Journal of Colloid and Interface Science 484 (2016) 86–96

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

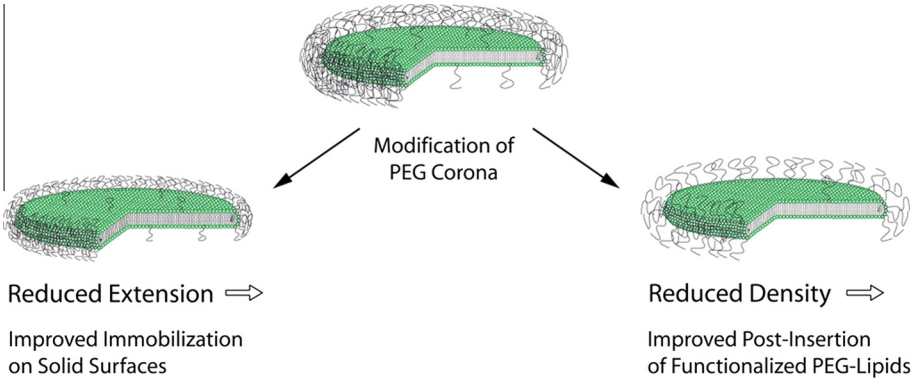
journal homepage: www.elsevier.com/locate/jcis

Optimization of lipodisk properties by modification of the extent and density of the PEG corona

Malin Morin Zetterberg [a], Sara Ahlgren [a], Víctor Agmo Hernández [a], Nagma Parveen [b], Katarina Edwards [a,*]

[a] Department of Chemistry – BMC, Uppsala University, Box 579, SE-752 37 Uppsala, Sweden
[b] Department of Physics, Biological Physics Division, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 May 2016
Revised 25 August 2016
Accepted 26 August 2016
Available online 27 August 2016

Keywords:
Lipid self-assembly
Bilayer disks
Biomimetic membranes
Surface immobilization
Drug delivery
Nanocarriers
Specific targeting

ABSTRACT

Lipodisks are nanosized flat, circular, phospholipid bilayers that are edge-stabilized by polyethylene glycol-conjugated lipids (PEG-lipids). Over the last decade, lipodisks stabilized with PEG of molecular weight 2000 or 5000 have been shown to hold high potential as both biomimetic membranes and drug carriers. In this study we investigate the possibilities to optimize the properties of the lipodisks, and widen their applicability, by reducing the PEG molecular weight and/or the density of the PEG corona.

Results obtained by cryo-transmission electron microscopy and dynamic light scattering show that stable, well-defined lipodisks can be produced from mixtures of distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylethanolamine conjugated to PEG of molecular weight 1000 (DSPE-PEG_{1000}). Preparations based on the use of DSPE-PEG_{750} tend, in contrast, to be polydisperse in size and structure. By comparing immobilization of lipodisks stabilized with DSPE-PEG_{1000}, DSPE-PEG_{2000}, and DSPE-PEG_{5000} to porous and smooth silica surfaces, we show that the amount of surface bound disks can be considerably improved by the use of PEG-lipids with reduced molecular weight. Further, a modified preparation protocol that enables production of lipodisks with very low PEG-lipid content is described. The reduced PEG density, which facilitates the incorporation of externally added ligand-linked PEG-lipids, is shown to be beneficial for the production of targeting lipodisks.

1. Introduction

Over the recent decades considerable attention has been focused on the development of new lipid based particles and systems for applications in the field of drug delivery and for use

* Corresponding author.
E-mail addresses: malin.zetterberg@kemi.uu.se (M.M. Zetterberg), sara.ahlgren@kemi.uu.se (S. Ahlgren), victor.agmo@kemi.uu.se (V. Agmo Hernández), nagma.parveen@chalmers.se (N. Parveen), katarina.edwards@kemi.uu.se (K. Edwards).

http://dx.doi.org/10.1016/j.jcis.2016.08.067
0021-9797/

as biomimetic membranes [1–4]. Some lipid structures have found important use in both areas, and among these the liposomes are the perhaps best-known example. Another nanosized lipid bilayer structure that has received significant attention is the lipodisk. Lipodisks, which were first described almost twenty years ago [5], form in aqueous mixtures of bilayer-forming phospholipids and micelle-forming PEG-lipids. The latter lipids have a long polymeric polyethylene glycol (PEG) moiety covalently attached to the lipid head-group. Under certain conditions the phospholipids and PEG-lipids partially segregate within the aggregates, resulting in a disk-like structure where the bilayer forming lipids constitute the flat part and the PEG-lipids reside predominantly at the highly curved rim. The accumulation of PEG-lipids at the lipodisk edge, which has been verified experimentally by, e.g., SANS measurements, can be understood in terms of the segregation promoting effects caused by the different spontaneous curvature, micromechanical properties, and limited miscibility of the lipodisk components [6–8]. A schematic illustration of the lipodisk structure is shown in Fig. 1. Similar to phospholipid liposomes, the lipodisks are very stable to dilution. In addition, the dense PEG polymer coat offers efficient steric stabilization that ensures excellent long-term stability of the lipodisks. By varying the lipid constituents and methods of preparation, the disks can be tuned in size and adapted to fit various pharmaceutical, biomedical and analytical applications.

Lipodisks have properties that render them interesting as carriers for several different classes of drugs. Investigations by Zetterberg et al. [9] have for example confirmed that the cationic amphiphilic peptide melittin can be formulated in lipodisks with maintained antibacterial effect. Results of the study show that, apart from allowing for a sustained peptide release, the lipodisks efficiently protect the peptide against enzymatic degradation. This finding has been further verified in recent studies by Gao et al., who, in addition, present *in vitro* and *in vivo* data suggesting that formulation in lipodisks leads to reduced hemolysis and increased anti-tumor activity of melittin [10] A study by Zhang et al. [11] suggests, moreover, that lipodisks hold potential for formulation and delivery of the anthracycline drug doxorubicin.

Several studies also support the applicability of lipodisks as model membranes in drug partition studies [12–20]. When used for this purpose, the disks have been immobilized to chromatographic media either passively, through the swelling of cross-linked gels in lipodisks suspensions [12–14], or actively *via* the use of functionalized PEG-lipids [15–17]. Drug partitioning studies have also been performed using lipodisks as a pseudo-stationary phase in capillary electrophoresis [18], and as model membranes in studies based on quartz crystal microbalance technique [19]. The possibility of incorporating membrane proteins in biomimetic lipodisks has, furhermore, been shown by Johansson et al. [13] and Duong-Thi et al. [21].

Lipodisks are known to readily form in lipid mixtures containing 1,2-distearoyl-*sn*-*glycero*-3-phosphoethanolamine-PEG with polymer molecular weight 2000, as well as 5000 Da (DSPE-PEG_{2000} and DSPE-PEG_{5000}). [6,7] Several advantages can be foreseen, however, if lipodisks could be produced using polymers of lower molecular weight. It has recently been shown that the targeting efficiency of liposomes can be improved by the use of shorter PEG-chains, probably due to improved exposure of the targeting agents [22]. It is plausible that targeting lipodisks would benefit from a shortening of the PEG-chains in a similar way. Moreover, for a given size of the bilayer part of the disk, shorter PEG-chains would reduce the total disk volume, and the smaller PEG corona could potentially enable a significantly tighter packing of the lipodisks upon immobilization to solid surfaces. The resultant increase in total membrane surface area is desirable, for instance, in order to increase the sensitivity of partition and interaction studies.

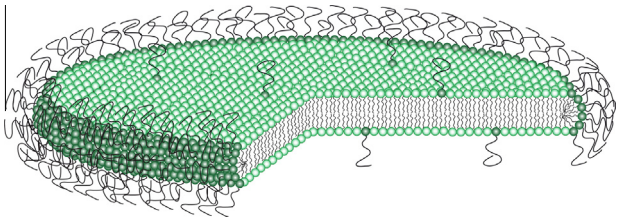


Fig. 1. Schematic illustration of a lipodisk. Bilayer-forming lipids are shown in light green and PEG-lipids are shown in dark green.

There are, however, some concerns involved with the use of PEG-lipids with shorter PEG-chains, since a shortening of the polymer chain-length will affect the molecular geometry of the PEG-lipid. A reduction in the PEG length tends to reduce the lipid spontaneous curvature and PEG-lipids with short polymer chains may therefore aggregate into non-micellar structures. Thus, DSPE-PEG_{350} is known to form lamellar structures [23] and structures of lamellar character have been reported to coexist with globular micelles in aqueous samples of DSPE-PEG_{750} [23,24]. Since production of lipodisks normally require addition of micelle-forming molecules to lipid bilayers [25,26], inclusion of PEG-lipids with short polymers may fail to induce lipodisk formation. One important aim of the present study was to investigate and, if possible, identify the lower limit for the PEG molecular weight that can be used for production of well-defined lipodisk samples.

The PEG-lipid content is another lipodisk parameter that can potentially be optimized to further improve the disks for different applications. Production of pure lipodisk samples typically requires inclusion of about 20 mol% PEG-lipid in the lipid mixtures [5,6]. Possible benefits can be foreseen, however, if the amount of PEG-lipid in the disks could be reduced. One such benefit is an increased range of possible disk sizes. Inclusion of large proportions of PEG-lipids leads to formation of small structures with high mean curvature [6], that is, lipodisks with a high amount of edge area. The edge-to-bilayer area ratio is inversely proportional to the lipodisk diameter and the highest value is therefore found in small disks. Consequently, if lipodisks can form in mixtures with low PEG-lipid content their total edge area should be small, and the disks are therefore expected to be large. One major obstacle needs to be overcome, however, in order to produce large lipodisks from mixtures containing low PEG-lipid amounts, *i.e.*, the formation of liposomes must somehow be circumvented. It is well documented that unless the PEG-lipid content in the mixtures corresponds to at least 20 mol%, the preparations always contain a fraction of liposomes [6]. In mixtures containing 10 mol%, or less, PEG-lipid, liposomes are the completely dominating aggregate structure. A second objective with the present study was therefore to explore if liposome formation can be prevented by modifications of the standard protocols used for lipodisk preparation. As will be described, our attempts were successful and by means of a method based on low-temperature sonication of the lipid/PEG-lipid mixtures we managed to obtain liposome-free lipodisk preparations with PEG-lipid content as low as 2 mol%. Finally, with the aim of highlighting the unique and advantageous properties of the sonicated disks, we investigate and verify their ability to incorporate externally added PEG-lipids modified with a targeting ligand.

2. Materials and methods

2.1. Materials

Dry powder of 1,2-distearoyl-*sn*-grycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-*glycero*-3-phosphoethanolamine-N-[met

hoxy(polyethylene glycol)-750] (DSPE-PEG_{750}), 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-1000] (DSPE-PEG_{1000}), 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) and 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (DSPE-PEG_{5000}) was purchased from Avanti Polar Lipids (Alabaster, AL). The *N*-hydroxysuccinimide ester of DSPE-PEG_{3400} (DSPE-PEG_{3400}-NHS) was purchased from Shearwater Polymers (Huntsville, AL). Octyl β-d-glucopyranoside (OG), chloramine-T and sodium metabisulfite was purchased from Sigma-Aldrich Chemical (Steinheim, Germany). ^{125}I was purchased from Perkin Elmer (Wellesley, MA). Epidermal growth factor (EGF) from mouse was obtained from Millipore (Molsheim, France). Sephadex G-150 and Sepharose CL-4B medium was purchased from GE Healthcare Lifescience (Uppsala, Sweden). Kromasil® porous silica particles with a diameter of 10 μm and 300 Å pore size was a kind gift from Akzo Nobel (Bohus, Sweden).

2.2. Lipid film preparation

The lipid samples used in this study were mixtures of DSPC and DSPE-PEG of varying polymer chain length. The PEG-lipids used were DSPE-PEG_{750}, DSPE-PEG_{1000}, DSPE-PEG_{2000} and DSPE-PEG_{5000}. Regardless of the preparation technique used the desired lipids were initially co-dissolved in chloroform and thereafter dried under a gentle stream of nitrogen gas. Remaining chloroform was then removed under vacuum overnight. Dry lipid films were stored in a nitrogen atmosphere at −20 °C until used.

2.3. Extrusion

The lipid film was dissolved in HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl pH = 7.40) to 10 mM and hydrated at 70 °C for 3 h with intermittent mixing. The dispersions were kept at 70 °C and extruded 20 times through a Whatman® polycarbonate filter with a pore size of 100 nm (GE Healthcare, Uppsala, Sweden) using a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL).

2.4. Sonication

The lipid film was dissolved in HBS giving a 10 mM lipid solution. The solution was hydrated at 70 °C for 3 h with intermittent mixing. A 1 mL aliquot was then transferred into a glass vial and the sample was sonicated at 0 °C for 45 min using a Soniprep 150 sonicator (MSE, London, England). Finally metal residues from the sonicator tip were removed by centrifuging the samples for 2 min at 268*g*.

2.5. Characterization of lipid samples

2.5.1. Cryo-transmission electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) was used to characterize the lipid samples. Cryo-TEM specimens were prepared by depositing a small drop (~1 μL) of the lipid sample on a copper grid covered with a holey polymer film. Thin liquid films (10–500 nm) were prepared by blotting the grid with a filter paper in a custom built climate chamber at 25 °C and close to 100% relative humidity (*RH*). The grid was then immediately plunged into liquid ethane held at a temperature just above its freezing point (−183 °C), resulting in vitrification of the liquid films. The specimens were kept at a temperature below −165 °C and protected against atmospheric conditions both during transfer from the climate chamber to the microscope and throughout the entire sample examination. A detailed description of the technique has been presented by Almgren et al. [27]. Cryo-TEM investigations were performed using a Zeiss TEM Libra 120 Transmission Electron Microscope (Carl Zeiss AG, Oberkochen, Germany). Observations were made in zero loss bright-field mode at an accelerating voltage of 80 kV. Digital images were recorded under the low dose conditions with a TRS slow scan CCD camera system (TRS GmbH, Germany) and iTEM software (Olympus Soft Imaging Solutions GmbH, Germany). An underfocus of 1–2 μm was used to enhance the image contrast. For all samples a large number images were studied to qualitatively determine the aggregate structures and the micrographs presented in this article are representative images for each sample. Quantitative size determinations were made by manually measuring more than 600 randomly selected disks in the micrographs by use of the measuring tool built into the iTEM software.

2.5.2. Dynamic light scattering

The aggregate size distributions in the lipid samples were analyzed using dynamic light scattering (DLS). The experimental setup consisted of a Uniphase He-Ne laser (Milpitas, CA) emitting vertically polarized light with a wavelength of 632.8 nm operating at 25 mW. The scattered light was detected at 90° scattering angle using a Perkin Elmer (Quebec, Canada) diode detector connected to an ALV-5000 multiple digital autocorrelator (ALV-laser, Vertriebsgesellschaft, Germany).

2.6. Lipodisk immobilization on silica

In order to determine the effects caused by changes in the PEG molecular weight, lipodisks were immobilized on different silica surfaces. Immobilization was in all cases performed using lipodisks composed of DSPC supplemented with 20 mol% DSPE-PEG_{1000}, DSPE-PEG_{2000} or DSPE-PEG_{5000} respectively.

2.7. Porous silica particles

For lipodisk immobilization to porous silica, 200 μL aliquots of disk suspensions (10 mM lipid concentration) were added to 25 mg of Kromasil® silica in Eppendorf tubes. Immobilization was performed passively under stirring at room temperature (approximately 21 °C) for 19 h. The silica samples were thereafter centrifuged (2 min at 268*g*) and washed five times with 1 mL MQ water. In a next step the lipodisks were removed from the silica beads by addition of 1 mL 120 mM OG, and subsequently stirred for 24 h to ensure complete lipodisk solubilization. The phospholipid content in the solution was thereafter determined from the amount of phosphorous using a method previously described by Paraskova et al. [28].

2.8. QCM-D measurements

The passive immobilization of lipodisks on smooth silica sensors was followed using a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). A QCM-D E1 (Q-Sense, Gothenburg, Sweden) instrument thermostated at 21 °C and with a controlled sample flow of 150 μL min^{-1} was employed for all experiments. Frequency and dissipation data was collected from the fundamental sensor frequency (5 MHz) as well as the 3rd, 5th, 7th, 9th, 11th and 13th overtones. Prior to use, the silica QCM-D sensors were cleaned according to the procedure recommended by the supplier. Briefly, the sensors were treated for 10 min in an UV/Ozone chamber (BioForce Nanosciences Inc, Ames, Iowa), followed by 30 min rinsing in SDS 2%. The sensors were then rinsed with MilliQ water, dried with a nitrogen stream and, finally, treated again for 10 min in the UV/ozone chamber. After cleaning, the sensors were directly mounted in the QCM-D instrument. The system was then equilibrated with the same buffer as the one used for lipodisk

preparation. Once a stable baseline was obtained, a 0.25 mM lipodisk suspension was loaded onto the system, and left to react with the surface until a stable signal was obtained. The system was then rinsed with buffer to remove any unbound material. The immobilized mass was calculated from the values obtained after rinsing.

Quantitative determinations of the immobilized mass were based on the approach proposed by Agmo Hernández et al., [17] which is, in turn, a linearization of the model by Voinova et al. [29]. for the formation of thin viscoelastic films on QCM-D sensors. The linearization is based on the following relationship:

$$\frac{\Delta f}{n} = -\frac{m_d f_0}{t_q \rho_q} + \frac{\pi \eta_1 (f_0)^2}{\mu_1}(n\Delta D) \quad (1)$$

where m_d is the adsorbed mass surface density, n is the overtone number, f_0 is the fundamental oscillation frequency, t_q and ρ_q are, respectively, the thickness and density of the quartz crystal, and η_1 and μ_1 are the viscosity and elastic modulus of the adsorbed layer. A plot of $\Delta f\, n^{-1}$ vs. $n\Delta D$ at different values of n is therefore a line with a y-intercept equal to $-(m_d f_0)\,(t_q \rho_q)^{-1}$. All reported values were calculated from Eq. (1) and the $\Delta f\, n^{-1}$ and $n\Delta D$ values obtained from at least four overtones.

2.9. MP-SPR

Multi-parametric surface plasmon resonance (MP-SPR) measurements were performed with a SPR Navi 220A (BioNavis, Finland) using the SPR Navi control software. The used sensors were silica coated gold plasmon surfaces on glass substrates. The sensors were cleaned by first thorough washing with 10 mM SDS solution for at least 10 min, followed by thorough rinsing with millipore water. The sensors were then dried and kept in UV-Ozone chamber for 10 min. Measurements were performed at 21 °C. The surface plasmon resonance was monitored at wavelengths 670 nm and 785 nm for a scanning interval between 39° and 78°. The chips were first equilibrated with a phosphate buffered saline (PBS) at 50 μL min^{-1}. The lipodisk suspensions (0.25 mM in PBS) were then injected at the same flow rate for 8 min to two parallel surface areas of the sensor. After 10 min, the sensors were rinsed with PBS. The bound mass was calculated from the following equation [30],

$$m_d = \frac{\delta}{S(dn/dC)}\Delta\deg \quad (2)$$

where δ is the decay length of the intensity of the evanescent field associated with the SPR excitation (109 nm and 154 nm for wavelengths 670 nm and 785 nm, respectively), S is the bulk sensitivity parameter, dn/dC is the refractive index increment (0.14 mL/g, as calculated from reported values for lipids and PEG [31,32]), and Δdeg is the measurement response. Sensitivity parameter values (109.6785 degree/ RIU at 670 nm and 91.42605 deg/RIU at 785 nm) were estimated from a calibration curve plotting Δdeg vs. the independently determined refractive index of five glycerol-water mixtures with varying glycerol concentration. Eq. (2) applies only for films much thinner than the decay wavelength δ, a condition that is fulfilled if adsorption of a lipodisk monolayer is assumed.

2.10. Post-insertion of targeting agents into lipodisks

The amount of targeting agent that could be incorporated into preformed lipodisks was quantified using PEG-lipids conjugated with ^{125}I-labelled EGF. The radiolabeling of EGF has been described in detail by Kullberg et al., [33] and the conjugation of EGF to PEG-lipids by Kullberg et al. [34] In short, 25 μg epidermal growth factor (EGF) was first labelled with ^{125}I by mixing the two components with chloramine-T in PBS at room temperature. The reaction was terminated after 60 s by addition of sodium metabisulfite. The ^{125}I-labelled EGF was then incubated with 300 μg DSPE-PEG_{3400}-NHS at room temperature overnight, resulting in a fraction (5%) of DSPE-PEG_{3400} lipids with radiolabeled EGF molecules covalently bound to the distal end of the polymer chain (^{125}I-EGF-PEG-lipids). The EGF-PEG-lipids were thereafter separated from unconjugated EGF using a small (PD-10 size) column, packed with Sephadex G-150. Incorporation of the EGF-PEG-lipids into the lipodisks was performed by mixing preformed disks with EGF-PEG-lipids in a 4–1 lipid molar ratio and incubating the samples at 37 °C for 24 h. The lipodisks were then separated from unincorporated EGF-PEG-lipids by running the sample on three consecutive Sepharose CL-4B columns (PD-10 size). 500 μL fractions were collected and the ^{125}I distribution in the fractions was determined with an automatic gamma counter (1480 Wallac Wizard, Perkin Elmer, Wellesley, MA). From the ^{125}I-distribution after separation the post-insertion yield could then be calculated. To ensure that no structural rearrangements occurred in the lipodisks, all samples were analyzed with cryo-TEM before and after the post-insertion step.

2.11. In vitro cell studies

In vitro binding specificity of ^{125}I-labelled EGF-targeted lipodisks was studied on cultured A-431 cells (squamous cell carcinoma cell line, CLR 1555, ATCC, Rocksville, MD, USA), overexpressing the human epidermal growth factor receptor (EGFR). To avoid internalization of the receptor, the experiment was performed at 4 °C. All procedures were performed on ice. Two groups of cell dishes (3 dishes/time point; diameter, 3.5 cm; $\sim 3 \times 10^5$ cells/dish) were incubated with lipodisks (1.5 μM total lipid amount) in cell media. In one group, the EGFRs were pre-saturated for 5 min with 100 nM EGF. The total volume in all dishes was 1 mL. Cells were incubated for 4 or 8 h at 4 °C before incubation medium was collected. The cells were washed three times with cold serum-free medium followed by treatment with 0.5 mL trypsin-EDTA solution for 10 min at 37 °C. When cells were detached, 0.5 mL cell media was added to each dish, and the cells were re-suspended and collected. The radioactivity associated with the cells and medium, respectively, was measured in the gamma counter. To distinguish statistical differences, data from cell assays were analyzed using an un-paired, two tailed t-test ($p < 0.05$).

3. Results and discussion

3.1. Lipodisks with reduced PEG corona volume

In order to investigate the possibility to prepare lipodisks by use of PEG-lipids with reduced polymer chain length, three series of samples based on DSPE-PEG_{2000}, DSPE-PEG_{1000} and DSPE-PEG_{750}, respectively, were prepared by extrusion (see Materials and Methods for details) and analyzed by cryogenic transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS). When evaluating and comparing data obtained with cryo-TEM and DLS it is important to keep in mind that these two techniques, although complementary, give information about different sample parameters. Thus, while both methods report on the particle size, the collected data are not directly comparable. In cryo-TEM the PEG chains are, due to the poor contrast between the polymer and the vitrified water, invisible to the electron beam. As a result, the cryo-TEM micrographs only display the lipid core of the disks and the measured size does not include the PEG-corona (see Fig. 1 for a schematic picture of the lipodisk structure). In case of

DLS, on the other hand, the apparent hydrodynamic radius retrieved from the measurements corresponds to the "equivalent sphere radius" and should not be mistaken for the actual disk radius. By use of, e.g., the model described by Mazer et al. [35] it is in principle possible to convert the apparent hydrodynamic radius into a disk radius. This operation requires, however, knowledge about the disk thickness (including the contribution from the PEG corona) and, since reliable data concerning this parameter is largely lacking for our systems, we refrain from any such attempts.

Keeping the above-mentioned limitations in mind, some interesting conclusions about the aggregate size and structure in the different systems can be drawn from the cryo-TEM and DLS investigations. The micrographs shown in Fig. 2 display the structures observed by cryo-TEM in extruded samples composed of DSPC and DSPE-PEG_{1000}. As seen in Fig. 2a, the aggregate structure in samples containing 5 mol% PEG-lipid is completely dominated by liposomes. Since the cryo-TEM sample preparation was performed at 25 °C, *i.e.* well below the gel-to-liquid crystalline phase transition temperature of DSPC (55 °C), the bilayers are in the gel phase state and the liposomes display an uneven, almost polygonal, shape [36]. Apart from liposomes, a small number of lipodisks can be observed in the samples (inset Fig. 2a). The number of lipodisks gradually increases with increasing PEG-lipid concentration, while at the same time the population of liposomes becomes smaller and eventually disappears. Moreover, as the PEG-lipid content increases, the lipodisk radii progressively decrease (Fig. 2b–d). In line with previously published data [6], the cryo-TEM investigations revealed a very similar structural behavior in samples where DSPE-PEG_{1000} was replaced with DSPE-PEG_{2000} (see Supplementary Data). A deviating behavior is displayed, however, by samples containing the shortest PEG-lipid, DSPE-PEG_{750}. As shown in Fig. 3, DSPE-PEG_{750} is, similar to DSPE-PEG_{2000} and DSPE-PEG_{1000}, clearly able to induce lipodisk formation. The samples appear, however, less homogeneous in both size and structure. Thus, structures that may be classified neither as liposomes nor lipodisks were frequently observed (see, e.g., the presence of a thick band-like structure in Fig. 3a). Further, keeping in mind that large lipodisk, in contrast to small ones, tend to be observed mostly face-on (see note in legend to Fig. 2), it is obvious that the lipodisks shown in Fig. 3b display a great variation in size. In addition, micrographs obtained from samples containing 30 mol%, or more, DSPE-PEG_{750} disclose a population of large, undefined, structures not seen in systems containing the two other PEG-lipids. These structures (marked with arrows in Fig. 3c) are most likely related to the lamellar fragments previously reported by Johnsson et al. [24] to coexist with globular micelles in dilute aqueous dispersions of DSPE-PEG_{750}. The presence of the fragments, together with the overall inhomogeneous nature of the samples indicates that DSPE-PEG_{750} is not an ideal PEG-lipid for the production of lipodisks.

The results of the DLS measurements support the conclusions drawn from the cryo-TEM investigations. Thus, data displayed in Fig. 4 indicate that two distinct populations of particles, *i.e.* liposomes and lipodisks, coexist in the samples when the PEG-lipid concentration corresponds to 5 and 10 mol%. The monomodal size distributions found at 20 mol% PEG-lipid suggest, in line with the cryo-TEM results, that the liposomes have essentially disappeared at this concentration. Further, the shift of the lipodisk peaks towards smaller apparent hydrodynamic radii seen with increasing PEG-lipid concentration corresponds with the lipodisk size reduction observed in the cryo-TEM analysis. The data shown in Fig. 4c confirm moreover the existence of a population of large structures (>200 nm), presumably identical to the lamellar fragments shown in Fig. 3c, that is unique to samples containing 30 mol% DSPE-PEG_{750}.

One important parameter that might be affected by the choice of PEG-lipids is the colloidal stability of the lipodisks. Stable systems are for example crucial for the development of pharmaceutical formulations with long shelf-lives, and also of high importance in analytical applications that require measurements over long periods of time. The colloidal stability of lipodisks is largely governed by the magnitude of the steric repulsion provided by the PEG-polymers, and changes in the PEG molecular weight will affect the range and magnitude of the steric barrier [37]. Previous studies have verified excellent long-term stability of lipodisks stabilized by DSPE-PEG_{5000} [12] and measurements carried out in the present study show that efficient steric stabilization is obtained also with PEGs of considerably lower molecular weight. Thus, by repeatedly characterizing lipodisk samples containing 20 and 30 mol% of DSPE-PEG_{1000} and DSPE-PEG_{2000}, respectively, by cryo-TEM and

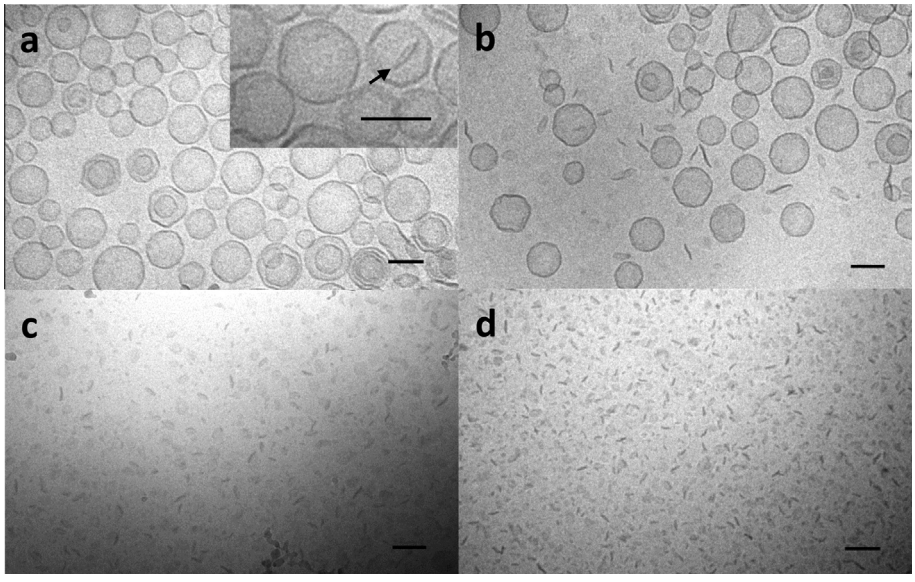


Fig. 2. Cryo-TEM micrographs of 10 mM DSPC/DSPE-PEG_{1000} dispersions prepared by extrusion. The lipid/PEG-lipid molar ratio is 95:5 (a), 90:10 (b), 80:20 (c) and 70:30 (d). The arrow in (a) indicates a lipodisk. Note that the image of a lipodisk depends on its orientation with respect to the incoming electron beam. Thus, as viewed face-on the lipodisks appear as circular objects, while they give the impression of short rods when observed edge-on. Scalebar = 100 nm.

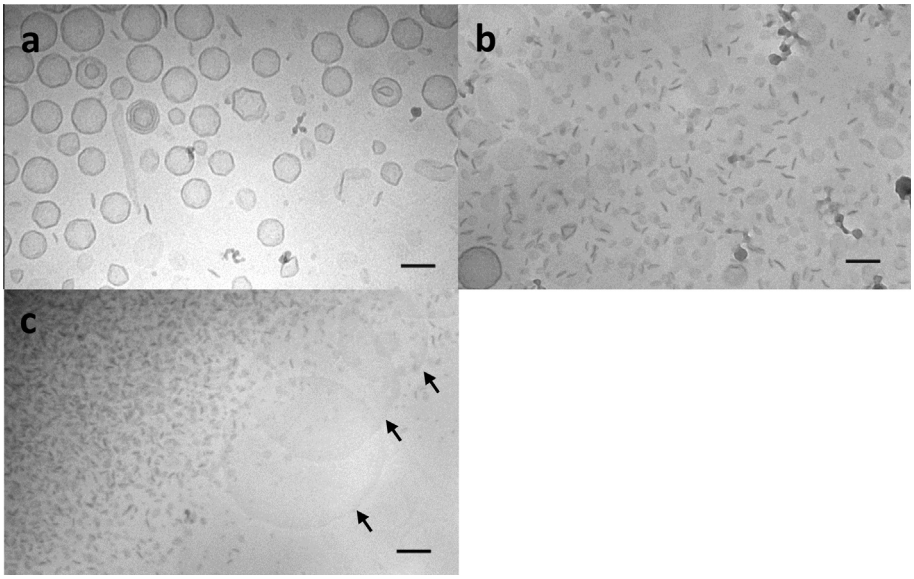


Fig. 3. Cryo-TEM micrographs of 10 mM DSPC/DSPE-PEG_{750} dispersions prepared by extrusion. The lipid/PEG-lipid molar ratio is 90:10 (a), 80:20 (b) and 70:30 (c). The arrows in (c) indicate the large undefined structures found in samples containing 30 mol% DSPE-PEG_{750}. Scalebar = 100 nm.

DLS, we could confirm that no structural rearrangement occurred in any of these samples within the 2 month time period of our study (a comparison of DLS data obtained for fresh and stored samples can be found as Supplementary material).

3.2. Effect of chain-length on lipodisk immobilization

Apart from controlling the colloidal stability, the extent of the steric barrier provided by the PEG-polymers can be expected to influence also other aspects of lipodisk behavior. It is for instance plausible that changes in the thickness of the PEG layer could affect the immobilization of lipodisks to solid surfaces. More specifically, reduced PEG molecular weight might allow for tighter packing of the lipodisks on the surface, which, in turn, could promote immobilization of a larger number of disks. As mentioned in the introduction, immobilized lipodisks and proteolipodisks can be used, e.g., in partition studies and fragment screening. Increasing the amount of immobilized disks would increase the sensitivity in these, and other applications. In order to test the hypothesis that the PEG molecular weight affects the immobilization behavior we investigated and compared the passive immobilization of lipodisks stabilized by 20 mol% DSPE-PEG_{5000}, DSPE-PEG_{2000}, and DSPE-PEG_{1000}, respectively, to silica surfaces. These experiments are based on the rather surprising observation that lipodisks spontaneously bind to clean silica surfaces at room temperature. The reason for this behavior is an interesting subject for future research. Relevant for this report is the fact that the binding was reproducible and irreversible at the working conditions.

Prior to the immobilization experiments the particle size in the three different lipodisk preparations was determined from cryo-TEM analyses. The lipodisks used are shown in Figs. 2c and 5. The measurements suggest that the diameter of the lipid part of the lipodisks stabilized by DSPE-PEG_{5000}, DSPE-PEG_{2000}, and DSPE-PEG_{1000} correspond to 23.1 ± 7.3 nm, 26.0 ± 10.4, and 25.4 ± 6.6 respectively. The similar size of the bulk lipid part of the disks is advantageous, since it means that possible differences in the immobilization behavior confidently can be traced back to differences in the size or properties of the PEG corona.

The effect of the PEG-length on the amount of disks adsorbed onto silica substrates was determined with three independent approaches. First, the binding of lipodisks to porous silica particles was determined with the help of phosphorous analysis to determine lipid concentration. Secondly, the binding of lipodisks to flat and smooth silica surfaces was followed with multi-parametric surface plasmon resonance (MP-SPR), as well as with quartz crystal microbalance with dissipation monitoring (QCM-D). As shown in Fig. 6, changing the length of the PEG-lipid used for the lipodisk preparation dramatically affected the lipodisk binding to silica, regardless of how the binding was determined.

The effect is particularly obvious in the case of immobilization of the disks to porous silica. As can be seen in Fig. 6a, the amount of lipid associated with the particles is tripled upon reduction of the PEG molecular weight from 5000 to 2000. Moreover, immobilization of disks stabilized by the even shorter DSPE-PEG_{1000} results in an amount of bound lipid that is almost six times higher than observed in the case of disks stabilized with DSPE-PEG_{5000}. These results are clearly in line with the suggestion that a less extended PEG corona should enable a more condensed packing of the lipodisks on the surface. It cannot be excluded, however, that disks with smaller overall size also have a higher probability of penetrating the small particle pores (c.a. 30 nm) and therefore have access to a larger fraction of the total silica surface. This issue is avoided in the QCM-D and MP-SPR experiments measuring lipodisk binding to smooth flat surfaces.

As observed in Fig. 6b and c, both MP-SPR and QCM-D data unequivocally show that a larger number of lipid molecules (and therefore, of lipodisks) binds to the surface when the PEG chains are shorter. Furthermore, the relative difference in the amount of immobilized lipodisks containing PEG_{1000} and PEG_{2000} is similar when determined on smooth silica with MP-SPR and in porous silica particles using phosphorous analysis. This suggests that the accessibility to the pores is not a limiting condition for the binding of these two types of lipodisks to the porous silica used. Lipodisks containing PEG_{5000}, on the other hand, are underrepresented in the porous silica, suggesting that they have limited access to the pores.

An increase in the amount of immobilized lipodisks with decreasing polymer length could be observed also with QCM-D. However, in this case the relative difference in the amount of bound lipodisks was smaller. This is due to the fact that the response from QCM-D, in contrast to the data received from MP-SPR and phosphorous analysis, also includes the coupled water in

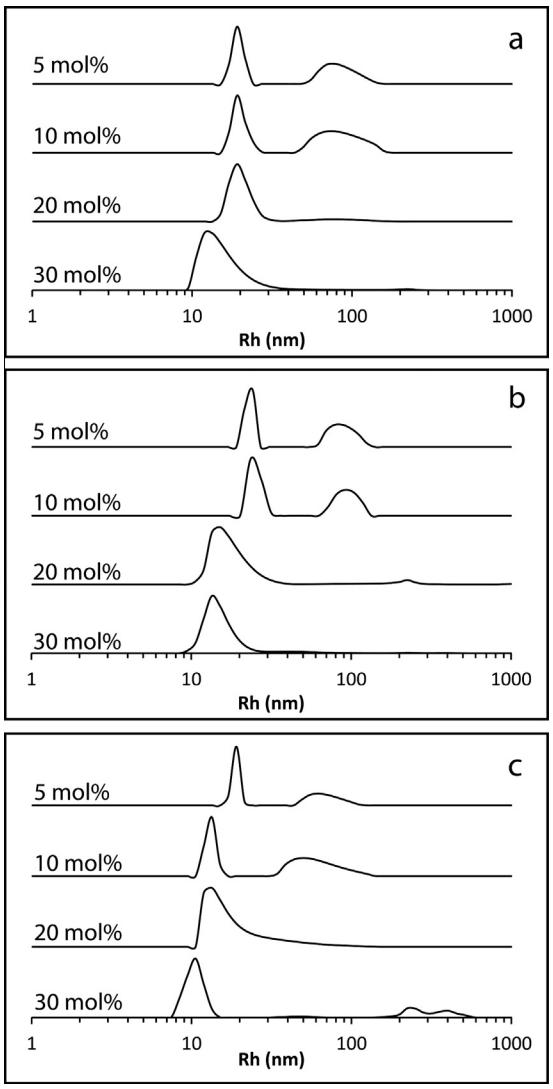


Fig. 4. Apparent hydrodynamic radii distribution (volume weighted) obtained from DSPC/DSPE-PEG$_{2000}$ (a), DSPC/DSPE-PEG$_{1000}$ (b) and DSPC/DSPE-PEG$_{750}$ (c) dispersions with increasing PEG-lipid amount. The total PEG-lipid contents in the samples are listed to the left.

the immobilized film. Since the amount of coupled water increases with the length of the PEG chains [38], the apparent weight of the lipodisks increases accordingly.

Taken together, the results obtained strongly support the hypothesis that surface immobilization of lipodisks can be improved by reducing the polymer chain length of the PEG-lipid used for disks stabilization.

3.3. Lipodisks with low PEG-lipid amounts

Although lipodisk formation usually starts already in the range of 5–10 mol% PEG-lipid, a considerable amount of liposomes is also present in these samples (see, e.g., Fig. 2b) [5,6,12]. In order to produce pure, essentially liposome-free, lipodisk samples, the PEG-lipid concentration typically needs to be increased to about 20 mol%. As mentioned in the introduction, several situations can be envisioned where lipodisks with lower PEG-lipid content could prove advantageous.

A commonly used method in lipodisk production is ultrasonic probe sonication. When performed at a temperature above the main transition temperature for the bilayer forming lipid, this method tends to produce lipodisks that are slightly smaller than those obtained by extrusion [39]. The general structural behavior in the lipid/PEG-lipid dispersions is not changed, however, and sonicated samples containing 10 mol%, or less, PEG-lipid are dominated by liposomes (see Fig. 7a). During our investigation of alternative liposome preparation protocols, we made the interesting discovery that sonication of DSPC/PEG-lipid mixtures at 0 °C, *i.e.* well below the transition temperature of DSPC, resulted in pure lipodisk samples already at a PEG-lipid content as low as 2 mol%.

Figs. 7 and 8 show the aggregate structure found by cryo-TEM in mixtures of DSPC and DSPE-PEG$_{2000}$ and DSPE-PEG$_{1000}$, respectively, after sonication at 0 °C. As can be deduced from the micrographs, inclusion of 2 or 5 mol% PEG-lipid results in liposome-free samples containing large lipodisks. The cryo-TEM micrographs indicate that the disks are larger in samples containing 2 mol% PEG-lipid (121 ± 52 nm and 77 ± 33 nm for lipodisks containing DSPE-PEG$_{1000}$ and DSPE-PEG$_{2000}$, respectively) than in samples containing 5 mol% PEG-lipid (72 ± 33 nm and 65 ± 30 nm for lipodisks containing DSPE-PEG$_{1000}$ and DSPE-PEG$_{2000}$, respectively). Moreover, the disks were found to be larger when the shorter DSPE-PEG$_{1000}$ was used as edge-stabilizer. Importantly, sonication of pure DSPC samples resulted in optically clear solutions that rapidly became opaque, and cryo-TEM characterization of the turbid samples revealed the existence of large bilayer sheets (Fig. 7d). It can therefore be concluded that inclusion of PEG-lipid is required in order to obtain lipodisks. Moreover, for mixtures containing DSPC and 5 mol% DSPE-PEG$_{2000}$, control experiments were carried out in which the sonication time was reduced from 45 to 10 min. Cryo-TEM investigation confirmed that these samples were structurally identical to the samples shown in Fig. 7b. The possibility of disk formation as a result of lipid degradation induced by the sonication process can thus be ruled out.

A likely explanation for the formation of the large lipodisks can be found if we consider the fact that the samples, at the start of the sonication procedure, consist of dispersed lipid bilayers in the gel phase state. Upon sonication the bilayers are shattered into small fragments, which, due to the high bending rigidity of bilayers in the gel phase state [40], cannot close up on themselves to form liposomes. Because of the exposed hydrophobic edges, the small

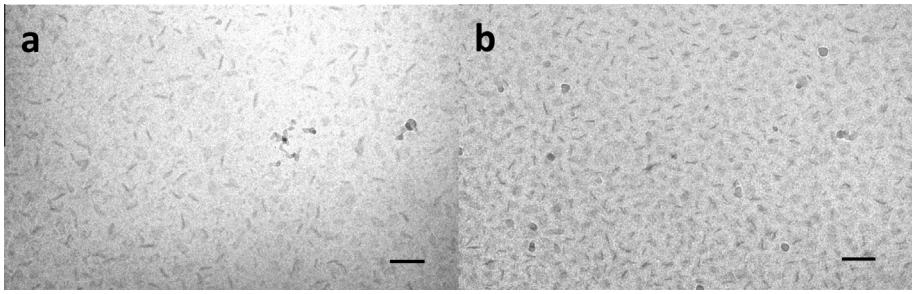


Fig. 5. Cryo-TEM micrographs of 10 mM DSPC/DSPE-PEG$_{2000}$ (80:20) (a) and DSPC/DSPE-PEG$_{5000}$ (80:20) (b) dispersions prepared by extrusion. Scalebar = 100 nm.

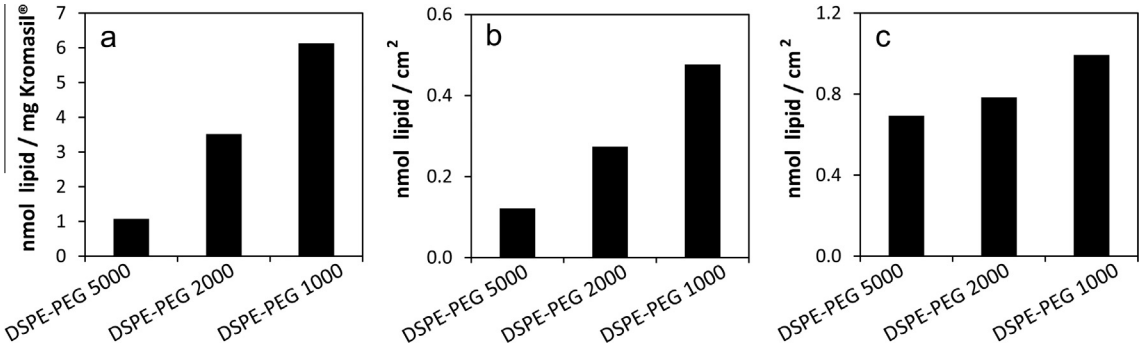


Fig. 6. Total phospholipid amount (both DSPC and DSPE-PEG$_n$) immobilized on silica surfaces determined by multiple techniques. The graphs represent the amount of lipodisks immobilized on porous Kromasil® silica (a) and on smooth silica surfaces using MP-SPR (b) and QCM-D (c). The lipodisks used were composed of DSPC/DSPE-PEG$_n$ (80:20) with n = 1000, 2000 and 5000 respectively. The data in (c) are not corrected for coupled water.

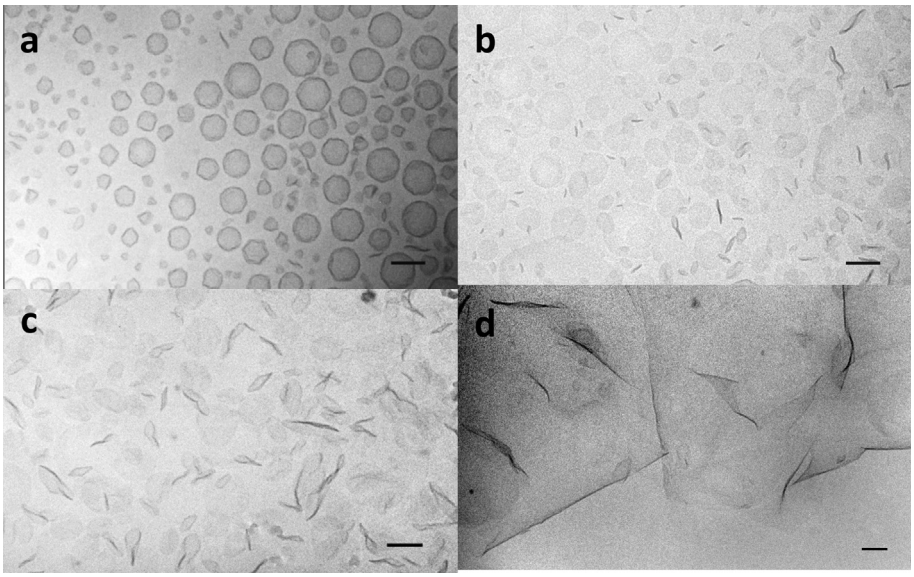


Fig. 7. Cryo-TEM micrographs of 10 mM DSPC/DSPE-PEG$_{2000}$ mixtures prepared by sonication. The lipid molar ratio and sonication temperature was 95:5/70 °C (a), 95:5/0 °C (b) and 98:2/0 °C (c), respectively. The image in (d) shows a pure DSPC sample sonicated at 0 °C. Scalebar = 100 nm.

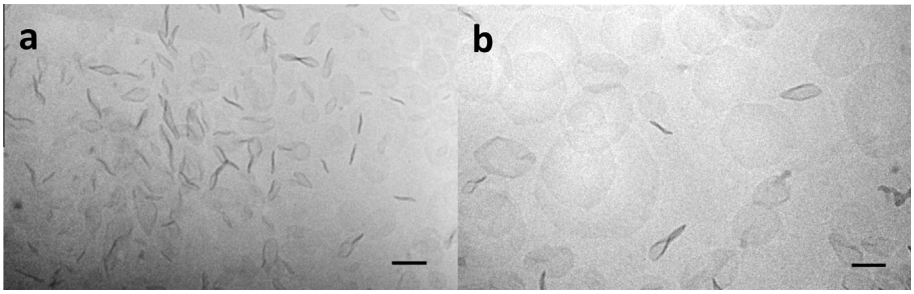


Fig. 8. Cryo-TEM micrographs of 10 mM DSPC/DSPE-PEG$_{1000}$ dispersions prepared by sonication at 0 °C. The lipid/PEG-lipid molar ratio is 95:5 (a) and 98:2 (b). Scalebar = 100 nm.

bilayer pieces are highly energetically unfavorable. Subsequent to the sonication, and in the absence of PEG-lipids, the bilayer fragments therefore fuse into the bilayer sheets shown in Fig. 7d. In the presence of PEG-lipids, which preferentially accumulate at the fragment edges, fusion is sterically hindered, however. Thus, after a small, and likely PEG-lipid concentration dependent, number of fusion events the sterically stabilized fragments become "trapped" into the lipodisks shown in Figs. 7 and 8. The mechanism described above infers that the lipodisks formed as a consequence of low temperature sonication are far from equilibrium structures, and this justifies questions about their stability.

Hence, the stability of lipodisks containing 2 and 5 mol% of DSPE-PEG$_{2000}$ and DSPE-PEG$_{1000}$, respectively, was investigated over time. As expected, the disks were found to be less stable than conventional lipodisks prepared with high amounts of PEG-lipids, and storing them for long periods eventually led to fusion and formation of bilayer sheets. Characterization using cryo-TEM and DLS showed no structural change of the lipodisks containing 2 mol%

PEG-lipids after 24 h storage at 0 °C, but reinvestigation of the samples after one week revealed the existence of large bilayer sheets. As can be anticipated, the lipodisks containing 5 mol% PEG-lipid were considerable more stable and no change in size or structure was noted during the first two weeks following preparation. Analysis carried out a further four weeks later showed, however, that bilayer sheets had begun to form also in this sample. Noteworthy, the stability of the lipodisks was found to be similar irrespective of whether DSPE-PEG_{2000} or DSPE-PEG_{1000} was employed in the sample preparation.

The fact that the sonicated lipodisks with time slowly fuse into larger structures implies that the disk rim is not fully saturated with PEG-lipids. This may at first seem suboptimal but could for certain lipodisk applications actually prove to be beneficial. It is for instance plausible that the low PEG-lipid density would be an advantage in applications that require, or benefit from, insertion of functionalized PEG-lipids into preformed lipodisks. The experiments described in the following section were carried out in order to verify this hypothesis.

3.4. Post-insertion of functionalized PEG-lipids into preformed lipodisks

During the last decades significant attention has been focused on the possibilities to increase drug efficacy and reduce the risk of unwanted side effects by use of nanocarriers that are able to specifically target selected tissue or cells. Nanocarriers with cell-targeting capacity can be achieved by linking targeting agents, e.g., ligands with high specificity for selected receptors, to the surface of the carrier. In case of liposomes, the drug carriers are usually provided with targeting ligands by utilization of the "post-insertion" technique [41–43]. In this technique, micelles formed from PEG-lipid-linked ligands are incubated with preformed liposomes to convert them into ligand-targeted liposomes. A major advantage of this approach is that the ligands, which are often delicate, need not be subjected to the rather harsh conditions used during liposome production (e.g. exposure to organic solvents or high temperature, freeze-thawing, sonication, etc.). The post-insertion technique is unfortunately not readily compatible with conventional lipodisks, since the high PEG-lipid content prevents the incorporation of satisfactory amounts of the externally added ligand-linked PEG-lipids. As discussed in the previous section, there is reason to believe that lipodisks prepared by low temperature sonication are more sparsely decorated with PEG and therefore able to incorporate higher amounts of externally added PEG-lipids. This could potentially open up for the production of targeting lipodisks by means of the "post-insertion" technique.

In order to test if the low temperature sonicated lipodisks possess properties that promote "post-insertion" of PEG-lipid-linked targeting agents, we performed studies based on the use of PEG-lipids conjugated to epidermal growth factor (EGF). EGF was chosen as targeting agent since it has been well established that liposomes provided with EGF-linked PEG-lipids are able to efficiently target tumor cells that express the epidermal growth factor receptor [44,45]. In order to allow for high sensitivity quantification, the PEG-lipid-linked EGF was radiolabelled with ^{125}I (see Methods section for details).

The amount of EGF-PEG-lipids that can be added to preformed lipodisks was investigated and compared using lipodisks composed of DSPC/DSPE-PEG_{2000} with 5 and 20 mol% PEG-lipid, respectively. The former lipodisks were prepared by low temperature sonication, whereas the latter were produced by extrusion (cryo-TEM micrographs of the lipodisks are shown in Figs. 5a and 7b, respectively). The lipodisks were allowed to incubate with externally added ^{125}I-labelled EGF-PEG-lipid for 24 h at 37 °C and the material was then run through a gel filtration column. As seen in Fig. 9, which shows the distribution of ^{125}I in the different fractions collected after the separation, the material was eluted in two peaks. The lipodisks, being the biggest aggregate structure in the samples, elute first and the signal in the first peak corresponds to the fraction of radiolabeled ^{125}I-EGF-PEG-lipids that have inserted into the disks. Due to their larger size, lipodisks produced with 5 mol% PEG-lipid elute slightly earlier (peak centered around fraction 22) than the disks with 20 mol% PEG-lipid (peak centered around fraction 27). The second peak in the chromatograms, which originates from non-inserted ^{125}I-EGF-PEG-lipids in the form of PEG-lipid micelles, is for both lipodisk preparations centered around fraction 41. After compensating for the peak overlap, assuming two normally distributed populations, the post-insertion yield was calculated from the relative signal between the peaks corresponding to lipodisks and PEG-lipid micelles, respectively. The results confirm that incorporation indeed is facilitated by the much lower PEG polymer density in the lipodisks with 5 mol% PEG-lipid. Considering the fact that the lipid bilayers are in the gel phase state at 37 °C, is it reasonable to assume that the PEG-lipids added by post-insertion will distribute preferentially to the disk rim, rather than to the highly condensed flat part of the disks. Since the disks with low amount of PEG-lipid are much larger than the disks with high amounts of PEG-lipid (diameter ~64 nm and ~26 nm, respectively, as determined by cryo-TEM), the edge/planar area ratio will be lower for the lower PEG-lipid content disks. An estimation of the amount of post-inserted EGF-PEG-lipids per lipid disk was carried out based on the determined lipid bilayer mean diameter, the headgroup area of the DSPC lipids, and the total DSPC and incorporated EGF-PEG-lipids concentrations. These calculations show that approximately 35 EGF-PEG-lipids per disk are incorporated in the 5%-PEG samples, while only an average of 2.3 EGF-PEG-lipids per disk are incorporated into the 20%-PEG samples. The amount of edge area per disk, on the other hand, is approximately 2.5 times larger for the 5%-PEG samples. This gives a density of post-inserted molecules (defined as the number of EGF-PEG-lipid molecules per edge area unit) 6 times higher in the larger lipodisks found in the 5%-PEG samples.

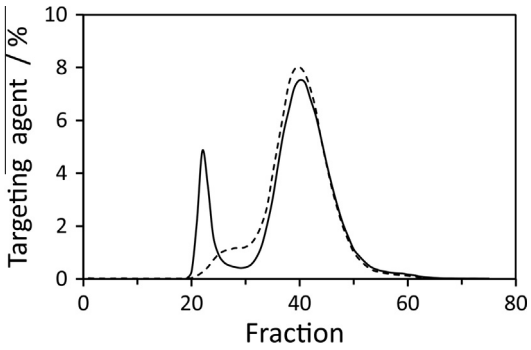


Fig. 9. Distribution of ^{125}I-EGF-PEG-lipids after post-insertion in lipodisks composed of DSPC/DSPE-PEG_{2000} in molar ratio 95:5 (solid line) and 80:20 (dashed line). Depending on disk size lipodisks elute approximately between fraction 20 and 33. Unincorporated PEG-lipids elute as micelles in the large peaks centered at fraction 41.

To verify that the "post-insertion" technique resulted in lipodisks capable of active targeting, the *in vitro* binding specificity of the large EGF-targeted lipodisks, *i.e.*, those produced from 5%-PEG samples, was tested on EGFR-expressing A431 tumor cells. Data displayed in Fig. 10 indicate that the EGF-targeted disks interact with and bind to the tumor cells in a time-dependent manner ($p = 0.0003$). Importantly, the fact that binding of the disks is almost completely suppressed upon pre-blocking of the cells with an excess of free EGF confirms that the binding is EGFR specific (4 h: $p < 1.82 \times 10^{-5}$, 8 h: $p < 2.8 \times 10^{-5}$).

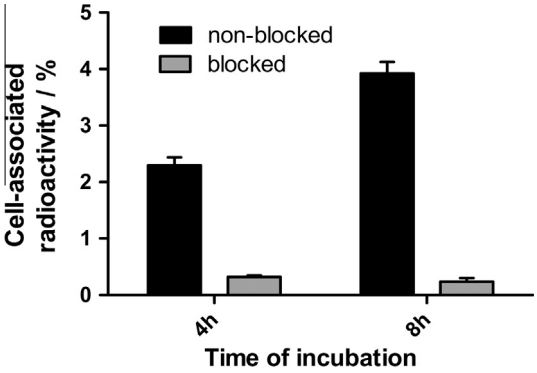


Fig. 10. Binding specificity of ^{125}I-labelled EGF-targeted lipodisks to A431 tumor cells. One group of cells (blocked cells) was pre-treated with saturating amounts of non-labelled EGF before the addition of ^{125}I-labelled EGF-targeted lipodisks. To avoid internalization of EGFR, the experiments were performed at 4 °C. Cell-associated radioactivity was calculated as percentage of total added radioactivity. Data are presented as mean ± range (n = 3).

4. Conclusions

The results of this study show that DSPE-PEG$_{1000}$ and DSPE-PEG$_{750}$, similar to the closely related PEG-lipids DSPE-PEG$_{5000}$ and DSPE-PEG$_{2000}$, can be used to produce lipodisks. Utilization of DSPE-PEG$_{750}$ results, however, in preparations that tend to be inhomogeneous in terms of both aggregate size and structure. Lipodisks based on DSPE-PEG$_{1000}$ display, on the other hand, a structural behavior very similar to that observed with DSPE-PEG$_{5000}$ and DSPE-PEG$_{2000}$. Comparative studies of lipodisk adsorption behavior on silica surfaces confirmed a negative correlation between the PEG molecular weight and the amount of disks immobilized on the surface. This finding implies that the number of immobilized lipodisks can be increased and optimized by utilization of lipodisks stabilized by DSPE-PEG$_{1000}$, rather than by DSPE-PEG$_{2000}$ or DSPE-PEG$_{5000}$.

The data collected show, moreover, that it is possible to produce lipodisks with very low PEG-lipid content. Thus, by modification of the preparation protocols, lipodisks can be prepared with as little as 2 mol% PEG-lipid. Importantly, the disk preparations are liposome-free and lipodisks with 2 or 5 mol% PEG-lipid are substantially larger than those prepared with higher concentrations of PEG-lipid using conventional methods. As expected, the low PEG lipid content impairs the long-term stability of the disks. The sub-saturating PEG-lipid concentrations enable, on the other hand, incorporation of increased amounts of externally added ligand-linked PEG-lipids. Results from cell studies show that large EGF-targeted lipodisks can be successfully produced by this technique. The feasibility of the "post-insertion" technique could prove important not only for the production of cell- or tissue-targeting lipodisks, but also for other applications where addition of functionalized PEG-lipids to preformed lipodisks is necessary or desirable.

Taken together, the findings of the present study show that it is possible to influence several important properties of PEG-stabilized lipodisks by means of variations in the volume and density of the PEG corona. Thus, by a deliberate choice of PEG molecular weight and lipodisk preparation protocol the lipodisks can be optimized and tailored for various applications as model membranes and drug delivery vehicles. The flexibility in size and lipid composition, as well as the protein-free nature of the disks, opens up for the use of PEG-stabilized lipodisks as an advantageous alternative to other frequently used discoidal lipid nanostructures, such as bicelles [46], Nanodiscs [47] and SMALPs [48].

Acknowledgements

J. Eriksson (Department of Chemistry – BMC, Uppsala University, Sweden) is gratefully acknowledged for skillful technical assistance with the cryo-TEM analysis. F. Höök (Department of Physics, Biological Physics Division, Chalmers University of Technology, Sweden) is gratefully acknowledged for assistance in the collection and evaluation of MP-SPR data. Help from J. Rosenquist and K. Reijmar (Department of Chemistry – BMC, Uppsala University, Sweden) with the production of targeting lipodisks is greatly appreciated. Financial support was received from the Swedish Research Council (621-2011-3524) and the Swedish Cancer Society (CAN 2014/617).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcis.2016.08.067.

References

[1] A.M. Carmona-Ribeiro, Biomimetic nanoparticles: preparation, characterization and biomedical applications, Int. J. Nanomed. 5 (2010) 249–259.
[2] B. Mandal, H. Bhattacharjee, N. Mittal, H. Sah, P. Balabathula, L.A. Thoma, G.C. Wood, Core–shell-type lipid–polymer hybrid nanoparticles as a drug delivery platform, Nanomed. Nanotechnol. Biol. Med. 9 (2013) 474–491.
[3] U.H.N. Durr, R. Soong, A. Ramamoorthy, When detergent meets bilayer: birth and coming of age of lipid bicelles, Prog. Nucl. Magn. Reson. Spectrosc. 69 (2013) 1–22.
[4] I.G. Denisov, Y.V. Grinkova, A.A. Lazarides, S.G. Sligar, Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size, J. Am. Chem. Soc. 126 (2004) 3477–3487.
[5] K. Edwards, M. Johnsson, G. Karlsson, M. Silvander, Effect of polyethyleneglycol-phospholipids on aggregate structure in preparations of small unilamellar liposomes, Biophys. J. 73 (1997) 258–266.
[6] M. Johnsson, K. Edwards, Liposomes, disks, and spherical micelles: aggregate structure in mixtures of gel phase phosphatidylcholines and poly(ethylene glycol)-phospholipids, Biophys. J. 85 (2003) 3839–3847.
[7] M.C. Sandström, E. Johansson, K. Edwards, Structure of mixed micelles formed in PEG-lipid/lipid dispersions, Langmuir 23 (2007) 4192–4198.
[8] A. Lundquist, P. Wessman, A.R. Rennie, K. Edwards, Melittin-Lipid interaction: a comparative study using liposomes, micelles and bilayer disks, Biochimica Et Biophysica Acta-Biomembranes 1778 (2008) 2210–2216.
[9] M.M. Zetterberg, K. Reijmar, M. Pranting, Å. Engström, D.I. Andersson, K. Edwards, PEG-stabilized lipid disks as carriers for amphiphilic antimicrobial peptides, J. Control. Release 156 (2011) 323–328.
[10] J. Gao, C. Xie, M. Zhang, X. Wei, Z. Yan, Y. Ren, M. Ying, W. Lu, RGD-modified lipid disks as drug carriers for tumor targeted drug delivery, Nanoscale 8 (2016) 7209–7216.
[11] W.P. Zhang, J. Sun, Y. Liu, M.Y. Tao, X.Y. Ai, X.N. Su, C.F. Cai, Y.L. Tang, Z. Feng, X. D. Yan, G.L. Chen, Z.G. He, PEG-stabilized bilayer nanodisks as carriers for doxorubicin delivery, Mol. Pharm. 11 (2014) 3279–3290.
[12] E. Johansson, C. Engvall, M. Arfvidsson, P. Lundahl, K. Edwards, Development and initial evaluation of PEG-stabilized bilayer disks as novel model membranes, Biophys. Chem. 113 (2005) 183–192.
[13] E. Johansson, A. Lundquist, S.S. Zuo, K. Edwards, Nanosized bilayer disks: attractive model membranes for drug partition studies, Biochimica Et Biophysica Acta-Biomembranes 1768 (2007) 1518–1525.
[14] E. Boija, A. Lundquist, K. Edwards, G. Johansson, Evaluation of bilayer disks as plant cell membrane models in partition studies, Anal. Biochem. 364 (2007) 145–152.
[15] A. Lundquist, S.B. Hansen, H. Nordström, U.H. Danielson, K. Edwards, Biotinylated lipid bilayer disks as model membranes for biosensor analyses, Anal. Biochem. 405 (2010) 153–159.
[16] E. Meiby, M.M. Zetterberg, S. Ohlson, V.A. Hernandez, K. Edwards, Immobilized lipodisks as model membranes in high-throughput HPLC-MS analysis, Anal. Bioanal. Chem. 405 (2013) 4859–4869.
[17] V.A. Hernandez, K. Reijmar, K. Edwards, Label-free characterization of peptide-lipid interactions using immobilized lipodisks, Anal. Chem. 85 (2013) 7377–7384.
[18] E. Boija, A. Lundquist, M. Nilsson, K. Edwards, R. Isaksson, G. Johansson, Bilayer disk capillary electrophoresis: a novel method to stud drug partitioning into membranes, Electrophoresis 29 (2008) 3377–3383.
[19] K. Vainikka, K. Reijmar, G. Yohannes, J. Samuelsson, K. Edwards, M. Jussila, M.L. Riekkola, Polyethylene glycol-stabilized lipid disks as model membranes in interaction studies based on electrokinetic capillary chromatography and quartz crystal microbalance, Anal. Biochem. 414 (2011) 117–124.

[20] M.V. Lindén, K. Meinander, A. Helle, G. Yohannes, M.L. Riekkola, S.J. Butcher, T. Viitala, S.K. Wiedmer, Characterization of phosphatidylcholine/polyethylene glycol-lipid aggregates and their use as coatings and carriers in capillary electrophoresis, Electrophoresis 29 (2008) 852–862.
[21] M.-D. Duong-Thi, M. Bergström, K. Edwards, J. Eriksson, S. Ohlson, J. To Yiu Ying, J. Torres, V. Agmo Hernandez, Lipodisks integrated with weak affinity chromatography enable fragment screening of integral membrane proteins, Analyst (2016).
[22] J.F. Stefanick, J.D. Ashley, T. Kiziltepe, B. Bilgicer, A systematic analysis of peptide linker length and liposomal polyethylene glycol coating on cellular uptake of peptide-targeted liposomes, ACS Nano 7 (2013) 2935–2947.
[23] A.K. Kenworthy, S.A. Simon, T.J. McIntosh, Structure and phase-behavior of lipid suspensions containing phospholipids with covalently attached poly (ethylene glycol), Biophys. J. 68 (1995) 1903–1920.
[24] M. Johnsson, P. Hansson, K. Edwards, Spherical micelles and other self-assembled structures in dilute aqueous mixtures of poly(ethylene glycol) lipids, J. Phys. Chem. B 105 (2001) 8420–8430.
[25] E. Johansson, M.C. Sandström, M. Bergström, K. Edwards, On the formation of discoidal versus threadlike micelles in dilute aqueous surfactant/lipid systems, Langmuir 24 (2008) 1731–1739.
[26] S. Beugin, K. Edwards, G. Karlsson, M. Ollivon, S. Lesieur, New sterically stabilized vesicles based on nonionic surfactant, cholesterol, and poly (ethylene glycol)-cholesterol conjugates, Biophys. J. 74 (1998) 3198–3210.
[27] M. Almgren, K. Edwards, G. Karlsson, Cryo transmission electron microscopy of liposomes and related structures, Colloids Surf. A-Physicochem. Eng. Aspects 174 (2000) 3–21.
[28] J.V. Paraskova, E. Rydin, P.J.R. Sjöberg, Extraction and quantification of phosphorus derived from DNA and lipids in environmental samples, Talanta 115 (2013) 336–341.
[29] M.V. Voinova, M. Rodahl, M. Jonson, B. Kasemo, Viscoelastic acoustic response of layered polymer films at fluid-solid interfaces: continuum mechanics approach, Phys. Scr. 59 (1999) 391–396.
[30] L.S. Jung, C.T. Campbell, T.M. Chinowsky, M.N. Mar, S.S. Yee, Quantitative interpretation of the response of surface plasmon resonance sensors to adsorbed films, Langmuir 14 (1998) 5636–5648.
[31] M.P. Jonsson, P. Jonsson, F. Höök, Simultaneous nanoplasmonic and quartz crystal microbalance sensing: analysis of biomolecular conformational changes and quantification of the bound molecular mass, Anal. Chem. 80 (2008) 7988–7995.
[32] T. Tumolo, L. Angnes, M.S. Baptista, Determination of the refractive index increment (dn/dc) of molecule and macromolecule solutions by surface plasmon resonance, Anal. Biochem. 333 (2004) 273–279.
[33] E.B. Kullberg, N. Bergstrand, J. Carlsson, K. Edwards, M. Johnsson, S. Sjöberg, L. Gedda, Development of EGF-conjugated liposomes for targeted delivery of boronated DNA-binding agents, Bioconjug. Chem. 13 (2002) 737–743.
[34] E.B. Kullberg, Q.C. Wei, J. Capala, V. Giusti, P.U. Malmström, L. Gedda, EGF-receptor targeted liposomes with boronated acridine: growth inhibition of cultured glioma cells after neutron irradiation, Int. J. Radiat. Biol. 81 (2005) 621–629.
[35] N.A. Mazer, G.B. Benedek, M.C. Carey, Quasi-elastic light-scattering-studies of aqueous biliary lipid systems - mixed micelle formation in bile-salt lecithin solutions, Biochemistry 19 (1980) 601–615.
[36] M. Andersson, L. Hammarström, K. Edwards, Effect of bilayer phase-transitions on vesicle structure and its influence on the kinetics of viologen reduction, J. Phys. Chem. 99 (1995) 14531–14538.
[37] A.K. Kenworthy, K. Hristova, D. Needham, T.J. McIntosh, Range and magnitude of the steric pressure between bilayers containing phospholipids with covalently attached poly(ethylene glycol), Biophys. J. 68 (1995) 1921–1936.
[38] S. Lusse, K. Arnold, The interaction of poly(ethylene glycol) with water studied by H-1 and H-2 NMR relaxation time measurements, Macromolecules 29 (1996) 4251–4257.
[39] M.C. Sandström, E. Johansson, K. Edwards, Influence of preparation path on the formation of discs and threadlike micelles in DSPE-PEG(2000)/lipid systems, Biophys. Chem. 132 (2008) 97–103.
[40] K.R. Mecke, T. Charitat, F. Graner, Fluctuating lipid bilayer in an arbitrary potential: theory and experimental determination of bending rigidity, Langmuir 19 (2003) 2080–2087.
[41] D.L. Iden, T.M. Allen, In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach, Biochimica et Biophysica Acta-Biomembranes 1513 (2001) 207–216.
[42] J.N. Moreira, T. Ishida, R. Gaspar, T.M. Allen, Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity, Pharm. Res. 19 (2002) 265–269.
[43] S. Zalipsky, N. Mullah, J.A. Harding, J. Gittelman, L. Guo, S.A. DeFrees, Poly (ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains, Bioconjug. Chem. 8 (1997) 111–118.
[44] E.B. Kullberg, M. Nestor, L. Gedda, Tumor-cell targeted epidermal growth factor liposomes loaded with boronated acridine: uptake and processing, Pharm. Res. 20 (2003) 229–236.
[45] A. Fondell, K. Edwards, L.M. Ickenstein, S. Sjöberg, J. Carlsson, L. Gedda, Nuclisome: a novel concept for radionuclide therapy using targeting liposomes, Euro. J. Nucl. Med. Mol. Imag. 37 (2010) 114–123.
[46] U.H.N. Dürr, M. Gildenberg, A. Ramamoorthy, The magic of bicelles lights up membrane protein structure, Chem. Rev. 112 (2012) 6054–6074.
[47] S. Denisov, S.G. Sligar, Cytochromes in P450 in nanodiscs, Biochim. Biophys. Acta - Proteins Proteomics 1814 (2011) 223–229.
[48] T.J. Knowles, R. Finka, C. Smith, Y.P. Lin, T. Dafforn, M. Overduin, Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer, J. Am. Chem. Soc. 131 (2009) 7484–7485.