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Note

Lipid membrane composition influences drug release from dioleoylphosphatidylethanolamine-based liposomes on exposure to ultrasound

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ABSTRACT

The effect of membrane composition on calcein release from dioleoylphosphatidylethanolamine (DOPE)-based liposomes on exposure to low doses of 1.13 MHz focused ultrasound (US) was investigated by multivariate analysis, with the goal of designing liposomes for US-mediated drug delivery. Regression analysis revealed a strong correlation between sonosensitivity and the non-bilayer forming lipids DOPE and pegylated distearoylphosphatidylethanolamine (DSPE-PEG 2000), with DOPE having the strongest impact. Unlike most of the previously studied distearoylphosphatidylethanolamine (DSPE)-based liposomes, all the current DOPE-based liposome formulations were found stable in 20% serum in terms of drug retention.

A novel approach to improve delivery of anticancer drugs to tumour cells is to combine liposomal cytostatics with ultrasound (US) treatment of tumour tissue. The strategy is based on the potential of US to both induce local drug release from the liposome carrier and increase intracellular drug uptake. For reviews see Schroeder et al. (2009), Pitt et al. (2004). In order to maximize the benefit of such combined treatment, the liposome formulation should fulfill two main requirements: (1) retention of the liposome drug payload in blood circulation, thus allowing sufficient tumour accumulation and, (2) sufficient drug release from the liposomes on US exposure. We suggest that these requirements may be met by engineering the liposome membrane composition.

Recently, we demonstrated that incorporation of DSPE into liposomes significantly enhanced the *in vitro* release of doxorubicin (DXR) or sonosensitivity on exposure to 40 kHz US (Evjen et al., 2010). It was also observed, that most of the liposome formulations exhibiting high sonosensitivity also displayed high serum-induced drug leakage. Thus, further optimization of the membrane composition was required (Evjen et al., 2010).

In the current study the influence of membrane composition on sonosensitivity of DOPE-based liposomes was investigated by multivariate analysis using high frequency, focused US (1.13 MHz). DOPE represents an even more pronounced non-bilayer forming characteristic than its saturated lipid analogue DSPE (Israelachvili, 1991), which might further perturb the liposome membrane upon US exposure, enhancing liposomal drug release. Moreover, high frequency US is regarded more clinically relevant than 40 kHz US due to improved focusing ability and minimised damage to surrounding healthy tissue.

A D-optimal mixture design was employed to elucidate the effects and interplay of the lipid membrane components DOPE, distearoylphosphatidylcholine (DSPC), DSPE-PEG 2000 and cholesterol on sonosensitivity and serum stability, respectively. Eleven liposome batches containing the drug marker calcein were prepared, including triplicates of the centre point formulation. The levels of the lipid variables are shown in Table 1.

The liposome batches were prepared by the thin film hydration and sequential extrusion technique similarly as previously described by Evjen et al. (2010), except that isotonic sucrose solution containing 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) (pH 7.4) and 50 mM calcein was used to hydrate the dry lipids films, giving a lipid concentration

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Table 1
Overview of the lipid variables investigated.

Lipid variables	Range (mol%)
DOPE	52–72
DSPC	5–20
DSPE-PEG 2000	3–8
Cholesterol	20–35

of 16 mg/ml. The lipid hydration, liposome extrusion and thawing process were performed at 25 °C, above the gel-to-liquid crystalline phase transition temperature of the phospholipid blends. Untrapped calcein was removed by dialysis against an isotonic sucrose solution containing 10 mM HEPES and 0.01 w/v% sodium azide (herein defined as sucrose/HEPES solution) as previously described by Evjen et al. (2010). All phospholipids were purchased from Genzyme Pharmaceuticals, Liestal, Switzerland. Cholesterol, calcein, HEPES, TritonX-100® solution, sucrose and sodium azide were obtained from Sigma–Aldrich, Oslo, Norway.

Determination of size and size distribution of the dialyzed liposomes were performed using photon correlation spectroscopy (Nanosizer, Malvern Instruments, Malvern, UK) similarly as previously described by Evjen et al. (2010). The liposome batches showed mean intensity-weighted hydrodynamic liposome diameter in the range of 81–89 nm and polydispersity index (P.I.) below 0.15, indicating a narrow size distribution.

US-release measurements were performed using a custom built US-setup providing a standardized and precise US dosimetry, as described by Somaglino et al. (2010). In brief, a 1.13 MHz focused US transducer was used to generate cavitation. A needle hydrophone was inserted into the liposome sample vial and placed into degassed water in front of the transducer to detect cavitation. The chosen US dosimetry parameters were: 25% duty cycle, 200 Hz pulse repetition frequency, spatial peak-temporal average intensity (Ispta) = 5500 W/cm^2.

Prior to US exposure, liposome dispersions were diluted in a 1:500 (v/v) ratio with sucrose/HEPES solution. The release extent of calcein was monitored in terms of relief of calcein-mediated fluorescence self-quenching in the extraliposomal phase and concomitant increase in fluorescence intensity, and release extent quantification was performed according to the Düzgünes et al. (2003). The fluorescence measurements were performed using a 176-fluorescence spectrometer from Ocean Optics (model QE65000 Duiven, Netherlands). The excitation and emission wavelength of calcein were 490 and 550 nm, respectively.

Fig. 1 shows a typical calcein release profile of DOPE-based liposomes during exposure to increasing cavitation doses. Release increased with the cavitation dose, and at the highest dose an almost complete release of calcein was obtained. It should be noted that no detectable passive leakage of calcein was observed for non-US treated DOPE-based liposomes.

Moreover, the DOPE-based liposome formulations also showed significantly higher drug release extents than DSPE-based liposomes at similar cavitation doses. At a cavitation dose of 2000, the most sonosensitive DSPE-based liposome formulation previously studied (DSPE:DSPE-PEG 2000:cholesterol 62/8/30 mol%), released 34 ± 2% of the drug payload versus 49 ± 4% for the DOPE-based formulation.

At the highest cavitation dose applied, the DOPE-based liposomes released almost all the entrapped calcein (Fig. 1). Hence, to better detect variations in release extent between the different formulations, the data used for multivariate modelling were generated at a low cavitation dose of 2000. At this dose, the extent of release varied from 30 to 64% among the different liposome formulations (Table 2).

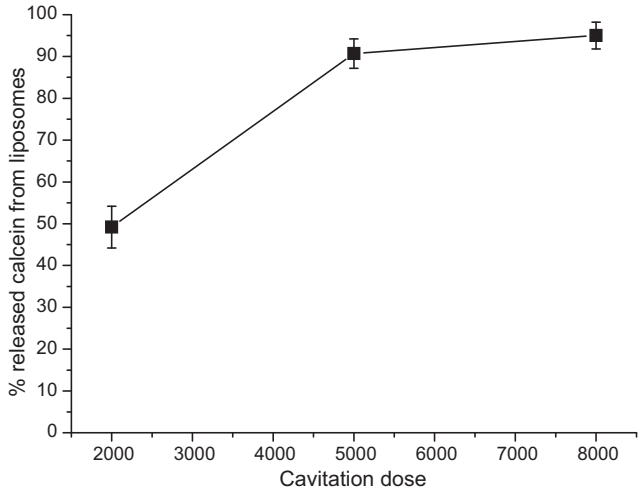

Fig. 1. Release of calcein from DOPE-based liposomes during exposure to 1.13 MHz US. Membrane composition: DOPE/DSPC/DSPE-PEG 2000/Chol 58/11/5/26 mol%. Bars represent the mean and standard deviation (SD) of triplicate US measurements.

The multivariate data analysis was performed with the software Unscrambler® (version 9.6, Camo Technologies Inc.). Partial least square regression (PLS) analysis using full cross validation was performed to identify the lipid variables with significant influence on sonosensitivity. The uncertainty testing was performed at a probability level of 0.05. The derived regression model described 89% of the variation in the data set. The precision of the model showed a root mean squared error of calibration of 3.3%.

Both the non-bilayer forming lipids DOPE and DSPE-PEG 2000 significantly increased sonosensitivity (Fig. 2). The positive correlation between DSPE-PEG and liposome sonosensitivity is in agreement with previous findings by Lin and Thomas (2003). The strong modulating effect of DOPE on sonosensitivity might be related to the non-bilayer characteristics of the lipid, which might induce membrane perturbations or local phase conversions from a lamellar to a reversed hexagonal phase upon US exposure. Such mechanisms may account for the demonstrated enhanced US-mediated liposomal drug release. By contrast, cholesterol displayed a negative correlation to sonosensitivity, i.e. higher levels of cholesterol reduced sonosensitivity. In general, the main effects of the lipids on sonosensitivity were comparable to previous data obtained with DSPE-based liposomes exposed to 40 kHz US (Evjen et al., 2010). The DSPE-PEG 2000 and cholesterol effects were, however, more pronounced in the current study.

The model revealed a significant interaction effect between DSPC and DOPE on sonosensitivity (Figs. 2 and 3), implying that

Table 2
Percent calcein released from liposomes at a cavitation dose of 2000 (1.13 MHz US). The mean and SD of triplicate US measurements are given.

Batch no.	DOPE:DSPC:DSPE-PEG 2000:CHOLESTEROL (mol%)	% calcein release
1	52:5:8:35	42 ± 1
2	52:20:8:20	45 ± 3
3[a]	52:10:3:35	49 ± 9
4	72:5:3:20	53 ± 4
5	52:20:3:25	30 ± 1
6	57:20:3:20	50 ± 6
7	67:5:8:20	64 ± 5
8	57:5:3:35	34 ± 1
9	58:11:5:26	49 ± 4
10	58:11:5:26	50 ± 4
11	58:11:5:26	48 ± 5

[a] The formulation batch was identified as an outlier by the PLS model and was therefore excluded from the design.

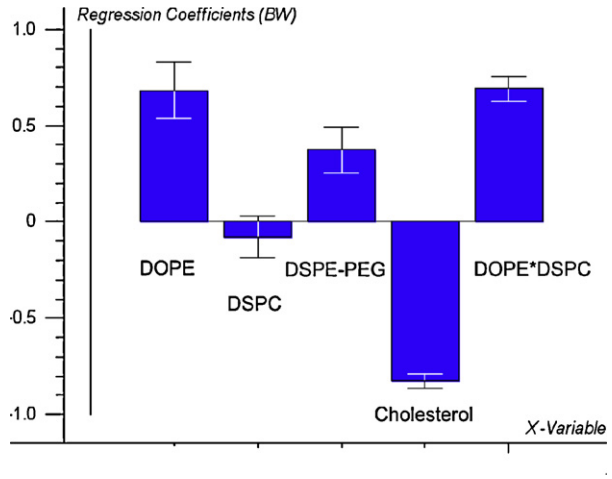

Fig. 2. Regression coefficients ($p < 0.05$) of the partial least square regression analysis of calcein release at a cavitation dose of 2000 (1.13 MHz US). (The amplitude of the regression coefficients indicates the degree of their positive or negative impact on liposome sonosensitivity.)

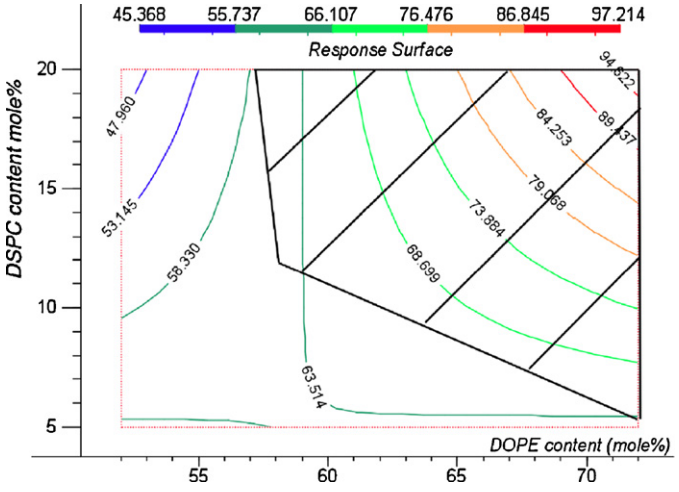

Fig. 3. Response surface plot showing the percentage US-mediated release of calcein from liposomes as a function of DOPE and DSPC content (mol%) (1.13 MHz US, cavitation dose 2000). DSPE-PEG 2000 and cholesterol levels are held constant at 8 and 20 mol%, respectively. The marked area is not included in the model.

for membranes containing low levels of DOPE, increased levels of DSPC reduced sonosensitivity. At higher levels of DOPE, the DSPC content had no significant influence on sonosensitivity (Fig. 3). This could be due to the strong modulating effect of DOPE, meaning that above a certain level of DOPE the DSPC level was not able to reduce sonosensitivity.

Application of sonosensitive liposomes for US-mediated drug delivery requires that the liposomes are able to retain its drug content on the way to the tumour. *In vitro* serum stability studies were performed to investigate whether the DOPE-based liposomes were able to retain their entrapped calcein under simulated biological conditions. The studies were performed according to experimental methods previously described (Evjen et al., 2010). In brief, calcein leakage from liposomes was studied by incubation of the liposomes in sucrose/HEPES solution containing 20% serum of fetal bovine origin (Autonorm, sero, Billingstad, Norway) for 6 h at 37 °C. Time-dependent leakage of liposomal calcein was quantified by fluorescence measurements as described above. Triplicate samples were measured for each formulation. All of the investigated liposome formulations showed less than 5% leakage of calcein after 6 h incubation. In general, the DOPE-based liposomes were markedly more stable in 20% serum than the previously investigated DSPE-based liposomes. Based on these results all of the investigated DOPE-based liposome formulations were found suitable for further testing in animals.

In conclusion, the current study demonstrated efficient release of calcein from DOPE-based liposomes on exposure to low doses of focused and high frequency US. The study has provided insight into key membrane parameters that affect liposome sonosensitivity. A strong correlation between sonosensitivity and the non-bilayer forming lipids DOPE and DSPE-PEG 2000 was demonstrated. All of the DOPE-based liposome formulations studied displayed both acceptable sonosensitivity and serum stability. The results obtained are promising for further evaluation of DOPE-based liposomes for US-mediated drug delivery *in vivo*.

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