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# Influence of preparation path on the formation of discs and threadlike micelles in DSPE-PEG$_{2000}$/lipid systems

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**Abstract**

In a recent study we showed that the surfactant 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[methoxy(polyethylene glycol)-2000 (DSPE-PEG$_{2000}$) induce mixed micelles of either threadlike or discoidal shape when mixed with different types of lipids. In certain lipid systems the discoidal micelles adapt sizes large enough to be characterized as bilayer discs. The discs hold great potential for use in various biotechnical applications and may *e.g.* be used as model membranes in drug/membrane partition studies. Depending on the application, discs with certain characteristics, such as a particular size or size homogeneity, may be required. These factors can in our experience be influenced by the preparation method. In this study we systematically investigated three different PEG-lipid/lipid mixtures prepared by four commonly used preparation techniques. The techniques used were simple hydration, freeze-thawing, sonication and detergent depletion, and the aggregate size and structure was analyzed by cryo transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS). Our results show that the type and size of the micellar structure found, as well as the structure homogeneity of the preparation, can be modified by the choice of preparation path.


*Keywords:* Bilayer disc; Phospholipids; PEG-lipid; Cryo-TEM

## 1. Introduction

Poly ethylene glycol (PEG)-lipids are commonly used to sterically stabilize lipid aggregates, such as liposomes [1–3]. Their presence, however, may seriously influence the aggregate structure. High concentrations of PEG-lipids will, in most phospholipid/PEG-lipid mixtures, induce the formation of mixed spherical micelles [4–11]. Previous studies have shown that two fundamentally different types of mixed micellar aggregates may form at intermediate PEG-lipid to lipid ratios. Depending on the characteristics of the lipid mixture the PEG-lipids induce formation of either long threadlike or discoidal micelles [7,10,12,13]. Threadlike micelles are, for example, commonly observed in PEG-lipid/lipid samples where the lipid is in the liquid crystalline phase, while discs are observed in such mixtures supplemented with cholesterol [7,12]. Further, discoidal micelles are formed in cases where the lipid is in the gel phase [10,12,13]. From previous studies it appears that components, as well as environmental conditions, that increase the monolayer bending modulus and decrease the spontaneous curvature of the mixture promote the formation of discs [12]. As discussed before [10,12] formation of the discoidal micelles requires partial component segregation. For curvature reasons the PEG-lipids accumulate at the hemispherical disc edge whereas the bilayer forming lipids reside preferentially in the flat part of the disc.

The discoidal micelles, which in certain lipid systems adapt sizes large enough to merit their characterization as bilayer discs, hold great potential in various biotechnical applications. The discs have *e.g.* been utilized as model membranes in drug/membrane partitioning studies [13–15]. Results of these studies suggest that the discs, due to their stable and open structure, in many cases are superior to liposomes as model membranes. Ongoing studies indicate, furthermore, that the discs are well suited for structure/function studies of proteins and peptides, and that they hold further promise as carriers of various therapeutic agents. Depending on the application discs with

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certain characteristics, such as a particular size or size homogeneity, may be required. These factors can in our experience be influenced by the preparation method. In previous studies different sample compositions and preparation techniques have been used in order to study the aggregate structures formed in PEG-lipid/lipid mixtures, but no systematic investigation has so far been done concerning the effect of preparation path. In the present study we have chosen to focus on three different lipid systems, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), egg phosphatidylcholine (EPC)/cholesterol and EPC, all supplemented with a fixed amount of 25 mol% 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (DSPE-$PEG_{2000}$). The samples were prepared by four commonly used preparation techniques, simple hydration, freeze-thawing, sonication and detergent depletion, and the aggregate size and structure were analyzed by cryo transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS). Cryo-TEM is well suited for observation of structurally polydisperse samples where it is hard, or even impossible, to interpret data from indirect techniques such as light scattering. Light scattering is, however, a valuable supplement to cryo-TEM and can be used to detect small changes in size that are difficult to assess based on cryo-TEM investigation.

## 2. Materials and methods

### 2.1. Materials

Egg phosphatidylcholine (EPC) was obtained from Lipid Products (Nutfield, UK). 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-$PEG_{2000}$) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol and octyl glucoside were purchased from Sigma-Aldrich (Stockholm, Sweden). Sephadex G50 from Amersham Biosciences (Uppsala, Sweden). Minicon-B15 concentrators were from Millipore (Bedford, MA). Spectra/Por 7 dialysis membrane was from Sigma-Aldrich (Stockholm, Sweden). All other salts and reagents were of analytical grade and were used as received.

### 2.2. Sample preparation

Three different sample compositions were used, DPPC:DSPE-$PEG_{2000}$ (75:25 mol%), EPC:DSPE-$PEG_{2000}$ (75:25 mol%) and EPC:cholesterol:DSPE-$PEG_{2000}$ (35:40:25 mol%). Lipids in desired ratios were codissolved in chloroform and the solvent was removed in a gentle stream of $N_2$-gas followed by further evaporation in vacuum over night. The dried lipid films were hydrated in HEPES-buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) using one of four different preparation methods. 1) The sample was hydrated at 55 °C for approximately 30 min with intermittent mixing. 2) The sample was subjected to eight freeze-thaw cycles, frozen in $N_2$(l) and thawed to 55 °C with intermittent vortex mixing. 3) The sample was sonicated for 10 min using a Soniprep 150 sonicator (MSE Scientific Instrument, England). During sonication EPC based samples were kept in ice-cold water and DPPC based samples were kept above the transition temperature of DPPC [16]. 4) The sample was prepared by a detergent removal method. Octyl glucoside (OG) was used as solubilizing agent and added, dissolved in buffer, to the dry lipid film to give a final lipid to detergent ratio of 1:10 in the micelles (21.5 mM extra OG, *i.e.* corresponding to the OG cmc [17], was included in the sample). The sample was hydrated at approximately 55 °C for 30 min with intermittent vortex mixing and then allowed to equilibrate at room temperature with stirring for 1.5 h. The sample was then applied to a Sephadex G50 column (volume 100 mL) with an elution flow rate of 0.7 mL/min. The lipid eluate was then concentrated on a Minicon-B15 to approximately 1.5 mL and thereafter dialysed for at least 36 h in 500 mL HEPES-buffer, which was exchanged every 12 h.

Lipid concentrations were 10 mM except in the dialysed samples where the final concentrations were around 25 mM.

### 2.3. Cryo-transmission electron microscopy

Cryo-TEM images were obtained in a Zeiss EM 920 A transmission electron microscope (Carl Zeiss Inc., Oberkochen, Germany) operating at 80 kV. A small drop of sample was placed on a copper grid coated with a polymer film and the excess solution was removed by a filter paper producing a thin film. This procedure was performed in a custom-built environmental chamber under controlled temperature (25 °C) and humidity. Immediately after film preparation the grid was plunged into liquid ethane. The vitrified sample was then transferred into the microscope. During both transfer and viewing procedure the temperature was kept below 108 K to prevent ice crystal formation and sample perturbation. A more detailed description of the cryo-TEM procedure can be found elsewhere [18,19].

All samples were stored in the dark at room temperature for 24 h before analysis.

### 2.4. Dynamic light scattering

The light scattering setup consists of a Uniphase He–Ne laser emitting vertically polarized light with a wavelength of 632.8 nm operating at 25 mW. The detector is a Perkin Elmer diode detector connected to an ALV-5000 autocorrelator built into a computer. Analysis of data was done using the program GENDIST [20]. The autocorrelation function obtained from DLS measurements gives the relaxation rate $\Gamma$ from which the translational diffusion coefficient can be obtained as $D=\Gamma/q^2$. $q$ is the magnitude of the scattering vector, $q=(4\pi n_s/\lambda)\sin(\theta/2)$ with $n_s$ being the refractive index of the solution, $\lambda$ the wavelength of the radiation, and $\theta$ the scattering angle. $\theta$ was set at 90°. For a spherical particle the diffusion coefficient is related to the hydrodynamic radius, $R_h$, through the Stokes–Einstein relation, $D=k_BT/6\pi\eta R_h$, with $k_B$ being the Boltzmann constant, $T$ the temperature, $\eta$ the viscosity of the solvent.

The samples investigated were diluted to 4 mM in HEPES-buffer and stored 24 h in the dark at room temperature before analysis. All samples, with the exception of hydrated and

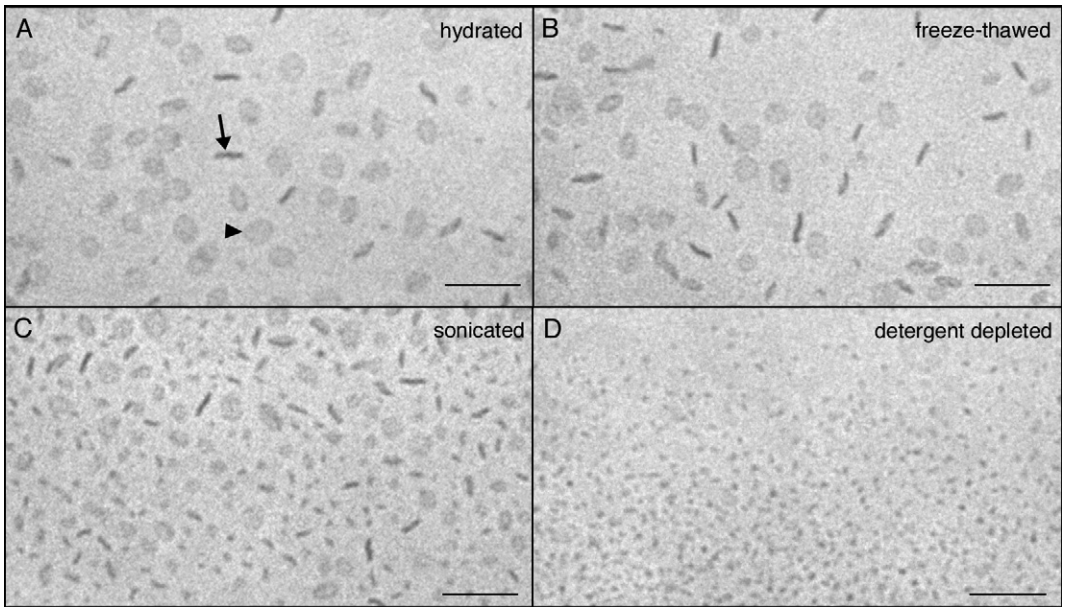


Fig. 1. Cryo-TEM images of DPPC:DSPE-PEG$_{2000}$ (75:25 mol %) mixtures. The samples were prepared by A) hydration, B) freeze-thawing, C) sonication and D) detergent depletion. The arrowhead indicates a disc positioned face-on and the arrow indicates disc positioned edge-on. Scale bars indicate 100 nm.

freeze-thawed EPC/DSPE-PEG$_{2000}$, were prepared in triplicate and measured with DLS.

## 3. Results

### 3.1. DPPC/DSPE-PEG$_{2000}$ samples

Irrespective of preparation method used all DPPC/DSPE-PEG$_{2000}$ samples contained almost exclusively discs of a relatively homogeneous size (Fig. 1). The actual size of the discs varied however depending on the preparation path.

The sample hydrated at 55 °C (preparation path 1, see sample preparation section) contained apart from discs (Fig. 1A) a small population of large bilayer flakes. The observations made from cryo-TEM correspond well with DLS data, which indicate the presence of two populations with hydrodynamic radii corresponding to 20 and 300 nm respectively[1] (Table 1).

The freeze-thawed sample was very similar in appearance to the hydrated sample and contained mainly discs of a fairly homogeneous size (Fig. 1B). The value of $R_h$ corresponds to 19 nm (Table 1). Also in this case a small population of larger aggregates was present as observed by both cryo-TEM and DLS (Table 1).

Sonicated DPPC/DSPE-PEG$_{2000}$ mixtures contained somewhat smaller discs compared to the previously discussed samples (Fig. 1C). As reported in Table 1, DLS indicated a hydrodynamic radius of 15 nm and, similar to the other preparation methods, sonication resulted in a small fraction of larger aggregates.

The aggregates generated by the detergent depletion method were considerably smaller than those found in samples prepared by the other methods (Fig. 1D). The small size prevented detailed determination of aggregate shape from the cryo-TEM images. Light scattering, as well as cryo-TEM, revealed, furthermore a very small population of large bilayer flakes to be present in the sample. DLS data suggested an aggregate radius corresponding to 9 nm (Table 1).

Irrespective of preparation path, storage for 1 week at room temperature did not change aggregate size or structure in the samples noticeably as judged from cryo-TEM investigations. However, the value of $R_h$ for the small particles increased from 9 to 12 nm in the detergent depleted samples (Table 1).

Table 1
Hydrodynamic radius [nm] as determined from DLS data

| | DPPC/DSPE-PEG$_{2000}$ | EPC/chol/ DSPE-PEG $_{2000}$ | EPC/ DSPE-PEG$_{2000}$ |
|---|---|---|---|
| Hydrated | 20±3/292[a] | 77±11 | 162[b] |
| Hydrated 1 week | 20±3/199±72 | 77±10 | 162[b] |
| Freeze-thawed | 19±2/432[a] | 52±4 | 101[b] |
| Freeze-thawed 1 week | 19±2/261±97 | 33±10 | 90[b] |
| Sonicated | 15±2/307[a] | 30±3 | 24±2 |
| Sonicated 1 week | 15±1/161±29 | 30±2 | 27±3 |
| Detergent depleted | 9±1/144±18 | 13±2/181±64 | 12±1/121±41 |
| Detergent depleted 1 week[c] | 12±1/136±21 | 15±1/351±19 | 15±0/113±7 |

Numbers of samples analyzed, $n=3$ if not otherwise stated.
In some of the samples two populations were detected. The hydrodynamic radii of the two populations are separated with a slash sign in the table.
[a]The larger aggregates were not detected in all samples.
[b]$n=1$.
[c]$n=2$.

[1] It is important to remember that the size obtained from DLS data corresponds to the equivalent sphere hydrodynamic radius of the particles. Hence the mean particle size determined for samples containing discs or other nonspherical aggregates cannot be directly deduced from the data.

### 3.2. EPC/cholesterol/DSPE-PEG$_{2000}$ samples

Hydrated samples contained mostly discs of varying size but a considerable amount of liposomes were also present (Fig. 2A). The liposomes were unilamellar with a size range from approximately 100 nm to 300 nm in diameter as estimated from the cryo-TEM investigation. The discs varied in size from around 15 nm in diameter to 200 nm in diameter as estimated from the cryo-TEM images. The apparent hydrodynamic radius determined using DLS was 77 nm (Table 1). After storage for 1 week at room temperature the apparent size of the sample did not change (Table 1) and this was also verified by means of cryo-TEM (results not shown).

Freeze-thawed samples were dominated by discs but liposomes were also frequently observed (Fig. 2B). The amount of liposomes appeared to be slightly less compared to the hydrated sample. The hydrodynamic radius determined using DLS was 52 nm (Table 1). Storage for 1 week at room temperature resulted in the formation of smaller discs with a $R_h$ corresponding to 33 nm in radius (Fig. 2C, Table 1).

Sonicated EPC/cholesterol/DSPE-PEG$_{2000}$ samples contained mostly discs but again some liposomes were also present (Fig. 2D). DLS data indicated an apparent average radius of 30 nm (Table 1). As determined from the cryo-TEM images the size distribution of the discs was narrower than in the hydrated or freeze-thawed samples. One week of storage did not alter the sample appearance or apparent aggregate size.

Sample preparation using detergent depletion resulted in the formation of small aggregates with a radius of 13 nm (Table 1). The majority of the aggregates appeared to be discoidal but there were also aggregates that were too small to be described in detail from a shape perspective using cryo-TEM (Fig. 2E). Further, a minor population of both small and large liposomes was found and in addition few large bilayer flakes were present in the samples. After 1 week of storage cryo-TEM revealed discs of a slightly larger size compared to those found in the fresh sample (Fig. 2F). DLS studies showed only a minor increase in apparent hydrodynamic radius from 13 to 15 nm (Table 1). A small population of large aggregates was still present in the stored sample.

### 3.3. EPC/DSPE-PEG$_{2000}$ samples

Hydrated EPC/DSPE-PEG$_{2000}$ mixtures were structurally polydisperse and contained unilamellar liposomes, both small and very large, in coexistence with micellar aggregates (Fig. 3A). The shape of the micelles varied from spherical, or slightly elongated, to long threadlike. Further, large bilayer

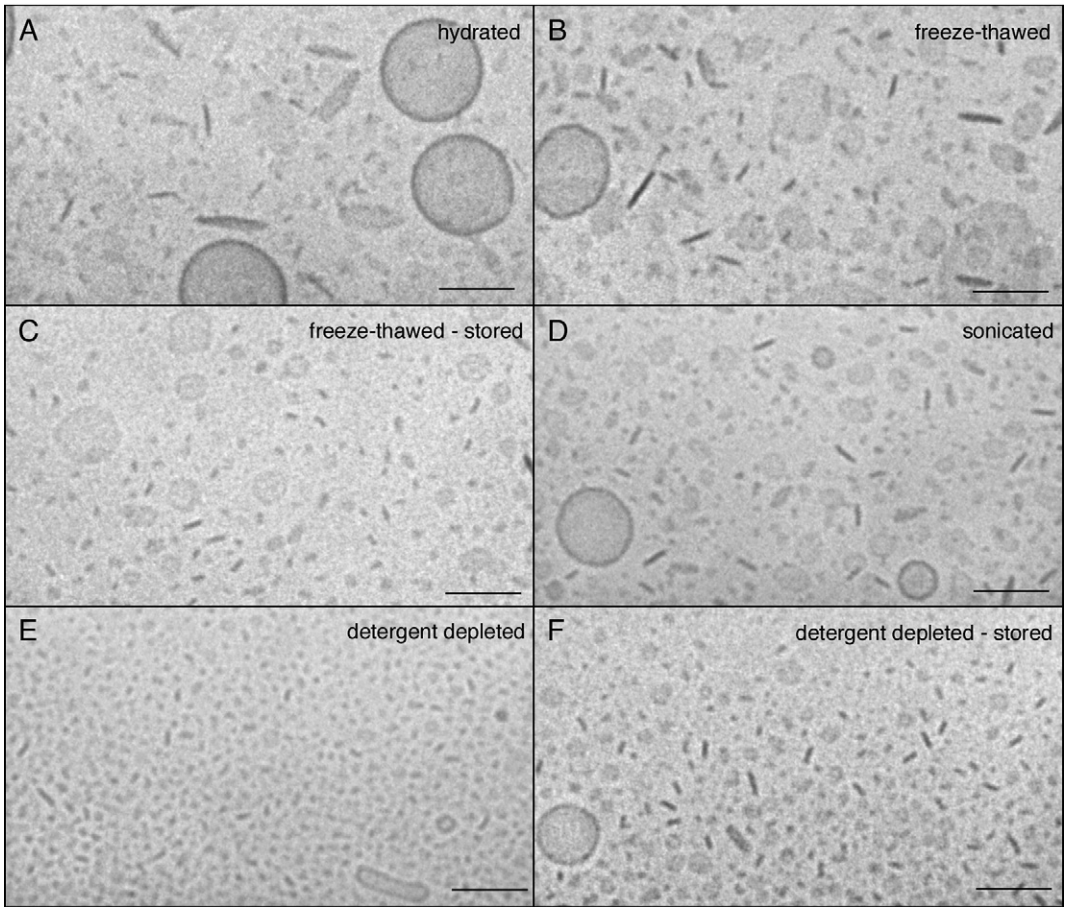


Fig. 2. Cryo-TEM images of EPC:cholesterol:DSPE-PEG$_{2000}$ (35:40:25 mol %) mixtures. The samples were prepared by A) hydration, B) freeze-thawing, C) freeze-thawing followed by 1 week of storage at RT, D) sonication, E) detergent depletion and F) detergent depletion followed by 1 week of storage at RT. Scale bars indicate 100 nm.

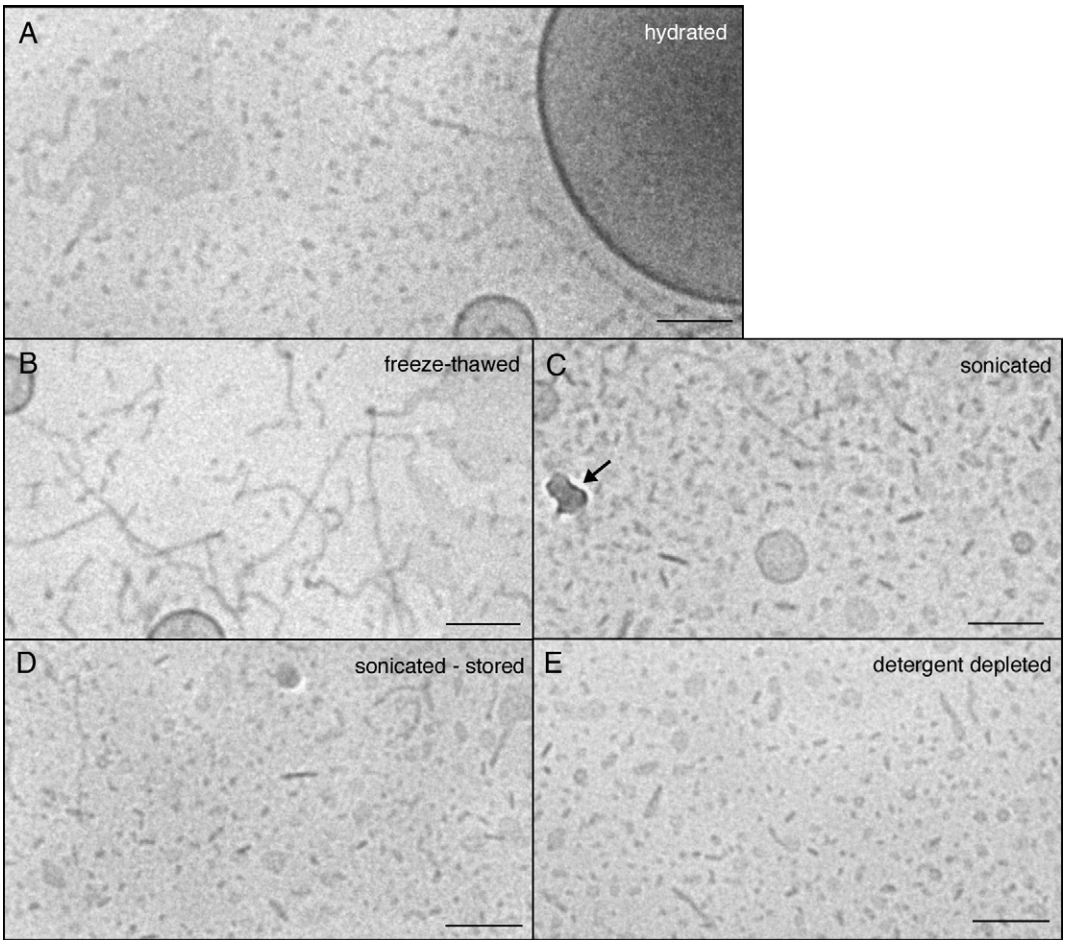


Fig. 3. Cryo-TEM images of EPC:DSPE-$PEG_{2000}$ (75:25 mol %) mixtures. The samples were prepared by A) hydration, B) freeze-thawing, C) sonication, D) sonication followed by 1 week of storage at RT and E) detergent depletion. The arrow denotes an ice crystal. Scale bars indicate 100 nm.

flakes with cylindrical extensions were frequently observed. The apparent radius of the aggregates as determined using DLS was 162 nm (Table 1). Storage for 1 week at room temperature did not change the aggregate structure noticeably.

The appearance of the samples changed when the freeze-thawing procedure was used. A considerably larger amount of long threadlike micelles was displayed in the cryo-TEM images (Fig. 3B). Further, the liposomes were generally smaller in size. The apparent hydrodynamic radius as determined by DLS was 101 nm (Table 1). Cryo-TEM revealed no change in the sample appearance after storage for 1 week at room temperature. DLS data indicate, however, a slight decrease in $R_h$ value (Table 1).

Sonication of the EPC/DSPE-$PEG_{2000}$ mixtures resulted in the formation of mainly discoidal structures (Fig. 3C). There were also a fraction of small liposomes present, as well as a few very long threadlike micelles. The discs were relatively similar in size and the apparent radius determined with DLS was 24 nm (Table 1). When storing the sample for 1 week more threadlike micelles of varying length were formed and irregular bilayer flakes were occasionally observed (Fig. 3D).

Detergent depleted samples contained small aggregates, including both irregular and relatively circular discs (Fig. 3E). Further, some large bilayer flakes and a few liposomes were observed. The DLS investigation revealed two populations with $R_h$ values corresponding to 12 and 121 nm, respectively (Table 1). After 1 week the sample appeared unchanged based on cryo-TEM inspection but the small aggregates had, according to DLS measurements, increased slightly in size to 15 nm.

## 4. Discussion

The type of micellar structures found in the three different lipid systems investigated in the present study agree with those found in previous studies [12]. All DPPC/DSPE-$PEG_{2000}$ samples contained disc-shaped micelles, but their size varied with the preparation method. EPC/cholesterol/DSPE-$PEG_{2000}$ mixtures also formed discoidal micelles. The latter mixtures contained in addition a significant amount of liposomes. EPC/DSPE-$PEG_{2000}$ samples were structurally polydisperse, in particular when prepared by simple hydration. Importantly, threadlike micelles were observed in all EPC/DSPE-$PEG_{2000}$ samples, apart from those prepared by detergent depletion. The disc-shaped aggregates found in the latter samples were as a rule more or less irregular.

Freeze-thawing clearly influences the aggregate structure in EPC/cholesterol/DSPE-$PEG_{2000}$ and EPC/DSPE-$PEG_{2000}$ mixtures. The most prominent effect is seen in the latter lipid

mixture where freeze-thawing drastically reduces the aggregate polydispersity and produces samples that contain mainly long threadlike micelles. The reduced aggregate polydispersity caused by freeze-thawing can also be seen by comparing reference [10] with [12] where EPC/DSPE-PEG samples were prepared with or without freeze-thawing, respectively. In order to understand this effect it should be pointed out that the PEG-lipid distribution most likely differs between the various aggregates observed in the hydrated EPC/DSPE-PEG sample (Fig. 3A). It is, for instance, plausible that the globular micelles have a higher PEG-lipid/lipid ratio than the liposomes. Freeze-thawing may be expected to lead to a more homogeneous component distribution and thus a structurally less polydisperse appearance. This effect may also explain the partial disappearance of liposomes in EPC/chol/DSPE-$PEG_{2000}$ mixtures upon freeze-thawing of the hydrated sample. Another effect of freeze-thawing was the decrease in liposome size found in the EPC/DSPE-$PEG_{2000}$ sample. This is a documented effect of freeze-thawing for liposomes in the liquid crystalline phase, which has been shown to apply also for liposomes containing a relatively high amount of PEG-lipids [21–24].

A general observation is that sample preparation using sonication or detergent depletion generates aggregates with a smaller size as compared to the other preparation paths. Sonication is a high-energy input method, which hence may force the amphiphilic molecules to assemble into structures deviating from the equilibrium state, such as for instance very small aggregates [25]. Relaxation into larger aggregates will in the present case most likely be prevented or at least delayed by the high amount of PEG-lipid used in the preparations. The steric hindrance originating from the polymer chains will effectively prevent aggregate contact and fusion [26]. The same reasoning may be applied to the small aggregates found in detergent depleted samples where the PEG-lipids may be expected to hinder direct fusion of the aggregates during the course of gradual detergent removal.

An interesting, and somewhat surprising, finding was that preparation protocols based on sonication or detergent depletion tended to reduce the propensity for formation of threadlike micelles in the EPC/DSPE-$PEG_{2000}$ mixtures. As seen in Fig. 3C and E the majority of the aggregates instead adopted a discoidal, albeit quite irregular, shape. Sonication is known to increase the risk of lipid degradation, about 5% of the lipids are de-esterified when sonicated for up to 1 h [25], which may alter the characteristics of the lipid monolayer to such extent that the aggregate structure is affected. However, considering that sonication was minimized to only 10 min and performed in ice-cold water, it is not very likely that the amount of degradation products potentially produced would be high enough to allow for the large difference in aggregate shape as observed between *e.g.* freeze-thawed and sonicated samples [25]. A more plausible explanation may be found when considering the high amount of energy that is dissipated into the sample during sonication and that this may lead to formation of aggregates that are far from equilibrium. Potentially, the energy input is high enough to pay for the partial component segregation needed in order to make disc formation possible [12]. Upon storage of the sonicated EPC/DSPE-$PEG_{2000}$ mixtures more threadlike micelles are formed. This may be interpreted as a result from reorganization and relaxation towards an equilibrium aggregate structure. From the micrograph presented in Fig. 3D it is clear, however, that discoidal micelles are still the dominating aggregates after 1 week of storage. As already discussed, structural transitions are most likely slowed down by the high amount of PEG-lipid.

The small aggregate structures formed in the detergent depleted EPC/DSPE-$PEG_{2000}$ samples are also disc shaped rather than threadlike. A rod-shaped micelle of small size would have a very high average curvature compared to a longer threadlike micelle. When growth from initial small globular micelles into larger assemblies is hindered by the presence of PEG-lipids it may thus be more energetically favourable to partially segregate EPC and PEG-lipid components and form discs. Importantly, we found that detergent depleted EPC/DSPE-$PEG_{2000}$ samples containing only 15 mol% PEG-lipid contained threadlike micelles in coexistence with discs (not shown). This suggests that growth of the initially globular micelles into threadlike micelles is possible at lower DSPE-$PEG_{2000}$/EPC ratios.

The fact that it is possible to prepare discs based on EPC/DSPE-$PEG_{2000}$ lipid mixtures is interesting from an application point of view and helps extend the possibilities to prepare discs of varying lipid composition. It is also interesting and important to point out that the present investigation shows that discoidal, rather than threadlike, micelles form in DPPC/DSPE-$PEG_{2000}$ and EPC/chol/DSPE-$PEG_{2000}$ samples irrespective of preparation method. This gives an indication of the discs as an equilibrium structure, or at least a very long-lived metastable structure, in these systems.

## 5. Concluding remarks

In order to utilize PEG-stabilized discs in a broad range of biotechnical applications means to customize their lipid composition, size and size homogeneity are desirable. In previous studies we have shown that the composition of the discs can be varied and *e.g.* tailored to reflect that of a biological membrane [14,15]. Further, the size of the discs can be altered by changing the PEG-lipid content [13]. In this study we show that the disc size and particle homogeneity is influenced, and can be modified, also by the choice of preparation path.

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