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Cryo-TEM investigation of phase behaviour and aggregate structure in dilute dispersions of monoolein and oleic acid

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Abstract

Cryo-transmission electron microscopy (cryo-TEM) was used to image the microstructure in dilute sonicated dispersions of monoolein and oleic acid. The aim of the study was to explore how different experimental parameters, such as sample composition, total lipid concentration, pH, and ageing affect the phase behaviour and aggregate structure. Our investigations show that a rich variety of lamellar and non-lamellar structures, including liposomes and particles of cubic and inverted hexagonal phase, may form depending on the experimental conditions. The results are analyzed and discussed in relation to existing phase diagrams and earlier investigations concerning phase- and structural behaviour in monoolein/oleic acid/water systems.

Keywords: Monoolein; Oleic acid; Aggregate structure; Liposomes; Dispersed liquid crystalline phases; Cryo-TEM; Vaccines

1. Introduction

The area of lipid and surfactant self-assembly has over the years received well-deserved attention from a multidisciplinary science community encompassing chemists, biologists and physicists. In the pharmaceutical field, interest in the use of micelles, microemulsions and dispersed liquid crystalline phases formed by biocompatible surfactants is rapidly growing. As a consequence, the demand has increased for systems where particle size, curvature, charge, and other physico-chemical properties are well defined and possible to control (Mele et al., 2004).

Monoolein (MO), a monoglyceride traditionally used as emulsifier and food additive, has during the last decades received significant attention for applications in the pharmaceutical area (Larsson, 2000; Ganem-Quintanar et al., 2000). This polar lipid is essentially insoluble in water but self-associates and may, depending on water content, form a reversed micellar (L_2), a lamellar (L_α), or a bicontinuous cubic (C) phase of either the diamond (space group *Pn*3*m*) or gyroid (space group *Ia*3*d*) type (Lindblom et al., 1979; Hyde et al., 1984; Qiu and Caffrey, 2000).

Several publications have reported on the ability of the MO/water system to solubilize both hydrophilic and lipophilic substances and pointed out the potential of this system for controlled drug delivery purposes (Shah et al., 2001). A number of studies have focused on modifications in the MO/water phase behaviour induced by addition of a third component (Lawrence, 1994; Larsson, 2000; Ganem-Quintanar et al., 2000), and the observed changes in phase behaviour have been correlated to properties, such as polarity, geometric shape and concentration, of the additive (Caboi et al., 1997; Pitzalis et al., 2000; Caboi et al., 2001).

Apart from constituting promising candidates for drug delivery, MO-based dispersions have lately emerged as attractive systems also for the delivery of vaccines. The interest is here primarily sparked by the need to improve the immunogenicity of vaccines containing weak or non-traditional antigens, such as recombinant proteins, peptides, and DNA (O'Hagan and Rappuoli, 2004). In particular, MO/oleic acid (OA) dispersions have demonstrated good potential as mucosal vaccine adjuvants

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(Schröder and Svenson, 1999; Haile et al., 2004; Haile et al., 2005; Devito et al., 2004; Hinkula et al., in press).

In order to understand the parameters that may influence stability and performance of the abovementioned and related MO-based adjuvant/delivery systems, detailed information concerning phase behaviour and aggregate structure is needed. MO/OA dispersions intended for drug and vaccine delivery are as a rule prepared from buffered lipid/aqueous solutions. Further, in order to reduce particle size the dispersions are commonly subjected to high frequency ultrasound sonication. Although a number of studies have addressed the phase- and structural behaviour in aqueous dispersions of MO/OA and MO/sodium oleate (Borné et al., 2001; Borné et al., 2003) no systematic investigations have so far been performed concerning the buffered and sonicated dispersions of relevance for vaccine delivery.

In the present study, we use cryo-transmission electron microscopy in order to investigate the microstructure in sonicated dispersions of MO/OA as a function of pH, lipid composition, and lipid concentration. The results point out the sensitivity of the MO/OA/H_2O-system to changes in experimental conditions and stress the need for careful control of, in particular, pH during preparation of MO/OA particles intended for use in pharmaceutical and/or biotechnical applications.

2. Materials and methods

2.1. Materials

Monoolein (MO), Rylo MG 19, Pharma (Item number 173403) (Lot 220236) was obtained from Danisco Ingredients, Denmark. The monoglycerides content was greater than 90%. Oleic acid (OA) Palmolyn 100 FG (number 7084-8) was obtained from Hercules Incorporated, USA, and had the following composition: oleic acid (92%), linoleic acid (5%) and saturated acid (2%).

2.2. Sample preparation

The samples were prepared by weighting appropriate amounts of lipids according to the desired molar ratio of each assay. Typically, MO:OA molar ratios of 1:9; 3:7; 5:5; 7:3 and 9:1 were used at a final lipid concentration ranging from 1.0 to 5.0 wt.%. The dispersions were prepared by melting the lipid mixture at 40 °C in a beaker, where after 10 ml of MilliQ water was added. The mixtures were probe sonicated for 3 min at an amplitude of 14 μm under cooling in a water bath. Alternatively, the lipid mixture was hydrated with 10 ml of 0.2 M Tris–buffer, next the pH was adjusted to 7.4 or 8.0 with 5 M NaOH, and the dispersion was then sonicated as described above.

Unless otherwise stated, all samples appeared homogeneous and were imaged by cryo-TEM within 96 h after sonication.

2.3. Cryo-transmission electron microscopy

The cryo-TEM investigations were performed with a Zeiss EM 902A transmission electron microscope (Carl Zeiss NTS, Oberkochen, Germany). The instrument was operating at 80 kV and in zero loss bright-field mode. Digital images were recorded under low dose conditions using a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and the analySIS software (Soft Imaging System, GmbH, Münster, Germany). In order to visualize as many details as possible, an underfocus of 1–2 μm was used to enhance the image contrast. A more comprehensive description of the method for sample preparation and image recording is described in Almgren et al., 2003. The samples were equilibrated at 25 °C and approximately 99% relative humidity within a climate chamber. A small drop (∼1 μL) of sample was deposited on a copper grid covered with a perforated polymer film. Excess liquid was thereafter removed by means of blotting with a filter paper, leaving a thin film of the solution on the grid. Immediate after blotting the sample was vitrified in liquid ethane, held just above its freezing point. The samples were kept below −165 °C and protected against atmospheric conditions during both transfer to the TEM and examination.

3. Results

3.1. Aggregate structure in pure oleic acid dispersions

The phase- and structural behaviour of OA/water systems is known to be strongly dependent upon the protonation state of the fatty acid (Düzgünes et al., 1985; Cevc et al., 1988; Edwards et al., 1995). Before we began our investigations of the structural behaviour in mixtures of MO and OA it was therefore essential to establish the aggregate structure in pure sonicated OA dispersions at the relevant pH.

The micrographs presented in Fig. 1 reveal that relatively minor alterations of pH may lead to significant structural changes. As seen from Fig. 1a, samples buffered at pH 8.0 are dominated by uni- and bilamellar liposomes of predominantly spherical shape. Liposomes are present also at pH 7.4 but their shape is now frequently elongated or tubular. Fig. 1b discloses another interesting, and perhaps more important, structural difference. At pH 7.4 the bilayers of bi- and multilamellar structures have developed a tendency to connect and form catenoidal fusion pores, often referred to as interlamellar attachments (ILAs). Some of these structures have for clarification been marked with arrows in Fig. 1b (compare also Fig. 2a). ILAs have been documented in several lipid systems and are commonly interpreted as intermediates formed during the transition from L_α to inverted cubic phase (Siegel et al., 1989; Frederik et al., 1991; Siegel et al., 1994; Siegel, 1999; Johnsson and Edwards, 2001). It is noteworthy that extensive ILA formation between lamelleas in multilayer structures may give rise to particles with a very complex inner morphology (see for instance the multilamellar structures at the bottom of Fig. 1b).

At pH values between 5.0 and 6.5 the OA samples were difficult, or impossible, to disperse and no attempts were therefore made to visualize the aggregate structure under these conditions.

3.2. Structure in MO/OA dispersions at pH 8.0

Once the structure in pure OA dispersions had been determined, we set out to investigate how addition of MO affected

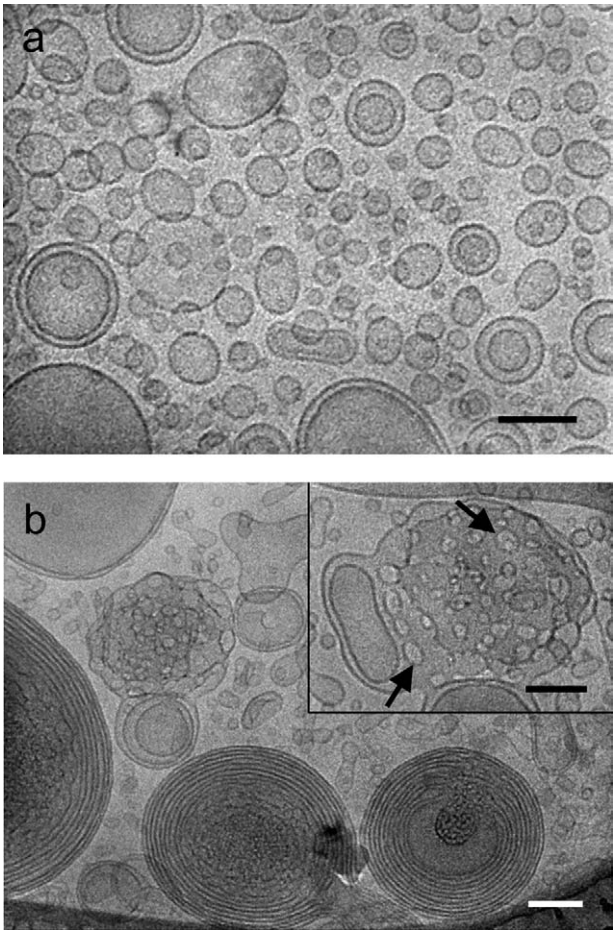

Fig. 1. Cryo-TEM micrographs showing aggregate structure in sonicated dispersions containing 5 wt.% oleic acid at pH 8.0 (a) and 7.4 (b). Arrows in (b) denote ILAs. Bar = 100 nm.

the structural behaviour. We began by exploring the aggregate structure in MO/OA mixtures prepared at pH8.0.

A series of samples with total lipid concentration corresponding to 5 wt.% and MO:OA molar ratios equal to 1:9, 3:7, 5:5, 7:3, and 9:1, respectively, were dispersed by sonication and thereafter analyzed by cryo-TEM.

Interestingly, the cryo-TEM analysis revealed that inclusion of MO promotes ILA formation. This tendency could be observed at MO:OA ratios as low as 1:9 (Fig. 2a) but it should be pointed out that ILAs and related transition structures were rare at low to intermediate concentrations of MO. At MO:OA ratios corresponding to 5:5, we occasionally observed complex transition intermediates of the type shown in Fig. 2b. These structures became more numerous with increasing MO content and eventually the presence of cubic phase was evident. The micrographs showed, however, that liposomes strongly dominate the aggregate structure in samples containing up to 70 mol% MO. Fig. 2c corresponds to a sample having MO:OA ratio 9:1 and shows liposomes and intermediate structures in coexistence with large particles of dispersed cubic phase. The latter closely resemble the structures observed by means of cryo-TEM in aqueous dispersions of pure MO (Borné et al., 2002, 2003).

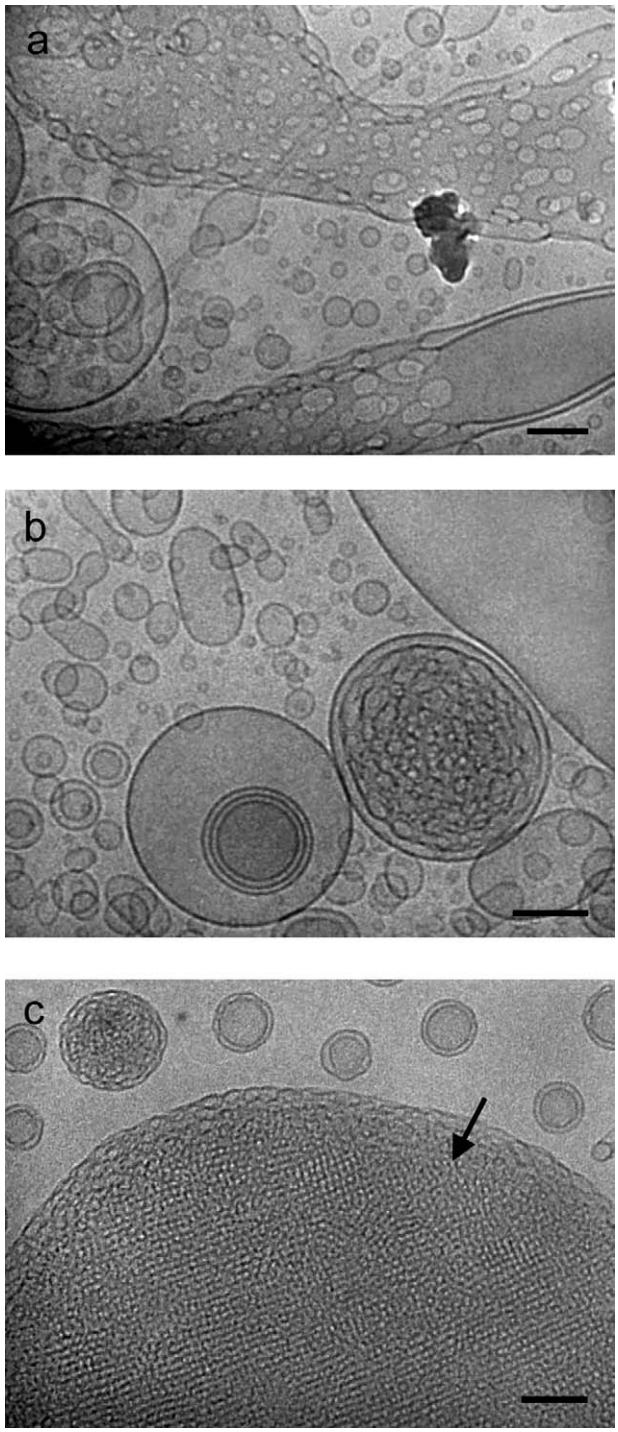

Fig. 2. Aggregate structures found in MO/OA dispersions buffered at pH 8.0: (a) MO:OA ratio 1:9, 95 wt.% H_2O, (b) MO:OA ratio 5:5, 98 wt.% H_2O, (c) MO:OA ratio 9:1, 95 wt.% H_2O. Arrow in (c) denotes a particle of dispersed cubic phase. Bar = 100 nm.

In order to investigate the influence of lipid concentration on the aggregate structure, we prepared a series of samples with MO:OA ratio 5:5 and total lipid concentrations corresponding to 1, 2, 3, and 4 wt.%, respectively (not shown). All samples were clearly dominated by liposomes and displayed, similar to the 5 wt.% lipid dispersion, a tendency for ILA-formation (see, e.g. Fig. 2b).

3.3. MO/OA dispersions at pH 7.4

Recalling that a slight drop in pH from 8.0 to 7.4 gives rise to a significant change in the sample morphology for pure OA dispersions (compare Fig. 1a and b), we anticipated a strong pH dependence also in the MO/OA system. The micrographs shown in Fig. 3 reveal that changes in pH indeed have a strong influence on the type of structures formed. Complex intermediate structures were frequently observed to coexist with liposomes in samples having MO:OA compositions 1:9 and 3:7 (Fig. 3a and b), and large particles of seemingly well-developed cubic phase appeared already at equimolar concentration of MO and OA (Fig. 3c). The MO:OA 5:5 dispersion contained, in addition to cubic particles, also a considerable amount of liposomes. At MO:OA compositions corresponding to 7:3, and higher, conventional unilamellar liposomes were only rarely observed and the majority of lipids were found in cubic particles and complex intermediates (results not shown).

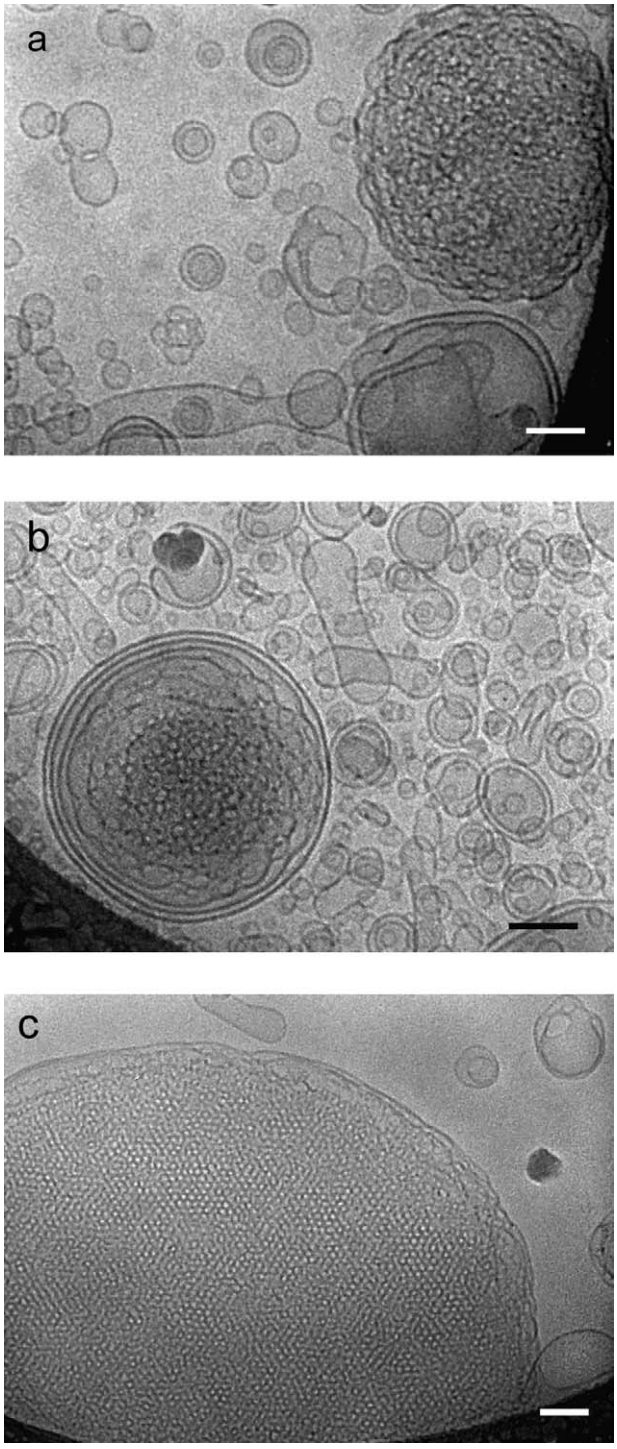

Fig. 3. Aggregate structures found in MO/OA dispersions buffered at pH 7.4: (a) MO:OA 1:9, 95 wt.% H_2O, (b) MO:OA 3:7, 95 wt.% H_2O, (c) MO:OA 5:5, 98 wt.% H_2O. Bar = 100 nm.

3.4. Mixtures of MO and OA dispersed in pure water

In the above-described investigations, we did not detect any obvious signs of inverted hexagonal (H_{II}) phase. Particles displaying structural features characteristic of H_{II}-phase were, on the other hand, observed when certain proportions of MO and OA were dispersed in pure water. As described below, the behaviour of the MO/OA/H_2O system differed from that of the buffered systems also in several other ways.

Lipid mixtures containing above 50 mol% OA could not be dispersed in pure water by means of the sonication procedure employed in the present study. Samples with equimolar concentrations of MO and OA were also somewhat hard to disperse but repeated sonication produced samples that appeared homogenous and stable over a time period of at least 2 months. Cryo-TEM revealed for this composition the presence of dark, roughly circular structures that sometimes exposed a peculiar honeycomb-like pattern (Fig. 4a). Dispersions with MO:OA ratio 6:4 were inherently unstable and went through a macroscopic phase separation shortly after the sonication procedure. Cryo-TEM analysis of the lower phase showed coexistence between very electron dense particles of more or less hexagonal shape and oil-like droplets of varying size (Fig. 4b). The former displayed the curved striations and/or textures of hexagonal symmetry characteristic of H_{II}-phase (Gustafsson et al., 1997) (compare also Figs. 4c, and 5f, g). The upper oil-like phase was not further analyzed. No macroscopic phase separation was observed for dispersions with MO:OA ratio 7:3. As seen in Fig. 4c, the presence of dense striated structures, which most probably represents H_{II}-phase, was characteristic at this composition. It is noteworthy, however, that the cryo-TEM analysis indicated the presence also of lamellar phase; accordingly several small liposomes can be seen in the background of Fig. 4c. Liposomes of various sizes were, moreover, found at MO:OA ratios 9:1. For this composition, we detected no H_{II} particles but frequently observed compact roughly spherical aggregates with a more open structure (Fig. 4d). Similar structures have been documented, and interpreted as cubic phase precursors, in sonicated dispersion containing MO and the block copolymer poloxamer 407 (Gustafsson et al., 1997) and in mixtures of dioleoylphosphatidylethanolamine and poly(ethylene glycol)-lipids (Johnsson and Edwards, 2001).

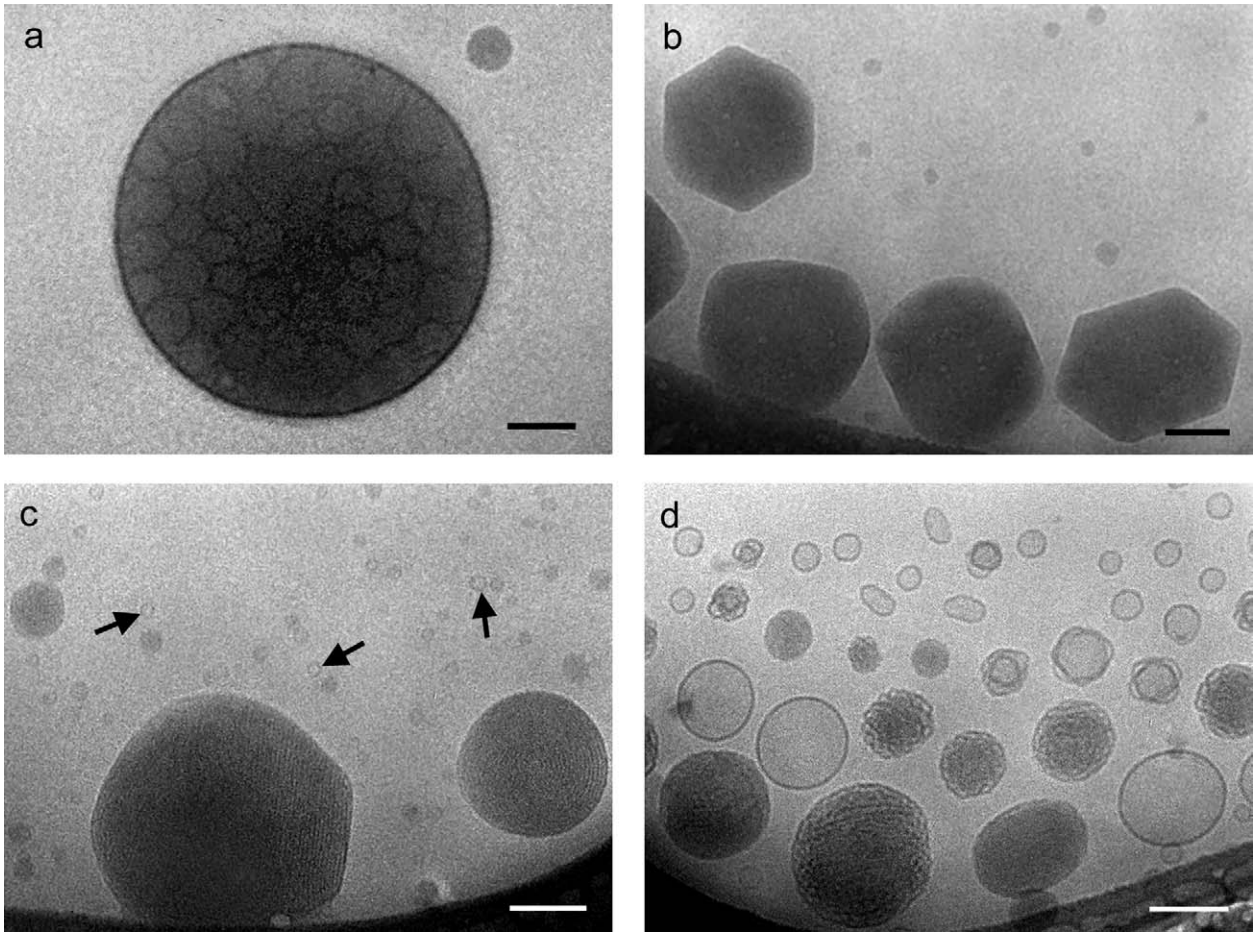

Fig. 4. Micrographs revealing structures formed in the MO/OA/H_2O-system: (a) particles displaying honeycomb-like pattern observed at MO:OA ratio 5:5, (b) particles of dispersed HII-phase found in phase separated samples with MO:OA ratio 6:4, (c) coexistence between small liposomes (marked with arrows) and particles of dispersed HII-phase observed at MO:OA ratio 7:3, and (d) liposomes and complex transition structures seen at MO:OA ratio 9:1. Water content was 99 wt.% for all samples. Bar = 100 nm.

3.5. Effect of ageing

In order to investigate the long-term stability of the dispersions a selected number of the above-mentioned samples were reanalyzed after 2 months incubation at 25 °C. Cryo-TEM micrographs obtained from the aged samples are displayed in Fig. 5 and reveal that some important structural changes take place over time.

Fig. 5a and b show the aggregate structure in two samples belonging to the series buffered at pH 8.0. Some differences between fresh and aged samples are evident. Subsequent to ageing the ILAs previously observed at MO:OA ratio 1:9 (see Fig. 2a) have disappeared and intact uni- and multilamellar liposomes now constitute the only structures present (Fig. 5a). Comparison of micrographs obtained from fresh and aged dispersions with MO:OA ratio 9:1 did not expose any differences in the type of aggregates present. As seen in Fig. 5b, the aged dispersion still contained a mixture of lamellar and cubic phase. The size and number of the cubic phase-particles were, however, greater in the aged sample. In line with this observation, ocular inspection of the aged sample revealed the presence of particulate material.

The effect of ageing was investigated also for two samples prepared at pH 7.4. Similar to the case at pH 8.0, we detected less ILAs and intermediate structures when samples containing low amounts of MO were reanalyzed 2 months after preparation. As seen in Fig. 5c, the MO:OA 3:7 dispersion now contained almost exclusively liposomes. Samples with MO:OA ratio 7:3 displayed, on the other hand, no significant changes in aggregate structure. Particles of seemingly well-ordered cubic phase still coexisted with more or less complex intermediate structures (Fig. 5d) and, as for the fresh sample, no signs of macroscopic phase separation were noticed.

Micrographs from the aqueous unbuffered system are shown in Fig. 5e–g. After 2 months of incubation the MO:OA 5:5 dispersion still looked homogeneous to the naked eye. Cryo-TEM confirmed that the aggregate structure had not changed noticeably. As seen in Fig. 5e, the micrographs expose again dense objects with an approximately circular shape. Reanalysis of the MO:OA 9:1 dispersion revealed, however, some interesting effects of ageing. From ocular inspection it was clear that the aged sample, in contrast to the fresh dispersion, contained macroscopic particles. The structural analysis provided information as to the origin of these particles. As shown in Fig. 5f

and g, the sample still contained a few liposomes but the majority of lipids were now found in large particles of H_{II}-phase. It is likely that the particulate material observed upon storage of the dispersion represents macroscopic pieces of this phase.

4. Discussion

The binary MO/water system is at temperatures around 25 °C dominated by an extensive region of cubic liquid crystalline phases (Lindblom et al., 1979; Hyde et al., 1984; Qiu and Caffrey, 2000). At water contents above 40 wt.% an inverted bicontinuous cubic phase, belonging to space group *Pn*3*m*, exists in equilibrium with the aqueous phase. The cubic phase may be partially fractionated into particles that are small enough for visualization by means of cryo-TEM (Borné et al., 2003). Dispersion of the cubic phase into long-lived submicron particles requires, however, the presence of a stabilizing agent and typically tri-block copolymers, such as poloxamer 407, are used for this purpose (Gustafsson et al., 1997). The structural behaviour in the MO–water system is not expected to depend on pH (although, as discussed below, alterations in pH may affect the rate of MO hydrolysis).

Concerning OA it is important to realize that the protonation state of the fatty acid to a large extent governs the type of microstructures formed and that variations in pH thus may have a considerable influence on the structural behaviour. Pure fatty acid/soap systems display rich and complex phase behaviour in water solution (Small, 1986) and a number of phase diagrams have been presented for the oleic acid/Na-oleate(NaO)/water system (Stenius et al., 1984; Engblom et al., 1995; Small, 1968). The information contained in these diagrams indicates that, for dilute solutions, three liquid crystalline phases may be found in equilibrium with the aqueous phase. These correspond, with increasing oleic acid/oleate molar ratio, to a lamellar (L_α) phase,

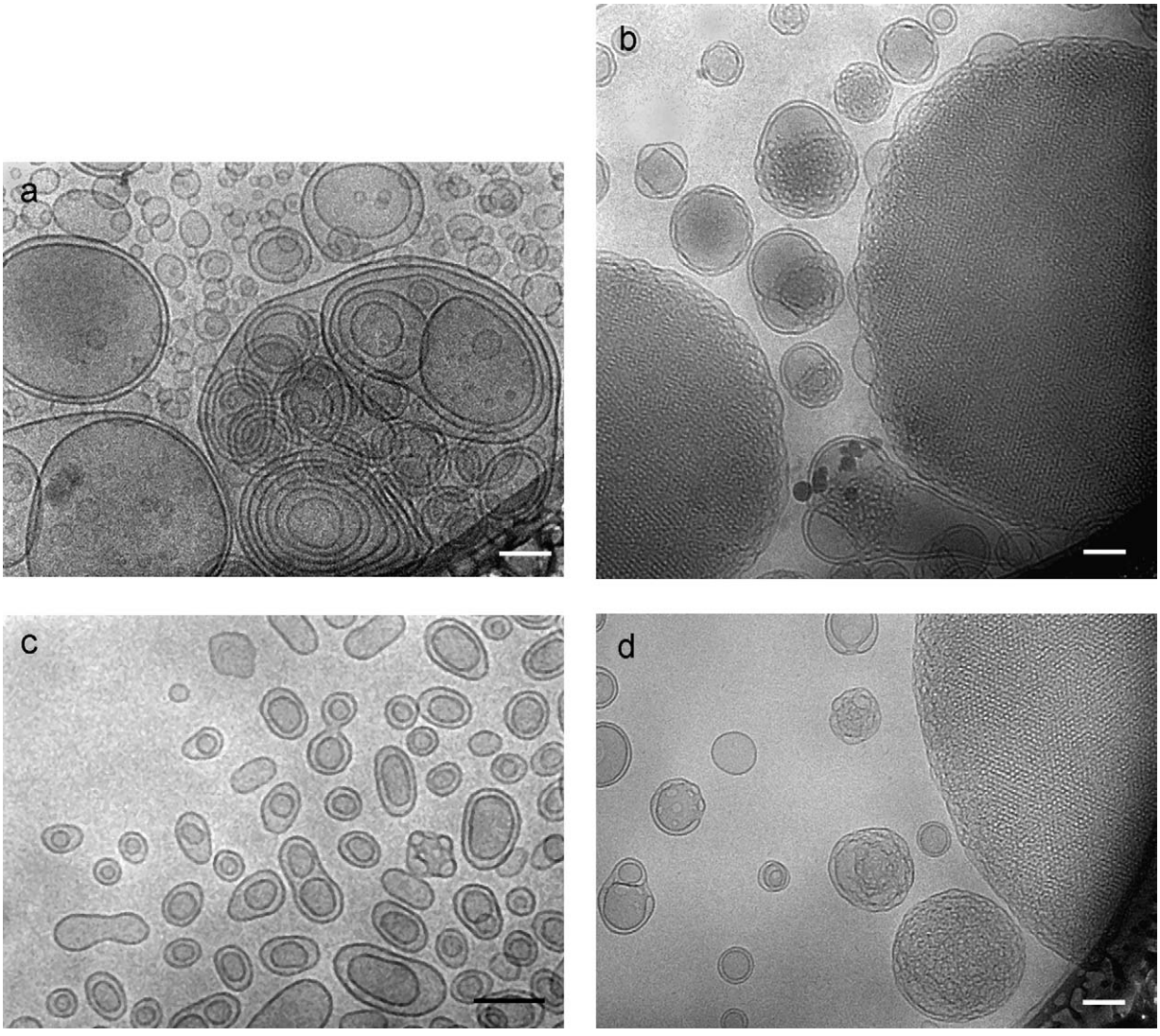

Fig. 5. Structures observed after 2 months incubation at 25 °C of MO:OA dispersions prepared in buffer at pH 8.0 (a), (b) or pH 7.4 (c), (d) and water (e)–(g), respectively. The micrographs display: (a) liposomes at MO:OA ratio 1:9, (b) dispersed cubic phase at MO:OA ratio 9:1, (c) mainly liposomes at MO:OA ratio 3:7, (d) transition structures and particles of cubic phase at MO:OA ratio 7:3, (e) structures with honeycomb pattern at MO:OA molar ratio 5:5, (f) and (g) particles with characteristics typical of dispersed H_{II}-phase at MO:OA ratio 9:1. The water content in the samples was 95 wt.% (a)–(d), or 99 wt.% (e)–(g). Bar = 100 nm.

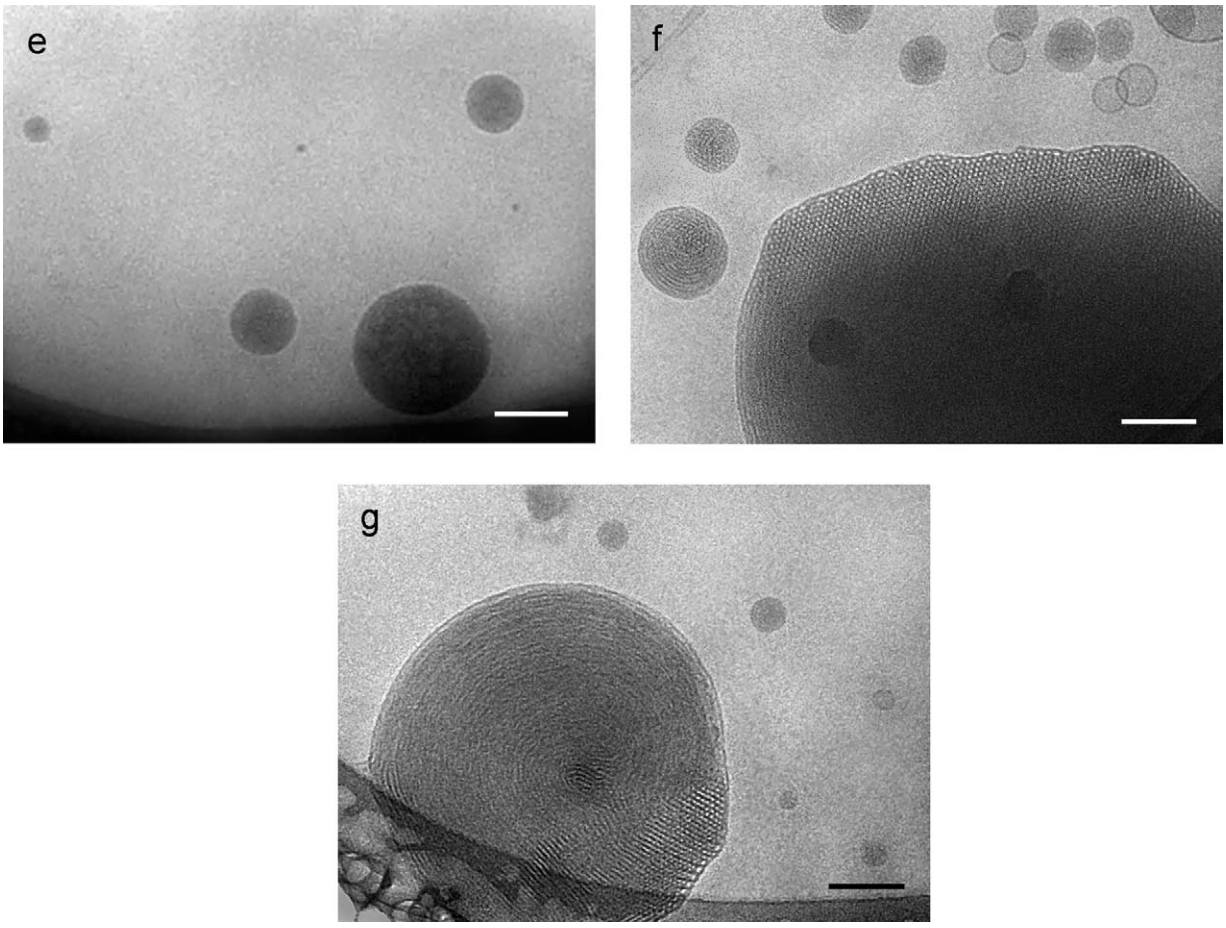

Fig. 5. (*Continued*).

an inverted hexagonal (H_{II}) phase, and an isotropic cubic (C_{mic}) phase composed of closed packed reversed micelles. Cryo-TEM has previously been employed to investigate the aggregate structure in very dilute OA/NaO/water-systems (Edwards et al., 1995). Data from this study show that an interesting sequence of structural transformations takes place upon acidification of dilute NaO solutions. At high pH, where the fatty acid is close to fully ionized, globular or long thread-like cylindrical micelles may form depending on the NaO concentration. As the pH is reduced to values around 9 unilamellar liposomes start to appear and then constitute the dominating aggregate structure until the pH has been reduced to values around 8. Further acidification of the fatty acid/soap mixture induces the formation of non-lamellar structures and at pH values corresponding to 7.4 particles of inverted hexagonal phase are present in coexistence with liposomes.

These above observations are in qualitative agreement with the cryo-TEM data obtained for aqueous dispersions of OA in the present study. At pH 8.0, the sonicated dispersions contained well-separated and intact liposomes (Fig. 1a), whereas, at pH 7.4 there was a strong tendency for inter-bilayer interaction and formation of non-lamellar structures (Fig. 1b). Notably, fully developed fusion pores, such as those displayed in Fig. 1b, are according to recent theories not expected to form during the transition from L_α to H_{II} phase. Rather, the transition has been suggested to proceed directly from intact L_α-phase lamellae to H_{II}-tubes via formation, and subsequent aggregation, of hemi-fused structures termed *trans*-monolayer contacts (TMCs) (Siegel, 1999). Interestingly, rupture of the bilayer diaphragm located in the center of a TMC converts the structure to a fusion pore, i.e. an ILA. TMCs have consequently been postulated as intermediates in the formation of both inverted hexagonal and cubic phase (Siegel, 1999; Cherezov et al., 2003; Malinin and Lenz, 2004). The reason why fusion pores and related precursors of cubic phase, rather than the expected H_{II}-phase, were found in the presently investigated OA dispersion still remains to be clarified. It may be hypothesized, however, that excess energy introduced during the sonication procedure may promote rupture of TMCs into ILAs and thus prevent TMC aggregation and subsequent lipid arrangements necessary for development of H_{II}-phase.

The results of the present study show that the propensity for non-lamellar structures increases upon addition of MO to the OA system. At pH 8.0 the liposomal system proceeds with increasing MO:OA ratio straight into a three-phase region where the aqueous and lamellar phases stand in equilibrium with a not yet identified cubic phase. As judged by the structural intermediates revealed in the present study the latter is most likely bicontinuous in its nature. It is in this context interesting to note that the large particle revealed in Fig. 2c is very similar in appearance to the

dispersed particles of cubic *Pn*3*m* phase observed by cryo-TEM in the MO/H_2O system (Borné et al., 2002, 2003).

At pH 7.4, where a tendency for non-lamellar structures exists already in the pure OA/water system, less MO is needed to produce the cubic phase. It is interesting to compare the behaviour observed in the buffered systems with the phase diagrams determined for MO/NaO/water and MO/OA/water, respectively. According to recently published data (Borné et al., 2001), the ternary MO/NaO/water system is dominated by a large lamellar phase, which at high water content stands in equilibrium with the aqueous solution phase. No reversed phases are observed until the MO content exceeds values corresponding to about 55 mol% of total lipid. At this point a cubic bicontinuous phase appears in coexistence with the lamellar- and the solution phase. With increasing MO concentration a two-phase area composed of cubic- and aqueous phase is eventually entered and, for fresh samples, no further phase changes are expected. Previous investigations have not detected any lamellar phase (L_α) in the MO/OA/water system (Borné et al., 2001). Instead a narrow area of micellar cubic (C_{mic}) phase has been identified. The aqueous solution phase has been proposed to exist in equilibrium with either this phase and/or H_{II}-phase, before the cubic bicontinous phase finally appears at comparably high MO:OA ratios. Since the buffered dispersions investigated in the present study contain a mixture of ionized and protonated fatty acids a phase behaviour somewhere in between that found for the MO/NaO/water and MO/OA/water systems may be anticipated. The micrographs presented in Section 3 give support to this hypothesis. As evidenced by the abundance of liposomes found in the dispersions an L_α-phase is present at both pH 8.0 and pH 7.4. As expected, the size of the lamellar region decreases with decreasing pH.

Somewhat surprising, and contrary to previously published phase diagrams (Borné et al., 2001; Borné et al., 2003) our investigations indicate that a lamellar phase is present also in the unbuffered MO/OA/water-system. We observed small unilamellar liposomes together with particles of H_{II}-phase in samples having MO:OA ratio 7:3. Moreover, numerous comparably large liposomes were observed in samples with MO:OA ratio corresponding to 9:1. Further investigations are required, however, in order to confirm the existence of a lamellar phase and to determine its exact location and extension in the ternary MO/OA/water phase diagram.

Apart from the unexpected presence of liposomes, some additional structural features of the MO/OA/water system deserve to be mentioned and discussed. First, for samples with MO:OA ratio 5:5 the cryo-TEM analysis revealed particles that displayed an unusual honeycomb-like pattern. Micrographs exhibiting similar structures have to our knowledge not been published before and we have at present no straightforward explanation for these peculiar particles. It is tempting, however, to assume that the particles have a three-dimensional structure that is similar to the multiniosome particles revealed by freeze-fracture electron microscopy in mixtures of sorbitan monooleate, cholesterol and dicetyl phosphate (Sternberg et al., 1995). The phase diagram presented by Borné et al. (2001) suggests that samples with equimolar concentration of MO and OA reside in a three-phase region composed of aqueous- and L_2-phase in equilibrium with the cubic (C_{mic}) phase. The latter is built from two types of quasi-spherical reversed micelles and has been classified as belonging to space group *Fd*3*m* (Luzzati et al., 1992). Since a slightly different MO preparation was used in our study, i.e. with different monoglyceride content, no direct comparisons can be made with the system investigated by Borné et al. It appears reasonable, however, to assume that a similar C_{mic} phase should be present also in our system and it is possible that the honeycomb-like structures in some way are connected to the presence of this phase. A second aspect that needs to be discussed is the significant change in aggregate structure observed upon aging of MO/OA dispersions with high content of MO. Fresh samples with MO:OA ratio 9:1 contained, as mentioned above, a large proportion of liposomes. In addition, we detected in these samples a complex type of disordered particles that most likely represent some relatively long-lived intermediate structures. Particles of similar appearance have been classified as precursors of cubic bicontinuos phase (Johnsson and Edwards, 2001; Gustafsson et al., 1997). Interestingly, upon incubation of the MO/OA dispersion for 2 months at 25 °C the aggregate structure changed markedly. Cryo-TEM images of the aged dispersion showed that the dominant structures were large particles of well-developed H_{II}-phase. It is possible that the differences between fresh and aged samples simply reflect the fact that phase equilibration and formation of H_{II}-phase require comparably long time for completion when the MO content is high. A more likely explanation is, however, that the sample composition changes over time. Similar to other ester bound lipids containing unsaturated acyl chains MO is sensitive to hydrolysis (Muria et al., 2002). With time the MO component is thus broken down to glycerol and free fatty acid and as a consequence the MO:OA ratio changes. The rate of ester hydrolysis increases at elevated temperatures and the extent of hydrolysis is, furthermore, expected to escalate at both acidic and basic conditions. Some important time and temperature dependent changes in the phase behaviour of the MO/NaO/water system have previously been reported and discussed in terms of a hydrolysis effect (Borné et al., 2001). More specifically, the cubic (C) one-phase area found at high MO concentrations was observed to shift its position and shrink in size when the samples were incubated for 2 months at 25 °C, or a few days at 37 °C. Interestingly, as a result of the changes in the cubic phase boundaries H_{II}-phase may appear in dilute dispersion with high MO:NaO ratio. No systematic investigations concerning the effect of ageing have to our knowledge been carried out for the MO/OA/water system. Based on the existing phase diagram (Borné et al., 2001) together with the results displayed in Fig. 4, it appears however logical that a hydrolysis induced decrease in the MO:OA ratio would increase the propensity for H_{II}-phase also in the MO/OA/water-system. Further experiments, including determinations of glycerol content in aged samples and investigations of phase behaviour in the quaternary MO/OA/H_2O/glycerol system, are needed in order to verify this hypothesis.

It is in this context interesting to recall that we did not observe any signs of H_{II}-phase when samples with pH 8.0 and MO:OA ratio 9:1 were reanalyzed after 2 months incubation at 25 °C. Part of the explanation may be that the rate of hydrolysis is somewhat

slower in the buffered system. Another, more important, difference must however be considered when comparing the buffered and unbuffered systems. In the latter, the fatty acid produced upon MO hydrolysis may change the relative amounts of protonated and ionized fatty acids in the system. This complication is, however, absent in the buffered systems where MO hydrolysis is not expected to change the overall protonation state of the fatty acid. In this case, the sole effect of hydrolysis would be a straightforward reduction in MO:OA molar ratio of the sample. The analysis of fresh MO/OA dispersions buffered at pH 8.0 did not, for any MO:OA ratio below 9:1, indicate the presence of H_{II}-phase. Thus, we cannot exclude that a significant amount of hydrolysis occurred during the 2 months incubation period. In fact, the structural change observed upon ageing of samples with low MO:OA ratio could be explained by a hydrolysis-induced reduction of the MO content. After incubation the MO:OA 1:9 sample displayed no ILAs or other transition structures and contained, like the pure OA sample at pH 8.0, exclusively intact liposomes. We have at present no straightforward explanation as to why the same behaviour was observed also at pH 7.4 where the pure OA sample showed a clear tendency to form non-lamellar structures.

In summary, the results of the present study show that a range of aggregate structures may form in dilute sonicated dispersions containing monoolein and oleic acid. Samples buffered at pH 7.4 and 8.0 are dominated by liposomes and particles of cubic phase. In addition, a range of more or less complex intermediate structures where captured by cryo-TEM in these dispersions. Judging from the micrographs, it appears that the whole composition range studied, i.e. MO:OA ratios between 1:9 and 9:1, is located within a three-phase area containing aqueous-, lamellar and cubic phase. Changing the pH between 7.4 and 8.0 or varying the lipid composition, either directly or via ageing of the samples, may alter the nanostructure and relative amount of L_α- and C-phase. No evidence was discovered, however, to suggest the presence of any additional liquid crystalline phases in the buffered systems. Dispersions prepared from unbuffered MO/OA/water-mixtures were generally less stable and displayed a more composite phase- and structural behaviour. In particular, particles of well-developed H_{II}-phase were, depending on the MO:OA ratio, observed to coexist with either oil droplets or liposomes. The information disclosed in the present report show that sonicated MO/OA dispersions as a rule are fairly polydisperse in both size and structure. Moreover, apart from liposomes, no long-lived submicron particles of well-defined liquid crystalline phase could be produced at pH 7.4 or 8.0. This fact, together with the well-documented sensitivity of the MO/OA system to slight changes in pH, should be kept in mind when designing formulations intended for drug delivery or other pharmaceutical/biotechnical applications.

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References

Almgren, M., Edwards, K., Karlsson, G., 2003. Cryo-transmission electron microscopy of liposomes and related structures. Colloid Surf. A 174, 3–21.

Borné, J., Nylander, T., Khan, A., 2001. Phase behaviour and aggregate formation for the aqueous monoolein system mixed with sodium oleate and oleic acid. Langmuir 17, 7742–7751.

Borné, J., Nylander, T., Khan, A., 2002. Effect of lipase on monoolein-based cubic phase dispersion (cubosomes) and vesicles. J. Phys. Chem. B 106, 10492–10500.

Borné, J., Nylander, T., Khan, A., 2003. Vesicle formation and other structures in aqueous dispersions of monoolein and sodium oleate. J. Colloids Interf. Sci. 257, 310–320.

Caboi, F., Nylander, T., Razumas, V., Talaikyté, Z., Monduzzi, M., Larsson, K., 1997. Structural effects, mobility and redox behaviour of Vitamin K1 hosted in the monoolein-water liquid crystalline phases. Langmuir 13, 5476.

Caboi, F., Amico, G.S., Pitzalis, P., Monduzzi, M., Nylander, T., Larsson, K., 2001. Phase diagrams and mesophases characterisation in monoolein–water-additive systems. Chem. Phys. Lipids 169, 47–62.

Cevc, G., Seddon, J.M., Hartung, R., Eggert, W., 1988. Phosphatidylcholine–fatty acid membranes. I. Effects of protonation, salt concentration, temperature and chain-length on the colloidal and phase properties of mixed vesicles, bilayers and non-lamellar structures. Biochim. Biophys. Acta 940, 219–240.

Cherezov, V., Siegel, D.P., Shaw, W., Burgess, S.W., Caffrey, M., 2003. The kinetics of non-lamellar phase formation in DOPE-Me: relevance to biomembrane fusion. J. Membr. Biol. 195, 165–182.

Devito, C., Zuber, B., Schroder, U., Benthin, R., Okuda, K., Broliden, K., Wahren, B., Hinkula, J., 2004. Intranasal HIV-1-gp160-DNA/gp41 peptide prime-boost immunization regimen in mice results in long-term HIV-1 neutralizing humoral mucosal and systemic immunity. J. Immunol. 173, 7078–7089.

Düzgünes, N., Straubinger, R.M., Baldwin, P.A., Friend, D.S., Papahadjopoulus, D., 1985. Proton-induced fusion of oleic acid–phosphatidylethanolamine liposomes. Biochemistry 24, 3091–3098.

Edwards, K., Silvander, M., Karlsson, G., 1995. Aggregate structure in dilute aqueous dispersion of oleic acid/sodium oleate and oleic acid/sodium oleate/egg phosphatidylcholine. Langmuir 11, 2429–2434.

Engblom, J., Engström, S., Fontell, K., 1995. The effect of the skin penetration enhancer Azone on fatty acid–sodium soap–water mixtures. J. Control. Rel. 33, 299–305.

Frederik, P.M., Burger, K.N.J., Stuart, M.C.A., Verkleij, A.J., 1991. Lipid polymorphism as observed by cryo-electron microscopy. Biochim. Biophys. Acta, 133–141.

Ganem-Quintanar, A., Quintanar-Guerrero, D., Buri, P., 2000. Monoolein: a review of the pharmaceutical applications. Drug Dev. Ind. Pharm. 26, 809–820.

Gustafsson, J., Wahren-Ljusberg, H., Almgren, M., Larsson, K., 1997. Submicron particles of reversed lipid phases in water stabilized by a non-ionic amphiphilic polymer. Langmuir 13, 6964–6971.

Haile, M., Schröder, U., Hamasur, B., Pawlowski, A., Jaxmar, T., Källenius, G., Svenson, S.B., 2004. Immunization with heat-killed *Mycobacterium bovis* bacille Calmette-Guerin (BCG) in Eurocine L3 adjuvant protects against tuberculosis. Vaccine 22, 1498–1508.

Haile, M., Hamasur, B., Jaxmar, T., Gavier-Widen, D., Chambers, M.A., Sanchez, B., Scröder, U., Källenius, G., Svensson, S.B., Pawlowski, A., 2005. Nasal boost with adjuvanted heat-killed BCG or arabinomannan-protein conjugate improves primary BCG-induced protection in C57BL/6 mice. Tuberculosis 85, 107.

Hinkula, J., Devito C., Zuber, B., Benthin, R., Ferreira, D., Wahren, B., Schroder, U., in press. A novel DNA adjuvant, N3, enhances mucosal

and systemic immune responses induced by HIV-1 DNA and peptide immunizations. Vaccine.

Hyde, S.T., Andersson, S., Ericsson, B., Larsson, K., 1984. A cubic structure consisting of a lipid bilayer forming an infinite periodic minimum surface of the gyroid type in the glycerolmonooleate–water system. Z. Kristallogr. 168, 213–219.

Johnsson, M., Edwards, K., 2001. Phase behaviour and aggregate structure in mixtures of dioleoylphosphatidylethanolamine and poly(ethylene glycol)-lipids. Biophys. J. 80, 313–323.

Larsson, K., 2000. Aqueous dispersions of cubic lipid–water phases. Curr. Opin. Colloids Interf. Sci. 5, 64.

Lawrence, M.J., 1994. Surfactant systems: their use in drug delivery. Chem. Soc. Rev. 23, 417.

Lindblom, G., Larsson, K., Johansson, L., Fontell, K., Forsen, S., 1979. The cubic phase of monoglyceride–water systems. Arguments for a structure based upon lamellar units. J. Am. Chem. Soc. 101, 5465–5470.

Luzzati, V., Vargas, R., Gulik, A., Mariaini, P., Seddon, J.M., Rivas, E., 1992. Lipid polymorphism: a correction. The structure of the cubic phase of extinction symbol Fd—consists of two types of disjointed reverse micelles embedded in a three-dimensional hydrocarbon matrix. Biochemistry 31, 279–285.

Malinin, V.S., Lenz, B.R., 2004. Energetics of vesicle fusion intermediates: comparison of calculations with observed effects of osmotic and curvature stresses. Biophys. J. 86, 2951–2964.

Mele, S., Murgia, S., Caboi, F., Monduzzi, M., 2004. Biocompatible lipidic formulations: phase behaviour and microstructure. Langmuir 20, 5241–5246.

Muria, S., Caboi, F., Monduzzi, M., Ljusberg-Wahren, H., Nylander, T., 2002. Acyl migration and hydrolysis in monoolein-based systems. Prog. Colloid Polym. Sci. 120, 41–46.

O'Hagan, D.T., Rappuoli, R., 2004. Novel approaches to vaccine delivery. Pharm. Res. 21, 1519–1530.

Pitzalis, P., Monduzzi, M., Krog, N., Larsson, H., Ljuserg-Wahren, H., Nylander, T., 2000. Characterization of the liquid-crystalline phases in the glycerol monooleate/diglycerol monooleate/water system. Langmuir 16, 6358–6365.

Qiu, H., Caffrey, M., 2000. The phase diagram of the monoolein/water system. Equilibrium and metastability aspects. Biomaterials 21, 223–234.

Schröder, U., Svenson, S.B., 1999. Nasal and parenteral immunizations with diphtheria toxoid using monoglyceride/fatty acid lipid suspensions as adjuvants. Vaccine 17, 2096–2103.

Shah, J.C., Sadhale, Y., Chilukuri, D.M., 2001. Cubic phase gels as drug delivery systems. Adv. Drug Deliv. Rev. 47, 229.

Siegel, D.P., Burns, J.L., Chestnut, M.H., Talmon, Y.T., 1989. Intermediates in membrane fusion and bilayer/nonbilayer phase transitions imaged by time-resolved cryo-transmission electron microscopy. Biophys. J. 56, 161–169.

Siegel, D.P., Green, W.J., Talmon, Y.T., 1994. The mechanism of lamellar-to-inverted hexagonal phase transitions: a study using temperature-jump cryo-electron microscopy. Biophys. J. 66, 402–414.

Siegel, D.P., 1999. The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion. Biophys. J. 76, 291–313.

Small, D.M., 1968. A classification of biological lipids based upon their interaction in aqueous systems. J. Am. Oil Chem. Soc. 45, 108–119.

Small, D.M. (Ed.), 1986. Handbook of Lipid Research, vol. 4. Plenum Press, New York (Chapters 8 and 9).

Stenius, P., Palonen, H., Ström, G., Ödberg, L., 1984. Surfactants in solution. In: Mittal, K.L., Lindman, B. (Eds.), Surfactants in Solution, vol. 1. Plenum Press, New York, pp. 153–174.

Sternberg, B., Moody, M.F., Yoshioka, T., Florence, A.T., 1995. Geodesic surfactant structures. Nature 378, 21.