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Rapid report

Low amounts of PEG-lipid induce cubic phase in phosphatidylethanolamine dispersions

Rumiana Koynova [a,*], Boris Tenchov [a], Gert Rapp [b]

[a] *Institute of Biophysics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[b] *European Molecular Biology Laboratory - Outstation Hamburg, D-22603 Hamburg, Germany*

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Abstract

By using time-resolved X-ray diffraction we demonstrate that low amounts (5–10 mol%) of a phospholipid with two saturated hydrocarbon acyl chains 14 carbon atoms long and PEG550 chain covalently attached to its phosphoethanolamine polar head group, DMPE(PEG550), induce spontaneous formation of a cubic phase with lattice constant 20.5 nm (cubic aspect #8, space group Im3m) in aqueous dispersions of dielaidoylphosphatidylethanolamine (DEPE). This phase displays a highly resolved X-ray diffraction pattern with 17 low-angle reflections. The cubic phase was found to intrude in the temperature range between the lamellar liquid crystalline (L_α) phase and the inverted hexagonal phase (H_{II}) known to form in pure DEPE/water dispersions. A higher DMPE(PEG550) amount of 20 mol% was found to eliminate the non-lamellar phases in the temperature scale up to 100°C. DMPE grafted with PEG5000 only shifts the L_α-H_{II} transition of DEPE to higher temperatures but does not promote formation of cubic phase. These findings indicate that, consistent with their bulky head groups, the PEG-lipids decrease the tendency for negative interfacial mean curvature of the DEPE bilayers.

Keywords: Liposome; Mesophase; PEG-lipid; Phase transition; TRXRD

Using liposome preparations for in vivo drug delivery is a long-standing goal of numerous scientific researches. Some of the problems to be solved upon developing such preparations include increasing their time in blood circulation and controlled-release formulations. Important recent advances in liposome design in this respect represent incorporation into liposomes of phospholipids with poly(ethylene glycol) (PEG) chains covalently attached to their polar head group (PEG-lipids) for prolongation of the circulation time [1–4], and utilising of cubic mesomorphic liquid crystalline phases formed in lipid/water systems for controlled drug release [5,6].

Here we report an intersection of these two approaches in liposome design which may prove useful for further investigations in the field of controlled drug delivery. By using time-resolved X-ray diffraction we demonstrate that low amounts (5–10 mol%) of phospholipid grafted with PEG-550 induce spontaneous formation of cubic phase of space group Im3m in aqueous dispersions of dielaidoylphos-

Abbreviations: DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DMPE(PEG550), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 550]; DMPE(PEG5000), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 5000]

* Corresponding author. Fax: +359 2 9712493. E-mail: rkoynova@bgearn.acad.bg

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phatidylethanolamine (DEPE). The cubic phase was found to intrude in the temperature range between the lamellar liquid crystalline (L_α) phase and the inverted hexagonal phase (H_{II}) known to form in pure DEPE/water dispersions. Such knowledge is of basic scientific interest as well, since the physico-chemical properties of the recently developed PEG-lipids and their interactions with membrane lipids are still not well studied.

1,2-Dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 550] (DMPE(PEG550)), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 5000] (DMPE(PEG5000)) (Avanti Polar Lipids, Birmingham, AL) were used without further purification. The DEPE/PE(PEG) samples were prepared by mixing appropriate amounts of lipids as chloroform solutions, the chloroform was removed by rotary evaporation under nitrogen and the lipid mixtures were dried under vacuum for at least 48 h. Double distilled deionized water was added and the dispersions were hydrated overnight at 20°C. Samples were homogenised by at least 10 successive cycles of freezing to −20°C, followed by thawing at room temperature and vortexing during the thawing step. The lipid concentration was 10 wt%. The samples were filled into glass capillaries ($d = 1.0$ mm) (Hilgenberg, Malsfeld, Germany), flame sealed, and stored at room temperature for 1–2 days before measurements.

Time-resolved X-ray diffraction experiments were carried out on beam line X13 of the EMBL outstation at DESY in Hamburg. In brief this camera comprises a double focusing monochromator-mirror arrangement [7] with an X-ray wavelength of 0.15 nm. X-ray reflections in the small- and wide-angle regimes were recorded simultaneously using a data-acquisition system described recently [8]. Detectors were calibrated using dry rat tail tendon collagen (long spacing 65 nm) and Ag-behenate in the SAXS and *p*-bromobenzoic acid in the WAXS region. Data were normalised for incident intensity and analysed using the interactive data evaluating program OTOKO [9]. Diffraction patterns were recorded during heating-cooling scans at scan rates of 0.5–2 C°/min as previously described [10,11]. Temperature cycling at 10 C°/min was also applied.

Aqueous dispersions of DEPE were observed to form lamellar gel (L_β) phase at low temperatures, with lamellar repeat period $d = 6.5$ nm at 35°C, lamellar liquid crystalline (L_α) phase at intermediate temperatures ($d = 5.5$ nm at 40°C and 5.2 nm at 60°C), and inverted hexagonal (H_{II}) phase at high temperatures, with a lattice constant $a = 2d/\sqrt{3} = 7.5$ nm at 65°C and 7.3 nm at 75°C. The L_β-L_α transition was at 37.8°C and the L_α-H_{II} transition at 63.0°C, in good agreement with published data [12,13].

Addition of 5 mol% DMPE(PEG550) results in (i) decrease of the temperature of the L_β-L_α transition by ca. 1 C°; (ii) increase of the L_α-H_{II} transition temperature by ca. 10 C°, and (iii) considerable broadening of the SAXS lamellar reflections of both L_β and L_α phases which is attributed to strong perturbation of the interlamellar correlation. Interestingly, the SAXS reflections of the H_{II} phase in DEPE + 5 mol% DMPE(PEG550) mixture are similarly sharp as those in the pure DEPE dispersion. Also, the lattice constant of the H_{II} phase in the mixture coincides with that in pure DEPE at the same temperatures. Thus, at 75°C, $a = 7.3$ nm in the 95:5 (mol/mol) mixture. Concurrently with the H_{II} phase, additional trace reflections appear at small angles, better seen upon cooling. During the first heating-cooling course, these reflections are weak but become more pronounced with temperature cycling and gradually start dominating over the H_{II} phase reflections. Thus, after 10 cycles between 52.5 and 76.5°C, reflections at 15.9, 11.2, 9.2, 8.0 and 7.1 nm are observable, with the spacing ratios $\sqrt{2}$:$\sqrt{4}$:$\sqrt{6}$:$\sqrt{8}$:$\sqrt{10}$, characteristic for the cubic phases of cubic aspects #6 (single space group Pn3n) and #8 (space group of highest symmetry Im3m) [14].

In the aqueous dispersion of DEPE/DMPE(PEG550) mixture containing 10 mol% DMPE(PEG550), the L_β-L_α transition is shifted down in temperature by additional 1.5°C and takes place at 35.2°C upon heating at 1 C°/min. Upon further heating, first traces of a non-lamellar phase appear at 87°C. The spacings of these reflections are characteristic for a combination of a hexagonal phase with (100) reflection at 6.2 nm, and another non-lamellar, possibly cubic phase with the most intense reflections at 14.9, 10.5 and 8.6 nm. Pausing of heating at 89.8°C and equilibration of the sample at this temperature results in relatively fast disappearance of the

H_{II} phase and increasing intensity of the cubic phase. After 15 min incubation, the reflections of the H_{II} phase have disappeared from the X-ray pattern. The spacings of the remaining small-angle scattering reflections are in the ratio: √2 (strong):√4 (strong):√6 (very strong):√8 (weak):√10 (strong):√12 (very weak):√14 (strong):√16 (weak):√18 (strong):√20 (medium):√22 (weak):√24 (very weak):√26 (weak):√30 (weak):√32 (medium):√42 (medium):√48 (weak) (Fig. 1), consistent with cubic aspect #8, extinction symbol I—, with space group of highest symmetry Im3m [14]. The indexing of the diffraction pattern from Fig. 1 is shown in Fig. 2 as a plot of the reciprocal d-spacing vs. $\sqrt{(h^2+k^2+l^2)}$. It gives a straight line passing through the origin, that supports the cubic phase identification. The reciprocal slope of this line, giving the cubic unit cell lattice parameter, is $a = 20.5$ nm. Subsequent continuation of heating above 89.8°C leads to reappearance of the H_{II} phase at ca. 93°C, which coexists with the cubic phase up to 100°C. The lattice of the Im3m cubic phase shrinks upon heating, after the H_{II} phase appears, and at 100°C its lattice constant is 18.1 nm. Upon cooling, the reverse phase sequence $H_{II} + Q_{II} \rightarrow Q_{II} \rightarrow L_\alpha \rightarrow L_\beta$ is observed.

In a DEPE/DMPE(PEG550) sample containing 20 mol% DMPE(PEG550), an L_β-L_α transition takes place at 34.8°C. Only the L_α phase is observed up to 100°C. Equilibration at high temperatures (87°C and

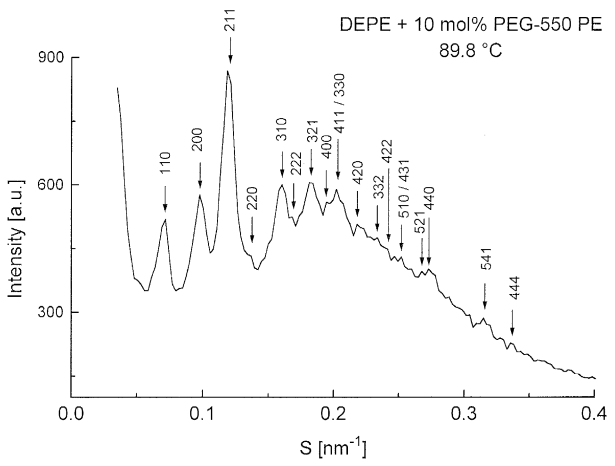

Fig. 1. Small-angle X-ray diffraction pattern of the hydrated DEPE + 10 mol% DMPE(PEG550) mixture recorded for 1 min at 89.8°C after heating at 1 C°/min and equilibration at this temperature for 20 min.

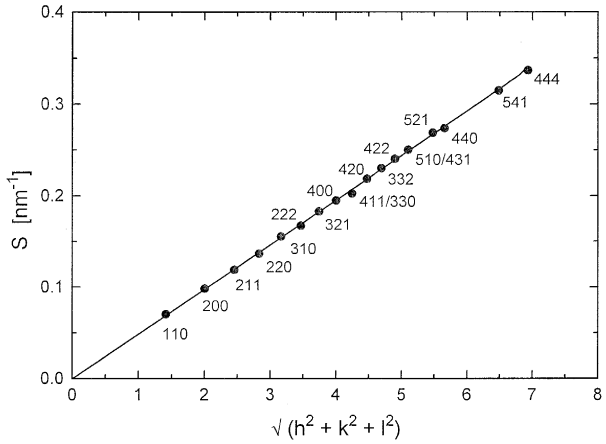

Fig. 2. Indexing of the X-ray diffraction pattern of the hydrated DEPE + 10 mol% DMPE(PEG550) mixture at 89.8°C as cubic aspect #8, space group Im3m. The lattice parameter obtained as the reciprocal slope is $a = 20.5$ nm.

100°C) for 15 min also does not result in any transformation of the L_α phase to a non-lamellar phase.

The observed propensity of DMPE(PEG550) to both increase the L_α-H_{II} transition temperature and promote formation of a cubic phase in a temperature range between the L_α and the H_{II} phases indicates that it decreases the tendency for negative interfacial mean curvature of the DEPE bilayer thus increasing the rate of formation of cubic phase. This behavior is consistent with the bulky hydrophilic head group of this PEG-lipid.

We examined also the effect of another PEG-lipid with a much bigger head group, DMPE(PEG5000), on the phase behavior of DEPE aqueous dispersion. Addition of 0.5, 1, and 2.5 mol% DMPE(PEG5000) to DEPE shifts the L_α-H_{II} transition upwards in temperature but does not induce formation of other mesomorphic phases. In a preparation with 2.5 mol% DMPE(PEG5000) the L_α-H_{II} transition is at 77°C and the lattice constant of the H_{II} phase is 6.9 nm at 80°C. Equilibration of these DEPE/DMPE(PEG5000) samples at different temperatures near and above the L_α-H_{II} transition temperature for 15–20 min does not produce observable changes in the H_{II} phase. Higher concentrations of DMPE(PEG5000) (5 and 10 mol%) rule out the L_α-H_{II} transition from the temperature scale up to 100°C. It is thinkable that the far bigger head group of DMPE(PEG5000) shifts the delicate

hydrophilic/hydrophobic balance regulating the phase behavior in lipid/water systems away from the region in which formation of cubic phases is favorable.

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