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ORIGINAL PAPER

Impact of Free Fatty Acids and Phospholipids on Reverse Micelles Formation and Lipid Oxidation in Bulk Oil

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Abstract Association colloids such as phospholipid reverse micelles could increase the rate of lipid oxidation in bulk oils. In addition to phospholipids, other surface active minor components in commercial oils such as free fatty acids may impact lipid oxidation rates and the physical properties of reverse micelles. In this study, the effects of free fatty acids on changes in the critical micelle concentration (CMC) of 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) in stripped corn oil (SCO) were determined by using the 7,7,8,8-tetracyanoquinodimethane solubilization technique. Different free fatty acids including myristoleic, oleic, elaidic, linoleic and eicosenoic were added at 0.5 % by wt along with the DOPC into the bulk oils. There was no significant effect of free fatty acids with different chain length, configuration and number of double bonds on the CMC value for DOPC in bulk oil. However, increasing concentrations of oleic acid (0.5 to 5 % by wt) caused the CMC value for DOPC in bulk oils to increase from 400 to 1,000 μmol/kg oil. Physical properties of DOPC reverse micelles in the presence of free fatty acids in bulk oils were also investigated by the small angle X-ray scattering technique. Results showed that free fatty acids could impact on the reverse micelle structure of DOPC in bulk oils. Moreover, free fatty acid decreased pH inside reverse micelle as confirmed by the NMR studies. The oxidation studies done by monitoring the lipid hydroperoxide and hexanal formation revealed that free fatty acids exhibited pro-oxidative activity in the presence and absence of DOPC. Different types of free fatty acids had similar pro-oxidative activity in bulk oil.

Keywords Reverse micelles · Bulk oil · Critical micelle concentration · Lipid oxidation · Free fatty acids · Phospholipids

Introduction

Bulk oils contain various kinds of surface active components such as free fatty acids, monoacylglycerols, diacylglycerols, phospholipids and polar amphiphilic products arising from lipid oxidation, such as lipid hydroperoxides, aldehydes, ketones, and epoxides. Moreover, commercial oils contain an amount of water that still remains after the refining process. This would provide an oil–water interface where the surface active components would self-aggregate into association colloids such as reverse micelles, which are thermodynamically more favorable than dispersed surfactant monomers in the oil or water [1].

Reverse micelles are nanometer-sized aggregates consisting of a water core surrounded by surfactants in nonaqueous media such as bulk food oils. Surfactant molecules arrange themselves in the way that polar head groups point inward to the water core and nonpolar tails point outward to bulk oil phase. Reverse micelles are in thermodynamic equilibrium with the surrounding medium. Thus, changes of the composition of the lipid medium, or the

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concentration of surface active molecules could lead to exchange of surface active substances between the reverse micelles and the medium. This could alter the structure and characteristic of reverse micelles. Several researchers have observed that the reverse micelle droplet size increased with increasing the water-to-surfactant ratio (ω_0) [2–4]. Additionally, the presence of co-surfactants could affect the reverse micelle size and shape by altering the optimum curvature of the system. Chaiyasit and co-workers [3] reported that cumene hydroperoxide and oleic acid caused the reverse micelle size to decrease in a sodium bis(2-ethylhexyl) sulfosuccinate (AOT) reverse micelle model system while phospholipids caused the reverse micelle size to increase. Reverse micelles could only be formed when the critical micelle concentration (CMC) of surfactants is exceeded. The CMC value for phospholipids is affected by the phospholipid composition and solvent medium. The higher composition of phosphatidylcholine in mixed phospholipids lowered the CMC value [5]. The dilution of the oil with hexane caused the CMC of phospholipids to decrease as compared to that of undiluted oil systems due to the greater hydrophobic repulsive forces between hexane and amphiphilic phospholipids [5].

Phospholipids are surface active components present in refined edible oils at concentration less than 0.03 mmol phosphorus/kg oil [1]. Crude oil contains phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and phosphatidylserine even after the degumming process. Phospholipids were found to show antioxidant activity in bulk oils and their antioxidant activity was attributed to metal chelating, lipid hydroperoxide decomposition activity, and free radical scavenging ability [6]. However, phospholipids at high concentration act as pro-oxidants by decreasing surface tension of oil leading to increased diffusion rate of oxygen into the oil [6]. Phospholipids have been reported to form reverse micelles in bulk oils in the presence of small quantity of water [7–11]. The formation of phospholipid reverse micelles has been observed to involve in lipid oxidation in bulk oils in several aspects. A number of studies have suggested that reverse micelles formed by dioleoyl phosphatidylcholine (DOPC) accelerated oxidation rate in bulk oils [7, 10, 12]. On the other hand, DOPC reverse micelles showed synergistic effect with some antioxidants in inhibiting oxidation. For example, phospholipids enhanced free radical scavenging activity of tocopherol in bulk oil as reverse micelles formed by phospholipids enhanced the accessibility of tocopherol into the aqueous microenvironment where the oxidation reactions can be concentrated [13]. This result was in agreement with Chen and co-workers [12] who recently reported that antioxidant activity of α-tocopherol and Trolox at low concentration (10 μM) increased in the presence of DOPC reverse micelles in bulk oil. Moreover, the presence of reverse micelle structures in bulk oils also had impact on the antioxidant effectiveness of phenolics compounds with different hydrophobicity such as chlorogenic acid and hexadecyl chlorogenate as reported by Laguerre and co-workers [11]. DOPC reverse micelles showed antagonistic effect with chlorogenic acid but did not impact the antioxidant activity of hexadecyl chlorogenate. The authors suggested that reverse micelle structures could promote the partitioning of chlorogenic acid into the water core, where chlorogenic acid could reduce the pro-oxidative effect of the metals. Antioxidant activity of hexadecyl chlorogenate was not affected by DOPC reverse micelle suggesting that the hydrophobicity of hexadecyl chlorogenate prevented it from partitioning into water phase and thus did not affect the pro-oxidant activity of metals.

Another surface active component that has an impact on lipid oxidation in bulk oil is free fatty acids. Since free fatty acids cause foaming and decrease the smoke point of the oil, they are removed from crude oil by neutralization using caustic alkali [14]. However, refined edible oils still contain free fatty acids at concentrations ranging from 1.0 to 140 mmol free fatty acid/kg oil [1]. Free fatty acids are well established as pro-oxidants in bulk oils by enhancing pro-oxidant activity of transition metals [15] and promoting the decomposition of lipid hydroperoxide [16]. Moreover, free fatty acids could enhance lipid oxidation in oil by decreasing the surface tension of oil leading to increasing the diffusion rate of oxygen from the headspace into the oil [6]. Chaiyasit and co-workers [9] also reported that oleic acid was able to accumulate at the oil/water interface in bulk oil and decrease the pH of the aqueous phase then promoting acid catalyzed lipid hydroperoxide decomposition. Free fatty acids could impact the physicochemical properties of association colloids in bulk oils or even form reverse micelles in bulk oil as they are surface active with a hydrophilic lipophilic balance of around 1[17].

In this paper we attempt to add to the current knowledge by studying the combination effect of free fatty acids and phospholipids on reverse micelles formation and lipid oxidation in bulk oil. This system could imitate structures in real bulk oils which contain various kinds of surface active components providing a better understanding of the complexity of association colloids and their impact on lipid oxidation.

Materials and Methods

Materials

Corn oil was purchased from a local retail store and stored at 4 °C. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dibutyryl-*sn*-glycero-3-phosphocholine (DC_4PC) were acquired from Avanti Polar Lipids, Inc. (Alabaster, AL,

USA). Silicic acid (100–200 mesh), activated charcoal (100–400 mesh), 7,7,8,8-tetracyanoquinodimethane (TCNQ), barium chloride, ammonium thiocyanate, iron (II) sulfate heptahydrate, sodium metavanadate ($NaVO_3$), myristoleic acid, oleic acid, linoleic acid, elaidic acid, eicosenoic acid and methyl oleate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Medium-chain triacylglycerols (MCT, Miglyol) were obtained from Sasol North America Inc. (Houston, TX, USA). Chloroform and *n*-hexane (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was used in all experiments. Glassware was submerged in 2 M HCl overnight to remove metals, followed by rinsing with deionized water before use.

Methods

The first part of the study is to determine the CMC of DOPC in bulk oil with addition of (a) different free fatty acids including myristoleic acid (14:1), oleic acid (18:1, *cis*), elaidic acid (18:1, *trans*), linoleic (18:2) and eicosenoic acid (20:1) at 0.5 % (by wt), then (b) determine the effect of oleic acid at concentrations varied from 0.5 to 5.0 % (by wt) and (c) determine the effect of methyl oleate (from 0.5 to 5.0 % by wt). The second part of the study determined the pH inside the reverse micelles of DOPC in the absence and presence of 3 % (by wt) oleic acid by using ^{51}V-NMR spectroscopy. The third part of the study is to confirm the reverse micelle structure of DOPC through small angle X-ray scattering (SAXS). The last part of the study is to determine the lipid oxidation in bulk oil with addition of (a) DOPC (at 1,000 μmol/kg oil) in the absence or presence of different free fatty acids at 0.5 % by wt (b) DOPC (at 200 and 1,500 μmol/kg oil) in the absence or presence of oleic acid at 3 % by wt (c) DC_4PC at 1,000 μmol/kg oil in the absence or presence of oleic acid at 0.5 % by wt. The lipid oxidation lag time was determined by monitoring the lipid hydroperoxides and hexanal formation.

Preparation of Stripped Corn Oil

Stripped corn oil was prepared as described by Boon et al. [18]. Briefly, silicic acid (100 g) was washed three times with a total volume of 3 L of distilled water and dried at 110 °C for 20 h. A chromatographic column (3.0 cm internal diameter × 35 cm height) was then packed sequentially with 22.5 g of silicic acid, followed by 5.63 g of activated charcoal and another 22.5 g of silicic acid. Thirty grams of corn oil was dissolved in 30 mL of *n*-hexane and passed through the column by eluting with 270 mL of *n*-hexane. The container used to collect the triacylglycerols was held in an ice bath and covered with aluminum foil to retard lipid oxidation during stripping. The solvent presented in the stripped oils was removed with a vacuum rotary evaporator (RE 111 Buchi, Flawil, Switzerland) at 37 °C and traces of the remaining solvent were evaporated under a nitrogen stream. The water content of the oil was determined using the Karl Fisher Coulometer (756 KF Coulometer connected to 703 Ti Stand, Metrohm, Herisau, Switzerland). The SCO was kept at −80 °C for subsequent studies.

Determination of Critical Micelle Concentrations in Bulk Oils

The CMC of free fatty acids and those of DOPC in the presence and absence of free fatty acids in bulk oils were determined by using the TCNQ solubilization technique [19]. Briefly, the bulk oil was prepared from a mixture of MCT and SCO (3:1, by wt; MCT was used as a non-oxidizable lipid). Various amounts of free fatty acids ranging from 0.1 to 5.0 % (by wt) were added into bulk oil, then the mixture was magnetically stirred for 12 h. To study the effect of free fatty acids on the CMC of DOPC, the DOPC (1–2,000 μmol/kg oil) was mixed with the oils for 12 h prior to adding free fatty acids at concentrations ranging from 0.5 to 5.0 % (by wt) followed by addition of 5 mg of TCNQ/5 g oil and mixing for 5 h. The excess TCNQ was removed by centrifugation at 2,000 *g* for 20 min and subsequent decanting. The absorbance was measured at 480 nm using a spectrophotometer (Shimadzu 2,014, Tokyo, Japan). The CMC was determined as the inflexion point in the curve plotting absorbance as a function of DOPC concentration (semi-log plot) [11].

^{51}V-NMR Spectroscopy

Sodium metavanadate solution at 200 mM was freshly prepared by dissolving $NaVO_3$ in deionized water. The solution was heated on the hotplate (approximate temperature of 100 °C) and stirred magnetically until the $NaVO_3$ was completely dissolved. After cooling, 18 μL of sodium metavanadate solution was pipetted into 2 g of bulk oil containing DOPC (1,000 μmol/kg oil) in the absence or presence of 3 % (by wt) oleic acid. Then, the samples were sonicated on an ice bath using a 1/8″ probe sonicator (Model FB505, Fisher Scientific, Pittsburgh, PA, USA) at 40 % amplitude with 0.05 s/pulse for 1 min. To prepare standard pH solutions, the sodium metavanadate solution was mixed with 0.04 M Britton Robinson buffer (pH 1.5, 3.1, 5.0 and 6.6) at 18:1 ratio according to the volume ratio of sodium metavanadate solution: water in bulk oil. ^{51}V NMR spectra were recorded on a Bruker Avance 400 at 105.2 MHz with the spectral window of 64.9 kHz, a pulse angle of 30°, and acquisition time of 0.126 s with a relaxation delay of 0.500 s. The pH of DOPC reverse

micelle in bulk oil was determined by comparing the ^{51}V-NMR spectra of vanadium in bulk oil with those of the standard pH solutions [20–22].

Small-angle X-ray Scattering (SAXS) Measurement

SAXS measurements were performed on the oil samples using a Rigaku Molecular Metrology SAXS instrument (Rigaku, Inc.) operating at 45 kV and 0.67 mA. The instrument generates X-rays using a microfocus Cu X-ray tube with point source (focal spot 30 × 30 μm^2) of which the CuK$_\alpha$ line at 0.1542 nm. Samples were placed into the 1-mm outer diameter quartz capillary (Hampton Research, Aliso Viejo, CA, USA) and were positioned inside the sample chamber. The whole system was evacuated by vacuum pump. After passing through the samples, the scattered x-rays were collected by a 2D multiwire detector with a sample-to-detector distance of 1,477 mm. The actual distance from the sample to the detector was calibrated using silver behenate. The 2D scattering patterns were recorded on the samples for 3 h and then integrated, using the program POLAR, to one-dimensional scattering function $I(q)$, where q is the length of the scattering vector defined by $q = (4\pi/\lambda)$ sin $(\theta/2)$, λ is the wavelength and θ is the scattering angle.

Samples Preparation for Oxidation Study

Either DOPC or DC$_4$PC was added to the bulk oil (a mixture of MCT/SCO, 3:1, by wt) using chloroform as the carrier which was removed by evaporation under nitrogen at room temperature. The samples were magnetically stirred at a speed of 1,000 rpm in a 20 °C incubator room for 12 h. Each of the free fatty acids including myristoleic acid, oleic acid, linoleic acid, elaidic acid, and eicosenoic acid was added and stirred for 12 h to obtain homogenous samples. Samples (1 mL) were aliquoted into 10-mL GC headspace vials (Supelco), capped with aluminum lids having PTFE/silicone septa and stored at 55 °C in the dark.

Measurement of Lipid Oxidation

Lipid Hydroperoxides

Lipid hydroperoxides were measured using a method adapted from Shanta and Decker [23]. The bulk oil samples (20 μL) were weighed and dissolved in 2.8 mL of methanol/butanol solution (2:1, v/v). A mixture of 15 μL of 3.94 M ammonium thiocyanate and 15 μL of 0.072 M ferrous solution was used as an indicator. The ferrous solution was obtained from the supernatant of a mixture of one part of 0.144 M $FeSO_4$ and one part of 0.132 M $BaCl_2$ in 0.4 M HCl. After 20 min of incubation at room temperature, the absorbance of the samples was measured at 510 nm using a spectrophotometer (Genesys 20, Thermospectronic, Waltham, MA, USA). The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve.

Headspace Hexanal

Headspace hexanal was measured using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan) [18]. Samples (1 mL) in 10-mL glass vials capped with aluminum caps with PTFE/silicone septa were preheated at 55 °C for 8 min in an autosampler heating block. A solid-phase microextraction (SPME) fiber needle (50/30 μm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA, USA) was injected into the vial for 2 min to absorb volatiles and then was transferred to the injector port (250 °C) for 3 min. The injection port was operated in split mode, and the split ratio was set at 1:5. Volatiles were separated on an Equity-1 column (30 m × 0.32 mm × 1 μm film thickness, Supelco, Bellefonte, PA, USA) at 65 °C for 10 min. The carrier gas was helium set at a flow rate of 15 mL/min. A flame ionization detector was used at a temperature of 250 °C. Hexanal concentrations were determined from peak areas using a hexanal standard curve.

Statistical Analysis

All experiments were conducted in triplicate samples. Data were presented as means ± standard deviations. Data results were analyzed by analysis of variance (ANOVA) using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). The differences between mean values were compared using Duncan's multiple-range test with significance defined as $p \leq 0.05$.

Results and Discussions

Effect of Free Fatty Acids with and Without DOPC on Reverse Micelle Formation

The CMC is one important characteristic of surface active components. It is the concentration at which the surface active molecules begin to aggregate into association colloids. Due to the fact that physical properties of solutions such as surface tension, electrical conductivity, turbidity and osmotic pressure depend upon whether the surfactant molecules are dispersed as monomers or micellar aggregates, the CMC can be determined by monitoring the abrupt change of these physical properties when the CMC is exceeded [17]. In this experiment, the CMC was

determined by using TCNQ solubilization technique. The charge transfer interaction between DOPC and TCNQ occurred when the concentrations of DOPC exceed the CMC, leading to an increase in the solubility of TCNQ which can be observed by an increase in absorbance at 480 nm. The concentration where the absorbance abruptly changes was identified as the CMC. We initially investigated the ability of free fatty acids to form reverse micelles. The free fatty acids themselves did not show ability to form reverse micelle structure at concentration range from 0.1 to 5 % (by wt) in bulk oil as determined by the TCNQ method (data not shown). From previous studies we know that DOPC forms reverse micelles in bulk oil in the presence of small amounts of water [7, 8, 10–12]. As DOPC is a zwitterion with a head group containing negatively charged phosphate and positively charged amine. Changing the pH of the system could alter the net charge and surface activity of DOPC thus free fatty acids could alter the ability of DOPC to form structures [24]. Therefore, we investigated the effect of free fatty acids on the ability of DOPC to form reverse micelles in bulk oil by determining changes in the CMC of DOPC. Figure 1 shows that the CMC of DOPC in SCO in the absence of added free fatty acids was at 400 μmol/kg oil. The CMC of DOPC has been reported to depend on oil type and water content. Chen and co-workers [7] revealed that the CMC of DOPC in stripped soybean oil containing 200 ppm water was around 650 μM. Laguerre and co-workers [11] reported that the CMC of DOPC in SCO equaled 65 μM with an endogenous water content of <40 ppm. In our system, the water content was 464.4 ± 51.8 ppm which was in the range of the amount of water in commercial oil (200–865 ppm) [1].

From Fig. 1, we observed that all types of free fatty acids including myristoleic acid (14:1), oleic acid (18:1, *cis*), elaidic acid (18:1, *trans*), linoleic acid (18:2), and eicosenoic acid (20:1) added at 0.5 % (by wt) did not impact the CMC of DOPC. However, as shown in Fig. 2, increasing fatty acid concentrations up to 5 % could alter the CMC of DOPC. For example, the CMC of DOPC in oil containing oleic acid at 0.5 and 1 % (by wt) was not different from that of the control which contained only DOPC. However, with the addition of 3 and 5 % (by wt) of oleic acid, the CMC of DOPC increased to 800 and 1,000 μmol/kg oil, respectively. We presume that the effect of oleic acid on the CMC of DOPC could relate to the net charge on DOPC head group which is highly pH dependent [24]. The addition of free fatty acids could alter the pH of the system then increase the positive charge on phospholipid head group. The protonation of the DOPC could increase repulsion between the head group of DOPC leading to an increase in the CMC. To test our hypothesis, oleic acid was substituted with methyl oleate which is an ester form of oleic acid without a carboxyl group. The

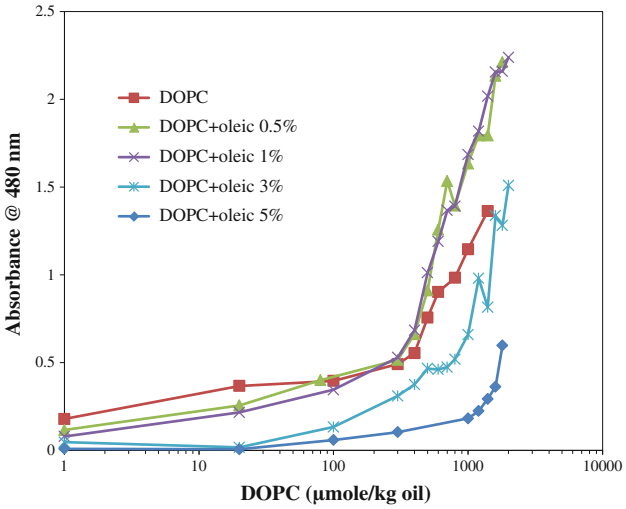

Fig. 2 Determination of critical micelle concentration of DOPC in bulk oil with addition of oleic acid at 0.5, 1, 3 and 5 % (by wt)

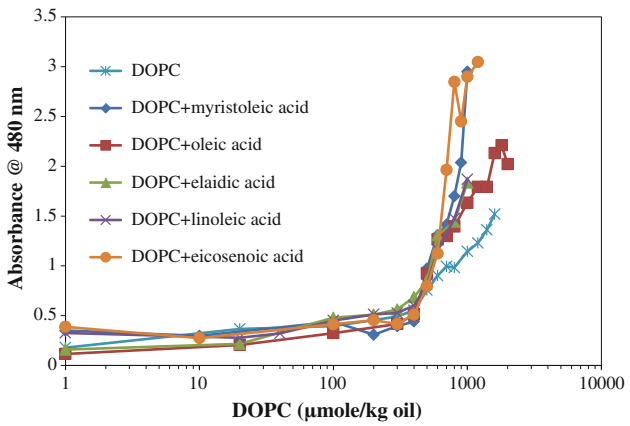

Fig. 1 Determination of critical micelle concentration of DOPC in bulk oil with the addition of different free fatty acids at 0.5 % (by wt)

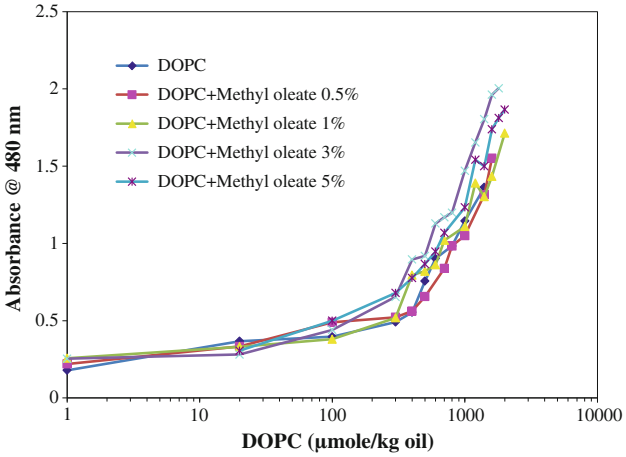

Fig. 3 Determination of critical micelle concentration of DOPC in bulk oil with addition of methyl oleate at 0.5, 1, 3 and 5 % (by wt)

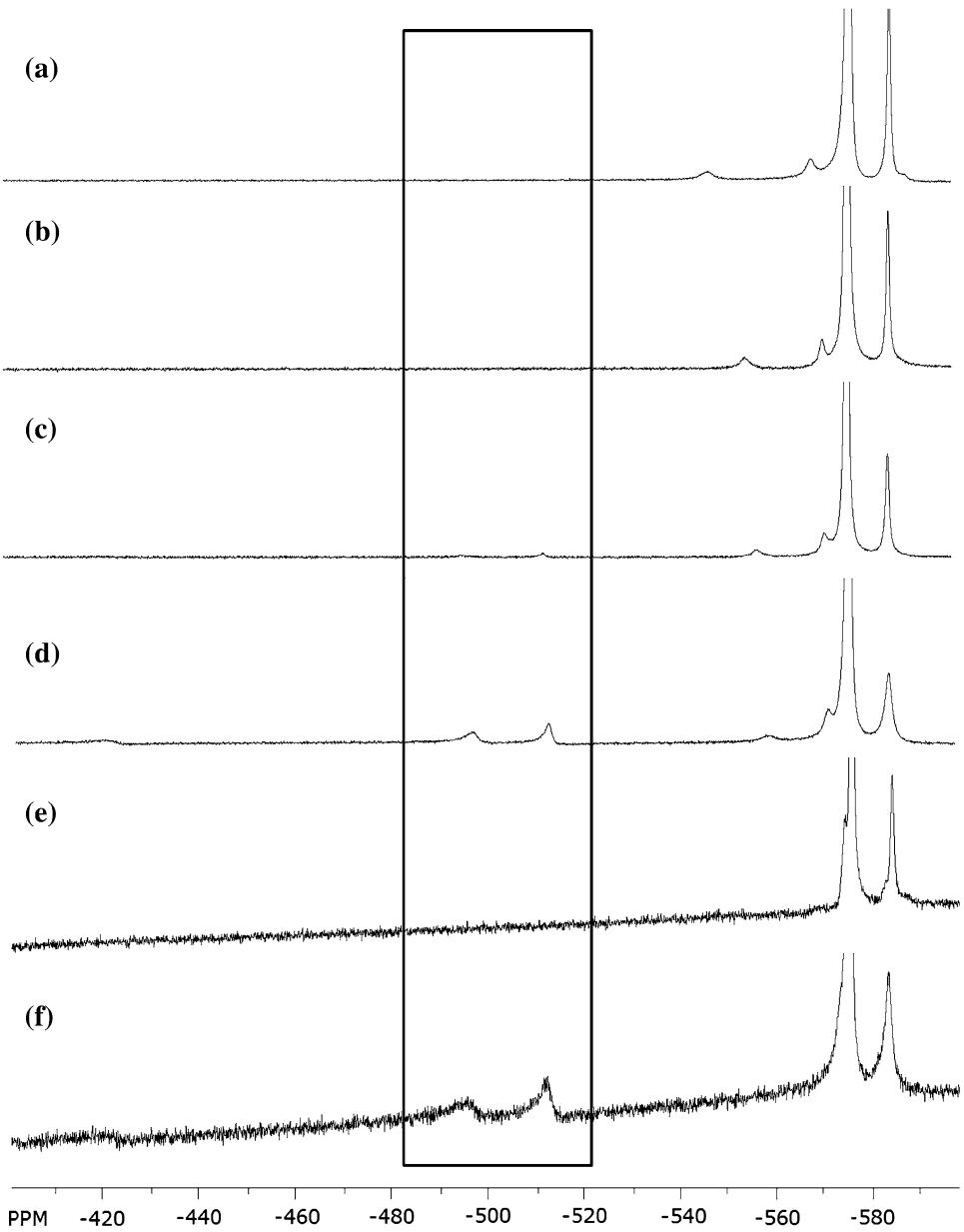

Fig. 4 ^{51}V-NMR spectra of 200 mM sodium metavanadate in Britton Robinson buffer pH: **a** 6.6, **b** 5.0, **c** 3.1 and **d** 1.5, **e** DOPC (1,000 μmol/kg oil) reverse micelle in stripped corn oil without oleic acid, **f** DOPC reverse micelles in stripped corn oil with 3 % (by wt) oleic acid in bulk oil

result showed that methyl oleate at concentrations up to 5 % did not influence the CMC of DOPC (Fig. 3). This confirmed that the ability of oleic acid to impact the CMC of DOPC depends on the concentration of oleic acid and the presence of the free carboxylic group in the molecule.

To investigate the influence of free fatty acid on the pH inside reverse micelle, where the DOPC head group resides, we used sodium metavanadate ($NaVO_3$) as a probe that measures the proton concentration in the water pool of the reverse micelle. The speciation of vanadate is highly pH dependent and can be monitored using ^{51}V-NMR spectroscopy [20–22]. Changes in the signal chemical shift and intensities reflect the protonation state and the relative concentration of each vanadate species, respectively. Figure 4 shows the ^{51}V-NMR spectra of vanadate species in aqueous solutions at different pH and in DOPC reverse micelle in bulk oil in the absence and presence of 3 % (by wt) of oleic acid. As the overall concentration of the vanadium nuclei probes in DOPC reverse micelle in bulk oil was much lower than that in the aqueous solutions, the signals from the reverse micelle samples were relatively weak. The ^{51}V-NMR spectrum of vanadate species in DOPC reverse micelle in bulk oil was consistent with that of the aqueous solution of sodium metavanadate at pH above 5.0. In the presence of 3 % (by wt) of oleic acid in combination with DOPC in bulk oil, the spectrum changed towards those observed in sodium metavanadate solutions at pH below 3.0.

However, one should bear in mind that there are some limitations to quantitatively determining pH in reverse micelles in bulk oil by using a vanadium probe. For example, the ^{51}V chemical shift has been reported to change with the size of the reverse micelles. The chemical shift in the larger reverse micelles is closer to that in bulk solution. This is attributed to different behavior of water molecules in small reverse micelles and in bulk solution [21, 25]. Moreover, protonation and oligomerization of vanadium are also sensitive to factors such as ionic strength, concentration, temperature and solvent polarity, which could cause slight shifts in the speciation observed in reverse micelles [20]. Nevertheless, this novel vanadium probe is a useful tool to investigate environments inside reverse micelles that are not readily accessible to direct measurements. The results from this study suggest that free fatty acids are able to accumulate at the oil/water interface and reduce the pH inside reverse micelles. It has been reported that the intrinsic pK_a of the phosphate group of phosphatidylcholine monolayer and bilayers were 0.8 and 2.6, respectively [26, 27]. Thus, it is possible that 3 % oleic acid could alter pH and thus the charge of the DOPC head group thus altering repulsive and/or attractive forces among the surface active molecules, leading to an increased CMC of DOPC. Also, as free fatty acids could reside at the oil/water interface, they could compete with DOPC for the interface and cause the CMC of DOPC to increase.

The formation of the reverse micelle structure of DOPC in bulk oil was confirmed by SAXS as shown in Fig. 5a. The SAXS profile shows the Bragg peak at $q = 1.4$ nm^{-1}. This corresponds to a d-spacing of 4.49 nm, according to $d = 2\pi/q$. When the particles align themselves into a highly ordered arrangement, the Bragg peak indicates the distance between the aligned particles. The scattering pattern has equal intensities along concentric circle around the incident beam when the samples were randomly oriented (isotropic). The size and shape of reverse micelles can vary depending on the type of surfactants and surrounding medium. In the case of water/DOPC/bulk oil system, reverse micelles have been reported to form in spherical shape [7]. However, when the 0.5 % oleic acid was added into the bulk oil without DOPC, no peak was observed (Fig. 5b). This is in agreement with the CMC result which revealed that oleic acid at this concentration did not show the ability to form structure in bulk oil as determined by the TCNQ technique. Moreover, no Bragg peak could be seen in the SAXS profile of DOPC in the presence of 0.5 % (by wt) of oleic acid (Fig. 5c). The TCNQ study (Fig. 1) revealed that oleic acid at this concentration did not affect the CMC value of DOPC. As oleic acid is also surface active, it could reside at the oil/water interface. This made it plausible that oleic acid could act as a co-surfactant and

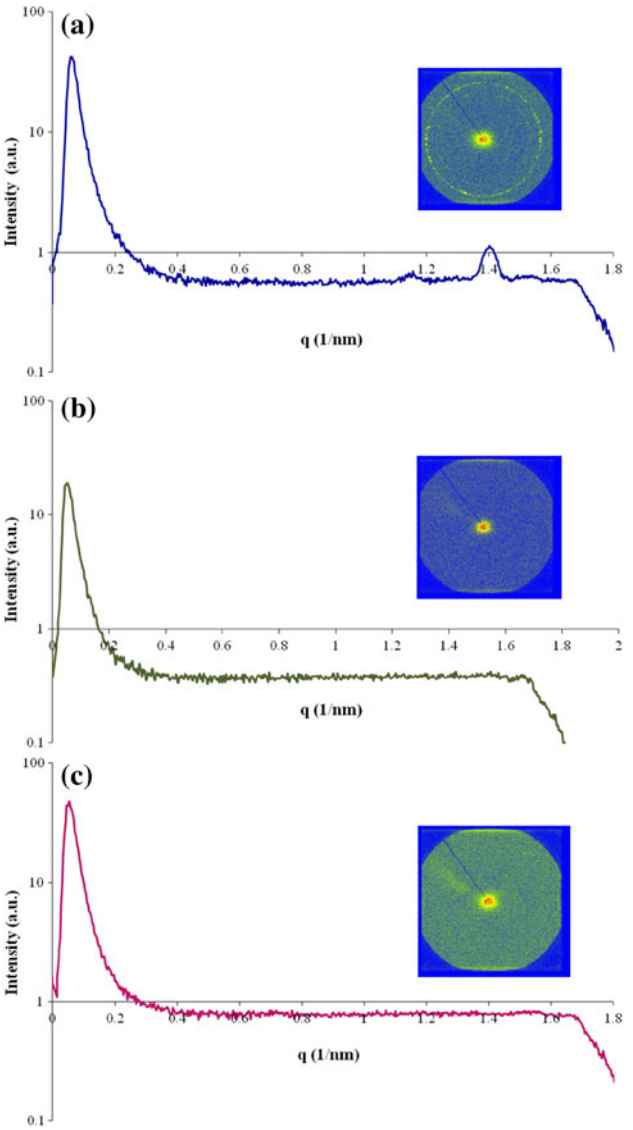

Fig. 5 SAXS pattern of: (a) DOPC (1,000 μmol/kg oil) in bulk oil, **b** oleic acid (0.5 %,by wt) in bulk oil and, **c** DOPC (1,000 μmol/kg oil) in the presence of 0.5 % (by wt) of oleic acid in bulk oil

alter the physical structure of the DOPC reverse micelle because of its difference in molecular geometry. For example, oleic acid could change the size of the reverse micelles. Chaiyasit and co-workers [3] previously reported that the addition of oleic acid decreased AOT reverse micelle size in hexadecane system. In addition, when water content is constant, increasing concentration of surface active components in the system could also decrease the reverse micelle size [2]. Moreover, oleic acid could impact the size of the reverse micelles by altering pH that could alter the charge of DOPC thus affecting packing properties at the oil–water interface. If the resulting size was out of the detection limits of SAXS, it is possible that a Bragg peak would not be detected. The accessible angular range

for SAXS that we used corresponds to dimension between about 4 and 100 nm.

Effect of Free Fatty Acids and DOPC on the Oxidation Stability of Bulk Oil

Minor components in bulk oils have strong impact on lipid oxidation. Some of these components are surface active and could affect lipid oxidation through their ability to form physical structures in the presence of small amounts of water. Among those surface active components, free fatty acids are known as pro-oxidants, while phospholipids have been reported to be both pro-oxidative and antioxidative in bulk oils. In oil stripped of its minor components, phosphatidylcholine such as DOPC have been reported to form reverse micelles and exhibit pro-oxidant activity [7, 10, 12]. In the presence of free fatty acids, it is possible that DOPC reverse micelles could negatively impact lipid oxidation by enhancing the pro-oxidant activity of free fatty acids in bulk oils. Therefore, the combination effect of free fatty acids and DOPC on lipid oxidation in bulk oil was investigated in this experiment. Different types of free fatty acids were added at 0.5 % (by wt) in the presence and absence of DOPC in bulk oil. The concentration of 0.5 % fatty acid was chosen since this concentration did not change the CMC of the DOPC reverse micelles and thus reverse micelles would be in the oil in both the presence and absence of fatty acids. Lipid oxidation was determined by following the lipid hydroperoxide and hexanal formation over the time. The length of lag phase of lipid hydroperoxide and hexanal indicate the lipid oxidation rate in which the shorter lag phase has the higher oxidation rate. As reported in Table 1, the lag phase for the control oil without DOPC was 20 days. The addition of DOPC at 1,000 μmol/kg oil reduced the lag time to 13 days indicating that DOPC was pro-oxidative as previously reported [7, 10, 12]. At concentrations above its CMC, DOPC could form reverse micelles which are able to increase lipid oxidation rates by attracting pro-oxidative factors such as lipid hydroperoxides and transition metals to the water–oil interface [3, 7]. In addition, DOPC can accelerate lipid oxidation through its surface activity which can reduce the surface tension and increase the oxygen diffusion rate from the headspace to the oil [6]. Free fatty acids at 0.5 % (by wt) exhibited strong pro-oxidant activities regardless of the type of free fatty acids by decreasing the lag phase to 9–10 days. Several mechanisms have been proposed to explain the pro-oxidative effects of free fatty acids. Miyashita and Takagi [16] reported that free fatty acids could accelerate decomposition of lipid hydroperoxides and could bind metals to make them more pro-oxidative. Moreover, Mistry and Min reported that free fatty acids could reduce surface tension and increase oxygen diffusion rate from headspace into the oil [28]. There was no significant difference in lag time observed for bulk oil containing free fatty acids in the absence or presence of DOPC. These data suggest that the presence of DOPC did not alter the pro-oxidant activity of free fatty acids at this concentration. Similar trends were also noticed for hexanal formation, whose lag phases are shown in Table 1.

Table 1 Lag time of lipid hydroperoxide and hexanal formation in bulk oil containing different free fatty acids (0.5 % by wt) without or with addition of DOPC (1,000 μmol/kg oil) during storage at 55 °C in the dark

Fatty acids	Lag time (days)			
	Lipid hydroperoxide formation		Hexanal formation	
	Without DOPC	With DOPC	Without DOPC	With DOPC
Control	20[a]	13[b]	20[a]	14[b]
Myristoleic acid (14:1)	9[c]	9[c]	9[c]	9[c]
Oleic acid (18:1, *cis*)	10[c]	10[c]	10[c]	10[c]
Elaidic acid (18:1, *trans*)	10[c]	10[c]	10[c]	10[c]
Linoleic acid (18:2)	9[c]	9[c]	9[c]	9[c]
Eicosenoic acid (20:1)	9[c]	9[c]	9[c]	9[c]

[a,b,c] Represent significantly different for mean value sharing different letter in each row ($p \leq 0.05$, $n = 3$)

Our previous reverse micelle formation study revealed that increasing the concentration of oleic acid up to 3 % (by wt) caused the CMC of DOPC to increase to 1,000 μmol/kg oil. To investigate whether DOPC at concentrations below and above its CMC would impact the pro-oxidant activity of free fatty acids differently, DOPC at 200 and 1,500 μmol/kg oil were added to the bulk oil in combination with oleic acid at 3 % (by wt). Figure 6 shows that the duration of lag phase decreased with increasing the concentrations of DOPC compared to the control. The addition of DOPC at 200 and 1,500 μmol/kg oil decreased the lag phase of both lipid hydroperoxide and hexanal formation to 14 and 10 days, respectively. Oleic acid by itself at 3 % (by wt) showed strong pro-oxidant activity by reducing the lag phase of lipid hydroperoxide and hexanal to 5 days. However, no significant differences in pro-oxidant activity were found between the bulk oil containing 3 % (by wt) of oleic acid without and with DOPC at 200 and 1,500 μmol/kg oil again suggesting that the presence of a DOPC reverse micelle had little impact on the pro-oxidant activity of free fatty acids. This could be attributed to the molar ratio of free fatty acids used in this model system where free fatty acids were over 70 times higher than that of DOPC (e.g. oleic acid (3 % by wt) equal to 1.06×10^5 μmol/kg oil compared to DOPC at 1,500 μmol/kg oil. In commercial refined oils free fatty acids would also be in large excess to phospholipids at a

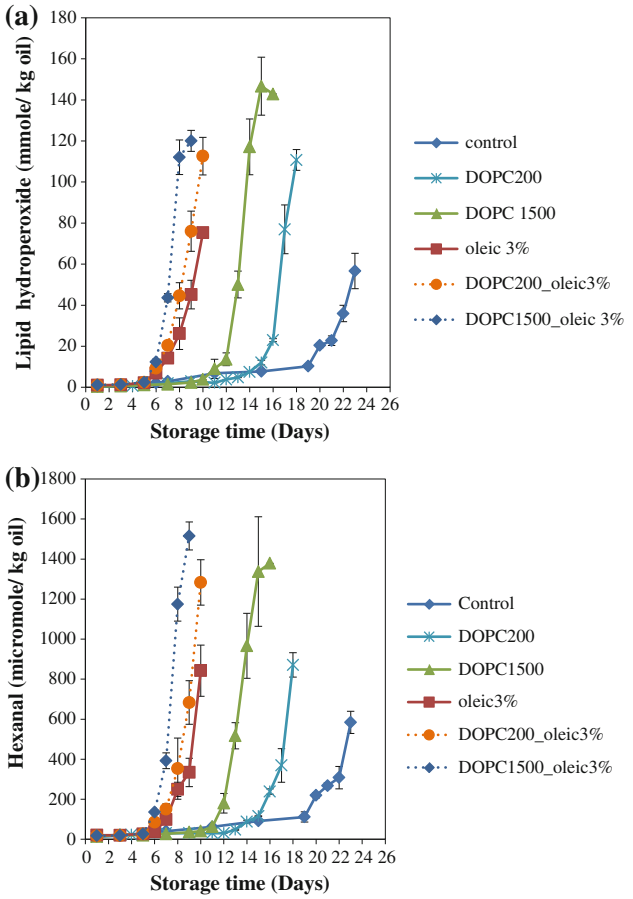

Fig. 6 Formation of lipid hydroperoxide (**a**) and hexanal (**b**) in bulk oil containing oleic acid (3 % by wt) without or with addition of DOPC at 200 and 1,500 μmol/kg oil during storage at 55 °C. Data points represent means ($n = 3$) ± standard deviations. Some *error bars* lie within data points

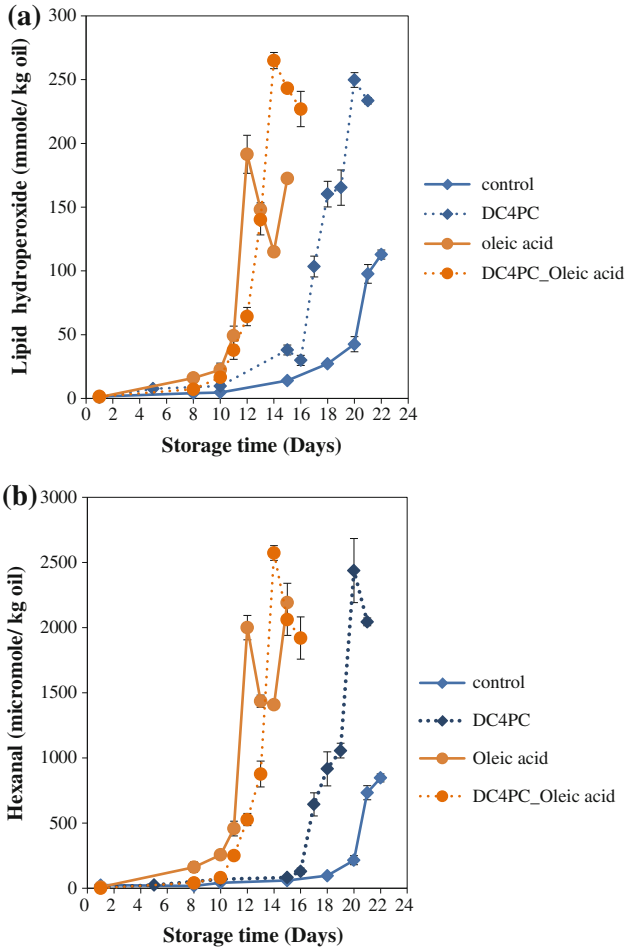

Fig. 7 Formation of lipid hydroperoxide (**a**) and hexanal (**b**) in bulk oil in the presence/absence of oleic acid (0.5 % by wt) with/without addition of DC_4PC (1,000 μmol/kg oil) during storage at 55 °C. Data points represent means ($n = 3$) ± standard deviations. Some *error bars* lie within data points

ratio of 10–50 [1]. Thus, it is plausible that the majority of free fatty acids reside in the bulk oil phase and not in the reverse micelles and thus exert their pro-oxidative activity by catalyzing the decomposition of lipid hydroperoxides and forming pro-oxidative complexes with metals, regardless of the existence of DOPC [6, 16, 28].

To confirm that the presence of reverse micelle structure had no impact on the pro-oxidant activity of free fatty acids, an experiment was conducted with and without addition of DC_4PC (at 1,000 μmol/kg oil) to bulk oil containing 0.5 % (by wt) of oleic acid.. DC_4PC is a phosphatidylcholine containing an identical head group as DOPC but with different fatty acyl residues. By having butyric acid as the hydrophobic tails, DC_4PC does not form reverse micelles in bulk oil according to both CMC and the SAXS results (data not shown). This finding is in agreement with other reports that short chain phospholipids are likely present in the form of monomers [12, 29]. Figure 7 shows that the presence of DC_4PC decreased the lag phase of lipid hydroperoxide and hexanal formation as compared to that of control. The possible explanation for pro-oxidant activity of DC_4PC is that the negative charge on the head group of DC_4PC could attract transition metals and increase their pro-oxidative activity [12]. However, no significant differences in the lipid hydroperoxide and hexanal formation were observed between the oils containing oleic acid with and without added DC_4PC. These findings again suggest that free fatty acids accelerate lipid oxidation in the manner that independent on the existence of reverse micelle structures.

Conclusions

The rate and mechanism of lipid oxidation in bulk oils containing association colloids likely depends on the total amount and the ability of surfactants to form aggregates. Free fatty acids were shown to possess lower surface

activity compared to DOPC as observed from the inability of free fatty acids to form aggregates according to the CMC and SAXS results. Free fatty acids were found to partition into DOPC reverse micelles as seen by the ability of free fatty acids to decrease the pH of the aqueous phase of the micelles and change the CMC of DOPC at high fatty acid concentrations. Reverse micelles formed by DOPC could increase the lipid oxidation rate. However, the pro-oxidant activity of free fatty acids is independent of the presence or absence of reverse micelles.

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