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Nanosized self-emulsifying lipid vesicles of diacylglycerol-PEG lipid conjugates: Biophysical characterization and inclusion of lipophilic dietary supplements

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ABSTRACT

Hydrated diacylglycerol-PEG lipid conjugates, glyceryl dioleate-PEG12 (GDO-PEG12) and glyceryl dipalmitate-PEG23 (GDP-PEG23), spontaneously form uni- or oligolamellar liposomes in their liquid crystalline phase, in distinct difference from the PEGylated phospholipids which form micelles. GDP-PEG23 exhibits peculiar hysteretic phase behavior and can arrange into a long-living hexagonal phase at ambient and physiological temperatures. Liposomes of GDO-PEG12 and its mixture with soy lecithin exchange lipids with the membranes much more actively than common lecithin liposomes; such an active lipid exchange might facilitate the discharging of the liposome cargo upon uptake and internalization, and can thus be important in drug delivery applications. Diacylglycerol-PEG lipid liposome formulations can encapsulate up to 20–30 wt.% lipophilic dietary supplements such as fish oil, coenzyme Q10, and vitamins D and E. The encapsulation is feasible by way of dry mixing, avoiding the use of organic solvent.

1. Introduction

Liposomes are self-assembled nanoparticles in which lipid bilayers encapsulate a volume of aqueous solution. They have been considered for a long time as one of the most promising delivery systems; their similarity to cell membranes makes them useful as a biomembrane model as well [1–3]. Because of their biocompatibility, biodegradability, low toxicity and low immunogenicity, the most valuable application of liposomes is in drug delivery, especially as intravenous drug carrier. Their pronounced instability in biological environments, such as in blood circulation, has been the reason for developing specific steric stabilization by grafting inert hydrophilic polymers such as PEG to their surface. Indeed, incorporation of PEG-lipid conjugates into the lipid bilayers (stealth liposomes) can significantly increase the liposome stability and prolong, by several orders of magnitude, their blood circulation times after systemic administration [4]. Thus, the grafted polymer acts as a steric barrier and reduces the level of plasma protein binding and uptake by phagocytic cells.

Problems associated with the liposome preparation include colloidal instability, difficulties with scale-up, sterilization, and irreproducibility between batches. The methods for liposome preparation typically include dissolving lipids in organic solvent, with subsequent solvent removal and hydration, which result in the formation of multilamellar vesicles (MLV). For some applications, small unilamellar vesicles (SUV) are preferred, which can be produced from MLV by various techniques such as sonication, extrusion through membranes, French press extrusion, etc. [3,5]. Processes like solvent removal, extrusion and homogenization may expose liposomal components to extreme, possibly damaging, conditions such as elevated pressures and temperatures, and high shear. The solution of some of these problems is provided by the recently introduced diacylglycerol-PEG lipid conjugates [6–9]. They spontaneously form liposomes in aqueous media due to their appropriate packing parameters, and exhibit steric stability due to the grafted polymer. Here we report results on the physico-chemical properties of two such compounds comprising dioleate or dipalmitate hydrophobic cores, and PEG12 or PEG23 hydrophilic moieties, respectively: glyceryl dioleate-PEG12 (GDO-PEG12) and glyceryl dipalmitate-PEG23 (GDP-PEG23) (Fig. 1). Their encapsulation capacity for lipophilic dietary supplements is also examined (although, in the context of drug carriers, the term "encapsulation" is more frequently used to indicate the presence of cargo inside the aqueous inner compartment of liposomes, generally it should not be limited to that; here we used it to indicate the inclusion of hydrophobic drugs in the core of the lipid bilayer).

2. Materials and methods

2.1. Materials

Glyceryl dioleate-PEG12 (GDO-PEG12) (BioZone Laboratories, Inc. Pittsburg, CA 94565), glyceryl dipalmitate-PEG23 (GDP-PEG23)

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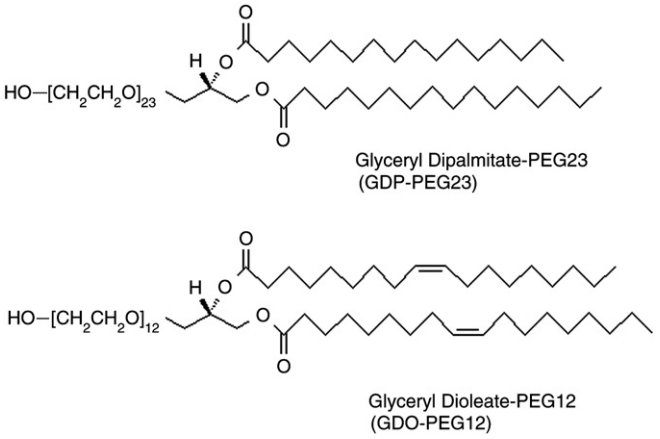

Fig. 1. Structures of the PEG lipids used in the study.

(Biopharma Scientific, Inc. San Diego, CA 92121) (Fig. 1), Liposome Concentrate (LipConc) (GDP-PEG23/soybean lecithin 1:1 wt/wt) (Biopharma Scientific, Inc. San Diego, CA 92121), 1,2-Dipalmitoyl-sn-3-phosphocholine (DPPC), 1,2-Dioleoyl-sn-3-phosphoethanolamine (DOPE) from Avanti Polar Lipids (Birmingham, AL); Coenzyme Q10 (CoQ10), Vitamin E, Vitamin D (Fisher Scientific), Fish Oil, Immune Oil (Pharmax LLC).

2.2. Sample preparation

Dispersions of lipids and lipid / dietary supplement mixtures were prepared in two different ways: (i) by dissolving the compounds in organic solvent (typically chloroform), followed by the solvent removal under argon and overnight evacuation; aqueous phase was then added followed by vortex-mixing. Typically, several cycles of freezing–thawing have been applied to the lipid aqueous dispersions, in order to attain maximum hydration and sample homogeneity. (ii) Dry preparation: lipid dispersions were prepared by direct addition of aqueous phase to the lipid; for mixed samples, the compounds were initially mixed in dry state, then aqueous phase was added, followed by vortex-mixing (in mixtures, wt/wt is used to indicate lipid/lipid or lipid/oil weight ratio). For preparing dispersions of GDP-PEG23, which is solid at room temperature, both lipid and aqueous phase were heated to 60 °C prior to mixing; at that temperature GDP-PEG23 was a transparent viscous fluid. For the lipid exchange experiments, equal volumes of dispersions with the same weight fraction of lipid have been mixed. The lipid concentration of the dispersions was typically 2 wt.% for DSC samples, 20 wt.% for the X-ray experiments and 0.01–0.1 wt.% for the light scattering samples.

2.3. Differential scanning calorimetry (DSC)

Measurements were performed using a VP-DSC Microcalorimeter (MicroCal Inc., Northampton, MA) at 30 °C/h. Thermograms were analyzed using OriginLab (Northampton, MA) software.

2.4. Synchrotron small-angle X-ray diffraction (SAXD)

Measurements were performed at Argonne National Laboratory, Advanced Photon Source, DND-CAT (beamline 5-IDD) and BioCAT (beamline 18-ID), using 12 keV ($\lambda = 1.033$ Å) X-rays, as previously described [10]. The lipid concentration of the dispersions was 20 wt.%. Samples were filled into glass capillaries and flame-sealed. A Linkam thermal stage (Linkam Sci Instruments, Surrey, England) provided temperature control. Linear heating and cooling scans were performed at rates of 1–5 °C/min. Exposure times were typically ~0.5 s. Data were collected using a MAR-CCD detector. Sample-to-detector distance was 1.8–2 m. Diffraction intensity vs. Q plots were obtained by radial integration of the 2D patterns using the interactive data-evaluating program FIT2D [11]. Some samples with longer exposure time were checked by thin layer chromatography after the experiments. Products of lipid degradation were not detected in these samples and radiation damage of the lipids was not evident from their X-ray patterns.

2.5. Dynamic light scattering

Measurements were performed with a Brookhaven Instrument with BI-200SM goniometer and BI-9000 digital correlator (Brookhaven, NY). Lipid dispersions in PBS were prepared at 100 µg/ml. Measurements were carried out at 37 °C. Borosilicate glass, 250 µl, 3×30 mm, flat bottom tubes were used. Delay times of 10 µs–1 s were examined. The correlation data were fitted with quadratic cumulants, using the algorithm provided with the instrument.

2.6. Transmission electron microscopy

2.6.1. Fixation and embedding

Samples were washed with PBS and fixed with 2% Glutaraldehyde (GA) in 0.1 M Cacodylate buffer, pH 7.3 at 4 °C for 30 min. The material was washed in the Cacodylate buffer and post-fixed in 2% OsO_4 for 30 min at 4 °C and then thoroughly washed again. Cells were dehydrated in ethanol solutions at increasing concentrations of 60%, 70%, 80%, 95% and 100% followed by infiltration with Epoxy embedding medium, transferred into fresh Epoxy resin and polymerized at 64 °C for 48 h. Sections of 65 nm thickness were cut on a Leica ultramicrotome, stained with Uranyl acetate and post-stained with Reinold's. Thin sections were examined on Philips CM12 transmission electron microscope at 100 kV and micrographs were taken on film.

3. Results and discussion

3.1. Phase behavior of diacylglycerol-PEG lipid conjugates

3.1.1. GDP-PEG23

Aqueous dispersion of GDP-PEG23 was prepared by direct mixing of lipid and water at 60 °C, followed by overnight equilibration at room temperature. Dispersion exhibits double endotherm (two sequential endotherms) upon initial heating (Fig. 2), with specific heat maxima at 13.5 °C and 28 °C. The peak at 13.5 °C increases its enthalpy upon low-temperature incubation, while the peak at 28 °C

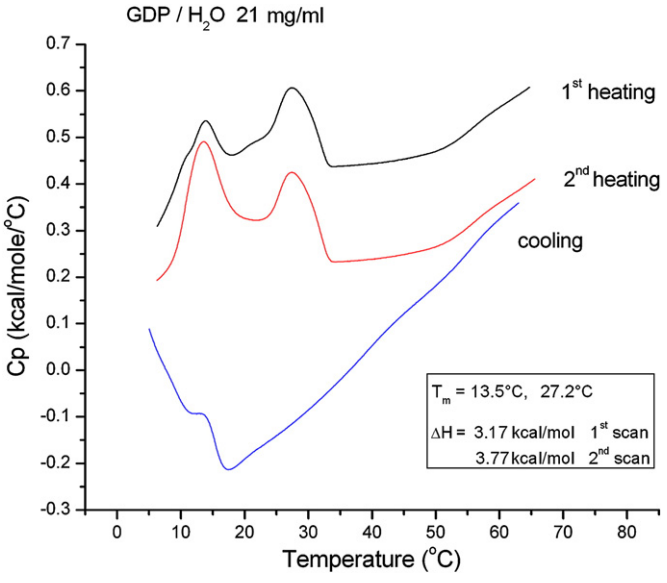

Fig. 2. Heating and cooling thermograms of a GDP-PEG23 aqueous dispersion.

dominates upon immediate reheating, and also on the cooling thermograms. The observed hysteresis is suggestive for phase metastability. In PBS (50 mM phosphate buffer, 100 mM NaCl, pH 7.2), the temperatures of the endotherms are close to those in water. A similar complex transition pattern has been reported for other saturated diacylglycerol-PEG conjugates [7].

According to the small-angle X-ray diffraction data, GDP-PEG23 forms a lamellar phase at room temperature, with lamellar repeat period $d = 9.6$ nm (Fig. 3A). This is considerably larger than the lamellar spacings of other saturated dipalmitoyl glycerolipids (e.g., $d \sim 6.4$ nm for DPPC, $d \sim 6.0$ nm for DPPE [12]). The difference is apparently due to the voluminous PEG-chains conjugated to the glycerol backbone (Fig. 1). Noteworthy is the peculiar diffraction intensity distribution, with a strongly dominating lamellar second order, suggestive of an unusual electron density distribution, as well as the broad hump underlying the lamellar reflections. Upon heating, the lamellar reflections disappear, and only the broad hump remains at temperatures above 35 °C. Such diffraction humps are indicative of small aggregates, lacking repeat order, such as uni- or oligolamellar liposomes (or micelles), with uncorrelated bilayers. Since GDP-PEG23 comprises saturated palmitoyl chains, the recorded transition presumably reflects the gel-liquid crystalline (melting) of the lipid hydrocarbon chains. Considering the large difference in the scan rate of the calorimetric and X-ray diffraction experiments, the temperature discrepancy between the major endothermic event at ~28 °C, and the X-ray data, recording the disappearance of the ordered lamellar phase above 30 °C, could be anticipated.

Upon cooling, the lamellar phase does not restore. A highly ordered diffraction pattern, with spacings in the ratio of $1:1/\sqrt{3}:1/2$, characteristic of a hexagonal phase arrangement, appears at ~40 °C (Fig. 3B). It persists on cooling to ~10 °C, and converts to a strongly disordered, uncorrelated lamellar phase. The lamellar-hexagonal transition is reversible: the hexagonal phase is observed in the temperature interval ~20–50 °C during the subsequent heating scan.

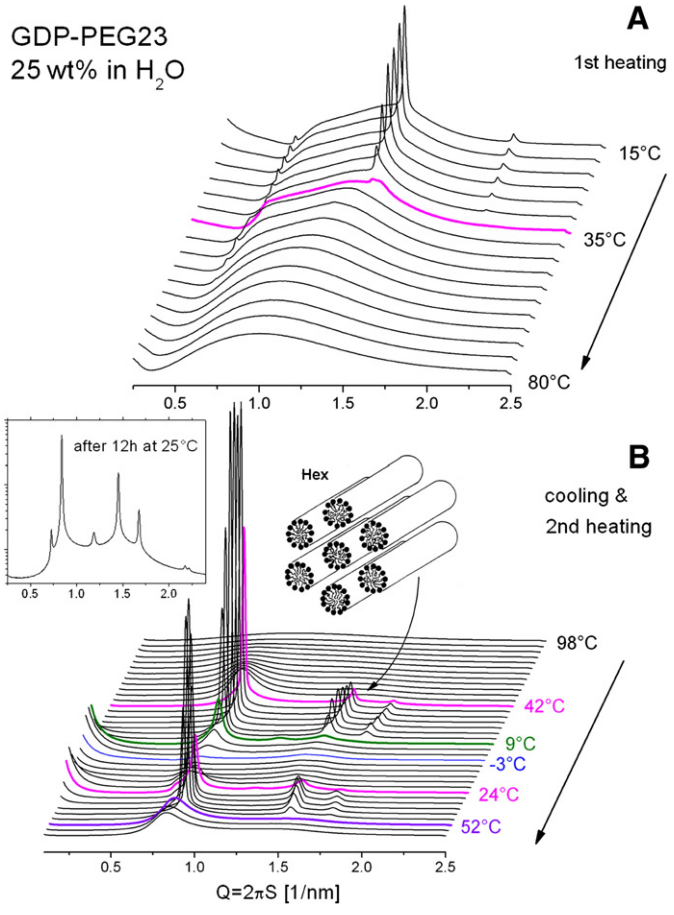

Fig. 3. SAXD patterns of a GDP-PEG23 aqueous dispersion, recorded upon initial heating (A), and subsequent cooling–heating cycle (B).

The observed hexagonal phase is characterized by a relatively large unit cell dimension, $a = 2d_{10}/\sqrt{3} = 8.3$ nm, by almost 2 nm larger than that of dipalmitoleoyl phosphatidylethanolamine, comprising the same 16C hydrocarbon chainlength [13]. From the diffraction data only, it is impossible to distinguish between normal (oil-in-water, type I) and inverted (water-in-oil, type II) hexagonal phase. Considering the voluminous PEG-chains conjugated to the lipid glycerol backbone, however, it seems hardly possible to accommodate the inverted hexagonal phase. The observed phase sequence is also suggestive of a normal type hexagonal phase. Upon equilibration at room temperature for 12 h, cubic phase developed in coexistence with the hexagonal phase (Fig. 3B, inset). The observed reflections are insufficient for its unambiguous identification, but from the initial reflections ratio as $1/\sqrt{6}:1/\sqrt{8}:1/\sqrt{14}$ it might be supposed to be the Ia3d (gyroid) phase [14].

The phase behavior of the PEG-diacylglycerol conjugates is substantially different from that of the PEG-phospholipid conjugates, which do not form lamellae, but have been reported to arrange into micelles in their liquid crystalline state [15]. A likely reason for the difference is the lack of an electric charge in the PEG-diacylglycerol conjugate headgroups. Indeed, the PEG-derivatization of phospholipids leaves an uncompensated negative charge at the phosphate group, thus significantly enhancing the effective size of the headgroup due to the electrostatic repulsion, making way for micelles as a favored packing mode of these molecules. At the same time, the PEG-diacylglycerol compounds studied here are uncharged, with thus effectively smaller headgroups, which makes it possible for them to pack into bilayers, and to spontaneously form unilamellar liposomes (self-emulsifying compounds).

Because of the peculiar and complicated phase behavior of GDP-PEG23, exhibiting phase transition above room temperature, and a strong temperature hysteresis, a 1:1 w/w mixture of GDP-PEG23 with soy lecithin was prepared, further referred to as Liposome concentrate (LipConc). The LipConc mixture was found to combine the spontaneous liposome forming ability of the PEG lipid with the lack of a phase transition in the physiologically relevant temperature range, typical for the natural extracts (see below). According to the calorimetric data, it undergoes a melting transition at ~12 °C (data not illustrated). At room and physiological temperatures, it is arranged into highly swollen, uncorrelated lamellae (see X-ray patterns below) similar to those of the liquid crystalline GDP-PEG23. Indeed, such uncorrelated, irregular bilayers could be seen in the micrographs (Fig. 4).

3.1.2. GDO-PEG12

Aqueous dispersions of GDO-PEG12 do not exhibit calorimetric transitions in the temperature interval 5–95 °C. It is typical for lipids with unsaturated oleoyl hydrocarbon chains to exhibit gel-liquid crystalline transitions at temperatures below 0 °C [16].

The X-ray diffraction pattern of GDO-PEG12 dispersions is dominated by a broad hump centered at ~5 nm (Fig. 5A), characteristic of small disordered aggregates, possibly liposomes. They coexist with a small portion of highly swollen lamellar arrays, as judged by the minor diffraction peak at ~11.9 nm. The latter peak disappears at ~47 °C upon heating, and the system seems fully disordered and non-diffracting at higher temperatures. On cooling, the swollen lamellae are replaced by highly ordered lamellar phase at temperatures below 46 °C, with a lamellar repeat period $d = 5.6$ nm.

In equimolar mixture with DOPE (frequently used as a "helper lipid" in drug delivery lipid formulations) GDO-PEG12 exhibits the phase sequence: lamellar → bilayer cubic (Pn3m) → hexagonal → micellar cubic (Fd3m) → micellar phase upon heating from 20 °C to 90 °C (Fig. 5B).

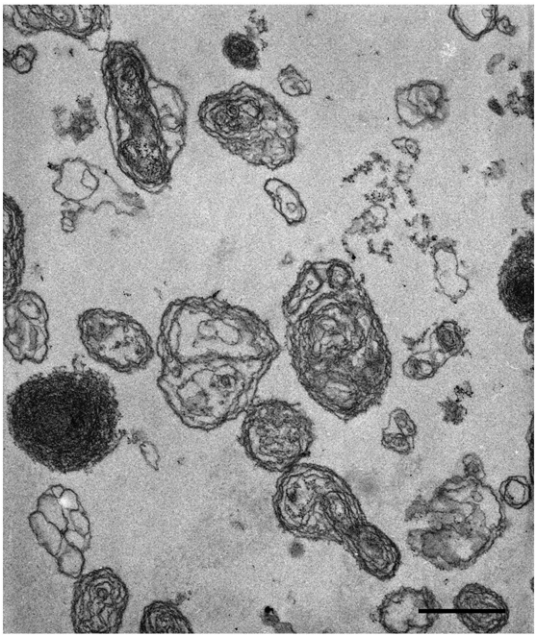

Fig. 4. Thin section of LipConc MLV embedded in Eponate resin; bar—1 μm.

3.2. Liposome size

Both GDP-PEG23 and GDO-PEG12 form liposomes spontaneously upon hydration. Their size and morphology were examined by dynamic light scattering (DLS) and electron microscopy.

At 25 °C large, micron-size aggregates form in GDP-PEG23 (Fig. 6A). Upon heating above the melting transition they reorganize into liposomes of ~130 nm mean diameter (Fig. 6B). The liposome sizes of GDO-PEG12 are somewhat larger, with 180–200 nm diameter (Fig. 6C); strong (over twofold) size decrease was observed with the GDO-PEG12 liposomes on heating from 25 to 60 °C. In PBS dispersions the particle sizes were similar to those in water. These sizes are considerably larger than those reported for sonicated phosphatidylcholine vesicles, d~15–20 nm, but smaller than the multilamellar phosphatidylcholine vesicles forming spontaneously [17]. The polydispersity index range for the GDP-PEG23 and GDO-PEG12 liposomes was typically 0.2–0.4.

The liposomes prepared using LipConc are smaller (100–200 nm) than those of GDO-PEG12. Their size is also much less sensitive to temperature relative to the GDO-PEG12 liposomes, yet still more sensitive than regular phosphatidylcholine unilamellar liposomes [18].

3.3. Liposomes prepared by GDO-PEG12 or LipConc exchange lipids with the membranes much more actively than common lecithin liposomes

Differential scanning calorimetry was used to examine the lipid exchange between model phosphatidylcholine membranes and liposomes of GDO-PEG12 or LipConc. In mixtures, transition temperatures are informative about the mode of mixing (i.e., the homogeneity of the mixture) and depend on composition. For miscible components, the transition temperature can be used as a measure of composition. Thus, the changes of the lipid phase transition parameters (temperature, shape) upon consecutive temperature scans were used as an indication of lipid mixing between aggregates, as described earlier [19].

The usual approach to lipid mixing includes preparation of molecular mixtures in organic solvent, with subsequent solvent removal and hydration. This procedure is believed to produce essentially equilibrium mixtures, but it is not quite biologically relevant, since in biological systems, lipid exchange can take place in an aqueous medium. We thus used lipids premixed in chloroform as reference samples for complete, equilibrium lipid mixing. These control samples were compared to samples in which lipid aqueous dispersions were prepared separately, then mixed *extempore* in water, and successive heating–cooling thermograms through their solid–liquid crystalline phase transition were recorded. Since transition thermodynamic parameters are strongly sensitive to the composition, they were taken as an indication for lipid mixing [19].

Mixture of DPPC and GDO-PEG12 prepared by premixing of the lipids in chloroform exhibits phase transition centered at 38 °C (Fig. 7A, top). When DPPC and GDO-PEG12 liposomes were mixed as aqueous dispersions at 5–10 °C and immediately scanned in the calorimeter, a double endotherm (two peak) was recorded, with peaks at 38 °C and 41 °C (Fig. 7A). A transition temperature of 41 °C is characteristic for the pure DPPC dispersions [16], and is thus a

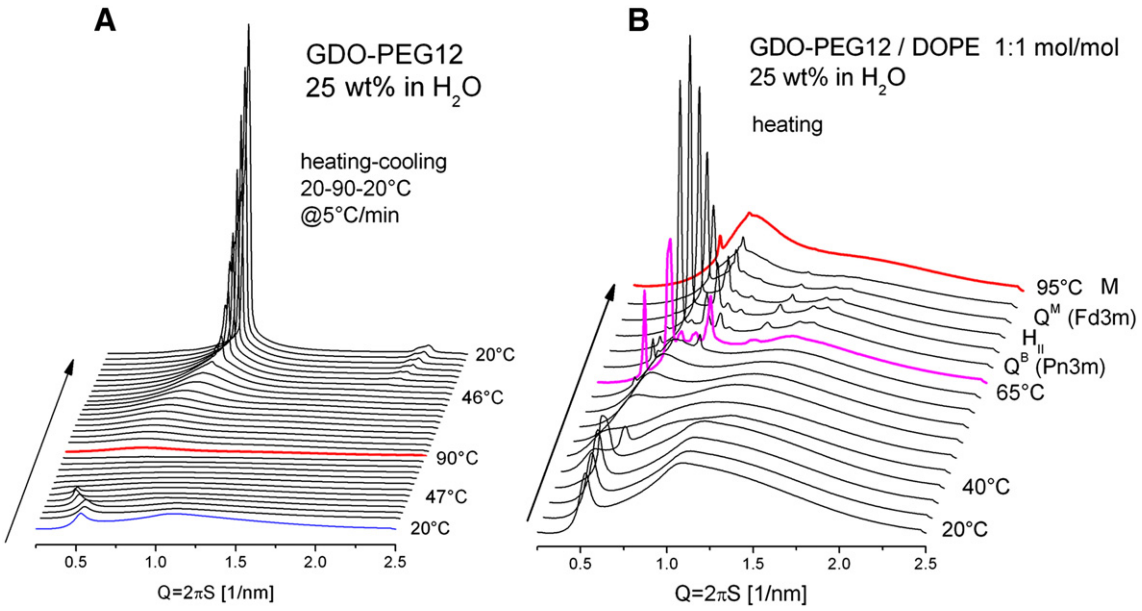

Fig. 5. SAXD patterns of a GDO-PEG12 aqueous dispersion (A) and GDO-PEG12/DOPE mixture (B), recorded upon temperature scans.

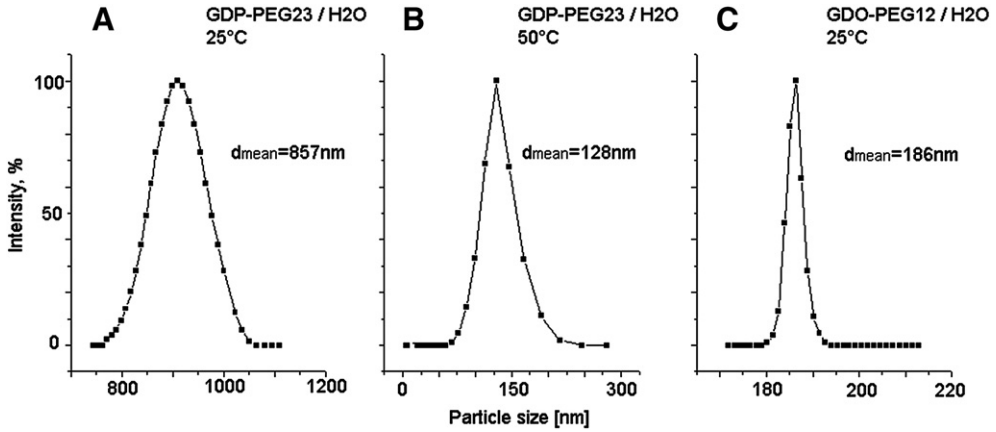

Fig. 6. Particle size distributions of aqueous dispersions of GDP-PEG23 at 25 °C (A) and at 50 °C (B), and GDO-PEG12 at 25 °C, as recorded by dynamic light scattering.

measure of the relative amount of unmixed DPPC in the sample. During subsequent heating scans, the peak at 41 °C disappeared, and only the peak at 38 °C, virtually equivalent to that of the chloroform-premixed sample, was observed during the 3rd heating scan. Variations of the lipid concentration between 1 and 10 wt.% did not influence the rate of change of the transition temperature. These results are compatible with monomeric exchange through the aqueous medium and not upon vesicle collision [19].

For comparison, a similar experiment was performed with liposomes of DPPC and the non-PEGylated DOPC (Fig. 7B). The chloroform-premixed DPPC/DOPC sample exhibits a broad transition at 31 °C (Fig. 7B, top). The mixture of DPPC and DOPC aqueous liposome dispersions exhibits sharp endotherm at 41 °C and a small one at 35 °C, typical for the DPPC main transition and pretransition, respectively. Subsequent heating scans result in only a negligible increase in the main transition half-width, indicative of only an insignificant inclusion of DOPC admixtures in the DPPC liposomes. Lipid transfer is thus strongly suppressed between the phosphatidylcholine liposomes.

Relatively fast lipid exchange, although not as fast as that demonstrated by GDO-PEG12, was measured for LipConc liposomes as well (Fig. 7C).

Thus, exchange via lipid monomers is considerably more active for the PEG lipids than for the common phosphatidylcholines, presumably due to the higher monomer solubility of the PEGylated lipids [20,21]. Such an active lipid exchange is evidently an advantage for drug delivery applications, because it would facilitate the discharging of the liposome "cargo". Since the presence of the PEG-chains at the liposome surface would prevent close contact and fusion of liposomes with cell membranes (thereby prolonging the circulation time *in vivo*), it is necessary to have a mechanism of releasing the carried drug. The process of relatively facile lipid exchange reveals such an alternative mechanism for drug release from the PEG-lipid liposomes.

The observed strong temperature dependence of the GDO-PEG12 liposome size is perhaps also a consequence of the high lipid monomer concentration (CMC) in water and its pronounced temperature dependence.

3.4. Liposome digestion by plasma macrophages

Plasma monocytes were incubated in macrophage SFM media (12065-074) in 6 well plates in an incubator with a 5% CO_2, at 37 °C to mature to macrophages at 10^6 cells/well. The cells were incubated for an additional 60 min in the presence of 500 ng PEG-23 GDP liposomes followed by additional 15 min incubation with 0.25% Trypsin-EDTA. The macrophage suspension was centrifuged at 2000 rpm for 5 min. The pellet was fixed with 4% PFA in PBS overnight and processed for transmission electron microscopy (Fig. 8). The micrograph visualizes liposomes internalized within the macrophages.

3.5. Incorporation of lipophilic dietary supplements in LipConc liposomes

3.5.1. Fish oil and immune oil

Fish oil contains the omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid, precursors to eicosanoids that reduce inflammation throughout the body, and for this reason are highly recommended for a healthy diet.

At low concentrations (10–20 wt.%) fish oil stabilizes the LipConc bilayers (Fig. 9A). Thus, LipConc samples with 10 wt.% and 20 wt.% fish oil form expanded lamellar phase, with a lamellar repeat period of ~10 nm. The change of the bilayer structure and ordering is indicative for the inclusion of fish oil into the lipid bilayers. By presumably "dissolving" amongst the lipid molecules, rather than into the hydrophobic bilayer part, it effectively suppresses the intrinsic bilayer curvature imposed by the bulky PEG chains. At 35 wt.% fish oil, bilayers are still observable into the diffraction pattern, coexisting with aggregates giving rise to the hump at ~5–5.3 nm, presumably unilamellar liposomes (Fig. 9A). At 50 wt.% and 70 wt.% fish oil the system is strongly disordered. The hydrated pure fish oil samples do not exhibit diffraction patterns—they presumably form oil drops, so diffraction was not expected. Thus the samples with more than 20 wt.% fish oil are supposedly phase separated, with coexisting LipConc liposomes incorporating ~20 wt.% fish oil, and fish oil drops most likely decorated with a lipid shell.

Similarly to the fish oil, addition of 20 wt.% immune oil produces bilayer stabilization (Fig. 9A). Thus immune oil at this concentration is apparently incorporated into the LipConc bilayers.

The samples for these experiments were prepared by mixing of the lipid and fish oil as chloroform solutions, with subsequent chloroform evaporation and hydration (because this way it is easier to precisely control the ratio of the components in small samples). Control samples with 20 wt.% and 30 wt.% fish oil in LipConc prepared by mixing of the dry substances and subsequent hydration exhibited the same (even better) ordering and supplement incorporation (Fig. 10).

We thus conclude that fish oil can be incorporated in LipConc liposomes up to ~20–30 wt.%. Dry mixing the two components is possible and gives identical results to the mixing from organic solvent.

3.5.2. Coenzyme Q10

Coenzyme Q (CoQ), also known as ubiquinone, is a biologically active quinone with an isoprenoid side chain, related in structure to vitamin K and vitamin E. The various kinds of Coenzyme Q can be distinguished by the number of isoprenoid side chains they have. The most common CoQ in human mitochondria is CoQ10. Because of its ability to transfer electrons and therefore act as an antioxidant, CoQ10 has become a valued dietary supplement. CoQ10 has been widely used for the treatment of heart disease (especially heart failure), gum diseases, and also breast cancer.

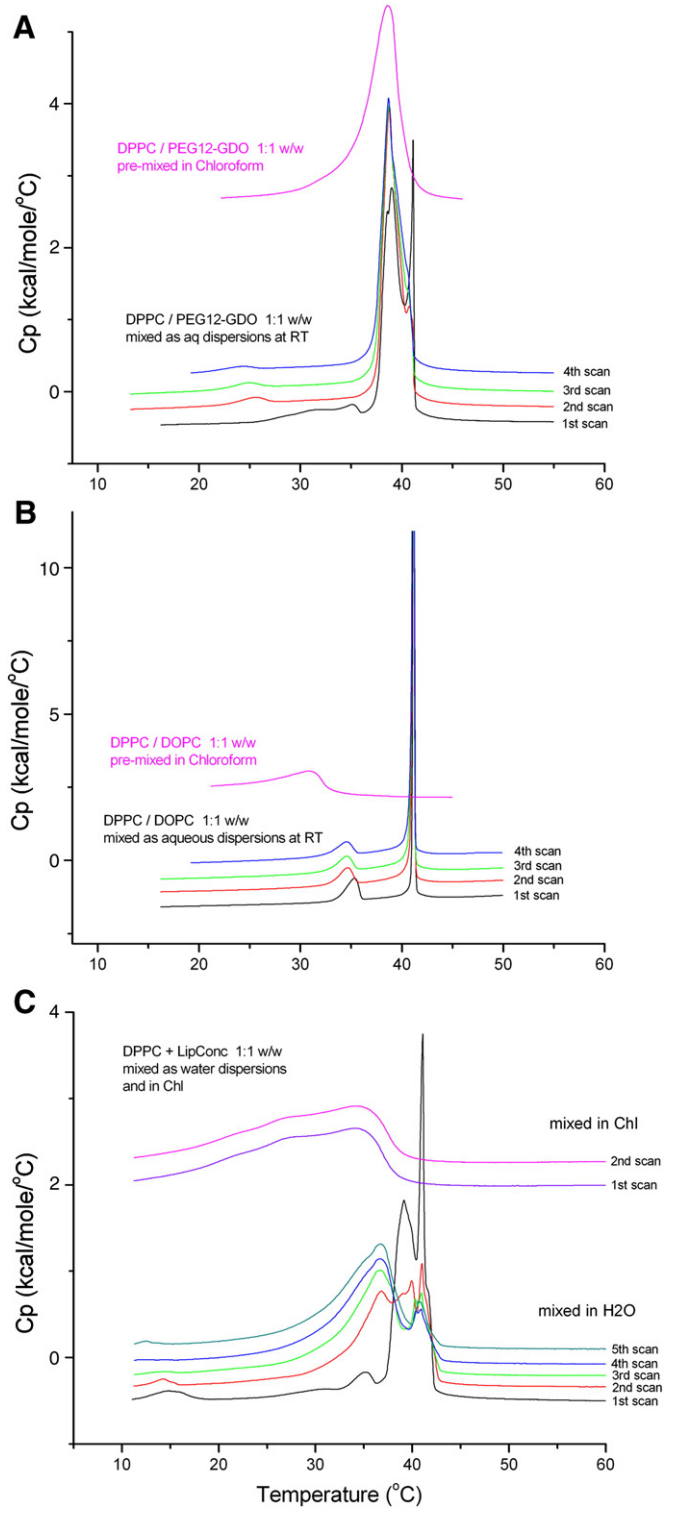

Fig. 7. Heating thermograms of DPPC/GDO-PEG12 (A), DPPC/DOPC (B) and DPPC/LipConc (C) mixtures, prepared as separate dispersions and mixed extempore, or premixed in chloroform, as indicated (thermograms have been vertically moved in order to facilitate the figure readability).

The pure hydrated CoQ10 organizes into well-ordered bilayers, with a lamellar spacing $d = 5.7$ nm, at room temperature and at 37 °C (Fig. 9B, top). Upon heating, the lamellar ordering is lost at ~60 °C, and no diffraction pattern is observed above that temperature—the sample probably transforms into micelles or oil drops after melting, as previously reported [22]. The transition is slowly reversible—several hours equilibration at room temperature are necessary before the lamellar diffraction pattern reappears.

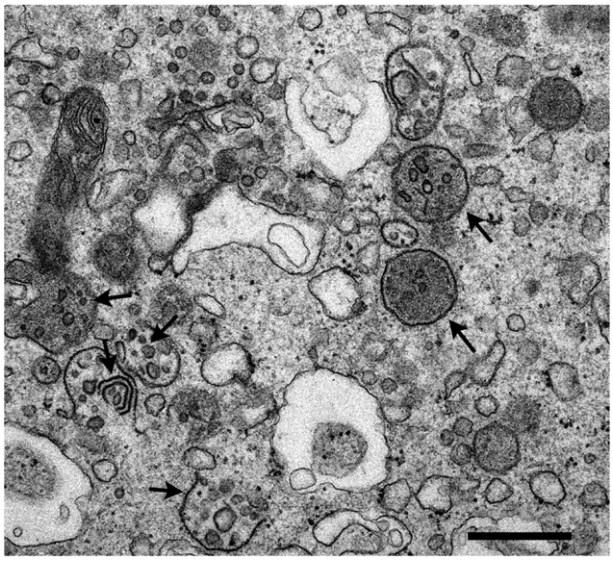

Fig. 8. Thin section of Eponate embedded sample of plasma macrophages incubated with PEG-23 GDP liposomes for 60 min in 5% CO_2 incubator at 37 °C; arrows point at the internalized liposomes; bar = 500 nm.

When mixed with LipConc, 10 wt.% and 20 wt.% CoQ10 stabilize the liposome bilayers, and, similarly to the fish oil, appear incorporated into the bilayer (Fig. 9B). At 35 wt.% CoQ10, according to the diffraction pattern the sample seems phase separated and a phase with CoQ10-saturated lipid bilayers coexists with (nearly) pure CoQ10 lamellae. After heating to above 60 °C, the components mix, and stay mixed upon cooling down to room temperature for several hours. At a higher concentration of CoQ10 (50 and 70 wt.%) it seems immiscible with the liposomes even after heating. Thus, an immiscibility composition range at >20 wt.% CoQ10 obviously exists in the LipConc/CoQ10 phase diagram. This result is in accord with previously published data for DPPC/CoQ10 mixtures [23].

The samples were prepared by mixing the components as chloroform solutions, with subsequent chloroform evaporation and hydration. Since the source CoQ10 was a powder, dry mixing test has not been performed.

Thus, we conclude that CoQ10 can be incorporated in LipConc liposomes up to ~20–25 wt.%; for a higher concentration (~35 wt.%) it would necessary to heat the dispersion to >60 °C, and to lyophilize it immediately upon cooling to room temperature, within several hours, in order to avoid phase separation.

3.5.3. Vitamin D

Vitamin D (VitD) refers to a group of lipophilic prohormones, the two major forms of which are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). The term vitamin D also refers to metabolites and other analogues of these substances. Vitamin D3 is produced in skin exposed to sunlight, specifically ultraviolet B radiation. Vitamin D plays an important role in the maintenance of several organ systems. It regulates the calcium and phosphorus levels in the blood by promoting their absorption from food in the intestines and by promoting reabsorption of calcium in the kidneys; it promotes bone formation and mineralization and is essential in the development of an intact and strong skeleton; it also affects the immune system by promoting immunosuppression and anti-tumor activity.

The hydrated VitD samples do not exhibit ordering, as manifested by the lack of small-angle diffraction. Addition of 10, 20, and 35 wt.% of VitD to the LipConc liposomes strongly influenced the bilayer ordering (Fig. 9C) and was thus indicative of inclusion of the drug into

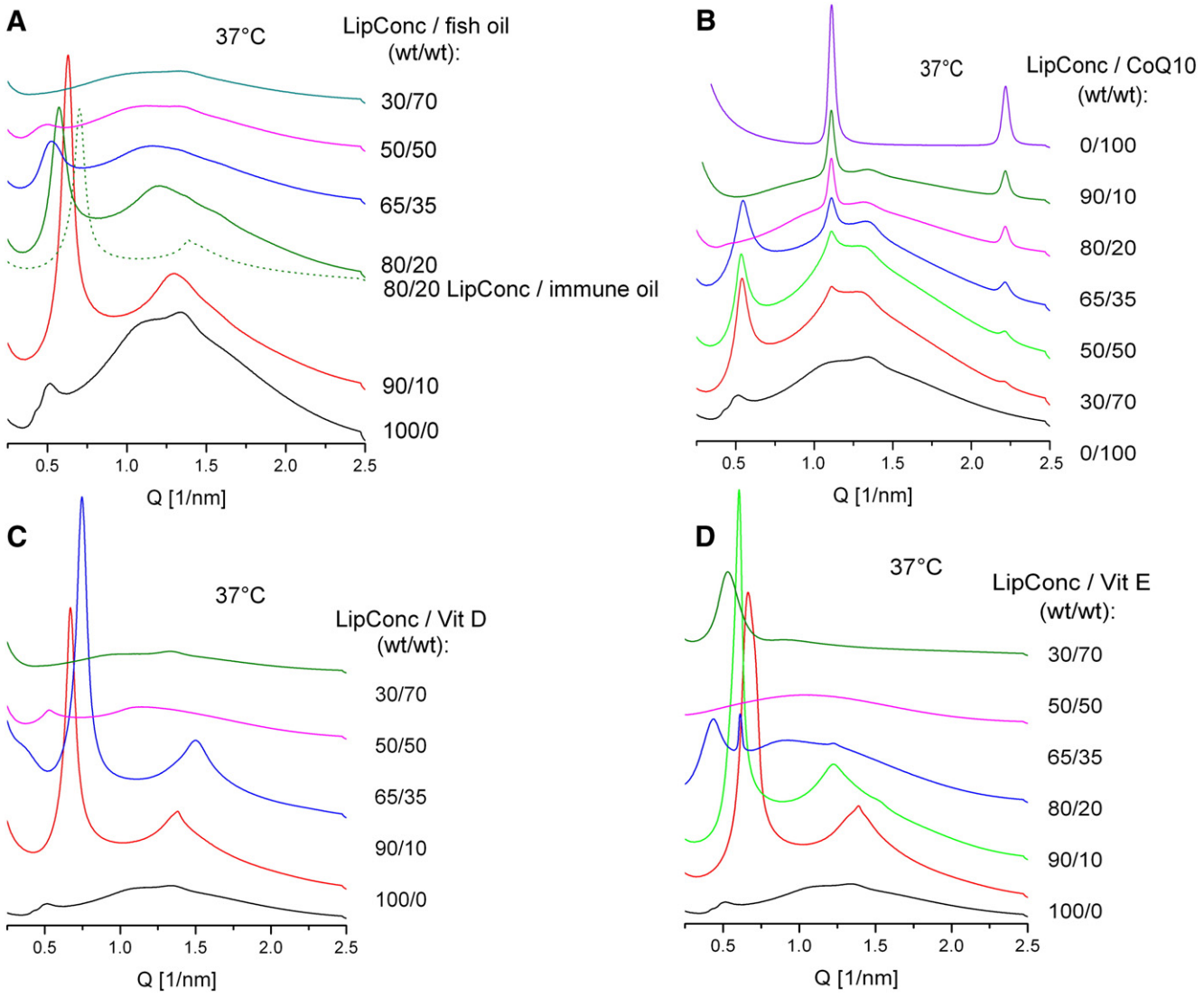

Fig. 9. SAXD patterns of: (A) LipConc/fish oil (and immune oil—dotted line); (B) LipConc/CoQ10; (C) LipConc/VitD; and (D) LipConc/VitE mixtures at different compositions, recorded at 37 °C.

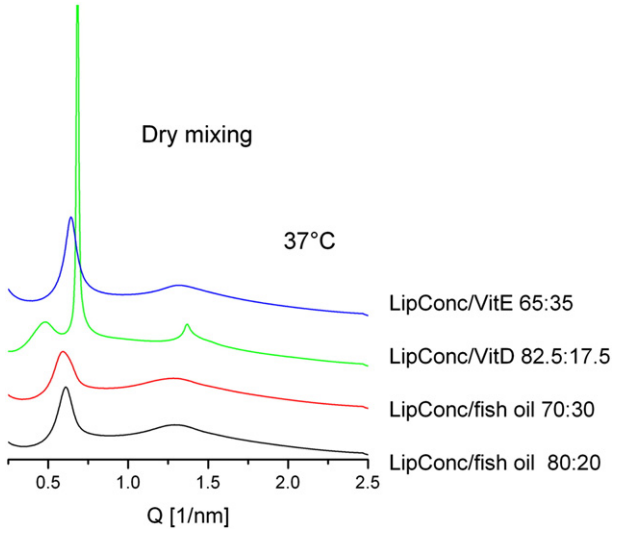

Fig. 10. SAXD patterns at 37 °C of mixtures of LipConc with fish oil, VitD and VitE, prepared by direct mixing of the compounds and subsequent hydration, without chloroform premixing.

the bilayers. Higher concentrations (50 and 70 wt.%) did not display lamellar ordering anymore (Fig. 9C).

The above samples were prepared by mixing of the lipid and drug as chloroform solutions. Control samples with 20 wt.% and 40 wt.% VitD were prepared by mixing of the dry substances. The 20 wt.% sample exhibited the same organization as that mixed in chloroform. Lamellar ordering was not observed at 40 wt.% VitD dry mixed with LipConc (Fig. 10). Hence, VitD can be incorporated in LipConc liposomes up to ~35 wt.%. Dry mixing of the two components is possible at those concentrations.

3.5.4. Vitamin E

Tocopherol, or vitamin E (VitE), is a lipophilic vitamin that is an important antioxidant; it is also essential in the formation of red blood cells. Tocopherol occurs in eight different isoforms; α-tocopherol is a form of vitamin E that appears to have the greatest nutritional significance.

The behavior of the LipConc/VitE mixture is more complicated than those of the other drugs above and required more experimentation.

Samples of 10 wt.% and 20 wt.% VitE behave similarly to those with the additives discussed above: they are well-ordered, with lamellar spacings d ~9.5–10 nm, and the drug appears incorporated into the liposome bilayers, as judged from the changes in the structural

parameters of the bilayers (Fig. 9D). At 35 wt.%, however, the sample is obviously phase separated into two components, and did not homogenize at any temperature up to 90 °C. The sample with 50 wt.% VitE was strongly disordered. Remarkably, a sample of 70 wt.% VitE again displayed good lamellar organization, but different of that at 10 wt.% and 20 wt.% VitE—with largely expanded lamellar period, d~12 nm. Noteworthy, this sample also looked very different from all other samples—very dense white sticky substance. The pattern of the pure hydrated VitE was also peculiar and difficult to interpret. Obviously, the phase diagram of this mixture is complicated and requires special investigation, which was out of the scope of the present study. Anyway, it appears safe to incorporate up to 20 wt.% VitE into the LipConc liposomes.

3.5.5. Fish oil, VitD and VitE mix with LipConc in dry state in virtually the same way as in chloroform solution

For industrial applications, it is often technologically easier and safer if the liposome lipid and the additives could be dry-mixed, avoiding the troublesome mixing in organic solvent with subsequent solvent evaporation. With this motivation, we performed control experiments with mixing LipConc with fish oil, VitD, or VitE as dry substances, with subsequent hydration.

The X-ray patterns of these "dry" samples (Fig. 10) are virtually the same as those of samples in which the components have been mixed as chloroform solution, with subsequent chloroform evaporation and subsequent hydration. There was no indication for phase separation, or differences in the structural organization suggestive for a different component ratio when applying the two different protocols. This was taken as indicative for identical sample organization in the two preparations.

4. Conclusions

- Hydrated GDP-PEG23 and GDO-PEG12 both form lamellar liquid crystalline phase, and morphologically arrange into uni- or oligolamellar liposomes; the liposomes form spontaneously upon hydration. This is in distinct difference from the PEGylated phospholipids which only form micelles but not liposomes, and is possibly due to the lack of electric charge in the PEG-diacylglycerol conjugate headgroups. The formation of liposomes might be advantageous for drug delivery because of their higher encapsulation capacity.
- GDP-PEG23 exhibits peculiar phase behavior and can form a long-living hexagonal phase at ambient and physiological temperatures. This feature might be important for drug delivery applications, since nonlamellar phase formation is known to facilitate the release of the content of lipid vehicles.
- The GDO-PEG12 liposomes are bigger than the sonicated phospholipid vesicles; their size exhibits pronounced temperature dependence.
- GDO-PEG12 and LipConc liposomes exchange lipids with the membranes much more actively than common lecithin liposomes, presumably due to the higher monomer solubility (CMC) of the PEGylated lipids. Such an active lipid exchange might be an advantage for drug delivery applications, because it would facilitate the discharging of the liposome cargo upon uptake and internalization.
- Liposomes of LipConc formulation might be used for incorporation and delivery of lipophilic dietary supplements.
- Fish oil can be incorporated in LipConc liposomes up to ~20–30 wt.%. Dry mixing of the two components is feasible and gives identical results to the mixing in organic solvent.
- CoQ10 can be incorporated in LipConc liposomes up to ~20–25 wt.%; for incorporation of higher concentration (~35 wt.%) it would be necessary to heat the dispersion to >60 °C, and to lyophilize it immediately upon cooling to room temperature, within several hours, in order to avoid phase separation.
- Vitamin D can be incorporated in LipConc liposomes up to ~35 wt.%. Dry mixing of the two components is achievable at those concentrations.
- Vitamin E can be incorporated in LipConc liposomes up to ~20 wt.%.

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