Lipids

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Self-Assembly Structure Formation during the Digestion of Human Breast Milk**

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Abstract: *An infant's complete diet, human breast milk, is the basis for its survival and development. It contains water-soluble and poorly water-soluble bioactive components, metabolic messages, and energy, all of which are made bioavailable during the digestion process in the infant's gastrointestinal tract. Reported is the first discovery of highly geometrically organized structures formed during the digestion of human breast milk under simulated in vivo conditions using small-angle X-ray scattering and cryogenic transmission electron microscopy. Time of digestion, pH, and bile salt concentration were found to have symbiotic effects gradually tuning the oil-based environment inside the breast milk globules to more water-like structures with high internal surface area. The structure formation is necessarily linked to its function as carriers for poorly water-soluble molecules in the digestive tract of the infant.*

Human breast milk is the key to the development and survival of humans. It contains a variety of essential bioactive components, metabolic messages, and energy.[1–5]

Fat, of which approximately 98% are triglycerides, is a critical component of breast milk, thus providing energy and nutrients which are key to the development of the brain and central nervous system in the infant.[1,2,9–11] Triglycerides are molecules with a glycerol backbone esterified with fatty acids on the two outer (sn-1 and 3) and the middle (sn-2) positions. Processes such as growth of membrane structures, cell division, and myelination require the synthesis of large amounts of new membrane material, which results in a high demand for such lipids. In human breast milk, triglycerides and other poorly water-soluble components such as fat-soluble vitamins are emulsified in small fat globules stabilized by an outer layer of mainly proteins and phospholipids.[12]

In the gastrointestinal tract (GIT), lipases transform the milk triglycerides into smaller-molecular-weight, more hydrophilic and bioavailable components to enable absorption into the circulatory system. Gastric lipase secreted by the gastric mucosa is mainly responsible for hydrolysis of the triglycerides at one of the outer ester bonds, thus liberating free fatty acids and diglycerides. These products are mechanically sheared in the stomach and alter the interfacial composition of the emulsion droplets for subsequent pancreatic lipase digestion in the small intestine.[13–16] Fat digestion continues in the small intestine with pancreatic lipase hydrolyzing ester bonds on both outer positions (sn-1 and sn-3), thus leading to a sn-2 monoglyceride and free fatty acids. Bile salt stimulated lipase (BSSL) from the infant pancreas also contributes to the release of fatty acids.[17] BSSL is also a component of human milk, secreted in the milk by the mammary gland.[18] Compared to pancreatic lipase, BSSL has higher activity towards hydrolyzing the ester bonds to yield long-chain polyunsaturated fatty acids from the milk triglycerides, which are key to the development of the infant's brain and central nervous system.[19,20]

The interfacial-active lipolysis products, together with phospholipids and bile salts (BS) secreted from the gall bladder into the small intestine, further facilitate an increase in the oil-water surface area and promote binding of lipase to the oil-water interface.[21,22] The pH of the food bolus gradually increases as it passes along the gastrointestinal tract from 1.0–8.0, thus leading to gradual deprotonation of the fatty acids accumulating at the oil-water interface.[23] Thus a complete understanding of the impact of pH and BS and lipase on the digestion process of human breast milk is imperative.

BS micelles also act as carriers for hydrophobic food components in the aqueous system of the GIT. However, in duodenal contents of newborn infants the bile salt concentration (ca. 1–5 mM) is much lower than in adults, and might even be below the critical micelle concentration (cmc of ca. 1 mM).[24–28] These compromised conditions raise the question of the existence of an alternative carrier and controlled release system to transport such essential molecules to secure survival under compromised bile salt conditions.

Herein we present the first study showing highly geometrically organized structures during the digestion of human breast milk. In vitro digestion of human breast milk under biologically relevant conditions revealed the formation of highly organized self-assembled structures within the breast milk fat globules, which can act as carriers and controlled

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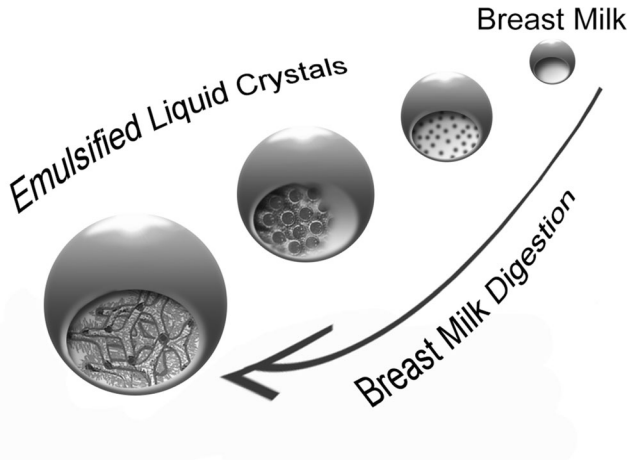
release systems for poorly water-soluble milk components in the aqueous system of the GIT. Structure formation was characterized in real-time using small-angle X-ray scattering (SAXS) on a synchrotron source. The structures were further visualized using cryogenic transmission electron microscopy (cryo-TEM) as a complementary method. The aims of this article are 1) to understand the structure formation during digestion in the absence of bile salt at various pH conditions (6.5, 7.0, and 7.5); 2) to investigate the self-digestion of milk in the absence of lipase; and 3) to study the influence of bile salts on the structures formed in human milk during digestion.

The self-assembly of the lipid digestion products inside the emulsion droplet drives the structure formation. Scheme 1 is an artistic description of changes inside the breast milk fat droplet during digestion. The observed biogenerated systems are similar to man-made dispersions of liquid crystalline surfactant phases discussed for applications such as controlled release of active ingredients and protein crystallization.[6–8] The oil-based environment inside the emulsion droplet is transformed into more water-like structures with a high internal surface area capable of carrying poorly water-soluble molecules in the digestive tract of the infant.



Scheme 1. The formation of highly ordered structures inside breast milk fat globules during digestion. The oil-continuous structures inside the globules are micellar (L_2 and Fd3m type), hexagonal (H_2 type), and bicontinuous cubic (Im3m type).

The time-resolved SAXS curves for the digestion of human breast milk at pH 6.5, where pancreatic lipase has its maximum activity, are presented in Figure 1. Prior to digestion, human breast milk is an oil-in-water emulsion (curve before lipase addition and at $t=0$, measured immediately after defrosting and equilibrating at 37 °C). Significant ordering of the molecules within the oil droplets occurred after addition of the lipase, thus leading to significant changes in the scattering profiles over time. After 30 seconds a broad correlation peak from oil-continuous micelles within the emulsion droplet (emulsified microemulsion; EME) appeared at $q=0.135$ Å^{-1}. At $t>7$ minutes equidistant peaks of a lamellar structure at $q=0.137$, 0.273, and 0.409 Å^{-1} (lattice constant $a=46$ Å) were seen. Lamellar

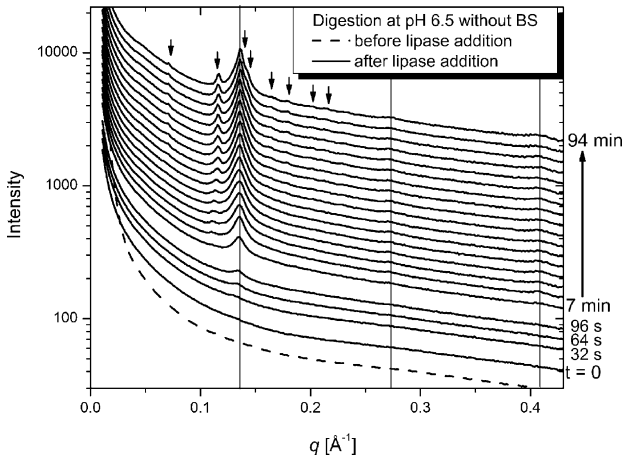


Figure 1. SAXS patterns for the digestion of human breast milk with lipase at pH 6.5 without added BS. The vertical lines highlight the constant peak positions of the lamellar phase. The arrows show the identifiable and further calculated theoretical Bragg peak positions for the Fd3m structure.

structures with similar dimensions have been previously reported in mixtures of DNA and DPPC in the presence of $CaCl_2$, all components of human breast milk.[29] After 14 minutes, Bragg reflections of the discontinuous micellar cubic (Fd3m type) phase grew at $q=0.071$, 0.117, 0.142, 0.165, 0.179, 0.200 Å^{-1}. The lattice constant of the Fd3m phase decreased slightly during the digestion from 157 to 153 Å. The titration curves following the release of fatty acid during the reaction show that most of the digestion occurs within the first 60 minutes. No major differences in the digestion kinetics were observed in the titration profiles for the different systems presented in Figure S1 in the Supporting Information.

Electrostatic interactions between deprotonated carboxylic groups render fatty-acid-containing colloidal structures highly pH sensitive.[23,30] The digestion of human breast milk at increased pH (7.0) is presented in Figure 2. Before the addition of lipase, the breast milk sample was kept at 37 °C for 1 hour to initiate the self-digestion by the breast milk's own lipase BSSL. Interestingly, peaks corresponding to a lamellar structure at $q=0.137$, 0.273, and 0.408 Å^{-1} were observed after 1 hour without added lipase. This structure formation in the absence of lipase seems unique to breast milk and has not been observed during the digestion of cow milk.[31] Yet this process appears very slow. After addition of pancreatic lipase to the same sample, transitions from EME to the discontinuous micellar cubic phase (Fd3m), inverse hexagonal (H_2), and Im3m type bicontinuous cubic phase were observed. The bicontinuous cubic phase existed between $t=20$ and 74 minutes of digestion, where over time the lattice constant gradually decreased from about 230 to 205 Å. The lattice constant of the coexisting H_2 phase present after $t=$ ca. 40 minutes was somewhat constant at 65 Å (see Figure S2 in the Supporting Information). The formation of these ordered geometric structures requires the transfer of water from the bulk into the lipid interior of the emulsion particle. Here, the water content increases from close to 0% in the triglyceride emulsions to around 33% in the bicontinuous cubic phase towards the end of digestion.[32]

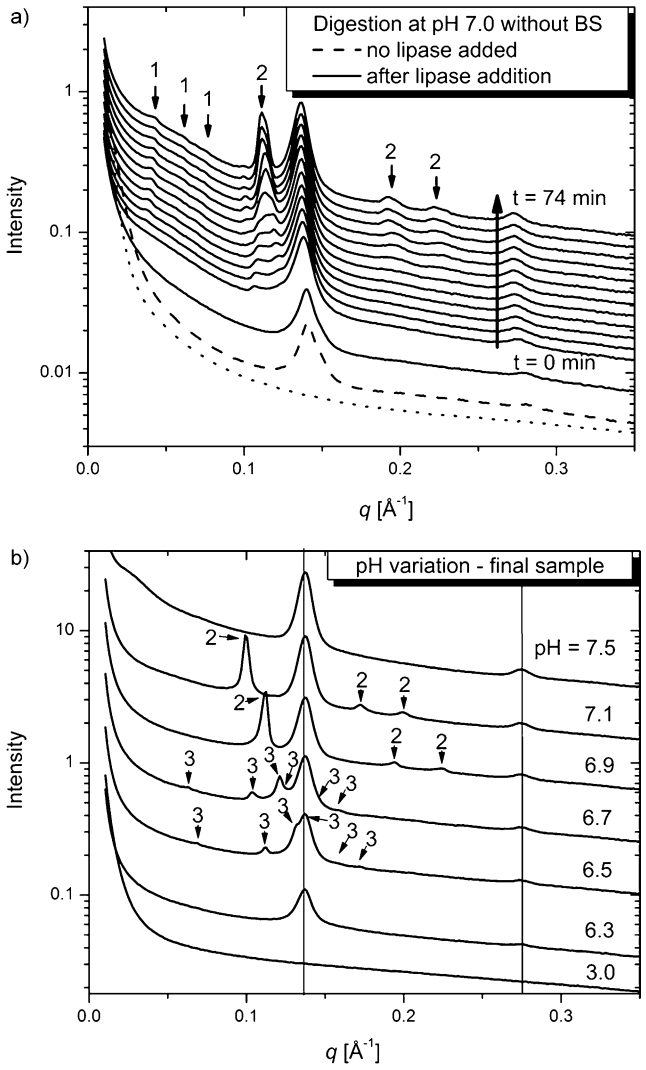


Figure 2. SAXS patterns for the digestion of human breast milk at pH 7.0 without addition of BS. a) The curves measured directly after defrosting (dotted line) and after 1 h of self-digestion of breast milk before lipase addition (dashed line) are also presented. Lipase was then added to the same sample to initiate further digestion (full lines from $t = 0$ min). b) pH variation in the final digestion sample is presented. The identifiable and further calculated Bragg peak positions are indexed as 1 for bicontinuous cubic structure (Im3m type), 2 for inverse hexagonal, and 3 for micellar cubic structure (Fd3m type).

The pH-response of milk structures was investigated on the final digestion mixture after 75 minutes. The pH environment of the GIT is highly dynamic, with the pH gradually increasing on the way through the GIT with additional gradients co-existing between the bulk and in proximity of absorptive cells in the intestine.[33] Varying the pH value of the milk sample by addition of 1M HCl/NaOH showed a strong influence on the ordering (Figure 2b). All highly organized structures diminish at pH 3. Upon increasing the pH value to 6.3 the lamellar phase in combination with a putative EME appeared and at pH > 7 structural transitions through the Fd3m and H_2 phases were observed. This is in agreement with the phase sequence observed upon increasing pH in equilibrium monoglyceride/fatty acid systems.[23] Contrary to the coexisting nonlamellar structures, the spacing in the lamellar phase is pH independent at 46 Å. Cryo-TEM images on this sample at pH 7.0 are presented in Figure S3 (see the Supporting Information). They indicated a complex mixture of structures and particle morphologies as expected. Particles with a mean diameter from one hundred up to several hundred nanometers in coexistence with anisotropic structures were apparent. Vesicles were also present at the surface of some particles.

The digestion of human breast milk was repeated at the elevated pH 7.5, thus representing the higher pH conditions also reported in the small intestine (see Figure S4 in the Supporting Information). Apart from a single Bragg reflection, which occurred intermediately during digestion, the scattering curves show the presence of vesicles. This finding confirms that increasing the pH value in the digestive tract forces the system towards more hydrophilic oil-water interfaces with decreasing mean curvature. Given that the pK_a value of long-chain fatty acids is expected to be between 6 and 7, the fatty acids would be mostly deprotonated at this pH value.[23] Decreasing the pH value in the final digestion sample from 7.5 to 7.0 resulted in the formation of the Im3m type bicontinuous cubic phase ($a = 208.5$ Å) and H_2 structure ($a = 66$ Å; see Figure S4). The lattice constants are in good agreement with those from the final sample digested at pH 7.0 (i.e. 208.5 Å for Im3m phase and 65 Å for H_2 phase). This indicates that the final digestion structure is independent of the pathway, and that the same structures will be found under the same conditions (pH and composition).

Biologically relevant BS concentration reflecting low and intermediate levels in the infant was added to the model digestive juice (1.25 mM and 2.5 mM). The influence of these bile salt concentrations on structure formation is presented in Figures S5 and S6 (see the Supporting Information). Similar to the digestion experiments presented above, Bragg peaks from ordered structures were formed in the breast milk before lipase addition because of self-digestion of the breast milk by BSSL within 1 hour. After lipase addition, the intensity of these peaks increased for both samples. An additional Bragg reflection, indicating the formation of H_2 structure, only appeared at the lower BS concentration. The modification of the structure in the presence of BS indicates an interaction with the internal particle structure. Increasing the BS concentration showed a similar effect to increasing the pH value, with the oil-continuous particle phase becoming more hydrophilic and organized.

For the first time, highly organized structures forming within the human milk fat globules were discovered. The structures are formed through the digestion of lipids by breast milk's own lipase BSSL and added pancreatic lipase under low BS concentrations relevant for infants. The major role of BS is the solubilization and transport of digestion products from the digesting milk fat droplet to the enterocytes for absorption to occur. The self-assembly of lipid digestion products within fat globules and composition of human breast milk is responsible for the structure formation. In the absence or at low concentrations of BS, such as in preterm infants, the larger oil-water interface areas in these structures might assure sufficient progress of digestion in these scenarios and

act as controlled release systems for nutrients and energy. The transfer of water into the digesting milk fat droplets suggests that in addition to hydrophobic molecules, hydrophilic milk components and the lipase might also be transported into the droplet interior. The pH dependence of the structure also indicates that they are nature's own pH-controlled release system for active molecules in the aqueous environment of the GIT, thus assuring the nutrient supply and for the healthy development of newborns.

Experimental Section

In vitro digestion studies of human breast milk were performed using a previously reported flow setup coupled to a quartz capillary to enable time resolved flow-through small-angle X-ray scattering for structural elucidation in real time.[30] The pooled human breast milk was supplied and studied at Mercy Hospital for Women Human Research Ethics Committee and the Monash University Human Ethics Committee in a multicenter approval (CF14/624-2014000188).

The digestion medium was drawn through a 1.5 mm diameter quartz capillary mounted in the X-ray beam at a flow rate of approximately 10 mL min^{-1} to avoid beam damage, through silicone tubing (total volume <1 mL) via a peristaltic pump. A pH-stat (Metrohm, Switzerland) titrated the digestion mixture with 0.2 M NaOH during digestion in order to maintain the system at the target pH in a thermostated glass vessel at 37 °C.

An X-ray beam with a wavelength λ of 1.1271 Å (11 keV) was used, with a sample to detector distance of 1575.3 mm providing a q-range from $0.01 < q < 0.6$ Å^{-1}, where q is the length of the scattering vector, defined by $q = 4\pi/\lambda \sin(\theta/2)$, θ being the scattering angle. The 2D SAXS patterns were acquired for 5 sec with 27 sec delay between frames, using a Pilatus 1M detector (active area 169×179 mm^2 with a pixel size of 172 μm) and integrated into the one-dimensional scattering function I(q). Hexagonal, cubic and lamellar space groups were determined by the relative positions of the Bragg peaks in the scattering curves, which correspond to the reflections on planes defined by their (hkl) Miller indices. More complete information concerning this point can be found elsewhere.[23,30]

Further details on the materials, digestion experiments, and cryo-TEM measurements are presented in the Supporting Information.

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