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Research paper

Distearoylphosphatidylethanolamine-based liposomes for ultrasound-mediated drug delivery

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ABSTRACT

The ability of ultrasound (US) to permeabilize phospholipid membranes has opened the potential of using US as a means to enhance delivery of anti-cancer drugs to tumour cells via liposomes. In this study, novel US sensitive or sonosensitive doxorubicin-containing liposomes based on 1,2 distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE) as the main lipid component are reported. A variety of lipid bilayer compositions was studied with respect to *in vitro* US triggered release of drug as well as serum stability in terms of drug retention, using experimental design. The multivariate data analysis indicated a strong correlation between DSPE content and sonosensitivity, both alone and in interplay with cholesterol. The most optimal formulation showed approximately 70% release of doxorubicin after 6 min of US exposure. This represented a 7-fold increase in release extent when compared to standard pegylated liposomal doxorubicin. The significant enhancement in sonosensitivity of the liposomes shows the potential of engineering liposomal lipid composition for US-mediated drug delivery.

1. Introduction

The main motivation for using liposomes as drug delivery systems for cytotoxic drugs is to improve the rather low therapeutic-to-toxicity ratio associated with conventional chemotherapy. This is achieved by the well-documented ability of liposomes to accumulate in tumour tissue due to the enhanced permeability and retention (EPR) effect [1–3]. The degree to which liposome accumulation will reflect drug delivery is, however, determined by the ability of the liposomes to remain stable in circulation and at the same time release their drug payload upon accumulation within tumour tissue. The latter is still a key challenge within the field of liposomology.

Several approaches have been attempted to enhance local drug delivery by triggered release from liposomes, including hyperthermia, enzymatic and pH strategies [4–10]. A new promising means is to combine ultrasound (US) with liposomal drug carriers. The strategy is based on the ability of US to permeabilize phospholipid membranes [11–13]. Thus, US exposure of tumour tissue comprising sonosensitive liposomes may not only induce drug release from the liposome carrier but also increase intracellular drug uptake. A few animal studies have reported a therapeutic benefit of combining US and liposomal cytostatics in tumour treatment, showing the potential of using this strategy within cancer therapy [14,15].

Although there is a considerable interest in ultrasound-mediated drug delivery, few have focused on improving the ultrasound sensitivity or sonosensitivity of liposomes, and the key factors behind sonosensitivity are currently not fully understood. It has, however, been shown that lipid membrane properties in general influence on ultrasound-induced changes in membrane permeability [16–19]. Lin and Thomas have demonstrated that inclusion of surfactants and polyethylene glycol in conventional phosphatidylcholine (PC)-based liposomes enhanced sonosensitivity with respect to drug release *in vitro* [16].

In the current study, we have investigated the sonosensitivity of liposomes containing the non-bilayer forming lipid 1,2 distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE). Unlike PC, DSPE has an inverted cone-shaped geometry defined by a large head group and long acyl chains. Our hypothesis is that inclusion of DSPE within lipid bilayers would affect sonosensitivity of liposomes.

Moreover, in order to develop sonosensitive liposomes for drug delivery applications, sonosensitivity must be combined with a

Abbreviations: US, ultrasound; Chol, cholesterol; DSPE, 1,2 distearoyl-sn-glycero-3-phosphatidylethanolamine; DSPE-PEG, 1,2 distearoyl-sn-glycero-3-phosphatidylethanolamine-N-(methoxy(polyethylene glycol)-2000); DSPC, 1,2 distearoyl-sn-glycero-3-phosphatidylcholine; HSPC, hydrogenated (soy) L-α-phosphatidylcholine; DXR, doxorubicin; E, phosphatidylethanolamine; L_α, lamellar liquid-crystalline phase; H_{II}, inverted hexagonal phase.

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high extent of drug retention in the blood circulation. Accordingly, doxorubicin-containing liposomes composed of varying mole percentages of DSPE as well as 1,2 distearoyl-sn-glycero-3-phosphatidylcholine (DSPC), 1,2 distearoyl-sn-glycero-3-phosphatidylethanolamine-N-(methoxy(polyethylene glycol)-2000) (DSPE-PEG) and cholesterol (Chol) were prepared, and the influence of the respective lipids on both sonosensitivity and serum stability was analyzed using multivariate data analysis.

2. Materials and methods

2.1. Materials

DSPE, DSPC and DSPE-PEG were purchased from Genzyme Pharmaceuticals, Liestal, Switzerland. Chol, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), ammonium sulphate, Triton X-100® solution and sucrose were obtained from Sigma Aldrich, Oslo, Norway. Serum of fetal bovine origin was obtained from Autonorm, Sero, Billingstad, Norway. Doxorubicin hydrochloride (DXR) was purchased from Nycomed, Asker, Norway. DXR-containing liposomes, Caelyx® (herein defined as reference liposomes) comprising hydrogenated soy phosphatidylcholine, DSPE-PEG and Chol (57:5:38 mol%) was supplied from the pharmacy at the Norwegian Radium Hospital, Oslo, Norway (European distributor Schering-Plough).

2.2. Experimental design

A full factorial design, including two centerpoints and one "random point", was used to systematically evaluate the effect of the lipids DSPE, DSPE-PEG and Chol on sonosensitivity and serum-induced drug leakage, respectively. This included the preparation and characterization of 11 different liposome batches. Except for the centerpoint formulation, each formulation was prepared once. The centerpoint formulation was prepared as two independent batches to reveal any variation in batch preparation. Triplicate US and serum stability measurements were performed for each liposome batch to detect any analytical variation. All variation in data was included in the confidence interval calculated by the regression models. The levels of the factors are shown in Table 1. DSPC could serve as a co-variable due to its low influence on both sonosensitivity and serum stability.

Partial least square regression (PLS) analysis was used to interpret the release data after 6 min of US and the leakage data after 6 h of liposome incubation in 20% serum at 37 °C (see Sections 2.7 and 2.8 for more details about the liposome tests). Regression models were calculated using full cross validation (Unscrambler® 9.6, Camo Technologies Inc.).

2.3. Liposome preparation

Liposomes of different membrane composition were prepared by the thin-film hydration method [20]. Briefly, lipids were dissolved in a chloroform/methanol mixture (9/1 v/v) at 60 °C and rotary evaporated to dryness under vacuum for 6 h. The resulting dried lipid films were hydrated with a 300 mM ammonium sulphate solution for 2 h followed by three freeze–thaw cycles in a dry ice/acetone/methanol mixture and water, respectively. The liposomes at a lipid concentration of 20 mg/ml were downsized by stepwise extrusion (Lipex extruder, Biomembrane Inc., Vancouver B.C., Canada) through Nucleopore polycarbonate filters with pore sizes of 800, 400, 200, 100 and 80 nm (Nuclepore, West Chester, PA, USA). The lipid hydration, liposome extrusion and thawing process were performed at 75 °C, which is above the gel-to-liquid-crystalline phase transition temperature of the phospholipids.

Table 1
Levels of the lipids investigated in the full factorial design. All lipid levels are given in mol%. DSPC is used as a filler to obtain 100 mol%.

Lipid variables	Level		
	−1	0	+1
DSPE	47	54.5	62
DSPE-PEG	3	5.5	8
Chol	20	25	30

2.4. Liposome entrapment of doxorubicin

DXR was remote loaded into liposomes using an ammonium sulphate transmembrane gradient [21]. Formation of the gradient was achieved by extensive dialysis of extruded liposomes against a 255 mM sucrose solution. The dialysis was performed by placing disposable dialysis bags (MW cut off 100,000 D) (Spectra/Por®, Float-A-Lyzer®, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) containing the liposome dispersion in a magnetically stirred dialysis solution for approximately 48 h (volume ratio liposome dispersion:dialysis solution, 1:100 v/v). Three consecutive equilibration periods with intermediate exchange of the dialysis solution were conducted prior to drug loading.

A solution of DXR hydrochloride was added to the liposome dispersion to give a final drug-to-lipid ratio of 1:16 (wt/wt). The final lipid concentration was 16 mg/ml. To obtain optimal entrapment efficiency, the liposome dispersions were, after DXR addition, incubated for 30 min under magnetic stirring at 75 °C. Any remaining free drug was removed by liposome dialysis against an isotonic sucrose solution containing 10 mM HEPES (pH 7.4), as described above.

2.5. Determination of entrapment efficiency

To estimate the percentage of drug entrapment, aliquots of both the dialyzed and the non-dialyzed liposome sample were diluted 1:500 (v/v) with an isotonic sucrose solution containing 10 mM HEPES (pH 7.4) and further dissolved with Triton X-100 surfactant solution in a ratio of 50:1 (v/v). The entrapment efficiency (%) was calculated according to:

$$(F_{en} - F_b)/(F_{tot} - F_b) * 100\% \quad (1)$$

where F_{en} is the fluorescence intensity of the dialyzed and surfactant-treated liposome sample, F_b is the initial background signal of the dispersion medium (isotonic sucrose/HEPES solution) and F_{tot} is the fluorescence intensity of the non-dialyzed and surfactant-treated liposome sample.

Fluorescence intensity measurements were performed using a fluorescence spectrometer from Ocean Optics (model QE65000. Duiven, Netherlands). The excitation and emission wavelength were 488 and 595 nm, respectively. Triplicate samples were measured.

2.6. Liposome characterization

The mean intensity-weighted hydrodynamic liposome diameter was determined by photon correlation spectroscopy at 23 °C and at a scattering angle of 90° (Nanosizer, Malvern Instruments, Malvern, UK). As an indicator for the width of the particle size distribution, the polydispersity index (PI) was given. Each liposome batch was diluted 1:200 (v/v) with 0.22-μm-filtered isotonic sucrose solution containing 10 mM HEPES (pH 7.4) and measured twice (unless indicated otherwise).

The Zeta potential of selected formulations (nos. 1, 8, 9, 11) was measured using a Malvern Zetasizer 2000 (Malvern Instruments,

Malvern, UK). The instrument was checked with the Malvern DTS0050 mobility standard prior to sample measurements. Liposome dispersions were diluted 1:30 (v/v) with 0.22-μm-filtered isotonic sucrose solution containing 10 mM HEPES (pH 7.4). The diluted liposome dispersions were injected into the measuring cell immediately after pH measurements. Five zeta potential measurements were performed on each duplicate sample.

2.7. US-mediated drug release

US-release measurements were conducted using a 40-kHz US transducer (VC 750, Sonic and Materials, Inc, Newtown, CT, USA) connected to a custom-built sample chamber similar to that disclosed in Huang and MacDonald [22]. For an illustration of the setup, see Supplementary data. The temperature in the sample chamber was kept constant at 25 °C by circulating water. The liposome dispersions were diluted in a ratio of 1:500 (v/v) with isotonic sucrose solution containing 10 mM HEPES (pH 7.4) just prior to the US experiments. The diluted liposome dispersions were exposed in a continuous mode (100% duty cycle) to 40-kHz US at an amplitude of 20%. At this amplitude, acoustic pressure measurements in the sample chamber gave ≈240 kPa (pk–pk). Pressure measurements were taken with a hydrophone (Bruel and Kjaer type 8103, Denmark). The release of DXR from liposomes could be monitored due to the relief of fluorescence self-quenching in the external liposomal phase and concomitant increase in fluorescence intensity [23,24]. Samples were taken out at 0.5, 1.0, 1.5, 2.0, 4.0 and 6.0 min of US exposure and the amount DXR calculated. The following equation was used for quantification of US-mediated drug release:

$$\%\text{Drug release} = (F_t - F_0)/(F_{\max} - F_0) * 100 \quad (2)$$

where F_t is the fluorescence intensity of the liposome sample after a given duration (t) of US, F_0 is the initial background fluorescence of the liposome sample prior to US and $F_{\max}$ is the fluorescence intensity after liposome solubilisation with surfactant (Triton X-100). The diluted liposome samples were solubilised with Triton X-100 at a ratio of 50:1 (v/v). The release test was performed on three independent samples of each liposome batch.

2.8. Serum stability

Liposome stability was studied using a well-established serum-induced leakage assay mimicking biological conditions [25–27]. Liposome dispersions, diluted 1:125 (v/v) with an isotonic sucrose solution containing 10 mM HEPES (pH 7.4) and 20% fetal bovine serum, were incubated up to 6 h at 37 °C. Time-dependent leakage of liposomal DXR was quantified by fluorescence measurements of serum samples further diluted 1:4 (v/v) with the previously mentioned sucrose solution, according to Eq. (2). The leakage test was performed on three independent samples of each liposome batch.

2.9. In vitro stability

Physical stability of selected liposome formulations was followed for up to 6 months storage at 4 °C. This testing included re-determination of mean liposome size and size distribution, sonosensitivity and retention of encapsulated DXR, using the analytical methods previously described.

3. Results

3.1. Liposome characterization

The liposome batches were characterized with respect to mean size, size distribution and DXR entrapment efficiency. Small-sized liposomes, slightly below 100 nm, are preferred to obtain long circulation in blood and consequently high extent of accumulation in tissues of increased vascular permeability, such as tumours. For an overview, see review by Drummond et al. [28]. Hence, the target size range was 80–90 nm for all formulations included in the design. The actual mean liposome size of the liposome batches ranged from 83 to 90 nm. The polydispersity index values were less than 0.14, indicating a narrow size distribution. For mean size and size distribution of liposomes after ultrasound exposure, see Table 2. Entrapment efficiencies of DXR were more than 92% (drug/lipid ratio of 1:16 (wt/wt)) for all formulations.

Zeta potential was measured for selected batches containing various levels of DSPE and DSPE-PEG, which might contribute to differences in surface charge of the liposomes [29]. The measured zeta potential for formulations nos. 1, 8, 9, 11 (see Table 2 for lipid compositions) ranged from −20.2 to −22.7 mV (with standard deviations less than 2.4 mV). In comparison, the zeta potential of the reference liposomes was measured to be −18.9 ± 0.8 mV. The slightly negative zeta potentials measured here are regarded typical for pegylated liposomes [29,30].

3.2. DSPE enhances sonosensitivity

Fig. 1 compares the US-mediated DXR release profiles of DSPE- and DSPC-based liposome formulations. The drug release extent of DSPC-based liposomes, after 6 min US, was increased from 9% to 69% by substituting 62 mol% DSPC with DSPE. Reference liposomes showed an approximately similar release profile as the DSPC-based liposomes (Fig. 1).

It should be noted that the liposome formulations showed no detectable leakage in the US chamber when not exposed to US, confirming that the observed release was only attributed to US stimuli.

Dynamic light scattering experiments were performed on both DSPE- and DSPC-based liposomes samples, both before and after 6 min exposure to US. Whilst the mean size and size distribution (PI) remained approximately unchanged for the DSPC-based liposomes after US exposure, an increased mean size and a broader size distribution were observed for the DSPE-based liposomes (Table 2). This data suggest a more pronounced destabilization of DSPE-based liposomes upon US exposure, which is in agreement with the enhanced sonosensitivity when compared to DSPC-based liposomes.

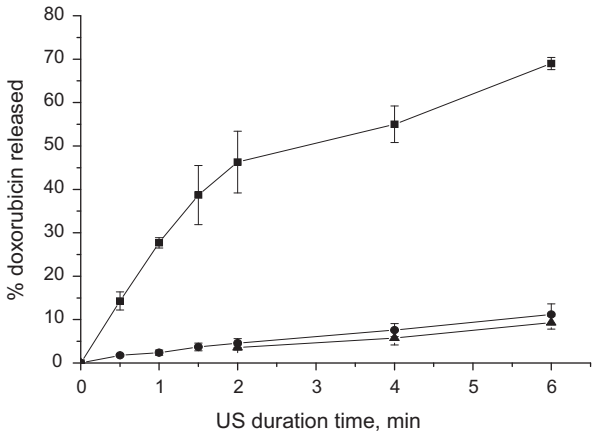

Fig. 1. US-mediated release profiles of DXR-containing liposomes consisting of the lipids; ■ DSPE, DSPE-PEG and Chol 62:8:30 mol%, ▲ DSPC, DSPE-PEG and Chol 62:8:30 mol%, and ● Reference liposomes (HSPC:DSPE-PEG:Chol 57:5:38 mol%). The mean and SD of triplicate measurements are given.

Table 2
Mean size and size distribution of DSPE- and DSPC-based liposomes before and after exposure to 6 min US. The mean and SD of triplicate measurements of independent samples are given.

Liposome formulation	Mean size (nm)		PI	
	Before US	After US	Before US	After US
DSPE:DSPE-PEG:CHOL (62:8:30 mol%)	84 ± 1	93 ± 0	0.11 ± 0.01	0.28 ± 0.03
DSPC:DSPE-PEG:CHOL (62:8:30 mol%)	87 ± 1	87 ± 1	0.11 ± 0.01	0.17 ± 0.01

3.3. Effect of lipid bilayer composition on sonosensitivity

Multivariate data analysis was applied to determine the lipid variables having an effect on the release extent after 6 min US (i.e. release modulators). An overview of sonosensitivity and serum stability data for the various formulations is given in Table 3 (batch nos. 1–11). Liposomes containing high levels of DSPE (62 mol%) displayed the highest release values. For a fixed level of Chol (30 mol%) and DSPE-PEG (8 mol%), an increase in DSPE content from 47 to 62 mol% increased the release extent with 51% points after 6 min of US (Table 3).

The derived multivariate model described approximately 97% of the variation in the data set. The standard deviation of the model, the root mean squared error of calibration, (RMSEC) was 1.8% (p value < 0.05). The release extent after 6 min US varied from 15% to 69% depending on membrane composition (Table 3). Within the range of formulations studied here, DSPE had the strongest impact on the model, showing a positive correlation to sonosensitivity (Fig. 2). The high sonosensitivity of the centerpoint samples confirmed the presence of curvature effects, which was assumed to be the squared effect of Chol (Fig. 2). Also an interaction effect between Chol and DSPE was observed (Figs. 2 and 3).

The interaction effect implied a larger impact of Chol on sonosensitivity at low contents of DSPE than at higher contents of DSPE (Fig. 3). Varying the content of DSPE-PEG did not significantly affect sonosensitivity within the tested design space (Fig. 3). The model predicted the optimal liposome composition with respect to sonosensitivity to contain 62 mol% DSPE, 8 mol% DSPE-PEG and 25 mol% Chol (Fig. 2).

3.4. Effect of lipid bilayer composition on serum stability

In vitro leakage data of DXR from the liposomes in 20% serum are presented in Table 3. The extent of leakage varied from 2% to 37% after 6 h of incubation, depending on membrane composition. The most stable formulation (DSPE:DSPE-PEG:Chol 62:8:30 mol%) showed approximately similar serum stability as the reference liposomes, with only 2% leakage after 6 h of incubation vs. 4% for the reference liposomes.

Table 3
Overview of sonosensitivity and serum stability for the different liposome formulations after 6 min of 40 KHz US and 6 h of incubation in 20% serum at 37 °C, respectively. The mean and SD of triplicate measurements are given.

Batch no.	DSPE:DSPE-PEG:Chol (mol%)	US-mediated DXR release (%)	Serum-induced DXR leakage (%)
1	47:3:20	34 ± 1	28 ± 3
2	47:3:30	15 ± 1	6 ± 1
3	47:8:20	37 ± 1	12 ± 5
4	47:8:30	19 ± 2	4 ± 1
5	54.5:5.5:20	45 ± 5	32 ± 1
6	54.5:5.5:25	57 ± 4	25 ± 6
7	54.5:5.5:25	62 ± 6	20 ± 1
8	62:3:20	60 ± 6	34 ± 3
9	62:3:30	60 ± 3	13 ± 2
10	62:8:20	64 ± 3	17 ± 1
11	62:8:30	69 ± 1	2 ± 2

Note: DSPC is used as filler lipid to obtain 100 mol%. The centerpoint formulation was prepared as two separate batches, no. 6 and 7.

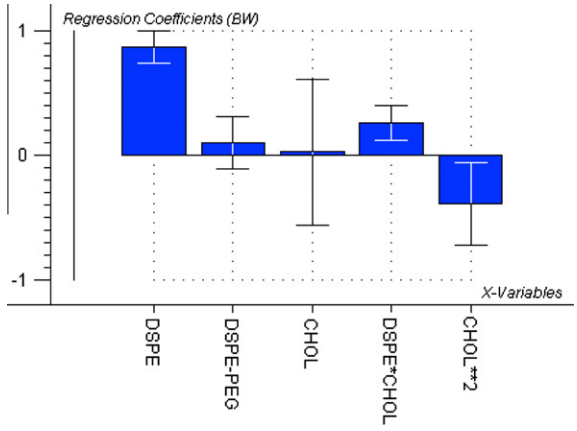

Fig. 2. Regression coefficients describing the significance of lipids on US-mediated DXR release after 6 min of US.

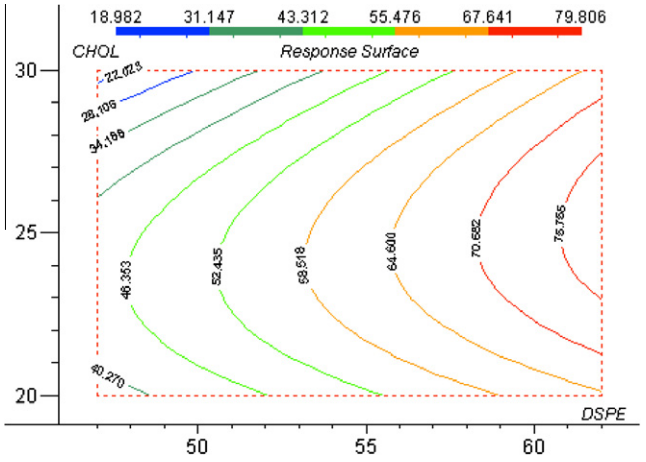

Fig. 3. Response surface plot of US-mediated liposomal DXR release as a function of DSPE and Chol levels (mol%). (6 min US data, DSPE-PEG level held constant at 8 mol%).

A multivariate data analysis of the 6 h leakage data was performed to elucidate the effect of lipid components on serum stability. The model described 88% of the variation in the dataset, which gave a RMSEC of 3.6%, (p value < 0.05).

The regression analysis confirmed Chol to be the most important lipid influencing on serum-induced drug leakage (Fig. 4). The strong correlation between Chol content and leakage extent was negative, implying that increasing levels of Chol reduced the leakage and improved stability. Also the squared effect of DSPE-PEG showed to have an impact on the model (Fig. 4).

It may be added that liposomes incubated in the buffer solution (without serum) showed no detectable drug leakage during 6 h incubation at 37 °C.

In order to investigate a plausible correlation between US-mediated release and serum-induced leakage, the two data sets were plotted versus each other (Fig. 5). Each data point is identified by its formulation number. It appeared to be an overall trend that formulations with high serum-induced leakage mostly also possessed good sonosensitivity. Nevertheless, there were a few formulations

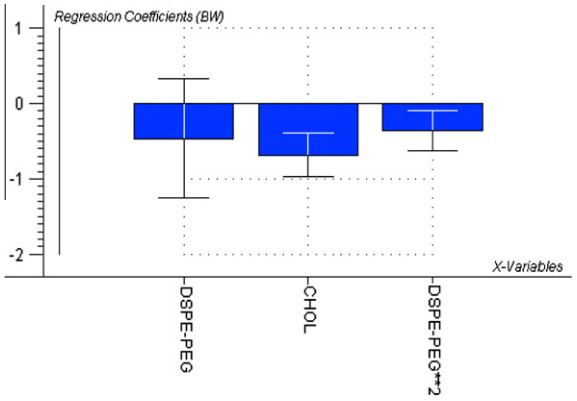

Fig. 4. Regression coefficients describing the significance of lipids on DXR leakage extent (%) after 6 h liposome incubation in 20% serum/37 °C.

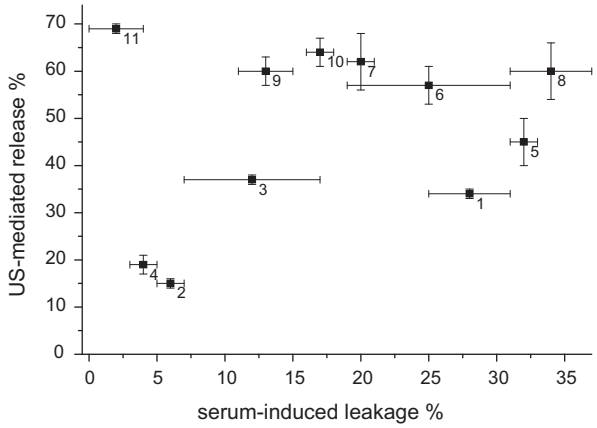

Fig. 5. US-mediated DXR release from liposomes after 6 min US versus serum-induced leakage after 6 h incubation in 20% serum/37 °C.

that were highly sonosensitive and still exhibited low or moderate serum-induced leakage, especially formulation no. 11. The two characteristics were not statistically correlated.

3.5. In vitro stability

Physicochemical properties important for the quality of the formulation such as liposome size, retention of encapsulated drug and sonosensitivity must be preserved at least from time of production to time of application. The formulation showing the overall best serum stability and sonosensitivity (no. 11, Table 3) was followed during 6 months of storage at 4 °C. Data on the above stability-indicating parameters after 6 months storage are shown in Table 4.

Table 4
In vitro stability of formulation no. 11 in terms of mean liposome size and polydispersity index, retention of encapsulated drug and sonosensitivity after 6 months of storage at 4 °C. Mean and SD of triplicate measurements are given (unless otherwise stated).

	Mean size (nm)	PI	US-mediated DXR release % (6 min, 40 kHz)	% DXR leakage
Date of production	88*	0.14*	69 ± 1	
6 months/4 °C	85 ± 1	0.14 ± 0.01	67 ± 4	0 ± 0

* Single measurement.

4. Discussion

To render sonosensitive liposomes useful as therapeutic tools, the liposome carrier should be stable *en route* to the target and turn instable only upon ultrasound exposure in the diseased volume. The goal of the current study was to develop sonosensitive liposomes that address these issues by optimizing the lipid bilayer composition vis-à-vis *in vitro* sonosensitivity and serum stability. Our main findings may be summarised as follows: (1) DSPE enhances sonosensitivity, (2) intermediate and high levels of Chol and DSPE, respectively, synergistically promote sonosensitivity, whilst (3) high levels of Chol improves serum stability.

DSPE showed the largest impact on sonosensitivity in the design space investigated, where DXR release extent increased markedly with increased levels of the lipid. At high levels of DSPE, up to a sevenfold increase in drug release was observed when compared to DSPC-based liposomes.

It should be noted that the US-mediated release extent of pegylated PC-based liposomes observed in the current study is somewhat less than reported in previous studies [16,18,31]. This might be due to certain differences in the experimental US setup such as frequency, duty cycle and distance between the transducer and the liposome sample.

We hypothesize that the mechanism behind the modulating effect of DSPE on sonosensitivity of liposomes is related to DSPE's ability to form inverted hexagonal structures under certain conditions, e.g. at high temperature, while DSPE-PEG is naturally prone to form micelles [32]. Combining these characteristics may promote induction of local defects or polymorphic phase transitions within micro-rafts or even in the whole liposome bilayer during ultrasound exposure, further leading to drug release. Liposomal phase transitions have earlier been described to be induced by pressure- and/or temperature-jumps [33–35]. The long acyl chains of DSPE occupy a large volume compared to the polar head group, which makes the lipid undergo a thermotropic phase transition from the lamellar liquid-crystalline to the inverted hexagonal phase ($L_\alpha \rightarrow H_{II}$) at temperatures above 80 °C [33–35]. Moreover, the theoretical gel-to-liquid-crystalline phase transition temperature is above 70 °C. In the current ultrasound experiments, sample temperature never exceeded 30 °C. Hence, it is unlikely that the drug release could be directly attributed to temperature effects. On the other hand, it cannot be excluded that extreme temperature and/or pressure jumps in the near vicinity of ultrasound-induced cavitating bubbles could induce drug release.

Moreover, the slightly positive effect of membrane-grafted DSPE-PEG on sonosensitivity of liposomes is in agreement with previous studies [16,17]. However, in the current study, no significant effects of varying DSPE-PEG concentration (3–8 mol%) were observed. This may indicate that above a certain level of pegylation, no significant added benefit is achieved for the DSPE-based formulations investigated here.

Chol displayed an interaction effect with DSPE, where an optimum in sonosensitivity was observed at intermediate Chol levels (25 mol%) and high DSPE levels (62 mol%). Moreover, the model indicated a squared effect of Chol, implying a nonlinear correlation between Chol level and sonosensitivity. In contrast, Lin et al. reported that the sonosensitivity of egg PC-based liposomes increased approximately proportionally with higher Chol concentrations [17]. This indicates that the effect of Chol on sonosensitivity may vary with different phospholipids.

There is little information in the literature regarding the impact of Chol on long-chain saturated PC/PE blends. However, the effect of Chol on phase transitions of PE-based membranes has been described in some reports [36–39]. In a study by McMullen et al., it was demonstrated that the lateral miscibility of Chol in saturated PE bilayers is limited and decreases with Chol level, resulting in

Chol-rich areas within the liposome membrane [38]. This has led us to speculate that increased sonosensitivity is observed up to around the point of Chol domain formation whereupon sonosensitivity detoriates. Cheetham et al. showed data for binary systems consisting of unsaturated dielaidoylphosphatidylethanolamine and Chol supporting this hypothesis. Here, the transition temperature of the lamellar liquid-crystalline to the inverted hexagonal ($L_\alpha \rightarrow H_{II}$) phase transition is slightly decreased at intermediate Chol content and increased at higher Chol levels [39]. However, whether lipid miscibility and/or phase transitions are factors describing the observed interaction effect between DSPE and Chol with respect to liposome sonosensitivity remain unresolved and are subject to further investigation.

The serum-induced drug leakage varied greatly with Chol level, where liposomes containing high levels of Chol were the most stable in terms of drug retention. This was confirmed by the regression analysis revealing a strong negative correlation between Chol and drug leakage. The observed effect could be explained by the well-known ability of cholesterol to tighten liposome membranes, increase mechanical stability and reduce the extent of opsonisation, thus minimizing loss of encapsulated drug [40].

Based on the chemometric model, the optimal lipid bilayer composition with respect to sonosensitivity was predicted to contain intermediate levels of Chol (25 mol%) and high levels of DSPE (62 mol%) and DSPE-PEG (8 mol%). This formulation would give approximately 80% drug release after 6 min of US (Fig. 3). However, stability issues pointed to formulations containing higher levels of Chol (30 mol%), (Fig. 5). Accordingly, within the design space investigated formulation number 11 was considered best featuring both high sonosensitivity and serum stability. The formulation showed approximately 70% drug release after 6 min of US and 2% leakage after 6 h of incubation in 20% serum. In addition, the acceptable storage stability of the formulation, implying no alteration in size, nor leakage of encapsulated drug and maintained sonosensitivity during the tested time frame of 6 months, was promising from a product point of view.

In conclusion, novel sonosensitive and serum-stable DXR-containing liposomes based on DSPE as the main lipid component were identified by optimization of lipid bilayer composition. The best formulation, containing high levels of DSPE and Chol, displayed a seven-fold increase in sonosensitivity when compared to reference liposomes. These data justify further investigation into the therapeutic effect in *in vivo* models.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2010.04.012.

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