Effect of fatty acids on self-assembly of soybean lecithin systems

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22 **ABSTRACT**

23 With the increasing interest in natural formulations for drug administration and
24 functional foods, it is desirable a good knowledge of the phase behavior of lecithin/fatty
25 acid formulations. Phase structure and properties of ternary lecithin/ fatty acids/ water
26 systems are studied at 37 °C, making emphasis in regions with relatively low water and
27 fatty acid content. The effect of fatty acid saturation degree on the phase microstructure
28 is studied by comparing a fully saturated (palmitic acid, C16:0), monounsaturated (oleic
29 acid, C18:1), and diunsaturated (linoleic acid, C18:2) fatty acids. Phase determinations
30 are based on a combination of polarized light microscopy and small-angle X-ray
31 scattering measurements. Interestingly, unsaturated (oleic acid and linoleic acid) fatty
32 acid destabilizes the lamellar bilayer. Slight differences are observed between the phase
33 diagrams produced by the unsaturated ones: small lamellar, medium cubic and large
34 hexagonal regions. A narrow isotropic fluid region also appears on the lecithin-fatty
35 acid axis, up to 8 wt% water. In contrast, a marked difference in phase microstructure
36 was observed between unsaturated and saturated systems in which the cubic and
37 isotropic fluid phases are not formed. These differences are, probably, a consequence of
38 the high Krafft point of the C16 saturated chains that imply rather rigid chains.
39 However, unsaturated fatty acids result in more flexible tails. The frequent presence of,
40 at least, one unsaturated chain in phospholipids makes it very likely a better mixing
41 situation than in the case of more rigid chains. This swelling potential favors the
42 formation of reverse hexagonal, cubic, and micellar phases. Both unsaturated fatty acid
43 systems evolve by aging, with a reduction of the extension of reverse hexagonal phase
44 and migration of the cubic phase to lower fatty acid and water contents. The kinetic
45 stability of the systems seems to be controlled by the unsaturation of fatty acids.

46 **KEYWORDS**

47 Soybean Lecithin; fatty acids; palmitic acid; oleic acid; linoleic acid; small-angle X-ray
48 scattering

49

50 **INTRODUCTION**

51 Phospholipids and fatty acids are mimic models of biological membranes because of the
52 formation of lamellar lyotropic liquid crystal. On the other hand, from the majority of
53 studies it becomes evident that dietary phospholipids have a positive impact on several
54 diseases and reduce side effects of some drugs. Both effects have partially been
55 explained by the delivery of their fatty acid residues for incorporation into the
56 membranes of cells involved in different diseases, e.g. immune or cancer cells.[1]
57 Lecithin is a highly compatible lipid and an important constituent of biological
58 membranes. The phase behavior in lecithin/water and related systems has been
59 extensively studied by Luzzati and coworkers.[2, 3] In light of the interest in the
60 applications of this kind of amphiphilic bilayer structure in fields such as natural
61 formulations for drug administration and functional foods, the interaction between fatty
62 acids and lipids has been studied.

63 Amphiphilic substances can destroy the lipid membranes and transform them into
64 surfactant–lipid micelles (with surfactants) or into hexagonal and cubic phases (with
65 fatty acids).[4] Lipids with cationic surfactants manifest extensive swelling of the lipid
66 lamellar phase, with a strong dependence of lipid–surfactant electrostatic interaction
67 effects on the lipid phase state.[5] Whereas, in the presence of nonionic surfactants such
68 as Triton X-100,[6] there is no detectable swelling of the lamellar phase. Furthermore,

69 interaction of a lipid with an anionic surfactant results in the breakdown of the lamellar
70 structures.[7, 8]

71 The interactions between lipid and polymer have also been studied.[8] In the case of
72 lecithin/ polyethylene glycol (PEG)/ water systems, neither a significant change in the
73 stability of the lamellar phase nor any effect on the chain length of the ethylene oxide
74 groups was detected.[9] Remarkably, the extent of swelling in the presence of a
75 hydrophobically modified PEG polymer (PEG 40 monostearate) [10] is more
76 comparable to the addition of ionic surfactants to the lecithin /water system than other
77 PEG [9, 11] or PEG-based surfactants [12].

78 Phase properties of systems with lecithin, water, and several oils have extensively been
79 studied, in particular for cyclohexane,[13] decane,[14] isooctane,[14] and different ester
80 derivatives of fatty acids such as isopropyl myristate,[15, 16] or isopropyl
81 palmitate.[17] Moreover, in the presence of fatty acid esters, there is a large portion of
82 the phase diagram occupied by a three-phase coexistence region consisting of water, oil,
83 and lamellar phase (in place of microemulsion as found in Winsor equilibrium).[13, 16,
84 17] The differences in the behavior have been explained by the different ability of the
85 oils to swell the lecithin tails.[17]

86 Other aqueous systems based on lecithin and biocompatible oils,[18] in the presence of
87 alcohols that decreases the rigidity of the lipid bilayers, form microemulsions and
88 emulsions,[19-24] which have been used in several cases to inspect formulations for
89 drug delivery.[25-27] The extension of the microemulsion phase decreases with
90 increasing alcohol chain length in systems composed of lecithin, water, alcohol and
91 triglyceride, soybean oil, miglyol 612 or oleic acid as oil phases. [15, 19, 20] The

92 stabilizing effect of lecithin with the fluidifying effect of ethanol can solubilize a large
93 amount of water in microemulsions based on long chain glycerides such as Peceol®
94 [28] (that also contains oleic, linoleic and other saturated acyl chains). This system
95 forms isotropic reverse L₂, reverse hexagonal phase and lamellar phases, as a function
96 of water content. Isopropyl myristate, soybean lecithin, water, ethanol and different
97 sugar-based surfactants can also form microemulsions in addition to other “gel” phases,
98 which microstructures were not studied.[29]

99 Furthermore, the addition of saturated fatty acids to phosphatidylcholine lipids has been
100 shown to promote the formation of reverse liquid crystalline structures. Thus,
101 phosphatidylcholine/ fatty acid mixtures in water may also form reverse hexagonal and
102 cubic phases at higher lecithin contents.[4] The longer the chain length in a homologous
103 series, the greater the preference for the reverse hexagonal phase at the expense of the
104 reverse bicontinuous cubic structure.[30] The fluid lamellar phase is suppressed in
105 phosphatidylcholine / fatty acid 1:2 (mol:mol) mixtures, being replaced by a solid-like
106 reverse hexagonal phase.[31] For chain length of C₁₆ and greater, the reverse hexagonal
107 phase is formed directly upon chain melting. Contrary to saturated fatty acids, oleic acid
108 exhibits quite different phase behavior, which was ascribed to a minor effect on the
109 hydration of the dipalmitoylphosphatidylcholine bilayer.[32] For mixtures of oleic acid
110 with dioleoylphosphatidylcholine or dioleoylphosphatidylethanolamine, it has been
111 recently reported that increasing the content of oleic acid increases the negative mean
112 curvature of the bilayer, with the occurrence of an reverse cubic phase *Fd3m*. [33]

113 However, a drawback in this field is the lack of a sufficient number of comprehensive
114 phase diagrams outlining the principles of the lipid-fatty acid interactions. In this paper,
115 we focus on the comparison of the phase structure of soybean lecithin systems with

116 fatty acids of the same chain length but different degree of saturation: monounsaturated
117 (oleic acid, C18:1) and polyunsaturated (linoleic acid, C18:2), and the fully saturated
118 palmitic acid (C16:0). The behavior has been studied at approximately the body
119 temperature, 37°C. Microstructure characterization has been performed by optical
120 microscope with crossed polarizers and small angle X-ray scattering (SAXS)
121 experiments.

122

123 **EXPERIMENTAL SECTION**

124 **Materials and Methods**

125 **Sample Preparation.** The zwitterionic phospholipid soybean lecithin (Epikuron 200®)
126 was obtained from Degussa and was used without further purification. This lecithin is
127 strictly a mixture of phosphatidylcholines with acyl chains of different lengths and
128 degrees of unsaturation. The main component (at 68–70 wt %) is linoleic acid. The
129 presence of high unsaturation (>84%) in the acyl chains brings down the melting
130 temperature below 25 °C. The lecithin contains about 0.8 wt % water, according to
131 Degussa. Fatty acids (99.2 % purity) from Sigma-Aldrich® are palmitic (PAL, C16:0),
132 oleic (OA, omega-9 C18:1) and linoleic (LA, omega-6, C18:2) and water was
133 deionized.

134 All the samples were prepared by weighing the appropriate amounts of each component
135 in small screw-cap vials. In order to mix the samples and remove bubbles, each mixture
136 was centrifuged inverting the vials many times per day for several days at 4000 rpm and
137 40°C, until macroscopic homogeneity was reached. Once the samples were ready, they
138 were stored in the dark in a water bath at 37°C to obtain the different ternary lecithin/

139 fatty acid/ water diagrams. Those are given in a triangular Gibbs representation, with
140 lecithin, water, and the respective fatty acid as the corners of the triangle. Sections of
141 interest at the lecithin-rich corner of the ternary diagrams are presented in weight
142 percent. All samples are labeled in this paper as content of lecithin/ fatty acid (in wt%).
143 Water composition is determined by the remaining weight to obtain 100 wt %.
144 Although theoretical interpretation could be easier using pure components, we prefer
145 the commercial multicomponent soybean lecithin because it is the more widely used in
146 everyday life in human and animal food, medicine, cosmetics, and pharmaceuticals.

147

148 **Polarized Light Microscopy.** Birefringent textures of lyotropic liquid crystals were
149 investigated by using a Nikon ECLIPSE 50i optical microscope thermostatted at 37°C
150 equipped with crossed polarizers. Lyotropic liquid crystal structures were assigned to
151 the different samples according to the observed textures; these observations were
152 pursued from two weeks to one year as the aggregation state of the samples change with
153 time. Selected samples in the single-phase regions were considered for further
154 characterization.

155

156 **Small-Angle X-ray Scattering (SAXS).** SAXS measurements were carried out using a
157 Kratky camera of small angle (M-Braun) coupled to a Siemens KF 760 (3KW)
158 generator. Nickel filtered radiation with wavelength 1.542 Å (Cu K_α line). The detector
159 was a linear PSD-OED 50 M-Braun. Temperature was controlled (37°C) by a Peltier
160 KPR AP PAAR model. Liquid samples were contained in a glass capillary 1 mm
161 diameter and 10 μwall thickness. Because of their high viscosity, the liquid crystalline
162 samples were inserted between two Mylar[®] sheets with a 1 mm separation.

163 The SAXS scattering curves are shown as a function of the scattering vector modulus,
164

$$165 \quad q = \frac{4\pi}{\lambda} \sin \frac{\theta}{2} \quad (1)$$

166

167 where θ is the scattering angle. The q values in our set-up ranged from 0.1 nm^{-1} to 6.0
168 nm^{-1} . The system scattering vector was calibrated by measuring a standard silver
169 behenate sample (> 98% RoseChemicals Ltd.). Because of the use of a linear beam the
170 scattering curves are mainly smeared by the convolution of the beam length with the
171 detector width. This smearing, mainly produces a widening and distortion of the peaks
172 without strong effects on the peak position.

173 The structure of the lyotropic liquid crystalline phases was determined from the relative
174 positions of the SAXS diffraction peaks. For the hexagonal (cylindrical assemblies
175 crystallized in a two-dimensional hexagonal lattice) structures, the position of the peaks
176 should obey the relationships $1:\sqrt{3}:\sqrt{4}:\sqrt{7}:\sqrt{9} \dots$. The lattice parameter a (distance
177 between the centers of adjacent cylinders) of the hexagonal structure can be obtained
178 from the position (q^*) of the first (and frequently most intense) diffraction peak given
179 by (2):

$$180 \quad a = \frac{4\pi}{q^* \sqrt{3}} \quad (2)$$

181 The internal radii (R_c) of the cylinders depends on the dispersed phase volume fraction
182 and the repetition distance as: [31]

$$183 \quad R_c = \sqrt{\frac{2\phi_c d^2}{\pi\sqrt{3}}} \quad (3)$$

184 Where d is the Bragg repetition distance $d = 2\pi / q^*$ and ϕ_c corresponds to the dispersed
185 phase volume fraction.

186 The corresponding area per molecule (A_m) can be calculated as

187
$$A_m = \frac{2V_c}{R_c} \quad (4)$$

188 where V_c corresponds to the dispersed phase volume per molecule.

189 The calculation of the area per molecule in the reverse hexagonal structure involved the
190 determination of the polar volume fractions. The volume of one molecule of lecithin, v_p
191 $\sim 1257 \text{ \AA}^3$ was calculated from the Epikuron 200 composition and using a value for the
192 polar headgroup of $326 \text{ \AA}^3$. [32] The contribution of the diverse hydrophobic volumes
193 has been obtained from the manufacturer's composition statements. Linoleic acid
194 volume is $516 \text{ \AA}^3$ and oleic acid volume is $525 \text{ \AA}^3$ according to their molecular weights
195 and liquid densities. The polar volume for the acids has been taken as $45 \text{ \AA}^3$. [34]

196 The microstructure of the micellar cubic phase has been determined applying the
197 Minamikawa and Hato model for the Fd3m phase. [35] The sizes of the reverse micelles
198 are based solely on the volume of the water pool plus the hydrated polar heads. This
199 volume has been defined considering the polar volume of the phospholipid,
200 corresponding to the choline+ glycerol and the carboxylic group of the fatty acid. The
201 continuous phase of the cubic liquid crystal corresponds to the hydrophobic residues of
202 the lipids implied in the structure; hence, both the phospholipid and the fatty acid
203 hydrophobic chains correspond to the continuous phase. The total surface corresponds
204 to the summation of the interface produced by the phospholipid and fatty acids and,
205 therefore, those intercalate with the phospholipids in the model.

206 For isotropic fluid samples, the SAXS curves were fitted to a water-in-oil core-shell
207 ellipsoid of revolution model with a hard-sphere structure factor with arbitrary volume
208 fraction and radii of interaction. [36] The area per molecule has been calculated based
209 only on lecithin molecules. The absolute scaling is correct to within a 10 % considering
210 the inner polar part formed by water + lecithin polar head groups.

211

212 **RESULTS AND DISCUSSION**

213 We have studied the ternary phase diagrams of systems containing lecithin/ fatty acids/
214 water, at 37 °C and with special emphasis in the high lecithin concentration region. The
215 effect of the degree of saturation in the alkyl chain and length of fatty acid have been
216 considered. For each fatty acid, a large number of samples have been prepared and
217 allowed to ripen for more than six months. Phase boundaries are delimited with errors
218 smaller than 3 wt %. Since several months are the time-window of interest in most
219 technical applications, we will illustrate the short-time phase appearance (at three-
220 months) and in a subsequent section, we discuss the long-time phase diagrams that are
221 attained at the time scale of 6/12 months. However, the liquid crystalline samples need
222 many months to attain "true" equilibrium, and structural transformations among
223 different phases may occur during aging.[10] Under this consideration, the phases
224 diagrams studied in this work may not correspond to the final thermodynamic
225 equilibrium. For this reason, we use the terminology "phase appearance" instead of
226 "phase behavior".

227 In the corner of low lecithin content, spherulites were easily recognized with polarized
228 optical microscopy because of Maltese cross textures. However, those lamellar

229 dispersions and emulsions are out of the scope of this work; they were no studied in
230 detail, and those zones of the triangle diagram are not shown. Many of those samples
231 show macroscopic phase separation in few days or weeks.

232

233 *Short-time phase appearance of soybean lecithin/fatty acid/water systems.* Fig. 1
234 shows sections of the soybean lecithin/ fatty acid/ water ternary diagrams observed at
235 short-time (3 months after sample preparation). The effect of the different degree of
236 saturation and the hydrophobic group on the phase microstructure are studied for a
237 series of fatty acids: fully saturated (palmitic acid, C16:0), monounsaturated (oleic acid,
238 C18:1) and diunsaturated (linoleic acid, C18:2). Phase boundaries were delimited using
239 both optical microscopy with crossed polarizers (by texture assignation) and small angle
240 X-ray scattering (SAXS). The ternary diagrams of lecithin-fatty acids mixtures revealed
241 the formation of a variety of non-lamellar structures (reverse hexagonal and cubic
242 crystalline regions, and reverse micellar solution) separated by multiphase regions,
243 where these liquid crystalline phases coexist. Multiphase samples are very viscous,
244 cloudy and birefringent fluids. Samples in the surroundings of cubic crystalline region
245 are solid-like, and they do not flow after inverting the vial. Analyses with
246 complementary techniques always detected the coexistence of the different liquid
247 crystalline phases with different proportions, but without macroscopic phase separation.

Figure 1. Ternary diagrams for lecithin/ fatty acid/ water systems, at 37 °C and obtained three months after sample preparation. From left to right: (a) palmitic acid; b) oleic acid; c) linoleic acid. Phase abbreviations are as follows: L_2 , reverse micellar solution phase; I_2 , reverse cubic phase; H_2 , reverse hexagonal phase; $L\alpha$, lamellar phase. The different pure regions are separated by multiphase regions with the coexistence of several liquid crystalline phases.

All those systems self-assemble into lamellar structures. However, the bilayer can incorporate very limited amount of fatty acid, less than c.a. 3 wt% (6 wt% for the palmitic acid system). A reverse hexagonal microstructure is preferred. A similar effect has been reported for other phospholipid bilayers containing phosphatidylcholine/fatty acid mixtures.[4, 31] The reverse hexagonal liquid crystalline regions have been delimited by optical microscopy textures (Fig. S1, in supporting information) and they are sustained by SAXS experiments (discussed below). The extension of the hexagonal liquid crystalline regions was found depend slightly on the fatty acid unsaturation

264 degree, being much larger for unsaturated fatty acids than for the saturated palmitic acid
265 (Fig. 1). The water uptake in the reverse hexagonal phase reaches c.a. 25 wt% for the
266 unsaturated fatty acids whereas this value is less than 10 wt% for the palmitic acid
267 system.

268 For the unsaturated fatty acid systems, an isotropic fluid phase was found along the
269 lecithin-fatty acid binary axis. It is designed as L_2 phase and it is made of disconnected
270 reverse micelles (mainly with globular morphology according to SAXS). Solubilization
271 of water in that L_2 isotropic region is nearly the same for both linoleic and oleic systems
272 (c.a. 8 wt%. water). However, a larger amount of lecithin is solubilized by the linoleic
273 acid (up to 60 wt%.) than by oleic acid (c.a. 50 wt%).

274 Between isotropic fluid and reverse hexagonal phase regions, a cubic phase is formed in
275 systems with unsaturated fatty acids: linoleic and oleic acids (Fig. 1). This phase is
276 clear, transparent, nonbirefringent and does not flow. The corresponding areas are quite
277 small compared with the reverse hexagonal regions. The presence of unsaturation and
278 its degree seem to be the most important effect since lecithin-palmitic acid system does
279 not form the cubic liquid crystalline phase and that phase is larger for the linoleic acid
280 (diunsaturated) than for oleic acid (monounsaturated).

281 Further characterization and phase boundary determinations have been done by SAXS
282 experiments. In Fig. 2a, the SAXS spectrum of a sample with a composition 85 %
283 lecithin / 13 % oleic acid/ 2 % water (in wt%.) in the reverse hexagonal phase is shown.
284 In the inset, the photomicrograph between crossed polarizers indicates the characteristic
285 fan-like hexagonal texture. Bragg peak reflections are indicated by arrows in the SAXS
286 pattern (Fig. 2a). The relative positions of the peaks resolved in the diffraction spectrum

287 correspond to the $1:\sqrt{3}:\sqrt{4}:\sqrt{7}:\sqrt{9}:\sqrt{12}$ relationship. They reveal the high ordering for the
288 bidimensional hexagonal (H_2) structure, with a very weak 2nd peak. This feature, the
289 second peak being weaker than the third peak, seems to be distinctive of all reverse
290 hexagonal phases encountered here with oleic and linoleic acid. This weakness should
291 be the result of a minimum in the cylinders from factor of these structures. The nearest
292 neighbor (cylinder) distance (a) and the area per molecule (A_m) were calculated as
293 described in the experimental section, and the results are given in Table 1. For both
294 unsaturated fatty acids, increasing the water content increases the area per molecule,
295 which could be due to the increase of the lateral repulsion between the headgroups
296 induced by a more complete hydration. At a same composition, values of all structural
297 parameters are slightly bigger for oleic acid samples than for the linoleic acid samples.
298 It could be a consequence of the effect of unsaturation on the chain length.

299

Figure 2. SASX spectra recorded 2 weeks after sample preparation. **a)** Sample 85 % lecithin/ 13 % oleic acid/ 2 % water (in wt%), in the H_2 region. Arrows show the characteristic reflections. **b)** Sample 55 % lecithin/ 37 % oleic acid/ 8 % water (in wt%), in the two-phase H_2 +cubic region. Lines show the reflection positions for an $Fd3m$ cubic phase. Both insets: photomicrographs of corresponding samples between crossed polarizers.

Table 1. Structural parameters of reverse hexagonal hphase in the oleic acid and linoleic acid systems. R_c , internal radii of the cylinders; A_m , the area per molecule; a , lattice parameter (or distance between adjacent cylinders).

Sample Lec/fatty acid/water (wt. %)	R_c (nm)	A (nm ²)	a (nm)
LINOLEIC ACID			
64/ 21/ 15	1.48	0.74	5.22
87/ 3/ 10	1.58	0.60	5.24
90/ 5/ 5	1.38	0.59	4.90
OLEIC ACID			
87/ 3/ 10	1.62	0.65	5.40
90/ 5/ 5	1.39	0.58	4.96
90/ 8/ 2	1.49	0.49	5.56

The complex polymorphic behavior obtained for these systems is shown in Fig. 2b. The SAXS spectrum of the sample of composition 55 % lecithin/ 37 % oleic acid/ 8% water (in wt %), located in the multiphase region, displays the relative reflection positions for a $Fd3m$ cubic phase (reflections at positions $\sqrt{3}$, $\sqrt{8}$, $\sqrt{11}$, $\sqrt{12}$, $\sqrt{16}$, $\sqrt{19}$, $\sqrt{24}$, $\sqrt{27}$, $\sqrt{32}$, $\sqrt{35}$, $\sqrt{36}$, $\sqrt{40}$, $\sqrt{43}$, $\sqrt{44}$, $\sqrt{48}$, $\sqrt{51}$, $\sqrt{56}$...) [37]. The main peak slowly decreases and is related to other two peaks also decreasing at relative positions $\sqrt{3}:\sqrt{8}:\sqrt{11}$, which are characteristic reflections of the face-centered cubic space group $Fd3m$. Microstructure of this reverse cubic phase is formed by two types of reverse micelles with two different radii, which have crystallized into a cubic lattice. $Fd3m$ structure involves 24 spherical

micelles per unit cell, with a bimodal size distribution composed of 8 larger micelles in a tetrahedral diamond-like lattice and 16 smaller micelles in tetrahedral clusters at the four octants of the cell. The polar radii of the two types of micelles of this structure are 2.1 nm for the small micelles and 2.5 nm for the large micelles. These values have been obtained using the model of Minamikawa and Hato [35] and using a mean area per molecule of 0.5 nm^2 . The same model produces a total minimum radius for the small micelles of 2.9 nm and 3.5 nm for the large ones, compatible with the large radii of the ellipsoids of the fluid (see below). Those radii are comparable with those obtained by Tyler et al. from electron density mapping of DOPC:DOG (1,2 oleoylphosphatidylcholine; 1,2-diacylglycerol), which are 3.5 and 4.6 nm for samples containing 19 % water and 1:2 molar ratio.[38] Although there are not apparently evidences in the SAXS spectra, according to the polarized light microscopy this cubic structure coexists with a H_2 liquid crystalline phase. Thus, the photomicrograph given in the inset shows optical birefringence (fan-like texture) attributed to the reverse hexagonal structure in the mixture, and black areas that are related to the isotropic matrix of the cubic phase. As indicated before, samples evolve and birefringence of that sample vanishes totally after 4 months, which was reflected in the SAXS curve at long-time study (with the disappearance of the peaks but preserving the overall form of the curve). Thus, the sample of composition 55 % lecithin/ 37 % oleic acid/ 8 % water (in wt%), (H_2 +cubic region) progresses by aging to a more disordered structure that could correspond to a glass.

The location of the pure cubic phase in the ternary diagram (between the micellar solution and the reverse hexagonal liquid crystalline phase) agrees well with the proposed microstructure.[39] This cubic structure is predominantly produced in the

346 presence of two differently hydrophobicity molecules, like in mixtures of diglyceride/
347 phosphatidylcholine[37] and the systems dioleoylphosphatidylcholine or
348 dioleoylphosphatidylethanolamine in the presence of oleic acid. In the last case, the
349 *Fd3m* cubic phase has been ascribed to an increase in the negative mean curvature of
350 the bilayer by the addition of the oleic acid. [33] The *Fd3m* microstructure allows for a
351 partial segregation of the two components between the two types of micelle: (i) those
352 with the smaller polar head molecules (i.e., the fatty acid) segregating preferentially to
353 the small micelles; (ii) and those with the phospholipid segregating mainly to the big
354 ones. Hence, the cubic phase position on the ternary diagram may be due to the
355 tendency of these reverse micelles to organize itself in a regular manner. The different
356 interfacial curvature between both types of micelles may favor the partial segregation of
357 the components in aggregates of different curvatures. In addition, the transverse
358 interactions between adjacent lipid layers (Van der Waals, hydration, fluctuation,
359 electrostatic) participate in stabilizing the structures. For reverse non-lamellar phases,
360 the layer separations (i.e. lattice parameters) and interfacial curvatures are strongly
361 coupled. [40]

362

363 ***Long-time phase appearance of soybean lecithin/ fatty acid/ water systems.*** All the
364 above-mentioned findings correspond to a time scale of three months. After about six
365 months of ripening, changes in the microstructure were observed (Fig. 3). At the time
366 window of six months, the extent of the hexagonal region is greatly reduced for both
367 unsaturated fatty acids systems while the isotropic phase L_2 is less affected. Reverse
368 hexagonal phase is constrained in the lecithin rich-corner, and it is formed at water
369 content lower than c.a. 17 wt. %. Lecithin-palmitic acid system showed no markedly

aging effect and for that reason, we do not consider it longer in this section. Nevertheless, the more notable effect is the displacement of the cubic region to higher lecithin and lower fatty acid contents (compare Fig. 1 and Fig. 3). This aging effect, as other authors suggested in different lipid structures,[41] may involve the acid hydrolysis of the ester bond present in the phospholipid chains, resulting in the formation of a fatty acid and a single-chain lipid that produce bilayer fragments[41]. This effect could explain the smaller amount of fatty acid needed for the cubic phase formation.

Figure 3. Ternary diagrams of the systems lecithin/ oleic acid/ water (a) and lecithin/ linoleic acid/ water (b), at 37°C with single-phase areas after six months of sample equilibration. Phase abbreviations are as follows: L₂, reverse micellar solution phase; I₂, reverse cubic phase; H₂, reverse hexagonal phase; L α , lamellar phase. The different pure regions are separated by multiphase regions with the coexistence of the different liquid crystalline phases.

The different phase boundaries were assigned after microscopic observation under polarized light. However, the system is very complex, and SAXS analysis was required. In this section, we describe the SAXS patterns of different phases after six months of sample equilibration. For the sample of composition 78 % lecithin/ 12 % linoleic acid/ 10 % water (in wt %) (Fig. 4a), the vertical lines show the characteristic *Fd3m* cubic spacing. However, in this sample we appreciated some optical birefringence, which is an indication of the coexistence of H₂ phase. Moreover, in the spectrum of sample 75 % lecithin/ 23 % linoleic acid/ 2 % water (in wt %.) only one peak is apparent in logarithmic scale (Fig. 4b). However, notably, in linear scale the other peaks are more apparent (see Supplementary information, Figure S2). According to the macroscopic observation, this is a clear, non-fluid, stiff, and isotropic phase, which could correspond to cubic phase. Nevertheless, the peak assignation could better correspond to the hexagonal diffraction pattern. The upper arrow in figure 4b could correspond to a different phase, probably cubic according its isotropic microscopy appearance. This is an example of the complex phase behavior of these systems with slowly reaching equilibrium.

403

404

405

406 **Figure 4.** Representative SAXS diffraction patterns of different phases after six months
 407 of samples preparation: a) 78 % Lecithin/ 12 % linoleic acid/ 10 % water (in wt%).
 408 Lines show the $Fd3m$ cubic spacing characteristic reflections. In this sample, because of
 409 optical birefringence, H_2 coexist; b) 75 % Lecithin/ 23 % linoleic acid/ 2 % water (in wt
 410 %.), in the border of cubic-reverse hexagonal phase. The downward arrows mark the

411 position of the peaks for the hexagonal order; the upward arrow may correspond to the
412 presence of a different phase.

413

414 Recorded SAXS spectra and the corresponding fitting model for samples in the reverse
415 micellar phase are given in Fig. 5. SAXS patterns for samples at the fluid isotropic
416 phase show a band around $0.16 \text{ \AA}^{-1}$. The slit-smeared curves have been fitted to a water-
417 in-oil ellipsoid of revolution model with a hard-sphere structure factor in which both the
418 volume fraction and radii of interaction have been let free. Of course, this is only a
419 crude approximation that should work well only for not very anisometric particles. In all
420 cases, there is a good agreement of fitting curves (lines in Fig. 5) with the experimental
421 spectra (symbols in the Fig. 5). The best results correspond to slightly prolate ellipsoids.

422

423 **Figure 5.** SAXS spectra of samples in the L_2 phase. Compositions are given in the inset.

424 a) System with linoleic acid; b) system with oleic acid. Symbols are the experimental
 425 data; lines are the fitting curves to a water-in-oil ellipsoid of revolution model with
 426 hard-sphere structure factor.

427

428 The effect of composition on the micellar aggregate size is summarized in Table 2.

429 Three volume fraction ϕ values are shown; the first column has been determined as the
 430 summation of the volumes of the polar heads plus the water pool (the corresponding

volumes for the phospholipid headgroup and carboxylic acid groups are given in the materials and methods sections). The second column, ϕ_{Lec} , corresponds to the summation of the water and the whole lecithin molecule. The third column, ϕ_{fit} , corresponds to the best fit of the spherical hard sphere potential. From those values we can infer that, because of the low hydration, not all lecithin participates in the dispersed phase but, rather, the continuous phase is constituted by most of the fatty acid plus the amount of not hydrated lecithin. If we consider the hydration of lecithin to involve as a very minimum two molecules of water, one per charged group, then 2% water allows for the hydration of lecithin to reach a total volume fraction around 0.4. This value coincides quite well with the fitted volume fraction. Therefore, the area per molecule (A_m) has been calculated based on the geometry and the consideration of only lecithin and water forming the core of the reverse micelles. This implies considering the fatty acid as the continuous medium and, implicitly, the non-micelle lecithin. Further, the absolute scaling is correct to within a 10 % with these assumptions. For both fatty acids, series of micellar samples with the same amount of water are considered (2 wt% water). At fixed water content, there is not a clear dependence of the geometrical parameters with lecithin or fatty acid content variation. In addition, comparing samples with the same composition, the effect of the fatty acid unsaturation degree on the micellar size is not obvious. There is a slight tendency to increase particle volume as the volume fraction of the dispersed phase increases. The length of the ellipsoids is only about 2-4 times their thickness, but this length seems to increase as the cubic phase boundary is approached.

455

456 **Table 2.** Composition, volume fraction and geometric parameters of the water-in-oil
 457 ellipsoid of revolution (axis $a=b$ and c) and area of headgroup of lecithin per molecule
 458 (A_m).

Sample Lec/ fatty acid (wt. %)	ϕ^1	ϕ_{Lec}^2	ϕ_{fit}^3	$a=b^4$ (nm)	c^5 (nm)	A_m^6 (nm ²)
LINOLEIC ACID						
40/ 58	0.116	0.39	0.37	1.12	1.8	0.88
50/ 48	0.142	0.49	0.41	1.17	4.7	0.76
55/ 43	0.155	0.54	0.43	1.12	5.2	0.79
60/ 38	0.168	0.59	0.42	1.25	4.5	0.72
OLEIC ACID						
50/ 48	0.140	0.49	0.37	1.25	3.2	0.73
55/ 43	0.154	0.54	0.42	1.27	4.5	0.70
60/ 38	0.167	0.59	0.43	1.12	5.2	0.79
85/ 13	0.236	0.85	0.42	1.35	4.8	0.69

459 1 Lecithin polar head plus water volume fraction
 460 2 Total lecithin volume plus water volume fraction
 461 3 Volume fraction from the fitting of a hard sphere model interaction.
 462 4 Minor radii of the prolate ellipsoidal micelles corresponding to the polar core.
 463 5 Major radii of the prolate ellipsoidal micelles corresponding to the polar core.
 464 6 Area per molecule corresponding to the lecithin molecules located at the polar non polar interface of the
 465 inverse micelles calculated from the geometric parameters.
 466

467 CONCLUSIONS

468 When comparing the phase diagrams of the three lecithin/ fatty acid/ water systems, the
469 most striking feature is the destabilization of the lamellar phase and its replacement by
470 the reverse hexagonal phase induced by the unsaturation of the fatty acid. Small
471 differences were observed between the phase diagrams of the unsaturated oleic and
472 linoleic fatty acids. For the unsaturated fatty acids, the overall ternary diagram is
473 dominated by a small lamellar, medium cubic and large hexagonal region. A second
474 marked difference in the ternary diagrams between the saturated and unsaturated
475 systems is the absence of the cubic and isotropic fluid phases in the former. Thus,
476 unsaturated fatty acids (C18:1 and C18:2) show similarly complex phase diagram
477 features while saturated fatty acid (palmitic acid, C16:0) shows simpler features. The
478 differences probably are a consequence of the high Krafft point of the C16 saturated
479 chain. Additionally, phospholipids generally contain at least one unsaturated chain and,
480 therefore, it is very likely that unsaturated fatty acids result in a better mixing with the
481 phospholipid chains thus favoring the transition to reverse non-lamellar phases such as
482 the hexagonal, cubic and micellar phases. Long chain fatty acids adsorb preferentially
483 with their polar groups near the lipid headgroups, and their hydrophobic parts embedded
484 within the hydrocarbon chain region.[40]

485 Three similarities of the unsaturated fatty acids were found with respect to other
486 comparable systems[30]:

487 1) The occurrence of non-lamellar phase induced by the presence of fatty acids at
488 relatively high concentrations; 2) the limited presence (or near absence) of the lamellar
489 phase; and 3) the presence of unstable phases. The latter expressed in our studies as the
490 displacement of the boundary of the isotropic fluid region, cubic and hexagonal regions.

491 The unsaturation of fatty acid is crucial for the stability of phases. As reported for other
492 lecithin systems, the unsaturated fatty acids (oleic and linoleic acids) experience aging
493 effects with cubic phase formed at smaller fatty acid content and significant shrinkage
494 of the hexagonal phases. This aging can be due to acid hydrolysis of the ester linkages
495 present in the phospholipid chains, resulting in the formation of fatty acid and
496 lysophospholipid that produce bilayer fragments.[41] Consequently, the presence of free
497 fatty acid in the system increases due to aging, which makes that liquid crystalline
498 phases apparently migrates to areas in the phase diagram with a lower amount of fatty
499 acid. The complex phase behavior of lecithin/ fatty acid mixtures needs further studies
500 to get a comprehensive knowledge of the lipid-fatty acid interaction.

501

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572 **ASSOCIATED CONTENT**

573 **Supporting information.** Figures relevant to the photomicrograph with crossed
574 polarizers of different liquid crystalline structures and systems (Figure S1) and details
575 of the low intensity data for SAXS pattern (Figure S2). This material is available free of
576 charge via the Internet at <http://www.sciencedirect.com>

577

578 **AUTHOR INFORMATION**

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582

583 **Notes**

584 The authors declare no competing financial interest.

585

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594 **ABBREVIATIONS**

595 PAL, palmitic acid; OA, oleic acid; LA, linoleic acid; L₂, reverse micellar solution
596 phase; I₂, reverse cubic phase; H₂, reverse hexagonal phase; L α , lamellar phase; SAXS,
597 small-angle X-ray scattering.

598

599

600 SUPPLEMENTARY INFORMATION

601

602 **Figure S1.** Photomicrographs between crossed polarizers for reverse Hexagonal
603 samples, at 37°C. Magnification 20x. Left) 89% Lecithin/ 9% linoleic acid/ 2% water
604 (in wt. %); Right) 85% Lecithin/ 13% oleic acid/ 2% (in wt. %). Samples related to
605 short-time phase behavior, with equilibration time lower than 3 months.

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609

610 **Figure S2.** SAXS pattern for sample 75% Lecithin/ 23% linoleic acid/ 2% water (in wt.
 611 %), in the border of cubic-reverse hexagonal phase. The red line corresponds to the
 612 right axes and shows the detail of the low intensity data after adjacent averaging with 5
 613 points. The down arrows show the peaks for a hexagonal structure. The upwards arrows
 614 could correspond to a second phase, possibly cubic according its microscopy
 615 appearance.

616