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How docetaxel entrapment, vesicle size, zeta potential and stability change with liposome composition–A formulation screening study

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ABSTRACT

Limitations of the anticancer drug product Taxotere® have encouraged researchers to entrap the active ingredient docetaxel (DTX) into nanocarriers such as liposomes. However, until now no DTX-liposome formulation has reached the clinic. Hence, in the present study, different Soy-PC based DTX-liposome formulations were screened in an attempt to identify lipid-compositions with promising DTX-entrapment (DTX-EE). Various other quality attributes, such as vesicle size and morphology, poly dispersity index (PDI), zeta potential (ZP), stability and *in vitro* drug release were also investigated. In an initial study, the inclusion of charged lipids within the liposome bilayer was observed to have a positive effect on DTX-EE. Thus, cationic DOTAP (1,2-Dioleoyl-3-trimethylammonium-propane) and anionic DMPG (1,2-Dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) lipids were selected for further investigations. With anionic DMPG, only a temporary rise in EE was gained with $\geq$ 20% (w/w) DMPG in Soy-PC lipid-based liposomes, whereas a concentration-dependent increase in EE was observed with cationic DOTAP. A DTX-EE $>$ 95% was obtained with only 5% (w/w) DOTAP in Soy-PC, while neutral liposomes formed from Soy-PC alone, gave 41.5% DTX-EE. In the stability study, a DOTAP concentration $>$ 10% (w/w) in Soy-PC was found to facilitate a stable DTX-EE $>$ 90% after 12 weeks storage. The positive effect of cationic lipids on the EE was confirmed when replacing cholesterol (CHOL), initially shown to suppress DTX-entrapment, with cationic 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]Cholesterol (DC-CHOL). Here, DTX-EE was improved from 29.8% to 92.0% (w/w) with 10% (w/w) CHOL and DC-CHOL in Soy-PC, respectively. Finally, PEGylation of DOTAP-liposomes with DSPE-PEG2000 and DSPE-PEG750 reduced the DTX-EE relative to DOTAP-liposome with no PEGylation. As with the DMPG-liposomes, a temporarily raised affinity between DTX and liposomes was obtained with anionic DSPE-PEGylation of Soy-PC liposomes, however, this effect was not maintained after 4 weeks storage. However, in a dialysis set-up, cationic DOTAP-liposomes released DTX to a higher extent than PEGylated liposomes. Thus, the optimal formulation with regard to storage stability and *in vivo* performance need to be investigated further, applying conditions that are closer to mimic the *in vivo*-situation. Applying the Dual Asymmetric Centrifugation (DAC) method in liposome production appears favourable due to its good reproducibility. The observed increase in DTX entrapment with cationic lipids or PEGylation appears scalable into pilot manufacturing scale.

Abbreviations: ACN, acetonitrile; CER, ceramide; $CHCl_3$, chloroform; CHOL, cholesterol; DAC, dual asymmetric centrifugation; DC-CHOL, 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DMPE, 1,2-dimyristoyl-sn-glycero-3-phosphoetanolamine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol); DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DTX, docetaxel; EE, entrapment efficiency; PEG, polyethylene glycol; PC, phosphatidylcholine; MeOH, methanol; PDI, polydispersity Index; PEG750, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-750]; PEG2000, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine; POPE, 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphoetanolamine; DPPG, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; ZP, Zeta potential.

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1. Introduction

Cancer is the most life-threatening disease worldwide, accounting for nearly 10 million deaths in 2020 (Ferlay et al., 2020). Chemotherapy is widely used in the treatment of many forms of cancer and various classes of drugs are used such as alkylating agents, platinum compounds, anti-metabolites, anthracyclines and taxanes. However, the success of treatment with chemotherapy is often hampered by dose limiting side-effects and development of resistance to treatment (Gote et al., 2021; Grimaldi et al., 2019; Zheng et al., 2021). Drug delivery systems (DDS) in cancer therapy aim to improve the therapeutic index of chemotherapy. The targeted benefits of the DDS will differ with the limitations of the chemotherapeutic drug selected for the formulation, but these limitations typically include poor solubility and/or stability, and lack of specificity (Øverbye et al., 2017; Sætern et al., 2004; van Eerden et al., 2020). DDS in the nano-size range are called "nano-carriers". When the chemotherapeutic agent is entrapped in nano-carriers, they will hide their cargo, and the pharmacokinetic of the drug will depend on the carrier and not the drug its selves. However, this is only a valid statement if the stability of the drug carrier in the biological environment is sufficiently high, and the drug is not prematurely released from the carrier prior to reaching the targeted cancerous tissue (Cauzzo et al., 2020; Pedziwiatr-Werbicka et al., 2020).

Liposomes are the most successful nano-sized drug delivery system investigated with regard to reaching the clinic as a commercial product (Ahmed et al., 2018; Allen and Cullis, 2013; Kaur et al., 2014; Kraft et al., 2014; Rommasi and Esfandiari, 2021). These artificial Phospholipid (PL) vesicles are obtained from PLs dispersed in water, where the vesicle membrane might incorporate lipophilic compounds, and/or encapsulate the aqueous core housing water-soluble cargoes/drugs. However, the incorporation of lipophilic drugs into the liposome membrane is often limited, as the drug affinity to the membrane need to be sufficiently high to be retained in the membrane in a high concentration and for a satisfactorily long period of time with respect to storage, but also with respect to retaining the drug after administration to the patient (di Cagno et al., 2011; Flaten et al., 2013; Sætern et al., 2004). Certain liposome surface modifications (*e.g.* PEGylation) might help oppose the unwanted uptake by the mononuclear phagocyte system (MPS) *in vivo* (Gabizon et al., 2003; Klibanov et al., 1990).

Docetaxel (N-debenzoyl-N-tert-butoxycarbonyl-10-deacetyl) (DTX)), is a chemotherapeutic drug belonging to the taxane family. It is clinically active against several cancers such as ovarian carcinoma, prostate cancer, gastric cancer, head and neck cancer, breast cancer and lung cancers (Crown and O'Leary, 2000; Immordino et al., 2003; Morse et al., 2005). DTX is practically insoluble in water (0.025 μg/mL) (Farhan Sohail et al., 2018), and this is, why the commercially available product, Taxotere®, contains surfactants and co-solvents (polysorbate 80 and ethanol). Unfortunately, these co-solvents cause serious and sometimes dose-limiting adverse effects (da Silva et al., 2018; Gregoriadis et al., 1990; Ma, 2013; Ten Tije et al., 2003). Thus, new formulations with DTX in nanocarriers might be an attractive approach for improved clinical success of DTX (Al Saqr et al., 2021; Bowerman et al., 2017; Chang et al., 2018; Li and Qi, 2014; Li et al., 2021; Mahalingam et al., 2014; Pereira et al., 2016; Tan et al., 2012). Although DTX-in-nanoparticles is not a novel idea, significant hurdles to its broader use are currently remaining. Among these are the optimization of the liposome composition with regard to stability and drug entrapment efficiency (EE); at the same time establishing procedures that include a well-controlled and reproducible manufacture technology. With respect to the last hurdle, Dual Asymmetric Centrifugation (DAC) has recently been established as a method to prepare liposomes in a reproducible manner (Ingebrigtsen et al., 2016; Massing et al., 2008, 2017). This new method seems promising as it is gentle, allows small batch sizes and is conducted in closed containers, making it a suitable formulation screening method (Ingebrigtsen et al., 2017a; Massing et al., 2008).

Thus, in this study we aimed to screen different membrane lipid-compositions for their effect on DTX-entrapment (EE). After an initial screening study where we used the traditional probe sonication set-up for the size reduction of the liposomes, the further investigations were conducted with liposomes prepared with the DAC-method. The liposomal solubilization of DTX, corresponding to the DTX-EE, was determined by removing the unentrapped DTX by centrifugation. The remaining drug (and initially also PLs) in the supernatant were quantified by HPLC and a colorimetric quantification of PC, respectively. All liposome dispersions were also characterized with respect to liposome size, zetapotential (ZP) and polydispersity index (PDI). The drug release rate of selected liposome formulations, as well as a commercial DTX-product, was studied in diluted state from a dialysis bag, to compare how well the drug was retained in the vesicles. Finally, the most promising liposome formulations were stored for up to 12 weeks at 4 °C for stability assessments and the effect of PEGylation of the most promising formulation was also investigated.

2. Materials and methods

2.1. Materials

DTX was obtained from Euroasian chemicals pvt Ltd., Mumbai, India. 3β[N-(N',N'-dimethylaminoethane)-carbamoyl]Cholesterol hydrochloride (DC-CHOL), N-Hexanoyl-D-erythro-sphingosine/Ceramide C6 (CER C6), N-Lauroyl-D-erythro-sphingosine/Ceramide C12 (CER C12), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000]) (DSPE-PEG2000) and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-750] (DSPE-PEG750) were purchased from Avanti Polar Lipids Inc., Alabaster, AL, USA. Soybean phosphatidylcholine, Lipoid S100 (Soy-PC), 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPG), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and Lipoid S100 (Soybean lecithin, > 94% phosphatidylcholine) were all from Lipoid GmbH, Ludwigshafen, Germany. 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-Dipalmitoyl-sn-glycero-3-phosho-(1′-rac-glycerol) (DPPG sodium salt) were purchased from Genzyme pharmaceuticals, Sygena Facility, Liestal, Switzerland. Cholesterol (CHOL), 1,2-Dioleoyl-sn¬-glycero-3-phosphocholine (DOPC) and 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Sigma Aldrich Co, St. Louis, USA, whereas 1-Palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) and 1-Palmitoyl-2-oleyl-sn-glycero-3-phosphoethanolamine (POPE) were obtained from Sigma Aldrich Co, Japan. Infusion concentrate of DTX; Docetaxel Kabi 20 mg/mL (Fresenius Kabi Norway AS, Halden, Norway) and human albumin (Albunorm® infusion 20% (w/v) from Octapharma AG (Lachen, Switzerland) were purchased from the Hospital Pharmacy, University Hospital of North Norway, Tromsø, Norway. MilliQ water was freshly prepared from our Millipore Water purification system with Millipak® M 0.22 μm filter (LOT NO C5MA58154), from Merck KGaA, Darmstadt, Germany. Distilled water (dH_2O) was freshly prepared by the distillation unit, Distinction D4000, Bibby Sterilin Ltd., Staffordshire, UK. Acetonitrile (≥ 99.9%) (ACN) and methanol (≥ 99.9%) (MeOH) were both from VWR Chemicals, BDH Prolabo, France, and chloroform (99.0–99.4% $CHCl_3$), formic acid (CH_2O_2), Fiske Subbarow reducer, ammonium molybdate tetrahydrate $(NH_4)Mo_7O_{24} \cdot 4H_2O$ and a phosphorus standard solution, KH_2PO_4 (with 0.65 mM phosphorous), were purchased from Sigma Aldrich Chemie GmbH, Steinheim, Germany. Sulfuric acid (analytical grade reagent) was from May and Baker Ltd, Dagenham, England.

2.2. Liposome preparation

2.2.1. Lipid film formation

As a first step, a lipid film containing 200 mg lipids (controls) or 200 mg lipids/20 mg DTX (DTX-liposomes) was formed. The selected lipids were dissolved in $CHCl_3$ and/or MeOH (Table 1), whereas DTX was

Table 1
Overview of the DTX-Soy-PC liposome compositions with molar lipid ratios and the transition temperatures (Tm) of the second lipid added, solvent(s) applied for dissolving the lipids, and duration of sonication of the liposome dispersion during size reduction. All formulations contained 20 mg docetaxel and 200 mg lipids.

No.	Composition (mol%)	Tm (°C)*	Solvent (v/v)	Sonication time (min)
1	DTX/Soy-PC (9/91)	(−20 to 30)	MeOH	3.0 and 2.0
2	DTX/Soy-PC/CHOL (7/62/31)	–	$CHCl_3$:MeOH (2:1)	3.0
3	DTX/Soy-PC/DOPE (9/72/19)	−16	$CHCl_3$:MeOH (2:1)	1.5
4	DTX/Soy-PC/DOPC (9/73/18)	−20	MeOH	3.0
5	DTX/Soy PC/PEG2000 (9/72/20)	65	$CHCl_3$	0.5
6	DTX/Soy-PC/DOTAP (9/72/20)	–	$CHCl_3$:MeOH (2:1)	3.0
7	DTX/Soy-PC/CER C6 (7/30/62)	–	$CHCl_3$	2.0
8	DTX/Soy-PC/CER C12 (8/66/26)	–	$CHCl_3$	2.0
9	DTX/Soy-PC/DPPG (8/72/19)	41	$CHCl_3$:MeOH (2:1)	0.5
10	DTX/Soy-PC/DMPG (9/71/20)	23	$CHCl_3$:MeOH (2:1)	0.5
11	DTX/Soy-PC/DMPC (9/71/20)	24	MeOH	0.5
12	DTX/Soy-PC/DMPE (8/70/21)	50	$CHCl_3$	4.0
13	DTX/Soy-PC/POPC (9/73/19)	−9	$CHCl_3$:MeOH (4:1)	4.0
14	DTX/Soy-PC/POPE (9/72/19)	25	$CHCl_3$:MeOH (6:1)	2.0

*Literature values for the Tm of the Soy-PC (Formulation 1) or the second added lipid (Formulation 3–5 and 9–14) from www.avantilipids.com Abbreviations: CER, Ceramide; $CHCl_3$, Chloroform; CHOL, Cholesterol; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPE, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); DPPG, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; DTX, docetaxel; MeOH, methanol; PEG2000, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine, POPE, 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphoetanolamine; Soy-PC, Soy phosphatidylcholine.

added from a 20 mg/mL DTX-stock solution in MeOH. The organic solvents were removed using a Rotavapor R-124, a Water Bath B-480 with a Vac V-500 Vacuum Pump system and a Vacuum Controller B-721 (Büchi Labortechnik, Switzerland). The water bath was preheated to keep a temperature higher than the lipids transition temperature, Tm (Table 1), and the pressure was decreased gradually from 1005 to 55 mBar, at a rotation speed of 80 rpm. The lipid films were formed in 100 mL round bottom flasks for making sonicated liposome dispersions (Section 2.2.2) and in a 30 mL brown coloured injection vial when made for further processed by DAC (Section 2.2.3). Suitable organic solvents were selected with respect to the solubility of the respective lipids and DTX (Table 1).

For the lipids films prepared in the follow-up studies, PEGylated lipids (DSPE-PEG750, DSPE-PEG2000) were dissolved in $CHCl_3$, whereas a 25 mg/mL stock solutions with Soy-PC was made in MeOH. Stock solutions of DOTAP, DMPG, CHOL and DC-CHOL were prepared with $CHCl_3$, $CHCl_3$/EtOH (1:2) and $CHCl_3$/MeOH (2:1) as solvents, respectively. In the same study (Section 3.6), the same lipid ratio between the main lipid Soy-PC with PEGylated lipids was maintained for the PEGylated liposomes as for the non-PEGylated liposomes with DOTAP (10% (w/w)), by adjusting for the mass-contribution from PEG (polyethylene glycol) on the total molecular mass of the PEG-lipid. The amount of lipids added to the lipid film is shown in Table 2.

2.2.2. *Liposome size reduction by sonication*

The lipid film was hydrated adding 10 mL preheated and filtrated (0.2 μm) dH_2O. Thereafter, the formed lipid dispersion was vortexed using a MS2 Minishaker Vortex mixer (IKA-Werke, Staufen im Breisgau, Germany) to form a liposome dispersion with a lipid concentration of 20 mg/mL and a DTX concentration of 2 mg/mL. The liposome dispersions were left at room temperature for 1 hour and thereafter stored overnight at 4 °C. For size reduction, the liposome dispersion was transferred to a 45 mL falcon tube. The 13 mm probe of the Sonics Vibra Cell high intensity ultrasonic processor (VS 754, 750 Watt, from Sonic and Materials, USA) was inserted into the center of the sample and the tubes were placed in an ice bath. The amplitude was 40%. The duration of the sonication depended on the lipid composition and varied from 0.5 to 4 min (Table 1). Max duration of the sonication intervals was two minutes with a one-minute cooling step. A liposome size of approximately 100 nm was targeted.

2.2.3. *Liposome size reduction using dual asymmetric centrifugation (DAC)*

The DAC was applied for making most of the liposomes in this study. The lipid film (Section 2.2.1) was hydrated with 300 μL dH_2O to form a VPG with a lipid concentration of 400 mg/mL and a DTX concentration of 40 mg/mL. The VPG was added 500 mg zirconium oxide beads ($\varnothing$ = 1.4 mm) before vortexing. Like for the liposome dispersions processed by the sonication method (Section 2.2.2), the VPGs were stored overnight at 4 °C before further processing/size reduction. The size reduction was carried out in a Speedmixer (DAC 150.1 FVZ-K Speedmixer, Synergy Devices Ltd., High Wycombe, UK). All samples were processed for 6 × 5 min (in total 30 min) at maximum speed, corresponding to 3500 rpm. Finally, the VPGs were diluted into liposome dispersions by adding filtered dH_2O. The liposome dispersion had a volume of 2 mL and a lipid- and DTX concentration of 100 and 10 mg/mL, respectively.

2.3. *Liposome characterization*

The liposome dispersions were allowed to equilibrate in the refrigerator overnight, and the next day centrifuged at 3000 rpm/min (corresponding to 1800 g) for 20 min at 25 °C to separate the unentrapped drug (DTX) from the liposomes. For this purpose, a Biofuge stratos centrifuge (Heraeus Instruments, Oslo, Norway) with a Heraeus rotor

Table 2
The amount of lipids and docetaxel added to the different liposome formulations in the PEGylation study (Section 3.6).

Composition (mol%)	Lipid (mg/sample)				DTX (mg/sample)
	Soy-PC	DOTAP	DSPE-PEG750	DSPE-PEG2000	
DTX/Soy-PC (9/91)	200	–	–	–	20
DTX/Soy-PC/DOTAP (9/81/10)	180.0	20.0	–	–	20
DTX/Soy-PC/PEG750 (9/86/5)	190.0	–	21.8	–	20
DTX/Soy-PC/PEG2000 (9/86/5)	190.0	–	–	40.0	20
DTX/Soy-PC/DOTAP/PEG750 (9/76/10/5)	170.0	20.0	21.8	–	20
DTX/Soy-PC/DOTAP/PEG2000 (9/76//10/5)	170.0	20.0	–	40.0	20

Abbreviations: DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DSPE-PEG750/PEG750, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-750]; DSPE-PEG2000/PEG2000, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]; DTX, docetaxel; Soy-PC, Soy phosphatidylcholine.

#3048 and #3047 was used. For process control, the liposome size, polydispersity index (PDI), zeta-potential (ZP), DTX-concentration, and in the case of the formulation screening also PC-concentration (Section 2.3.2), were measured both prior to ("Total sample") and after centrifugation (in the supernatant), applying the same methods as described in the coming sections.

2.3.1. Liposome size, zeta-potential and morphology

Liposome size, PDI and ZP were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Since the liposome dispersions prepared by DAC was five times more concentrated than the sonicated liposome samples, theses samples had to be diluted correspondingly to achieve the suitable concentration range for the further characterizations. The sonicated liposome dispersions were diluted 1:100 (v/v) with dH_2O before analyses of size and PI (1:800 (v/v) for the bigger DAC-liposomes), and 1:10 with freshly filtered tap water (v/v) to measure the ZP (diluted 1:20 (v/v) for the DAC-liposomes). Analyses were performed in triplicates with an equilibration time of 180 s to allow the sample to stabilize. Drive-voltage and number of runs were automatically determined by the instrument. All measurements were made in triplicates and with 10 – 20 runs for each measurement for the PDI and size, whereas for the ZP up to 100 runs were performed. The count rate and correlation function were monitored to rule out any disturbances from dust contamination or sedimentation and aggregation of particles. An attenuator index of 7 was regarded optimal for the measurements. A PDI value < 0.25 was judged satisfying. The results were presented with intensity weighted distributions. Some formulations were also selected for morphological characterization and studied by transmission electron microscopy (TEM). For this, a previously described method from our group was applied (Hemmingsen et al., 2021). In short, Liposomes were deposited onto carbon-coated grids for 5 min, washed with double-distilled water, and stained with 3% uranyl acetate and 2% methylcellulose (1:9) for 2 min. The samples were picked up with a loop and dried before the images were taken using a transmission electron microscope HT7800 Series (Hitachi High-Tech Corp., Tokyo, Japan), The machine was operated at an accelerated voltage of 100 kV coupled with a Morada camera.

2.3.2. Lipid content and recovery

The concentration of phosphatidylcholine (PC) in the liposome dispersions was determined by a method modified from the original Bartlett assay (Bartlett, 1959), described in detailed in previous publications from our group (Ingebrigtsen et al., 2017b, 2016). In brief, concentrated sulfuric acid was added to diluted liposome samples prior to the digestion of organic matter in a wet ashing process, carried out in a heating cabinet at 160 °C. After 3 h, 30% water-free hydrogen peroxide was added to assist the digestion process further, and the samples were heated for 1.5 more hours. When all samples reached room temperature, a solution of 0.22% ammonium molybdate was added together with Fiske-Subbarow reducer. Finally, the samples were heated at 100 °C for seven additional minutes. The concentration of phosphorus was determined by colorimetric analysis at 830 nm, detecting the blue coloured 4-amino-2-naphthyl-4-sulfonic acid. The total PC concentration was calculated by relating measured absorbance to the absorbance value for a stock solution of known PC-concentration. The PC recovery was calculated from Eq. (1), comparing the relative concentration of PC in the liposome dispersion before ("total sample") and after (supernatant) centrifugation.

Eq. (1). Recovery of phosphatidylcholine (PC)

$$Recovery\ PC\ (\%) = \left(\frac{PC\ conc.\ Supernatant\ \left(\frac{\mu g}{mL}\right)}{PC\ conc.\ Total\ sample\ \left(\frac{\mu g}{mL}\right)}\right) x\ 100\% \tag{1}$$

2.3.3. Docetaxel quantification by HPLC

The concentration of DTX in the liposome dispersion before and after centrifugation was determined by high performance liquid chromatography (HPLC) with a Waters e2795 Separations Module a Waters 2489 UV/Visible detector. The selected wavelength was $\lambda = 232$ nm. We used a C18 column: XSELECT CSH column XP, 2.5 μm 3.0 × 75 mm and a C18 XSELECT CSH guard column (Waters, Dublin, Ireland). The injection volume and the flow rate were 10 μL and 1.0 mL/min., respectively. Mobile phase A, contained Milli-Q-water with 0.1% formic acid and Mobile phase B, contained acetonitrile (ACN) with 0.1% formic acid. The mobile phase was linearly changed from 20 to 90% mobile phase B during the first 8 min and then kept at 90% mobile phase B for two minutes, before returned to the initial 80/20 A:B composition for two minutes prior to injecting the next sample. Temperature of both the column and sample were 25 ± 1 °C. The DTX-retention time was at 7.7 min. The concentrations ranged of the standard curve was from 20 to 280 μg/mL, and the curve attained an R^2-value ≥ 0.99.

2.3.4. Calculation of drug recovery and entrapment

Before DTX quantification, the DTX-liposomal dispersion was diluted with MeOH to reach concentrations within the calibration curve. In the formulation screening study involving sonicated liposome dispersions, every liposome batch was diluted separately in squintuplicate (five preparations of each sample), and every sample was injected twice into the HPLC. In the last part of the study, involving DAC-prepared liposomes, every formulation was prepared in triplicate batches, if not stated differently. Standard deviations were calculated from the mean of the results from the three batches ($n = 3$), if not specified differently.

The amount of DTX-recovery in the supernatant was compared with the amount of DTX present in the total sample before centrifugation. The recovery of DTX was calculated using Eq. (2), whereas the DTX entrapment efficiency (EE) of the sonicated liposomes (Section 2.2.2) was calculated as the ratio between the final concentration of DTX and PC in the liposome dispersion after centrifugation (Eq. (3)).

Eq. (2). Docetaxel recovery

$$Recovery\ DTX\ (\%) = \left(\frac{DTX\ conc.\ Supernatant\ \left(\frac{\mu g}{mL}\right)}{DTX\ conc.\ Total\ sample\ \left(\frac{\mu g}{mL}\right)}\right) x\ 100\% \tag{2}$$

Eq. (3). Docetaxel entrapment efficiency (EE) Sonicated liposomes

$$DTX\ EE\ (\%) = \left(\frac{Recovery\ DTX\ (\%)}{Recovery\ PC\ (\%)}\right) x\ 100\% \tag{3}$$

After the initial formulation screening study, the liposome size-reduction method was changed from sonication to DAC, and the study design was simplified, as the DTX-EE was regarded similar to the DTX recovery calculated for the sonicated liposomes, and PC-recovery assumed and from experience expected to be ~ 100% (Eq. (4)).

Eq. (4). Docetaxel entrapment efficiency (EE) DAC-liposomes

$$DTX\ EE\ (\%) = \left(\frac{DTX\ conc.\ Supernatant\ \left(\frac{\mu g}{mL}\right)}{DTX\ conc.\ Total\ sample\ \left(\frac{\mu g}{mL}\right)}\right) x\ 100\% \tag{4}$$

2.4. Stability of liposome after processed by DAC

To assess stability of the formulations, EE was retested after 4 weeks of storage at 4 °C, and in case of DOTAP-liposomes also after 12 weeks storage. At every time point the samples were centrifuged as described in Section 2.3. The concentration of DTX in the supernatant was quantified by HPLC as described in Section 2.3.3. EE was calculate using Eq. (4).

2.5. Drug release studies

The drug (DTX) release from PEGylated liposomes (Table 2), as well as a commercially available DTX-infusion concentrate (Docetaxel Kabi.,

20 mg/mL) were tested using a dialysis membrane set-up (Fig. 1). The dialysis membrane (Spectra/Por® 4, Spectrum Laboratories Inc, Rancho Dominquez, California, USA) was soaked in deionized water for 30 min. The liposome dispersion (approx. 2 mg/mL DTX) was centrifuged as explained in Section 2.3 to remove unentrapped drug into the pellet. The liposome supernatant was thereafter diluted 1:10, whereas the commercial product was diluted 1:20 (v/v) with dH_2O. A total volume of 3 mL of the diluted samples was transferred to the dialysis bag. The bag was thereafter inserted in a 500 mL Erlenmeyer flask filled to the top with PBS, pH 7.4 with 1% (w/v) human albumin (Albunorm®, Octapharma AG, Lachen, Switzerland). Sink condition was augmented by 1% human albumin (DTX solubility approx. 41 µg/mL), magnetic stirring and heating with a heating plate at temperature of 37 °C. DTX was quantified in the liposome dispersion prior to dialysis, and after one, three and 22 h of dialysis. Concentration of DTX remaining in the donor/dialysis bag was quantified by HPLC, as described in Section 2.3.3. The concentration at the different timepoint were calculated from the DTX-standard curve (Section 2.3.3), and the cumulative amount of remaining drug in the dialysis bag compared for the different formulations.

2.6. Statistical analysis

Results are given as mean ± SD. To determine the level of significance (*, p-value < 0.05) or substantial difference (** p-value < 0.01; *** $p < 0{,}001$; **** $p < 0.0001$), statistical analyses were performed by Students *t*-test and one-way ANOVA analysis using GraphPad Prism version 9.1.2 for Windows (GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

The anticancer drug docetaxel (DTX) has been in clinical use for decades, treating malignant conditions such as ovarian carcinoma, breast, and lung cancers. However, the presence of polysorbate and ethanol in the commercially available Taxotere® drug product has been associated with the risk of serious hypersensitivity reactions in patients. This has encouraged researchers to encapsulated DTX into nanocarriers, aiming at a more stable and clinically efficient formulation with less side effects (Swami et al., 2021; Tan et al., 2012). The focus of this study was on DTX-liposomal entrapment. DTX has a poor aqueous solubility (0.025 µg/mL), together with a poor membrane permeability (1 cm/s X 10^{-6}), which makes it a Class IV drug in the Biopharmaceutical Classification System (BCS) (Farhan Sohail et al., 2018). Thus, one of the main purposes of a liposomal DTX-formulation is to solubilize DTX in the liposome membrane. As a consequence of the poor aqueous solubility, non-entrapped DTX will precipitate in the liposome dispersion. Thus, to gain a stable formulation with therapeutically relevant DTX concentrations, a high DTX-loading capacity is desirable. Keeping in mind that the composition of the liposome membrane will determine the affinity of the DTX to the membrane, an optimized PL-membrane composition crucial, and will determine the stability of the liposome dispersion both during storage and after administration (Large et al., 2021; Maherani et al., 2012; Sætern et al., 2004).

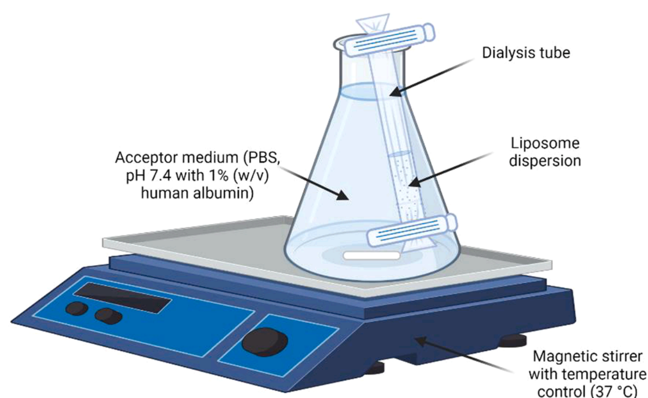

Fig. 1. The release testing set-up used for testing docetaxel release from liposome dispersions. Created with BioRender.com.

3.1. The formulation screening study

Liposome membranes can be formed from a variety of lipid blends, including PLs that differ with respect to phase transition temperature (Tm), charge and stability (Brandl, 2001). A liposome formulation screening study was conducted as a first step, aiming to identify promising lipid composition for DTX-liposomes with regard to gaining stable liposomes with a high DTX-EE. Fourteen different liposome formulations were tested (Table 3). The traditional thin-film hydration method (Bangham et al., 1965) was applied to obtain the lipid bilayers of phospholipids, before generating the final liposomes vesicles (Lasic, 1993). The hydration of the liposome bilayer was carried out with dH_2O, before further size reduction of the liposome vesicles by sonication (Szoka and Papahadjopoulos, 1980). Small-batch sizes (10 mL) were prepared and Soy-PC was used as the main lipid-ingredient. DTX was added in a 1:10 (w/w) drug:lipid ration (2 mg/mL DTX and 20 mg/mL lipid). This relatively high drug to lipid ratio, was applied to challenge the loading capacity of the membrane, since otherwise all formulations would incorporate the total amount of DTX present. Previous

Table 3
Docetaxel-liposomal characteristics; size, poly dispersity index (PDI), zeta potential (ZP) and lipid recovery (%) after centrifugation.

No.	Composition (mol%)	Size (nm)	PDI (AU ± SD)	ZP (ζ)	Lipid recovery (%)
1[a]	DTX/Soy-PC (9/91)	81.8 ± 3.5	0.24 ± 0.01	−0.2 ± 0.1	85.9 ± 0.05
2[a]	DTX/Soy-PC/CHOL (7/62/31)	58.0 ± 3.4	0.21 ± 0.01	−2.1 ± 0.4	97.3 ± 0.04
3	DTX/Soy-PC/DOPE (9/72/19)	91.8 ± 0.6	0.24 ± 0.00	−5.5 ± 0.5	84.4
4	DTX/Soy-PC/DOPC (9/73/18)	78.1 ± 0.5	0.28 ± 0.01	−2.6 ± 0.2	89.3
5	DTX/Soy PC/ PEG2000 (9/72/20)	97.6 ± 0.2	0.36 ± 0.01	−3.3 ± 0.3	95.1
6	DTX/Soy-PC/DOTAP (9/72/20)	78.0 ± 0.3	0.29 ± 0.00	76.3 ± 0.9	88.8
7	DTX/Soy-PC/CER C6 (7/30/62)	97.0 ± 0.8	0.24 ± 0.00	−1.9 ± 0.0	89.8
8	DTX/Soy-PC/CER C12 (8/66/26)	104.0 ± 0.7	0.42 ± 0.06	−1.3 ± 0.1	89.3
9	DTX/Soy-PC/DPPG (8/72/19)	98.7 ± 0.8	0.24 ± 0.01	−31.5 ± 0.6	90.7
10	DTX/Soy-PC/DMPG (9/71/20)	109.4 ± 0.7	0.25 ± 0.00	−31.9 ± 0.4	95.9
11	DTX/Soy-PC/DMPC (9/71/20)	182.6 ± 9.0	0.21 ± 0.00	−2.3 ± 0.1	84.6
12	DTX/Soy-PC/DMPE (8/70/21)	81.5 ± 0.3	0.86 ± 0.03[b]	−6.6 ± 0.2	99.2
13	DTX/Soy-PC/POPC (9/73/19)	86.0 ± 1.0	0.21 ± 0.00	−0.3 ± 0.1	95.7
14	DTX/Soy-PC/POPE (9/72/19)	97.0 ± 0.8	0.32 ± 0.02	−5.6 ± 0.5	97.4

Abbreviations: CER, Ceramide; Chol, Cholesterol; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPE, 1,2-dimyristoyl-sn-glycero-3-phosphoetanolamine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); DPPG, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; DTX, docetaxel; PEG2000, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine; POPE, 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphoetanolamine; Soy-PC, Soy phosphatidylcholine; ZP, Zeta Potential.

[a] $n = 3$ (average of the results validation batches).

[b] The estimated diameter of the liposome vesicles is too polydisperse, that means the PI values that exceeds 0.7 are not valid.

publications have reported that a 1:10 drug-to-lipid ratio will challenge the loading capacity of the liposome membrane (Pereira et al., 2016), whereas others have applied a far lower drug-to-lipid ratio to assure a high relative EE (Liang et al., 2007; Qiu et al., 2008; Zhang et al., 2014).

Two liposome formulations, contained Soy-PC (Formulation 1, Table 3) or a mixture of Soy-PC and cholesterol (CHOL) with a in 5:1 (w/w) total lipid:CHOL ratio (Formulation 2, Table 3), respectively, were first prepared in triplicate to judge the reproducibility of the preparation method, and the reliability of the DTX-EE assessment (Fig. 2A). The liposome size (Fig. 2B) and PDI, as well as the ZP were also measured for all batches (Table 3). The obtained DTX-EE from these two formulations was significantly different ($p < 0.0001$) with a decreased EE seen with CHOL present in the PL-bilayer (Fig. 2A). Although liposome size was different for the two formulations (Fig. 2B), they were judged to be in the same size-range. Also, since EE of lipophilic drug like DTX is not expected to be sensitive to liposome size, something that is a well-known issue for hydrophilic (entrapped) drugs, where the volume of the aqueous inner compartment of the liposome vesicles is very much determinant for the drug EE (Massing et al., 2017), this difference in EE can most likely be attributed to liposome composition.

Since the sonication conditions (time) were different for the two formulations (Table 1), this difference was explained by varying sonication settings, and not by the different lipid compositions. However, the liposome size was interesting to know in order to judge whether or not the obtained liposomes could be successfully separated from free DTX by centrifugation, retaining the small DTX-liposomes dispersed in the supernatant after centrifugation and removing non-dissolved DTX (crystals) in the pellet. The standard deviation between batches was satisfying and the method considered to be reliable and suitable for its intended purpose, *i.e.* to identify the best PL-composition with regard to DTX-EE.

The EE capacities for the Soy-PC:CHOL and the Soy-PC formulation were 25.2 ± 3.3% (mean ± SD) and 103.3 ± 3.0% (mean ± SD), respectively (Fig. 1A). The main function of CHOL in liposome membranes is to increase the rigidity of the membrane to leak less hydrophilic cargo from the aqueous core of the vesicle (Briuglia et al., 2015; Maherani et al., 2012). The effect of CHOL on the DTX-EE has been studied earlier with contradicting results. Both Muthu and colleagues (Muthu et al., 2011) and Pereira and co-workers (Pereira et al., 2016) reported that including small amounts of CHOL in the liposome bilayer increased the DTX-loading capacity, whereas others observed the opposite effect (Naik et al., 2010; Yousefi, 2009), which is in line with our observations (Fig. 2A).

As also bigger particles in the form of PL-precipitates, liposomes- and liposome aggregates, could be removed from the liposome dispersion during the centrifugation process in addition to precipitated (crystalline) DTX, we also quantified the PC-content in the supernatant after centrifugation, and compared it to the liposome dispersion prior to centrifugation, to determine the "lipid recovery (%)" Eq. (1)). In this way, PC-loss was taken into account when EE was calculated (Eqs. (2) and (3). Although the lipid recovery varied between 84 and 97% (Table 3), the PC-recovery was not judged to make the final EE-values more reliable. Therefore, EE was instead calculated directly from the DTX recovery in the supernatant in the next step of the study (Eq. (4)).

The results from the 14 formulations studied, their composition, ZP, EE and the transition temperature (Tm) of the added lipid or main lipid (Soy-PC), when that was the only lipid ingredient, are presented in Fig. 3. Detailed values for the DTX-EE and DTX-recovery after centrifugation are summarized in Supplement (Table S1).

Since the rigidity of the liposome membranes is known to vary with saturation and length of the hydrocarbon tails of the PL, PLs with varying features with regard to acyl chain length and saturation were included as the "second lipid" embedded in 20 (w/w)% in Soy-PC; oleic (18:1) in formulation 3, 4, 6, 13 and 14, stearic (18:0) in formulation 5, palmitic (16:0) in formulation 9, and myristic (14:0) in formulation 10, 11 and 12.

The main lipid, Soy-PC, is a natural PL-blend isolated from soybeans, rich in phosphatidylcholines. Soy-PC was selected because excipients of natural origin are (i) favourable with respect to upscaling, (ii) accessible in larger scale than synthetic lipids, (iii) well accepted by regulatory authorities as well as (iv) less expensive than the synthetic lipids (van Hoogevest and Wendel, 2014). Soy-PC contains lecithin with varying acyl chain compositions, both with respect to chain length and saturation; from 16:0 to 18:3. The fact that Soy-PC contain a mixture of different PL constituents might explain that no direct correlation was observed between the Tm of the added lipid and the observed DTX-EE (Fig. 3). In a previous study, Pereira and coworkers (Pereira et al., 2016) conclude that unsaturated lipids (*i.e.* DOPC) showed the highest DTX-EE as compared to saturated lipids (DPPC and DSPC). Obviously, to see an effect of the synthetic lipids with regard to DTX-EE, these lipids must show a relatively big advantage or disadvantage over Soy-PC lipids, which in our case constituted 80% (w/w) of the lipid membrane.

The lipids that differed significantly in structure from lipids present in Soy-PC gave the clearest effect, *i.e.* CHOL, CER, DOTAP. Ceramides (CER-C6 and CER-C12) were included in this study, since CER has been demonstrated to have a cytotoxic effect, *i.e.* when co-delivered with doxorubicin (Chen et al., 2019; Øverbye et al., 2017; Sriraman et al., 2016). CER belong to the lipid class called Sphingolipid. Sphingolipids are formed from a long-chain sphingosine base by acetylation, and are biological active regulators in cell growth, differentiation, and death (Alrbyawi et al., 2019). Unfortunately, both CER-C6 (Formulation 7) and CER-C12 (Formulation 8), showed very disappointing

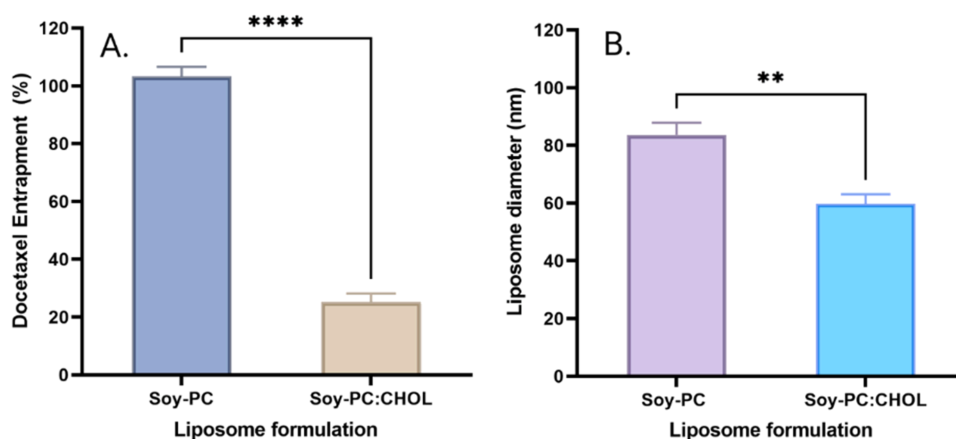

Fig. 2. Liposomal docetaxel entrapment efficiency (panel A) and liposome Size (panel B) of Soy-PC and Soy-PC:Cholesterol (Soy-PC:CHOL, 5:1 w/w) liposomes. The value denotes the mean of three separate experiments ± SD. (Stars of significance, unpaired *t*-test $^{**}p < 0.01$; $^{****}p < 0.0001$).

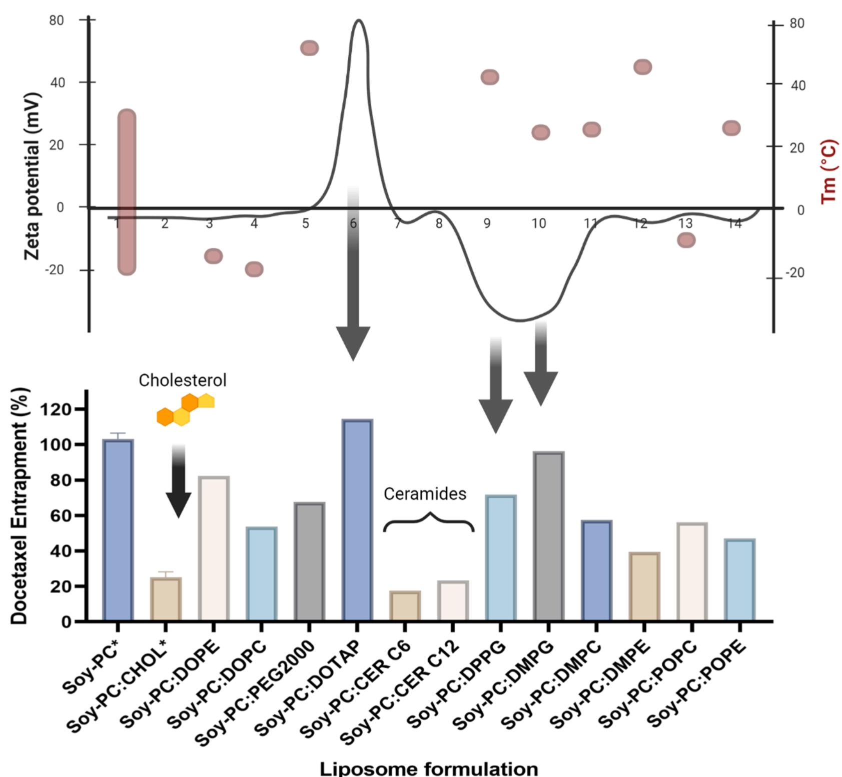

Fig. 3. The docetaxel entrapment efficiency in liposome formulations with a 1:10 (w/w) docetaxel:lipid ratio size reduces by probe sonication, and the formulations respectively zeta potential and literature values on the transition temperatures (Tm) from the main lipid (Soy-PC, formulation 1) and the extra lipid added in 20% (w/w) in Soy-PC (formulations 2–14). (n = 1, whereas *n = 3). Abbreviations: CER, Ceramide; CHOL, Cholesterol; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPE, 1,2-dimyristoyl-sn-glycero-3-phosphoetanolamine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); DPPG, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; PEG2000, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine; POPE, 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphoetanolamine; Soy-PC, Soy phosphatidylcholine.

DTX-entrapment; 17.5 and 23.3%, respectively (Fig. 3). The low DTX-entrapment might be due to the fact that CER have very tight packing properties. As CER has small polar head groups, they are known to be able to replace CHOL from PL-bilayers (Megha et al., 2007).

DOTAP (Formulation 6) is a cationic liposome-forming lipid. Cationic liposomes have so far mainly been used for various applications in gene delivery (Lou et al., 2020). The negatively charged nucleic acids and the cationic liposome membranes forms so-called “lipoplexes”, that help in transfecting target cells. However, DOTAP and positively charged liposomes have as well been found to have favourable features in cancer therapy, with their proven vascular targeting ability proposed to allow a more efficient inhibition of the angiogenesis (Schmitt-Sody et al., 2003; Thurston et al., 1998). Recently, DTX-liposomes with and without SIRT1 shRNA, a nucleic acid based anticancer therapeutic agent, were designed as liposomes and lipoplexes, respectively, with DOTAP combined in different ratios with DOPE, CHOL and PC as membrane-forming lipids (Swami et al., 2021). They reported promising results with regard to anticancer efficacy of these formulations both *in vitro* and *in vivo* in mice (breast cancer model), but these formulations had a far lower ZP than formulation 6, since the negatively charged nucleic acids neutralize DOTAP in the formulation. Formulation 10 with 20% (w/w) anionic DMPG (Formulation 10) showed, like the positively charged DOTAP formulation, a promising DTX-EE, having a ZP of −31.9 ± 0.4 mV and 76.3 ± 0.9 mV, respectively (Fig. 3). The apparent positive effect of the charged lipids, DOTAP and DMPG on DTX-EE, might be related to ZP, which is a function of surface charge, and an indication of the dispersion’s stability, as increased ZP improve the physical stability of liposomal dispersions due to the repulsion forces acting between charged vesicles.

Sterically stabilized liposomes are often applied to obtain an improved *in vivo* stability (longer circulation in the blood stream), and a more sustained release of the drug from liposomes (Feng, 2006; Gabizon et al., 2003), Formulation 5 was therefore made with DSPE-PEG2000. This formulation showed an EE of 67.7%, and neither a beneficial nor an unfavorable effect from PEG could be deducted from this single experiment.

With the sonicated liposomes no correlation between the size and EE could be seen since this method is hard to control and different durations of sonication were applied. The results obtained with the sonicated liposomes (Fig. 3), confirmed that lipid composition made a difference with regard to EE, since a big EE-variation was seen between formulations; 17.5% (CER- liposomes) and 110.2% (DOTAP-liposomes). The most evident observations made were the negative effect of CHOL and CER on DTX-EE, and the positive effect of cationic lipids (DOTAP). The EE of the most promising formulation, formulation 6, containing 20% (w/w) of the cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), was 114.6%. The higher EE than 100% might be due to an overestimation of the lipid-loss during centrifugation (Eqs. (2) and (3)). The same was the case for the Soy-PC-formulation (Formulation 1), where an EE of 110.3 ± 0.4% was obtained.

With respect to further *in vivo* application, we therefore decided to take a closer look at the effect of PEGylation (Sections 3.6 and 3.7). Having in mind that charged lipids facilitate clearance and liver accumulation (Levchenko et al., 2002), the next step should be to investigate further the effect of PEGylation on the *in vivo* performance of the liposomes. However, the first step was to verify the positive effects observed with the charged lipids; DOTAP and DMPG, on DTX-entrapment, as well as the negative effects observed with CHOL.

3.2. Optimization of the dual asymmetric centrifugation (DAC) method

Although the suitability of this sonication method for the purpose of screening of different formulations was judged good, the probe sonication method had some obvious limitations, as the span of the sonication cycles varied with the rigidity of the PL-membranes to assure the suitable liposome sizes. A more reproducible method with regard to obtaining liposome sizes in a range that assure high lipid recovery after centrifugation would allow to skip PC-quantification as part of the DTX-EE quantification procedure. Thus, for the further investigations, the Dual Asymmetric Centrifugation (DAC) method (Ingebrigtsen et al., 2017b) was applied (Section 2.2.2). DAC provides safe handling of the samples in a closed container, good reproducibility, and gentle

processing (Massing et al., 2008). An initial optimization of the DAC-method revealed that DAC-processing should be carried out for 6 × 5 min at maximum speed (3500 rpm). With these settings, typically a liposome size below 200 nm and a PDI smaller than 0.2 were obtained.

3.3. The effect of DOTAP and DMPG on DTX-liposomes

DOTAP- and DMPG-liposomes were prepared comprising 0, 2.5, 5.0, 10.0 and 20% (w/w) of the charged lipid. The observed EE, ZP and liposome size are shown in Fig. 4. The DTX-EE in the Soy-PC-formulation (0% DOTAP or DMPG, Fig. 4) was poorer here than what was seen in the screening study (Fig. 3). This might be explained by the change in the processing method. In the case of DMPG, no positive effect on the DTX-EE could be observed with less than 20% DMPG in the bilayer. On the contrary, a negative effect of the negatively charged lipid was observed with lower concentrations, although this negative effect was not significant (Fig. 4A) Cationic DOTAP, on the other hand, showed an increase, DTX-EE with increased DOTAP concentration in the bilayer. However, an EE-plateau seemed to be reached with close to 100% DTX-EE with only 5% (w/w) DOTAP. For both the DOTAP and the DMPG lipids, the biggest liposome sizes were obtained with 2.5 and 5.0% (w/w) concentrations of this charge lipid. The liposomes ZP was as expected changing with changing concentrations of both DOTAP and DMPG, but the effect was non-linear, and leveled out as the content of the charge lipid reached a concentration > 5–10% (w/w) (Fig. 4B).

A DTX-EE plateau was reached at 10% (w/w) DOTAP concentration in the bilayer, and this also correlates well with the ZP observed for the same DOTAP-formulations (Fig. 4A and B).

As shown in Fig. 4, DOTAP- and DMPG-liposome dispersions were stored at 4 °C for 12 weeks, and how well the liposome membrane was retaining DTX investigated after 4 and 12 weeks by repeating the centrifugation process to remove unentrapped drug. As can be seen in Fig. 5B, 20% (w/w) DMPG-liposomes only provided a temporarily increase in the DTX-EE as compared to the neutral Soy-PC liposomes. On the other hand, DOTAP-containing liposomes (Fig. 5A) show an improved DTX-EE also after 12 weeks storage when more than 10% (w/w) DOTAP was included in the formulation. All liposome preparations were made in distilled water, and at neutral pH, and since DTX is expected to be neutral at this pH, an improved EE in charged liposomes cannot be explained by electrostatic interactions between the drug and the lipid bilayer.

From these results, it was concluded that only the cationic lipid (DOTAP) had a favorable effect on DTX EE.

3.4. Comparing the effect of neutral and positively charged cholesterol

From the initial screening study, CHOL seemed to expel DTX from the liposome membrane (Fig. 2). To reveal how the positive effect of cationic lipids observed with DOTAP could counterbalance the negative effect from CHOL, CHOL-liposomes, Soy-PC-liposomes and liposomes with cationic CHOL, DC-CHOL (3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol) were prepared for comparison. CHOL is often used as stabilizer in the liposome bilayer (Briuglia et al., 2015). Cationic DC-CHOL is like DOTAP frequently used in nanoparticle system, and mostly for nucleic acid delivery, like mRNA (Lou et al., 2020). The negative effect of CHOL was less pronounced here as compared to in the screening study (Fig. 2A), and only significant ($p < 0.05$) when 10% (w/w) CHOL was added to the formulation. This can be explained by the

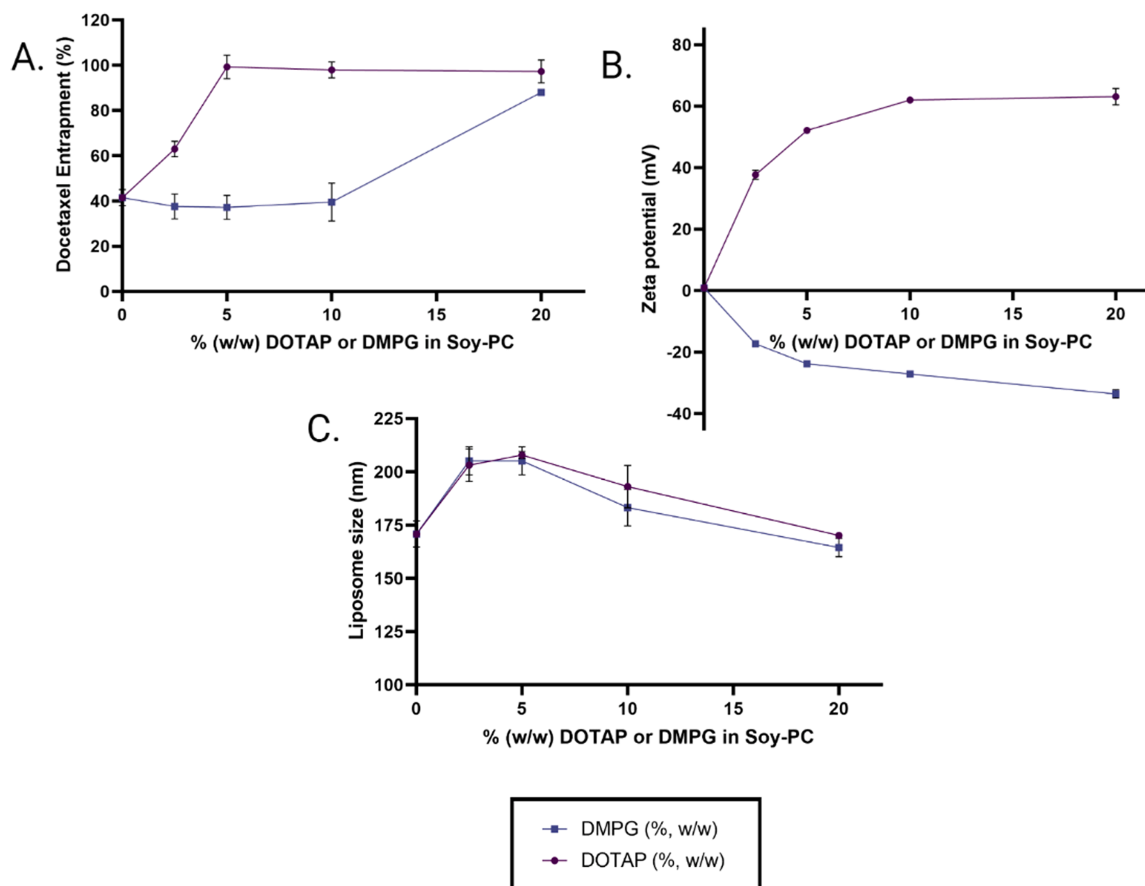

Fig. 4. The change in docetaxel entrapment (A), zeta potential (B) and liposome diameter (C) with increasing concentrations (% (w/w)) of cationic DOTAP lipids and anionic DMPG lipids. (mean ± SD, $n = 3$). Abbreviations: DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); Soy-PC, Soy phosphatidylcholine.

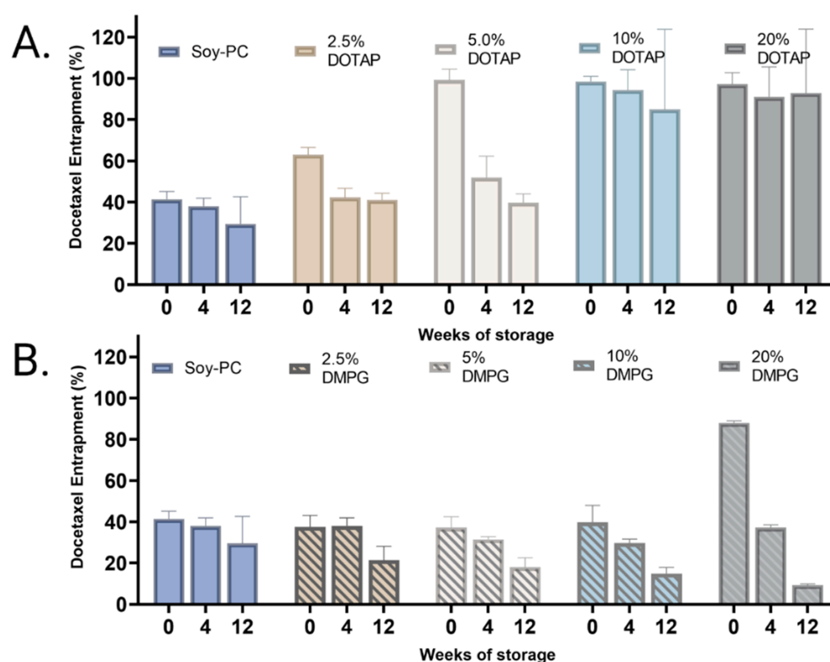

Fig. 5. Docetaxel entrapment with increasing DOTAP (Panel A) and DMPG content (Panel B) in Soy-PC liposomes. Storage time up to 12 weeks ($n = 3$) (Results presented as Mean ± SD). Abbreviations: DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); Soy-PC, Soy phosphatidylcholine.

fact that Soy-PC-liposomes in this part of the study and when prepared by DAC gave a lower DTX-EE than those that were sonicated for size reduction. For the cationic formulations, a higher EE is obtained with both 2.5 and 10% (w/w) DC-CHOL as comped to Soy-PC liposomes (Fig. 6).

These results indicate that it is the cationic charge of the liposome bilayer and not the lipid moiety of DOTAP that makes the main difference to the EE of the lipid bilayer, and that the positive ZP also can overcome the negative effect of the Chol moiety of the molecule that clearly reduces the affinity of the membrane towards DTX.

3.5. The effect of docetaxel on the liposome size and zeta potential

As there seems to be a correlation between ZP, liposome size and PDI with the observed DTX-EE, we also wanted to compare these liposome features by preparing both DTX-liposome (1:10 DTX:lipid ratio) and empty liposomes (lipids only). When doing so, the liposome size appeared quite similar for the different formulations, although the more successful formulations with regard to DTX-EE (Soy-PC, Soy-PC: DOTAP and Soy-PC: DC-CHOL) showed an increase in liposome size when DTX was added to the formulation ($p < 0.05$), whereas with the less successful CHOL- and DMPG- containing liposomes no size-effect was observed by adding DTX (Fig. 7A). When comparing the liposome size for liposomes without DTX, positively charged DOTAP and DC-CHOL seem to also give increased liposome sizes as compared to the Soy-PC-liposomes ($p < 0.05$), with an additional increase in liposome size differences is seen when DTX is added. This is the opposite effect to what was observed by Pereira et al. (2016), who observed a reduced liposome size with increasing DTX loading in DOPC:CHOL (2:1 molar ratio) liposomes. In this previous study, they prepared their liposomes with extrusion, and all liposomes had a negative ZP of around −8.8 to −14.9.

Not only will the lipid charge affect the ZP of the formulation, but also drug content, particle size and dispersion media might have an affect (Brgles et al., 2008). The very high ZP of our DOTAP-liposomes (Soy-PC-DOTAP and Soy-PC-DC-CHOL) was reduced when DTX was added to the liposome bilayer, whereas the neutral (Soy-PC and Soy-PC-CHOL) and the anionic liposomes (Soy-PC-DMPG) had a higher ZP with DTX in the formulations as compared to the DTX-free

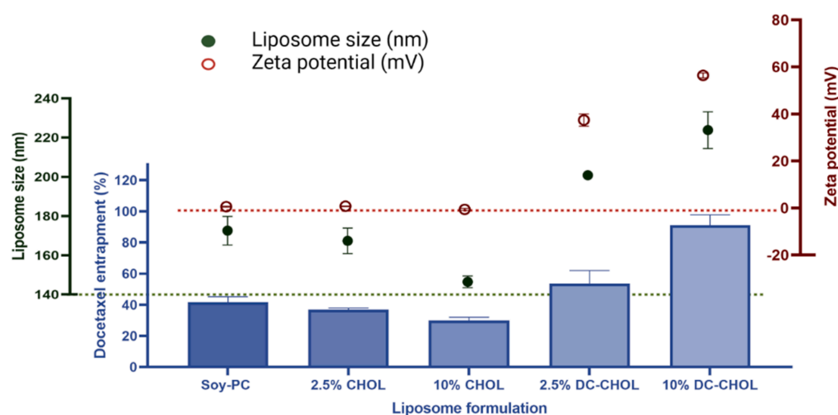

Fig. 6. Docetaxel entrapment, zeta potential and liposome size with neutral Cholesterol (CHOL) and cationic DC-Cholesterol (DC-CHOL). ($n = 2$, except Soy-PC where $n = 3$). Abbreviations: CHOL: Cholesterol, DC-CHOL: 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol, Soy-PC: Soy phosphatidylcholine.

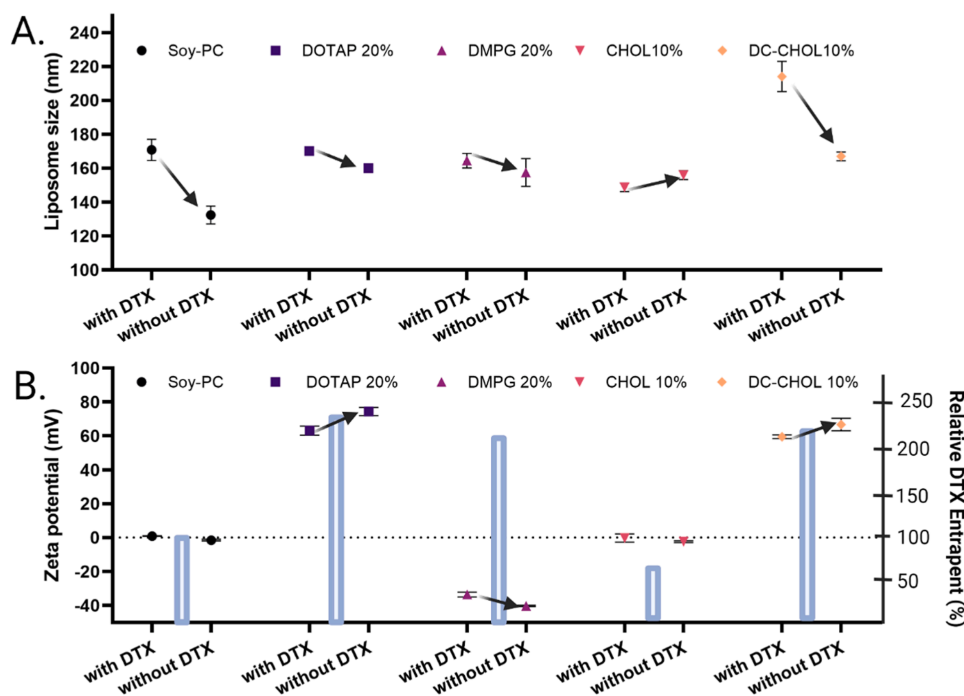

Fig. 7. Docetaxel liposomess and DTX-free liposomes; their relative DTX-entrapment (%) relative to Soy-PC liposomes, and size (A) and zeta potential (B) and how this change when DTX is present and not present in the formulation (mean ± SD). $n = 3$, except for CHOL and DC-CHOL formulations where $n = 2$. Abbreviations; CHOL, Cholesterol; DC-CHOL, 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl] Cholesterol; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DTX, Docetaxel; DMPG, 1,2-Dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); Soy-PC, Soy-phosphatidylcholine.

formulations (Fig. 7B). Thus, DTX seem to act neutralizing on the surface charge of the liposomes. This same neutralizing effect was not reported by Pereira et al. (2016), but in this case also only weak negatively ZP was reported as compared to what was the case in our formulations. The PDI of all formulations were low (Table S2, Supplement), and no obvious or systematic change in PDI was observed when adding DTX for any of the formulations. Thus, preparation of liposomes with DAC gave liposomes with a low PDI. Both DMPG and DOTAP shows a concentration-related change in ZP, but not as linear as one could expect from literature (Smith et al., 2017).

Liposomes were prepared by two different methods in the initial screening study and the optimization studies; sonication and dual asymmetric centrifugation (DAC) (Massing et al., 2017), respectively. This means that the dynamics of vesicle formation also was different from the initial screening study, where size reduction was conducted with sonication. With both methods small batches could be prepared, ideal for the screening purpose. However, the DAC method has several advantages, and when it comes to reproducibility, we realized that when using this method also other parameters such as ZP and liposomes sizes could be investigated with regard to support the DTX-EE of the different formulations.

3.6. *The effect of PEGylation on DTX-liposomes*

A well-established strategy to improve stability of nanomedicines is to conjugate polyethylene glycols (PEGs) to the surface of these nanosized drug carriers for steric stabilization. The non-covalent or covalent conjugation to PEG-molecules (with polymer chains usually in the 2–40 kDa range) build up S-shaped structures on the surface of the carriers. These structures increase the carriers' hydrodynamic size and water solubility and leads to a decreased self-aggregation and interaction with blood proteins and cells of the mononuclear phagocyte system (MPS). This ultimately gives an improved stability allowing longer storage time, and entail decreased renal-, proteolytic- and phagocytic clearance with consequent increase of circulation time after i.v. administration. Thus, PEGylated liposomes are attractive with regard to obtaining reduced adverse effects, and an overall increase of applicability and therapeutic index (Park et al., 2022).

In the initial screening study (Fig. 3), DSPE-PEG2000 was included in 20% (w/w) in Soy-PC in Formulation 5. This formulation showed a moderate DTX-entrapment of 67.7%, which was lower than that of formulations with DOTAP and DMPG, and lower than that observed with Soy-PC only. However, since this result was obtained with sonicated liposomes, and was based on the performance of one batch only, we wanted to have a closer look into the effect of PEGylation when combined with the so far most promising liposome formulations with positively charges DOTAP. Two different PEGylation chain lengths were included; DSPE-PEG750 and DSPE-PEG2000 lipids. Since 10% (w/w) DOTAP gave promising DTX-EE and stability results, with comparable DTX-entrapment and stability as the 20% (w/w) DOTAP in Soy-PC formulation (Fig. 5), we decided to include 10% (w/w) DOTAP, with and without comparable concentrations of DSPE from the DSPE-PEG conjugate lipids (Table 2). As previously in this study, liposome batches contained 20 mg DTX, corresponding to a 1:10 (w/w) drug:lipid ratio. As expected, the hydrophilic surface-decoration of the liposomes with PEGylation increased the liposome size, but only for the longer PEG-chains, namely PEG2000. These liposome formulations showed a significant increase in size compared to both Soy-PC liposomes and Soy-PC:DOTAP liposomes ($p < 0.05$). However, the effect was more pronounced for the DOTAP-liposomes, where liposome size increased from 280 ± 19 nm to 356 ± 29 nm with PEG2000 (Fig. 8B). This means that the vesicle diameter with PEG-2000 was in the higher range of what is acceptable for i.v. application, and not acceptable for sterile filtration using a 0.22 μm-filter. PEG750 liposomes with and without DOTAP did not show the same size increase but had a diameter of 168 ± 24 nm and 186 ± 2 nm, respectively. Results were obtained from three independent baches of every formulation. PEGylated liposomes had as expected a negative ZP, from the negatively charged DSPE lipid anchor (Fig. 8C). The longer PEG chains (PEG2000) also neutralized the charge from DSPE more than the shorter PEG (PEG750), as well as from the positively charges DOTAP, when applied in combination. Charge and size were therefore as expected, but DTX-entrapment observed was more surprising, as the improved entrapment seen with PEGylation compared to neutral Soy-PC liposomes was not additive but neutralized the positive effect of DOTAP (Fig. 8A).

Again, we wanted to compare the stability of the formulation to

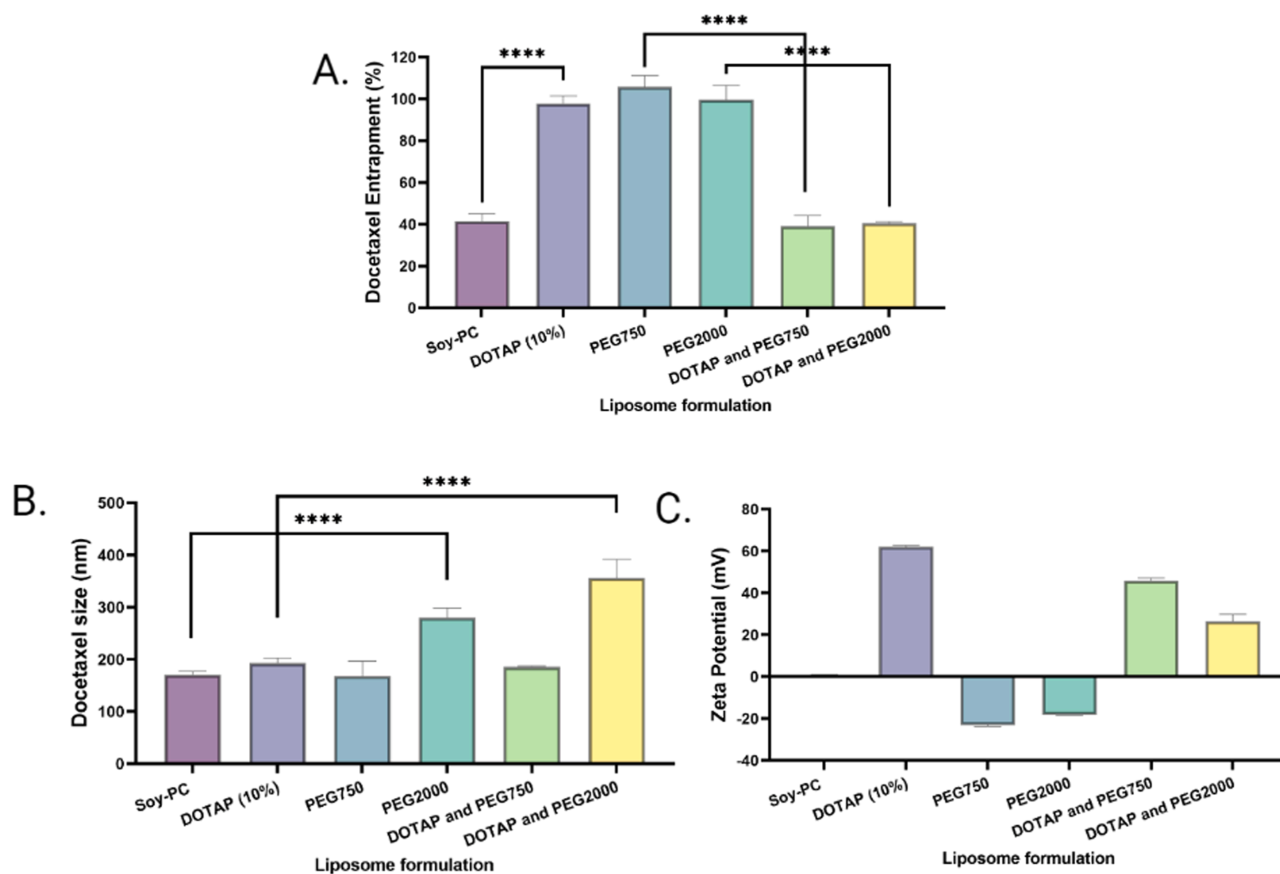

Fig. 8. The effect of two different PEGylation polymer length when applied alone in Soy-PC and in combination with 10% DOTAP. Values for docetaxel entrapment (A) liposome size (B) and zeta potential (C) are compared to Soy-PC liposomes and 10% (w/w) DOTAP-liposomes. (mean ± SD, $n = 3$) Abbreviations; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PEG750, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-750]; PEG2000, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000]; Soy-PC, Soy-Phosphatidylcholine.

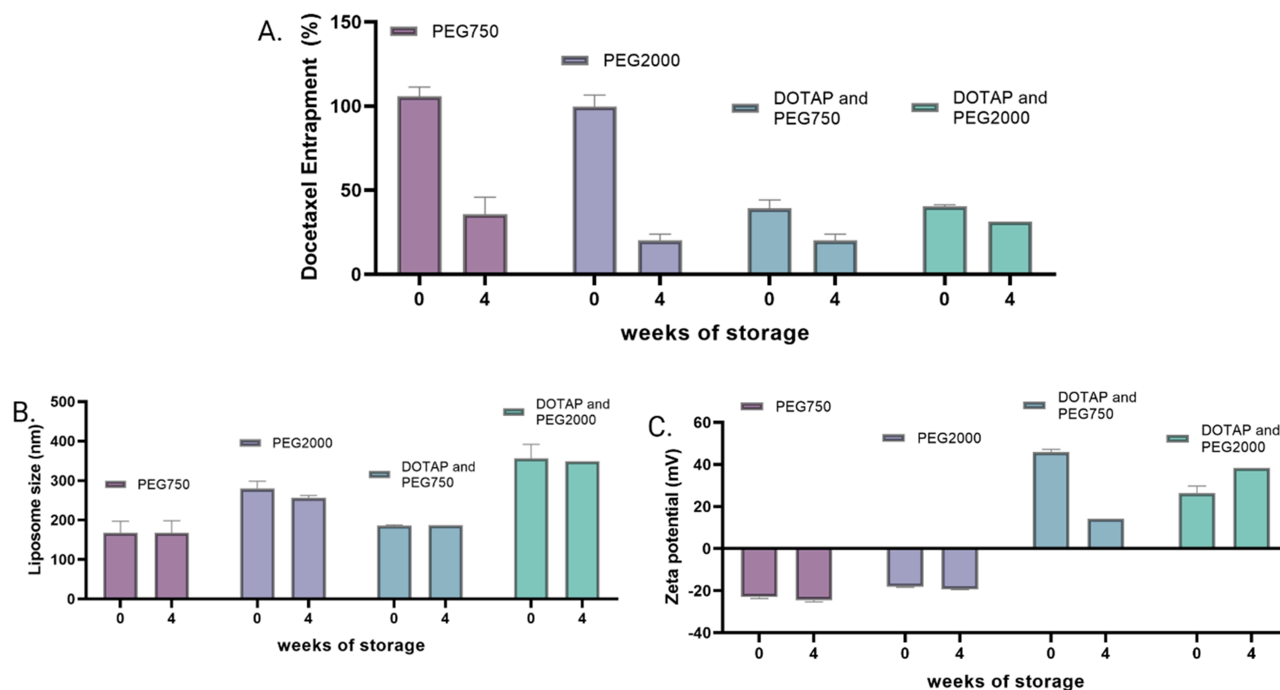

Fig. 9. Docetaxel entrapment (A), liposome size (B) and zeta potential (C) for PEGylated liposomes stored for 4 weeks (mean ± SD, $n = 3$). Abbreviations; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PEG750, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-750]; PEG2000, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000].

evaluate how the storage stability differed between the formulation. As with DOTAP and DMPG liposomes in Section 3.4, we observed, that during storage, DTX-entrapment changed less with DOTAP than with the negatively charged vesicles (in this case PEGylated liposomes) (Fig. 9A) All formulations maintained their original vesicle size during the 4 weeks of storage (Fig. 9B), and the same was true with regard to ZP for the PEGylated liposomes without DOTAP. With PEG750 a significant drop in ZP was observed during storage, whereas with DOTAP2000 a moderate rise in ZP was observed. Thus, it seems the charge needs some time to equilibrate/PEGylation to stabilize. Both PEGylated liposomes had a big drop in DTX-EE during the 4 weeks of storage, and consequently the final entrapment was on the same level for all formulations after storage, and between approx. 20 and 30%.

3.7. Drug release study

For the liposome formulations to obtain a passive targeting of solid tumors trough the enhanced permeability and retention effect (EPR-effect), the carriers should not only escape the reticuloendothelial system, but also have the ability to hold the drug until reaching its target site (Shi et al., 2017). In a dialysis set-up, liposome formulations as well as a commercial DTX-infusion concentrate (Docetaxel Kabi 20 mg/mL) were diluted in dH_2O before immersed in a dialysis bag that was placed in the acceptor medium, consisting of PBS (pH 7.4) with 1% (w/v) albumin. Albumin was added to maintain sink conditions (as described in Section 2.5). The DTX-release from these formulations were followed by determining the concentration of DTX in the dialysis bag over time. The start concentration of the drug relied on the initial entrapment efficiency of the drug in the formulations (Fig. 10), as the supernatant of the centrifuged liposome dispersion (removing unentrapped drug into the pellet) was diluted 1:10 (v/v) in dH_2O. The diluted sampled were analyzed by HPLC to determine the initial concentration of the drug, thereafter by withdrawing 500 μL sample from the dialysis bag after one and three hours. At the end of the study, after 22 h of dialysis, the DTX-concentration was also determined. The initial concentrations of these samples were as expected from previous studies with the same formulations (Fig. 8), demonstrating again the very good reproducibility of the obtained entrapment in liposomes when prepared by DAC. The cumulative amount of drug remaining and not released into the acceptor media was calculated for all formulations and the results are shown in Fig. 10. From these results, we could see that as expected, DTX-release into the acceptor media is mainly taking place the first hour(s). For the commercial product (Docetaxel Kabi™), there is a concentration dependent release, that continues throughout the 22 h of testing. For the liposome formulations a different release kinetics is observed, and especially with PEGylated liposomes the DTX -concentration is close to constant after the first hour, when the free drug is released from the dispersion. Since the initial concentration of DTX is very low for the cationic liposome containing both DOTAP and PEG, these are anyhow less promising than the Soy-PC-PEGylated liposomes, which, in addition to showing a promising DTX-EE, also hold the drug well after the initial burst release of only loosely associated drug, accounting for approx. 26 ± 1% (w/w) of the initial concentration of the drug for both these PEGylated formulation. During the 22 h of dialysis, both PEG-formulations retained a similar final amount of DTX in the dialysis bag of 13.4 ± 2.3 mg/mL (PEG750) and 13.8 ± 2.5 mg/mL (PEG2000), respectively. The DOTAP-PEG liposomes lost approx. 35 ± 1% (w/w) of the initial concentration of the drug during the first hour and had a final concentration of 4.6 ± 0.1 mg/mL (DOTAP-PEG750) and 4.5 ± 0.1 mg/mL (DOTAP-PEG2000) after 22 h of dialysis. Thus, combined DOTAP and PEGylation is not a good blend for DTX-liposomes. Even plain Soy-PC liposomes, which have a higher DTX-entrapment than the DOTAP:PEGylate liposomes seem to be preferred; also since the very low release of DTX is hard to distinguish at the low DTX-concentrations reached for the liposomes with the poorest DTX-EE. The plain DOTAP-liposomes continued to release DTX after 3 h dialysis and did not maintain the DTX concentration similarly to the PEGylated liposomes. However, DOTAP-liposomes released less drug than the commercial product. In conclusion, the stability of the formulations with acceptable DTX-solubilization potential can be ranked in the following order regarding the release of DTX into an aqueous environment; PEG2000 = PEG750 > DOTAP > Docetaxel Kabi™. However, the *in vivo* correlation remains to be assessed, and should be investigated first *in vitro* using closer to *in vivo* conditions with regard to dispersion media, as well as by conducting further investigations *in vivo*.

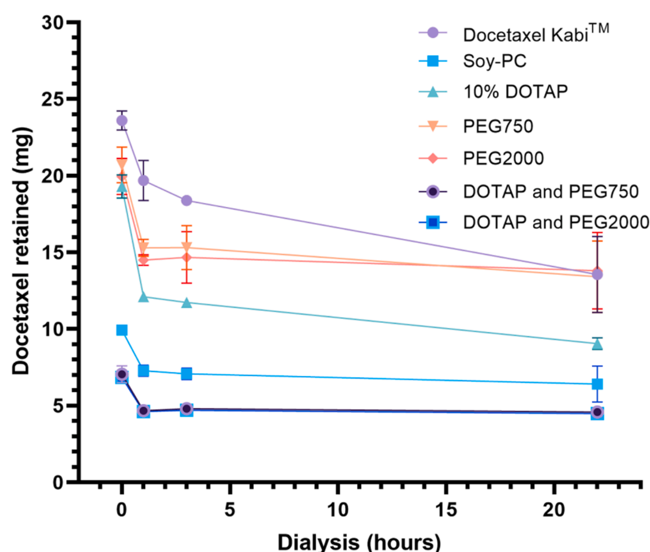

Fig. 10. The docetaxel concentration retained in the dialysis bag expressed before the dialysis started, during dialysis (one and three hours), and after ending the dialysis (22 h), for six different liposome formulations; plain Soy-Phospholipid liposomes (Soy-PC), Soy-PC-liposomes with a 1:10 w/w DOTAP/Soy-PC (10% DOTAP), PEGylation Soy-PC liposomes (PEG750 and PEG2000) and PEGylated Soy-PC:DOTAP liposomes (DOTAP and PEG750, DOTAP and PEG2000). The release of the commercial infusion concentrate (Docetaxel Kabi™) was included as a control. (mean ± SD, $n = 3$). Abbreviations; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; PEG750, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-750]; PEG2000, 1,2-distearoylsn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000].

4. Conclusions

The liposome entrapment of the poorly water-soluble drug docetaxel (DTX) was found to be highly sensitive to lipid composition. Our novel findings include the observed positive effect of cationic DOTAP- and DC-CHOL lipids on DTX-entrapment. Anionic DMPG lipid showed a temporary increase in the attraction of DTX, which was not retained during storage, whereas both, cholesterol and ceramides decreased the affinity of the liposome bilayer towards DTX. PEGylated liposomes were observed to give a positive effect on DTX-entrapment, but to have a negative effect when added to DOTAP-containing liposomes. These effects could be accurately determined due to the highly reproducible liposome preparation DAC-method, enabling us to both observe the effect of formulation on DTX-entrapment, as well as the effect of DTX on liposome size and surface charge. The further studies should explore more the stability of the liposomes in closer to *in vivo* conditions and the effect of formulations on cytotoxicity to select the most promising formulations for clinical development and scaling up.

CRediT authorship contribution statement

Ann Mari Holsæter: Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Visualization, Project

administration, Supervision. **Kristina Wizgird:** Validation, Investigation. **Iselin Karlsen:** Validation, Investigation. **Jeanette Frimand Hemmingsen:** Validation, Investigation. **Martin Brandl:** Conceptualization, Writing – review & editing, Resources, Supervision. **Nataša Škalko-Basnet:** Conceptualization, Writing – review & editing, Resources, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2022.106267.

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