10 captured: (i) the broad peak at high-q, shifted towards lower q values over time, more markedly for TO than for TC, (ii) the large plateau forming at intermediate q range, and (iii) the decrease of the intensity over time at low-q, with a flattening for the lower q values at long digestion times.



Figure 6: Background subtracted SAXS intensity profiles at selected times during the digestion of the BLG emulsions. Successive curves are multiplied by a factor of 10 for clarity. The continuous black line represents the best fit by combining a spherical form factor, a vesicle form factor, a hard sphere repulsion structure factor and a power law.

The fitted structural parameters are reported in figs. 7-10. As reported in the previous interpretations, the emulsifier type has only a minor effect on all results. Fig. 7 shows that micelles and vesicles coexist, but micelle is always the most abundant assembly formed during digestion. Moreover, its number density changes only marginally during digestion, whereas the number density of vesicles drops substantially. Overall, less variation is seen for the TC systems.

The number density of micelles is almost constant while the number density of vesicles decreases at the end of the digestion. For the TO systems, there is a decrease of the number density of micelles while the number density of vesicles increases again after an initial decrease. These correspondences show that some vesicles split into micelles and reciprocally some micelles form vesicles. Quantitatively, the ratio of the micelle/vesicle number densities is very close to that reported in vivo (between $10^5 - 10^6$),²³ showing that the number density determination is accurate. Also, the significant curve inflections appear around 1000-2000 s, what agrees with some characteristic times identified in fig. 2.

Fig. 8 shows the evolution of the hard sphere repulsion length during digestion. For the TC systems, the value is again close to the intermediate length in table 1, confirming it represents the molten lipid arrangement. During digestion, it does not change significantly, in agreement with the previous interpretations. For the TO systems, the first value matches the intermediate length in table 1, then it increases very quickly to a maximum and decreases again during digestion. This is in agreement with the shifting reported in the previous interpretations. However, the exact arrangement it represents is unclear.¹⁷ We suspect it is not fully separable from the mixed micelle characteristic length.

Fig. 9 shows the micelle diameter as a function of digestion time. For all systems it starts around the same value $(28 \pm 1 \text{ Å})$, which matches the diameter of the sodium glycodeoxycholate micelle.⁷ As digestion proceeds, this diameter remains constant for the TC systems, whereas it increases for the TO systems. The values are in good agreement with those reported in the IFT interpretation, and are similar to those for mixed micelles in the literature.^{7,11,24} As already mentioned, the typical value is close to the hard sphere repulsion length, so both might be confounded in the broad peak, thus the mixed micelle diameter might not be strictly exact. Nevertheless, these results confirm that mixed micelles are smaller in the TC systems than in the TO systems.²⁴

Fig. 10 shows the evolution of the vesicle diameter during digestion. It starts around 56 ± 3 Å for all systems and increases as digestion proceeds. This first value is small compared to those of the literature, but the values reached at long digestion times are similar.^{7,23-27} This discrepancy might come from the fact that all vesicle measurements of the literature were made at equilibrium, that is after a long period enabling vesicle growth. Moreover, this size depends on the exact system

used, and most of them contained phosphatidylcholine,²³⁻²⁵ which is known to increase vesicle size as the length of its fatty acids increases.²⁸ Besides, the vesicle size in the TC systems is much smaller than in the TO systems. This trend was already reported, although less marked.²⁴

Because both the number density and the diameter of micelles and vesicles vary, fig. S5 shows the total volume of these objects per unit dispersion volume. This essentially confirms that micelles were more abundant, always representing more volume. The same trends apply, although less marked than in fig. 7 (except for the TO systems), what shows that object coalescence also occurred.



Figure 7: Evolution of the number density of micelles and vesicles during digestion for all systems (parameters obtained from equation 10 fitting).



Figure 8: Evolution of the hard sphere repulsion length during digestion for all systems (parameter obtained from equation 10 fitting).



Figure 9: Evolution of the micelle diameter during digestion for all systems (parameter obtained from equation 10 fitting).



Figure 10: Evolution of the vesicle diameter during digestion for all systems (parameter obtained from equation 10 fitting).

Overall, the lipid digestion data of this SAXS study and their interpretations all show that digestion process is very fast, the first changes occurring after 2-3 min. They also demonstrate that the major assembly formed by digestion is mixed micelle, although vesicle is also formed very quickly, but in a lower proportion. This is in agreement with the observation that vesicle is usually an intermediate assembly before mixed micelle dominates.^{7,29,30} However, this is in contrast with the results of Salentinig et al. for similar systems,^{31,32,33} reporting vesicle as the major assembly formed in the aqueous phase. The main difference in their studies is the relative quantity of bile salt, either much higher or much lower than the one used in the present study, representative of the normal adult physiology.³⁴ This could explain the discrepancy, as they reported vesicles at high quantity of bile salt, whereas they reported internal structures in emulsion droplets (inverse micelles or liquid crystalline phases) at low quantity or in the absence of bile salt.^{31,32,33} This is reminiscent of the results of Patton and Carey, showing that vesicles originate from the formation of a lamellar liquid crystalline phase starting from the surface of droplets followed by the extrusion of undigested core triglyceride.² In the absence of bile salt, this extrusion was not seen,² so the lamellar liquid crystalline phase may have expanded to the droplet core, forming internal structures.

To confirm our results, a transmission electron microscopy (TEM) study was conducted for the BLG emulsions, in the exact same conditions as those of the SAXS study. Some TEM micrographs acquired as a function of digestion time are given in the supplementary information, essentially showing droplets at the earliest stage (just after lipase and bile salt addition), then vesicles after 15 min digestion (results not shown) and up to the experiment end (90 min). The objects are quite polydisperse, as was expected and taken into account in the shape-dependent model. Moreover, the collection of TEM micrographs confirmed the formation of a complex system of assemblies during digestion, with large objects hiding the small ones in the micrographs. Thus a quantitative size distribution could not be calculated but an estimation of the dimensions of individual objects could be obtained. From 60 min digestion of the TO emulsion, the smallest spherical objects have a size range below 10 nm, close to the micellar size, comforting the sphere geometry used for micelles. At this digestion time, singular morphologies of 100-200 nm were seen, described as droplets filled with a spherical internal structure of micellar size, but this was only seen once for this specific time and TO system. Thus, in our conditions, this structure seems to be uncommon, possibly resulting from a local lack of bile salt.

Conclusion

The SAXS data and interpretations presented here allow a time-resolved description of the assemblies formed during the *in vitro* intestinal digestion of triglyceride emulsions. The main opinion that it consists in the coexistence of micelles and vesicles was comforted, with micelle being the major assembly. Their mean sizes were determined using a shape-dependent model to describe the SAXS data in the whole q range. However, the very high resolution of synchrotron SAXS revealed a broad peak, characteristic of the lipid molecular arrangement itself, confounding the interpretation of the mixed micelles. Despite that, the interpretation based on shape-independent and shape-dependent models gave excellent results in the multi-scale description of the dispersed objects. Thus, future synchrotron SAXS studies should concentrate on the low-q range in order to resolve emulsion droplets rather than include the high-q range (higher than the expected micellar range). Concerning the emulsions, this study confirmed that the type of triglyceride greatly influences the digestion kinetics, as well as the size of the

assemblies formed by the lipid digestion products. In contrast, the two emulsifiers studied here only had minor effects.

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Graphical abstract: The assemblies formed during *in vitro* intestinal digestion of triglyceride emulsions were monitored using time-resolved synchrotron SAXS.



Figure S1: Schematic of the *in vitro* digestion setup coupled to the SAXS capillary.



Figure S2: Full data for the spatiotemporal SAXS profiles (background is not subtracted) of TC-BLG (left) and TO-BLG (right) emulsions during *in vitro* intestinal digestion. The first digestion profile was obtained 100 s after the digestive fluid injection, then the normal time step was 20 s (see fig. 2 for exceptions).



Figure S3: Background subtracted SAXS intensity profiles for TC emulsion stabilized by BLG after 2440 s of the digestion experiment (blue square). The green dotted line indicates the form factor of spherical micelles (P_{mic}) with a radius R_{mic} of 13 Å. The hard sphere structure factor arising from the micelles, $S_{HS}(q)$, is indicated by the purple line. The red dotted line corresponds to the form factor of vesicles (P_{ves}) of inner radius R_{ves} of 69 Å and 16 Å shell thickness (dR_{ves}). The maximum at $q \approx 0.13$ Å⁻¹ in the experimental data is well-represented by the primary oscillation of the form factor of vesicles. The black line corresponds to the best fit obtained on the basis of equation 9 (a) or equation 10 (b).



Figure S4: Background subtracted SAXS intensity profiles at selected times during the digestion of the NaO emulsions. Successive curves are multiplied by a factor of 10 for clarity. The continuous black line represents the best fit by combining a spherical form factor, a vesicle form factor, a hard sphere repulsion structure factor and a power law.



Figure S5: Evolution of the total volume of micelles and vesicles per unit dispersion volume during digestion for all systems (parameters obtained from equation 10 fitting).



TC-BLG ref





TC-BLG t0



TO-BLG t0



TC-BLG t30

TO-BLG t30



TC-BLG t90



Supplementary information: Transmission electron microscopy micrographs of the BLG reference emulsions and at selected times (min) during *in vitro* digestion. All scales were harmonized to enable a direct visual comparison of the objects size.

Methods: Transmission electron microscopy (TEM). For each specimen, a droplet of emulsion was placed on a carbon-coated TEM copper grid (Quantifoil, Germany) previously submitted to a glow-discharge to ensure hydrophilicity. The sample was then negatively stained with a 2 % aqueous solution of uranyl acetate (Merck, Germany), and air-dried before electron microscopy investigation. Observations were performed using a JEM-1230 microscope (Jeol, Japan) operated at an acceleration voltage of 120 kV and equipped with a LaB6 filament. All the micrographs were recorded on a 1.35 K x 1.04 K x 12 bit ES500W erlangshen CCD camera (Gatan, USA).