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Effect of temperature, water content and free fatty acid on reverse micelle formation of phospholipids in vegetable oil

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Abstract

The self-assembly of phospholipids in oil, specifically lecithin in rapeseed oil, was investigated by combining experimental and computational methods. The influence of temperature, water, and free fatty acids on the onset of lecithin aggregation in the rapeseed oil was determined using the 7,7,8,8-tetracyanoquinodimethane dye (TCNQ) solubilization method and the size and shape of the self-assembled lecithin structures were investigated by small-angle X-ray scattering and cryogenic transmission electron microscopy. In the absence of excess water in the system (0.03 wt-% water in oil), stable cylindrical lecithin reverse micelles were observed above the critical micelle concentration (CMC). Comparing the aggregation response in room temperature and at 70°C revealed that CMC decreased with increasing temperature. Furthermore, already a modest amount of added water (0.3 wt-% water in oil) was sufficient to induce the formation of lamellar lecithin structures, that phase separated from the oil. In low water content, oleic acid suppressed the formation of lecithin reverse micelles whereas in the presence of more water, the oleic acid stabilized the reverse micelles. Consequently, more water was needed to induce phase separation in the presence of oleic acid. Molecular dynamics simulations indicated that the stabilizing effect of oleic acid resulted from oleic acid enhancing phospholipid solubilization in the oil by forming a solvating shell around the phosphate head group. The findings showed that the response of the mixed surfactant system is a delicate interplay of the different components and variables. The significance of the observations is that multiple parameters need to be controlled for desired system response, for example towards vegetable oil purification or phospholipid based microemulsions.

Keywords: Reverse micelles, phospholipids, free fatty acid, vegetable oil, molecular simulations

1. Introduction

Reverse micelles (RM) have attracted much attention in technology and sciences since they have a broad range of applications ranging from nanoparticle synthesis templates to drug delivery carriers and oleogels (1-4). Reverse micellar structures form via self-assembly of amphiphilic molecules in nonpolar medium and the main feature of this supramolecular system is that RMs tend to solubilize small molecules and other hydrophilic material. Specifically reverse micellar assemblies of phospholipids have raised interest as model environments for biological membrane-bound proteins (5), as drug transport systems (6, 7), and as confined chemical reaction platforms (8) but also because of their effect on preservation and usability of vegetable oils both as biofuel ingredients and in alimentation (9, 10).

In vegetable oils, phospholipids influence oxidation and contribute toward the formation of colloidal aggregates and precipitates (10, 11). The diversity in surface-active components and oil compositions leads to a diverse response to water content and formation of a variety of physical surfactant structures (12-14). In general, first various reverse micellar aggregates form and at a certain water-to-lipid ratio, the reverse micelles are not stable anymore but instead large, lamellar structures that precipitate, are formed. Furthermore, phospholipid based assemblies influence significantly the quality of edible oils (11, 14, 15). The effect can be positive but most often, the presence of phospholipids in vegetable oils leads to undesired oil properties, such as reduced preservation due to oxidation and foul taste, smell, or appearance (10, 11, 16).

Phospholipids and other surface-active components, such as, fatty acids, monoglycerides and diglycerides are removed in vegetable oil purification through a process called degumming (17, 18). In this process, the addition of water to the oil promotes the aggregation of the amphiphilic species and induces phase separation, enabling easy removal of the surfactants (mainly phospholipids), carbohydrates, and water out of the bulk triglyceride phase, *i.e.* the oil (19). However, a water content that exceeds the point of precipitation can result in the formation of water droplets in the oil phase, which disturbs the degumming process. Thus, understanding the phospholipid aggregation response in terms of sensitivity to water addition is highly relevant for plant oil purification in general but also for engineering vegetable oil characteristics for alimentary products. Both the aggregation response and the macroscopic phase behavior of lecithin–nonpolar solvent systems have been shown to be

dependent on, and controllable by, *e.g.* oil and lecithin composition, temperature, water content, and various solution additives (12, 20-26). Specifically, the effect of free fatty acids on the RM formation and aggregate structure is of high practical interest because free fatty acids are naturally occurring minority components in plant oils and influence lipid decomposition. Kittipongpittaya *et al.* (26) examined 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) in stripped corn oil and found that oleic acid can act as a cosurfactant and increase the CMC of DOPC reverse micelle formation in the oil. They also associated the presence of free fatty acids with a decrease of pH in the RMs. Furthermore, free fatty acids can enhance lipid oxidation (23, 26, 27). Additionally, the effects of several free fatty acid species with soybean lecithin assembly phases have been explored, yet not in oil, and not considering the effect of water content (28). Studies considering both the effect of water and free fatty acids on lecithin aggregation are scarce: A very recent investigation examines the effect of water on the association of minority components in bulk corn oil, but the focus was on lipid oxidation and not on the synergistic effect of water and the minority components, including fatty acids, on the association of phospholipids (26). Furthermore, free fatty acids have been found to affect the emulsification characteristics of lecithin, indirectly suggesting an interplay of water and free fatty acids on phospholipid association (29, 30). Considering the strong impact of these two major control factors calls for more systematic mapping of their effect on the phospholipid assembly.

While reverse micelles of phospholipids have been rather extensively studied in various organic solvents, like cyclohexane, benzene or toluene (31-33), very few investigations have used oil as the nonpolar media (13, 14, 26, 27). Nevertheless, both experimental results and simulations have shown that the nature of the media has a strong influence on the aggregation behavior of phospholipids (34-37). Notably, vegetable oils can solubilize considerably more water than cyclohexane or the other solvents that have been more frequently studied in the literature (13, 38, 39). In light of the current literature, the response of phospholipid aggregates in vegetable oils is quite likely to differ significantly from the response in other apolar solvents.

The ternary mixtures of phospholipids, free fatty acid, and water in oil are very complex, and thus unravelling the interplay of the surface-active components is challenging based purely on experimental methods. However, recently computational modelling efforts in this area have emerged as simulational characterization provides a tool to acquire additional data on the micellar local structures, which can assist the interpretation of experimental data. Recently, Vierros *et al.* examined a phosphatidylcholine lipid based lecithin model in cyclohexane in the presence of water via all-atom molecular dynamics simulations and identified potential pitfalls in the modelling of phospholipid

assemblies in apolar media (40). They also studied the interactions governing lecithin aggregate morphology in cyclohexane in comparison to those in, *e.g.* heptane (37). Abel *et al.* have examined phospholipid reverse micelle structure and dynamics in benzene via similar methodology (41). Additionally, a basic molecular dynamics characterization of triglycerides-phospholipid-water emulsion system and the surfactant interface exists (42, 43).

Here, we have combined detailed molecular dynamics simulations with systematic experimental investigations to gain a deeper understanding of the influence of temperature, water, and free fatty acid content on the self-assembly of phospholipids in a real plant oil, namely rapeseed oil. More specifically, we used the (7,7,8,8-tetracyanoquinodimethane dye) TCNQ solubilization method to determine the effect of temperature, added fatty acids and water on the RM formation, while small-angle X-ray scattering (SAXS) and cryogenic transmission electron microscopy (cryo-TEM) were used to determine size and shape of the micelles and lamellar structures. All examined parameters affected the self-assembly of the phospholipids in oil, but the most remarkable effect was the combined effect of free fatty acids and water. To the best of our knowledge, this is the first time the all these parameters are systematically investigated in a vegetable oil and in combination with computational methods.

2. Materials and methods

2.1 Materials

Food grade rapeseed oil (Euro Shopper, Bordeaux, France) was purchased from a local super market. The rapeseed oil was composed of 97.1 wt.-% triglycerides, 0.6 wt.-% of free fatty acids and contained less than 0.6 ppm of phosphorous. The triglyceride composition of the rapeseed oil was 63.20% C18:1 (triolein), 19.80% C18:2, 9.90% C18:3, 4.40% C16:0, 1.50% C18:0, and 1.00% C20:1. Oleic acid was purchased from VWR Chemicals (Radnor, Pennsylvania, USA) and it consisted of 84.5 wt.-% oleic acid, 12.5 wt.-% linoleic acid and 3 wt.-% of other fatty acids, mainly palmitic acid and stearic acid. Granular L-Alpha-Lecithin was purchased from Acros Organics (New Jersey, USA). The composition of lecithin was approximately 23 wt.% phosphatidylcholine, 20 wt.% phosphatidylethanolamine, 14 wt.-% phosphatidylinositol, 8 wt.-% phosphatidic acid, 8 wt.-% minor phospholipids, 8 wt.% sugars, 15 wt.% glycolipids, 3 wt.% triglycerides and 1 wt.% moisture. 7,7,8,8-tetracyanoquinodimethane dye (TCNQ) was purchased from Sigma-Aldrich Co. (Missouri, USA). The Millipore water used was purified using a Synergy UV water purification system (Millipore SAS,

Molsheim, France). All lipid materials were stored in a freezer to avoid oxidation and changes in moisture and only the required amount was defrosted prior to the experiments.

2.2 Material drying

All lipid materials including rapeseed oil, oleic acid and lecithin were dried before use and the final moisture content was determined using the Karl-Fischer titration DL38 (Mettler-Toledo, LLC, Ohio, USA. Rapeseed oil and oleic acid were dried using molecular sieves and lecithin was dried using silica granules.) The measured moisture content of these components were 0.03 %, 0.04 % and 0.63 %, for rapeseed oil, oleic acid and lecithin respectively. These values were further used to calculate the final moisture contents in samples.

2.4 Determination of critical micelle concentration (CMC) using TCNQ technique

The CMC of lecithin in absence or presence of oleic acid and water was determined using the 7, 7, 8, 8-tetracyanoquinodimethane (TCNQ) solubilization method (44). The principle of the TCNQ method is that the reversed micelles solubilize the TCNQ dye leading to a rapid change in color around the CMC. A stock solution of lecithin in oil was prepared by solubilizing lecithin in rapeseed oil for 3 hours at 70 °C using magnetic stirring and cooled to room temperature. The samples were prepared by diluting desired amounts of the lecithin-rapeseed oil stock solution into the pure rapeseed oil, whereupon oleic acid, water and TCNQ-dye (12 mg/12 g of sample) was added. Samples were then agitated for 5 hours. For samples at 70 °C, 3 hours agitation time was sufficient. After agitation, the excess of TCNQ was removed using centrifugation at 800 g for 20 min (Megafuge 1.0, Heraeus Sepatech GmbH, Germany). The absorbance spectra of the samples were measured from the upper phase using a UV-spectrophotometer at the wavelength 480 nm (Model UV-1800, Shimadzu Corporation, Kyoto, Japan). All experiments were repeated at least twice, some three times, and the reproducibility was good.

2.5 Determination of aggregate properties using small angle X-ray scattering (SAXS)

The structural properties of reversed micelles and possible presence of liquid crystals was determined using small angle X-ray scattering (SAXS). Samples for SAXS analysis were prepared following exactly the same procedure of lecithin solubilization and mixing as the samples for TCNQ experiments, except that the TCNQ dye wasn't added to the mixture. More specifically, two samples were prepared, one sample consisted of 1 wt.-% lecithin solubilized in a rapeseed oil containing 0.03 wt.-% water and another one with 1 wt.-% lecithin and H₂O-to-Oil weight ratio of 0.21 wt.-%. A

precipitate formed in the second sample and that sample was centrifugated and both the precipitate and the supernatant was analysed. After the sample preparation, 0.9 mm thick samples were placed between two 7 μm kapton foils. The kapton foil was attached to the washer which was then placed in the steel sample holder. The liquid samples were placed into the washer using a pipette. The washer was then sealed with a flat metal piece with another kapton foil attached to it and a rubbery ring between the metal washer and the flat metal piece. These were then screwed tightly together. The samples were measured for 30 minutes using customized SAXS system operating in vacuum at room temperature or in helium atmosphere at 70 °C. The system had a Bruker MICROSTAR rotating anode X-ray source (Bruker, Billerica, Massachusetts, USA) with Montel collimating optics (CuK α radiation $\lambda = 1.54 \text{ \AA}$ (Incoatec GmbH, Geesthacht, Germany)). The X-ray beam was further collimated using four sets of 4-blade slits (JJ X-Ray, Lyngby, Denmark). The diffraction pattern of the sample was collected on a HiStar 2-D area detector (Bruker, Billerica, Massachusetts, USA) and the sample to detector distance was 0.59 m. One-dimensional SAXS data were obtained by azimuthally averaging the 2D scattering data. The magnitude of the scattering vector is given by $q = 4\pi/\lambda \sin\theta$, where 2θ is the scattering angle and to calibrate the q -range, silver behenate (AgBeh) was used as a standard.

2.6 Cryogenic transmission electron microscopy (cryo-TEM)

Cryo-TEM imaging was used to further characterize the aggregate conformation in the same samples that were prepared for SAXS characterization. Samples were vitrified onto Quantifoil 3.5/1 holey carbon copper grids (Quantifoil Micro Tools GmbH, Großlobichau, Germany). The vitrification was done using an automated FEI Vitrobot (FEI, Hillsboro, Oregon, USA) in which they were blotted three times for four seconds to remove excess sample from the sample grid. After this, the vitrification of the sample was done by immersing it into liquid ethane/propane mixture at -185 °C. After plunging into liquid ethane/propane, the solid thin film formed in the carbon copper grids was immersed in liquid nitrogen to avoid sample contamination. The samples were then transferred into JEOL JEM-3200FSC cryo-transmission electron microscope (JEOL Ltd., Tokyo, Japan). The microscope operated at 300 kV voltages. Cryo-TEM-images were taken in bright field mode using zero loss energy filtering (omega type) with the slit width of 20 eV. Micrographs were recorded using a Gatan Ultrascan 4000 CCD camera and analyzed using Gatan Digital Micrograph software (Gatan, Inc., Pleasanton, California, USA).

2.7 Computational methods

The GROMACS 4.6 simulation package (45, 46) with the empirical CHARMM force field (47, 49) and the CHARMM modified TIP3P water model (47, 48) were used for the simulations. The simulations examine a model phospholipid-triglyceride-oleic acid system consisting of 1,2-dioleylphosphatidylcholine (DOPC) in triolein with varying mass percentage of oleic acid and a small amount of water (to match experimental hydration). DOPC and triolein are chosen as the phospholipid and the triglyceride species to model lecithin and the rape seed oil because oleic acid is the dominant triglyceride component of the oil, because it provides a the chemistry match with the oleic acid (model consistency) and because PC lipids are the dominant component of the lecithin in the experiments.

The simulations were performed in the NPT ensemble using the stochastic velocity rescaling thermostat (50) with reference temperature of $T=70\text{ }^{\circ}\text{C}$ and isotropic Parrinello-Rahman barostat (51) with reference pressure $p=1\text{ bar}$. Depending on the examined free fatty acid concentration, the simulated systems contain 53-106 triolein and 0-165 oleic acid molecules, 1 DOPC and 4 or 0 water molecules (to see the effect of a small amount of water in the simulations results). The simulations were run for 100 ns and a minimum of 50 ns was discarded as initial relaxation period in the analysis. VMD was used in molecular visualizations (52). Additional simulations protocol specifics and initial configurations preparation details are provided in the Supplementary Information (SI).

3. Results and Discussion

3.1. Effect of temperature on lecithin reverse micelles

The temperature is one of the crucial parameters affecting surfactant interactions and subsequently CMC. In this work, the effect of temperature on the self-assembly of the lecithin RMs was investigated via performing the measurements at two different temperatures, room temperature (RT) and $70\text{ }^{\circ}\text{C}$. The stability of lecithin at these working temperatures was evaluated using Differential Scanning Calorimetry (DSC) confirming that no conformational changes of lecithin in rapeseed oil at temperatures ranging between $10\text{ }^{\circ}\text{C}$ and $90\text{ }^{\circ}\text{C}$ occurred, see the thermogram in SI (**Fig. S1**). At RT and $70\text{ }^{\circ}\text{C}$, the CMC of lecithin in rapeseed oil having moisture content of 0.03 wt.-% was subsequently determined using the TCNQ solubilization technique. Lecithin demonstrated a higher CMC at RT than at $70\text{ }^{\circ}\text{C}$, 0.082 wt.-%, corresponding to 0.092 mol.-% and 0.059 wt.-% (0.066 mol.-%), respectively (**Fig. 1**). In agreement with our findings, Cui *et al.* found that the CMC of the phospholipid components, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in soybean oil decreased with increasing temperature (53).

Fig. 1. TCNQ absorbance as a function of lecithin concentration in rapeseed oil at room temperature (RT) and 70 °C. Solid lines represent the best fit to the data, while dotted lines indicate the CMC of lecithin at respective temperature.

3.2 Effect of oleic acid on lecithin self-assembly

If more than one surface active agent is present in the system, the assembly phase behavior of phospholipids in the oil may drastically change. Free fatty acids are naturally occurring minority components in many vegetable oils and their presence affects both phospholipid assembly characteristics (11, 26, 54) and the degumming process during oil purification (55). Oleic acid was selected as a model of free fatty acid to investigate its impact on lecithin self-assembly in oil because it is liquid at room temperature. Pure oleic acid did not form reverse micelles in the oil at any of the studied concentrations (0.03–80 wt.-%) at room temperature (SI **Fig. S2**). However, presence of oleic acid had a profound effect on the association of lecithin reverse in oil both at RT and at 70 °C at constant water content of 0.03 wt.-%. More specifically, an increase of oleic acid concentration from 0 wt.-% to 20 wt.-%, increased the CMC of lecithin from 0.055 wt.-% (corresponding to 0.062 mol.-%) to 0.20 wt.-% (0.22 mol.-%) at 70 °C (**Fig. 2**). At low concentration of oleic acid (< 5 wt.-%), the CMC of lecithin was not significantly affected by the presence of oleic acid. At a concentration of oleic acid exceeding 20 wt.-%, no CMC could be detected below 3 wt.-% lecithin and a higher concentration of lecithin could not be dissolved in the oil. Thus, the effect of varying concentration of oleic acid up to 20 wt.-% is shown in the figure. The trends in RT were similar and the CMC increased from 0.092 mol.-% without oleic acid to 0.36 mol.-% at 10 % oleic acid, while at higher oleic acid concentrations micelles did not form within the concentration range of lecithin that could be dissolved in oil. In line with our findings, Kittipongpittaya *et al.* (26) also found an increase in

CMC of DOPC in stripped corn oil at RT, but they did not study oleic acid concentrations above 5% or at elevated temperatures.

Fig. 2. Critical micelle concentration (CMC) of lecithin as a function of oleic acid concentration in rapeseed oil at 70 °C as determined using TCNQ technique.

To further investigate the reason for the influence of oleic acid on the reverse micelle formation of lecithin in rapeseed oil, molecular simulations on a model system consisting of DOPC, triolein, and varying amount of oleic acid in the presence of no water or 4 water molecules (0.08 wt.-% concentration of water) were performed. The molecular simulations demonstrated the interactions between oleic acid and the other components including triglycerides, other oleic acid molecules, and DOPC when adjusting the water-to-lipid molar ratio (W_o) to 4 (**Fig. 3A**). Notably, no significant difference in the interactions was seen in comparison to the unhydrated system where W_o was set to 0. This is because $W_o = 4$ hydration conditions is still a very low amount of water and the hydrogen bonding capability of the lipid head group significantly exceeds the amount of oleic acid that binds with it. The intermolecular interactions between lipid molecules and water were evaluated via the hydrogen bonds formed between these molecules. At low concentration of oleic acid, the oleic acid molecules interacted with all components in the system proportional to their concentration, but most efficiently surrounding the DOPC head group. Notably, DOPC head group is more polar than the triolein ester groups and hence the acid group of oleic acid preferentially binds to the DOPC head. The preferential binding can be seen in the lipid phosphate group binding already 2 oleic acids at the lowest studied, i.e. 5 wt% oleic acid concentration (**Fig. 3B**). At elevated oleic acid concentrations, the oleic acid molecules had some tendency of clustering through increasing propensity of oleic-oleic

acid interactions at the cost of triglyceride-oleic acid contacts. However, no larger aggregates of oleic acid, such as oleic acid micelles, persistently formed, within the simulations time scale. This observation supported the experimental data in SI (**Fig. S2**) and fully confirmed that the oleic acid did not form reverse micelles at any range of concentrations studied here.

The interactions between oleic acid and the DOPC lipid phosphate and oleic acid and the DOPC lipid ester group, as measured by hydrogen bonding, increased linearly with the oleic acid mass fraction (**Fig. 3B**). The oleic acid bound dominantly to the phosphate group and forms a shell around the lipid head group. Notably, water molecules can hydrogen bond with the oleic acid molecules already bonded with the lipid head group. The binding pattern and the formation of the oleic acid shell is visualized in the simulation snapshot (**Fig. 3D**). This leads to enhanced solubilization of DOPC in accordance with the experimental data. However, although oleic acid binding to the lipid head group increases with increasing oleic acid mass concentration, oleic acid does not displace water molecules bound to the phospholipid. The water-phosphate and water-ester interactions remain at the same level of hydrogen bonding density throughout the examined oleic acid mass fraction range. Instead, oleic acid can also bind to the water molecules hydrating the phospholipid head group, see the simulation snapshot in **Fig. 3D**.

Interestingly, the simulation systems contained 4 water molecules per surfactant, yet **Fig. 3B** shows approximately one water molecule bound to a phospholipid molecule in the simulated system. An analysis of the water partitioning in the system presented in **Fig. 3C** reveals that the water is in contact also with the triglycerides and the oleic acid in the system. The amount of water interacting with oleic acid and triglycerides reflects the mass fractions of each solvent component. However, a relatively larger fraction of water binds to oleic acid than to triolein. Furthermore, in all simulated oleic acid fractions, the portion of water partitioned to the triglycerides – oleic acid mixture remains approximately constant, *i.e.* insensitive to the oleic acid fraction.

Fig. 3 Molecular dynamics simulations results of (A) oleic acid hydrogen bonding with the different components of the model system, (B) oleic acid and water hydrogen bonding to the lipid phosphate and the ester groups, and (C) water interactions with the oleic acid, triglyceride, DOPC and itself measured by hydrogen bonding all as a function of oleic acid mass fraction in the system. Water-to-lipid molar ratio is 4 in the system. (D) The snapshot visualizes a typical oleic acid binding configuration (shell build-up).

The rapeseed oil examined in the experiments contained 97 wt-% of triglycerides of varying composition and traces of free fatty acids and the lecithin is a mixture of various phospholipids, as well as other components. PC lipids, such as the DOPC examined in the simulations, make up approximately one quarter of the lecithin composition in the experiments. However, it is important to note that also the other main lipid components have the same phosphate group to which the oleic acid dominantly binds in the simulations. Thus, the observed trends are likely to hold also for the other lipid components participating in the micellar formation in the experiments. Nevertheless, due to slightly different hydrogen-bonding capability of the ethanolamine and inositol groups, the magnitudes may differ. The trace amount of free fatty acids in the oil could in principle compete for the PC head group as oleic acid binding sites, and consequently reduce the influence of the oleic acid. Nevertheless, the simulations data highlighted that the oleic acid assembles around the hydrophilic head group of lecithin, thus making it more hydrophobic and increasing its solubility in oil.

3.3 Effect of increasing water concentration on lecithin self-assembly

Besides temperature, and free fatty acids, also the amount of added water influence self-assembly of lecithin. Typically small water-to-lipid ratios lead to reverse micelle formation, while in some cases gelation may occur at a higher water-to-lipid ratio, depending on the chemical nature of the organic media and the lecithin composition (20). Furthermore, an excess of water has been shown to induce phase separation in the reverse micelle systems (19). Both gelation and a sudden phase separation may have a vast impact on, for example, purification of natural oils. Thus the effect of water content on the self-assembly of lecithin was investigated at RT and 70 °C at a constant lecithin concentration of 1 wt.-%, that was clearly above CMC (**Fig. 4A**). It is worth noting that already at a H₂O-to-lecithin weight ratio of 14 wt.-%, phase separation occurred at room temperature. At 70 °C, the reaction between lecithin and the fluorescence dye, TCNQ led to very dark colors (**Fig. 4C**). Consequently, the UV absorbance was above 1 for all samples. Since the absorbance is not increasing linearly with the amount of micelles at absorbance values above 1, these results are not shown. However, comparison of the photographs corresponding to the sample series at RT (**Fig. 4B**) and 70 °C (**Fig. 4C**) reveals that the trend at elevated temperatures is qualitatively similar to the response at RT. It is difficult to detect exactly at what H₂O-to-lecithin weight ratio the phase separation occurs, but precipitation was at least detected at H₂O-to-lecithin weight ratio of 14 wt.-% corresponding to H₂O-to-oil weight ratio of 0.14 wt.-% at RT (**Fig. 4B**) and at H₂O-to-oil weight ratio of 0.21 wt.-% at 70 °C (**Fig. 4C**).

The water saturation point of the system at room temperature occurred at H₂O-to-lecithin weight ratio of 43 wt.-%, in accordance with previous findings (19). At a higher water concentration, free water was found in the oil, which led to an increase in the UV absorbance at H₂O-to-oil weight ratio of 0.53 wt.-% or higher (**Fig. 4A**). The water saturation point of lecithin is dependent on the temperature, as can be qualitatively seen from photographs in **Fig. 4B** and **4C**.

Fig. 4. TCNQ absorbance plotted as a function of H₂O-to-ecithin weight ratio (lower x-axis) and H₂O-to-oil weight ratio (upper x-axis), in rapeseed oil, with 1 wt.-% lecithin at RT (A). X refers to the water saturation point. Photographs of the corresponding samples after TCNQ addition at (B) RT and (C) 70° C. The numbers on top of the samples representing the H₂O-to-oil weight ratio in wt.-%.

3.4 Structure of reverse micelles and phase separated aggregates

SAXS and cryo-TEM were used to determine structural properties and morphology of lecithin reversed micelles and to further understand what drives the phase separation at elevated water content. In **Fig. 5**, Cryo-TEM and SAXS patterns for 1 wt.-% lecithin in rapeseed oil with 0.03 wt.-% water (*i.e.* no added water and the moisture content originated from the materials themselves) are shown. This corresponds to the second tube in **Fig. 4B** and a sample rich in reverse micelles, with no indication of phase separation. The fast Fourier transform (FFT, inset **Fig. 5A**) allows evaluation of the symmetrical structure of reverse micelles and shows cylindrical structures, implicating that the reverse micelles are cylindrical rather than purely spherical. The corresponding SAXS patterns (**Fig. 5B**) indicated no ordered structures, like hexagonal or lamellar phases that are structures known to

lead to phase separation (19, 34). The energetically most favorable packing depends both on steric factors related to the structure of the lecithin molecules as well as on the interactions in the system. Most importantly, the surfactant packing geometry is governed by the attraction between the hydrophobic tails and the apolar medium, and the repulsion between the apolar medium and the polar head-group of lecithin, as well as, the water dynamics at the micellar interface. These interactions are strongly influenced by the properties of the apolar medium, amount of water in the system and the presence of any other surface-active components. The influence of the nonpolar solvent along with other chemical components, *i.e.* water and additives, on phospholipid self-assembly is illustrated by the large variety of lecithin morphologies reported in literature (13, 19, 56).

Fig. 5. Cryo-TEM images (A) and SAXS patterns (B) of the 1 wt.-% lecithin in rapeseed oil containing 0.03 wt.-% water at RT (Inset figure: Fast Fourier Transform (FFT) of cylindrically packed structure). The scale bar of the image is 50 nm.

It is worth noting that very subtle differences in the oil phase affect the formed reverse micellar structures (36). Additionally, detailed structural analysis of phospholipids in vegetable oils at varying water content is lacking. To obtain structural information of the formed aggregates, Cryo-TEM and SAXS were employed to investigate the structures observed at a higher water content, *i.e.* H₂O-to-oil weight ratio of 0.21 wt.-%. A sample where phase separation clearly occurred, consisting of 1 wt.-% lecithin in rapeseed oil at room temperature with H₂O-to-oil weight ratio of 0.21 wt.-% was centrifuged and both the precipitate and the soluble phase were characterized. A lamellar crystalline structure was clearly observed from cryo-TEM image of the precipitated phase (**Fig. 6A**) and as indicated by FFT image (inset of **Fig. 6A**), this led to a lamellar structure with a d-spacing of 5.05 nm. This agreed well with the Bragg peaks of a lamellar structure at 1:2 leading to a distance between the lamellar planes of 5.03 nm ($d=2\pi/q$) observed using SAXS (**Fig. 6A**). We suggest that water is bound to the phospholipid head groups leading to swelling of the lamellar structures but possibly also that free water is present in between the hydrophilic phospholipid head groups. The values for the spacing observed here are very close to previously reported values for similar systems (34). In contrast, only a very weak stripe pattern, corresponding to lamellar structure, was observed in the cryo-TEM image of the soluble phase (**Fig. 6B**). In addition, the intensity peaks of FFT lamellar structure of the soluble phase was much weaker than that of the precipitated phase, thus it was hard to calculate d-spacing for each X-ray reflection. Correspondingly, only a very small peak was observed in the SAXS spectrum of the soluble phase, suggesting a much lower concentration of lamellar structures. Thus, it is worth noting that the formation of a lamellar phase led to phase separation and phospholipids could in this way be removed from the oil phase. Only a small amount of lamellar structures remained in the oil after centrifugation. A possible explanation for the formation of lecithin lamellar liquid crystalline structures is that the water addition might increase the volume of lecithin head groups, thereby leading to elongation of the reverse micelles, by affecting the packing parameter of the lecithin at the water-oil interface. Packing parameter considerations (57) reveal that this promotes first an increase of the aggregation number of the reverse micelles and further, at a critical water-to-lipid ratio, lamellar assembly. Hence, with swollen head groups, the lecithin cannot maintain the soluble reversed micelle shape due to the energetically unfavorable packing and it is forced to form lamellar liquid crystal structures with lower solubility in rapeseed oil. The polar head-group area observed in the lamellar structures may stay constant, but in general it depends on water-to-lipid ratio (34, 35).

Fig. 6. SAXS patterns of (A) the precipitated phase and (B) the soluble phase of 1 wt.-% lecithin in rapeseed oil at RT at H₂O-to-oil weight ratio of 0.21 wt.-%. Inset figures: cryo-TEM and FFT of (A) the precipitated and (B) the soluble or continuous phase. The Bragg peaks demonstrated lamellar phase with reflections corresponding to (001) and (002) planes.

3.5 Effect of water, oleic acid and temperature on lecithin self-assembly

As shown above, water played an essential role in inducing phase separation of phospholipids from oil, while oleic acid enhanced the solubility of phospholipids in oil, thus suppressing formation of RM and increasing the CMC, while elevated temperature led to lower CMC. Thus, the interplay of these parameters, *i.e.* water, oleic acid, and temperature, was investigated using the TCNQ solubilization method. In **Fig. 7**, the influence of the oleic acid concentration on the self-assembly of lecithin at varying H₂O-to-lecithin weight ratios is shown. Lecithin concentrations that contained

abundantly RM before water or oleic acid addition were chosen for the experimental series being performed at RT and 70 °C, namely 1 wt.-% and 0.2 wt.-%, respectively (**Fig. 7A** and **7B**). The corresponding photographs of the samples are shown in Supplementary Information (**Fig. S3**). At 70 °C, a lower concentration of lecithin was used since at 70 °C and 1 wt.-% of lecithin, the reaction with TCNQ resulted in very dark colour and almost all the experimental points had UV absorbance above 1, thus eventually deviating the Beer-Lambert's law (58). Without added water the absorbance, decreased upon addition of higher concentration of oleic acid into the w/o emulsion system, suggesting a lower concentration of lecithin reverse micelles, in good correlation with the solubilizing effect of oleic acid shown earlier (**Fig. 2**). Comparing the system free from fatty acid (black circles) with the systems containing 5-20 wt.-% of oleic acid (white, red, and green hollow circles in (**Fig. 7A**), it can be noted that without oleic acid the abundance of reverse micelles decrease rapidly already at a H₂O-to-lecithin weight ratio of 9 wt.-%. The absorbance at lower water content was well above 1 and is thus shown only as a dashed line in **Fig. 7A**. In contrast, the absorbance, and consequently the concentration of RMs, was much more stable in the presence of oleic acid up to H₂O-to-lecithin weight ratio around 14 wt.-% (**Fig. 7A**). At H₂O-to-lecithin weight ratios higher than about 14, the abundance of reverse micelles decreased rapidly for all the systems containing oleic acid at RT. This indicates that the lecithin precipitated as a lamellar phase due to the presence of water, the precipitation is evident in Supplementary Information (**Fig. S3**). Precipitation occurs at a considerably higher H₂O-to-lecithin weight ratio in the presence of oleic acid than in the absence of it. Thus, oleic acid is proposed to have deferred the phase separation process by increasing the lecithin solvation into the rapeseed oil, thereby subsequently delaying the formation of lamellar structures. The effect of oleic acid concentration was not significant at RT.

Fig. 7. TCNQ absorbance as a function of H₂O-to-lecithin weight ratio (wt.-%) in rapeseed oil containing (A) 1 wt.-% of lecithin at RT and (C) 0.2 wt.-% of lecithin at 70 °C. UV absorbance values >1 are shown for qualitative comparison as dashed lines. B is a zoom in of the system at RT. 0, 5, 10, and 20 represent the concentration of oleic acid (in wt.-%), while X refers to the water saturation point for the system without added oleic acid

Adding oleic acid into 0.2 wt.-% lecithin solubilized in rapeseed oil at 70 °C (**Fig. 7B**) generally decreased the concentration of the lecithin reverse micelles, similar to observations at RT and 1wt.-% lecithin (**Fig. 7A**). Note that the measurement points at high absorbance are also shown but should be used only as qualitative indication of the high amount of RMs present, thus the dashed line. The higher the oleic acid concentration, the stronger the decrease in RM concentration was. However, the lecithin RM concentration decrease with addition of varying concentrations of oleic acid differed from observations at RT. At 70 °C, almost no RM form in 20 wt.-% oleic acid, see the steady, very low absorbance in accordance with the shift in CMC noted in **Fig. 2**. Slightly higher absorbance values were found at 5 and 10 wt.-% oleic acid, respectively, where some RM should be present before adding excess of water (**Fig. 2**). Contrary to the results at RT, no clear phase separation was seen at 70 °C; the absorbance remained high with the 5 and 10 wt.-% oleic acid sample series until the water saturation point was achieved. This clearly shows that temperature plays an important role in determining the structure of the micelles. At RT, the water saturation point (indicated with X) appeared similar in all systems while at 70 °C it shifted to higher water-to-lecithin ratios with increasing oleic acid concentration.

These findings further indicate that free fatty acids could exhibit a dual function in nonpolar phospholipid systems. In the absence of excess water, they solubilized phospholipids in oil, thus suppressing the formation of RMs, while in the presence of certain concentration of water they may act as cosurfactants stabilizing the reverse micelles and subsequently, hindering formation of phospholipid – water lamellar structures that precipitate out of the oil.

4. Conclusions

Temperature, water and free fatty acid content was found to strongly influence the self-assembly of lecithin in rapeseed oil. Lecithin was found to form cylindrical reverse micelles above CMC in the oil. At low water content, increasing the temperature from RT to 70 °C shifted the CMC to lower values. Furthermore, oleic acid assembled around the polar head group of lecithin compounds increasing their solubility in oil and suppressing the formation of reverse micelles, thus shifting the CMC to higher lecithin concentrations. At lecithin concentrations above CMC, addition of a critical amount of water resulted in the formation of lamellar structures. The formation of lamellar structures led to phase separation, efficiently removing RM from the oil phase. The oleic acid was found to have a dual effect on the lecithin aggregation; at very low water concentration it inhibited RM formation by increasing lecithin solubilization in oil, while at slightly higher water concentrations it acted as a co-surfactant stabilizing the RMs. The onset of phase separation started at significantly higher water

concentrations in the presence of oleic acid. Additionally, the results show that very small shifts in water content or presence of other surfactants may profoundly change the behavior of phospholipids in oil. This outlines the importance of varying system components one at the time in future related studies, as well as, the need for characterization of the solution composition including water content. Additionally, individual components of these systems may interact in a very complex way that cannot be predicted if only one parameter is changed. To our knowledge, we provide the first systematic comparison of water and free fatty acid content on lecithin aggregation in plant oils. Besides demonstrating the interplay of the components, the findings could be of importance for optimizing properties of, *e.g.*, oleogels, alimentary oils, as well as, lecithin and plant oil based transdermal drug delivery emulsions.

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Highlights