Dual centrifugation as a novel and efficient method for the preparation of lipodisks


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ABSTRACT

Polyethylene glycol (PEG)-stabilized lipodisks have emerged as innovative, promising nanocarriers for several classes of drugs. Prior research underscores the important role of lipid composition and preparation method in determining the lipodisk size, uniformity, and drug loading capacity. In this study, we investigate dual centrifugation (DC) as a novel technique for the production of PEG-stabilized lipodisks. Moreover, we explore the potential use of DC for the encapsulation of two model drugs, curcumin and doxorubicin, within the disks. Our results show that by a considerate choice of experimental conditions, DC can be used as a fast and straightforward means to produce small and homogenous lipodisks with a hydrodynamic diameter of 20–30 nm. Noteworthy, the technique works well for the production of both cholesterol-free and cholesterol-containing disks and does not require pre-mixing of the lipids in organic solvent. Furthermore, our investigations confirm the efficacy of DC in formulating curcumin and doxorubicin within these lipodisks. For doxorubicin, careful control and optimization of the experimental conditions resulted in formulations displaying an encouraging encapsulation efficiency of 84% and a favourable drug-to-lipid ratio of 0.13 in the disks.

1. Introduction

Due to their biomimetic properties and non-toxic, biocompatible nature, nanoparticles built from lipid bilayers have found frequent use as both model membranes and vehicles for drug delivery. Uni- and multilamellar liposomes, i.e., spherical lipid particles consisting of an aqueous core surrounded by one or multiple self-closed lipid bilayers, constitute the to date most widely used class of lipid-based nanoparticles. Nevertheless, depending on the application, alternative types of nanoparticles may offer better opportunities. As one example, the closed, hollow structure of liposomes can prove disadvantageous in experiments probing analyte-membrane interactions, since a large portion of the surface area is initially hidden from interaction with the surrounding aqueous environment (Johansson et al., 2007, 2005). Further, the spherical shape, rather large size, and comparably high rigidity may render liposomes suboptimal as nanocarriers for certain drugs, such as tumour-targeted anticancer agents. In line with this, accumulating evidence suggests that non-spherical shape, deformable morphology, and small size all are features that enhance the ability of nanoparticles to target and penetrate deep into tumour tissue (Ding et al., 2019; Niora et al., 2020; Zhu et al., 2019). Hence, unless the anticancer drugs belong to the category which requires or benefits from encapsulation in the aqueous core of liposomes, there are good reasons to consider alternative nanocarriers.

Polyethylene glycol (PEG)-stabilized lipodisks constitute a class of nanoparticles that are closely related to liposomes. Thus, they consist of self-assembled lipid bilayers, and are built from the same lipid components that are typically used to construct PEGylated liposomes. In lipodisks the bilayer is not self-closed, however, but stabilized into a discoidal shape by the edge-active PEG-lipids (Fig. 1). Formation of the flat, circular lipodisks is observed when PEG-lipids are mixed with lipids in the gel or liquid ordered phase state, and the concentration of the former exceeds the bilayer saturation limit (Edwards et al., 1997; Johnsson and Edwards, 2003; Sandstrom et al., 2007; Viitala et al., 2019). The disks are surrounded by a curved rim composed predominately of PEG-lipids (Lundquist et al., 2008), and their size can be changed and adapted by varying the PEG-lipid concentration (Johansson et al., 2005; Johnsson and Edwards, 2003) or PEG molecular weight (Morin Zetterberg et al., 2016). Due to their high PEG-lipid content, lipodisks display excellent structural stability and
typically remain unchanged upon storage for several months at 4 °C (Johansson et al., 2005; Morin Zetterberg et al., 2016). Numerous studies have reported on the applicability of PEG-stabilized lipodisks as versatile model membranes for, e.g., drug partition studies and investigations involving amphiphilic peptides and membrane proteins (see Zetterberg et al. (Morin Zetterberg et al., 2016), and references therein). During the last decade, lipodisks have received increasing attention also as vehicles for drug delivery. Thus, lipodisks have been employed for single and dual delivery of several different classes of anticancer drugs, including conventional chemotherapeutic drugs and anticancer peptides (Ahlgren et al., 2017b; Ahlgren et al., 2017a; Fang et al., 2019; Gao et al., 2016; Lundsten et al., 2020; H. Wang et al., 2019a; Wang et al., 2018; L. Wang et al., 2019b; Zhang et al., 2018, 2014). Noteworthy, two recently published studies involving head-to-head comparisons of lipodisks and PEGylated liposomes conclude that lipodisks display greater tumour accumulation and considerably more efficient tumour penetration (Dane et al., 2022; Huang et al., 2022). Data reported by Dane et al. more over verify a significantly extended circulation half-life for lipodisks, as compared to liposomes (Dane et al., 2022). A number of recent studies furthermore point towards lipodisk provoking a weaker anti-PEG IgM response and being less susceptible to accelerated blood clearance (ABC) phenomena than PEGylated liposomes (Grad et al., 2020; Wang et al., 2022).

A variety of different preparation protocols, including methods based on, e.g., simple hydration of thin lipid films (Johansson et al., 2005; Sandström et al., 2008), probe sonication (Ahlgren et al., 2017a; Johansson et al., 2007; Sandström et al., 2008; Wang et al., 2018), detergent depletion (Ahlgren et al., 2017b, 2017a; Lundsten et al., 2020; Sandström et al., 2008), ethanol injection (Dane et al., 2022), and microfluidic technology (Levy et al., 2021), have been used to produce lipodisks. The selection of preparation protocol, as well as the choice of lipid composition, has been shown to affect the size and size heterogeneity of the lipodisks. Protocols built on detergent depletion using size exclusion chromatography tend for instance to generate particularly small disks (Ahlgren et al., 2017a; Sandström et al., 2008), and the use of cholesterol supplemented lipid mixtures typically result in disks that are comparatively large and rather heterogeneous in size (Johansson et al., 2007, 2005; Sandström et al., 2008). Apart from preferences regarding nanoparticle size or homogeneity, other circumstances may be decisive for the choice of preparation method or lipid composition. Thus, protocols that in some step include dissolution of the lipid components in organic solvents may not be compatible with the incorporation of proteins, or other sensitive molecules, in the disks. Also, the intended application may require a lipodisk concentration that is higher than what is possible to achieve with conventional preparation protocols.

Dual centrifugation (DC) has emerged as a novel, fast, aspic and convenient technique for the preparation of liposomes (Koehler et al., 2021; J. K. Koehler et al., 2023b; Massing et al., 2008). When using this method, a highly concentrated aqueous dispersion of the lipids is homogenized to produce a vesicular phospholipid gel (VPG), which can then either be stored or converted to a conventional liposome dispersion by simple dilution. The VPG-production is performed “in-vial” using a specially equipped centrifuge that allows not only high-speed centrifugation but also forces the sample vial to turn around a second rotation axis (Massing et al., 2017). The resulting fast movement of the viscous sample back and forth from the bottom to the top of the vial, together with the presence of added ceramic beads, ensures efficient homogenization of the dispersion. Noteworthy, although the sample is exposed to a large number of homogenization events, each of these involve only moderately strong shear forces. Hence, DC-homogenization is gentler than high-pressure homogenization, and thus less likely to induce, e.g., lipid degradation (Massing et al., 2008). Further, in contrast to most other liposome-production methods, liposome preparation by DC does not require premixing of the lipid components in an organic solvent. Thus, even when working with complex lipid compositions including cholesterol the VPGs can be produced from dry lipids after hydration with a small volume of aqueous buffer (J. Koehler et al., 2023a).

Previous studies have confirmed that PEGylated liposomes can be produced by DC (J. Koehler et al., 2023a; Koehler et al., 2021). Given the compositional similarities between PEGylated liposomes and PEG-stabilized lipodisks, it’s reasonable to assume that DC could be used as a fast and convenient means to produce also the latter. There are some noteworthy differences, however, between the two types of nanoparticles. Compared to PEGylated liposomes, lipodisks contain a considerably higher proportion of PEG-lipids with high water-binding capacity. At the same time, lipodisks are smaller and have, in contrast to liposomes, no water filled core. These dissimilarities can be foreseen to affect the lipid-to-water ratios and forces needed to successfully produce the nanocarriers by DC-homogenization. Hence, it is not obvious that the experimental conditions found optimal for the production of liposomes (J. Koehler et al., 2023a) apply for lipodisks.

In the current study, we have explored the potential of DC as a novel production method for lipodisks. To this end, we have carried out systematic studies to investigate how different parameters, such as lipid composition, lipid concentration, and the duration of the homogenization process affect the particle size and morphology in the preparations. Further, using curcumin and doxorubicin as model compounds, we have made initial investigations regarding the possibilities of exploiting DC-homogenization as an efficient method for the formulation of anticancer drugs in lipodisks.

2. Materials and methods

2.1. Materials

Curcumin (≥98.0 % pure; HPLC grade), Ammonium molybdate, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, ≥99.5 %), Triton™ X-100 and Dimethyl sulfoxide (DMSO; ≥99.9 %) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Doxorubicin hydrochloride (≥99.60 % pure) was purchased from MedChemExpress (MCE, New Jersey, USA). Potassium antimony (III) oxide tetrata trihydrate was obtained from Merck KGaA (Darmstadt, Germany). Phospholipids including Hydrogenated egg phosphatidylcholine (HEPC ≥98.0 %; Lipoid EPC-3), N-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (MPEG-2000-DSPE ≥ 98.0 %; Lipoid PE 18:0/18:0 – PEG 2000) were generously provided by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol, Sodium chloride (NaCl), Ascorbic acid, and HPLC-grade organic solvents were purchased from VWR International (Darmstadt, Germany). Amicon® Ultra centrifugal filters (Ultracelet®, MWCO 3 K) were obtained from Merck Millipore (Carrigtwohill, Ireland). Ultrapure Milli-Q water (Type 1; Synergy® UV dispenser, MilliporeSigma™, UK) was used for all the experiments. HEPES buffered saline (HBS; 20 mM HEPES + 150 mM NaCl; pH 7.4) was freshly prepared, sterile-filtered, and stored in the fridge until further use.
2.2. Methods

2.2.1. Preparation of lipodisks by DC

The lipodisk formulations were prepared using a dual centrifuge (DC, ZentriMix 380R, Andreas Hettich GmbH & Co KG, Tuttlingen, Germany), as described previously (Koehler et al., 2021). Based on the formulation, samples were prepared either by a dry lipid mixture or using the in-vial lipid film method. The two formulations prepared and analysed are HEPC:Cholesterol:DSPE-PEG$_{2000}$ with a molar ratio of 35:45:20 and HEPC:DSPE-PEG$_{2000}$ with a molar ratio of 80:20. Formulations are hereafter denoted as HEPC:Chol:DSPE-PEG$_{2000}$ and HEPC:DSPE-PEG$_{2000}$.

2.2.1.1. Preparation of lipodisks using dry lipid mixture. Briefly, accurately weighed amounts of lipids to produce a final lipid concentration of 10–60 % w/v in the formulations, were taken in 2 mL conical screw cap vials (Sarstedt AG & Co. KG, Nümbrecht, Germany). 600 mg zirconium oxide beads with a diameter of 1.5 mm (SiLibeads® Type ZY-P Pharma, Sigmund Lindner GmbH, Germany) were added to each sample and the calculated amount of HBS buffer (20 mM HEPES, 150 mM NaCl; pH 7.4) were then added to the vials. Depending on the lipid content used, a phospholipid gel with a final batch size of 50 mg was prepared by mixing 5–30 mg of lipid mixture and 45–20 µL of aqueous buffer (i.e., HBS). Due to the density of the buffer and phospholipid being close to 1 g mL$^{-1}$, we calculated that dispersion of 1 mg lipid per 100 µL corresponds to 1 % lipid concentration (J. Koehler et al., 2023a). Phospholipid gels were then produced by homogenizing the lipids at 2350 rpm for 5–60 min.

In case of cholesterol-containing lipid mixtures, the centrifuge was pre-cooled to 4 °C before sample preparations. When using mixtures without cholesterol, precautions were made to avoid exposing the sample to temperatures below the gel-to-liquid crystalline transition temperature ($T_{m}$). Thus, the vials and DC plates were pre-heated by
placing them in a heating oven (UF30plus, Memmert GmbH & Co. KG, Schwabach, Germany) set at 65 °C for 45 min. Second, the centrifuge was prewarmed by running it empty at a setting of 40 °C for 45 min. These measures, in combination with the fact that the sample, due to the significant homogenization forces, heats up by around 20 °C in comparison to the temperature specified on DC, ensured that the samples were above 60 °C throughout the homogenization process.

The formed lipid gels were diluted with HBS buffer by low-speed DC or by gently vortexing the samples. In order to avoid foaming and potential loss of PEG-lipid, care was taken to completely fill the vials, i.e., add buffer up to 2 mL, during the dilution step.

2.2.2. Preparation of drug-loaded lipodisks by probe sonication

Lipids in desired molar ratios were weighed directly into the clean glass vials and were then dissolved in chloroform. The required volume of the stock solutions was transferred to the DC vials (Sarstedt AG & Co. KG, Nümbrecht, Germany). The organic solvents were then evaporated and lipodisk prepared using the dry lipid mixture (Sarstedt AG & Co. KG, Nümbrecht, Germany). The different lipids were carefully weighed in the 2 mL conical screw cap vials (Sarstedt AG & Co. KG, Nümbrecht, Germany). The lipids were then dissolved in an organic solvent mixture (chloroform: ethanol 2:1; v/v) and were thoroughly vortexed. The organic solvent was thereafter evaporated under a gentle stream of N₂ gas to obtain dry a lipid film. The samples were further dried overnight in a vacuum oven (3608-1ce, Lab-Line Instruments Inc, Melrose Park, Illinois, USA) overnight to remove the residual solvents. The dried lipid film was hydrated with 2 mL HBS (pH 7.4) at 65 °C for approximately 45 min with intermittent vortexing. The lipid dispersion was then sonicated for 5 and 10 min for cholesterol-free and cholesterol-containing formulations respectively, using a probe-sonicator (Soniprep 150, MSE Scientific Instruments, London, UK). During the sonication, cholesterol-based formulations were maintained in ice-cold water while the cholesterol-free formulations were kept above the transition temperature of the dominant lipid (i.e., HEPC). The formulations were centrifuged at 380 g for 2 min to remove any metal remnants produced from the sonicator tip. The prepared lipodisks were stored at 4 °C until further analysis.

2.2.3. Preparation of drug-loaded lipodisks by probe sonication

Lipids in desired molar ratios were weighed directly into the clean glass vials and were then dissolved in chloroform. Based on the required drug-to-lipid ratio, calculated quantities of the stock solutions of doxorubicin or curcumin were pipetted into the lipid mixture. The organic solvents were evaporated first under the gentle stream of N₂ gas and then in a vacuum oven (3608-1ce, Lab-Line Instruments Inc, Melrose Park, Illinois, USA) overnight to remove the residual solvents. The dried lipid film was hydrated with 2 mL HBS (pH 7.4) at 65 °C for approximately 45 min with intermittent vortexing. The lipid dispersion was then sonicated for 5 and 10 min for cholesterol-free and cholesterol-containing formulations respectively, using a probe-sonicator (Soniprep 150, MSE Scientific Instruments, London, UK). During the sonication, cholesterol-based formulations were maintained in ice-cold water while the cholesterol-free formulations were kept above the transition temperature of the dominant lipid (i.e., HEPC). The formulations were centrifuged at 380 g for 2 min to remove any metal remnants produced from the sonicator tip. The prepared lipodisks were stored at 4 °C until further analysis.

2.2.4. Physicochemical characterizations

2.2.4.1. Dynamic light scattering (DLS). The number-weighted average hydrodynamic diameter (Dh) of the lipodisks was measured by frequency power spectrum (FPS) combined with laser-amplified detection using a Nanotract wave (Microtrac MRB GmbH, Germany). The instrument is equipped with a 3 mW solid-state laser diode with a wavelength of 780 nm and backscattered light detection at 180°. The particle diameter and polydispersity index (PDI) were measured after diluting the sample to a final lipid concentration of 1 mM with HBS (viscosity: 0.89 mPa·s; refractive index: 1.33). The diameter obtained from the measurements corresponds to the “equivalent sphere” diameter and is therefore not identical to the actual disk diameter.

2.2.4.2. Cryogenic transmission electron microscopy. Cryo-EM samples were prepared at a controlled temperature (25 °C) and high humidity in a custom-built environmental chamber. A minute volume of the specimen was deposited on a copper grid (300 mesh, Agar Scientific, Essex, UK) coated with a custom-made holey polymer film. After blotting away excess liquid with a filter paper, the sample was rapidly vitrified by plunging the grid into liquid ethane, maintained just above its freezing point. The prepared grid was then placed in a Gatan CT3500 holder and transferred to a Zeiss TEM Libra 120 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany) for examination. Throughout the
transfer and viewing procedures, the sample was consistently main-
tained at a temperature below – 160 °C. The microscope, operating at
80 kV in zero-loss bright-field mode, captured digital images under low-
dose conditions using a BioVision Pro-SM CCD camera from Proscan
elektronische Systeme GmbH (Scheurig, Germany) (Almgren et al.,
2000; Morin Zetterberg et al., 2016).

2.2.4.3. Quantification of lipid contents. The total amount of the lipid in
lipodisk formulations was determined by analyzing the phosphorous
content of the samples similar to that described previously (Paraskova
et al., 2013). Briefly, the calculated amounts of the lipodisk samples
were pipetted in phosphorous-free clean glass vials. The vials were then
placed in a laboratory ashing furnace (CSF 1200, Carbolite Gero Ltd,
Sheffield, UK) at 550 °C for at least 5 h to calcinate the samples. Post
digestion, the vials were allowed to cool down and the obtained dry
ashes were dissolved in 2 mL Milli-Q water. Afterwards, 0.5 mL of a
freshly prepared solution consisting of seven parts reagent A (2.74
mg × mL⁻¹ of K(SbO)C₄H₆₅H₂O, 40 mg × mL⁻¹ of (NH₄)₆MoO₄·4H₂O
and 2.5 M H₂SO₄; 1:3:10) and three parts reagent B (0.1 M C₆H₆O₆) was
added to the vials. The vials were then carefully vortexed and allowed to
settle for at least 15 min. The phosphorous contents were then quantified
at λ_max = 882 nm using a UV–VIS spectrophotometer (HP 8453, Agilent
Technologies, Santa Clara, USA). The standard curve for phosphorous
was constructed using a phosphorous standard solution (KH₂PO₄, 0.65
mM; Sigma-Aldrich, St. Louis, MO, USA). It is important to note that the
cholesterol, which is the phosphorous-free component, was assumed to
contribute in line with the original molar ratios in the formulation when
calculating the final lipid content (Grad et al., 2020).

2.2.4.4. Encapsulation efficiency (EE %). The encapsulation efficiency
(EE %) of the curcumin-containing formulations was determined using a
method based on solvent extraction. Briefly, 1 mL of freshly prepared
lipodisk formulation was centrifuged at 11,500 g for 15 min using a
Biofuge pico centrifuge (Heraeus Instruments, Osterode, Germany) to
separate the disks from the unloaded drug. Post centrifugation, the supernatant (containing drug-loaded lipodisks) and the pellet (containing unloaded drug) were separated. Thereafter, 0.15 mL of supernatant was diluted to 3 mL using DMSO supplemented with 20 % v/v HBS buffer. The amount of curcumin encapsulated was quantified by measuring the absorbance at $\lambda_{\text{max}} = 435$ nm using a UV–VIS spectrophotometer (HP 8453, Agilent Technologies, Santa Clara, USA). The separated pellet was dissolved in 1 mL of DMSO. 0.15 mL of dissolved pellet was diluted to 3 mL with DMSO containing 20 % v/v HBS buffer. The amount of curcumin was then quantified as described previously. DMSO supplemented with 20 % v/v HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) was used as blank. The standard curve for the curcumin was constructed in the same solvent system and the molar extinction coefficient ($\varepsilon$) was determined. Measurements of a saturated solution confirmed that the solubility of curcumin in the HBS buffer was very low, i.e., in the range of 1.0—1.4 $\mu$M. Phosphorus analysis moreover verified that the lipid content in the pellet was negligible. The EE % was then determined using the formula: (Ali et al., 2021)

$$\text{EE} \% = \frac{\text{Amount of drug encapsulated}}{\text{Total drug}} \times 100$$ (1)

The EE % from the doxorubicin-containing formulations was, due to doxorubicin’s comparatively high aqueous solubility, determined using a slightly modified version of the solvent extraction method. Briefly, 1 mL of lipodisk dispersion was centrifuged at 11,500 $g$ for 15 min. The resulting pellet (containing precipitated unloaded drug) was dissolved in 1 mL of 30 mM Triton™ X-100 solution. 0.5 mL of supernatant (containing drug-loaded lipodisks and some dissolved unloaded drug) was added to Amicon® Ultra centrifugal filters (Ultracel®, MWCO 3 K; Merck Millipore, Carrigtwohill, Ireland) and centrifuged again at 16,089 $g$ for 25 min. After the filtrate (containing dissolved unloaded drug) was separated, the filter was inverted into a new Eppendorf tube and centrifuged again at 6093 $g$ for 2 min to get the lipodisk concentrate (containing the drug-loaded lipodisks). 30 $\mu$L of the concentrate was then diluted to 0.5 mL with HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4). Subsequently, 125 $\mu$L of each of the filtrate, lipodisk concentrate, the pellet and the supernatant from the original formulation were diluted separately to 2.5 mL using 30 mM Triton™ X-100 solution. The absorbance was then recorded at $\lambda_{\text{max}} = 480$ nm using the UV–VIS spectrophotometer (HP 8453, Agilent Technologies, Santa Clara, USA). The doxorubicin standard curve was generated in 30 mM Triton™ X-100 solution and the molar extinction coefficient ($\varepsilon$) was then calculated. The same solvent system was used as blank. The EE % for doxorubicin was determined using the following formula while taking into consideration the possibility of potential loss of some of the drug and lipid to the filters:

$$\text{EE} \% = \frac{[\text{Doxorubicin}]_{\text{encapsulated}}/\text{[Lipid]}_{\text{Concentrate}}}{[\text{Doxorubicin}]_{\text{total}}/\text{[Lipid]}_{\text{total}}} \times 100$$ (2)

Here, $[\text{Doxorubicin}]_{\text{total}}$ represents the total amount of doxorubicin present in the formulation (encapsulated + free), whereas $[\text{Doxorubicin}]_{\text{encapsulated}}$ represents the doxorubicin that is encapsulated in the lipodisks. The latter is calculated from what is left in the concentrate fraction, corrected by the free drug found in the filtrate. Since the Amicon® filtration procedure concentrates the lipodisk formulation, Eq [2] uses the [drug]/[lipid] ratio in order to translate it to Eq [1].

3. Results and discussion

3.1. Characterisation of lipodisks prepared by dual centrifugation

3.1.1. Preparations based on HEPC:Chol:DSPE-PEG$_{2000}$

Previous studies show that the morphology, size and size polydispersity of DC-produced liposomes can be tailored and adapted through deliberate adjustments of the conditions used during the...
homogenization process (J. Koehler et al., 2023a; Koehler et al., 2021).
We deemed it plausible that the characteristics of lipodisk preparations in a similar fashion could be modified and optimized by a considerate choice of the experimental conditions. Thus, we began our investigations by exploring how changes in some key parameters affected the size and structure of the lipid self-assemblies found in the formulations.

3.1.1.1. Effect of homogenization time. As a first step, we analysed how changes in the duration of the initial homogenization step affected the morphology and homogeneity of the lipid particles present in the final preparations. To this end, a series of samples with 40 % lipid concentration were first homogenized by DC for different periods of time. The resulting lipodisk gels were then dispersed in buffer, and thereafter analysed by cryo-EM. The images shown in Fig. 2 illustrate the differences observed between the preparations. Samples homogenized for 5 min were dominated by lipodisks but also contained some irregular bilayer patches, as well as a small population of unilamellar liposomes with diameters from about 50 to 150 nm (Fig. 2A). The latter often appeared to encapsulate some lipodisks in their aqueous core. Note-worthy, the lipodisks were rather heterogeneous in size. As judged by the cryo-EM images, which do not reveal the lipodisk PEG-corona, the bilayer part of the disks displayed diameters from about 150 nm down to around 10 nm. Given that the core of a DSPE-PEG2000 micelle can be estimated to be about 6.4 nm (Johnsson et al., 2001), it cannot be excluded that the smallest structures observed represent particles that are more or less exclusively composed of PEG-lipid. Increasing the time for homogenization to 15 min had a clearly noticeable effect on the particle homogeneity. A few liposomes were still detected in the cryo-EM analysis but no irregular bilayer patches were observed. Also, the lipodisks assumed a narrower size distribution and disks with diameters around 10 nm, or above 100 nm, were now only rarely observed (Fig. 2B). Increasing the homogenization time to 30 min did not lead to any visible changes in size or homogeneity of the lipodisks (Fig. 2C). The images suggested, however, a slightly larger population of unilamellar liposomes than what was observed after 15 min homogenization. Further extension of the homogenization time to 60 min leads to some major structural changes in the samples. Thus, in addition to lipodisks, the images revealed a significant number of large liposomes and irregular bilayer patches. The cryo-EM analysis also disclosed the presence of some large, contrast rich, amorphous assemblies (Fig. 2D). The size of these structures was often found to be in the micrometer range.

In order to complement the cryo-EM analysis, the samples described above were also measured by DLS. In case of samples homogenized for 5, 15 and 30 min, the number weighted size distributions displayed a single narrow peak corresponding to particles with a mean hydrodynamic diameter in the range between 25 and 30 nm (Fig. 3A). As expected from the cryo-EM analysis, the DLS measurements returned inconclusive and irreproducible results for the samples that were homogenized for 60 min (results not shown).

The results reported above confirm that cholesterol-containing lipodisks can be efficiently produced by DC. Our findings show, however, that the duration of the homogenization step is of crucial importance for the homogeneity of the preparations. Homogenization times that are too short risk leading to insufficient mixing of the lipid components and, as shown by the cryo-EM analysis, result in lipodisks with a broad size distribution. Excessively long homogenization times may, on the other hand, result in samples that, apart from lipodisks, contain significant amounts of non-desired structures, such as liposomes and the large amorphous assemblies shown in the inset of Fig. 2D. It can be speculated that the appearance of these unwanted structures is linked to a partial lipid degradation taking place during the prolonged homogenization. The latter may lead to compositional changes, including loss of PEG-lipid, that, together with the high energy input, help explain the shift from lipodisks towards liposomes and large assemblies of, as yet, uncharacterized components. It can be concluded that in order to minimize the risk of lipid degradation, while at the same time ensuring sufficient lipid mixing, a homogenization time in the interval between 15 and 30 min is optimal for the production of lipodisks based on HEPC and cholesterol.

3.1.1.2. Influence of lipid concentration. We next set out to explore the influence of lipid concentration. Based on the results reported in the previous section the time for homogenization was kept constant at 15 min, while the lipid concentration was varied between 10 and 60 %. Cryo-EM analyses confirmed that lipodisks were the dominating structures in all cases, but also disclosed some important lipid concentration-dependent differences between the samples. Thus, some unusually large
Fig. 7. Cryo-EM images of HEPC: DSPE-PEG\textsubscript{2000} lipodisks prepared by DC for 15 min with a lipid concentration corresponding to: (A) 10 % (B) 20 % (C) 50 % and (D) 60 %. Arrows indicate irregular lamellar sheet (s) and discoidal structures (d). The lipid gels were dispersed in HBS (pH 7.4) before visualization. The scale bar represents 100 nm.
liposomes, as well as aggregates of apparently non-dispersed lipid material, were observed in samples prepared with 10 % lipid (Fig. 4A). The presence of these structures is corroborated by the considerably larger mean hydrodynamic diameter and wider particle size distribution returned by DLS for the 10 %, as compared to the 40 %, sample (Fig. 3B). Preparations based on the use of 20 % lipid contained apart from lipodisks and some small liposomes, also a population of irregular bilayer patches/fragments (see inset Fig. 4B) that were not observed in samples prepared with 40 % lipid (Fig. 2B). In case of samples prepared with 50 % lipid, the cryo-EM analyses revealed lipodisks with a broad size distribution. Thus, similar to what was observed with 40 % lipid, lipodisks with sizes in the range from 20 to 80 nm were the dominating structures, but disks with diameters around 200 nm were also frequently observed in the micrographs (Fig. 4C). Also noteworthy, in contrast to the observations made for the 40 % samples, no liposomes were detected in the preparations based on 50 % lipid. A comparison of the DLS data retrieved for the corresponding samples indicates that the use of the higher lipid concentration indeed results in particles with somewhat larger mean hydrodynamic diameter and wider size distribution (Fig. 3B). In case of samples prepared with 60 % lipid concentration the cryo-EM images revealed a few small lipodisks but most of the material was found in the form of large discoidal or irregular bilayer structures (Fig. 4D). In line with this, DLS suggested an increase in particle mean hydrodynamic diameter from 30 to 95 nm as the lipid concentration was changed from 50 to 60 % (Fig. 3B).

Based on the results of the cryo-EM and DLS investigations it can be concluded that the most homogeneous preparations in terms of particle size and structure were obtained when the lipid concentration was set to 40 % during the homogenization step. The size and structural heterogeneity observed for preparations based on 20 and, in particular, 10 % lipid is likely a consequence of the sample viscosity being too low to ensure efficient homogenization. The poor result noted in case of preparations based on 50 and, especially, 60 % lipid concentration is, on the other hand, most likely due to a shortage of water. As previously discussed in connection with the use of DC for liposome preparation (J. Koehler et al., 2023a), there exists a maximum lipid concentration above which there is simply not enough water available to hydrate the lipids and allow them to assemble into the desired nanostructures. It is in this context worth noting that while lipodisks, in contrast to liposomes, do not have a water-filled core, the disks contain a high content (20 mol%) of PEG-lipids with high water binding capacity. More specifically, each PEG

3.1.2. Preparations based on HEPC:DSPE-PEG2000

In the same manner as done for the cholesterol containing lipodisks,
we performed systematic studies to optimize the conditions for the production of cholesterol-free lipodisks consisting of HEPC and DSPE-PEG<sub>2000</sub> alone.

The cryo-EM pictures displayed in Fig. 5 show the structures observed in samples with 40 % lipid concentration homogenized for 5, 15, and 30 min. Irrelevant of the homogenization time, all samples contained lipodisks. Use of the shortest homogenization time resulted, however, in samples that in addition to lipodisks also displayed some larger, irregular bilayer structures (Fig. 5A).

The analyses revealed no major differences between samples homogenized for 15 and 30 min (Fig. 5B and 5C). In both cases, the micrographs suggested a homogenous population of lipodisks with diameters between 20 and 40 nm (excluding the PEG corona). The observations of similar lipodisk sizes and size distributions were supported by DLS measurements (Fig. 6A). Due to the previously mentioned potential risk of lipid degradation, homogenization times longer than 30 min were not investigated. Noteworthy, in contrast to what was observed in the presence of cholesterol (Fig. 2) all investigated samples appeared completely devoid of liposomes. DLS measurements moreover confirmed a generally smaller mean particle size in the absence, as compared to the presence, of cholesterol (compare Fig. 6A and 3A).

In order to complement the above-described investigations of the cholesterol-free preparations, we explored the effects brought about by varying the lipid concentration used in the homogenization step. For these experiments, the duration of the homogenization process was set to 15 min. As revealed by the micrographs shown in Fig. 7, preparations free of liposomes, and completely dominated by lipodisk with sizes in the range of 30–60 nm, were obtained when the lipid concentration was either decreased to 10 and 20 % (Fig. 7A and 7B, respectively) or increased to 50 % (Fig. 7C). The particle size and morphology remained essentially unchanged also when the lipid concentration was increased to 60 % (Fig. 7D). Thus, albeit the micrographs occasionally revealed the presence of some irregular or discoidal bilayer patches, small, homogenous lipodisks were the clearly dominating structures in all the investigated samples. In line with this, DLS measurements returned data suggesting only a modest increase in mean particle size with increasing lipid concentrations (Fig. 6B).

It may appear somewhat surprising that the use of lipid concentrations as high as 60 % resulted in samples dominated by small, homogeneous lipodisks. Similar to the case with the cholesterol containing preparations, simple calculations confirm that the amount of water present in the 60 % samples during the homogenization process is below what is required for full hydration of the lipid and PEG-lipid components. It is therefore unlikely that a gel consisting of small, well-formed lipodisks can be produced at this lipid concentration. It is in this context worth noting, however, that small lipodisks of uniform size have been shown to form upon simple hydration of cholesterol-free lipid films composed of PEG-lipids and long-chained saturated phosphatidyl choline lipids (Sandström et al., 2008). This indicates that the lipodisks found in the cholesterol-free samples may represent equilibrium structures. Hence, even if the low water content prevents conversion of the lipid material into small, well defined lipodisks during the homogenization step, it is reasonable to expect that lipodisks of small and uniform size will form upon dilution of the samples. In case of samples supplemented with cholesterol, the situation is obviously different and simple hydration is not enough to transform the lipid material in the gels into small lipodisks.

### 3.2. Drug-loaded lipodisks

Having mapped and optimized the experimental conditions for lipodisk production, we continued by exploring the utility of DC for the formulation of two different drugs, curcumin and doxorubicin, in the disks. Both drugs have previously been shown suitable for incorporation in lipodisks (Ahlgren et al., 2017a; Feng et al., 2019; Zhang et al., 2014).

#### 3.2.1. Disks loaded with curcumin

For the experiments involving curcumin, we followed a protocol in which the lipids (40 %) and curcumin were co-homogenized by DC for 15 min, whereafter the lipodisk gels were diluted with buffer and dispersed by low-speed DC. The resulting lipodisk dispersions were investigated by cryo-EM, measured by DLS, and analysed in terms of lipid and curcumin content (see section 2.2.4 for details).

The choice of lipid composition was found to have an effect on both the sample homogeneity and the lipodisk encapsulation efficiency (EE). As noted previously for the drug-free preparations, formulations devoid of cholesterol were completely free of liposomes, and contained lipodisks of more uniform size than did the cholesterol-supplemented formulations (Fig. 8A and 8B). The presence of cholesterol did on the other hand lead to somewhat higher EE (Table 1). Thus, for samples prepared with an initial drug-to-lipid molar fraction of 0.2, the encapsulation efficiency increased from 21 to 31 % upon inclusion of cholesterol in the preparations. A further increase of the EE to 66 % was achieved by decreasing the initial drug-to-lipid fraction in the cholesterol-supplemented preparations to 0.06 (Table 1). This improvement suggests that the presence of high quantities of curcumin during the homogenization step either decreases the disks’ capacity to accommodate the drug, or, more likely, leads to a lower proportion of curcumin being available for incorporation into the disks. An explanation for the latter could be that the use of high quantities of curcumin leads to less efficient, and perhaps incomplete, disintegration of the crystalline drug material. This hypothesis is supported by the fact that DC-prepared formulations analysed directly after dilution of the gels, i.e. without prior separation of unencapsulated

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**Table 1**

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>Method</th>
<th>Lipid&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Lipid&lt;sup&gt;b&lt;/sup&gt; (mM)</th>
<th>Initial D/L&lt;sup&gt;c&lt;/sup&gt;</th>
<th>D&lt;sub&gt;h&lt;/sub&gt; (nm)</th>
<th>PDI&lt;sup&gt;d&lt;/sup&gt;</th>
<th>EE (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; DC</td>
<td>40</td>
<td>9.8 ± 0.2</td>
<td>0.20 ± 0.01</td>
<td>21 ± 5</td>
<td>0.05</td>
<td>31 ± 2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; DC</td>
<td>40</td>
<td>7.4 ± 0.3</td>
<td>0.22 ± 0.01</td>
<td>21 ± 5</td>
<td>0.04</td>
<td>21 ± 4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; DC</td>
<td>40</td>
<td>9.8 ± 0.8</td>
<td>0.06 ± 0.00</td>
<td>24 ± 5</td>
<td>0.05</td>
<td>66 ± 2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; DC</td>
<td>40</td>
<td>9.6 ± 0.5</td>
<td>0.06 ± 0.01</td>
<td>25 ± 5</td>
<td>0.04</td>
<td>72 ± 2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; DC</td>
<td>40</td>
<td>9.8 ± 0.3</td>
<td>0.06 ± 0.00</td>
<td>26 ± 6</td>
<td>0.06</td>
<td>55 ± 2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; DC</td>
<td>40</td>
<td>9.8 ± 1.3</td>
<td>0.06 ± 0.00</td>
<td>25 ± 6</td>
<td>0.05</td>
<td>57 ± 2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; Sonication</td>
<td>–</td>
<td>9.5 ± 0.2</td>
<td>0.20 ± 0.01</td>
<td>34 ± 9</td>
<td>0.06</td>
<td>16 ± 4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; Sonication</td>
<td>–</td>
<td>3.9 ± 0.0</td>
<td>0.18 ± 0.01</td>
<td>39 ± 11</td>
<td>0.08</td>
<td>32 ± 0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HEPC:Chol:DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt; Sonication</td>
<td>–</td>
<td>1.0 ± 0.3</td>
<td>0.19 ± 0.00</td>
<td>32 ± 8</td>
<td>0.06</td>
<td>70 ± 2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Lipid concentration used for DC homogenization

<sup>b</sup> Lipid concentration in the final formulation (mean ± SD)

<sup>c</sup> Initial drug-to-lipid molar fraction (mean ± SD)

<sup>d</sup> Hydrodynamic diameter (mean ± SD) determined by DLS

<sup>e</sup> Polydispersity index calculated from hydrodynamic diameter as (SD/mean)<sup>2</sup>

<sup>f</sup> Formulation homogenized for 15 min

<sup>g</sup> Formulation homogenized for 30 min
drug by conventional centrifugation, occasionally displayed structures that likely represent crystalline drug (inset of Fig. 8B).

In an attempt to further increase the EE, we co-dissolved the lipid and curcumin components in a mixture of chloroform and ethanol, and subsequently formed a dry “lipid film” before proceeding as before with the DC homogenization. As shown in Table 1, for preparations with an initial drug-to-lipid fraction of 0.06 the EE increased to 72 %, as a result of the pre-mixing in organic solvent. Increasing the homogenization time from 15 to 30 min did, on the other hand, have the general effect of decreasing the EE. Thus, the EE dropped to 56 and 55 % for samples prepared with and without pre-mixing in organic solvent, respectively.

For comparison, curcumin was also formulated in cholesterol-supplemented lipodisks by use of a preparation protocol based on probe sonication (see Materials and Methods for details). When employing this method, the lipid components were first co-dissolved with curcumin in the organic solvent. Probe sonication of samples with a total lipid concentration of 9.5 mM, and initial drug-to-lipid molar fraction corresponding to 0.2, resulted in an EE of 16 % (Table 1). The EE was thus lower than what was noted for formulations with the same initial drug-to-lipid molar fraction prepared by DC. Upon decreasing the lipid concentration to 3.9 mM the EE increased to 32 %, i.e., close to what was obtained with DC. A further increase of the EE to 70 % was achieved by lowering the lipid concentration to 1.0 mM. These findings indicate that the lipodisks have a high loading capacity for curcumin (maximum drug-to-lipid molar fraction in disks $\geq 0.13$), but that the drug is less efficiently encapsulated in the disks when performing the sonication with a higher, as compared to lower, drug and lipid content in the samples. As judged by Cryo-TEM and DLS, neither the lipodisk size homogeneity nor the proportion of liposomes present in the preparations varied notably depending on the lipid concentration. Thus, in all cases the lipodisks displayed a similar and rather broad size distribution, and the proportion of liposomes was comparable (Table 1, Fig. 8C, Fig. S1). It hence appears that the lower EE noted for formulations prepared with higher lipid concentrations can be traced back to the higher initial drug content of the samples.

From the data collected for the cholesterol-supplemented curcumin formulations it can be concluded that preparation based on sonication enabled a drug-to-lipid fraction in the disks that was about two times higher than what could be achieved using DC (0.13 as compared to 0.06). The use of DC enabled the production of formulations that were clearly superior to those produced by sonication in terms of particle size and structural homogeneity. It should in this context be mentioned that the use of lipid concentrations lower than 40 % was found to be suboptimal for the production of cholesterol-containing lipodisks by means of DC (see Section 3.1.1.2).

3.2.2. Disks loaded with doxorubicin

A similar protocol as used for curcumin was employed to prepare doxorubicin-loaded lipodisks by DC. Thus, doxorubicin and lipids (40 %) in varying molar fractions were co-homogenized by DC for 15 min and the resulting gels were thereafter diluted and dispersed in buffer. Also, since initial cryo-EM analyses indicated the presence of some aggregated drug/lipid material in formulations based on HEPC:Chol:DSPE-PEG$_{2000}$ (Fig. S2A), we limited the investigations to formulations based on HEPC:DSPE-PEG$_{2000}$, for which no observations of such aggregated material were made.

Cryo-EM and DLS investigations confirmed that the doxorubicin formulations prepared by use of HEPC:DSPE-PEG$_{2000}$ were free of liposomes and contained lipodisks of small and uniform size (Fig. 9, Table 2). As noted for the curcumin-containing formulations, the initial drug-to-lipid molar fraction had a significant effect on the EE. Hence, encapsulation efficiencies corresponding to 64 and 77 % were obtained for formulations with initial drug-to-lipid molar fractions of 0.13 and 0.06, respectively (Table 2). Thus, also in case of doxorubicin it appears that the proportion of drug available for incorporation in the disks tends to decrease when the DC homogenization is performed in the presence of
high drug quantities. In order to explore this matter further, we investigated the effects brought about by reducing the lipid concentration used in the homogenization step from 40 to 10 %. As shown in Table 2, this modification of the protocol resulted in an EE of 84 % for formulations prepared with an initial drug-to-lipid molar ratio of 0.15. Since this EE translates to a drug-to-lipid fraction in the disks of 0.13, it is highly unlikely that the inferior EE obtained when using 40 % lipid, and an initial drug-to-lipid fraction of 0.13, is due to saturation of the disks with doxorubicin. The reason for the negative correlation between EE and lipid concentration must thus be sought elsewhere. As discussed in connection with the curcumin formulations, a likely explanation for the observed trend is that ineffective, or incomplete, disintegration of the crystalline drug material during the homogenization step leads to suboptimal EE.

For comparative reasons, we also prepared some doxorubicin-containing formulations by means of the method based on probe sonication. Cryo-TEM and DLS analysis verified that the formulations were completely dominated by small homogeneous lipodisks (Fig. S2B, Table 2). As shown in Table 2, an EE corresponding to 99 % was obtained when using a lipid concentration of 3.9 mM and an initial drug-to-lipid molar fraction corresponding to 0.06. This can be compared to the EE of 77 % determined for formulations prepared by DC using the same initial drug-to-lipid molar fraction and 40 % lipid concentration. The superior EE obtained when using sonication again suggests that the high drug quantities employed in the DC preparation resulted in disks with less than maximal doxorubicin content.

Some important conclusions can be drawn from the results presented in section 3.2. First, lipodisks are capable of encapsulating substantial amounts of both curcumin and doxorubicin. The data collected in Tables 1 and 2 indicate a maximum drug-to-lipid molar fraction in the disks corresponding to at least 0.13 in case of both drugs. Second, DC can be used as a straightforward means to produce lipodisk-based formulations of the two drugs. Our findings indicate, however, that a conscious choice of lipid composition and drug-to-lipid molar ratio in the starting material is important to ensure high encapsulation efficiencies. An important observation is that the use of high lipid concentrations during the DC homogenization may result in limited possibilities to fully utilize the drug loading capacity of the lipodisks. In case of formulations based on the use of cholesterol-supplemented lipodisks, this limitation appears difficult to circumvent. The situation is different, however, when cholesterol is omitted from the formulations. In this case, the DC homogenization can be performed at low enough lipid concentration to ensure that both the EE and the drug-to-lipid molar fraction in the lipodisks is kept high.

4. Concluding remarks

The present study represents the first report on the successful use of dual centrifugation to produce small and homogeneous PEG-stabilized lipodisks. The preparation of lipodisks by DC, which in principle can be done under aseptic conditions, turned out to be fast and straightforward. As shown for curcumin and doxorubicin, the technique allowed for the production of lipdisk-based drug formulations with high encapsulation efficiencies. The results and insights gained from this initial systematic study opens up for the screening for optimal conditions for efficient and robust entrapment of curcumin, doxorubicin, and other drugs in small and uniform lipodisks. Such screening can include variations in lipodisk lipid composition and initial drug-to-lipid ratios with the purpose to identify conditions that lead to a satisfactory balance between the loading efficiency and the amount of drug encapsulated in the lipodisks. Investigations of the impact of the aqueous phase may also be warranted as part of the optimization procedure. Here, different pH-values, buffer capacities, and buffers can be explored and compared in terms of their effect on the drug-lipodisk interactions. Also, the effect of the addition of other ions or substances can be tested in the search for conditions leading to an optimal lipodisk formulation. As one example, potential issues connected to recrystallization of the drug might be counteracted by the addition of alcohol. The presence of alcohol may, on the other hand, alter the structure and micro-mechanical properties of the bilayer and, as a consequence, have an undesirable effect on the drug release behavior or drug loading capacity of the disks. In conclusion, DC enables fast and simple preparation of drug-loaded lipodisks and has the potential to become a valuable tool in the continued development of lipodisks as versatile platforms for efficient and safe drug delivery.

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CRediT authorship contribution statement

Sajid Ali: Investigation, Methodology, Writing – original draft, Writing – review & editing. Jonas K. Koehler: Formal analysis, Investigation, Methodology, Writing – review & editing. Luis Silva: Data curation, Investigation, Methodology, Writing – review & editing. Lars Gedda: Data curation, Investigation, Validation, Writing – review & editing. Ulrich Massing: Methodology, Resources, Writing – review & editing. Katarina Edwards: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: S. A., J.K., L.S., L.G. and K.E. declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. U.M. is also an employee of Andreas Hettich GmbH & Co. KG. The company had no role in the design.
of the study, in the collection, analysis or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

Data availability
Data will be made available on request.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.123894.

References


