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RESEARCH PAPER

Structural Aspects of Digestion of Medium Chain Triglycerides Studied in Real Time Using sSAXS and Cryo-TEM

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ABSTRACT

Purpose The purpose of this study was to investigate the colloidal structures formed on digestion of medium chain triglyceride (MCT) with a specific objective of identifying and characterizing a previously reported vesicular phase, which has been linked to supersaturation and anomalous digestion kinetics, and to evaluate the influence of lipid mass and enzyme inhibition on self assembled structure.

Methods MCT was digested *in vitro* and nanostructure was monitored in real time using synchrotron small angle X-ray scattering (sSAXS), and morphology was studied using cryogenic transmission electron microscopy (cryo-TEM).

Results Formation of the putative vesicular phase formed on digestion of MCT was confirmed and its structural attributes were determined. Vesicle formation was dependent on lipid mass and bile salt concentration. The use of enzyme inhibitor for offline analysis of lipolysis samples did influence structural aspects of the digestion medium when compared to real time evaluation.

Conclusions The formation of a vesicular phase was directly linked to the kinetics of lipid digestion. Vesicle formation is linked to lipid mass, or more specifically the ratio of lipid to bile salts present in the digestion mixture. Inhibition of lipase to halt digestion during sampling for offline analysis must be done with caution as structural aspects were shown to differ for the MCT digests with and without inhibitor present.

KEY WORDS cryogenic transmission electron microscopy · *in vitro* lipolysis · lipid digestion · lipid-based drug delivery · synchrotron small angle X-ray scattering

ABBREVIATIONS

Cryo-TEM	Cryogenic transmission electron microscopy
LCT	Long chain triglyceride
MCT	Medium chain triglyceride
SAXS	Small angle X-ray scattering
sSAXS	Synchrotron small angle X-ray scattering
TBU	Tributyrin units

INTRODUCTION

Many new drug compounds are poorly soluble in water, leading to limited dissolution in the gastrointestinal (GI) tract, presenting a barrier to efficient absorption and bioavailability. Lipid based formulations are increasingly seen to be a solution to address this issue (1).

There are a number of physicochemical and biological mechanisms by which lipid-based formulations may increase the bioavailability of poorly water soluble drugs. Arguably, of most importance is the ability of lipids to boost the solubilisation capacity of gastrointestinal fluids, providing a mechanism for drug to remain in a solubilized state prior to absorption. When lipids are digested in the GI tract, lipolytic products combine with endogenous amphiphilic molecules (primarily bile salts and phospholipids) to form colloidal phases such as liquid crystals, vesicles and micelles (2–8). If appropriate quantities and type of lipid are present in the gut, these structures can be critical to maintaining drug in

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solution rather than precipitating during dispersion of the formulation and digestion of the lipid components. Despite the importance of these structures in determining the fate of drug in the pre-absorption gastrointestinal milieu, the colloidal aspects of lipid digestion and consequent disposition of drug *in vivo* are still not well understood.

Medium chain triglycerides (MCT) have attracted significant attention in the lipid-based drug delivery field, in part because they tend to provide greater solvency for drug in the pre-digested formulation. When MCT formulations are subjected to an *in vitro* lipolysis model (9), they often show a propensity to maintain poorly water soluble drugs in a solubilized state after complete digestion of the formulation. In fact, for highly lipophilic drugs such as halofantrine and cinnarizine, these formulations can provide an apparently supersaturated state in which the concentration of drug exceeds the solubility of drug in the blank pre-digested formulation (10). The supersaturation effect is highly dependent on the mass of lipid used in the closed *in vitro* model; reducing the mass of lipid reduces the propensity to support the high concentrations and leads to an increased proportion of drug precipitating during digestion (11). The effect is also dependent on the concentration of bile salts present and increasing the bile salt to lipid ratio also increases the likely level of drug precipitation. The supersaturation effect is of interest in optimizing lipid based formulations because engineering formulations to present drug in a state of high thermodynamic activity is an accepted strategy for driving passive drug absorption.

The supersaturation effect has been linked to the colloidal structures present during the digestion of MCT, with the formation of a putative 'vesicular phase' occurring during *in vitro* digestion of high concentrations of MCT (4,12). The 'vesicular' phase has been studied indirectly by size exclusion separation of model lipid digests where MCT digestion products were combined with low and high bile salt concentrations (4) and the vesicular phase present at low bile salt concentrations was shown to have high drug carrying capacity (4).

Thus the design of formulations for the generation of the apparent vesicular phase *in vivo* would be expected to be advantageous in terms of optimal conditions for drug solubilisation. Understanding, and more importantly controlling, structure formation *in vivo* is increasingly viewed as a crucial step in transforming lipid-based drug delivery beyond empirical formulation to rational design. However, to our knowledge the presence of the apparent vesicular phase has never been actually demonstrated nor its structure elucidated using contemporary structural techniques such as small angle X-ray scattering (SAXS). SAXS has been employed to characterise colloidal structures in equilibrium systems *via* a phase diagram approach, and dynamic digesting lipid systems (13,14). In recent years, there has been a move to structural studies in real time, by conducting *in vitro* lipolysis studies in a flow through mode at synchrotron facilities (15). Synchrotron X-rays are advantageous as they enable probing of structures in shorter time scales, have higher resolution, and have the potential for application to *in vivo* studies.

Various other techniques have been used to study digestion of dietary lipids, including light microscopy (2,16), freeze fracture electron microscopy (17,18) and quasielastic light scattering techniques (3,8). More recently cryogenic transmission electron microscopy (cryo-TEM) has successfully been utilised to observe colloidal structures during digestion of a pharmaceutical lipid-based formulation and also in human intestinal fluids (14,19–21). This technique is minimally invasive and advantageous as samples are preserved in their original state in the digested medium, avoiding issues associated with normal electron microscopy techniques, such as artefacts produced by staining, fixation and adsorption (22). However, the samples taken during digestion in past studies were treated with a lipase inhibitor and then imaged at a later time, leading to the possibility of artefacts.

Consequently, in this study, the structures produced during *in vitro* digestion of MCT have been elucidated using real time synchrotron SAXS (sSAXS), and samples retrieved from digestion for cryo-TEM were immediately vitrified without addition of lipase inhibitor. Specifically a high level of MCT (250 mg, ~50 mM) expected to form the vesicular phase, and low level (50 mg, ~10 mM) expected to form only micelles were investigated. Captex 355 was used as the triglyceride because it has been previously shown to support the supersaturation effect. The molar quantities of lipid stated in the case of Captex 355 are 'nominal' as it is a complex mixture, and were based on the equivalent mass of tricaprylin, as though tricaprylin were the only component. The composition of Captex 355 is elaborated on in the "Materials" section. Additionally, cryo-TEM was also conducted on digestion samples which were chemically inhibited to determine whether the presence of the lipase inhibitor induced any artefacts in understanding structural evolution in these digesting systems. The dynamic real time samples have been compared to equilibrium samples prepared by assembly of component monoglycerides and fatty acids for direct comparison of the two approaches.

MATERIALS AND METHODS

Materials

Captex 355 (MCT composed of 59% caprylic acid (C_8), 40% capric acid (C_{10}), < 1% lauric acid (C_{12}) as stated in the product information) was obtained from Abitec Corporation (Janesville, WI, USA) and used without further purification. Tris maleate (reagent grade), bile salt (sodium taurodeoxycholate >95%), monocaprylin (approx. 99%)

and caprylic acid (99+% by capillary gas chromatography) were purchased from Sigma Aldrich (St. Louis, MO, USA). Lipase inhibitor (4-bromophenylboronic acid, 4-BPB, >95% (high performance liquid chromatography)) was obtained from Fluka (Sigma Aldrich, Milwaukee, WI, USA) Phospholipid (dioleylphosphatidyl choline, DOPC, >94%) was obtained from Trapeze Associates Pty Ltd. (Clayton, Victoria, Australia). Pancreatin extract was purchased from Southern Biologicals (Nunawading, Victoria, Australia) and had USP grade pancreatin activity. Calcium chloride (>99%) was obtained from Ajax Finechem (Seven Hills, NSW, Australia). Sodium chloride (>99%) was purchased from Chem Supply (Gillman, SA, Australia). Water used was sourced from a Millipore water purification system using a Quantum™ EX Ultrapure Organex cartridge (Millipore, Australia).

Lipolysis Model and *In Vitro* Digestion

In vitro digestion studies were performed using a pH-stat auto titrator (Radiometer, Copenhagen, Denmark), illustrated schematically in Fig. 1, similar to previous reports (9,10,15,23). Digestion buffer was prepared with 50 mM Tris maleate, 5 mM $CaCl_2.2H_2O$, 150 mM NaCl and adjusted to pH 6.5. Bile salt (sodium taurodeoxycholate) and phospholipid (DOPC) concentrations, simulating intestinal fluid in the fasted state and fed state, were 5 mM:1.25 mM and 20 mM:5 mM respectively in digestion buffer, the mean values and 4:1 ratio representing intestinal contents after digestion (8,20). Lipid formulations were added to 9 mL of the fasted or fed simulated intestinal fluid in the thermostatted digestion vessel at 37°C.

Formulations were magnetically stirred for 5 min in simulated intestinal fluid for complete mixing and thermal equilibration and the pH was adjusted to 6.5 ± 0.003, chosen as a compromise between the optimum for pancreatic lipase activity pH (6–10) (24) and duodenal pH (5.9–5.5) (25). On addition of pancreatin (10000 TBU dry pancreatin powder) the pH-stat titrated the digestion mixture with 0.2 M NaOH in order to maintain the system at pH 6.5 ± 0.003. Digestion was allowed to proceed for 60 min, in which the degree of enzymatic digestion of the lipid was reflected in the volume of NaOH used to neutralize the fatty acids liberated during the digestion process. A blank digestion without lipid but

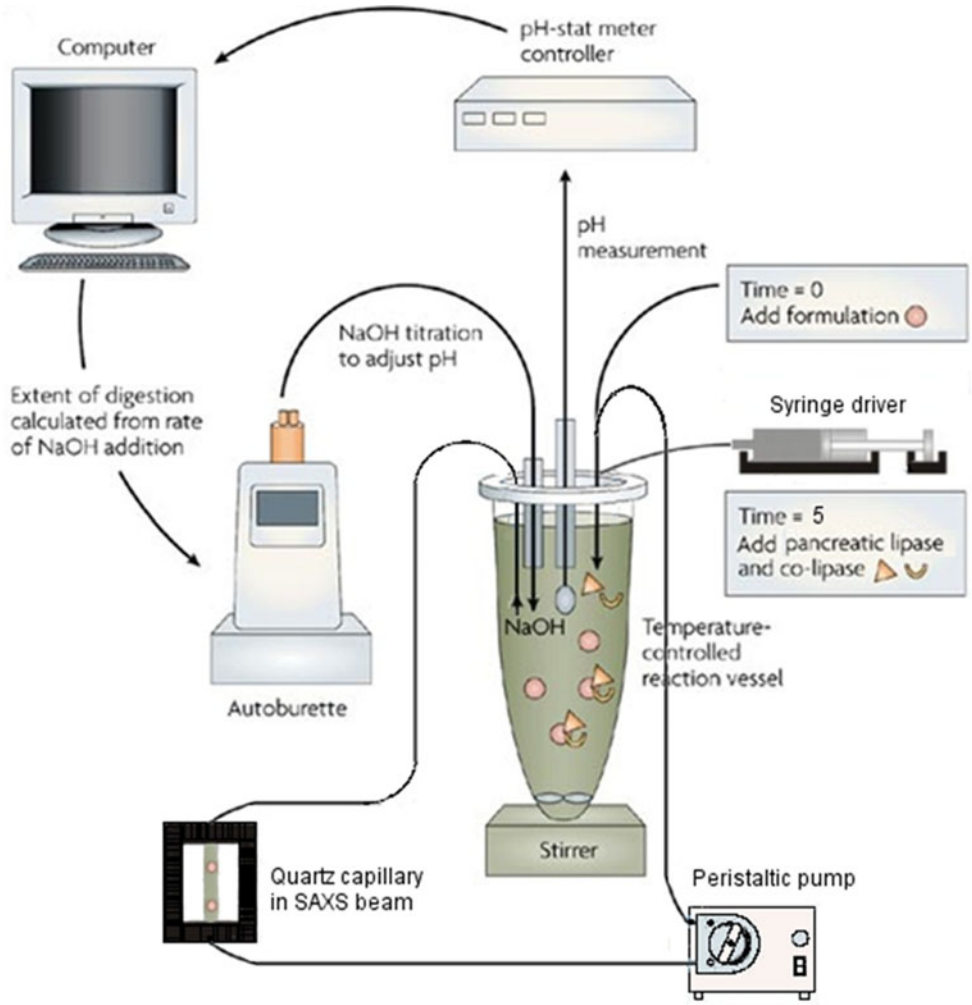

Fig. 1 Schematic of the *in vitro* digestion apparatus coupled to a synchrotron SAXS flow through cell with syringe driver for enzyme addition. Modified with permission from (15).

with bile salt micelles present was performed as a background experiment, to account for fatty acids that were produced from phospholipids and was subtracted from the profiles for the lipolysis experiments.

Synchrotron Small Angle X-ray Scattering and Flow Through *In Vitro* Digestion

SAXS measurements were performed at the Australian Synchrotron. For equilibrium studies, samples were placed in capillaries where they were inserted into a 37°C thermostatted metal heating block controlled by a Peltier system accurate to ± 0.1°C. An X-ray beam with a wavelength of 1.1271 Å (11 keV) was selected. The sample to detector distance was 1,015 mm, covering a q-range of 0.014–0.65 $Å^{-1}$. The 2D SAXS patterns were collected using a Pilatus 1 M detector (active area 169 × 179 mm^2 with a pixel size of 172 μm). Calibration was performed using silver behenate. Data was normalized the using ScatterBrain Analysis program available at http://www.synchrotron.org.au/index.php/aussyncbeamlines/saxswaxs/software-saxswaxs . The scattering pattern was converted to a plot of intensity *versus* scattering vector, q, using the equation $q=(4\pi/\lambda)\sin\theta/2$ (26). The periodicity of the lamellar phase was calculated using the equation $d=2\pi/q$ where q was the location of the lamellar peak. The average of three frames of each sample acquired before enzyme addition was subtracted as background for the dynamic digestions.

To monitor changes in nanostructure in real-time during digestion, the model was fitted with silicone tubing (total volume < 1 mL) to enable continuous flow of the digestion medium *via* peristaltic pump at a rate of 10 mL/min, through a 2 mm diameter quartz capillary. The capillary was fixed in the X-ray beam. A remotely operated syringe driver was used to deliver 1 mL of pancreatin extract over several seconds into the vessel to initiate digestion. A 5 s acquisition time per 30 s for up to 60 min was used to yield the required information in flow through mode depending on the kinetics of digestion. The computer software ScatterBrain Analysis was used to acquire and reduce 2D patterns to 1D curves.

Cryo-TEM Studies

A laboratory-built humidity-controlled vitrification system was used to prepare the samples for cryo-TEM. Humidity was kept close to 80% for all experiments, and ambient temperature was 22°C.

Copper grids (200-mesh) coated with perforated carbon film (Lacey carbon film: ProSciTech, Qld, Australia) were glow discharged in nitrogen to render them hydrophilic. Aliquots (4 μL) of the sample were pipetted onto each grid prior to plunging. After 30 s adsorption time the grid was blotted manually using Whatman 541 filter paper, for approximately 2 s. Blotting time was optimised for each sample. The grid was then plunged into liquid ethane cooled by liquid nitrogen. Frozen grids were stored in liquid nitrogen until required.

The samples were examined using a Gatan 626 cryoholder (Gatan, Pleasanton, CA, USA) and Tecnai 12 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) at an operating voltage of 120 kV. The sample holder operated at −175.5 ± 1°C. At all times low dose procedures were followed, using an electron dose of 8–10 electrons/$Å^2$ for all imaging. Images were recorded using FEI Eagle 4k × 4k CCD camera at magnifications ranging from 15 000× to 50 000×.

RESULTS

Evolution of Structure During Digestion of Captex 355 Under Fasted State Conditions (sSAXS)

On dispersion in simulated fasted intestinal conditions prior to addition of enzyme, Captex 355 forms a crude oily emulsion. The structural changes during a 60 min digestion of Captex 355 were monitored by sSAXS. For the digestion of 50 mM Captex 355 (250 mg) under fasted conditions, the scattering data in Fig. 2a indicated that initially only micelles were present, but that after 20–25 min, two peaks with spacing ratios in *q* of 1 and 2 became evident, indicating that a lamellar phase was gradually formed. The *q*-values of the peaks were at 0.2 and 0.4 $Å^{-1}$, indicating that the lamellar phase had a repeat distance of 30.1 Å.

The titration profile, obtained from consumption of NaOH over time due to fatty acid production, describes changes in chemical composition during digestion. Two distinct regimes of behaviour were evident from the solid line in Fig. 2b. An initial phase of digestion commencing on enzyme addition but slowed between 10 and 20 min, and a second phase in which at 20 min the digestion inexplicably accelerates with no intervention on the system, and continues until it has almost plateaued at 60 min when the monitoring was discontinued. The time scale of commencement of the second phase of digestion was very close to that of the appearance of the lamellar phase in the scattering data (Fig. 2a) suggesting a link between changing structure and the discontinuity in the digestion profile.

To further evaluate the link between structure and digestion kinetics, the intensity of the lamellar phase was calculated from the height of the first lamellar reflection, and plotted against time of digestion (open squares in Fig. 2b commencing at 30 min so that the intensity was resolved from the baseline). There was a very clear correlation between the rate of evolution of the lamellar phase, and the digestion kinetics.

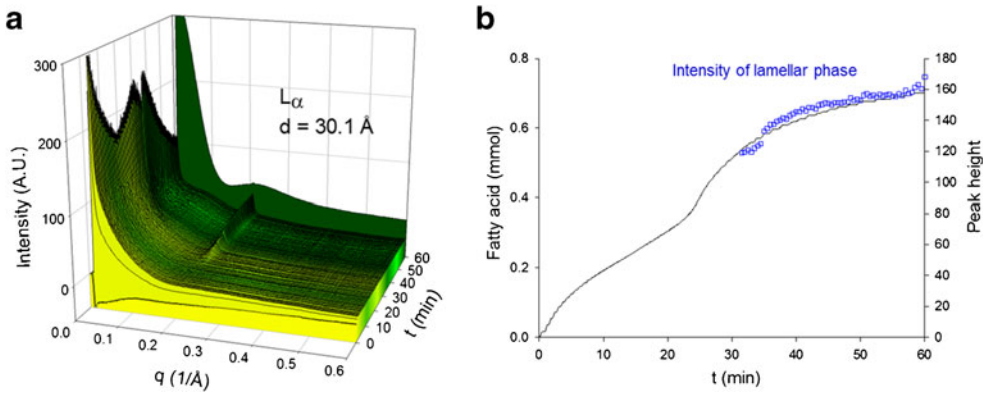

Fig. 2 Digestion of 50 mM Captex 355 (250 mg) in fasted state over 60 min at 37°C. (**a**) SAXS profiles over time during digestion. (**b**) Correlation between the digestion kinetics from the titration profile (*solid line*) and intensity of lamellar phase (*open squares*). The final frame in (**a**) indicates the scattering after addition extra bile salt to the digestion medium to a final concentration of 55 mM to induce conversion of the lamellar structure to micelles.

At the completion of digestion of 50 mM Captex 355, a high concentration of bile salt was introduced with the expectation that the lamellar phase structures transformed to swollen micellar structures. Indeed on introduction of bile salt to the digestion medium after 60 min (the final frame in Fig. 2a) such that the final bile salt concentration was 55 mM, the reflections indicating lamellar phase were abolished and a large broad micellar hump was evident.

During a 60 min digestion of 10 mM Captex 355 (50 mg) under the conditions previously described, there was no evidence of lamellar phase formation during digestion at the lower lipid content (Fig. 3).

Changes in Morphology of Colloidal Structure During Digestion of Captex 355 (Cryo-TEM)

Digestion buffer and fasted micelles were imaged as blanks, and both samples were clean with only micelles being observed in the fasted micelle sample (Fig. 4).

The images presented in Fig. 5 for the 50 mM Captex 355 digestion (250 mg) correlate well with what might be expected from the scattering data. The images are a representative sample of 306 images in total.

In the samples taken at 10 and 20 min, oil droplets 100–600 nm in size were present in close proximity to protein aggregates and long collagen strands several micrometers in length, and 100 nm in width, originating from the pancreatin extract. The collagen originating from the pancreatin extract was also reported by Fatouros and Mullertz, although they were referred to as "long, well-organised, ladder-shaped structures" (19,21). Micelles were also evident, which is in agreement with the scattering data. At the early stage of digestion in Fig. 5a and b, there were no signs of structures that would be consistent with scattering of a lamellar phase.

At 30 and 40 min, unilamellar vesicles and bilamellar vesicles (50–200 nm, some very spherical, others irregular/stretched, multicompartment vesicles) and large lamellar fragments a few hundred micrometers in length were visible in large numbers. Vesicles are dispersed lamellar phase, so the vesicles and lamellar fragments provide the scattering indicative of lamellar structures. At 30 min, oil droplet clusters were also present, however they were smaller (50–150 nm) and less numerous across the grid than those identified earlier on in the digestion. There were also protein aggregates and collagen strands. 3–4 nm micelles were seen as tiny black dots in all samples.

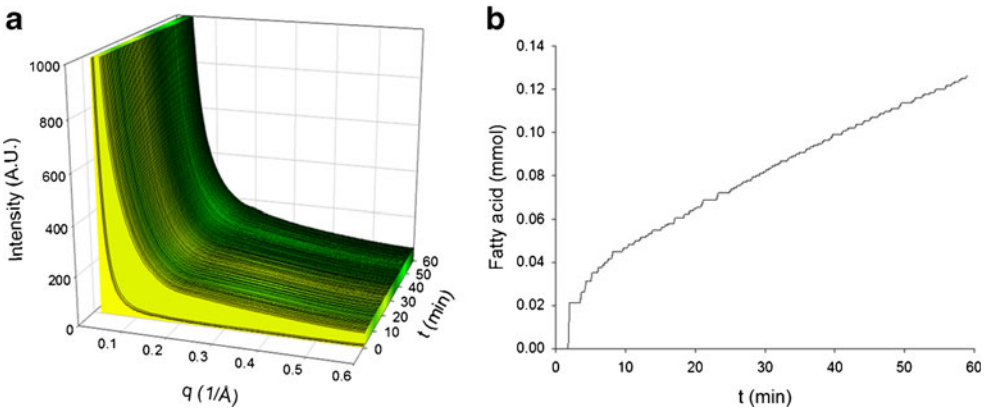

Fig. 3 Digestion of 10 mM Captex 355 (50 mg) in fasted state over 60 min at 37°C studied by SAXS (**a**) and digestion kinetics from the titration profile (**b**).

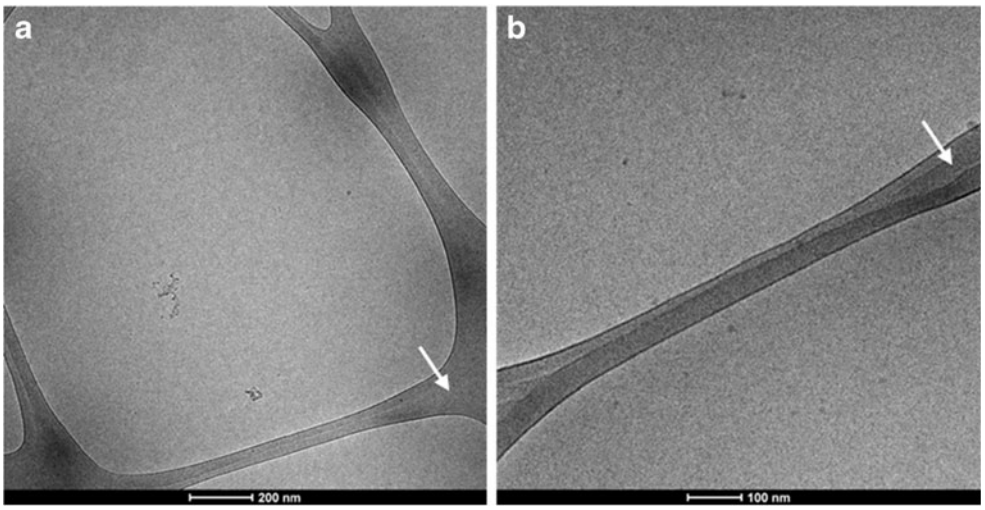

Fig. 4 Cryo-TEM images of (**a**) digestion buffer (no structures present) and (**b**) fasted bile salt + phospholipid micelles, where the black dots apparently indicate small micellar structures. *Arrows* indicate regions of the TEM grid.

The 50 mM digestion was repeated, and rather than immediately vitrifying the retrived samples, the lipase inhibitor (0.5 M 4-bromophenylboronic acid in methanol) was added, and the samples vitrified up to 24 h after digestion. The cryo-TEM images of the inhibited samples taken at the corresponding time points in Fig. 6 were broadly consistent with those in Fig. 5 from the real time study, however the abundant liposome structures present towards the end of digestion in the real time sample were less numerous in the inhibited sample. Thus lipase inhibition did appear to have a slight influence on the structures present in inhibited samples.

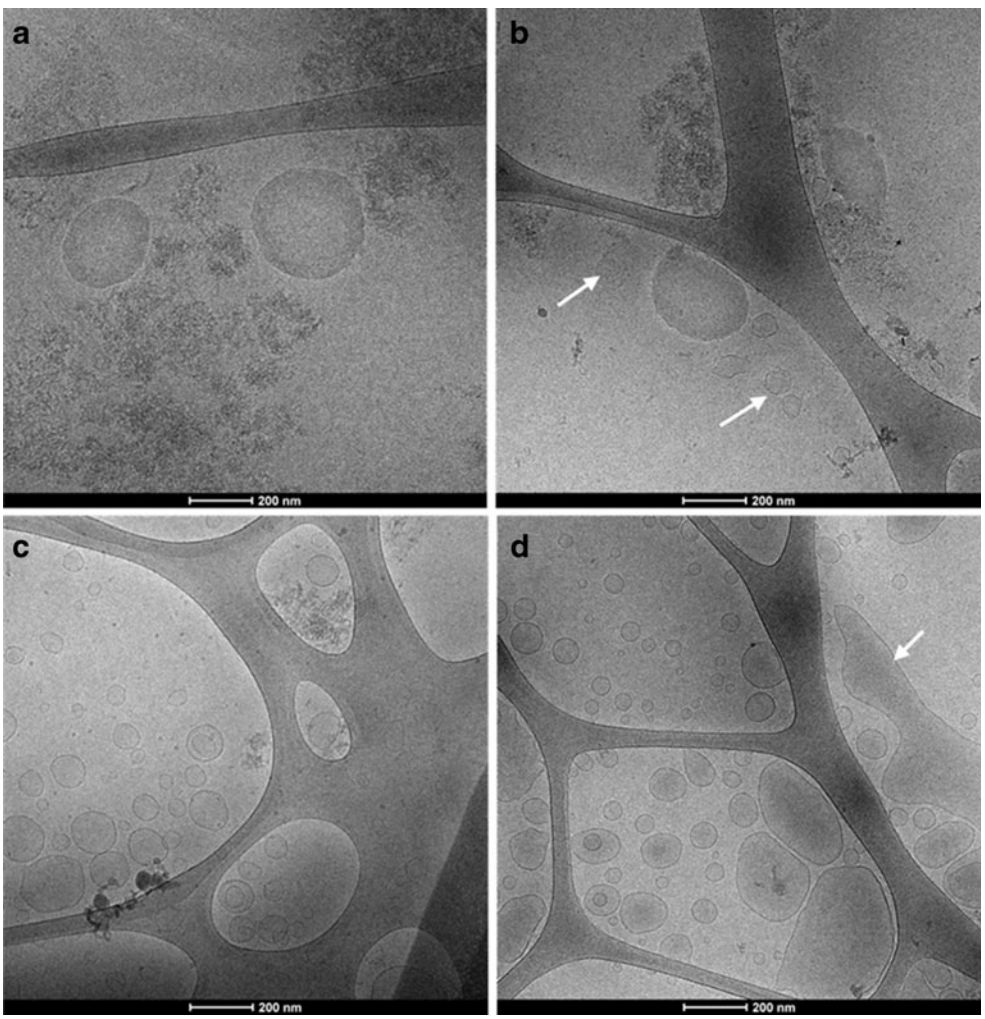

Fig. 5 Cryo-TEM images of the digestion medium during real time 50 mM Captex 355 (250 mg) digestion. (**a**) At 10 min, oil droplets approximately 200 nm in diameter and protein aggregates are evident. (**b**) At 20 min small faceted unilamellar vesicles 30–150 nm were apparent (indicated by *arrows*) in addition to a 200 nm oil droplet and protein aggregates. (**c**) At 30 min the appearance of unilamellar and bilamellar vesicles 50–200 nm increasingly dominate the structures present. (**d**) At 40 min large unilamellar and bilamellar vesicles 50–200 nm, and lamellar fragments a few hundred μm in length (indicated by *arrow*) were observed. Micelles approximately 3–4 nm in diameter were seen as *small black dots* in all samples, which were absent from digestion buffer (Fig. 4a).

Representative images (from 130 in total) obtained during the digestion of 10 mM Captex 355 (50 mg) are shown in Fig. 7. In the sample taken at 0 min (pre-enzyme addition), only oil droplets up to 2 μm in diameter were present. Following enzyme addition, collagen and protein aggregates were observed in close proximity in all samples. At 10 min (Fig. 7b), oil droplets 100–600 nm in size were present; at 20 min (Fig. 7c), there were 50–600 nm oil droplets present, while at 30 min, the oil droplet size had decreased to approximately 50 nm (Fig. 7d). At 40 min, no oil droplets were evident with only micellar structures in the field of view. As digestion proceeded, the micelle content indicated by the dark dots approximately 3–4 nm in diameter, increased.

Structure and Morphology in Equilibrium "Assembled" Systems Representing the Endpoint of Tricaprylin Digestion

Tricaprylin is a major component of Captex 355, and hence the structure formation by 'pure' tricaprylin and its digestion products is on interest to compare with the more complex lipid mixture. As a first step, equilibrium structures were prepared by addition of monoglyceride and fatty acid in bile salt and phospholipid mixtures to simulate the anticipated gastrointestinal state on digestion of tricaprylin. Systems were prepared by adding monocaprylin (MC) and caprylic acid (CA), in a 1:2 molar ratio expected on quantitative digestion of the triglyceride.

In both fasted and fed states (low and high bile salt and phospholipid concentrations), bile salt micelles coexisted with a lamellar phase (Fig. 8). In both the fasted and fed states, with increased lipid loading, the micelle content increased (indicated by the broad hump centred on approximately $q=0.16$ Å) as did the intensity of the sharp lamellar diffraction peaks. This indicates that within the concentration regime studied, there was no change in distribution of structures with increasing lipid load.

The low monocaprylin + caprylic acid system in bile salt micelles (12.5:25 mM ratio), prepared in fasted and fed state micellar systems were also analysed by cryo-TEM (Fig. 9). This system was intended to be indicative of complete digestion of the low Captex 355 digestion, although the concentration was

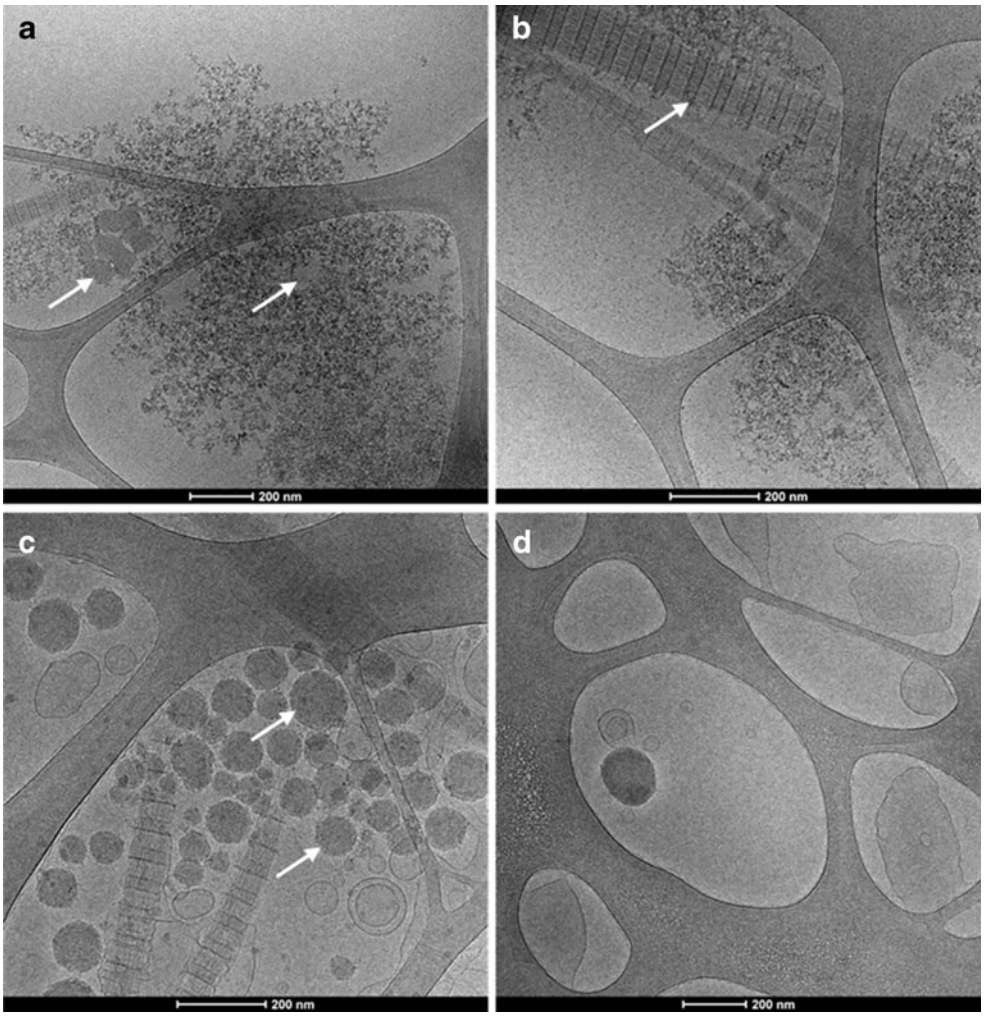

Fig. 6 Cryo-TEM images of inhibited samples of digestion medium during real time 50 mM Captex 355 (250 mg) digestion. (**a**) At 10 min oil droplets 100 nm in diameter (indicated by *arrows*) associated with protein aggregates and collagen strands are observed. (**b**) At 20 min oil droplets associated with protein aggregates and collagen strands (indicated by *arrow*) (present in the pancreatin) still appear to dominate the structures present. (**c**) At 30 min, unilamellar and bilamellar vesicles 50–200 nm in diameter, and oil droplets 50–150 nm in diameter are seen (indicate by the *arrows*), and in (**d**) at 40 min, lamellar fragments are seen and 150 nm bilamellar vesicles are observed, with one budding off from a 200 nm oil droplet.

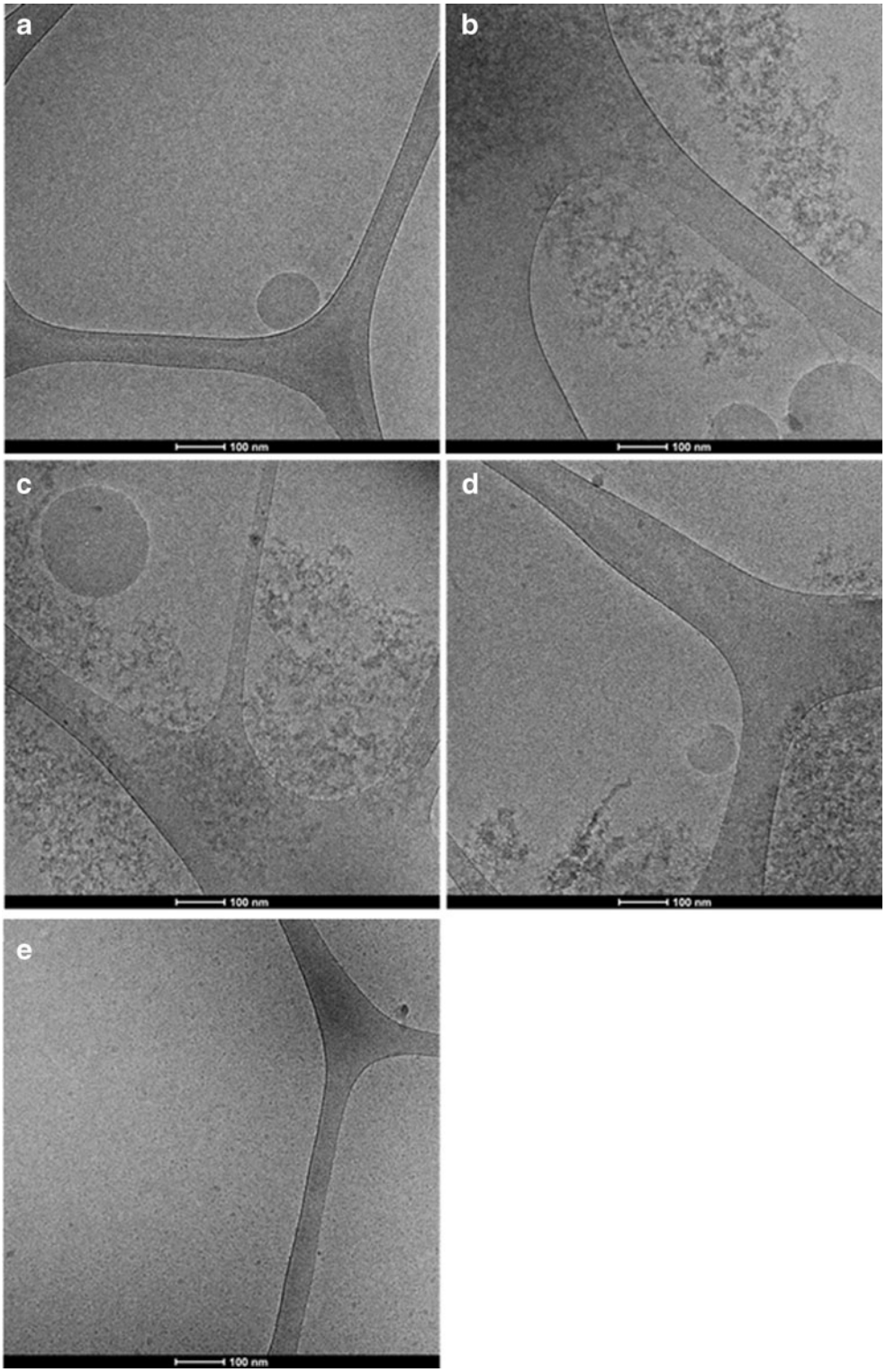

Fig. 7 Cryo-TEM images of the inhibited samples of digestion medium during real time digestion of 10 mM Captex 355 (50 mg). (**a**) Pre-digestion (0 min) showing a 100 nm oil droplet before enzyme addition. (**b**) After 10 min there are 100–200 nm oil droplets and protein aggregates. (**c**) At 20 min again 200 nm oil droplet and protein aggregates are present. At 30 min (**d**) a small residual 100 nm oil droplet remains with protein aggregates, and at 40 min (**e**) only micelles were evident.

slightly different (12.5 mM of equivalent triglyceride, compared to 10 mM Captex 355, and only C_8 lipids were present). In the fasted state, spherical and elongated liposomes 100–200 nm in diameter were present. Large lamellar fragments several hundred micrometers in length were also visible across the grid. This was consistent with the scattering data which a high intensity lamellar phase peak compared to the micellar hump. In the fed state, only micelles were evident which possessed a worm-like or thread-like structure. There was no co-existing lamellar phase observed in the imaging even though there were lamellar structures evident in the scattering profile for the fed low lipid system in Fig. 8.

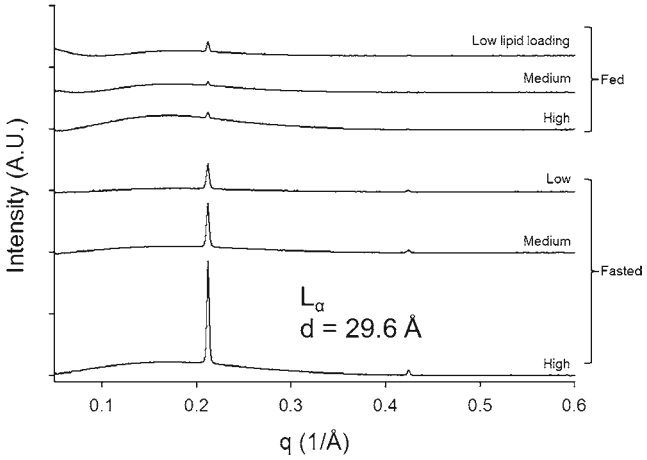

Fig. 8 SAXS profiles of equilibrium assembled monocaprylin + caprylic acid-containing fed and fasted systems. Systems contained MC + CA in a 1:2 mol ratio at high (50:100 mM), medium (25:50 mM) and low (12.5:25 mM) concentrations respectively. The high and low lipid 'assembled' systems are representative of the 250 and 50 mg Captex 355 digestions respectively. The d-spacing of the lamellar (L_α) structures present is indicated in the *inset*.

Evolution of Structure During Tricaprylin Digestion Under Fasted State Conditions

For comparison of the structures present during digestion of Captex to that of 'pure' tricaprylin, tricaprylin was also digested in the flow through synchrotron configuration. The structural changes during a 30 min digestion of 5 mM tricaprylin (equivalent to 23.5 mg/10 mL digest) were monitored by SAXS. Micellar content was seen to increase over time, however there was no co-existing lamellar phase (Fig. 10). This was consistent with the low lipid digestion with Captex 355 where no lamellar structures were present, whereas the assembled system at the lowest lipid level demonstrated strong lamellar scattering, and it was expected that this may exist at an equivalent lipid concentration in an assembled system, although the direct comparator system was not studied. Future experiments will determine the impact of increasing lipid load with pure tricaprylin and on lower (eg. 5 mM) assembled systems.

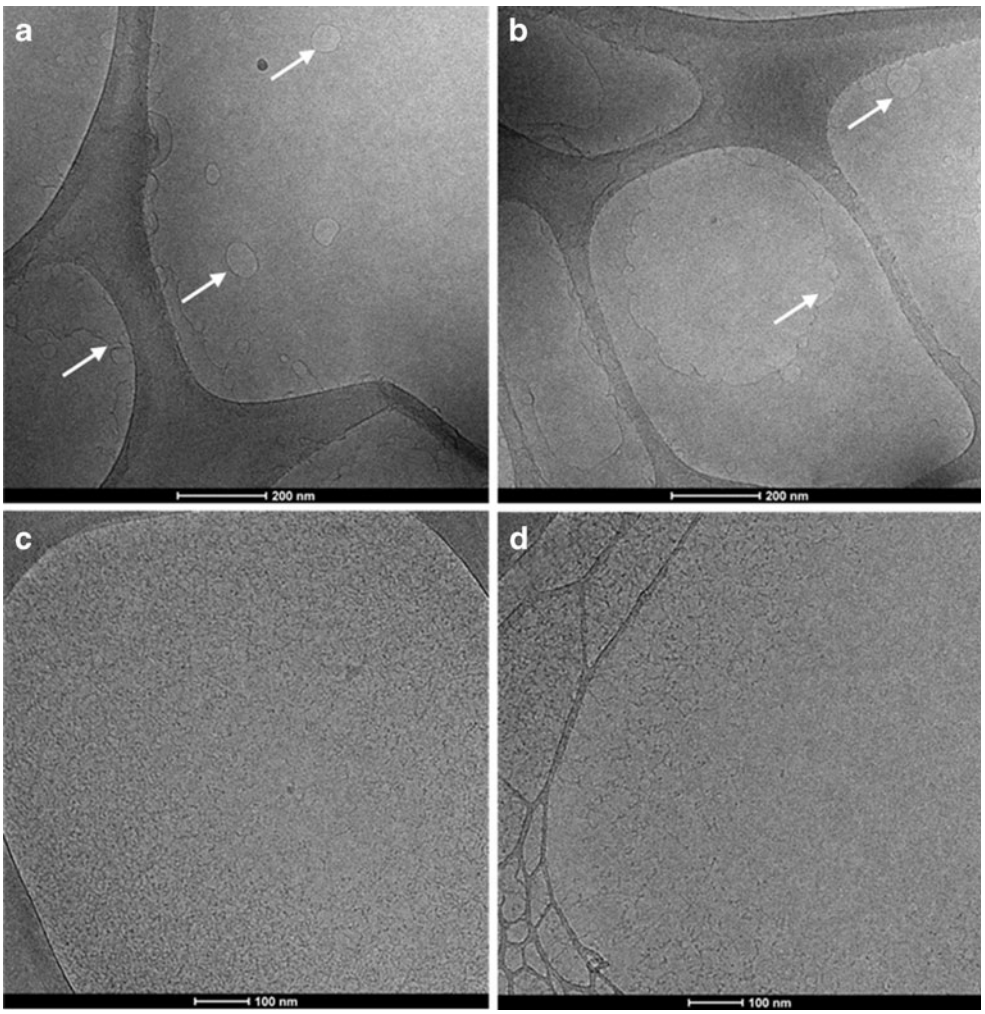

Fig. 9 Cryo-TEM images of the assembled monocaprylin + caprylic acid system at 12.5:25 mM ratio ('low' lipid ratio). (**a**, **b**) From samples assembled in the fasted state media, showing lamellar phase, as indicated by liposomes 100 nm in diameter (*arrows*), and large lamellar fragments. (**c**, **d**) Equivalent samples prepared in the fed state media showing micellar phase, indicated by the presence of *worm-like* or *thread-like micelles*.

DISCUSSION

The colloidal phases generated during lipid digestion are of interest due to their potential role in drug solubilisation, which may have implications for the performance of lipid-based drug formulations. Kossena *et al.* have demonstrated that these intermediate phases are important in drug solubilisation and trafficking (4).

Formation of Vesicular Phase is Linked to Digestion Kinetics

The lipid content was observed to influence the colloidal structures formed during digestion of Captex 355 in the fasted state. At low lipid level (50 mg, ~10 mM) only micelles were formed (Figs. 3 and 7), whereas a high lipid level (250 mg, ~50 mM) a lamellar phase was evident which was coincident with vesicle formation by cryo-TEM (Figs. 2 and 5). This is believed to constitute the first direct evidence of the generation of a vesicular phase during *in vitro* digestion of high concentrations of the MCT Captex 355. The propensity for the vesicular phase to support supersaturated drug concentrations during *in vitro* digestion is now clear from past studies; only high lipid load digestions exhibit the effect, and in low lipid loads, where micelles are the only colloidal structure present, high drug precipitation occurs. Thus the structure-digestion-performance link in this system was established.

The kinetics of the digestion of Captex 355 shows a two-stage digestion process. This is not normal during triglyceride digestion – neither tributyrin (27), often used to calculate the activity of pancreatin, nor soybean oil (9) (a long chain triglyceride comprised mainly of triolein with C_{18} unsaturated fatty acid chains) show such behaviour. This interesting phenomenon has been known for over a decade in previous studies (9) but no explanation has been evident prior to the findings in Fig. 2, where there is a direct temporal relationship between the onset of the lamellar phase formation and the discontinuity in the digestion kinetics.

The morphology of colloidal samples formed during digestion of a lipid-based formulation, and their similarity with *ex vivo* samples of human intestinal fluid has been previously studied by cryo-TEM and AFM (14,19–21). The sequence of phase changes observed in the current work was in good agreement with these studies and other reports (17), where at the beginning of lipid digestion, only oil droplets and protein were present, and unilamellar and bilamellar vesicles were seen to develop and dominate at the end of digestion. It has been reported that vesicles coexisting with worm-like mixed micelles should be present at the end of digestion, depending on bile salt levels (8,28,29). Micelles were present throughout digestion, and coexisted with unilamellar and multilamellar vesicles, which is in agreement with previous reports, however previous reports used long chain triglycerides (14,17,19).

In the current study, vesicles were seen to 'bud-off' from the surfaces of the oil droplets, suggesting the lipid digestion products were removed from the surface and detached to form vesicles and micelles. Müllertz *et al.* also observed a bilayer fragment loosely associated with an oil droplet in *ex vivo* samples (21). During digestion in the fasted state, only one trilamellar vesicle was observed, and unilamellar vesicles and bilamellar vesicles were found to be dominating. This is in agreement with previous findings, which reported that in the fed state, the higher levels of phospholipid favours formation of multivesicular structures (19,20). However, this appears to be in contrast to the SAXS data and cryo-TEM images for the fed-state 'assembled' monocaprylin + caprylic acid system in Figs. 8 and 9 which indicate reduced or absence of lamellar phase structures respectively.

The lattice dimensions for the lamellar spacing for the digested Captex 355 was 30.1 Å, which was slightly higher than that shown on Fig. 8 for the 'assembled' monocaprylin + caprylic acid system (29.6 Å). The lattice parameter for the lamellar phase formed in an equivalent 'assembled' C_{10} monoglyceride + fatty acid system was 34.6 Å (data not shown), confirming that the dimensions scaled with average lipid chain length as anticipated.

Segregation of lipids during the digestion process may be possible, for example leading to vesicles composed primarily of monoglyceride, and where fatty acid is incorporated into micellar structures. However, it is likely that the vesicles

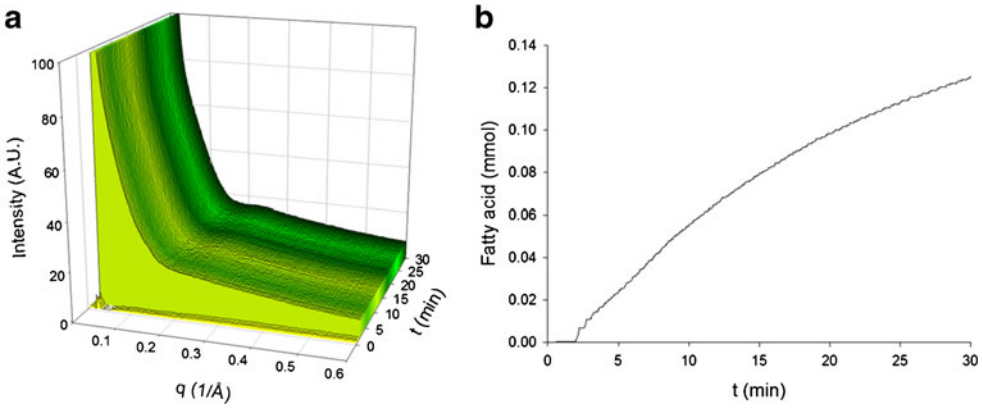

Fig. 10 Digestion of 5 mM tricaprylin (approx. 25 mg) in the fasted state over 30 min at 37°C studied by SAXS (**a**) and titration profile (**b**).

actually consist of a mixture of monoglyceride and fatty acid – not only because of the agreement with the expected lattice dimensions from the assembled equilibrium mixtures, but also due to the fact that the coexisting oil droplets indicates that it is generation of a separate phase with different local composition to the droplets that constitutes the vesicles, and not a phase change due to a global change in concentration.

"Assembled" Equilibrium Systems *vs In Vitro* Lipolysis and Sample Inhibition

The dynamic real time Captex 355 digestion samples were compared to equilibrium samples prepared by assembly of component monoglycerides and fatty acids for direct comparison of the two approaches. The low monocaprylin + caprylic acid system in bile salt micelles (12.5:25 mM ratio), prepared in fasted and fed state micellar systems exhibited a micellar phase and coexisting lamellar phase, as indicated in the scattering data. The cryo-TEM results however suggest that whilst there were large lamellar fragments in the fasted state, there were only worm-like or thread-like micelles present in the fed state. This suggests that "assembled" equilibrium systems have their limitations as they are not representative of the dynamic nature of the GI environment, thus dynamic lipolysis experiments measurements are more relevant. The vesicular phase was not apparent in fasted-state low lipid digestions, where the bile salt to lipid ratio was 1:2. The absence of lamellar structure in both the cryo-TEM images in Fig. 7 and the dynamic scattering data in Fig. 3 might be expected considering the higher bile salt to lipid ratio compared to the high lipid load digest, where the ratio was 1:10 . Previous work has shown further interaction of vesicles with bile salts results in the transition to mixed micelles (4,8,17,28). The finding is also consistent with the scattering data obtained after addition of 55 mM bile salt to the end point of the 50 mM lipid digestion in Fig. 2. The 'assembled' fed system did show some lamellar phase structure was present in the fed state where the ratio varied from approximately 1:0.6 to 1:2.5. The exact reason for this difference is not yet apparent, again highlighting the difficulties in extrapolation of data from equilibrium assembled systems to predict the likely structures formed during dynamic digestion processes.

The bile salt/phospholipid mixture used in both preparation of the assembled systems and in the dynamic digestion studies is a highly simplified version of the intestinal contents. The use of a complex bile salt mixture to better represent the likely *in vivo* content was of course possible, but studies have shown that increased complexity does not necessarily alter the behavior of such systems (30) and inter-person variability is high, meaning that the selected complex mixture may not be representative of bile in many cases.

The lipase inhibitor, 4-bromophenylboronic acid is often used to halt digestion when sampling is required during *in vitro* experiments to measure aspects such as drug precipitation or lipid composition. Whilst the inhibitor performs well, there has not been an attempt to directly confirm whether structural aspects are influenced by its presence. Cryo-TEM also revealed subtle differences between samples that were retrieved from digestion and immediately vitrified, compared to samples treated with a lipase inhibitor. Fewer liposome structures were observed in the end stages of digestion in the inhibited sample compared to the corresponding time-points in the real time samples. The lipase inhibitor is typically prepared in methanol. There is potential for the methanol, or less likely the inhibitor, to interact with those structures and either solubilize the lipids comprising the structures or induce a transition to other, likely micellar structures. Although the structure formed in this case were not dramatically different to the real time samples, care must certainly be taken in interpretation of samples containing enzyme inhibitor and the quantity of solvent used should be minimized.

Implications

The solubilization of drug has been shown to critically depend on colloidal structure and lipid comprising the structures (4,31). Consequently, an understanding of the structures that are present during digestion that give rise to beneficial effects, such as potential to support supersaturation of drug on digestion, are of interest. This level of structural understanding has potential to lead to rational engineering of formulations to generate specific structures on digestion to optimize the pre-absorptive environment. Until there is a clear understanding of the complex colloidal behavior of these relatively simple lipid formulations, development of more advanced and complex approaches such as self-nanoemulsifying systems (32–36) and solid matrix lipid-based formulations (37,38) will remain empirical by necessity. It remains to be seen whether the dynamic colloidal behaviour observed here and in other similar studies translates to the *in vivo* scenario, potentially leading to increased drug absorption and bioavailability.

CONCLUSION

Insights into the structural aspects of the digestion of medium chain triglycerides have been obtained by coupling the *in vitro* lipolysis model with sSAXS and cryo-TEM. A putative vesicular phase formed on digestion of MCT was confirmed and its structural attributes determined. The dependence on bile salt to lipid ratio demonstrated the lipid dependence on formation of the lamellar structures on digestion. Comparison of cryo-TEM images of real time samples compared with inhibited digestion samples demonstrated potential for

differing structures to be formed, highlighting the need to conduct real time structural investigations rather than relying on analysis of inhibited samples by offline techniques. The comparison with 'assembled' equilibrium samples, representative of the post-digestion environment also indicated some differences in structures formed, again highlighting the caution in interpretation of likely dynamic behaviour from 'static' samples.

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