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ORIGINAL PAPER

Organogel-Based Emulsion Systems, Micro-Structural Features and Impact on In Vitro Digestion

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Abstract Organogels based on edible oils and specific mixtures of phytosterols can serve as structured systems with a low saturated fat content. These low-SAFA organogels can be used also to create o/w emulsions. Little is known about the structures formed in these specific organogels and at the emulsion interface. We studied o/w organogels on different length scales to describe and understand their micro-structural features. Very basic processing conditions such as composition, temperature and storage time were taken into account. Two different types of structure were observed; at the smallest scale, long thin crystals are formed out of the oil phase into the continuous water phase. We propose that these are needle-like crystals. Next, tube-like structures are identified and can be visualized as tubular micelles. A model is proposed which fits the dimension (~ 7 nm) with the length scale of the molecular building blocks (TAGs and sterols). As edible fats from food products are enzymatically hydrolyzed in the gut prior to absorption, we also looked into the impact on the lipase reaction speed. Simple in vitro enzymatic hydrolysis experiments showed a slower enzymatic digestion. Organogel systems and emulsion made thereof have interesting food structuring properties with possible advantages in composition (low SAFA) and digestion speed.

Keywords Food structure · Organogel · Lipolysis · Micelles · Linear crystals · Phytosterols · Tubes · Tubules · SAFiN

All authors were full time employees or trainee (MC) of Unilever during actual execution of this study.

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Introduction

Structuring edible oils is a requirement for many food products such as vegetable oil based spreads (margarines) or dressings, and normally achieved with crystal networks formed from triacylglycerides (TAG) built mainly from saturated fatty acids (SAFAs) introduced in the oil phase [1]. In search for healthier products, alternative structuring agents would be beneficial, if they replace or lower the product SAFA content, do not contribute significantly to the product energy density themselves, while maintaining an adequate sensory profile, good product shelf-life stability and show consumer benefits. Reducing the SAFA content improves the nutritional profile of such products, as high SAFA intakes are linked to increased risk of cardiovascular disease [2].

One option explored to structure the edible oil phase is to use organogels. Organogels are organic liquids structured by low concentrations of relatively low molecular mass molecules. Organogels are known to structure organic materials

based on e.g., fatty acids, alcohols, or lecithins and are proposed for food or pharmaceutical formulations. Despite their interesting rheological properties and potential applications, questions remain on the micro-structures formed which make the gel [3]. Edible organogel systems are for example based on fatty alcohols/fatty acids or on combinations of certain phytosterols. Structuring properties of these latter systems have been claimed in the past [4–6]. Phytosterol based organogel systems are in particular interesting as they have good structuring properties, phytosterols are already common in the food chain, and higher intake of phytosterols is associated with blood cholesterol lowering [7–10].

However, structured edible oils are not consumed as such but further formulated into foods. Edible fats and oils are often consumed in the form of emulsion systems such as spreadable margarines (w/o systems) or mayonnaises and dressings (o/w systems). So far, little information is available on these organogel-based emulsion systems with respect to structure on a microscopic scale at or close to the droplet surface.

It is also relevant to understand what happens with these structures during the digestive processes. In particular, the rate and extent of fat hydrolysis by gastro-intestinal lipases could be influenced by the changed physico-chemical properties of the edible oil formulated into an organogel or organogel-based emulsion. These luminal lipases are true interfacial enzymes and the gastro-intestinal processing of TAG emulsions is a delicate process in which composition, size distributions and surface properties of the substrate play an important role, hence an impact of structure is not unlikely [11–13]. Influencing TAG digestion might have direct effects, immediately after absorption, as well as long term effects on TAG homeostasis [14].

To answer these questions we created model organogel systems, simple emulsions made thereof, and varied processing parameters such as time and temperature to understand the behavior and formation, relevant for actual food processing. Secondly, we studied the microstructure of the emulsion systems with different instrumental techniques to address different length scales. This report focuses on the structural description.

Finally, we looked into the fate of organogel-based emulsion (o/w) systems during simulated gastro-intestinal behavior. Hydrolysis of TAG from such systems under the influence of pancreatic enzymes was studied with a very simple model; and rate of digestion was analyzed as well as completeness of digestion.

Experimental Procedures

Materials and Sample Preparation

Olive oil (Bertolli), corn oil (Perfekt), and sunflower oil (Reddy) were bought in a local Dutch supermarket. Bile extract (porcine), Xanthan Gum, Triton X-100, potassium chloride, sodium chloride, and porcine pancreatin were all supplied by Sigma–Aldrich. Sodium hydroxide, tris(hydroxymethyl)-amino methane, calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$) were provided by Merck. γ-Oryzanol was obtained from the Tsuno Rice Fine Chemicals Company (Japan) and β-sitosterol from Forbes Medi-Tech Inc. (Canada).

Gel Preparation

Organogels were obtained by mixing 1:1 molar ratios of β-sitosterol:γ-oryzanol (4:6 weight ratios) in edible oil. The sterols (β-sitosterol and γ-oryzanol) were dissolved at 90 °C under continuous stirring in the hot oil. After 1 h, the oil–phytosterol mix was left to cool to room temperature to form the transparent (low) to slightly opaque (high sterol concentration) gel, which was then stored at 4 °C. Various amounts of the sum of phytosterols (8, 16, and 25 w/w%) were tested with the different oils.

Emulsion Preparation

Distilled water and Xanthan Gum (0.15 w/w%) were first mixed at high speed with an Ultra Turrax (T25 Basic, IKA Labortechnik). Subsequently, Triton X-100 (1 w/w%) was added. Next, melted gel (~90 °C) was poured into the aqueous phase and mixed again at high speed. The desired droplet size was about 2 μm. All the emulsions tested had a 10% fat-phase, including oil and phytosterols. Emulsion droplet sizes during emulsification were measured with a Mastersizer 2000 with Hydro S accessory (Malvern Instruments, UK). Final droplet sizes were derived from microscopy.

Conventional Light Microscope (LM)

A drop of the emulsion was placed on a microscope slide with a cover glass to create a thin layer of emulsion. Samples were observed at magnifications 10, 20 or 40, using a Zeiss Axioplan microscope in transmitted light mode (Zeiss, Oberkochen, Germany). Three observation modes were used: bright field, polarized light and phase contrast showing, respectively, absorption contrast, identification of molecular organized structures like for instance crystals, and contrast based on differences in refractive indices.

Confocal Scanning Laser Microscopy

Samples were stained with Nile Blue (NB, Janssen Chimica, Beerse, Belgium) as a fluorescent dye, which interacts both with lipids (based on solubility) and with proteins (based on affinity). The fluorescent properties of NB

change in relation to the environment of the dissolved dye: in an apolar, hydrophobic environment the dye fluoresces in green/yellow, in more hydrophilic regions, like hydrated proteins and carbohydrates the fluorescence is red. NB also gives rise to strong red fluorescence in the presence of non-protein emulsifiers at the interface of lipid and water. In water the fluorescence is quenched completely. Microstructure observations were made with the BioRad MRC1024 confocal microscope (BioRad Cell Division, Hemel Hempstead, UK) fitted to a Zeiss Axiovert 100 inverted microscope (Zeiss, Oberkochen, Germany) and equipped with an Ar/Kr mixed gas laser (Innova Enterprise Ion Laser, Santa Clara, CA, USA). The excitation wavelengths used were 488 and 647 nm. Fluorescence signals were collected over 605 ± 16 and 680 ± 6 nm. Lasersharp 2000 software (BioRad) was used for acquiring the images and Confocal Assistant (CAS ver 4.02, public domain software) to process the images.

Cryo-Scanning Electron Microscopy

The sample was frozen in melting ethane and the small frozen droplet was cryo-planed with a diamond knife in an ultra-microtome (Leica Ultracut UCT, Heidelberg, Germany). The microtomed sample was transferred to the Oxford CT1500 (Oxford Instruments, Oxon, UK) cryo-system, which is attached to the SEM. Ice is sublimated at −90 °C to expose the structures of the non-aqueous components in the sample. After the sublimation process the surface of the sample was covered by a thin layer of a mixture of gold and palladium to prevent charging in the SEM. Cryo-SEM-analysis was performed in a Jeol JSM6340F semi-in lens scanning electron microscope (Jeol, Tokyo, Japan) operating at 3.0 kV and a working distance of about 6 mm. The sample was kept at −180 °C during the observations.

Cryo-Transmission Electron Microscopy

Thin films of the suspensions were prepared on 200-mesh "lacey" carbon support grids (Ted Pella Inc. Redding, CA, USA) and vitrified by plunging into liquid ethane using the controlled environment vitrification system (CEVS) operated at saturated water vapor pressure to minimize dehydration and pre-cooling artifacts [15]. The vitrified samples were observed in a Tecnai Sphera TEM (FEI Company, Hilsborro, OR, USA) using 200 kV accelerating voltage and under low dose electron beam settings.

TAG Lipolysis Experiments

TAG enzymatic hydrolysis (lipolysis) experiments were performed in a DL53-Mettler-Toledo Titrator (Mettler-Toledo, UK) combined to a DG 111-SC combination pH electrode (VWR International, Poole, UK) and to a water bath (Lauda, Germany) to maintain the apparatus at 37 °C. Equipment control and data acquisition were performed with Mettler-Toledo LabX professional software (Mettler-Toledo, UK).

The conditions for the lipolysis experiments were selected so as to simulate the duodenal digestion step with respect to enzymes (porcine pancreatin) and bile salt concentrations. The amount of emulsion used at start of the lipolysis experiment was adjusted to introduce 150 mg of TAG into the systems. The rate of FFA release was expressed as a percentage relative to the maximal amount (100%) of FAs which can be generated from the *sn*-1 and *sn*-3 positions of the TAG participating in the reaction, thus correcting for the *sn*-1,3 specificity of the pancreatic lipase (EC 3.1.1.3).

Results and Discussion

The preparation of an o/w emulsion from phytosterol based organogel, in an aqueous solution of Xanthan in the presence of Triton X-100 as the emulsifier, results in the formation of small emulsion droplets (internal oil droplet structure not studied) and a structured water phase. The o/w emulsion system, contains 10% lipids, being 16% organogel (β-sitosterol:γ-oryzanol, 4:6 weight ratios in olive oil). The microstructure as observed in the light microscope under phase contrast conditions (Fig. 1) shows the presence of aggregates of small oil droplets (~2 μm) in the continuous aqueous Xanthan solution. Xanthan is not

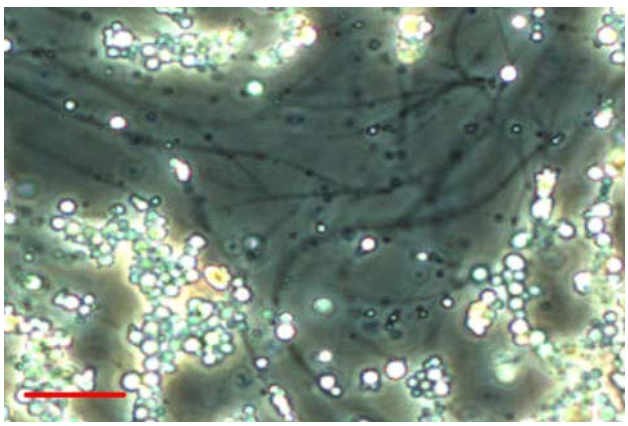

Fig. 1 Conventional light microscope pictures in phase contrast mode of an o/w emulsion system, 4 weeks after preparation, containing 10% lipids, being 16% organogel (β-sitosterol:γ-oryzanol (4:6 weight ratios) in olive oil. Small oil droplets cluster in the aqueous phase. Thread-shaped structures are present in the water phase. The view shows the typical nature of the fibers, having various thicknesses (marker equals 10 μm)

visible under light microscopy. In the aqueous phase, long strands are visible having a thickness distribution which ranges from near the resolution of the light microscope (~0.3 μm) up to approximately 2 μm. These strands are absent in emulsions made from olive oil without organogel. Therefore, it seems appropriate to identify these strands as β-sitosterol and γ-oryzanol containing structure elements.

Using the confocal microscope a clear distinction can be made between the oil (green) and emulsifiers and the strands (red) (Fig. 2). A brighter view of the red fluorescent structure elements is shown in the right image of Fig. 2 (same view represented in only gray scale). A broad distribution for the thickness of the strands is shown, ranging from the limit of resolution till ~2 μm. These strands are similar to that observed in phase contrast LM. The oil droplets are surrounded by a thin red fluorescent layer (only visible in the gray scale image). The components at the interface could represent the emulsifier system (Triton X-100) as well as β-sitosterol/γ-oryzanol. The red strands are absent in emulsion systems prepared without organogel.

Since CSLM is limited in spatial resolution, TEM of a thin frozen film of the emulsion was used to identify the nature of the thread-like structures. In Fig. 3 an area in the thin film is presented, showing the typical strands. In Fig. 4 another area is shown in which, next to the strands, a number of other features are present. For the interpretation of the images it has to be taken into account that the film is obtained by pressing a drop of emulsion liquid in the shape of a thin film. This results in some alignment of structures in the plane of the film and some induced alignment of features next to each other. Larger features are displaced to the thicker regions of the film and will therefore not be observed in the thin parts of the film. The following features can be clearly identified:

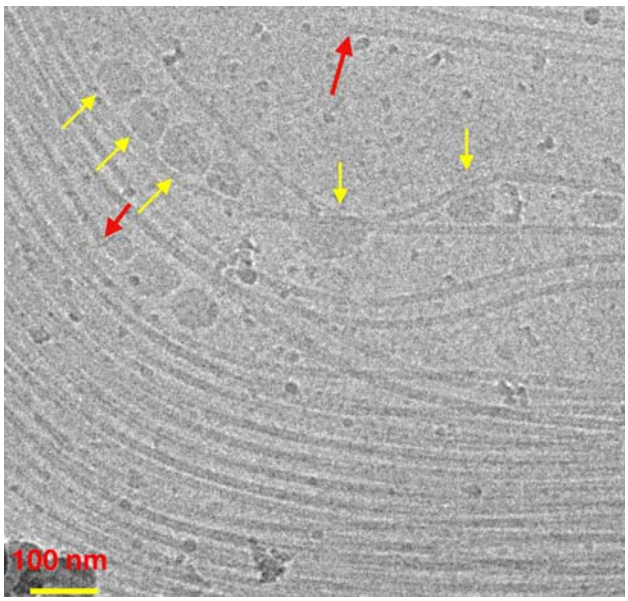

Fig. 3 Cryo-TEM observation of the 16% sterol organogel in corn oil emulsion. The image show the structure of a thin frozen film prepared from the emulsion. Thin strands are visible which are aligned as result of the preparation procedure

- Long thin strands having a thickness of approximately 10 nm (Figs. 3, 4). Close inspection of the thread-like structures shows that these structures are represented as a set of alternating dark, light, dark parallel lines, which is the representation in TEM of a hollow tube-like structure. These structures will further be referred to as tubes.
- Small, partly spherical structures having the same electron density as the tubes. These spheres are aligned with the tubes. The alignment may be artificial according to the discussion on the thin film formation given above (yellow arrows) (Fig. 3).

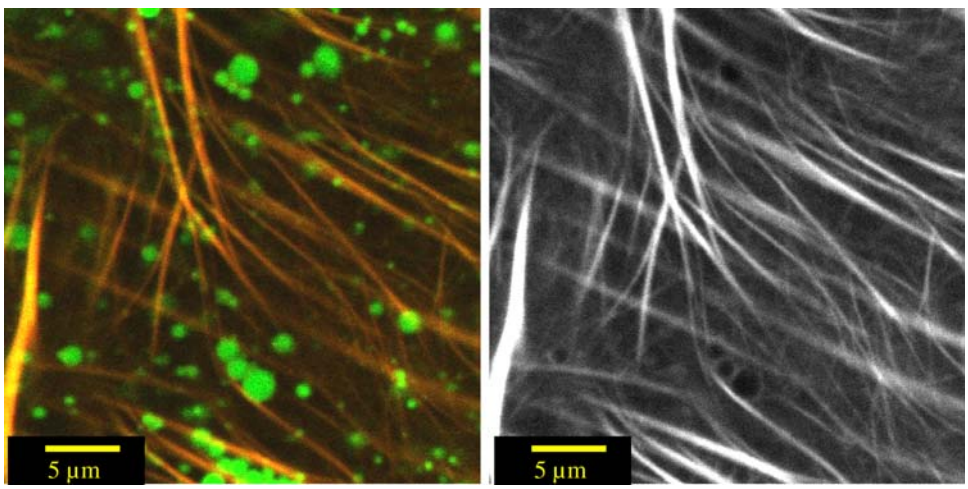

Fig. 2 Confocal images of an o/w emulsion system, containing 10% lipids, being 16% organogel (β-sitosterol: γ-oryzanol (4:6 weight ratios) in olive oil a 10% corn oil emulsion. The *left* image shows the combined *red*/*green* image in which *green* represents the corn oil and *red* the components at the oil/water interface and the thread-shaped structures in the continuous phase. In the *right* image the '*red* information' is shown separately

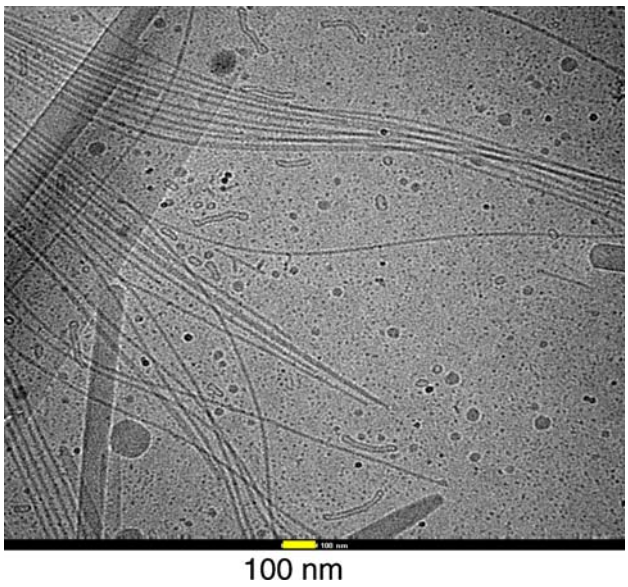

Fig. 4 TEM observations of 10% (16% sterol organogel in corn oil) emulsion showing the strands, long thin crystals (sterol crystals), spherical and worm-like vesicles and micelles (smallest features)

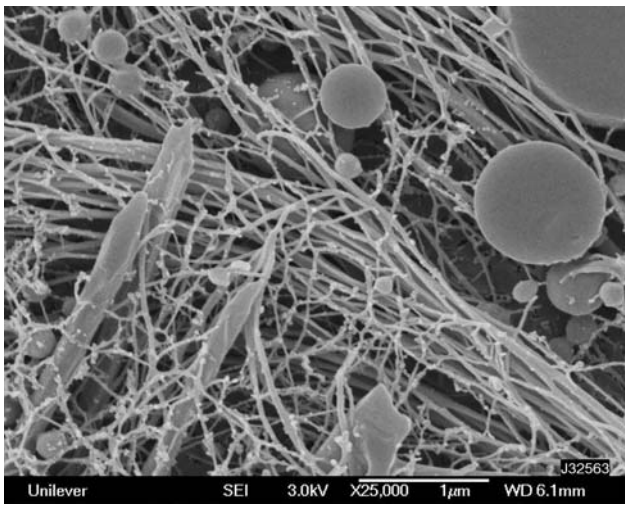

Fig. 5 Cryo-SEM observation of an olive oil based organogel emulsion (16% sterols). A bundle of fibers (*bottom*, *right*) which diverge into single fibers (*top*, *left*); some sterol crystals (sharp edged structures) and a few oil droplets (spherical shapes) are visible

- End points of the tubes (red arrows) (Figs. 3, 4).
- Small spheres having a diameter comparable to the size of the diameter of the tubes: these features are either tubes orientated perpendicular to the film or small vesicles/large micelles (Figs. 3, 4).
- Dark spherical features that are ice balls lying on the frozen film (Figs. 3, 4).
- Dark dots that are micelles (Fig. 4).
- Worm-like, short, thin structures that are worm-like micelles (Fig. 4).
- Very large, sharp edged features that are sterol crystals (Fig. 4).

In the thin film, as observed in cryo-TEM, the 3-D ordering of the structure elements has been disturbed as result of the thin film approach. Using cryo-SEM observation, after fast freezing of the specimen, followed by flat sectioning and sublimation of ice, allows observation of the 3-D architecture. The result of this approach is shown in Fig. 5. The following structure elements can be observed: (sectioned) oil droplets, sharp needle shaped sterol crystals, long smooth strands, being the tubes and a spider-web-like network of Xanthan gum. This network is artificial and is the result of displacement of Xanthan macromolecules during freezing of the sample, by which small ice crystals are formed. The tubes are present in this image as a bundle of tubes that fan out from bottom right to top left. The typical thickness of the single strands has been estimated to be around 10 nm. It is very likely that the tubes in the TEM images are the same features as the single tubes in the cryo-SEM images. The molecular composition of the strands has not been determined. Products were made in which various combinations of the components oil (TAG), Triton X-100, β-sitosterol and γ-oryzanol were used. Tubes can be formed even in the absence of oil.

The combination of Triton X-100 with β-sitosterol also results in the formation of tubes while this is not the case for the combination Triton X-100 with γ-oryzanol. It is hypothesized that the non-ionic surfactant Triton X-100 acts as a crystal habit modifier forcing the tubes to grow in one direction. The surfactant might also facilitate the growth of the tubes outside the lipid phase into the aqueous phase. Based on the TEM and SEM observations in combination with a model derived from the well-accepted theory on tubular micelle formation, a schematic representation of the tubes is shown in Fig. 6. In this model both sterols are present. Specific high-resolution studies of systems in absence of γ-oryzanol were not performed. The model shows that TAG molecules can be incorporated in the tubes, while not being a building block inside the center of the tube themselves.

This phenomenon of structural elements being formed in a one-dimensional growth, or even crystal-like thin strands has been described for other, including non-edible oil, organogel systems as well. These systems with spontaneously formed fibers, capable of structuring the oil phase, are termed "self-assembled fiber networks" (SAFiN) [16]. However, in this study we were able to demonstrate the formation of structural elements also in the aqueous phase and even in systems free of edible oils. Although we did not test in full detail the structuring aspects in oil-free systems, we believe the β-sitosterol–γ-oryzanol fibril systems are quite unique as they are one of the few

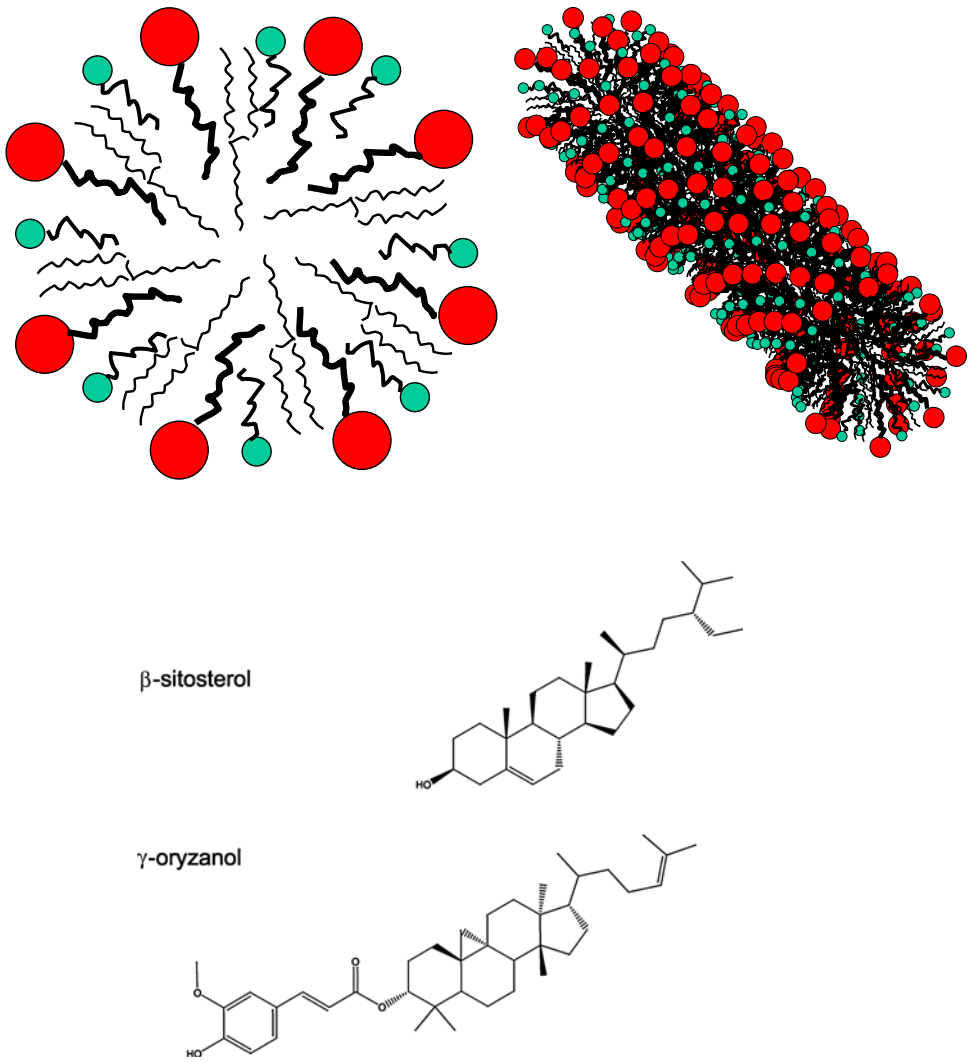

Fig. 6 Schematic diagram (hypothetic model) of the ordering of the two sterol species with triacylglycerides in a 10 nm tube structure. The molecules are drawn on scale approximately. *Red* γ-oryzanol, *Green* β-sitosterol; 'chair-model': triacylglycerides. The *right* image shows a schematic diagram of the tube structure; organization of the sterols with respect to each other may be more randomly

combinations capable of forming fibril structures in both aqueous and oil systems.

The formation of the tubes in the aqueous phase was found to be time and temperature dependent (Fig. 7). Storage of the emulsions at room temperature resulted in faster formation of the tubes compared to storage at 4 °C, based on observations of an increased presence of strand-like features in phase contrast light microscopy (Fig. 8). Storage at 50 °C resulted in an even higher abundance of the strand-like structures. It is concluded that the formation of the strands, and therefore also the tubes, is a diffusion-based mechanism in which the sterols or sterol-containing features move from the lipid phase into the aqueous phase. The driving mechanism is not clear but it can be hypothesized that sterol solubility in edible oils is further reduced with the transition from a pure organogel system to an organogel-based emulsion system. In hot dry vegetable oils, sterols are reasonably soluble but this drops to below 1% in oil, saturated with water (unpublished data). Tube structures have been reported to be present in neat organogel systems. Cryo-SEM has been used to visualize these tubes [17]. Thickness of the strands as observed in cryo-SEM are reported to be between 10 and 100 nm, suggesting that both single tubes as well as assembles of tubes were observed. X-ray diffraction [6, 18] has given evidence for the presence of 7.2 ± 0.1 nm tubes in organogel systems. This precise estimate of the thickness of the hollow tubes is in close correlation with the rough estimate of the thickness of the tubes in TEM. It has to be taken into account that both the cryo-SEM observations and the X-ray diffraction measurements were made of tubes in lipid environment while the TEM and SEM observations described in this paper are done in the aqueous phase.

More detailed X-ray diffraction studies established a tube wall thickness of 0.8 ± 0.2 nm. It is interesting to note that this dimension corresponds to the length of the long axis of the characteristic four-ring system in sterols.

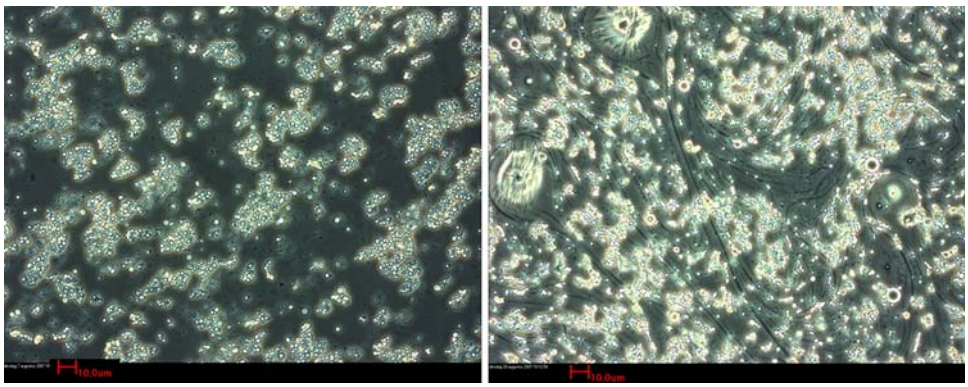

Fig. 7 Conventional light microscope pictures in phase contrast mode of an olive oil emulsion with addition of 16% sterol organogel (40× objective). The picture on the *left* is of a fresh emulsion and on the *right* the emulsion was 4-weeks old, stored at room temperature

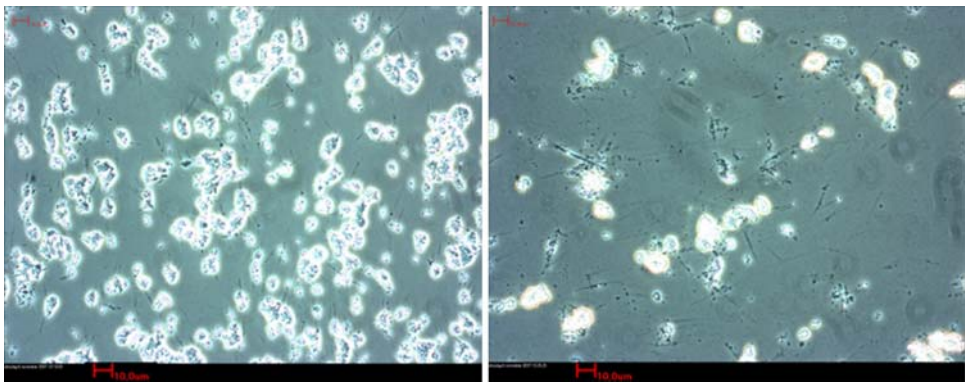

Fig. 8 Conventional light microscope pictures in phase contrast mode (40× objective) of an olive oil emulsion containing 16% sterol organogel, after 7 weeks. On the *left*, the emulsion was stored at 4 °C. On the *right* it was stored at room temperature

This observation provides circumstantial evidence that the tube wall is composed of the rigid elements of the sterol (ester) molecules [19].

One of the benefits that was envisaged in the use of organogels in an emulsion was controlled hydrolysis of TAG in mono- and di-acyl-glycerides and free fatty acids. In Fig. 9 results are shown of the lipolysis profile of a 10% lipid emulsion prepared with 16% organogel (on lipid basis). The graphs show the influence of temperature and age of the emulsion on the percentage release of free fatty acids. Emulsions that were stored for a longer period at high temperature shows a slower release of free fatty acids compared to emulsions that were stored shorter and at low temperature. Slower release correlates with more abundant presence of the strands as observed in phase contrast LM, as mentioned above.

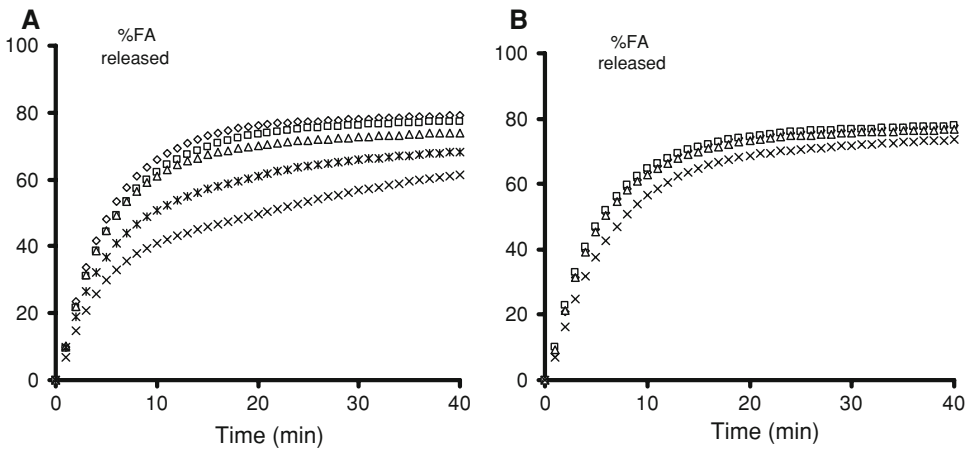

Fig. 9 Development of the lipolysis with age of a corn oil emulsion with a 10% fat-phase of which again 16% as sterol organogel. On the *left* (**a**), the emulsion was stored at room temperature. On the *right* (**b**), it was stored at +4 °C. Storage time series: fresh (*diamond*), 2 (*square*), 4 (*triangle*), 6 (*asterisk*, only for RT) and 10 (*cross*) weeks. The maximum lipolysis would be 84%. The 100% value is calculated from the total amount of fatty acids released from the *sn*-1 and *sn*-3 positions of the triacylglycerides, and also corrected for the amount of sterols replacing part of the TAGs in the emulsion

Based on the microscopy observations, reported here, the results of X-ray diffraction (XRD) measurements [6, 18] and the results of the lipolysis tests the following hypotheses have been formulated. These hypotheses take into account either the effect of the structuring of oil as an organogel or the incorporation of TAG in the tubes in the aqueous phase or both effects.

The first hypothesis is based on the assumption that the diffusion of the TAG molecules to the droplet surface would be restricted by the structure of the gel, thus slowing down the lipolysis rate by substrate limitation.

The second hypothesis is based on the idea that TAG is incorporated in the tubes which contain at least β-sitosterol. In that case TAGs would be protected inside these tubes from the action of lipase and thus would not undergo hydrolysis easily. This hypothesis needs direct measurement of the TAG content in the tubes, which was not performed within the scope of this work.

The third hypothesis is based on the idea that lipase may be inhibited in its action as result of adsorption of the lipase onto the tubes. It is known that luminal lipases are attracted to hydrophobic surfaces [20]. This may result in preventing or delaying the hydrolysis based on enzyme amount restriction.

Conclusion

O/w emulsions can be prepared from edible oils, which has previously been structured by addition of γ-oryzanol and β-sitosterol, forming an organogel structure. These organogel-based emulsion systems show retarded TAG hydrolysis by pancreatic lipase compared to emulsions prepared from edible oils not containing the sterols.

Nano-sized tubular structures are present in the aqueous phase of such organogel-based emulsion systems. The tubes are formed over time in the aqueous phase of the o/w emulsions, where the oil phase contains organogel. A co-surfactant, like Triton X-100 appeared to be indispensable for the formation of the tubes in the emulsions. The diameter of these tubular structures is about 10 nm, being approximately twice the length of TAG molecules, containing C16 and C18 fatty acid chains and the sterol molecules. The formation process is extended over several weeks when stored at 20 °C. At lower temperature the formation is slower.

Next to the tubes, dispersed crystals of the sterols are present. Crystals are observed more frequently in emulsion systems that are stored well below ambient temperatures (refrigerator).

It is suggested that sterols have to diffuse from the oil phase into the water phase, during and after the emulsification process, to form the tubular structures or crystals. Based on the differences of formation rate of the tubes the solubility and the diffusion rate of the sterols in both the lipid, especially in the presence of water, and aqueous phases is suggested to be a critical step.

No evidence has been built to support further or prefer one of the three hypotheses (i.e., oil structuring by organogel; incorporation of TAG in the tubes (being micelles formed by the sterols); or sterol induced lipase inhibition) are (the most) active in retardation of lipolysis by pancreatic lipase.

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