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Compositional inhomogeneity of drug delivery liposomes
quantified at the single liposome level

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Abstract
Liposomes are the most used drug delivery vehicle and their therapeutic function is closely linked to their lipid composition. Since most liposome characterization is done using bulk techniques, providing only ensemble averages, the lipid composition of all liposomes within the same formulation are typically assumed to be identical. Here we image individual liposomes using confocal microscopy to quantify that liposomal drug delivery formulations, including multiple component mixtures mimicking Doxil, display more than 10-fold variation in their relative lipid composition. Since liposome function is tightly regulated by the physicochemical properties bestowed by the lipid composition, such significant variations could render only a fraction of liposomes therapeutically active. Additionally, we quantified how this degree of compositional inhomogeneity was modulated by liposome preparation method, the saturation state of the membrane lipid, and whether anti-fouling
polyethylene glycol (PEG) conjugated lipids were added to the initial lipid mix or inserted after liposome formation. We believe the insights into the factors governing the degree of inhomogeneity offers the possibility for producing more uniform liposomal drug delivery systems, potentially increasing their therapeutic efficacy.

**Keywords**

Biomaterial composition  
Liposomal drug delivery systems  
Single liposome measurements  
Quantitative fluorescence microscopy  
Compositional inhomogeneity

1. **Introduction**

Liposomes are the most common nanoscale carrier used for medical applications [1-3]. Characterizing the lipid composition of drug delivery liposomes is important, since their function and therapeutic efficacy are directly linked to the physicochemical properties bestowed by the lipids making up the liposome [4, 5]. However, liposome characterization is typically done using bulk assays giving only an ensemble average read out, leading people to assume that all liposomes have identical lipid compositions. This concept has been challenged in recent years, with reports showing that liposomes in the same batch can display considerable inhomogeneities in their physicochemical properties [6-9]. These earlier reports have been based on simple model membrane systems, with an attempt to understand the basic principles underlying the phenomena, including how such compositional inhomogeneities would influence protein binding experiments. Consequently, little is known about the compositional inhomogeneity of applied lipid membrane systems, in particular liposomal drug delivery vehicles. Also, how parameters such as their membrane lipid composition...
and preparation method affect the compositional inhomogeneity of clinically relevant drug delivery liposomes remains largely unexplored.

A compositional inhomogeneity that is too high could lead to a larger fraction of the liposome population harboring properties that renders them suboptimal as drug delivery vehicles [10, 11]. This could be related to specific liposome compositions that might compromise e.g. liposome stability, targeting ability or circulation kinetics. Thus knowing how to reduce compositional inhomogeneity could potentially lead to more precise and controllable liposomal drug delivery systems, with increased therapeutic efficacy [12]. Here we have used a microscopy based single liposome assay to demonstrate that clinically relevant drug delivery liposomes display significant compositional inhomogeneity between individual liposomes. We studied how this inhomogeneity was affected by a number of liposomal structural features such as the saturation state of the membrane lipid and whether PEGylation was performed by adding PEG-lipids to the initial lipid mix or inserted after liposome formation. Also, we revealed that depending on the lipid composition, the liposome preparation method can significantly affect the compositional inhomogeneity. These findings highlight the importance of implementing the characterization of liposomal compositional inhomogeneity as a tool in the liposomal drug discovery process.

2. Materials & Methods

2.1 Materials

2-methyl-2-propanol (tert-Butanol) acquired from Honeywell Riedel-de Haën. Bovine Serum Albumin (BSA), Biotin labelled Bovine Serum Albumin (BSA-Biotin), Streptavidin (Strep), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonicacid, N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES) acquired from Sigma-Aldrich (Søborg, Denmark). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), L-α-phosphatidylcholine, hydrogenated Soy (HSPC), Cholesterol (Chol),
1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotin(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-Biotin), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2k), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DOPE-PEG2k), 23-(dipyrrometheneboron difluoride)-24-norcholesterol (Chol-TF) were all acquired from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA).

1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-Atto488 (DOPE-Atto488) and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-Atto655 (DOPE-Atto655) was acquired from Atto-Tec (Siegen, Germany).

2.2 Liposome and PEG-lipid preparations

We prepared six liposome formulations, details on specific lipid compositions and biophysical characterization can be found in Supplementary Fig. 1. We employed two liposome preparation techniques extensively used in pre-clinical development based on either lipid film rehydration [7] (LR) or lipid freeze-drying [13] (FD), both described in brief below.

For the liposomes prepared using the LR technique, lipids dissolved in chloroform were thoroughly mixed in a glass vial at the molar ratios described in Supplementary Fig. 1a. The solution was dried under nitrogen flow and incubated in vacuum for 4 hours.

For liposome formation using the FD method, lipids in powder forms were dissolved in tert-butanol:MQ water 9:1, mixed to the desired formulation ratios in glass vials and lyophilized overnight.

For both the LR and FD preparation methods, the dry lipids were re-hydrated in buffer heated to 65 °C made from 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 % glucose, pH 7.4 (HEPES buffer) to a concentration of 1 mM total lipid and put under 65 °C heating for 1 hour.
The liposomes were then subjected to ten freeze-thaw cycles to minimize multilamellarity by immersion in liquid nitrogen followed by thawing in a water bath. The size of the liposomes was controlled by extruding 21 times through a 100 nm Whatman filter (GE Healthcare) using an Avanti mini-extruder (Avanti Polar Lipids) on a heating block at 65 °C. Liposomes were transferred to glass vials, flash-frozen in liquid nitrogen and stored at -21 °C.

For PEGylated drug delivery liposomes, the incorporation of more than 0.5 mol% PEGylated lipid has previously been shown to greatly diminish the multilamellarity of liposome samples [14], which is needed to accurately assign the degree of inhomogeneity (DI) using the integrated intensity of the liposome. Additionally, the freeze-thawing step employed here is widely used and proven to effectively reduce multilamellarity [15]. We have previously confirmed, using electron microscopy imaging, that freeze-thawing can make the multilamellarity of the liposome preparations negligible for both the LR [16-18] and FD [19] preparation methods employed in this work. We have also shown that variation of membrane curvature within the ensemble does not skew the average liposome composition [7, 20, 21], and that surface immobilization does not perturb the spherical shape of the individual liposomes [22]. Additionally, we have previously shown that internal quenching of the fluorophores is not significant for the concentrations used in our experiments [16] and that the exact fluorophore pair concentration ratios does not affect the quantified DI [7].

For all liposomes, the total lipid concentration of the liposome stocks was determined by measuring the phosphorus concentration using inductively coupled plasma mass spectrometry (ICP-MS). Samples were diluted 200 times in an ICP-MS diluent (2% HCl, 10 ppb Ga) to fall within a set of standard samples from 25-100 ppb phosphorus, and phosphorus content measured on an iCAP™Q from Thermo Fisher Scientific (Waltham, MA, US).

Hydrodynamic diameter and polydispersity (PDI) of the liposomes were measured by dynamic light scattering using a ZetaSizer Nano ZS from Malvern Instruments (Malvern, Worcestershire, UK).
Liposomes were diluted to about 50 µM total lipids in a 10 mM HEPES, 139 mM NaCl buffer, and size measured as the average from 3 runs of 15 cycles (Supplementary Fig. 1b).

For the liposome systems without PEG-lipids in the initial lipid mixture we performed a PEG-lipid post-insertion as previously described [23]. We prepared a 1 nmol solution of either DSPE-PEG2k or DOPE-PEG2k by dissolving it in tert-butanol:MQ water 9:1, before lyophilizing overnight. Next, based on the lipid concentration from the ICP-MS measurements, we diluted the liposome samples and added a total volume of the liposome solution that would give a theoretical PEG surface density of 5 mol%, while keeping the concentration of the PEG-lipid around 2 µM and thus below the critical micelle concentrations [24, 25].

2.3 Single liposome assay
We used the single liposome assay as previously described [7, 16, 17, 26-28]. In brief, liposomes were labelled with 0.5 mol% of each member of pairs of reporter dyes (DOPE-Atto488 and DOPE-Atto655 or Chol-TF and DOPE-Atto655) and immobilized on a passivated glass surface (Fig. 1a).

For all liposomes we extracted the integrated intensity for each channel allowing us to calculate an intensity ratio value for each individual liposome. We then plotted a normalized intensity ratio histogram for each liposome system, which we fitted with a gaussian function. To quantify the variation in composition between the individual liposomes in the preparation we calculated the degree of inhomogeneity (DI) as the standard deviation (σ) divided by the mean (μ) from the gaussian fit:

\[ \text{DI} = \frac{\sigma}{\mu} \]

All intensity ratio histograms were produced from at least 500 and in general ~2500 single liposomes. This large number of data points ensured good statistics.

We emphasize that the DI quantification relies on the assumption that the incorporation of fluorophore-lipids are not systematically biased by errors such as fluorophore-fluorophore or...
fluorophore-membrane interactions. Additionally, it is important to note that the fluorophore pairs used to evaluate the DI are not a direct benchmark of the HSPC or POPC lipids, but serves as a generic tool for evaluating compositional inhomogeneity. Therefore, we also compared fluorophore reporter pairs believed to partition into the same membrane phase-state (DOPE:DOPE) and reporter pairs partitioning into different phase-states (DOPE:Cholesterol), if such occurs. Then we use identical fluorophore pairs in various membrane systems (HSPC vs POPC), for different PEGylation strategies (Premixed vs Post insertion) and for different preparation methods (Lipid rehydration vs Freeze-Drying) to be able to directly compare how these different and highly important aspects of liposomal drug delivery design and formulation affects compositional inhomogeneity.

2.4 Equipment and settings

For imaging liposomes we used a Leica TCS SP5 inverted confocal microscope and an oil immersion objective HCX PL APO CS 100x (Numerical Aperture 1.4). Detection of the TopFluor and Atto488 labels was performed at 495–580 nm (exc. 488 nm); detection of Atto655 labelled was performed at 640–750 nm (exc. 633 nm) using photomultipliers. In all cases, sequential imaging was used to avoid cross excitation. Images had a resolution of 2048 × 2048 pixels, with a pixel size of 25.2 nm and a bit depth of 10. Image analysis and data treatment were performed using custom-made routines in Igor Pro (Wavemetrics) and Fiji (ImageJ).

2.5 Statistical testing

We used the average DI values to compare the compositional inhomogeneity between the different liposome systems. All measurements were performed using between two and five independent liposome preparations, with DI values reported as the average ± standard error of the mean (SEM) of
N independent experiments (See Supplementary Table 1 for summary of all average DI values and information on N for individual systems). We used one-sided Student’s t-testing to evaluate the significance of the difference in the quantified DI values and drew comparisons among various liposome systems. In Supplementary Table 2 we assigned significance levels based on the premise: p ≤ 0.05 (*), p ≤ 0.01 (**) and p ≤ 0.001 (***) for all comparisons made in the text.

3. Results
3.1 Single liposome assay for studying compositional inhomogeneity of drug delivery liposomes
To study the compositional inhomogeneity of drug delivery liposomes we used a previously described single liposome assay [7, 16, 18, 28]. In brief, we labelled liposomes with pairs of various lipid fluorophores and immobilized single liposomes on a passivated glass surface (Fig. 1a). Using confocal microscopy we imaged hundreds of individual liposomes in parallel, in a high-throughput manner. Sequentially we imaged the two fluorescent channels before the integrated intensity of each channel was extracted for each individual liposome (Fig. 1b). The ratio of the integrated intensities allowed us to assign the molar ratio of the lipid fluorophores for each individual liposome. Assuming that all individual liposomes display an equal molar ratio of the lipid fluorophores, we would have detected a constant intensity ratio value, as a liposome with a high DOPE-Atto488 intensity should have a corresponding high DOPE-Atto655 signal. However as can be seen in the intensity surface plots in figure 1b, we found large variations in the intensity ratios between liposomes. To evaluate the variation in intensity ratios between individual liposomes we plotted an intensity ratio histogram (Fig. 2a). The large spread in the molar ratios of the reporter lipids was evident as the normalized intensity ratio values spanned more than an order of magnitude (from ~0.2 to 2). To quantify the compositional inhomogeneity we fitted the histogram with a gaussian function allowing us to calculate the DI value as the standard deviation divided with the mean of the fit [7, 29]. This value
represents the spread in the distribution and the higher the DI the more compositional inhomogeneity is displayed by the liposomal system. We will use the DI value to compare the compositional inhomogeneity between different liposomal drug delivery systems.

Figure 1 | Single liposome assay for studying the compositional inhomogeneity of drug delivery liposomes.

a Liposomes labelled with fluorescent pairs (DOPE-Atto488 and DOPE-Atto655) was immobilized on a Bovine Serum Albumin (BSA) passivated glass surface using biotin-streptavidin coupling and imaged sequentially using fluorescence confocal microscopy. b Micrographs of individual liposomes on a surface, the DOPE-Atto655 channel left and the DOPE-Atto488 channel right and zoom with surface intensity plots illustrating the significantly different ratio of labelled lipid components. Scale bars are 4 µm.

3.2 Lipid saturation state affects the compositional inhomogeneity of drug delivery liposomes
Lipid phase-state is a critical modulator of liposomal membrane permeability and circulation kinetics [30, 31], thus systems made from both saturated and unsaturated lipids are in clinical use [32]. Here we first studied how lipid saturation state affected the DI by preparing two liposome systems based on either HSPC or POPC lipids, representing a fully saturated or an unsaturated system respectively (see Experimental section and Supplementary Information for details on lipid compositions). We prepared HSPC and POPC liposomes labelled with a minute amount (0.5 mol%) of the lipid fluorophore pair DOPE-Atto488 and DOPE-Atto655 using the freeze-drying (FD) technique. Using the single liposome assay we quantified a DI value of 0.44 ± 0.02 for HSPC (FD) and a DI value of 0.24 ± 0.02 for POPC (FD) (Fig. 2b and Supplementary Table 1). To assess if these DI values corresponded to a genuine compositional inhomogeneity we quantified the experimental uncertainty on the DI value by performing a previously described control experiment [7, 8] where identical liposomes were imaged before and after refocusing, hereby finding DI uncertainty of 0.09 ± 0.01 (Supplementary Fig. 2). Thus, the DI values quantified for the HSPC and POPC systems are more than 2.5 fold higher than the DI uncertainty, suggesting that both liposome systems have significant differences in the lipid compositions between individual liposomes.
Figure 2 | Quantifying how lipid membrane composition and liposome preparation method affect compositional inhomogeneity.

a The variation in liposome intensity ratio values is quantified by fitting the normalized intensity ratio histogram with a gaussian function and calculating the DI value as the standard deviation, $\sigma$ (green) divided by the mean, $\mu$ (purple). b Quantified average DI values for HSPC (red) and POPC (grey) membrane systems prepared either by the FD or LR method using the DOPE-Atto488 and DOPE-Atto655 fluorescent lipid pair. c Gaussian functions depicting the intensity ratio distributions for the quantified average DI values for the HSPC (FD) (red) and POPC (FD) (grey) systems, illustrating how 68% of the POPC (FD) liposomes will differ by less than 24% from the mean molar ratio of the ensemble (shaded grey), whereas more than 58% of the HSPC (FD) liposome population will display a molar ratio that differs by more than 24% from the mean (red shaded area).

The DI value quantified for the HSPC (FD) systems was significantly higher than the DI value found for the POPC (FD) system, constituting a 83% increase (Supplementary Table 1). To illustrate the functional consequence of these variations in DI value we plotted a gaussian function with the
standard deviation determined as the DI for the HSPC (FD) and POPC (FD) systems (Fig. 2c). Employing the 68-95-97.7 rule, which describes the percentage of the population that falls within one, two, or three standard deviations around the mean value, we can quantify that 68 % of the POPC (FD) liposomes will differ by less than 24 % from the mean molar ratio of the ensemble (shaded grey area in figure 2c). In comparison, the majority of the HSPC (FD) liposome population, more than 58 % will display a molar ratio that differs by more than 24 % from the mean (red shaded area in figure 2c). Since liposome composition and function are tightly linked [4, 5], liposomes could potentially be rendered pharmaceutical inactive if they display a lipid composition outside a certain composition range. A consequence of the increased compositional variability might be a difference in the relative amount of liposomes that are pharmaceutically active for HSPC and POPC membrane systems. We did not observe a liposome size dependency of the DI for either the HSPC or POPC systems (Supplementary Fig. 3). Overall our data demonstrates that the membrane lipid saturation state can greatly affect the compositional inhomogeneity of liposomes, a finding that should be considered when preparing and comparing liposomal drug delivery systems with different compositions.

3.3 The preparation method affects the compositional inhomogeneity of drug delivery liposomes

Depending on the liposome composition a number of different preparation methods has been used to form drug delivery liposomes [33]. To study how two of the most used pre-clinical methods affected compositional inhomogeneity we compared HSPC and POPC liposomes formulated either by the FD method or a lipid film rehydration (LR) based method (See Experimentals for detailed description on preparation method). We quantified DI values of 0.24 ± 0.02 and 0.23 ± 0.01 for POPC (FD) and POPC (LR) respectively, demonstrating no significant preparation method dependent difference in the compositional inhomogeneity for the POPC system (Fig. 2b, grey and Supplementary Table 1 and
2). However for the HSPC system we quantified DI values of 0.44 ± 0.02 and 0.29 ± 0.02 for HSPC (FD) and HSPC (LR) respectively, representing a significant 52 % higher DI value for the FD as compared to LR preparation method. This demonstrates that for clinically relevant liposomal drug delivery systems, the choice of preparation method can directly affect liposome compositional inhomogeneity. Depending on the liposome system being developed, choosing the right preparation method might therefore constitute a central element for producing more precise and controllable drug delivery systems.

3.4 Membrane phase-state separation as a driver for compositional inhomogeneity

Next, to study how potential phase-separation during liposome formation influenced compositional inhomogeneity, we changed the fluorescent pair from the identical DOPE-Atto488:DOPE-Atto655 to the non-identical Chol-TF and DOPE-Atto655. It is known that the Chol-TF will partition to more ordered domains whereas the DOPE anchor will partition to the less ordered domains.[34] For the POPC<sub>Chol:DOPE</sub> membrane systems we quantified DI values of 0.24 ± 0.01 and 0.25 ± 0.01 for the FD and LR preparation methods respectively (Fig. 3a, light grey). These are almost identical to the DI values for the POPC<sub>DOPE:DOPE</sub> systems (Fig. 3a, dark grey, FD = 0.24 ± 0.02 and LR = 0.23 ± 0.01). This indicates that the compositional inhomogeneity of the POPC system is not affected by phase separation during the preparation steps, no matter if the FD or the LR method is employed. On the contrary, we found significantly increased DI values for the HSPC<sub>Chol:DOPE</sub> systems as compared to the HSPC<sub>DOPE:DOPE</sub> systems (Fig. 3a). We found DI values of HSPC<sub>Chol:DOPE</sub> (FD) = 0.62 ± 0.05 and HSPC<sub>Chol:DOPE</sub> (LR) = 0.37 ± 0.04, with the corresponding values of HSPC<sub>DOPE:DOPE</sub> (FD) = 0.44 ± 0.02 and HSPC<sub>DOPE:DOPE</sub> (LR) = 0.29 ± 0.02. This represents a 42 % and 37 % increase in DI value for FD and LR respectively, and suggests that membrane phase-state separation during liposome
preparation is contributing to significant compositional inhomogeneity of drug delivery liposomes made from saturated phospholipids, like the HSPC system.

**Figure 3**  | Quantifying how membrane phase-state and PEGylation strategy affect compositional inhomogeneity.

- **a** Quantified average DI values for HSPC (red) and POPC (grey) membrane systems prepared by either the FD or LR method using the Chol-TF and DOPE-Atto655 fluorescent lipid pair (light) or the DOPE-Atto488 and DOPE-Atto655 fluorescent lipid pair (dark).
- **b** Quantified average DI values for HSPC (red) and POPC (grey) membrane systems prepared wither by the FD or LR method using the DOPE-Atto488 and DOPE-Atto655 fluorescent lipid pair as a function of PEGylation strategy. Premixed represents liposome systems where the PEG-lipid was added to the initial lipid mixture whereas for the post-inserted systems the PEG-lipid was post-inserted in the outer liposome membrane after initial liposome formation.

**3.5 PEGylation strategy can regulate liposome compositional inhomogeneity**

A key prerequisite for the clinical efficacy of liposomal drug delivery systems is the ability to avoid the body’s natural clearance machinery. This is typically achieved by covering the liposomal surface
with the polymer PEG, so called liposome PEGylation [35]. PEGylation of the liposome surface can either be achieved by adding PEG conjugated lipids to the initial liposome lipid mixture or post-insert PEG-lipids after liposome formation [23, 24, 36]. To further expand on the knowledge of how PEGylation strategy affects liposome quality, we investigated how premixing versus post-inserting PEG-lipids might affect the compositional inhomogeneity. Therefore to compare with the previously presented liposomes, all prepared using the premixed strategy, we formulated both HSPC and POPC liposomes using the FD method where the DSPE-PEG2k and DOPE-PEG2k were removed from the initial lipid mixture and added after liposome formulation by post-insertion (see method for details). We measured no significant difference in the DI value for the premixed and post-inserted POPC liposome systems (POCP\textsubscript{premixed} = 0.24 ± 0.02 and POCP\textsubscript{post-ins} = 0.27 ± 0.01) (Fig. 3b, grey). However, for the HSPC system we quantified a significant 41 % reduction when comparing the premixing and post-insertion PEGylation strategy (HSPC\textsubscript{premixed} = 0.44 ± 0.02 and HSPC\textsubscript{post-ins} = 0.26 ± 0.01) (Fig. 3b, red). This demonstrates how compositional inhomogeneity of the HSPC system can be substantially reduced by adopting a PEG post-insertion strategy and in fact reach an inhomogeneity level similar to the low one consistently found for the POPC systems. Previously PEGylation by post-insertion has been discussed to be beneficial for both the ease of liposome fabrication[24] and enhanced drug encapsulation efficiency [36]. Here we add a new parameter to this list, by showing how PEG-lipid post-insertion has the potential to reduce compositional inhomogeneity, which could lead to more precise and controllable drug delivery systems.

4. Discussion

Despite the emergence of nanomedicine many decades ago, is has been argued that the number of nanomedicine based drugs that have reached the clinic is staggeringly low [37-39]. This translational gap has been proposed to be, at least in part, related to a lack of precision and sensitivity in the tools
employed to characterize the physicochemical properties of drug delivery liposomes [10, 37]. Due to technical limitations, most characterization of liposome properties have traditionally been done using bulk assays, giving an ensemble average read out of e.g. size and surface charge [40]. The recent emergence of assays capable of studying single liposomes however, offers the possibility to elucidate intra-sample inhomogeneities, facilitating an understanding of how these affect liposome function [10, 11, 41].

Here we showed that various elements of the design and preparation of drug delivery liposomes affect their compositional inhomogeneity. We studied liposome systems with a lipid composition closely resembling Doxil, the first FDA approved liposomal drug delivery product and still considered to be the gold standard [42]. Recently single liposome analysis of Doxil revealed considerable inhomogeneities in the drug encapsulation efficiency between individual liposomes, including a large portion of liposomes displaying no apparent encapsulated drugs [43]. These observations could potentially be linked to the variation in lipid composition we demonstrated in this work, highlighting the vast potential for optimizing the functional uniformity of drug delivery liposomes.

Modulating the lipid composition of drug delivery liposomes represents a well-established avenue for optimizing functional parameters of liposomes, such as recognition by opsonins, circulation half-life and membrane permeability to encapsulated drugs [30, 31]. One lipid parameter routinely modified is the saturation degree of the acyl chains [32], which, depending on the number and placement of double bonds in the acyl chains, can significantly affect the overall phase-state of liposomes [44]. Previously we have shown how lipid saturation degree can affect the inhomogeneity of liposome PEGylation [23], here we expand this by also showing that the compositional inhomogeneity varies between liposome systems produced from saturated (HSPC) versus unsaturated (POPC) lipid.
A critical parameter in liposomal drug delivery is the choice of preparation method [33]. Numerous methods are used across research groups and in industry, including ones based on organic solvent replacement [13], detergent dialysis [33] or ethanol injection techniques [45], which all offer unique advantages and limitations. Here we compare two methods extensively used in pre-clinical development based on lipid rehydration in aqueous media from either chloroform evaporated lipid films or freeze-dried powders. Previously, preparation method has been shown to affect the compositional inhomogeneity of model membrane and surfactant based systems [7, 8]. Here we expand this by also showing that clinically relevant liposomal systems, with a lipid composition mimicking Doxil, display preparation method based variations in the compositional inhomogeneity.

In order to create a truly compositional monodisperse nanometer sized liposome formulation, all lipid components need to be perfectly mixed throughout the preparation process [10]. Naturally, this requirement becomes increasingly difficult to fulfill as the number of lipid species included in the liposome composition are increased. Another correlative effect of increasing the compositional complexity is the potential for creating cooperative membrane effect such as phase separation. This could lead to lipid demixing during liposome formulation, which would substantiate compositional inhomogeneity if the lipid components display distinct partitioning into domains of different phase-state.[8, 46] The potential presence of such phase-separated domains could explain why we when employing a lipid reporter pair capable of partitioning to different phase-state domains, recorded a much higher DI for the HSPC:Chol:DOPE as compared to the the POPC:Chol:DOPE. It has previously been shown that POPC:Chol membrane systems, no matter the Chol concentration, resides far from any known domains in the phase-diagram displaying the coexistence of different phase-state [47]. This is supported by the non-significant differences in DI values for POPC:DOPE:DOPE and POPC:Chol:DOPE (Fig. 3a) as well as a POPC:DOPE:DPPE system (Supplementary Fig. 4). On the contrary, HSPC:Chol membrane systems have been shown to display different phase-state coexistence for Chol
concentrations below 40 mol%, putting our system (containing 39.5 mol% Chol) at the boundary. This suggests that preparation methods, like the ones used here, involving a solvent free step, believed to drive lipid demixing [8, 46], could for some lipid compositions introduce increased compositional inhomogeneity.

5. Conclusion
We have used a microscopy based single liposome assay to demonstrate that clinically relevant drug delivery liposomes display significant compositional inhomogeneity between individual liposomes. The DI was modulated by membrane lipid saturation state, the preparation method and the PEGylation strategy. As drug delivery systems are becoming more and more complex, but not necessarily more and more effective [48, 49], the concept of controlling compositional inhomogeneity will only be more critical going forward. We believe that the insights into the factors governing the DI presented in this study, offers the possibility for producing more uniform liposomal drug delivery systems. This could potentially lead to more precise and controllable liposomal drug delivery systems with increased therapeutic efficacy.

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Author Contributions
Thomas Lars Andresen: Funding acquisition, Conceptualization, Supervision, Writing – Review & Editing

Jannik Bruun Larsen: Conceptualization, Methodology, Investigation, Writing – Original Draft

Competing interest

The authors declare no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting Information

Details on lipid composition, biophysical characterization of liposome systems and experimental error determination. Summary of all quantified DI values and statistical testing.

References


