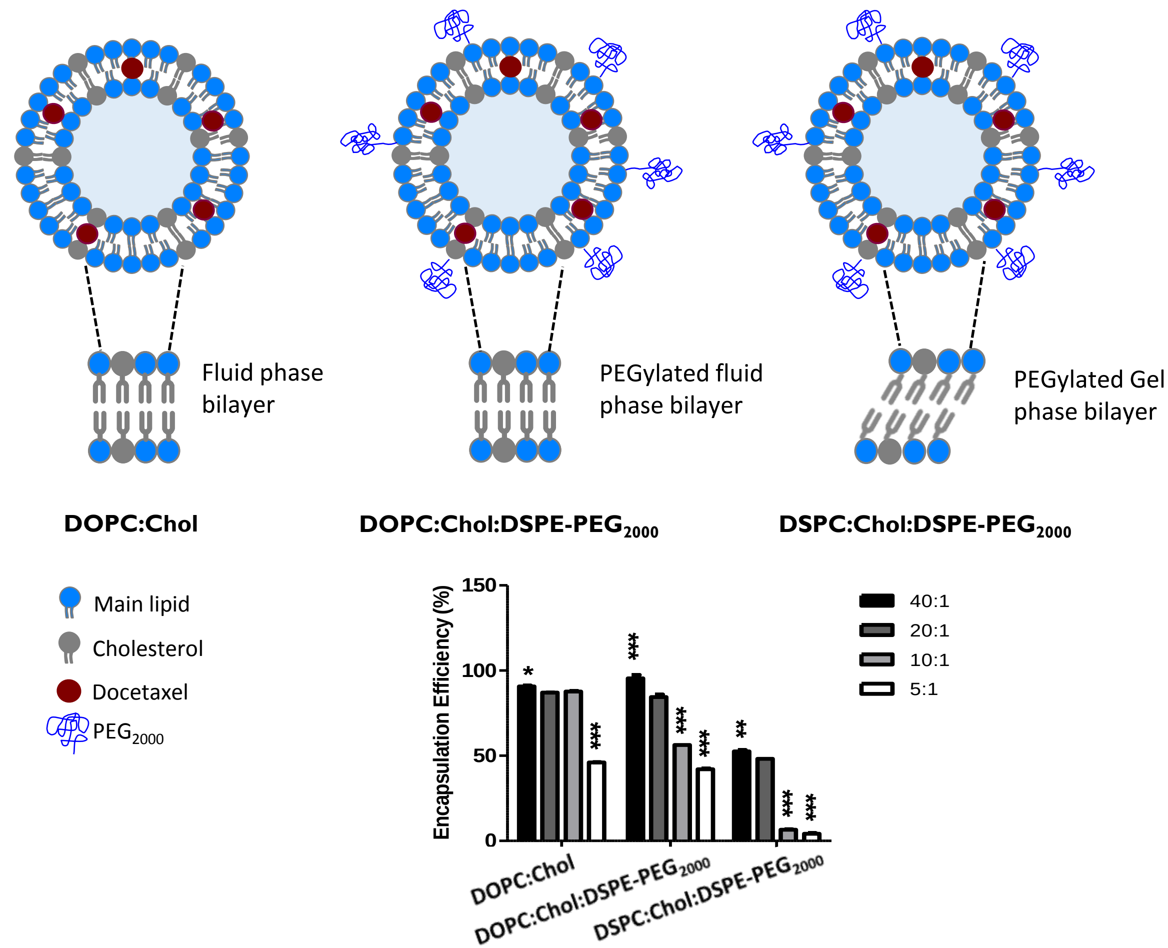
Fluid phase bilayer
PEGylated fluid phase bilayer
PEGylated Gel phase bilayer
DOPC:Chol
DOPC:Chol:DSPE-PEG2000
DSPC:Chol:DSPE-PEG2000
Main lipid
Cholesterol
Docetaxel
PEG2000
Encapsulation Efficiency (%)
150
100
50
0
40:1
20:1
10:1
5:1
DOPC:Chol
DOPC:Chol:DSPE-PEG2000
DSPC:Chol:DSPE-PEG2000

Docetaxel-loaded liposomes: the effect of lipid composition and purification on drug encapsulation and *in vitro toxicity*

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Abstract

Docetaxel (DTX)-loaded liposomes have been formulated to overcome DTX solubility issue, improve its efficacy and reduce its toxicity. This study investigated the effect of steric stabilisation, varying liposome composition, and lipid:drug molar ratio on drug loading and on the physicochemical properties of the DTX-loaded liposomes. Size exclusion chromatography (SEC) was used to remove free DTX from the liposomal formulation, and its impact on drug loading and *in vitro* cytotoxicity was also evaluated. Liposomes composed of fluid, unsaturated lipid (DOPC:Chol:DSPE-PEG$_{2000}$) showed the highest DTX loading compared to rigid, saturated lipids (DPPC:Chol:DSPE-PEG$_{2000}$ and DSPC:Chol:DSPE-PEG$_{2000}$). The inclusion of PEG showed a minimum effect on DTX encapsulation. Decreasing lipid:drug molar ratio from 40:1 to 5:1 led to an improvement in the loading capacities of DOPC-based liposomes only. Up to 3.6-fold decrease in drug loading was observed after liposome purification, likely due to the loss of adsorbed and loosely entrapped DTX in the SEC column. Our *in vitro* toxicity results in PC3 monolayer showed that non-purified, DTX-loaded DOPC:Chol liposomes were initially (24 h) more potent than the purified ones, due to the fast action of the surface- adsorbed drug. However, we hypothesize that over time (48 and 72 h) the purified, DTX-loaded DOPC:Chol liposomes became more toxic due to high intracellular release of encapsulated DTX. Finally, our cytotoxicity results in PC3 spheroids showed the superior activity of DTX-loaded liposomes compared to free DTX, which could overcome the DTX poor tissue penetration, drug resistance, and improve its therapeutic efficacy following systemic administration.

Keywords: Docetaxel, liposome, purification, spheroid, prostate cancer, PC3

1. Introduction

Taxanes are a key class of anticancer agents that are clinically used to treat a wide range of conditions, including breast and prostate cancers (Gelmon, 1994). They inhibit cell proliferation by inducing a sustained mitotic blockage at the metaphase/anaphase stage of the cell cycle, promoting polymerization of stable microtubules and preventing their disassembly (Horwitz, 1992; Lavelle et al., 1995). However, as shown by clinical trials, paclitaxel and docetaxel (DTX), predispose patients to toxicities such as neutropenia, peripheral neuropathy and hypersensitivity reactions, narrowing their therapeutic window (Rowinsky, 1997; Weiss et al., 1990). DTX, a member of the taxanes family, has been particularly promising as a therapeutic agent (Friedenberg et al., 2003) and is preferable to paclitaxel (PTX) due to its enhanced solubility in water and increased potency (Grant et al., 2003). Taxotere® is the commercially available formulation of DTX. Currently, the presence of Tween 80® (polysorbate 80) and ethanol (50:50, v/v) in Taxotere® formulation has been associated with serious hypersensitivity reactions in patients (Tan et al., 2012).

Encapsulation of anti-cancer drugs in nanoformulations, such as liposomes, can decrease drug clearance and reduce its associated toxicity (Crosasso et al., 2000). Liposomes are the most-clinically developed delivery systems. They are biodegradable, biocompatible and have the ability to encapsulate both hydrophilic and lipophilic drugs (Zhang et al., 2012). A phase I clinical trial was carried out with liposomal docetaxel for advanced solid tumour, and showed good tolerance profile and clinical benefits. As an outcome, a phase II trial has been planned with a dose of 85 mg/m^2 triweekly (Deeken et al., 2013). In the past few years, several efforts have been made to develop a successful nanocarrier for DTX. Muthu M. et al. engineered D-alpha-tocopheryl PEG$_{1000}$ succinate mono-ester (TPGS) coated liposomes for the delivery of DTX and quantum dot nanoparticles. These liposomes were prepared by the solvent injection method and presented DTX encapsulation efficiency up to 54 %, higher than those obtained with PEG-coated and conventional liposomes. Cellular uptake studies in C6 glioma brain cancer cells further revealed superior uptake of these liposomes compared to those obtained with PEG-coated and conventional liposomes (Muthu et al., 2012). In another study, Li X. et al. developed folate-poly (PEG-cyanoacrylate-co-cholesteryl cyanoacrylate) (FA-PEG-PCHL)-modified freeze-dried liposomes for targeted DTX chemotherapy, which displayed sustained release profile, promoted cell toxicity and apoptosis and enhanced the bioavailability at the tumour site (Li et al., 2011). In an attempt to target transferrin receptor (TfR) in cancer cells, Zhai et al. prepared DTX-loaded liposomes composed of hydrogenated soy phosphatidylcholine (HSPC)/ egg phosphatidylcholine (PC)/ cholesterol (Chol)/ mPEG$_{2000}$-DSPE by a post-insertion method. TfR-targeted liposomes loading DTX induced higher toxicity than the non-targeted liposomes in KB cells (Zhai et al., 2010).

Drug content, liposome size, liposome stability and blood circulation are key parameters that should be taken into account while developing liposome-based formulations (Senior and Gregoriadis, 1982). High drug loading is always desirable to accelerate the clinical translation of liposomal formulations, since high lipid concentrations may raise concerns of toxicity and reduce the viability of large-scale production (Straubinger and Balasubramanian, 2005). The present study focuses on investigating the effect of lipid composition, steric stabilisation (known as pegyaltion) and varying DTX to lipid ratio on DTX encapsulation into liposomes. The effect of purification using size exclusion chromatography (SEC) was also evaluated and the *in vitro* toxicity of non-purified and purified DTX-loaded liposomes was assessed in prostate cancer cell lines. In addition, prostate cancer (PC3) spheroids were used to demonstrate the enhanced toxicity of DTX-loaded liposomes in multicellular tumour spheroids.

2. Materials and Methods

2.1 Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG_{2000}) were a generous gift from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (Chol), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, ≥ 99.5 %), sodium chloride (≥ 99.0 %) and resazurin reagent (R7017) were purchased from Sigma-Aldrich (UK). Chloroform (HPLC grade), acetonitrile (HPLC grade), methanol (HPLC grade), ethanol (absolute 99.8 %) and sterile 0.2 µm pore size syringe filters were all obtained from Thermo Fisher Scientific (UK). Advanced Roswell Park Memorial Institute (RPMI) 1640 with 2 g/L D-glucose, 110 mg/L sodium pyruvate and non-essential amino acids, GlutaMAX™ supplement 200 mM, penicillin-streptomycin Solution liquid (10000 units/ml), 0.05 % Trypsin/EDTA, Trypan Blue Stain (0.4 %, 1:1 v/v) and Dulbecco's PBS (1X) were purchased from Invitrogen Gibco® Life Technologies (UK). Heat inactivated newborn fetal bovine serum (FBS) was obtained from First Link (UK). Docetaxel (DTX) was supplied by NCE Biomedical Co., Ltd (China).

2.2 Preparation of DTX-loaded liposomes

Liposomes were prepared by thin film hydration method as previously described (Al-Jamal et al., 2008). Briefly, stock solution of lipids, DTX and solvent, chloroform: methanol (4:1, v/v), were added to a 25 mL round bottom flask (Thermo Fisher Scientific UK). The lipids had a fixed concentration of 5 mM, while DTX concentration was varied to reflect the desired phospholipid to drug molar ratio. Liposomes were prepared at different lipid: DTX molar ratios (40:1, 20:1, 10:1 and 5:1). DOPC:Chol:DSPE-PEG_{2000}, DPPC:Chol:DSPE-PEG_{2000} and DSPC:Chol:DSPE-PEG_{2000} were prepared at molar ratio of 95:50:5, while DOPC:Chol liposomes were prepared at a molar ratio of 100:50. Total phospholipid: cholesterol molar ratio was kept at 2:1. The organic solvents were then removed under rotary evaporation at $T > T_c$ i.e. 60 °C for DOPC and DPPC and 65 °C for DSPC, at around 144 rpm which led to the formation a thin lipid film. The film was hydrated for 30 min with 1 mL of 0.2 µm filtered HEPES Buffer Saline (HBS) at 60 °C for DOPC and DPPC, and at 65 °C for DSPC formulation. In order to obtain small unilamellar vesicles (SUVs) from the hydrated lipids, extrusion technique was used. Extrusion was carried out at 60 °C for DOPC and DPPC liposomes and at 65 °C for DSPC liposomes. Briefly, the hydrated mixture was passed through a series of polycarbonate membranes of decreasing pore size (0.8 µm, 5 cycles; 0.2 µm, 15 cycles; 0.1 µm, 11 cycles) using a mini-extrusion device (Avanti Polar Lipids Inc, AL, USA). Samples were collected before the extrusion process in order to determine DTX encapsulation efficiency (EE) and the lipid loss. Liposomes were flushed with N_2 stream and stored at at 4 °C. The next day, liposomes were purified by SEC using PD-10 columns packed with Sephadex™ G-25 medium (GE Healthcare Life Sciences, UK). Following SEC, samples were left at least 2 h at RT before further characterization.

2.3 Physicochemical characterisation of DTX-loaded liposomes

Measurement of the hydrodynamic diameter and zeta potential (ZP) of the prepared formulations were performed using dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS (Malvern, UK; He-Ne laser). Disposable polystyrene cells and disposable plain folded capillary Zeta cells (Malvern, UK) were used. Suspensions were diluted in 0.2 µm filtered deionized water at ratios of 1:100 for size measurements and 1:10 for ZP measurements. All measurements were performed at 25 °C. Electrophoretic mobility was used to calculate the ZP using the Helmholtz-Smoluchowski equation. The hydrodynamic size was presented as the average value of 20 runs with triplicate measurements within each run, while ZP measurements were performed in quintuplicate.

2.4 Determination of DTX encapsulation efficiency and loading content

DTX was quantified either by high performance liquid chromatography (HPLC) or by UV-Vis spectrophotometry in order to evaluate which method was more sensitive and accurate.

2.4.1 HPLC

Briefly, 50 µL of DTX-loaded liposomes before extrusion, before purification and after purification were diluted 1:10 (v/v) in acetonitrile sonicated for 10 min to ensure complete liposome disruption. 20 µL were injected in an analytical HPLC column (Eclipse XDB-C18 4.6x150 mm, 5 µm, Agilent UK) and 15 min runs were performed in a mobile phase composed of water: acetonitrile (50:50, v/v). The flow rate was set at 1.0 mL/min and DTX was detected at λ= 230 nm. The calibration curve was linear in the range of 0.05-400 µg/mL with a correlation coefficient of R^2= 0.997. DTX encapsulation efficiency (EE) and drug loading were calculated using equations (1) EE= µg of encapsulated drug/ µg of total drug x 100 and (2) Drug loading= µg of drug/ mg of total lipid. Measurements were performed in triplicate and results were presented as mean ± SD.

2.4.2 UV-Vis spectrophotometry

Samples of DTX liposomes before extrusion and after extrusion (before purification) were withdrawn and made up with ethanol, maintaining a fixed concentration of 20 µg/mL. Ethanol was used to disrupt the liposomes and release DTX. A lambda 35 UV-Vis spectrophotometer (Perkin Elmer Lambda, USA) was used to determine the absorbance of DTX at λ= 230 nm. The calibration curve was linear in the range of 0-45 µg/mL with a correlation coefficient of R^2= 0.995. The obtained DTX concentrations were then used to determine DTX encapsulation efficiency before purification.

2.5 Phospholipid quantification by Stewart assay

Phospholipid content was determined in samples before extrusion, before purification and after purification according to the previous protocol established by Stewart (Stewart, 1980). Briefly, 2 mL of chloroform were transferred to 15 mL test tubes (Thermo Fisher Scientific, UK) and 20 µL of liposome samples were added to the organic solvent. Finally, 2 mL of 0.1 M ammonium ferrothiocyanate were transferred to the same tube and the components were vortexed vigorously for 15 s. For phase separation, the mixture was centrifuged at 1000 x g, 10 min and the organic phase recovered by careful pipetting. The lipid absorption was then measured using a Lambda 35 spectrophotometer (Perkin Elmer, USA) at 465 nm. Phospholipid content was determined using a calibration curve performed with empty liposomes. Triplicate samples were prepared for all measurements and blanks were used for baseline reference. The results were expressed as mean ± SD.

2.6 PC3 culture and maintenance

Androgen-independent PC3 cells (CRL-1435™) were purchased from American Type Culture Collection (ATCC®, USA). PC3 cells were cultured in Advanced RPMI 1640 supplemented with 10 % FBS, 50 U/mL penicillin, 50 µg/mL streptomycin and 1 % L-glutamine and maintained in a humidified chamber (BB15 CO_2 incubator, Thermo Fisher Scientific, UK) at 37 °C in 5 % CO_2. Cells were routinely grown in 75 cm^2 canted-neck tissue culture flasks and passaged twice a week using 0.05 % Trypsin/ EDTA when reaching 80 % confluence in order to maintain exponential growth.

2.7 Cytotoxicity of DTX-loaded liposomes in prostate cancer (PC3) monolayers

PC3 cells were trypsinized, stained with Trypan Blue (0.4 %, 1:1 v/v ratio) and counted using a hemocytometer. Cells were seeded at a seeding density of 1 x 10^4 cells/well in polysterene 96-well plates (Nunclon, Thermo Fisher Scientific, UK) in complete RPMI 1640 media. Next day, non-purified or purified DOPC:Chol or DOPC:Chol:DSPE-PEG_{2000} liposomes (prepared at 20:1 lipid:DTX molar ratio) containing 1-1000 nM of DTX were diluted in serum-free and antibiotics-free media and added to the cells. Untreated cells were used as a 100 % viability control. After 4 h incubation, media containing liposomes were removed, cells were washed with PBS and replenish with fresh media supplements with 10 % (v/v) FBS and 1 % (v/v) antibiotics. At 24, 48 and 72 h post-incubation, a resazurin cell viability assay was performed, which is based on the mitochondrial metabolic activity of live cells. Resazurin reagent was prepared as described by Walzl *et al.* (Walzl et al., 2014). Briefly, cells were incubated with 0.01 mg/mL resazurin solution for 4 h. After incubation, fluorescence (λ_{ex} = 544 nm, λ_{em} = 590 nm) was read using an automated FLUOstar Omega (BMG Labtech, UK) plate reader. Six replicates per condition were used. The results were expressed as the percentage of cell viability (mean ± SEM) and normalized to control untreated cells.

2.8 Cytotoxicity of DTX-loaded in PC3 multicellular tumour spheroids

PC3 multicellular tumour spheroid formation and growth were aided by coating polystyrene 96-well plates with 100 µL of 1 % (w/v) agarose in order to provide a non-adherent concave surface. PC3 cells were suspended using 0.05 % Trypsin/ EDTA and 5 x 10^3 cells/well were seeded onto the pre-coated plates in 200 µL of complete RPMI 1640 media. Spheroid growth was monitored daily and 50 % of media was replenished every 3 days. PC3 spheroids were grown for 7 days until they reached ~ 0.7 mm in diameter before use. For cytotoxicity assessment, spheroids were incubated with DOPC (± PEG) liposomes containing 1-10000 nM of DTX. For comparison purposes, spheroids were also incubated with 0.1-1000 nM of free DTX. Following treatment, free DTX toxicity and DTX-loaded liposomes toxicity were assessed at 48, 72 and 96 h by resazurin assay. Briefly, 100 µL of media was removed from each well and spheroids were incubated with 0.01 mg/mL resazurin solution for 4 h. After incubation, fluorescence (λ_{ex} = 544 nm, λ_{em} = 590 nm) was read using an automated FLUOstar Omega (BMG Labtech, UK) plate reader. Six replicates per condition were used. The results were expressed as the percentage of cell viability (mean ± SEM) and normalized to control untreated spheroids.

2.9 Statistical analysis

Statistical analysis was performed using a two-way analysis of variance (ANOVA). In all cases, post hoc comparisons of the means of individual groups were performed using Bonferroni´s test. A significance level of $p < 0.05$ denoted significance in all cases. Statistical analysis was performed using GraphPad Prism® version 6.0h (GraphPad Software Inc., CA, USA).

3. Results

3.1. Preparation and characterisation of DTX-loaded liposomes

In the present study, DTX-loaded, DOPC:Chol, DOPC:Chol:DSPE-PEG$_{2000}$, DPPC:Chol:DSPE-PEG$_{2000}$ and DSPC:Chol:DSPE-PEG$_{2000}$ liposomes were prepared using lipid film hydration and extrusion method. Liposomes were prepared at different lipid:DTX molar ratios (40:1, 20:1, 10:1 and 5:1) and purified using size exclusion chromatography (SEC). Cholesterol was included in all formulations at 50 mol % to stabilise the bilayer structure and improve liposome stability in serum (Drummond et al., 2008; Kirby and Gregoriadis, 1980). 5 mol % DSPE-PEG$_{2000}$ was incorporated in the formulation to sterically stabilise the liposomes and prolong their blood circulation *in vivo* (Lokerse et al., 2016).

Table 1

Table 1 represents the physicochemical properties (the size and the surface charge) of DTX-loaded, DOPC:Chol liposomes (100:50 molar ratio). Liposomes were formed at all different lipid:DTX ratios used. Interestingly, DTX-loaded liposomes, were smaller than the empty liposomes. Moreover, lowering lipid:DTX molar ratios consistently showed liposomes with smaller size and lower polydispersity index (PdI). The same trend was maintained following SEC purification step (Table 1). However, the purified liposomes exhibited slightly smaller size and lower PdI, which could be explained by the removal of unencapsulated DTX aggregates. No difference was observed in the zeta potential of all purified samples (Table 1), which was expected due to the incorporation of DTX into the lipid bilayer.

Table 2

Next, we evaluated the incorporation of DTX into sterically stabilised liposome formulations. Liposome fluidity was maintained by using DOPC:Chol:DSPE-PEG$_{2000}$ formulation. PEGylated liposomes were smaller in size (ranging from 112-126 nm) compared to non-PEGylated DOPC liposomes (ranging from 129-162 nm), across all lipid:DTX ratios (Table 2). The incorporation of DSPE-PEG_{2000} was confirmed by a slight increase in the negative charge of liposomes (increased from -5 mV to -9 mV) (Table 1 and 2). This could be explained by the presence of the carbamate linker that is used to couple PEG_{2000} chain to DSPE, resulting in a net negative charge on the phosphate moiety at physiological pH (Webb et al., 1998). Similar to previous results, a reduction in liposome size was observed by decreasing lipid:DTX molar ratios.

Table 3

PEGylated liposomes with higher phase transition, such as DPPC and DSPC exhibited similar patterns to DOPC:Chol:DSPE-PEG_{2000}, where smallest liposomes were obtained at low lipid:DTX molar ratio (5:1) (Table 3 and 4). Interestingly, purified DPPC:Chol:DSPE-PEG_{2000} and DSPC:Chol:DSPE-PEG_{2000} liposomes displayed lower PdI values across all the lipid:drug ratios tested in comparison to DOPC-based liposomes (Table 2). Such observation suggests that incorporation of DPPC and DSPC as the main lipid in the formulation resulted in narrowly-dispersed nanoparticles with no aggregation in water. Moreover, liposome size did not significantly change after purification as big drug aggregates, observed with DSPC and DPPC at 10:1 and 5:1 molar ratios, were lost during the extrusion step. All formulations were stable in size up to one-month storage at 4°C (Table A.1-A.4).

Table 4

3.2. The effect of size exclusion chromatography on DTX encapsulation into liposomes

Following characterising DTX-liposomes for size and surface charge, the encapsulation efficiencies (EE) of DTX into liposomes with different lipid compositions were determined using HPLC. Unsaturated lipid (DOPC:Chol) and PEGylated, unsaturated DOPC:Chol:DSPE-PEG_{2000}, and saturated DPPC:Chol:DSPE-PEG_{2000} and DSPC:Chol:DSPE-PEG_{2000} liposomes were prepared at different lipid:DTX molar ratios (40:1, 20:1 ,10:2, 5:1) using lipid film hydration and extrusion method. Figure 1a depicts the EE of DTX into liposomes, which was calculated as a ratio between the amount of drug encapsulated and the initial amount used. Non-PEGylated, unsaturated DOPC:Chol liposomes showed the highest EE between all formulations (around 90%) up to 10:1 lipid:DTX molar ratio, then dropped to 46.1 % at 5:1 molar ratio. Incorporating 5 mol % DSPE-PEG$_{2000}$ into DOPC:Chol liposomes affected the EE of DTX at lipid:DTX molar ratio of 10:1. The highest EE (> 95 %) was still observed at 40:1 lipid:DTX molar ratio, and the lowest percentage (< 40 %) at 5:1 ratio. This result highlights the effect of PEG$_{2000}$ on the EE of some drugs, which should be taken into account while designing sterically stabilised, long circulating liposomes for *in vivo* applications.

Strikingly, when saturated lipids (DPPC and DSPC) were used, lower EE were achieved. Highest EE (< 55 %) was obtained at high lipid:DTX molar ratio (40:1) with both formulations. Decreasing lipid:DTX molar ratio to 5:1 resulted in a significant reduction in the EE (< 10 %). In general, DSPC:Chol:DSPE-PEG$_{2000}$ liposomes encapsulated higher amount of DTX than DPPC-based formulation (Figure 1a). As mentioned previously, large precipitates were observed immediately after hydrating DPPC and DSPC formulations with high DTX contents (10:1 and 5:1 lipid: DTX molar ratios), which resulted in high DTX loss on the membrane filters during extrusion. Similar profile of EE was observed using UV-Vis spectrophotometry, however, overestimation of EE was observed with DOPC-based formulations. Most probably due to lipid interference at the applied wavelength (data not shown). This confirms that HPLC is more accurate technique to quantify the EE of DTX into liposomes, particularly following the purification step.

Figure 1

Following liposome purification using SEC, a significant reduction in EE was observed with all liposome formulations (data not shown). The EE could not be measured in DPPC and DSPC formulations as the amounts of the encapsulated DTX were below the detection limit of the machine (0.50 ng/mL). To check if the drastic reduction in EE after purification was due to liposome loss during SEC, a Stewart assay was carried out to quantify the phospholipids recovery after extrusion (before purification) and purification processes (Table A.5). All formulations showed lipid recoveries following SEC, ranging between 90-100 %. These results show that the lipid loss during SEC did not account for such a significant amount of drug that was lost during purification. We believe that if the drug was not fully embedded in the bilayer but was rather associated with the outer surface of the liposomes, it could be lost during the purification process, leading to a reduction in the EE without an accompanying decrease in the lipid content.

Figures 1b depicts the drug loading of DTX into liposomes, where the results were expressed as mol% of DTX/total lipid. It is important to highlight the different profiles obtained between EE and drug loading. Prior purification, DOPC:Chol liposomes showed rather similar drug EE across 40:1, 20:1 and 10:1 lipid:drug ratios and a marked reduction at 5:1 ratio (Figure 1a). However, this trend was not observed with drug loading, where the amount of DTX loaded into the liposomes increased with the decrease in the lipid:drug ratio (increased from 2.5 mol% DTX/total lipid to 9.7 mol% DTX/total lipid, at 40:1 and 5:1 ratios, respectively, Figure 1b). Same trend was observed with PEGylated DOPC:Chol:DSPE-PEG$_{2000}$ formulation, where the profile of the drug loading was inversely proportional to the EE. Thus, drug loading increased as lipid:drug ratio lowered, reaching a maximum of 9.7 $\pm$ 0.11 mol% DTX/total lipid at 5:1 ratio. When saturated DPPC lipid was used as the main lipid, drug loading ability of the nanocarrier was extremely poor independently of the initial amount of drug. Similarly, DSPC liposomes displayed drug loadings in the range of DPPC liposomes, although the highest lipid:drug ratios (40:1 and 20:1) were superior compared to 10:1 and 5:1 ratios. These results were in agreement with data obtained for DSPC EE before purification (Figure 1a). Following SEC purification, 1.6- and 3.6-fold decrease in drug loading was observed with both DOPC-based liposomes (Figure 1c), in line with the results obtained for the EE (data not shown). Due to the reduced loading capacity of DPPC and DSPC liposomes and because of the high drug lost during purification process, it was not possible to assess the drug loading for these formulation (Figure 1c). Overall, these results emphasise the importance of expressing the data as drug loading as well as EE, as in some cases expressing the results as EE could be misleading. For instance, DOPC-based formulations (conventional or stealth liposomes), although the highest EE were obtained for the lower lipid:drug ratios, liposomes prepared at 5:1 ratios encapsulated the highest amount of DTX, despite the high drug loss during extrusion and purification steps.

3.3. The effect of purification on the toxicity of DTX-loaded liposomes *in vitro*

In the previous section, the effect of SEC purification on DTX loading into liposomes was investigated. In this experiment, we studied the effect of purification on the toxicity of DTX-loaded liposomes *in vitro*. DTX-loaded liposomes were prepared by incorporating DTX into DOPC:Chol and DOPC:Chol:DSPE-PEG_{2000} liposomes at 20:1 lipid:DTX molar ratio. Other Liposomal formulations were not tested due to the poor encapsulation efficiency of DTX. Prostate cancer (PC3) cells were incubated with the same concentration of DTX, which was ranging between 1-1000 nM, as determined by the HPLC. Cells were incubated with non-purified, and purified DTX-loaded liposomes (DOPC:Chol and DOPC:Chol:DSPE-PEG_{2000}) for 4 h in serum-free media, then cells were washed and incubated with fresh serum-containing media for 24, 48 or 72 h. Cell viability was determined using the resazurin cell viability assay. Figure 2 depicts the dose-response curves and the relative IC_{50} (defined as the half maximal inhibitory concentration) and % I_{max} (defined as the maximum inhibition, here the lowest cell viability achieved) of DTX-loaded DOPC:Chol and DOPC:Chol:DSPE-PEG_{2000} liposomes at the different time points. As it can be seen in Figure 2a, at 24 h, DTX-loaded, DOPC:Chol liposomes had comparable potencies before and after purification (IC_{50} = 3.1 ± 0.5 nM *vs.* 2.3 ± 0.9 nM, respectively). However, the non-purified DOPC:Chol liposomes led to a significant higher cell death at DTX concentrations between 10-1000 nM (% I_{max}= 42.2 ± 0.7) compared to purified liposomes (% Imax = 54.8 ± 1) (Figure 2a). This observation could be explained by the rapid onset of loosely-attached DTX to the liposome surface, thus exhibiting a “free drug-like behavior”. DTX that was not fully embedded in the lipid bilayer would have a faster onset of action compared to the DTX that was fully encapsulated in the liposomes (i.e. purified samples), which encountered an extra barrier of liposome release before exerting its cytotoxicity. However, at 48 h the incubation time was long enough to allow the encapsulated DTX to be released intracellularly from DOPC:Chol liposomes, leading to a higher cytotoxicity compared to 24 h time point. At 48 h, the purified DTX-loaded DOPC:Chol liposomes were more potent than the non-purified ones (IC_{50} = 1.6 ± 0.1 nM *vs.* 5.2 ± 0.5 nM, respectively) (Figure 2b), which could be explained by the higher stability of DTX fully-encapsulated into liposomes, compared to loosely-associated DTX with the outer surface of the liposomes. Similarly, 72 h post incubation, purified DOPC:Chol liposomes were significantly more potent than non-purified DOPC:Chol liposomes (IC_{50}= 2.1 ± 0.1 nM *vs.* IC_{50}= 3.7 ± 0.1 nM, respectively) (Figure 2c). On contrary to non-PEGylated liposomes DOPC:Chol, DOPC:Chol:DSPE-PEG_{2000} showed no statistically significant toxicity profile between non-purified and purified liposomes, presumably due to the steric effect of PEG chains, which may delay internalization of DTX-loaded liposomes into cells. It is worth noting that at concentrations between 1-5 nM, PEGylated liposomes were less cytotoxic compared to the non-PEGylated equivalents, however, at higher concentrations the toxicity of PEGylated liposomes was similar to that achieved with non-PEGylated DOPC:Chol liposomes.

Figure 2

3.4 Toxicity of DTX-loaded liposomes in prostate cancer tumour spheroids

In order to assess the cytotoxicity of DTX-loaded liposomes in tumour spheroids, DTX-loaded, DOPC:Chol liposomes (conventional or stealth) were prepared at 20:1 lipid:drug molar ratio, purified using SEC, then incubated in complete media with PC3 prostate cancer spheroids for 48, 72 and 96 h. Spheroids were incubated with free DTX for comparison.

Overall, PC3 spheroids were more resistant to DTX treatment compared to PC3 monolayers. Prostate cancer spheroids incubated with free DTX did not show any dose-dependent or time-dependent toxicity profiles (Figure 3a). No significant difference in cytotoxicity viability was observed in cells incubated with DTX concentrations ranging between 1-10000 nM, where cell viability ranged between 50-60%. Moreover, cell death did not increase with prolonged exposure times (24-72 h). Shorter (24 h) and longer (96 h) DTX exposure times did not enhance the toxicity of free DTX (data not shown). This could be justified by the fast action of free DTX, combined with its poor penetration through the inner cell layers of the spheroids, which could explain the limited efficacy of DTX *in vivo*. In contrast to free DTX, both conventional (non-PEGylated) and PEGylated DTX-loaded liposomes exhibited dose- and time-dependent toxicity profiles. DTX-loaded liposomes showed lower cell death after 48 h incubation, compared to free DTX (Figure 3b & 2c, respectively). Similar observations were obtained in monolayers (data not shown). However, high cell death was observed with DTX-loaded liposomes at the concentrations of 100 and 1000 nM, at longer incubation time (72 and 96 h). This demonstrated the enhanced cell killing of DTX-liposomal formulations compared to the free DTX, under same conditions. These results could suggest the higher stability of DTX upon encapsulation into nanocarriers, as well as the enhanced permeability and diffusion of liposomes in the tumour tissue. Overall, PEGylated, DTX-loaded liposomes were less toxic to PC3 tumour spheroids, compared to non-PEGylated DTX-loaded liposomes, after 48 and 72 h. However, after 96 h incubation, PEGylated DTX-loaded liposomes at high concentrations (100, 1000 nM) induced reduction in the cell viability that was comparable with that achieved with conventional DTX-loaded liposomes. These findings highlight the potential use of liposomes to enhance the therapeutic efficacy of DTX *in vivo* by enhancing the drug accumulation and penetration in tumour tissues.

Figure 3

4. Discussion

Liposomes are the most clinically developed nanosystems to deliver cytotoxic drugs. DTX-loaded liposomes have been formulated to overcome DTX solubility issue, improve its efficacy and reduce its toxicity. However, in order for liposomes to be classed as an effective DTX delivery system, their drug content, *in vivo* stability and bioavailability of DTX at tumour site must all be balanced. A phase I clinical trial (Deeken et al., 2013) was carried out in patients with solid tumour and benefit was seen in 41 % of cases, using liposomal DTX consisting of DOPC:Chol :TMCL (90:5:5 % molar ratio) with an overall lipid:drug ratio of 33:1. Despite the known beneficial effects of PEG_{2000} to increase stability and prolong liposomes blood residency (Harashima et al., 1994; Johnstone et al., 2001; Yuan et al., 1995), it was not included in the formulation used in this clinical trial. Several studies showed that DTX encapsulation into liposomes was highly affected by the composition of the lipid bilayer (Immordino et al., 2003; Manjappa et al., 2013; Muthu et al., 2011; Naik et al., 2010). In this report, we systematically evaluated the effect of liposome composition, steric stabilisation, lipid:drug molar ratios, and the purification step on the final EE and drug loading of DTX into liposomes. Our results showed that the composition and the fluidity of the lipid bilayer had a strong influence on DTX encapsulation. Prior purification, fluid-lipid bilayer liposomes (DOPC:Chol) presented the highest EE across all lipid:drug ratios assessed (Figure 1a). Almost 100 % EE was achieved between 40:1 to 10:1 lipid:DTX molar ratios, however, lowering lipid:DTX molar ratio to 5:1 resulted in a lower EE (46 %). To correct for the incomplete DTX incorporation, drug loading was also calculated for all liposome formulations (Figure 1b). Interestingly, on contrary to the

EE results, DOPC-based liposomes showed the highest DTX loading at 5:1 lipid:DTX molar ratio, where 9.7 mol% DTX/total lipid was obtained, in comparison to 2.5-4 mol% DTX/total lipid at high ratios (40:1 and 20:1). Therefore, our results emphasise the importance of using drug loading, as more realistic and accurate measure of the effective drug dose entrapped in the liposomes.

Lipids with high phase transition temperature (T_m) are known to have longer plasma circulation (Gabizon and Papahadjopoulos, 1988). T_m represents a characteristic temperature of lipid phase change from gel to liquid phase and it is determined by the characteristic lipid structure. DPPC (T_m = 41.85 ˚C) and DSPC (T_m = 55 ˚C) have higher T_m than DOPC (T_m = - 17 ˚C) (Attwood et al., 2013; Prates Ramalho et al., 2011). Moreover, unsaturated lipids such as DOPC are more prone to oxidation, further decreasing stability. Although stability investigation was not carried out in this study, it is expected in line with literature, that saturated lipids, such as DPPC and DSPC will be more stable compared to unsaturated liposomal compositions (Gabizon and Papahadjopoulos, 1988; Huang et al., 1998). In our study, DSPC:Chol:DSPE-PEG_{2000} liposomes showed the highest DTX loading (2.46 mol% DTX /total lipid) at 20:1 lipid:drug ratio. This high loading was not maintained on increasing DTX concentrations in the liposome formulation (Figure 1b). The same trend was seen with DPPC liposomes, however the best formulation, 40:1, gave lower loading (0.91 ± 0.03 mol% DTX/total lipid) than any of the other lipid composition at the same lipid:drug ratio. Other studies reported higher extent of drug incorporation in DSPC liposomes as opposed to DPPC formulation (Anderson and Omri, 2004; Haeri et al., 2014). It was explained by the higher tendency of DPPC to form an interdigitated state during extrusion in comparison to DSPC, which decreases the space between the acyl chains, and therefore leads to increased DTX loss during extrusion and purification steps (Demetzos, 2008). DTX loading trend of both DPPC and DSPC formulations differed significantly compared to DOPC:Chol:DSPE-PEG_{2000}. Unlike the downward trend experienced with decreasing lipid:drug ratio, PEGylated DOPC formulations showed an increasing drug loading ratios tested (Figure 1b). This may be due to differences in the structure of these lipids which affect their drug accommodation behaviour at different DTX concentration. The presence of higher drug content in the rigid bilayers could lead to DTX precipitation in the bilayer and drug loss (Johnston et al., 2006). This result agrees with the literature, where a high lipid content (ca. 35:1) was required to maximise DTX loading into DSPC or DPPC liposomal formulations (Crosasso et al., 2000; Manjappa et al., 2013). To improve DTX encapsulation into rigid liposomes, a mix of DPPC and DSPC lipids could be used (Manjappa et al., 2013; Zhigaltsev et al., 2010). Furthermore, the cholesterol content may need to be lowered, as both molecules occupy the same region in the lipid bilayer (Lian and Ho, 2001; Zhang et al., 2005).

With respect to size distribution, all liposomes formulations maintained their homogeneity irrespective of varying lipid:drug ratio (Tables 1-4), with relatively smaller sizes with PEGylated liposomes. Interestingly, DTX-loaded liposomes resulted in a smaller size than empty liposomes. The differences in liposomal size are not significant throughout lipid:drug ratios 40:1, 20:1 and 10:1. Only lipid:drug ratio 5:1 presented lower size and this was consistent independently of the lipid composition used. Although further studies would be required to evaluate how this would affect the stability and drug release, we hypothesize that at the lower lipid:drug ratio the presence of DTX within the liposome bilayer is increasing the packing of the phospholipids (specially the more fluid ones composed of DOPC). A higher packing would allow increased curvature and therefore smaller size.

Complete removal of free drug is a prerequisite for accurate drug dosing. Due to the wide range of techniques used to separate unencapsulated drugs, EE largely vary between studies (Lane et al., 2015). Ultracentrifugation and dialysis are the main techniques that are used to remove unentrapped DTX from liposomal samples (Grabielle-Madelmont et al., 2003; Mozafari, 2010). There are many limitations to use ultracentrifugation to purify free drug from small liposomes, since small drug-loaded liposomes could be lost in the supernatant (Grabielle-

Madelmont et al., 2003; Meers Paul, 2011). Equally, drug adsorbed to the exterior of the liposomes might sediment along with the liposomes, resulting in an overestimation of the EE. Therefore, it is crucial to accurately quantify the amount of liposome-entrapped drug before studying its activity *in vitro* and *in vivo* behaviours. In the present work, SEC was the chosen for liposome purification as it is a well-established approach to separate hydrophobic liposome-entrapped drugs. Unexpectedly, huge drug loss (up to 60 %) was observed following purification (Figure 1c), which was not associated with lipid loss, as shown by the high lipid recovery (90-100 %) (Table A.5). This interesting result could be explained by the assumption that large amount of the drug was not fully incorporated within the lipid bilayer, and was rather weakly bound or adsorbed to the liposome surface. Prior purification, our EE was (92-100 %) which agrees with other studies where purification step was not introduced (Zhang et al., 2012). However, the EE of our purified liposomes is still much lower than DTX-liposomes purified using ultracentrifugation and dialysis (Immordino et al., 2003; Li et al., 2011; Muthu et al., 2011; Ren et al., 2016; Zhai et al., 2010). Our findings, demonstrated the importance of using a robust and reliable method to purify DTX-loaded liposomes, since injecting non-purified DTX-liposomes could lead to a substantial loss of DTX from the liposomes surface, leading to lower concentrations of DTX reaching to tumour tissues, which translates clinically into a lower therapeutic efficacy.

Purification step is not only necessary for accurate dosing but also to predict the toxicity of the DXT-loaded liposomes *in vitro* and *in vivo*. Interestingly, our results showed that while using the same concentrations of DTX, non-purified DOPC:Chol liposomes were more more cytotoxic than the purified ones at early time point (24 h), however, this effect was reversed at 48-72 h (Figure 2). These results support the hypothesis of the adsorbed drug to the liposome surface which exhibits a “free drug-like behaviour” at early time points, increasing the cytotoxicity in comparison to the encapsulated drug. The burst release of adsorbed drug from the nanoparticles surface was previously reported (B. Magenheim, 1993). Longer time points (48-

72 h) ensured sufficient time for DTX-loaded liposomes to be taken up and released DTX intracellularly, leading to a higher cell death. In addition to cell monolayers, and for the first time, our DTX-loaded liposomes, both conventional and sterically stabilised liposomes significantly enhanced the therapeutic efficacy of DTX in prostate cancer PC3 tumour spheroids, most likely due to the improved DTX solubility, diffusion and penetration into the inner layers of the spheroids (Chambers et al., 2014; Xu et al., 2014).

4. Conclusions

Our results demonstrated the effect of the lipid bilayer composition on DTX encapsulation. Liposomes composed of fluid, unsaturated DOPC lipid showed the highest DTX loading compared to rigid saturated DPPC and DSPC lipids. Steric stabilisation had minimum effect on DTX encapsulation into liposomes. Decreasing lipid:drug molar ratio from 40:1 to 5:1 led to an improvement in the loading capacities of DOPC-based liposomes only. Our study showed, and for the first time, that SEC is a reliable method to remove adsorbed DTX to the liposome surface, resulting in an accurate drug quantification. *In vitro* toxicity of non-purified and purified DTX-loaded DOPC-based liposomes further confirmed the relevance of using a robust and reliable means of liposome purification, which strongly impacts the DTX dose to be delivered to the tumour cells, and therefore DTX therapeutic efficacy. Finally, an improvement in DTX solubility and penetration was observed with DTX-loaded liposomes which translated in a dose- and time-dependent cell death in PC3 prostate tumour spheroids, in contrast to the limited efficacy of free DTX. Overall, our findings shed the light on the importance of selecting the right lipid composition and method of liposome purification when engineering a liposomal nanoformulation for DTX drug. Such parameters will impact the liposome drug entrapment ability and stability and, more importantly, will dictate the therapeutic efficacy and ultimately the success of DTX-loaded liposomal formulations *in vivo*.

5. Acknowledgments

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6. References

Al-Jamal, W.T., Al-Jamal, K.T., Tian, B., Lacerda, L., Bomans, P.H., Frederik, P.M., Kostarelos, K., 2008. Lipid-quantum dot bilayer vesicles enhance tumor cell uptake and retention in vitro and in vivo. ACS Nano 2, 408–418.

Anderson, M., Omri, A., 2004. The effect of different lipid components on the in vitro stability and release kinetics of liposome formulations. Drug Deliv 11, 33–39.

Attwood, S.J., Choi, Y., Leonenko, Z., 2013. Preparation of DOPC and DPPC Supported Planar Lipid Bilayers for Atomic Force Microscopy and Atomic Force Spectroscopy. Int J Mol Sci 14, 3514–3539.

B. Magenheim, M.Y.L. and S.B., 1993. A new in vitro technique for the evaluation of drug release profile from colloidal carriers - ultrafiltration technique at low pressure . Int. J. Pharm. 94, 115–123.

Chambers, K.F., Mosaad, E.M.O., Russell, P.J., Clements, J.A., Doran, M.R., 2014. 3D Cultures of prostate cancer cells cultured in a novel high-throughput culture platform are more resistant to chemotherapeutics compared to cells cultured in monolayer. PLoS One 9, e111029.

Crosasso, P., Ceruti, M., Brusa, P., Arpicco, S., Dosio, F., Cattel, L., 2000. Preparation, characterization and properties of sterically stabilized paclitaxel-containing liposomes. J Control Release 63, 19–30.

Deeken, J.F., Slack, R., Weiss, G.J., Ramanathan, R.K., Pishvaian, M.J., Hwang, J., Lewandowski, K., Subramaniam, D., He, A.R., Cotarla, I., Rahman, A., Marshall, J.L., 2013. A phase I study of liposomal-encapsulated docetaxel (LE-DT) in patients with advanced solid tumor malignancies. Cancer Chemother Pharmacol 71, 627–633.

Demetzos, C., 2008. Differential Scanning Calorimetry (DSC): a tool to study the thermal behavior of lipid bilayers and liposomal stability. J Liposome Res 18, 159–173.

Drummond, D.C., Noble, C.O., Hayes, M.E., Park, J.W., Kirpotin, D.B., 2008. Pharmacokinetics and in vivo drug release rates in liposomal nanocarrier development. J Pharm Sci 97, 4696–4740.

Friedenberg, W.R., Graham, D., Greipp, P., Blood, E., Winston, R.D., 2003. The treatment of multiple myeloma with docetaxel (an ECOG study). Leuk Res 27, 751–754.

Gabizon, A., Papahadjopoulos, D., 1988. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci U S A 85, 6949–6953.

Gelmon, K., 1994. The taxoids: paclitaxel and docetaxel. Lancet 344, 1267–1272.

Grabielle-Madelmont, C., Lesieur, S., Ollivon, M., 2003. Characterization of loaded liposomes by size exclusion chromatography. J Biochem Biophys Methods 56, 189–217.

Grant, D.S., Williams, T.L., Zahaczewsky, M., Dicker, A.P., 2003. Comparison of antiangiogenic activities using paclitaxel (taxol) and docetaxel (taxotere). Int J Cancer 104, 121–129.

Haeri, A., Alinaghian, B., Daeihamed, M., Dadashzadeh, S., 2014. Preparation and characterization of stable nanoliposomal formulation of fluoxetine as a potential adjuvant therapy for drug-resistant tumors. Iran J Pharm Res 13, 3–14.

Harashima, H., Sakata, K., Funato, K., Kiwada, H., 1994. Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes. Pharm Res 11, 402–406.

Horwitz, S.B., 1992. Mechanism of action of taxol. Trends Pharmacol Sci 13, 134–136.

Huang, Y.-Y., Chung, T.-W., Wu, C.-I., 1998. Effect of saturated/unsaturated phosphatidylcholine ratio on the stability of liposome-encapsulated hemoglobin. Int. J. Pharm. 172, 161–167.

Immordino, M.L., Brusa, P., Arpicco, S., Stella, B., Dosio, F., Cattel, L., 2003. Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing docetaxel. J Control Release 91, 417–429.

Johnston, M.J., Semple, S.C., Klimuk, S.K., Edwards, K., Eisenhardt, M.L., Leng, E.C., Karlsson, G., Yanko, D., Cullis, P.R., 2006. Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations. Biochim Biophys Acta 1758, 55–64.

Johnstone, S.A., Masin, D., Mayer, L., Bally, M.B., 2001. Surface-associated serum proteins inhibit the uptake of phosphatidylserine and poly(ethylene glycol) liposomes by mouse macrophages. Biochim Biophys Acta 1513, 25–37.

Kirby, C., Gregoriadis, G., 1980. The effect of the cholesterol content of small unilamellar liposomes on the fate of their lipid components in vitro. Life Sci 27, 2223–2230.

Lane, R.E., Korbie, D., Anderson, W., Vaidyanathan, R., Trau, M., 2015. Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing. Sci Rep 5, 7639.

Lavelle, F., Bissery, M.C., Combeau, C., Riou, J.F., Vrignaud, P., Andre, S., 1995. Preclinical evaluation of docetaxel (Taxotere). Semin Oncol 22, 3–16.

Li, X., Tian, X., Zhang, J., Zhao, X., Chen, X., Jiang, Y., Wang, D., Pan, W., 2011. In vitro and in vivo evaluation of folate receptor-targeting amphiphilic copolymer-modified liposomes loaded with docetaxel. Int. J. Nanomedicine 6, 1167–1184. doi:10.2147/IJN.S21445

Lian, T., Ho, R.J., 2001. Trends and developments in liposome drug delivery systems. J Pharm Sci 90, 667–680.

Lokerse, W.J., Kneepkens, E.C., Ten Hagen, T.L., Eggermont, A.M., Grull, H., Koning, G.A., 2016. In depth study on thermosensitive liposomes: Optimizing formulations for tumor specific therapy and in vitro to in vivo relations. Biomaterials 82, 138–150.

Manjappa, A.S., Goel, P.N., Vekataraju, M.P., Rajesh, K.S., Makwana, K., Ukawala, M., Nikam, Y., Gude, R.P., Murthy, R.S.R., 2013. Is an Alternative Drug Delivery System Needed for Docetaxel? The Role of Controlling Epimerization in Formulations and Beyond. Pharm. Res. 30, 2675–2693.

Meers Paul, A.P., 2011. Stress testing to determine liposome degradation mechanisms, in: Healthcare, I. (Ed.), Pharmaceutical Stress Testing: Predicting Drug Degradation. Informa Healthcare, pp. 4226–4446.

Mozafari, M.R., 2010. Nanoliposomes: preparation and analysis. Methods Mol Biol 605, 29–50.

Muthu, M.S., Kulkarni, S.A., Raju, A., Feng, S.-S., 2012. Theranostic liposomes of TPGS coating for targeted co-delivery of docetaxel and quantum dots. Biomaterials 33, 3494–3501. doi:10.1016/j.biomaterials.2012.01.036

Muthu, M.S., Kulkarni, S.A., Xiong, J., Feng, S.S., 2011. Vitamin E TPGS coated liposomes enhanced cellular uptake and cytotoxicity of docetaxel in brain cancer cells. Int J Pharm 421, 332–340.

Naik, S., Patel, D., Surti, N., Misra, A., 2010. Preparation of PEGylated liposomes of docetaxel using supercritical fluid technology. J. Supercrit. Fluids 54, 110–119.

Prates Ramalho, J.P., Gkeka, P., Sarkisov, L., 2011. Structure and phase transformations of DPPC lipid bilayers in the presence of nanoparticles: insights from coarse-grained molecular dynamics simulations. Langmuir 27, 3723–3730.

Ren, G., Liu, D., Guo, W., Wang, M., Wu, C., Guo, M., Ai, X., Wang, Y., He, Z., 2016. Docetaxel prodrug liposomes for tumor therapy: characterization, in vitro and in vivo evaluation. Drug Deliv 1–10.

Rowinsky, E.K., 1997. The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 48, 353–374.

Senior, J., Gregoriadis, G., 1982. Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of the phospholipid and cholesterol components. Life Sci 30, 2123–2136.

Stewart, J.C., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 104, 10–14.

Straubinger, R.M., Balasubramanian, S. V, 2005. Preparation and characterization of taxane-containing liposomes. Methods Enzym. 391, 97–117.

Tan, Q., Liu, X., Fu, X., Li, Q., Dou, J., Zhai, G., 2012. Current development in nanoformulations of docetaxel. Expert Opin Drug Deliv 9, 975–990.

Walzl, A., Unger, C., Kramer, N., Unterleuthner, D., Scherzer, M., Hengstschlager, M., Schwanzer-Pfeiffer, D., Dolznig, H., 2014. The Resazurin Reduction Assay Can Distinguish Cytotoxic from Cytostatic Compounds in Spheroid Screening Assays. J Biomol Screen 19, 1047–1059.

Webb, M.S., Saxon, D., Wong, F.M., Lim, H.J., Wang, Z., Bally, M.B., Choi, L.S., Cullis, P.R., Mayer, L.D., 1998. Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): effects on the pharmacokinetics of liposomal vincristine. Biochim Biophys Acta 1372, 272–282.

Weiss, R.B., Donehower, R.C., Wiernik, P.H., Ohnuma, T., Gralla, R.J., Trump, D.L., Baker Jr., J.R., Van Echo, D.A., Von Hoff, D.D., Leyland-Jones, B., 1990. Hypersensitivity reactions from taxol. J Clin Oncol 8, 1263–1268.

Xu, X., Farach-Carson, M.C., Jia, X., 2014. Three-dimensional in vitro tumor models for cancer research and drug evaluation. Biotechnol Adv 32, 1256–1268.

Yuan, F., Dellian, M., Fukumura, D., Leunig, M., Berk, D.A., Torchilin, V.P., Jain, R.K., 1995. Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. Cancer Res 55, 3752–3756.

Zhai, G., Wu, J., Yu, B., Guo, C., Yang, X., Lee, R.J., 2010. A transferrin receptor-targeted liposomal formulation for docetaxel. J. Nanosci. Nanotechnol. 10, 5129–36.

Zhang, H., Li, R.Y., Lu, X., Mou, Z.Z., Lin, G.M., 2012. Docetaxel-loaded liposomes: preparation, pH sensitivity, pharmacokinetics, and tissue distribution. J Zhejiang Univ Sci B 13, 981–989.

Zhang, J.A., Anyarambhatla, G., Ma, L., Ugwu, S., Xuan, T., Sardone, T., Ahmad, I., 2005. Development and characterization of a novel Cremophor EL free liposome-based paclitaxel (LEP-ETU) formulation. Eur J Pharm Biopharm 59, 177–187.

Zhigaltsev, I. V, Winters, G., Srinivasulu, M., Crawford, J., Wong, M., Amankwa, L., Waterhouse, D., Masin, D., Webb, M., Harasym, N., Heller, L., Bally, M.B., Ciufolini, M.A., Cullis, P.R., Maurer, N., 2010. Development of a weak-base docetaxel derivative that can be loaded into lipid nanoparticles. J Control Release 144, 332–340.

Table 1. **Physicochemical properties of DTX-loaded DOPC:Chol (100:50) liposomes prepared at different lipid:DTX molar ratios.** The hydrodynamic size, polydispersity index (PdI) and zeta potential (ZP) of liposomes before and after purification were measured by the Nanosizer ZS (Malvern, UK).

Lipid composition (molar ratio)	Lipid:DTX molar ratio	Before Purification: Size ± SD (d.nm) [§ n]	Before Purification: PdI ± SD [§±]	After Purification: Size ± SD (d.nm) [§ n]	After Purification: PdI ± SD [§±]	After Purification: ZP ± SD (mV) [§V]
DOPC:Chol (100:50)	no drug	156.4 ± 5.0	0.118 ± 0.04	160.9±60.9	0.147 ± 0.02	-5.6 ± 0.4
	40:1	157.9 ± 1.1	0.193 ± 0.01	145.1 ± 2.8	0.210 ± 0.03	-5.4 ± 0.6
	20:1	162.0 ± 1.6	0.202 ± 0.01	136.4 ± 7.0	0.224 ± 0.01	-8.7 ± 0.7
	10:1	145.1 ± 1.9	0.170 ± 0.02	140.8 ± 0.4	0.137 ± 0.01	-2.8 ± 0.4
	5:1	129.3 ± 0.3	0.093 ± 0.01	122.2 ± 1.6	0.088 ± 0.03	-3.8 ± 0.6

[§] Data shown as mean ± SD (*n* = 3); [†] measured by dynamic light scattering; [‡] electrophoretic mobility.

Table 2. **Physicochemical properties of DTX-loaded DOPC:Chol:DSPE-PEG$_{2000}$ (95:50:5) liposomes prepared at different lipid:DTX molar ratios.** The hydrodynamic size, polydispersity index (PdI) and zeta potential (ZP) of liposomes before and after purification were measured by the Nanosizer ZS (Malvern, UK).

		Before Purification		After Purification		
Lipid composition (molar ratio)	Lipid:DTX molar ratio	Size ± SD (d.nm) [§ n]	PdI ± SD [§±]	Size ± SD (d.nm) [§ n]	PdI ± SD [§±]	ZP ± SD (mV) [§V]
DOPC:Chol:DSPE-PEG$_{2000}$ (95:50:5)	no drug	132.3 ± 1.0	0.087 ± 0.04	142.6±42.6	0.157 ± 0.06	-11.0± 0.73
	40:1	126.7 ± 1.4	0.040 ± 0.02	133.1 ± 1.0	0.104 ± 0.01	-9.9 ± 1.5
	20:1	124.2 ± 1.8	0.030 ± 0.01	125.0 ± 4.1	0.051 ± 0.02	-9.7 ± 0.5
	10:1	117.8 ± 1.0	0.040 ± 0.00	127.4 ± 3.0	0.111 ± 0.03	-9.2 ± 0.5
	5:1	112.3 ± 2.4	0.086 ± 0.01	116.9 ± 2.4	0.131 ± 0.02	-9.4 ± 0.4

[§] Data shown as mean ± SD (n = 3); [†] measured by dynamic light scattering; [‡] electrophoretic mobility.

Table 3. **Physicochemical properties of DTX-loaded DPPC:Chol:DSPE-PEG$_{2000}$ (95:50:5) liposomes prepared at different lipid:DTX molar ratios.** The hydrodynamic size, polydispersity index (PdI) and zeta potential (ZP) of liposomes before and after purification were measured by the Nanosizer ZS (Malvern, UK).

		Before Purification		After Purification		
Lipid composition (molar ratio)	Lipid:DTX molar ratio	Size ± SD (d.nm) [§,†]	PdI ± SD [§,†]	Size ± SD (d.nm) [§,†]	PdI ± SD [§,†]	ZP ± SD (mV) [§,‡]
DPPC:Chol:DSPE-PEG$_{2000}$ (95:50:5)	no drug	158.5 ± 6.2	0.153 ± 0.03	145.1 ± 5.1	0.088 ± 0.02	-9.69 ± 7.4
	40:1	145.5 ± 0.9	0.147 ± 0.01	136.3 ± 0.6	0.047 ± 0.02	-12.6 ± 0.6
	20:1	134.2 ± 2.0	0.131 ± 0.02	127.3 ± 0.8	0.033 ± 0.01	-7.7 ± 0.5
	10:1	133.9 ± 3.3	0.115 ± 0.06	132.9 ± 1.6	0.057 ± 0.01	-9.8 ± 0.9
	5:1	118.4 ± 4.4	0.042 ± 0.02	115.1 ± 1.8	0.072 ± 0.02	-10.3 ± 1.1

[§] Data shown as mean ± SD (*n* = 3); [†] measured by dynamic light scattering; [‡] electrophoretic mobility.

Table 4. **Physicochemical properties of DTX-loaded DSPC:Chol:DSPE-PEG$_{2000}$ (95:50:5) liposomes prepared at different lipid:DTX molar ratios.** The hydrodynamic size, polydispersity index (PdI) and zeta potential (ZP) of liposomes before and after purification were measured by the Nanosizer ZS (Malvern, UK).

		Before Purification		After Purification		
Lipid composition (molar ratio)	Lipid:DTX molar ratio	Size ± SD (d.nm) [§,†]	PdI ± SD [§,†]	Size ± SD (d.nm) [§,†]	PdI ± SD [§,†]	ZP ± SD (mV) [§,‡]
DSPC:Chol:DSPE-PEG$_{2000}$ (95:50:5)	no drug	152.1 ± 3.7	0.069 ± 0.00	155.4 ± 3.2	0.073 ± 0.02	-9.25 ± 0.79
	40:1	132.9 ± 2.7	0.040 ± 0.02	133.0 ± 0.2	0.049 ± 0.02	-8.8 ± 0.3
	20:1	123.5 ± 2.3	0.021 ± 0.00	136.4 ± 3.6	0.094 ± 0.00	-9.5 ± 0.8
	10:1	125.7 ± 2.6	0.047 ± 0.02	131.0 ± 3.3	0.092 ± 0.01	-14.9 ± 1.0
	5:1	124.3 ± 2.2	0.067 ± 0.01	125.6 ± 0.9	0.063 ± 0.01	-12.9 ± 1.1

[§] Data shown as mean ± SD (n = 3); [†] measured by dynamic light scattering; [‡] electrophoretic mobility.

Figure captions:

Figure 1. Encapsulation efficiency and drug loading of DTX into liposomes with different lipid compositions. DOPC:Chol, DOPC:Chol:DSPE-PEG$_{2000}$, DPPC:Chol:DSPE-PEG$_{2000}$ and DSPC:Chol:DSPE-PEG$_{2000}$ liposomes were loaded with DTX at 40:1, 20:1, 10:1 and 5:1 lipid:drug molar ratios then purified using PD-10 column. (a) Encapsulation efficiency of DTX into liposomes before purification, as determined by HPLC; DTX loading efficiency in liposomes (b) before purification and (c) after purification. Liposomes were disrupted in 10:1 (v/v) MeOH:HBS, sonicated for 10 min and DTX content was quantified by HPLC. Lipid content was determined by Stewart's assay. Data shown as mean ± SEM (n = 3). Statistical analysis was performed using two-way ANOVA followed by Bonferroni *post-test* (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). Statistical analysis was performed using 20:1 lipid:DTX molar ratio of each formulation as a reference as this ratio is referred in the literature as the optimized ratio.

Figure 2. Cell viability of DTX-loaded, DOPC:Chol and DOPC:Chol:DSPE-PEG$_{2000}$ liposomes in PC3 cell monolayer. DTX-loaded liposomes were prepared at 20:1 molar ratio. Cells were seeded in 96-well plates ($1\text{x}10^4$ cells/well) and next day incubated in serum-free media with 1-1000 nM of DTX-loaded, non-purified DOPC:Chol liposomes (black solid circles) and purified liposomes (black open circles), and with DTX-loaded, non-purified DOPC:Chol:DSPE-PEG$_{2000}$ liposomes (grey solid triangles) and purified liposomes (grey open triangles). After 4 h incubation, liposome-containing media were removed and cells were replenished with complete media. Cell viability was assessed by resazurin assay at (a) 24 h; (b) 48 h; (c) 72 h post-incubation. IC_{50} and I_{max} results were summarized below each graph. Data shown as mean ± SEM (n = 6). Significance of IC_{50}/I_{max} between non-purified and purified DOPC:Chol ([a]< 0.001) and between non-purified and purified DOPC:Chol:DSPE-PEG$_{2000}$ liposomes ([b]< 0.001) was performed using two-way ANOVA followed by Bonferroni *post-test.* Abbreviations: IC_{50}, half maximal inhibitory concentration; % I_{max}, maximum inhibition (lower cell viability).

Figure 3. Cell viability of DTX-loaded, DOPC:Chol and DOPC:Chol:DSPE-PEG$_{2000}$ liposomes in PC3 multicelluarlr tumor spheroids. Purified DTX-loaded liposomes were prepared at 20:1 molar ratio. Cells were seeded in 1 % (w/v) agarose coated 96-well plates ($5\text{x}10^3$ cells/well) and after 7 days of growth spheroids were incubated in complete media with (a) free DTX (1-10000 nM); (b) DTX-loaded DOPC:Chol liposomes (1-1000 nM of DTX) or (c) DTX-loaded DOPC:Chol:DSPE-PEG$_{2000}$ liposomes (1-1000 nM of DTX). Cell viability was assessed by resazurin assay after 48 (black bars), 72 (grey bars) and 96 h (white bars). Data shown as mean ± SEM (n = 6). Statistical analysis was performed using two-way ANOVA followed by Bonferroni *post-test.* * denotes comparison between 48 and 72 h, $ denotes comparison between 72 and 96 h (***, $$$ $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

Figure 1

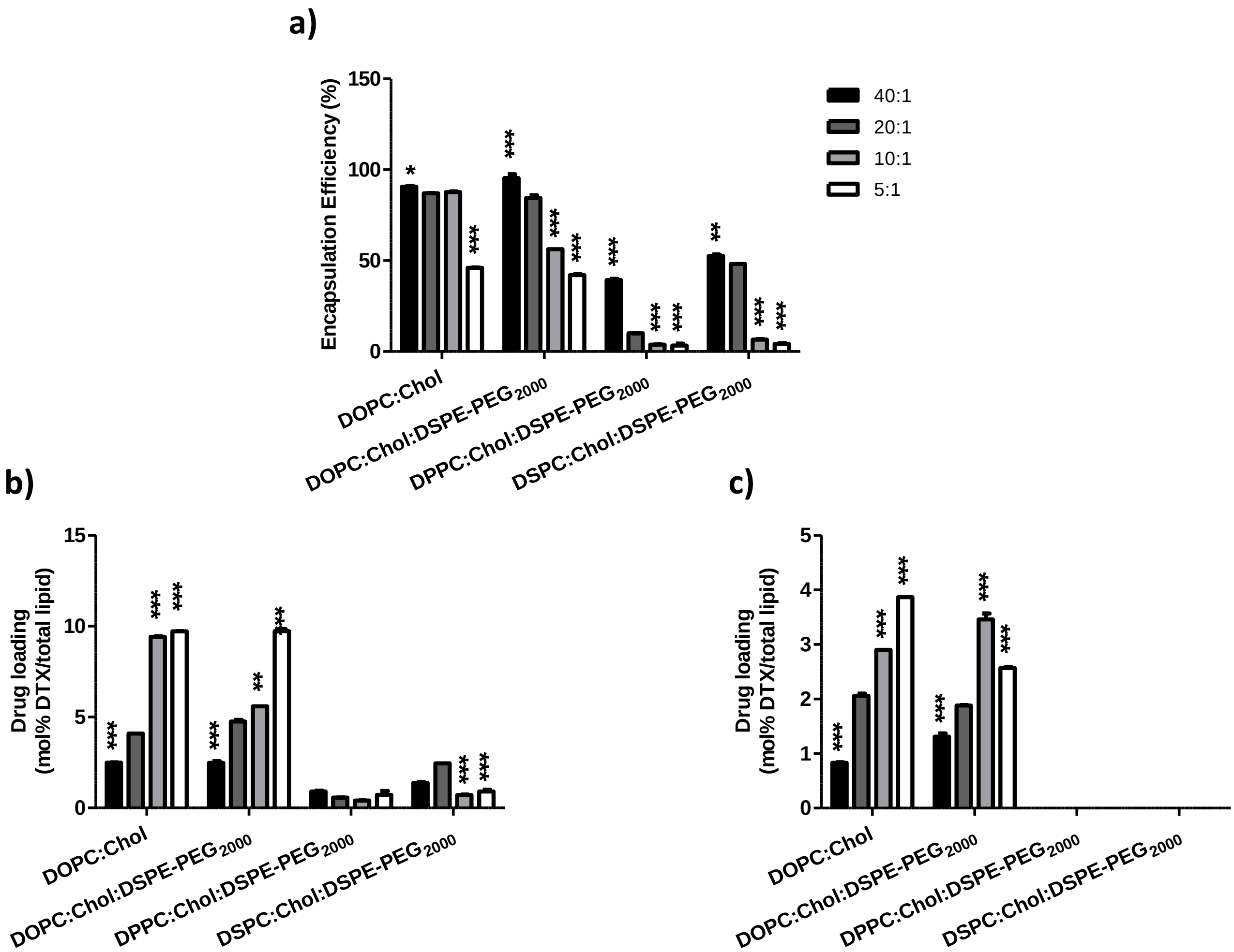
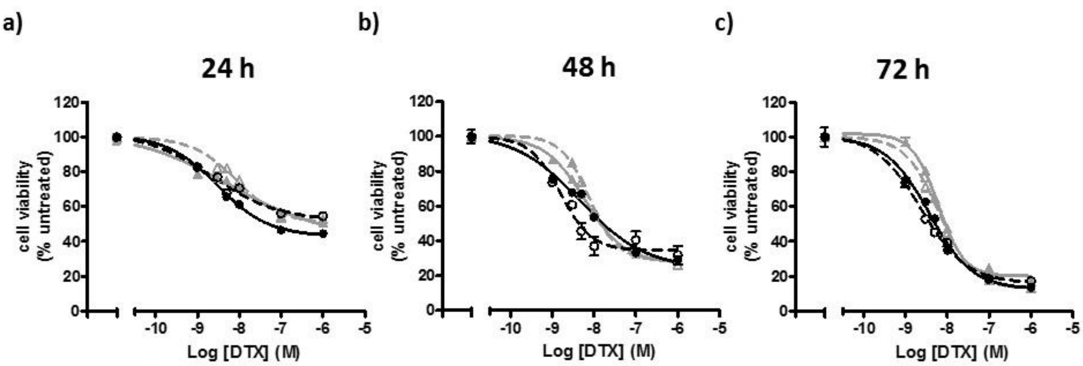


Figure 1

Figure 2



	IC_{50} (nM)	% I_{max}	IC_{50} (nM)	% I_{max}	IC_{50} (nM)	% I_{max}
● DOPC:Chol - purification	3.1 ± 0.5	42.2 ± 0.7	5.2 ± 0.5	25.1 ± 1.5	3.7 ± 0.1	12.7 ± 0.7
○ DOPC:Chol + purification	2.3 ± 0.9	54.8 ± 1.0[a]	1.6 ± 0.1[a]	34.7 ± 1.2[a]	2.1 ± 0.1[a]	16.1 ± 1.0[a]
▲ DOPC:Chol:DSPE-PEG_{2000} - purification	5.9 ± 1.2	44.9 ± 2.4	6.0 ± 0.2	27.5 ± 0.5	6.0 ± 0.2	20.1 ± 0.6
△ DOPC:Chol::DSPE-PEG_{2000} + purification	8.6 ± 0.8[b]	50.4 ± 1.0[b]	7.5 ± 0.2[b]	29.0 ± 0.5	6.4 ± 0.2	12.7 ± 0.7[b]

Figure 2

Figure 3

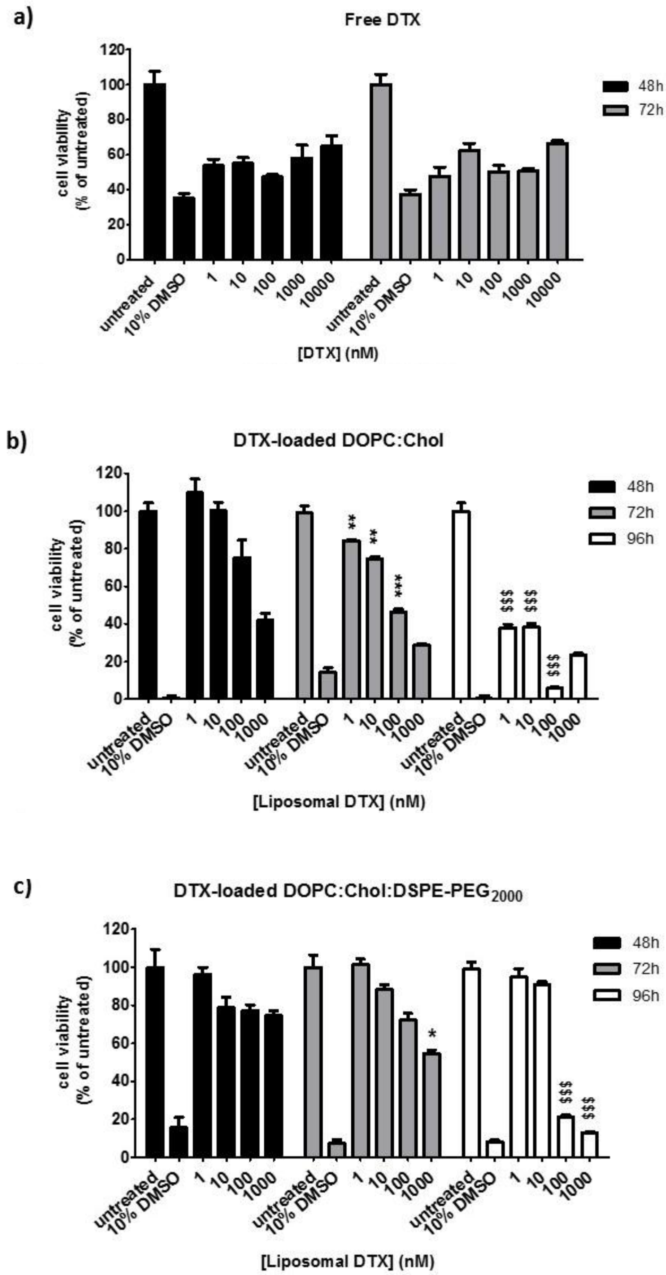


Figure 3