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Faculty of Health Sciences
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Sonosensitive liposomes for ultrasound-mediated drug delivery

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A dissertation for the degree of
Philosophiae Doctor

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CONTENTS

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Oslo, 5th April 2011. Tove Julie Evjen.

LIST OF PAPERS

The thesis is based on the papers listed below. They are referred to by their Roman numerals (I-V) in the text.

I Evjen TJ, Nilssen EA, Røgnvaldsson S, Brandl M, Fossheim SL, 2010. Distearoylphosphatidylethanolamine-based liposomes for ultrasound-mediated drug delivery. European Journal of Pharmaceutics and Biopharmaceutics 75:327-333.

II Hagtvet E, Evjen TJ, Olsen DR, Fossheim SL, Nilssen EA, 2010. Ultrasound enhanced antitumour activity of liposomal doxorubicin in mice. Journal of Drug Targeting. In press.

III Evjen TJ, Nilssen EA, Barnert S, Schubert R, Brandl M, Fossheim S, 2011. Ultrasound- mediated destabilization and drug release from liposomes comprising dioleoylphosphatidylethanolamine. European Journal of Pharmaceutical Sciences 42:380-386.

IV Evjen TJ, Nilssen EA, Fowler RA, Røgnvaldsson S, Brandl M, Fossheim S, 2011. Lipid membrane composition influences drug release from dioleoylphosphatidylethanolamine-based liposomes on exposure to ultrasound. International Journal of Pharmaceutics 406:114-116.

V Evjen TJ, Hagtvet E, Nilssen EA, Brandl M, Fossheim S, 2011. Sonosensitive dioleoylphosphatidylethanolamine-containing liposomes with prolonged blood circulation time of doxorubicin. Submitted manuscript.

ABBREVIATIONS

CryoTEM	Cryo-transmission electron microscopy
DEPC	1,2- dierucoyl-*sn*-glycero-3-phosphatidylcholine
DNPC	1,2-dinervonoyl-*sn*-glycero-3-phosphatidylcholine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine
DSPE-PEG 2000	1,2 distearoyl-*sn*-glycero-3-phosphatidylethanolamine- N-(methoxy(polyethylene glycol)-2000)
DSPE	1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine
DXR	Doxorubicin
H_{II}	Reversed hexagonal phase
HIFU	High intensity frequency ultrasound
HSPC	Hydrogenated-soy-phosphatidylcholine
L_{α}	Lamellar liquid crystalline phase
LFUS	Low frequency ultrasound
MVA	Multivariate data analysis
MPS	Mononuclear phagocyte system
PC	Phosphatidylcholine
PCS	Photon correlation spectroscopy
PE	Phosphatidylethanolamine
P.I	Polydispersity index
PLS	Partial least squares regression analysis
PP	Packing parameter
SOPE	1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine
T_c	Gel-to-liquid crystalline phase transition temperature
US	Ultrasound

ABSTRACT

Liposome encapsulation of cytotoxic drugs favours drug delivery to tumours and improves the therapeutic-to-toxicity ratio of conventional chemotherapy. A novel approach to further enhance the availability of liposomal drugs to tumour cells is to combine ultrasound (US) with US sensitive (sonosensitive) liposomes. US treatment of tumour tissue induces local drug release from the liposome carrier followed by increased drug uptake into tumour cells. The liposomes, however, should display properties that both favour high sonosensitivity and drug retention in the blood circulation prior to reaching the tumour tissue.

In the present study novel sonosensitive doxorubicin (DXR)-containing liposomes were developed. Inclusion of non-bilayer forming lipids in liposomal membranes was shown to significantly improve sonosensitivity. Two classes of liposomes based on distearoylphosphatidylethanolamine (DSPE) and dioleoylphosphatidylethanolamine (DOPE), respectively, were investigated. DOPE, displaying a more pronounced inverted cone shaped geometry than DSPE, was the most potent modulator of sonosensitivity. Inclusion of 25 mol% DOPE in liposome membranes comprising distearoylphosphatidylcholine, PEGylated phosphatidylethanolamine and cholesterol resulted in an up to 7-fold increased US-mediated DXR release *in vitro*. The postulated mechanism of drug release is disruption of PE-based liposomes on US exposure. We suggest that US energy triggers perturbations and/or lamellar to reverse hexagonal phase transitions in liposomal membranes comprising non-bilayer forming lipids, leading to drug release.

Selected liposome formulations were investigated in terms of blood pharmacokinetics in mice. Membrane composition was important for retaining DXR in blood circulation. Low levels of DOPE (25-32 mol%) were required to obtain long blood circulation times of DXR. Optimized DOPE-containing liposomes featured both sufficient blood circulation time and high *in vitro* sonosensitivity.

A significant tumour growth regression effect was demonstrated in tumour-bearing mice receiving prototype liposomes and US. The data support the concept that an US activated liposomal drug delivery system might enhance the therapeutic effect of chemotherapeutic drugs.

1. INTRODUCTION

1.1 Liposomes

Liposomes are spherical vesicles composed of an aqueous core surrounded by a membrane that is usually composed of phospholipids [1]. Phospholipids are amphiphiles, which form colloid dispersions in water. The hydrophilic part of the molecule tends to be in contact with the water, whilst the hydrophobic acyl chains are shielded against water in the interior of the membrane. The vesicles formed may consist of one or more concentric bilayers (lamellae), and have a size range from nanometers to micrometers (For a review see [2]). The composition of the aqueous core as well as a lipid membrane gives the liposome the ability to incorporate both hydrophilic and hydrophobic drugs. Hence, liposomes can be used as a drug delivery system of cytotoxic drugs in cancer treatment. To understand the behaviour of liposomes for drug delivery, some general features of phospholipids and phase behaviour are presented in the following section.

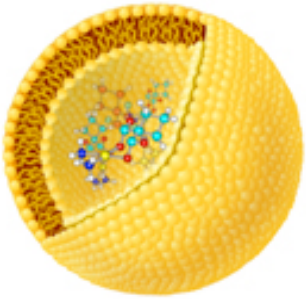
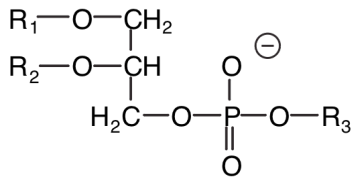


Figure. 1.1 *A schematic illustration of a liposome containing drug in the aqueous core. Epitarget©*

1.2 Phospholipids and aggregate structure

Phospholipids are the major components of biological membranes. The phospholipid molecule consists of two fatty acids, which are connected to a glycerol backbone with a polar phosphate headgroup via ester bonds (Figure 1.2).

Phospholipids are classified according to the type of polar headgroup, acyl chain length and degree of saturation. Phosphatidylcholines (PCs) are the most commonly used lipids in liposome formulations. PCs can be derived synthetically or from natural sources such as egg and soy. Phosphatidylethanolamines (PEs) are another group of phospholipids where PE substitutes the $-N^{+}(CH_3)_3$ polar headgroup with $-N^{+}(H_3)$ (For a review see [2]). Figure 1.2 shows the general structure of a phospholipid and the structure of distearoylphosphatidylcholine (DSPC), distearoylphosphatidylethanolamine (DSPE) and dioleoylphosphatidylethanolamine (DOPE) which are lipids used in this thesis.



DOPE: R_1=R_2= C18:1, R_3= -$CH_2CH_2^{\oplus}NH_3$

DSPE: R_1=R_2= C18:0, R_3= -$CH_2CH_2^{\oplus}NH_3$

DSPC: R_1=R_2= C18:0, R_3= -$CH_2CH_2^{\oplus}N(CH_3)_3$

Figure 1.2 *General structure of a phospholipid molecule and the structure of DOPE, DSPE and DSPC.*

The type of structure formed when phospholipids are dispersed in water is not only determined by the amphiphilic characteristics of the lipid, but also its shape, defined by the packing parameter (PP) [3]. The PP is defined as the ratio of the geometrical area of the hydrophobic to polar regions of the amphiphile, PP=v/l x a, where v is the volume of the molecule, a is the area of the polar head group, and l is the length of the hydrocarbon chains [3] (Figure 1.3).

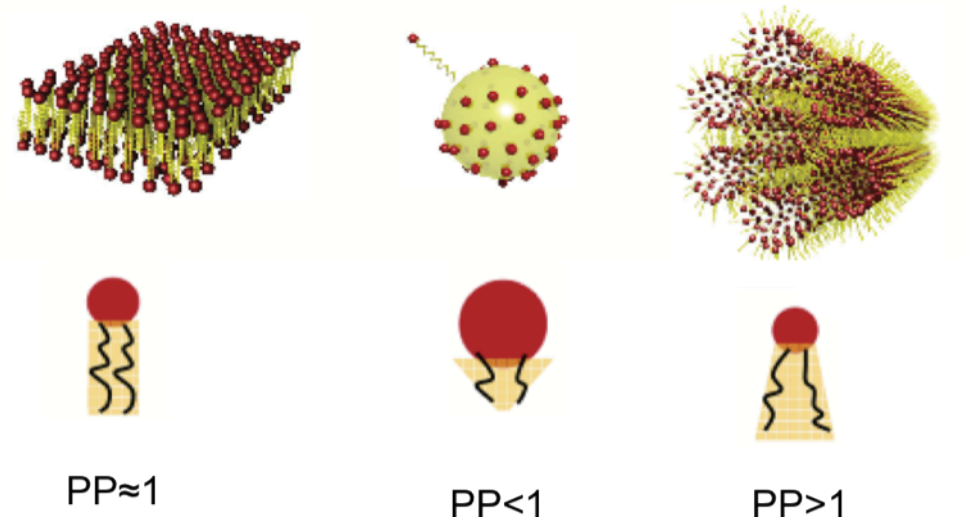


Figure 1.3*. Geometrical shape and packing parameters (PP) of lipids and the structures they form. Epitarget©*

Lipids like DSPC, where the ratio of hydrophobic tails to polar headgroup equals 1, have a natural tendency to form bilayers [3]. Lipids with a PP <1/3, e.g. fatty acids and lysolipids, form micelles [3]. In the case of non-bilayer forming lipids, like DOPE, the headgroup is small

compared to the bulky hydrocarbon chains (PP>1), which does not enable the lipid alone to form liposome bilayers at physiological pH. Instead DOPE forms reversed structures where the bulky hydrophobic part of the lipid imposes an orientation towards the aqueous phase [3]. At pH>8, the polar group deprotonates, increases its size due to increased hydration, and liposomes may be formed. Bilayers may also be formed when PEs are mixed with bilayer stabilizing lipids like DSPC or polyethylene glycol (PEG) grafted lipids, resulting in a net curvature of a bilayer [3,4].

The choice of lipid composition will to a great extent define the stability of the liposomes, including the gel-to-liquid crystalline phase transition temperature (T_c) and membrane permeability. T_c is a given temperature at which the hydrocarbon chains in the membrane melt from a tightly ordered gel phase to a liquid-crystal phase, which is more permeable to ions and molecules (For a review see [2]). At physiological temperature, liposomes composed of saturated phospholipids, which have a high T_c, will be more rigid than liposomes composed of unsaturated phospholipids, which renders the former less prone to drug leakage. Cholesterol is often used in liposomes to induce mechanical stability by tightening the membrane and reducing leakage of entrapped drug [5-7]. Liposomes composed of long saturated PCs, such as DSPC, and cholesterol are the most studied liposome formulations within drug delivery.

1.3 Liposomes in cancer treatment

The main rationale for developing liposomes as drug delivery systems for chemotherapeutic drugs is the unspecific distribution of most antineoplastic drugs in the body, giving a low therapeutic-to-toxic ratio. Liposome incorporation of drugs significantly alters the pharmacokinetics and biodistribution of the drugs [8]. Small liposomes, slightly below 100 nm in diameter, are able to circulate in blood reaching specific targets such as solid tumors [9-12]. The accumulation of liposomes into solid tumours is possible due to dissimilarities between healthy and cancerous tissues. Tumours are generally dependent on an increased blood supply compared to normal tissue because of the high turnover of neoplastic cells. Furthermore, the endothelium is often more permeable than normal endothelial linings because of larger gaps [12,13]. This gives nanosized drug carriers, like liposomes, the ability to diffuse into the interstitium of solid tumours [9,12-14]. At the same time the liposomes are retained in the tumour tissue due to reduced lymphatic drainage [13]. This increased accumulation is often referred to as the enhanced permeability and retention effect [12,13].

The extent of liposome accumulation in tumour tissue is largely determined by the blood circulation time of the liposome carrier, where long circulation times of the liposomal drug provide greater tumour delivery [9,14]. Extended blood circulation time of liposomes is

achieved by reducing the liposome size, preferably slightly below 100 nm in diameter, and by coating the liposomes with the hydrophilic polymer polyethylene glycol (PEG) [15]. The hydrophilic bulky PEG moiety prevents or minimizes interactions between plasma opsonins and the liposome surface due to steric hindrance, thereby retarding recognition of the liposomes by the mononuclear phagocyte system (MPS) [16].

1.4 Doxorubicin-containing liposomes

For chemotherapeutic drugs to be suitable for drug delivery by means of liposomes the drugs must be able to be efficiently loaded into the liposome where it is retained *en route* to the tumour. The anthracycline doxorubicin (DXR) is a suitable chemotherapeutic agent for liposomal drug delivery, mainly due to the efficient remote loading technique, resulting in a high drug-to-lipid ratio [17]. Most of the drug forms a crystalline-like precipitate in the liposomal aqueous core, lacking osmotic effects and thus contributing to the stability of the entrapment [17]. Encapsulation of DXR into PEGylated liposomes results in extended circulation time and a reduced volume of distribution compared to free DXR, thereby promoting tumour uptake [18]. PEGylated liposomal DXR is one of about 10 marketed liposome products, known as Doxil® in the USA and Caelyx® in Europe [18]. The formulation is approved for treatment of AIDS related kaposi sarcoma, metastatic breast cancer and recurrent ovarian cancer [18].

1.5 Triggered release

An inherent contradiction to the enhanced tumour delivery of liposomes exhibiting high drug retention is that drug availability to tumour cells may be severely limited. After the liposomes have accumulated in the tumour interstitial space, the encapsulated drug must be released before it can be sufficiently taken up by the surrounding neoplastic cells. A fast drug release from liposomes is especially important for fast growing tumours, where cells divide more rapidly than the liposomes can distribute to tumours and release their contents [8]. In the case of long circulating PEGylated liposomal DXR, the drug has shown to release slowly from the liposomes [19,20]. Thus, triggered release of the liposomal drug within the tumour area might lead to a substantial increase in drug availability and thus improve the therapeutic efficacy.

Throughout the last decades various approaches have been attempted to enhance local drug delivery to tumours by triggering drug release from liposomes, including hyperthermia, enzymatic and pH strategies [21-25]. The idea is that an environmental change will trigger liposome membranes to structural rearrangements that induce leakage of the encapsulated drug. Thermo-sensitive liposomes (TSL) combined with high intensity frequency ultrasound (HIFU), microwave or radiofrequency treatment are examples where localized hyperthermia in the tumour tissue induces drug leakage from TSL as a result of increased membrane permeability

above 37°C [26,27]. TSL have typically T_c in the range of 40-45 °C, which facilitates drug leakage at these temperatures and above [28]. A general limitation for thermo-sensitive formulations, however, has been premature leakage of the entrapped drug in blood circulation prior to reaching the tumour tissue, which in turn may limit the benefit of the strategy.

Recently, non-thermal ultrasound (US) has been suggested as an alternative non-invasive approach to enhance drug delivery of chemotherapeutics to solid tumours via liposomes [29,30].

1.6 Ultrasound-mediated drug delivery

US is in clinical use for diagnostic imaging and therapeutic purposes. High intensity frequency ultrasound (HIFU) is used to thermally ablate solid tumours such as uterine fibroids [31] and prostate cancer [32]. In recent years the potential of using US to enhance delivery of chemotherapeutics to solid tumours via drug carriers has been investigated [29,30,33,34]. Acoustic cavitation generated by US has shown to increase permeability of phospholipidmembranes, thus inducing both drug release from liposomes and enhancing drug uptake into cancer cells. For a recent review see Frenkel [30].

Acoustic cavitation may be defined as the growth, oscillation and collapse of small, stabilized gas bubbles under the influence of the varying pressure field of a sound wave in a fluid medium [33]. There are two distinct types of acoustic cavitation; stable, and inertial cavitation. Stable cavitation persists for repeatable acoustic cycles, where the bubble size is fairly stable. At increased US intensities, inertial cavitation may occur, where the bubbles grow faster, increase in size where they oscillate unstably, and finally collapse [33]. Inertial cavitation is considered to be the primary ultrasound cause for increasing cell-permeability and drug release from liposomes [30]. The underlying mechanism, although not fully established, is related to the collapse of microbubbles near the surface of the cells or liposome surface, which induce pores in the phospholipid membrane [33]. Hence, US focused to tumours upon accumulation of drug-containing liposomes may both induce release of the drug load from the liposome carrier and increase uptake into tumour cells, thus enhancing local drug delivery (Figure 1.4). The US energy deposited to tumours is monitored by controlling intensity and exposure time.

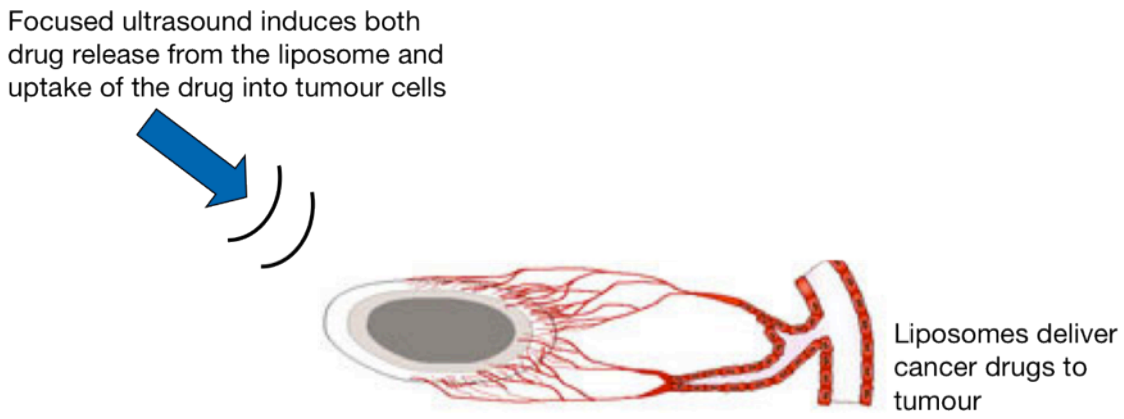


Figure 1.4 *Ultrasound-mediated drug delivery to solid tumours by means of sonosensitive liposomes. Epitarget ©*

1.7 Sonosensitive liposomes

US sensitive (sonosensitive liposomes) are liposomal drug carriers designed for releasing their drug load on exposure to US (Figure 1.5). Preclinical research in designing sonosensitive drug-containing vesicles is ongoing. Several studies have shown that gas-containing liposomes are highly sonosensitive [35-37]. However, gas-filled liposomes are typically micron-sized and thus too large to allow effective extravasation into tumour tissue [34]. Another limitation of gas-filled liposomes has been the rapid dissolution of the entrapped gas in the blood circulation with consequent loss of sonosensitivity.[34]

Although it has appeared to be a general assumption that gas is required to make drug carriers responsive to non-thermal US, a few studies have demonstrated that membrane composition can influence on liposome sonosensitivity [38-40]. Lin and Thomas found that inclusion of PEGylated distearoylphosphatidylethanolamines in liposome bilayers enhanced liposomal release of a drug marker upon exposure to low frequency US (LFUS) [38].

Recently, we have further explored the influence of liposome membrane composition on sonosensitivity with the intention of developing efficacious sonosensitive liposomes for US-mediated drug delivery. The formulation and characterization of the novel sonosensitive liposomes is the basis of this thesis.

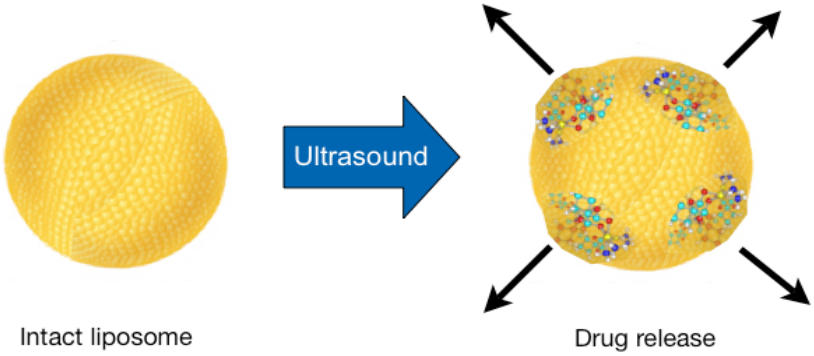


Figure 1.5 *Sonosensitive liposomes release their drug load on exposure to ultrasound. Epitarget ©*

2. AIM OF THESIS

The overall aim was to develop novel sonosensitive liposomes for US-mediated drug delivery to solid tumours.

Several specific aims were set for the liposome formulation work:

- Liposomes should allow for high and stable drug entrapment.
- Liposomes should efficiently release their drug load on US exposure.
- The liposomes should have good stability, both on the shelf and in serum.
- The liposomes should display long blood circulation time *in vivo* (1-2 days), enabling sufficient tumour accumulation. Liposome size should therefore be slightly below 100 nm in diameter.

The first section of the thesis includes formulation design of novel sonosensitive liposomes where the influence of liposome membrane composition on sonosensitivity is investigated (papers I, III, IV, V). Further, studies investigating potential mechanisms of drug release from liposomes on exposure to US are discussed (paper III). Thereafter follows evaluation of *in vitro* stability of liposomes, as well as *in vivo* blood circulation time of selected formulations in non-tumoured mice (paper V). In the last section a first proof of principle study of prototype liposomes in tumour-bearing mice is described (paper II).

3. MATERIALS AND METHODS

3.1 Materials

All phospholipids were purchased from Genzyme Pharmaceuticals, Liestal, Switzerland. Cholesterol, organic solvents, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), ammonium sulfate, calcein, sodium azide, 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. Caelyx® was obtained from the pharmacy at the Norwegian Radium Hospital, Oslo, Norway (European distributor Schering-Plough). For anesthesia of mice, a mixture of 2.4 mg/ml tiletamine and 2.4 mg/ml zolazepam (Zoletil®vet; Virbac Laboratories, Carros, France), 3.8 mg/l xylazine (Narcoxyl® vet; Roche, Basel, Switzerland) and 0.1 mg/ml butorphanol (Torbugesic®; Fort Dodge Laboratories, Fort Dodge, IA) was prepared and used. Physiological saline was supplied by Fresenius Kabi, Halden, Norway.

3.2 Methods

3.2.1 Liposome preparation and characterization

DSPE and DOPE-based liposomes of different membrane compositions were prepared by the thin-film hydration method and sequential extrusion technique (For reviews of methods see [41,42]. (See papers I-V for details regarding lipid membrane compositions). In brief, lipids were dissolved in chloroform/methanol (9/1 v/v) at 60 °C and rotary evaporated to dryness under vacuum. For preparation of calcein-containing liposomes, the dry lipid films were hydrated with isotonic sucrose solution containing 10 mM HEPES (pH 7.4) and 50 mM calcein to result in a lipid concentration of 16 mg/ml. For preparation of DXR-containing liposomes 300 mM ammonium sulfate solution was used for hydration, resulting in a lipid concentration of 20 mg/ml. The hydrated liposomes were submitted to three freeze–thaw cycles in a dry ice/acetone/methanol mixture and water, respectively (For a review of the method see [41]). The liposomes were reduced in size by stepwise extrusion (Lipex extruder, Biomembrane Inc., Vancouver B.C., Canada) through 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 temperatures above the nominal T_c 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 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 3 days (volume ratio liposome dispersion:dialysis solution, 1:100 v/v) with intermediate exchanges of the dialysis solution (For a review of the method see [41]).

Liposome entrapment of doxorubicin

DXR was remote loaded into liposomes using an ammonium sulfate transmembrane gradient as previously described [17], resulting in a concentration of 1 mg/ml DXR and 16 mg/ml lipids. The gradient was obtained by dialysis of DOPE-based liposomes against isotonic sucrose solution containing 10 mM HEPES (pH 7.4) (herein referred to as sucrose/HEPES solution). Non-buffered isotonic sucrose solution was used for dialysis of DSPE-based liposomes.

The dialysis was performed for approximately 48 h, with intermediate exchanges of the dialysis solutions as described above. To provide optimal loading efficiency, the DOPE- and DSPE-based liposome dispersions were, after DXR addition, further incubated under stirring for 60 min at 35 ◦C and for 30 min at 75 ◦C, respectively. Any remaining non-encapsulated DXR was removed by liposome dialysis against sucrose/HEPES solution containing 0.01w/v% sodium azide, as described above.

Determination of entrapment efficiency of DXR

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

Equation 1: $(F_{en} - F_b)/(F_{tot} - F_b) \times 100\%$

where F_{en} is the fluorescence intensity in the dialyzed and surfactant treated liposome sample, F_b is the initial background signal of the dispersion medium (sucrose/HEPES solution) and F_{tot} is the fluorescence intensity in 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 of DXR were 488 and 595 nm, respectively.

Liposome size measurements

The mean intensity-weighted hydrodynamic liposome diameter was determined by photon correlation spectroscopy (PCS). Prior to measurements the liposome dispersions were diluted 1:200 (v/v) with 0.22 μm filtered sucrose/HEPES solution. The measurements were performed

at 23 ºC and at a scattering angle of 90° (Nanosizer, Malvern Instruments, Malvern, UK). The width of the particle size distribution was expressed by the polydispersity index (P.I).

3.2.2 Low frequency ultrasound experiments

Low frequency US release measurements were conducted using a 40 kHz US transducer (VC 750, Sonic and Materials, Inc, Newtown, CT, USA) with a 19 mm diameter nonfocused probe connected to a custom-built sample chamber as previously described by Huang and MacDonald [37] (Figure 3.1). The temperature in the sample chamber was kept constant at 25 ºC by a water circulator system. The liposome dispersions were diluted in a 1:500 (v/v) ratio with sucrose/HEPES solution or sucrose/HEPES solution containing 20% (v/v) serum, respectively, just prior to the US experiments. The diluted liposome dispersions were exposed to 40 kHz US at a nominal intensity of 12 W/cm^2 in a continuous mode (100 % duty cycle) up to 6 min. Acoustic pressure measurements conducted with a Bruel and Kjaer hydrophone (Type 8103, Denmark) in the sample chamber gave 240 kPa (pk–pk). The temperature in the liposome samples never exceeded 30 °C during the US experiments, excluding the possibility of direct thermal effect of US on liposomal drug release.

The DXR or calcein release could be monitored due to the relief of fluorescence self-quenching in the external liposomal phase, and concomitant increase in fluorescence intensity [43]. Fluorescence measurements were carried out as described above. Release was calcluated using the following equation:

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

Where F_t is the fluorescence intensity in the liposome sample after a given duration (t) of US, F_0 is the initial background fluorescence of the diluted 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® solution at a 50:1 (v/v) ratio.

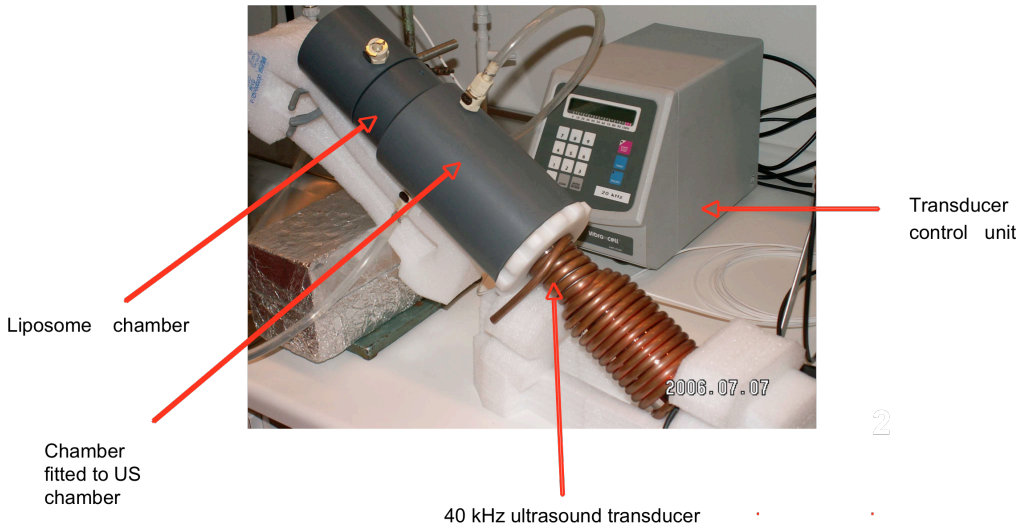


Figure 3.1 *Picture of the 40 kHz US set-up.*

3.2.3 High frequency ultrasound experiments

High frequency US release measurements were performed using a custom built US set-up providing a standardized US dosimetry, as described by Somaglino *et al.* [44] (Figure 3.2). The US experiments were conducted in Dr. Cyril Lafon's laboratory at INSERM U556, Lyon, France. 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. The liposome dispersions were diluted in a 1:500 (v/v) ratio with sucrose/HEPES solution just prior to the US experiments. US-mediated release of liposomal calcein and DXR was calculated according to Equation 2. The excitation and emission wavelength for the measurement of calcein were 490 and 550 nm, respectively.

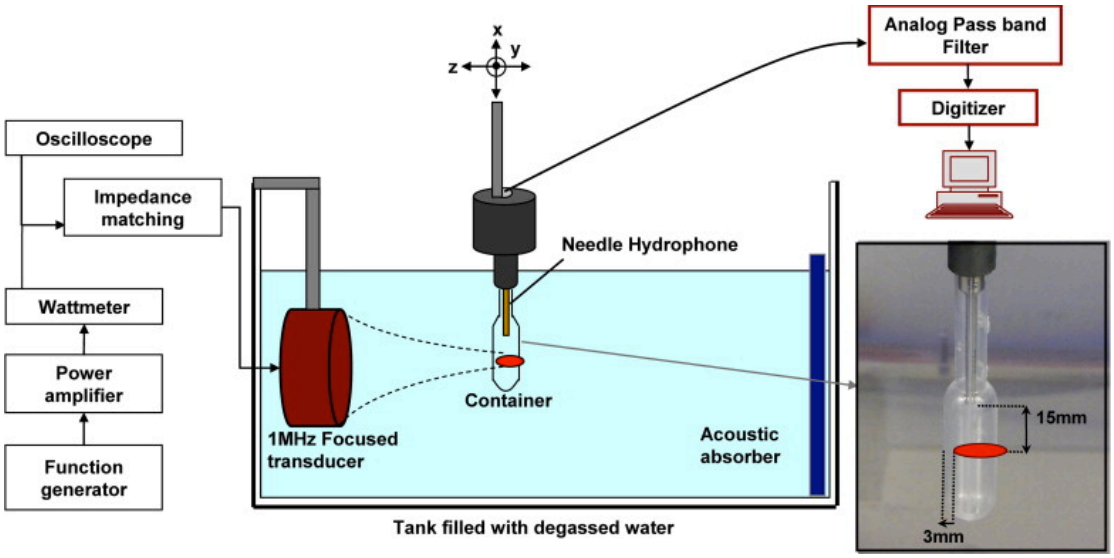


Figure 3.2 *Schematic drawing of the 1.13 MHz US setup. The drawing is adapted from Somaglino et al., with permission from Elsevier publisher.*

3.2.4 Cryo-TEM analysis

Cryo-TEM analysis was performed in collaboration with Prof. Rolf Schubert and Sabine Barnert at the Dept. of Pharmaceutical Technology, University of Freiburg, Germany. US-treated liposome dispersions for Cryo-transmission electron microscopy (CryoTEM) analysis were diluted 1:10 v/v with 0.22 μm filtered sucrose/HEPES solution prior to US. The non-treated liposome dispersions were not diluted prior to analysis.

Cryo-TEM investigations were performed according to methods described by Rank *et al.* [45], using a LEO 912 OMEGA electron microscope (Zeiss, Oberkochen, Germany) operating at 120 kV. A drop of the sample was placed onto a copper grid (Quantifoil® S7/2 Cu 400 mesh, holey carbon films Quantifoil Micro Tools GmbH, Jena, Germany). Excess solution was removed by a filter paper, leaving a thin liquid film with a thickness of 100-500 nm. The sample was then immediately shock-frozen by plunging it into liquid ethane. The vitrified sample was stored at 90 K in liquid nitrogen until it was loaded into a cryogenic sample holder (D626, Gatan Inc, Pleasanton, USA). The specimens were examined at -174 °C. Digital images with a magnification of 6300x or 12500x were recorded with a slow-scan CCD camera system (Proscan HSC 2). Minimal under-focus of the microscope objective lens was provided to obtain sufficient phase contrast [46].

3.2.5 Effect of US on chemical integrity of DXR, cholesterol and phospholipids

Assay and purity of DXR, and assay of cholesterol, DOPE, DSPE and DSPC before and after exposure of liposomes to 6 min 40 kHz US and 1.13 MHz US (cavitation dose of 200, 25% duty cycle, 200 Hz pulse repetition frequency, Ispta = 5500 W/cm^2), respectively, was analyzed by high performance liquid chromatography (HPLC). Vitas AS, Oslo, performed the analyses.

Five-point calibration curves were made from analysis of calibrators with known concentrations of DXR, cholesterol and phospholipids, respectively. For determination of DXR 25 µL of the liposome suspensions was extracted with 650 µl 2-propanol and water. After thorough sonication and mixing (5 + 15 min) and centrifugation (5 min, 4000 g at 10 °C), an aliquot of 3 µL was injected from the supernatant into the HPLC system. HPLC analysis was performed with a HP 1200 liquid chromatograph (Agilent Technologies, Palo Alta, CA, USA) with a HP1200 diode array detector (DAD) operated at 480 nm. DXR was separated from the matrix on a 4.6 mm x 50 mm reversed phase C18 column. The column temperature was 50 °C.

Cholesterol and phospholipids were determined by LC-UV and LC-MS, respectively. 20 µL of the liposome samples was extracted with 5000 µl 2-propanol, followed by sonication and mixing (5 + 5 min).

For quantification of cholesterol an aliquot of 20 µL was injected into the HPLC system. The HP1200 DAD operated at 204 nm. Cholesterol was separated from the matrix on a 4.6 mm x 150 mm reversed phase C18 column. The column temperature was 50 °C.

For quantification of phospholipids, an aliquot of 2 µL of the liposome suspensions was injected into the HPLC-Mass Spectrometry (HPLC-MS) system. HPLC-MS was performed with a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alta, CA, USA) with a HP LC/MSD SL mass spectrometer operated in single ion monitoring mode. Internal standards were used to adjust for sample injection volume and ion suppression. DSPC, DOPE and DSPE were separated from the matrix on a 4.6 mm x 50 mm reversed phase polymer column. The column temperature was 60 °C.

3.2.6 *In vitro* liposome stability

Storage stability

Physicochemical stability of liposomes was followed up to 6 months storage at 5 °C. The testing included re-determination of mean liposome size and size distribution, sonosensitivity, retention of encapsulated DXR, and chemical integrity of DXR, DOPE, DSPC, DSPE and cholesterol. See the above sections for methods.

Stability in serum

Liposome stability in serum was studied *in vitro* using a serum-induced leakage assay [28,47]. Liposome dispersions, diluted 1:125 v/v with sucrose/HEPES solution and 20% serum, were incubated at 37 °C up to 6 or 24 h. Time-dependent leakage of liposomal DXR or calcein was quantified by fluorescence measurements of serum samples further diluted 1:4 v/v with the sucrose/HEPES solution, according to Equation 2.

3.2.7 Animal studies

Animal studies were performed in collaboration with Eirik Hagtvet at the Department of Radiation Biology, Institute for Cancer Research, Oslo University Hospital, Norway. All animal studies were performed according to protocols approved by the National Animal Research Authority and in compliance with the European Convention for the Protection of Vertebrates Used for Scientific Purposes.

Male athymic nude Balb/c mice were provided by the Department of Comparative Medicine (animal facility), the Norwegian Radium Hospital. The mice were housed in transparent boxes with bedding material, fed *ad libitum* and kept under specific pathogen-free conditions. The temperature and relative humidity were kept constant at 20-21°C and 60%, respectively. At the end of the experiments all animals were euthanized by cervical dislocation.

Pharmacokinetic studies in non-tumoured mice

Non-tumoured mice were administered with 7 mg liposomal DXR/kg body weight. Standard liposomal DXR was administered at 14 mg/kg due to the doubled drug-to-lipid ratio of the formulation (1:8 drug/lipid) vs. DOPE-based liposomes (1:16 drug/lipid). Thus, the dosing was based on an identical lipid dose for all investigated liposome formulations, as the lipid dose (i.e. liposome dose) is generally known as a key factor influencing blood pharmacokinetics [8].

The liposomes were injected intravenously (i.v.) into the tail vein under anaesthesia induced by subcutaneous administration. Animals were sacrificed in groups of three at different time points post-injection (0.5, 1, 3, 8 12, 24 and 48 h). Blood samples were obtained by cardiac puncture using heparinized syringes and stored in heparinized tubes. All blood samples were kept on ice bath until storage at -80 °C. Extraction of DXR from blood was performed according to methods described by Gabizon *et al*. [48]. The extracted DXR was quantified by fluorescence measurements as previously described.

Therapy study in tumoured mice

CWR22 prostate adenocarcinoma, initially obtained from patients during surgery, was serially transplanted between mice. In brief, by blunt dissection through a skin incision a tumour

fragment (~2x2x2 mm) was subcutaneously implanted into the flank of 4-5 weeks old mice. The skin incision was sealed with topical skin adhesive. Approximately three weeks later a tumour xenograft of 5-7 mm in diameter had developed.

The animals were randomly allocated into 4 groups (n=8) according to different treatment regimes. A dose of 3.5 mg/kg liposomal DXR was given i.v. to anesthetized animals. The treatment groups included mice receiving 1) liposomal DXR, 2) liposomal DXR + US, 3) saline (0.25 ml/animal), 4) saline (0.25 ml/animal) + US.

Designated animals received US treatment at 24 h post-injection, when the tumour DXR concentration had reached peak levels. A 40 kHz ultrasonic transducer (Model VC 754, Sonic and Materials Inc., Newtown, CT, US) with a 19 mm diameter probe was partially submerged into a cylinder containing deionized and degassed water. The bottom of the cylinder was sealed with a latex membrane in firm contact with the skin covering the tumours located on an adjustable plate. A thin layer of US gel was placed between the skin and the latex membrane. The US probe was run at a nominal intensity of ~12 W/cm^2 for a duration of 4 min and with a 2 cm distance between the probe and skin. Preliminary tests were performed to ensure that the combination of probe-skin distance and duration of US exposure did not induce any visible local skin lesions. Tumour size was measured with 3-5 days intervals for 22 days using digital callipers (model B220S, Kroeplin, Schlüchtern, Germany). Tumour volume was calculated using the formula $(\pi/6)*length^2*width$ [49]. Individual tumour volumes were normalized to pre-treatment level on day 0.

3.2.8 Statistical methods

Multivariate data analysis (MVA) was performed with the software Unscrambler ® (version 9.6, Camo Technologies Inc). Partial least square regression (PLS) analysis using full cross validation was used to analyse significance of lipid variables in the MVA at a probability level (p) <0.05. Prior to calculations the variation of each variable was scaled to unite variance (using 1/SD as the scaling factor).

For statistical comparison of two means, a student t-test was used at significant levels of $p<0.05$ or $p<0.01$.

Differences in tumour growth delay between the four experimental groups in the therapy study were operationally represented by three between-group contrasts: 1) comparing the liposomal DXR groups and the saline groups, 2) comparing the US group with no-US within the liposomal DXR conditions and finally 3) comparing the US group with no-US within the saline conditions. The analysis was performed using normalised tumour volumes. The tumour growth delay was represented by developmental growth curves of linear and quadratic

polynomial contrasts, respectively, adjusted for unequal time intervals between the measurement points [50,51].

4. RESULTS AND DISCUSSION

4.1 Development of sonosensitive liposome membranes

The influence of liposome membrane composition on *in vitro* sonosensitivity was investigated. Traditionally, PC-lipids are used as the major membrane component in liposomes for drug delivery. However, PC-based liposomes (Caelyx®, herein defined as standard liposomal DXR) comprising HSPC, DSPE-PEG 2000 and cholesterol showed low *in vitro* sonosensitivity, where only 9 ± 2% of the entrapped DXR was released after 6 min of 40 kHz US exposure in sucrose/HEPES solution (paper I, Figure 4.2).

In an attempt to enhance responsiveness of liposome membranes to acoustic energy, non-bilayer forming PEs having an inverted cone shaped geometry were included in traditional liposome bilayers composed of DSPC, DSPE-PEG 2000 and cholesterol. The hypothesis was that US energy could trigger membrane perturbations and/or lamellar to reverse hexagonal phase transitions, leading to drug release (Figure 4.1).

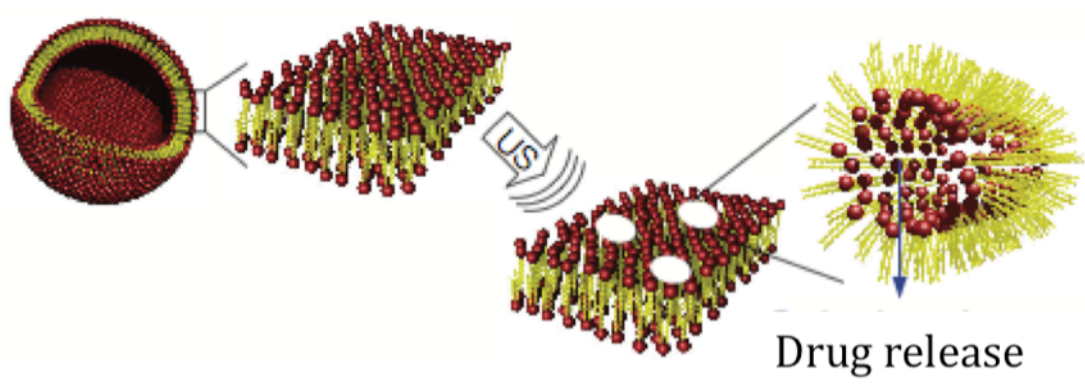


Figure 4.1 *Illustration of proposed mechanism for US-mediated drug release from liposomes containing non-bilayer forming lipids. US energy triggers lamellar to reverse hexagonal phase transitions in the liposome bilayer during US exposure, leading to drug release. Epitarget©*

4.1.2 DSPE-based liposomes

Initially, DXR-containing liposomes comprising DSPE, DSPC, DSPE-PEG 2000 and cholesterol were prepared and investigated in terms of *in vitro* sonosensitivity (paper I). The long saturated acyl chains of DSPE were considered to provide stability to the membrane *per se*, whilst the slight tendency of the lipid to form H_{II} phases was hypothesized to induce drug release upon US exposure.

Figure 4.2 shows increased liposomal drug release as a function of US exposure time. Inclusion of DSPE within the liposome bilayer resulted in a significantly enhanced sonosensitivity compared to traditional PC-based liposomes. For the particular formulations shown in Figure 4.2, liposomal DXR release after 6 min 40 kHz US showed a 7-fold increase by substituting DSPC with DSPE.

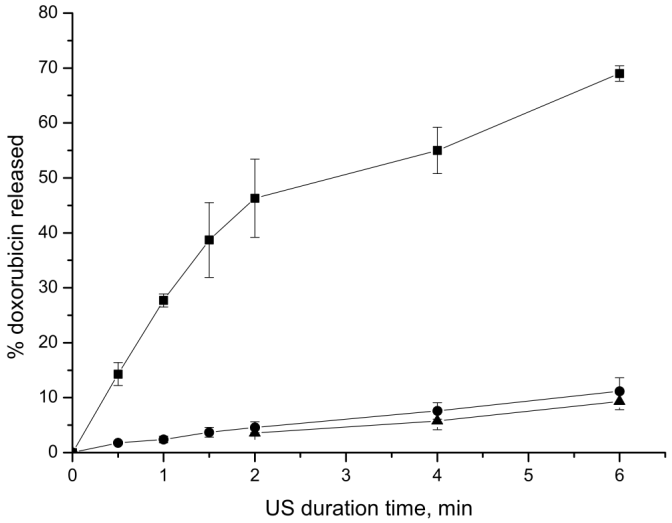


Figure 4.2 *US-mediated release profiles of DXR-containing liposomes consisting of the lipids; ■ DSPE, DSPE-PEG 2000 and cholesterol 62:8:30 mol %, ▲ DSPC, DSPE-PEG 2000 and cholesterol 62:8:30 mol %, and ●Standard liposomal DXR (HSPC:DSPE-PEG 2000:cholesterol 57:5:38 mol %). (The Figure is reprinted from paper I).*

As proposed above, the positive effect of DSPE on sonosensitivty was suggested to be related to the inverted cone shaped geometry of the lipid which upon US exposure promotes induction of local defects or polymorphic phase transitions within micro-rafts in the liposome bilayer, leading to drug release. The long saturated acyl chains of DSPE occupy a large volume compared to the small polar headgroup, which makes the lipid undergo a L_{α}-H_{II} phase transition at temperatures above 80 °C and/or at high pressure [52,53]. Pressure - and/or temperature jumps have previously shown to induce liposomal phase transitions [52-54]. The sample temperature never exceeded 30 °C during the US experiments, excluding direct thermal effects on drug release (paper I). However, it is likely that extreme temperature and/or pressure jumps in the near vicinity of cavitating bubbles could induce drug release from liposomes.

Influence of membrane composition on sonosensitivity of DSPE-based liposomes

Alterations in membrane composition of DSPE-based liposomes were further investigated to elucidate the effects, interplay and optimum levels of the different membrane lipids on

sonosensitivity (paper I). A full factorial design was employed where liposomes comprising different molar ratios of DSPE, DSPE-PEG 2000, DSPC and cholesterol were prepared. The correlation between the membrane lipids and DXR release after 6 min 40 kHz US exposure was explored using PLS analysis. The design is outlined in Table 4.1.

The actual mean intensity weight diameter of the formulations ranged from 83-90 nm, with P.I values less than 0.14, indicating narrow size distributions. Entrapment efficiencies of DXR were more than 92% for all formulations. The comparable mean sizes and loading efficiencies of the formulations should exclude potential influence of these factors in the MVA.

Table 4.1 *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 %. (The Table is reprinted from paper I)*

Lipid variables	**Level**		
	-1	0	+1
DSPE	47	54.5	62
DSPE-PEG 2000	3	5.5	8
Cholesterol	20	25	30

Sonosensitivity of DSPE-based liposomes was dependent on membrane composition, where release after 6 min 40 kHz US varied from 15 to 69 % for the different formulations (paper I). DSPE had the strongest impact on the model, showing a positive correlation to sonosensitivity (Figure 4.3). For a fixed level of cholesterol (30 mol%) and DSPE-PEG 2000 (8 mol%), an increase in DSPE content from 47 to 62 mol% increased the release extent with 51% after 6 min 40 kHz US (paper I).

The slightly positive effect of DSPE-PEG 2000 on sonosensitivity was in agreement with previous studies on PC-based liposomes by Lin and Thomas [38,39]. For the DSPE-based liposomes studied here, however, the effect of varying DSPE-PEG 2000 content (from 3-8 mol%) on sonosensitivity was not significant. This indicated that above a certain level of PEGylation, no added benefit on sonosensitivity was achieved.

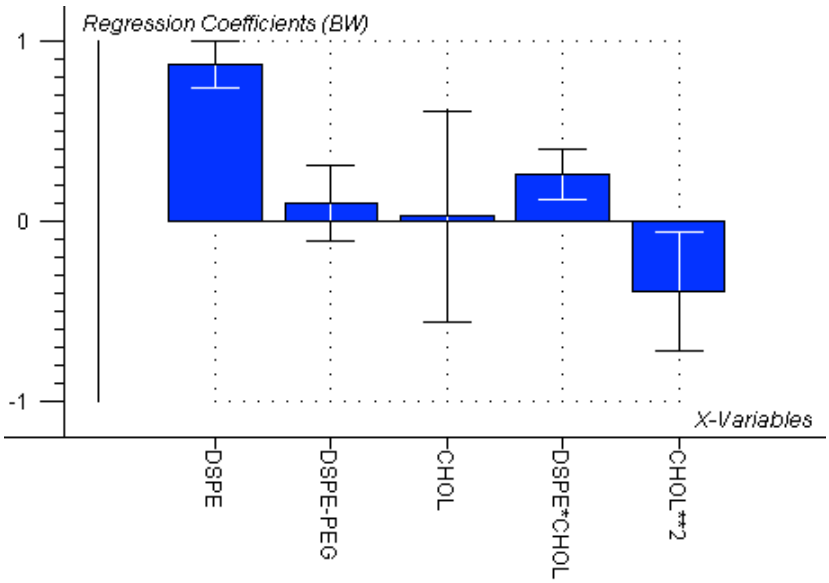


Figure 4.3 *Regression coefficients ($p<0.05$) of the partial least square regression analysis describing the significance of lipids on US-mediated DXR release after 6 min 40 kHz US. The amplitude of the regression coefficients indicates the degree of their positive or negative impact on liposome sonosensitivity. (The Figure is reprinted from paper I).*

Furthermore, the observed interaction effect between DSPE and cholesterol implied an optimum in sonosensitivitiy at intermediate levels of cholesterol and high levels of DSPE (Figure 4.4). The model also showed a squared effect of cholesterol, implying a negative and nonlinear correlation between cholesterol level and sonosensitivity. In contrast, Lin and Thomas reported that the sonosensitivity of PC-based liposomes increased approximately proportionally with higher cholesterol concentrations [39]. This indicates that the effect of cholesterol on sonosensitivity might vary with different phospholipids.

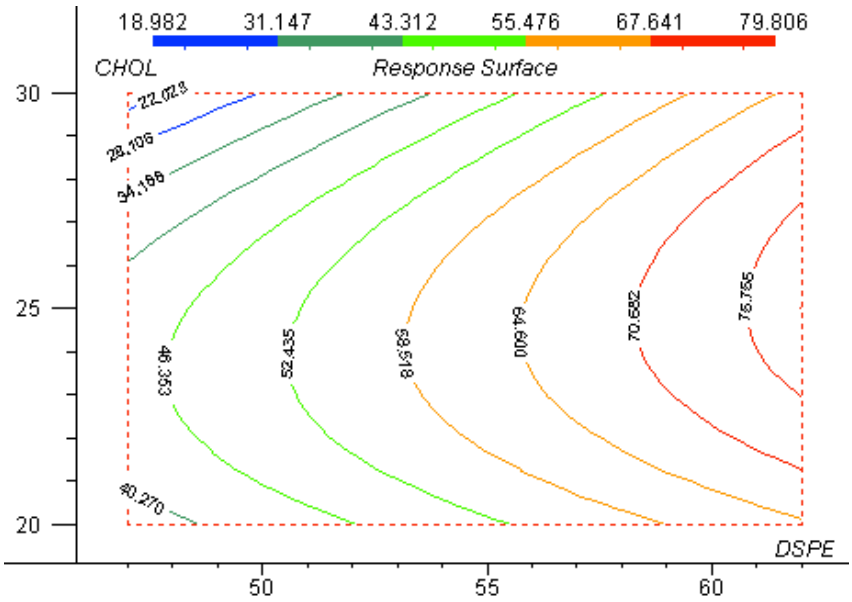


Figure 4.4 *Response surface plot of US-mediated liposomal DXR release as a function of DSPE and cholesterol levels (mol %). (6 min 40 kHz US data, DSPE-PEG 2000 level held constant at 8 mol %). (The Figure is reprinted from paper I).*

4.1.3 DOPE-based liposomes

The conditions for which PEs form H_{II} phases can be modulated by changing the degree of unsaturation in the acyl chains, where increased unsaturation increases the tendency of the lipid to form H_{II} structures [55]. Hence, to further test the relevance of non-bilayer forming lipids on sonosensitivity, liposomes comprising the unsaturated analogue of DSPE (DOPE) was investigated (paper III). DOPE has a more pronounced inversed conical shape than DSPE, as given by a higher PP value. Under physiological conditions (pH 7.4) DOPE tends to form H_{II} structures. Liposome bilayers can be formed, however, when DOPE is mixed with other stabilizing lipids such as PEGylated phospholipids [4,56].

The DOPE-based liposomes showed a further improved sonosensitivity compared to DSPE-based liposomes (paper III). As shown in Figure 4.5, the DOPE-based liposome formulation displayed approximately 95% DXR release after 6 min 40 kHz US exposure vs. 60% for the corresponding DSPE-based formulation. Both PE-based liposomes showed a several-fold increase in drug release as compared to standard liposomal DXR (Figure 4.5).

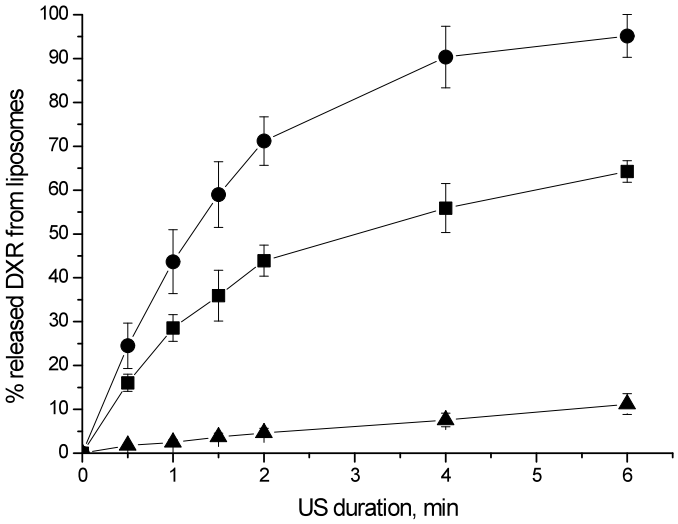


Figure 4.5 *DXR release profiles upon 40 kHz US exposure of ● DOPE-based liposomes (DOPE:DSPC:DSPE-PEG 2000:cholesterol; 62:10:8:20 mol%) in sucrose/HEPES solution. ■ DSPE-based liposomes (DSPE:DSPC:DSPE-PEG 2000:Cholesterol; 62:10:8:20 mol%) and ▲ standard liposomal DXR (HSPC:DSPE-PEG 2000:Cholesterol; 57:5:38 mol%) are included for comparison. (The Figure is reprinted from paper III).*

Influence of membrane composition on sonosensitivity of DOPE-based liposomes

A D-optimal mixture design was employed to systematically investigate the influence of the membrane lipids DOPE, DSPC, DSPE-PEG 2000 and cholesterol on sonosensitivity of DOPE-based liposomes on exposure to high frequency US (1.13 MHz) (paper IV). The levels of the lipid variables are shown in Table 4.2. 1.13 MHz US might be regarded more clinically relevant than 40 kHz US due to improved focusing ability and reduced damage to intermediate healthy tissue.

For practical reasons the liposomes in the study design contained calcein as a drug marker. Preliminary experiments showed no significant differences in sonosensitivity for DOPE-based liposomes comprising passively entrapped calcein or actively loaded DXR, respectively, indicating that sonosensitivity appeared to be attributable to membrane composition and not the type of entrapped drug or marker. The liposomes showed mean intensity weight liposome size diameters ranging from 81-89 nm, with P.I below 0.15, indicating narrow size distributions (paper IV).

Table 4.2 *Overview of the lipid variables investigated.*
(The Table is reprinted from paper IV).

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

Calcein release from liposomes increased with US (cavitation) dose (paper IV). All of the DOPE-based liposome formulations showed relatively high US-mediated release. Hence, the data used for multivariate modeling were generated at a low cavitation dose of 2000, which allowed for better detection in release variations for the different formulations. At this cavitation dose the extent of release varied from 30 to 64% depending on liposome membrane composition (paper III).

PLS regression analysis confirmed a positive correlation of the non-bilayer forming lipids DOPE and DSPE-PEG 2000 to sonosensitivity, where increased levels of the lipids increased release (Figure 4.6). By contrast, cholesterol showed a pronounced negative effect on sonosensitivity where increased levels of cholesterol reduced release (Figure 4.6).

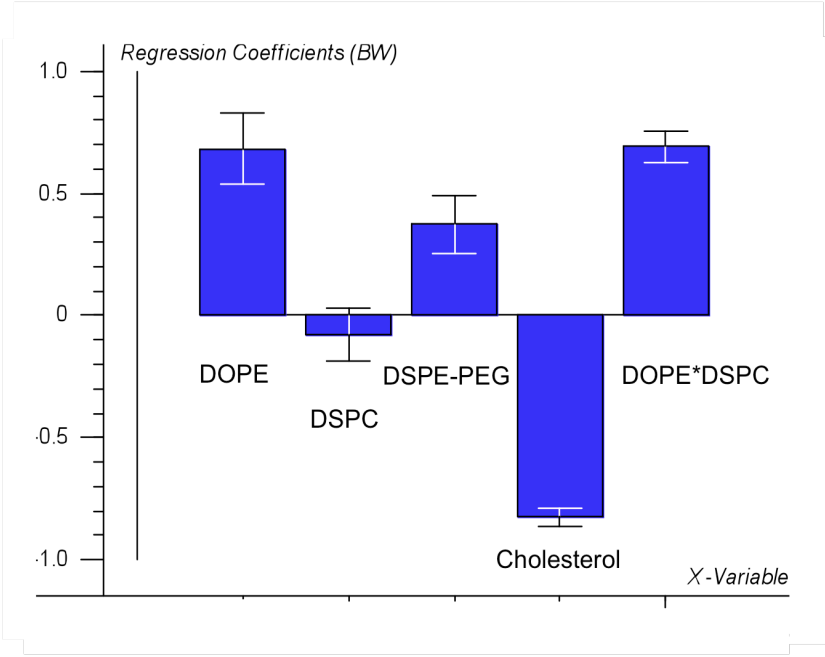


Figure 4.6 *Regression coefficients ($p<0.05$) of the partial least square regression analysis of calcein release extent from liposomes at a cavitation dose of 2000 (1.13 MHz US). The amplitude of the regression coefficients indicates the degree of their positive or negative impact on liposome sonosensitivity. (The Figure is reprinted from paper IV).*

The negative correlation of cholesterol to sonosensitivity might be due to increased lipid packing of the bilayer, which might in turn reduce sonosensitivity for example by reducing the ability of DOPE to undergo local phase conversions upon US exposure. On the other hand, cholesterol has previously been reported to induce H_{II} phase structure in mixtures of unsaturated PEs and bilayer-stabilizing phospholipids such as PCs [57].

The observed interaction between DSPC and DOPE on sonosensitivity implies that 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 (Figure 4.7). The optimal membrane composition contained high levels of DOPE and DSPE-PEG 2000 and low levels of cholesterol (Figure 4.7).

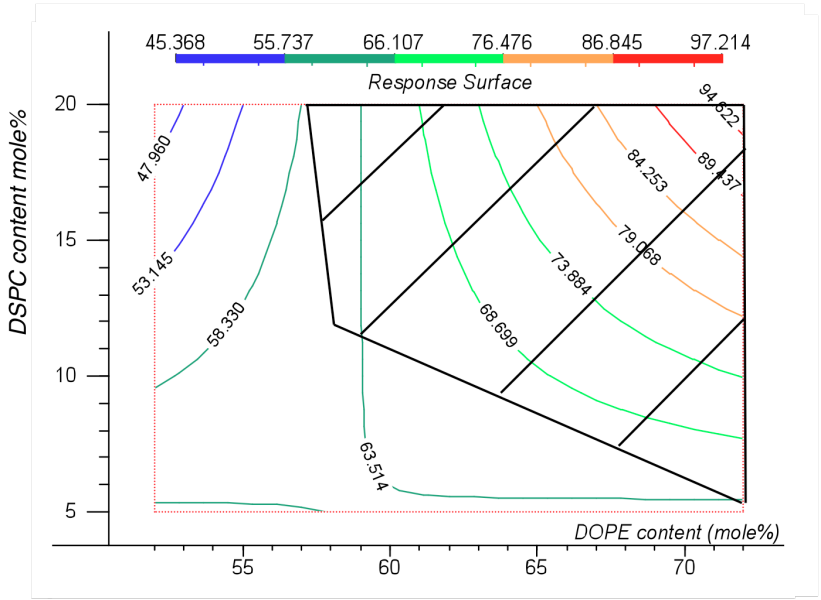


Figure 4.7 *Response surface plot showing the percentage US-mediated release of calcein from liposomes as a function of DOPE and DSPC content (mol%) (1.13 MHz US, cavitation dose 2000). DSPE-PEG 2000 and cholesterol levels are held constant at 8 and 20 mol%, respectively. The marked area is not included in the model. (The Figure is reprinted from paper IV).*

The chemometric studies of both DSPE- and DOPE-based liposomes indicate that the influence of lipid constituents on sonosensitivity are complex and may vary depending on the lipid mixture. Various underlying physicochemical properties such as lipid membrane packing, lipid miscibility and fluidity, depending on the lipid blend, are likely to influence on sonosensitivity.

In a recent study by Cavalcanti *et al.,* compressibility of DSPE- and DOPE-based lipid blends were evaluated by Brewster Angle Microscopy [58]. An increased compressibility was reported for DOPE-lipid blends than DSPE-lipid blends, suggesting a correlation between compressibility of liposome membranes and sonosensitivity [58]. However, the increased compressibility reported by increasing amounts of cholesterol in DSPE-based membranes did not fully support such a theory, as cholesterol in the current studies reduced sonosensitivity (Paper I). The polymorphic behavior of complex lipid blends and their effect on sonosensitivity needs further investigation.

4.1.4 DEPC-based liposomes

Based on the strong effect of PEs in improving liposome sonosensitivity, liposomes comprising various lipids characterized by a cone shaped geometry were investigated in terms of sonosensitivity, including liposomes based on long unsaturated PCs like dierucoylphosphatidylcholine (DEPC). (For patent applications see [59-61]). Increased acyl chain length and unsaturation is expected to increase the PP value and possible H_{II} formation [3]. An overview of the different formulations is given in appendix 1. There was a general trend that lipids with inverted cone shaped geometries (PP>1) increased sonosensitivity of liposomes. Micellar forming lipids (PP<1), like fatty acids, showed less influence on sonosensitivity.

In the case of DEPC, the PC headgroup area is larger than that of PE, but the long unsaturated acyl chains (22:1 cis PC) increase the volume of the hydrophobic part of the molecule and hence the PP value. The liposome formulation DEPC:DSPC:DSPE-PEG 2000:Cholesterol 52:5:8:35 mol% displayed high US-mediated DXR release profiles, where 74 ± 5% DXR (mean and SD of triplicate batches) was released after 6 min 40 kHz US in sucrose/HEPES solution. This represented a significant improvement in sonosensitivity comparing to DSPC-based liposomes (Paper I). The DEPC-based liposomes will not be further discussed in this thesis.

4.2 High frequency US versus low frequency US

HIFU might be considered more clinically relevant in tumour treatment than LFUS, due to improved focusing ability for tumours located deep in the body. Improved focusing implies minimal damage to surrounding healthy tissue. The different construction of the two US set-ups used in the current studies (40 kHz US and 1.13 MHz US) did not allow for a direct comparison between the liposome release data. However, the trends in sonosensitivity for the different liposomes investigated were similar irrespective of the US frequency used (Papers III and IV). Hence, for the purpose of sonosensitivity testing of liposomes *in vitro* both US set-ups were suitable. An advantage with the 40 kHz US set-up, however, was the possibility to perform release experiments in the presence of 20% serum. This was not feasible with the 1.13 MHz set-up, as foaming and coagulations were produced during the US run which made it difficult for the hydrophone to detect cavitation.

4.3 Sonosensitivity in serum-containing medium

To provide a closer simulation of biological conditions, sonosensitivity studies were performed in 20% serum (papers III and V).

The US-mediated drug release profiles (40 kHz US) in sucrose/HEPES solution containing 20% serum are shown in Figure 4.8. DSPE-based liposomes experienced a total loss

of sonosensitivity, whilst sonosensitivity of DOPE-based liposomes was essentially maintained (Paper III). It should be added that the loss of sonosensitivity in 20% serum was consistently seen for all of the DSPE-based liposome formulations investigated (data not shown).

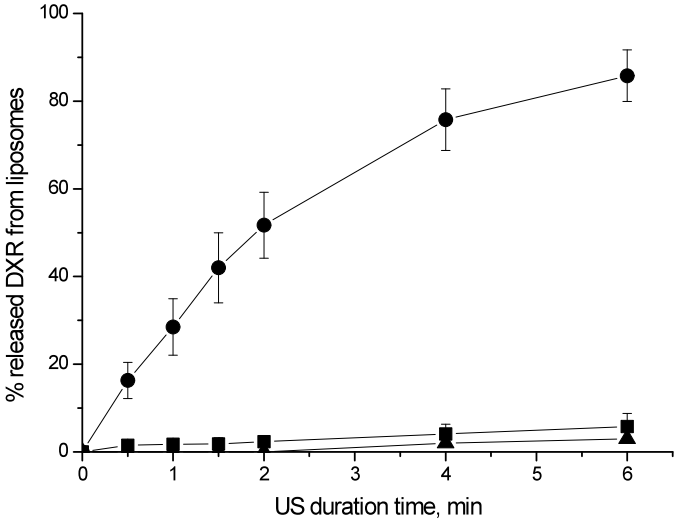


Figure 4.8 *Release profiles of DXR on 40 kHz US exposure of ● DOPE-based liposomes (DOPE:DSPC:DSPE-PEG 2000:Cholesterol; 62:10:8:20 mol%) and ■ DSPE-based liposomes (DSPE:DSPC:DSPE-PEG 2000:Cholesterol; 62:10:8:20 mol%) in sucrose/HEPES solution containing 20% serum. ▲ standard liposomal DXR (HSPC:DSPE-PEG 2000:Cholesterol; 57:5:38 mol%) is included for comparison. (The Figure is reprinted from paper III).*

The reasons for the loss of sonosensitivity of DSPE-based liposomes in serum remain unclear. One explanation could be interactions between serum proteins and DSPE-based membranes, which affect the polymorphism or arrangement of the lipidbilayer and hence sonosensitivity. It has been shown previously that the structural preferences of lipids in a lipid blend can be modulated by factors such as proteins [57].

Another factor that might influence on sonosensitivity is a reduction in the acoustic energy deposited to liposomes due to higher viscosity of the serum-containing medium. This could adversely affect release from DSPE-based liposomes whilst DOPE-based liposomes might be destabilized even at low acoustic energy.

Figure 4.9 shows the amount of DOPE required in liposomes (comprising a fixed level of cholesterol and DSPE-PEG 2000 of 40 and 8 mol%, respectively), to maintain sufficient sonosensitivity in 20% serum. High sonosensitivity was observed for formulations comprising between 25 and 52 mol% DOPE (Figure 4.9). Poor sonosensitivity was observed for liposomes

with 12 mol% DOPE, as evidenced by a significant reduction in DXR release (Figure 4.9), (paper V).

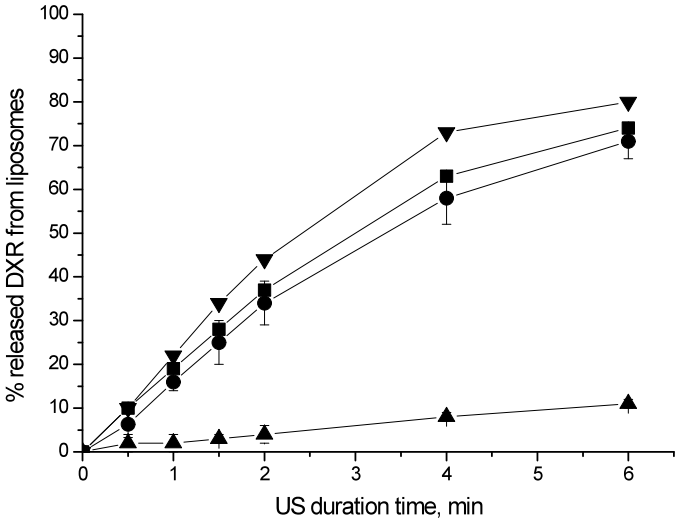


Figure 4.9 *Effect of DOPE level on US-mediated DXR release from liposomes in HEPES/sucrose solution containing 20% serum (40 kHz US). DOPE-levels: ▼ 32 mol%, ■ 52 mol%, ● 25 mol%, ▲ 12 mol%. (Cholesterol and DSPE-PEG 2000 levels: 40 and 8 mol%, respectively. DSPC level co varies). (The Figure is reprinted from paper V).*

Higher serum concentrations would theoretically better simulate plasma. Unfortunately, it was not feasible to perform release experiments in higher serum concentrations than 20% as serum reduces the fluorescence signal of DXR. However, 20% serum should still give a good indication of the impact of serum components on liposome sonosensitivity. The high sonosensitivity of DOPE-based liposomes in 20% serum shows promise for US-mediated drug release in tumour tissue (See section 6 for qualitative US-mediated release studies *in vivo*).

4.4 Mechanisms of US-mediated drug release

The mechanisms of US-mediated permeabilization of phospholipid membranes and drug release from liposomes have not been clearly elucidated in literature. To investigate the mechanism of US-mediated drug release of DOPE-based liposomes, liposome size and morphology were studied before and after 40 kHz US exposure by PCS and Cryo-TEM (paper III). The non-exposed samples showed mainly small unilamellar vesicles, slightly below 100 nm in diameter, with rod-like structures spanning the aqueous core indicating DXR precipitate (paper III). The US-treated liposomes displayed a significant change towards a broader size distribution, as shown by an increased P.I. A mixture of smaller and larger empty vesicles as well as non-

lamellar structures was observed (paper III). The results indicate US-induced destabilization and disruption of DOPE-based liposomes, explaining the high drug release. Similar results were observed for US-treated DSPE-based liposomes (data not shown).

It was of further interest to investigate whether US-mediated disruption of vesicles was a release mechanism that was specific for PE-based liposomes. Hence, the size and morphology of PC-based liposomes (standard liposomal DXR) was examined before and after US treatment with Cryo-TEM and PCS. To allow for a relative comparison between DOPE-based liposomes and standard liposomal DXR the formulations were investigated for a given release extent. However, when exposing low-diluted standard liposomal DXR to 12 min US, using the 40 kHz US set-up, less than 5% of the entrapped DXR was released from liposomes. To obtain sufficient drug release the US transducer had to be immersed directly into the liposome dispersions diluted in a 1/10 (v/v) ratio with 0.22 μm-filtered sucrose/HEPES solution. A water bath was used to keep the temperature below 30°C. This powerful US treatment resulted in approximately 60% DXR release from both DOPE-based liposomes and standard liposomal DXR after 2 and 6 min of US exposure, respectively.

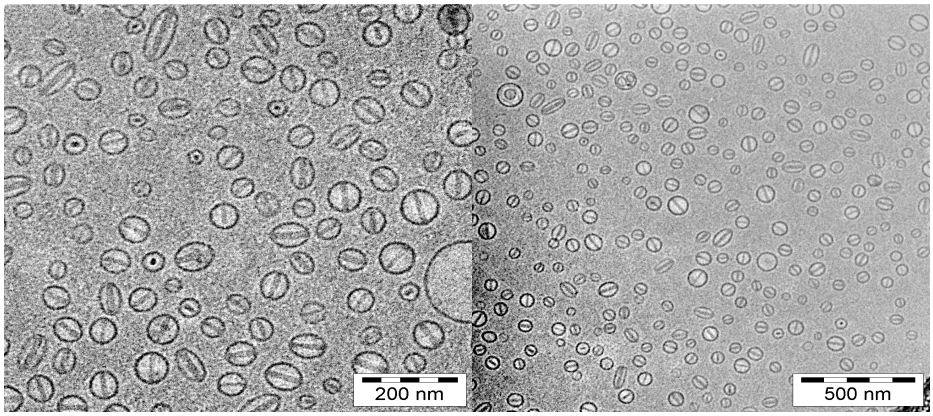
The structure of untreated DOPE-based liposomes and standard liposomal DXR was approximately similar (Figure 4.10 A, C). The liposome membranes are shown as the darker perimeters surrounding the inner aqueous compartment with precipitated DXR. After US treatment standard liposomal DXR showed, in contrast to the DOPE-based liposomes, no significant changes in liposomes size distribution or liposome structure (Table 4.3, Figure 4.10 B, D). The electron micrographs revealed mainly intact and empty vesicles, the latter confirming DXR release. In addition a few rod-like structures were seen, indicating DXR and/or cholesterol crystals (Figure 4.10 B). The results were in agreement with previous findings by Schroeder *et al.*, showing no significant changes in size or structure of PC-based liposomes exposed to low frequency US [62].

Table 4.3 *Mean size (intensity weighted) and P.I of standard liposomal DXR and DOPE-based liposomes before and after 40 kHz US exposure. DXR release for both liposome formulations is 60%.*

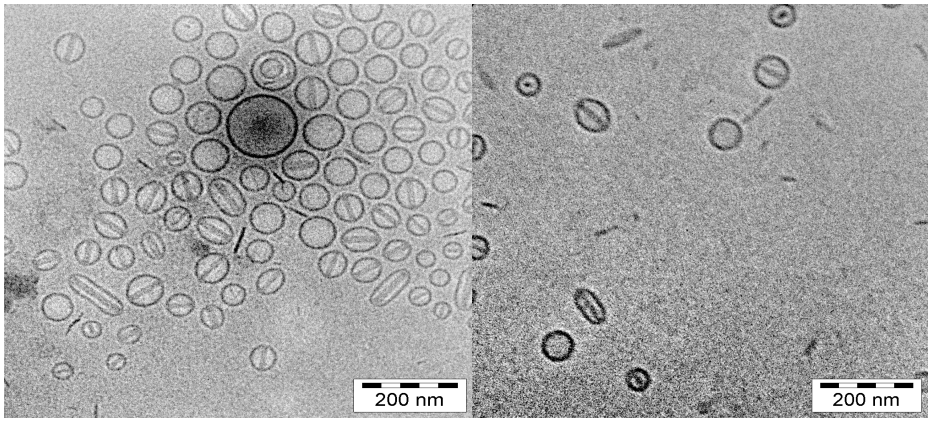
Liposome formulation	**Mean size, nm**		**P.I**	
	Untreated sample	US-treated sample	Untreated sample	US-treated sample
DOPE-liposomes	86 ± 2	90 ± 3 *	0.14 ± 0.03	0.25 ± 0.01
Standard liposomal DXR	74 ± 1	76 ± 1	0.10 ± 0.01	0.11 ± 0.01

* The PCS calculated two peaks with mean sizes of: 1) 120 nm, 2) 24 nm.

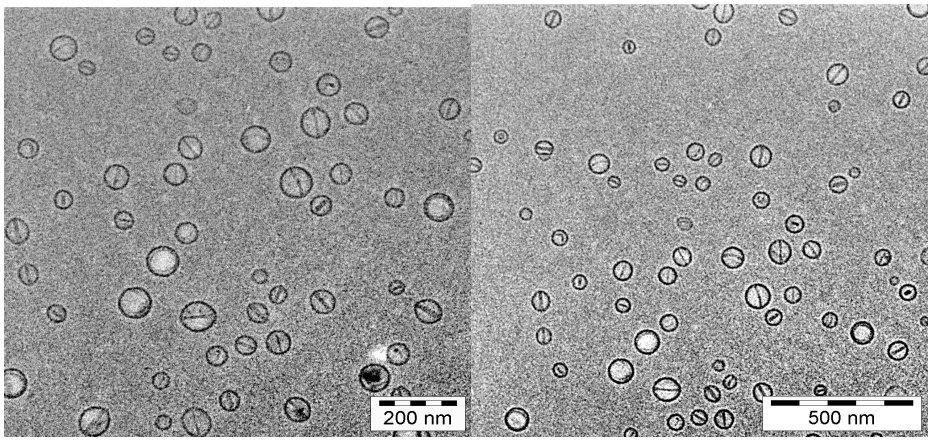
A)



B)



C)



D)

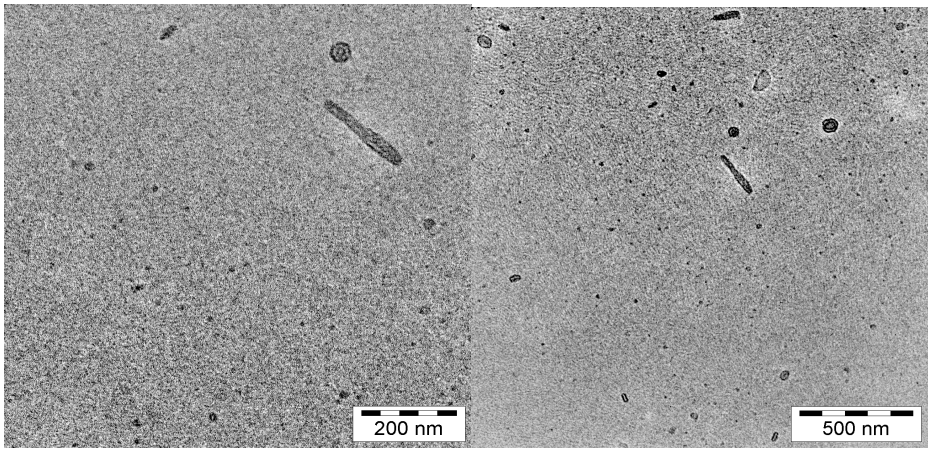


Figure 4.10 *Cryo-TEM micrographs showing lipid structures before and after US treatment of A) Non-treated standard liposomal DXR (HSPC:DSPE-PEG 2000:Cholesterol 57:5:38 mol%), B) US-treated standard liposomal DXR, C) Non-treated DOPE-based liposomes (DOPE:DSPC:DSPE-PEG 2000:Cholesterol; 62:10:8:20 mol%), D) US-treated DOPE-based liposomes. Both of the US treated liposome formulations have released approximately 60% of the entrapped DXR. The micrographs shown are representative for the whole specimen. Note that the US treated liposomes are diluted 1:10 v/v with sucrose/HEPES solution.*

The type of lipid structures formed in the US-treated DOPE-based liposome sample was difficult to identify from the micrographs. A large fraction of smaller structures was observed (Figure 4.10 D) indicating small vesicles or micelles. Due to the heterogeneous size distribution of the sample, the smaller structures were most likely underestimated in the calculation of mean particle size by PCS, which is a general limitation of the method [63].

The results indicated different release mechanisms for standard liposomal DXR and DOPE-based liposomes. This in turn might explain the large difference in sonosensitivity for

the two formulations. Previous reports have suggested that US-sensitization of PC-based liposomes induces reversible pore-like defects in the membrane allowing drug release [38,62]. The fact that the standard liposomal DXR investigated here remained mainly as intact empty vesicles after US release supports such a theory.

As already suggested, the US-mediated disruption of DOPE-based liposomes might be related to membrane phase conversions as DOPE adapts to a H_{II} phase upon exposure to acoustic energy. PEGylated phospholipids stabilize DOPE into a lamellar phase [4,56]. Hence, potential US-induced shedding of DSPE-PEG 2000 from the liposomes might destabilize the DOPE-containing bilayer due to phase conversions. US or pressure-driven exchange of PEGylated lipids with the external aqueous phase is possible as PEG-lipids have significantly greater water solubility than their parent lipids [64].

It should be added that ^{31}P nuclear magnetic resonance has been attempted to identify the type of lipid structures formed after US treatment of the DOPE-based liposomes, e.g. presence of potential H_{II} structures. Unfortunately, the results were inconclusive as isotropic phases made it difficult to distinguish and identify the different structures. Small angle X-ray scattering could be an alternative approach. However, potentially reversible and thermodynamically unstable lipid structures formed during US treatment may complicate the data interpretation. Simplified liposome models without DXR composed of for example only DOPE and DSPE-PEG 2000 could be used to reduce the complexity of the structures formed post US.

4.5 Chemical integrity of US treated doxorubicin and lipids

Chemical integrity of DXR-containing DOPE and DSPE-based liposomes before and after exposure to 40 kHz US and 1 MHz US, respectively, was analyzed by HPLC. The studies were performed to investigate whether potential sonochemical effects induced degradation of the lipids and/or DXR. The tested DOPE-based liposomes contained DOPE:DSPC:DSPE-PEG 2000:Cholesterol in molar ratios of 62:10:8:20 mol%. The tested DSPE-based liposomes contained DSPE:DSPE-PEG 2000:Cholesterol in molar ratios of 62:8:30 mol%.

The assays of DXR, cholesterol and phospholipids, respectively, were identical before and after US, indicating that the US treatments used did not degrade the molecules. No impurities/degradation products of DXR were detected. The results were in agreement with previous findings by Schroeder *et al.*, showing that LFUS do not affect the chemical integrity of DXR and lipids nor the biological potency of released DXR[62].

4.6 Storage stability

The storage stability of a liposomal drug formulation is an important pharmaceutical concern. Liposome storage stability includes colloid stability, drug retention and chemical integrity of both drug and lipids. For the sonosensitive liposomes it is important that these physicochemical properties are preserved at least from the time of production to time of application.

Hence, stability of selected DXR-containing DOPE and DSPE-based liposome formulations, considered as promising product candidates based on sonosensitivity and *in vitro* serum stability tests, was followed during 6 months storage at 5 °C. The tested DOPE-based liposomes contained DOPE:DSPC:DSPE-PEG 2000:Cholesterol in molar ratios of 62:10:8:20 mol% and 25:27:8:40 mol%. The tested DSPE-based liposomes contained DSPE:DSPC:DSPE-PEG 2000:Cholesterol in molar ratios of 62:0:8:30 mol% and 54.5:10:5.5:30 mol%.

The data showed no detectable leakage of liposomal DXR, no significant alteration in sonosensitivity, mean liposome size, P.I nor assay of phospholipids and DXR, during storage.

The high drug retention of the liposomes upon storage might be explained by the presence of cholesterol that reduces permeabilization of the liposome membrane and consequently drug leakage[8]. Furthermore, the small size of the liposomes and the PEGylated surface is expected to minimize or prevent liposome aggregation and/or fusion[8].

4.7 Serum stability

In vitro serum assays are often performed prior to *in vivo* studies to investigate liposome stability in terms of drug retention under simulated biological conditions. Serum stability strongly depends on liposome membrane composition and is induced by interactions between membrane phospholipids and serum proteins, such as albumin and lipoproteins [8,65].

Drug leakage from DSPE and DOPE-based liposomes was determined after 6 h incubation in sucrose/HEPES solution containing 20% serum at 37 °C (Papers I, II, III, IV, V). The DSPE-based liposomes, included in the full factorial design described in Table 4.1, showed variations in DXR leakage from 2 to 37%, depending on membrane composition (Paper I). PLS regression analysis examining the influence of the membrane lipids DSPE, DSPE-PEG 2000 and cholesterol on serum stability, revealed a major influence of cholesterol. At a constant level of DSPE and DSPE-PEG 2000 (47 and 3 mol%, respectively) a reduction in liposomal DXR leakage from 28 to 6% was obtained by increasing the cholesterol level from 20 to 30 mol% (paper I). The stabilizing effect of cholesterol might be explained by its known ability to tighten liposome membranes, reduce the extent of opsonisation and minimize loss of encapsulated drug [6].

Also DSPE-PEG 2000 had an influence on serum stability of DSPE-based liposomes. In general, PEGylation of liposomes is known to sterically prevent or minimize interactions between the liposome membrane and serum proteins, thus improving liposome stability. The squared effect observed for the DSPE-based liposome formulations implied a negative, but non-linear correlation of DSPE-PEG 2000 content on serum-induced drug leakage (Figure 4.11).

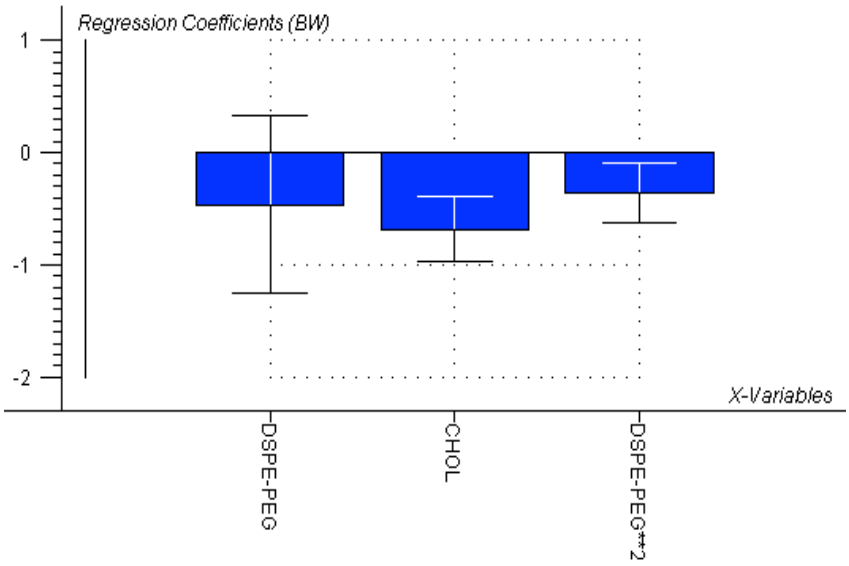


Figure 4.11 *Regression coefficients describing the significance of lipids on DXR leakage extent (%) from DSPE-based liposomes after 6 h incubation in 20% serum/ 37 °C. (The Figure is reprinted from paper I).*

The DOPE-based liposomes showed no detectable leakage of DXR nor calcein in serum, irrespective of membrane composition (Papers III,IV,V). Hence, the DOPE-based liposomes were stable in 20% serum compared to most of the DSPE-based liposome formulations (paper I). These data are contradictory to the general conception that liposome membranes comprising unsaturated lipids are more permeable and prone to drug leakage than liposomes composed of saturated lipids [66,67]. However, DSPE-based liposomes incubated in sucrose/HEPES solution without serum showed no DXR leakage, indicating that the leakage observed for DSPE-based liposomes in 20% serum was a result of serum components and not passive leakage (paper I).

The serum-induced leakage for selected DSPE-based formulations might be explained by poor lipid membrane packing or structural defects in the bilayer that causes penetration by proteins. The surface of small-sized liposomes below T_c often appears rigid and polyhedron-like, which makes the membrane more prone to opsonisation [28]. For liposomes in the liquid crystalline phase (above T_c), the membrane surface is typically smooth and curved, minimizing

interactions with proteins [28]. DSPE display a significantly higher T_c (74 °C) than DOPE (-16 °C) and should hence provide a more rigid bilayer at physiological temperatures. This can clearly be observed in Figure 4.12, where CryoTEM of DSPE- and DOPE-based liposomes are shown. The fluid DOPE-based liposome membranes appear smooth and curved, whilst the rigid DSPE-based liposomes are polyhedron-like. Inclusion of cholesterol (above 30 mol%) in liposomes has previously shown to give rise to a liquid ordered phase [28], which could explain the improved serum stability for DSPE-based liposomes with higher cholesterol levels.

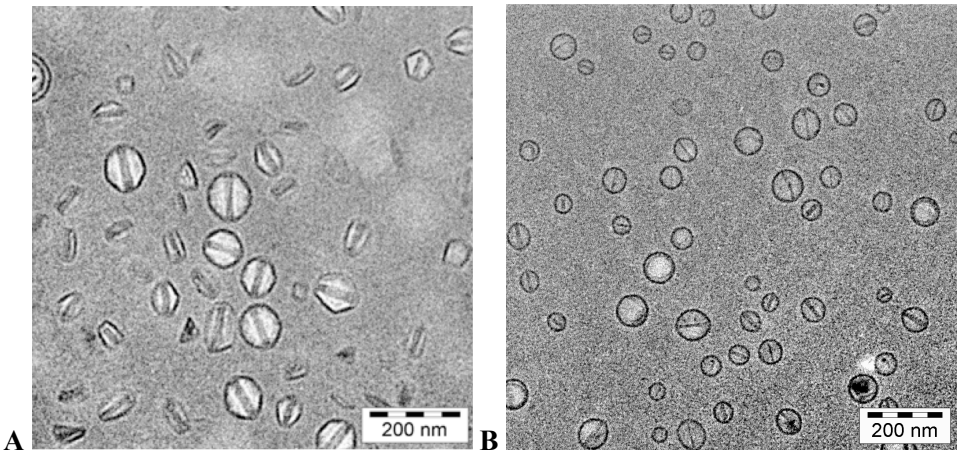


Figure 4.12 *CryoTEM of DXR-containing liposomes; A) DSPE-based liposomes (DSPE:DSPC:DSPE-PEG 2000:Cholesterol; 62:10:8:20 mol%) and B) DXR-containing DOPE-based liposomes (DOPE:DSPC:DSPE-PEG 2000:Cholesterol; 62:10:8:20 mol%). The micrographs shown are representative for the whole specimen.*

It should be emphasized that *in vitro* serum stability assays are not always indicative of *in vivo* stability in terms of liposomal drug leakage in blood circulation[28,8,67]. Hence, the most relevant studies of liposome stability are performed *in vivo*.

4.8 Pharmacokinetic studies

There is a well-established correlation between long blood circulation time and increased accumulation of liposomes in tumours [68]. Several biodistribution studies performed in tumour-bearing mice with PEGylated DXR-liposomes describe a peak DXR tumour concentration 1-2 days post injection [18,69,70]. Hence, for efficient US-mediated drug delivery to solid tumours, liposomes must retain their drug load while in circulation.

Blood pharmacokinetics of selected liposome formulations that combined both high *in vitro* sonosensitivity and serum stability was evaluated in non-tumoured mice (paper V). As only the blood pharmacokinetics were evaluated, it was found more practical to use healthy

mice instead of tumour-bearing mice in the study. Figure 4.13 shows the blood clearance profiles of the optimized DSPE-based liposome formulation in comparison to DOPE-rich liposomes and standard liposomal DXR. Despite good serum stability *in vitro*, the DOPE-rich liposomes (52-62 mol% DOPE) displayed a significantly faster DXR blood clearance in comparison to standard liposomal DXR and optimized DSPE-based liposomes (Figure 4.13) (paper V). At 24 h post-injection only 5% of the injected DXR dose for DOPE-based liposomes remained in blood, vs. 20% for standard liposomal DXR (Figure 4.13).

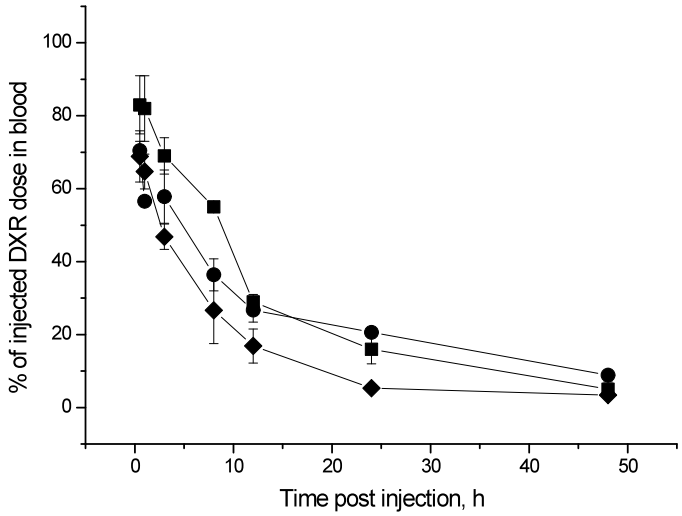


Figure 4.13 *Blood clearance of liposomal DXR in healthy mice.* ♦ *DOPE-based liposomes (DOPE:DSPC:DSPE-PEG 2000 2000:Cholesterol; 62:10:8:20 mol%),* ■ *DSPE-based liposomes (DSPE:DSPE-PEG 2000:Cholesterol 62:8:30 mol%) and* ● *standard liposomal DXR (HSPC:DSPE-PEG 2000:Cholesterol; 57:5:38 mol%).*

The relatively faster blood clearance of DOPE-rich liposomes might reflect leakage of DXR from the liposome carrier and/or premature uptake of the liposomes by the MPS. Previous studies have demonstrated faster blood clearance kinetics of liposomal drugs composed of unsaturated lipids like DOPE, due to high membrane permeability and drug leakage from the liposome carrier [66,71,72].

In an attempt to prolong blood circulation time of DOPE-containing liposomal DXR, the membrane composition was altered by both increasing the level of cholesterol and reducing the level of DOPE. Cholesterol has been shown to tighten liposome membranes, minimize opsonisation and decrease leakage of entrapped material [6,47,72,73].

An overview of the different liposome formulations prepared is presented in Table 4.4. All liposomes displayed DXR encapsulation efficiencies above 95%. Due to the general

influence of liposome size on pharmacokinetics[8], the comparable size of the formulations (Table 4.4) was essential to better assess the influence of the membrane composition *per se* on blood pharmacokinetics.

Table 4.4 *Overview of the different DOPE-containing liposome formulations evaluated in pharmacokinetic studies in mice. (The Table is reprinted from paper V)*

DOPE:DSPC:DSPE-PEG 2000:Cholesterol (mol%)	**Mean size, nm (P.I)**	**% DXR in blood circulation 24 h post i.v. injection.**
62:10:8.20	86 (0.14)	5 ± 1
52:5:8:35	86 (0.12)	3 ± 1
52:20:8:20	80 (0.10)	4 ± 1
52:0:8:40	86 (0.10)	2 ± 0
32:20:8.40	86 (0.10)	20 ± 3
25:27:8:40	87 (0.10)	17 ± 1
Standard liposomal DXR	76 (0.11)	21 ± 1

Increasing the cholesterol level from 20 to 40 mol% in DOPE-rich liposomes did not result in prolonged blood circulation time of DXR. The lack of a cholesterol stabilizing effect might be related to lipid packing constraints such as inhomogeneous distribution of cholesterol within the lipid membranes and/or poor cholesterol-phospholipid interactions, leading to high membrane permeability [5].

A significantly longer blood circulation time of DXR was obtained for liposomes with reduced levels of DOPE (32 and 25 mol%) (Figure 4.14). As the DOPE amount was reduced, DSPC was consequently increased (Table 4.4). Hence, the reduced blood clearance was most likely a result of both an increased DSPC level and reduced DOPE level. Previous studies have shown that saturated PCs impart order and rigidity to liposome membranes, increases the overall T_c and prolongs the blood residence time of liposomal drugs due to reduced liposomal drug leakage [5,66,74,72].

Biodistribution studies showed no increase in DXR uptake in liver, spleen and kidneys for DOPE-rich liposomes, which displayed the fastest blood clearance (paper V). Hence, the reduced blood clearance of DXR for intermediatelevel DOPE-containing liposomes was suggested to be due to less drug leakage from the liposomes in blood circulation and not reduced uptake by the MPS (paper V). Furthermore, DXR blood levels provide a good indication of liposome encapsulated drug, since DXR released in the bloodstream is rapidly distributed to other tissues and organs [48].

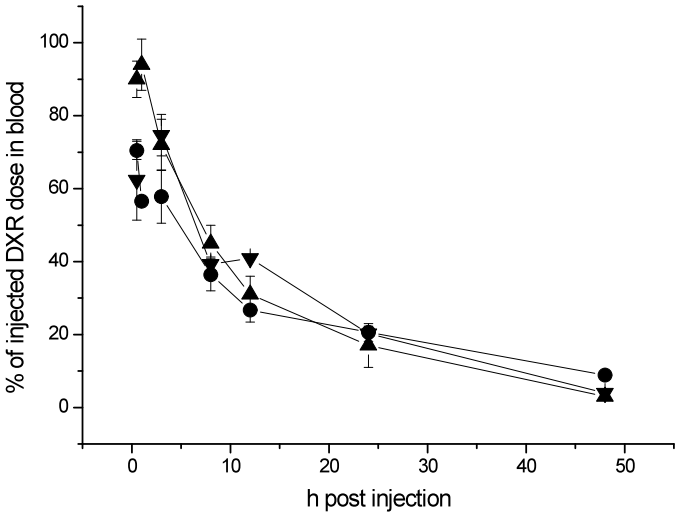


Figure 4.14 *Blood clearance kinetics of liposomal DXR in mice for liposomes containing the following DOPE levels; ▲ 25 mol% and ▼32 mol%. (Cholesterol and DSPE-PEG 2000 levels: 40 and 8 mol%, respectively. DSPC levels co varies with DOPE).* ● *standard liposomal DXR (HSPC:DSPE-PEG 2000:Cholesterol; 57:5:38 mol%) is included for comparison. (The Figure is reprinted from paper V).*

The liposomes containing 25-32 mol% DOPE, 8 mol% DSPE-PEG 2000 and 40 mol% cholesterol, showed approximately similar blood pharmacokinetics as standard liposomal DXR, where 20% of the injected DXR dose remained in blood circulation at 24 h post-injection (Figure 4.14). Furthermore, the reduction in DOPE content did not adversely reduce sonosensitivity (paper V). Considering both the blood clearance and sonosensitivity data in 20% serum, liposomes containing intermediate DOPE levels were the optimal formulations identified.

4.9 In vivo proof of principle study with prototype sonosensitive liposomes

Tumour regression was studied in mice bearing CWR22 prostate adenocarcinoma treated with prototype DSPE-based DXR-containing liposomes and 40 kHz US (paper II). The aim was to investigate whether an improved therapeutic effect could be obtained by combining sonosensitive liposomes and US. The liposome formulation used, however, (composed of DSPE:DSPC:DSPE-PEG 2000:Cholesterol 54.5:10:5.5:30 mol%) is regarded as a prototype formulation as it displayed lower *in vitro* sonosensitivity comparing to other formulations developed in later formulation work.

Tumours were exposed to 4 min 40 kHz US at 24 h post i.v. injection of 3.5 mg/kg liposomal DXR, coinciding with the peak DXR tumour concentration (paper II). Mice treated with liposomal DXR without US, saline, and saline + US, respectively served as controls.

Figure 4.15 shows the tumour growth curves of the different treatment groups vs. time. No difference between groups receiving saline and saline + US was observed, indicating that US alone did not inhibit tumour growth. Furthermore, no difference was found between liposomal DXR groups and the saline control groups. However, the groups representing liposomal DXR and US showed a significant slower tumour growth trend compared to the control groups ($p<0.05$).

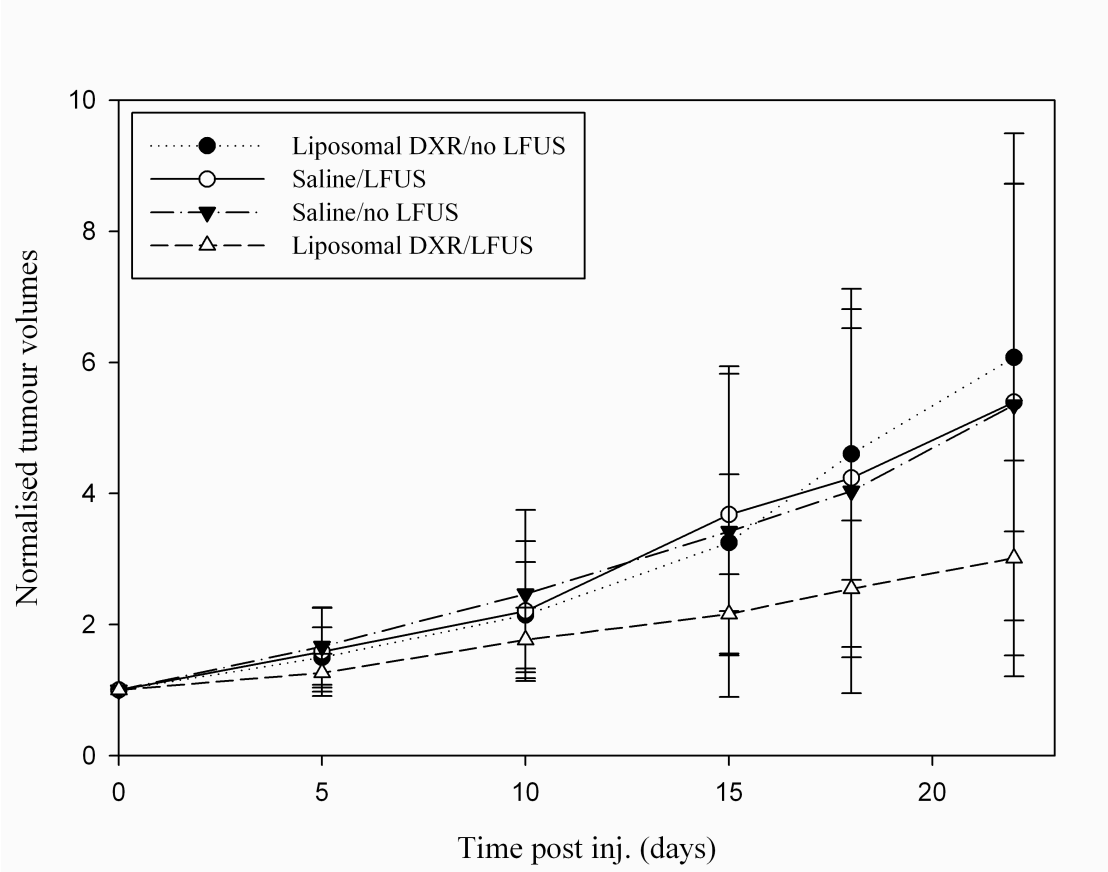


Figure 4.15 *Tumour growth evolution in mice receiving different treatments. The injected liposomal DXR dose was 3.5 mg/kg. (The Figure is reprinted from paper II).*

The fact that no anti-tumour effect was obtained with the group receiving liposomal DXR indicates a lack of tumouricidic concentrations of released DXR in tumours. The rationale for using a relatively low DXR dose of 3.5 mg/kg was to avoid therapy saturation levels where any beneficial effects induced by US would not be measureable. Only 10% of the injected DXR dose remained in the blood at the time of US treatment (24 h post-injection), implying a minor contribution of liposomal DXR in the tumour vasculature (paper II). Furthermore, if DXR were

retained within liposomes when entering the tumour tisssue, the bioavailability of DXR to tumour cells might be limited. The US-enhanced tumour regression response supports such a theory.

The tumour regression effect of combining US and liposomal drug is in consistency with previous studies [75,76]. The anti-tumour effect obtained at a low DXR dose indicates that this system may benefit in cases where low DXR doses are required due to toxicity problems. However, the formulation used in the current study was suboptimal in terms of sonosensitivity in 20% serum comparing to optimized DOPE-liposome formulations developed later on during formulation work (paper V). Hence, other factors than US-mediated drug release might explain the US-enhanced antitumour growth, such as US-induced tumour extravasation of liposomes and/or improved drug cellpermeability.

The mice were monitored throughout the therapy study for acute toxicity in terms of weight loss (paper II). All experimental groups experienced an initial weight loss independent of treatment, mainly due to the administration of anaesthesia. The maximum weight reduction was less than 10% for the group receiving liposomal DXR and US, which was considered acceptable (Figure 4.16). Previous toxicity studies with PEGylated liposomal DXR in mice have reported LD10 and LD50 to be 11.7 and 38.3 mg DXR/kg, respectively [77]. The US treatment *per se* did not produce any visible skin lesions or additional weight loss.

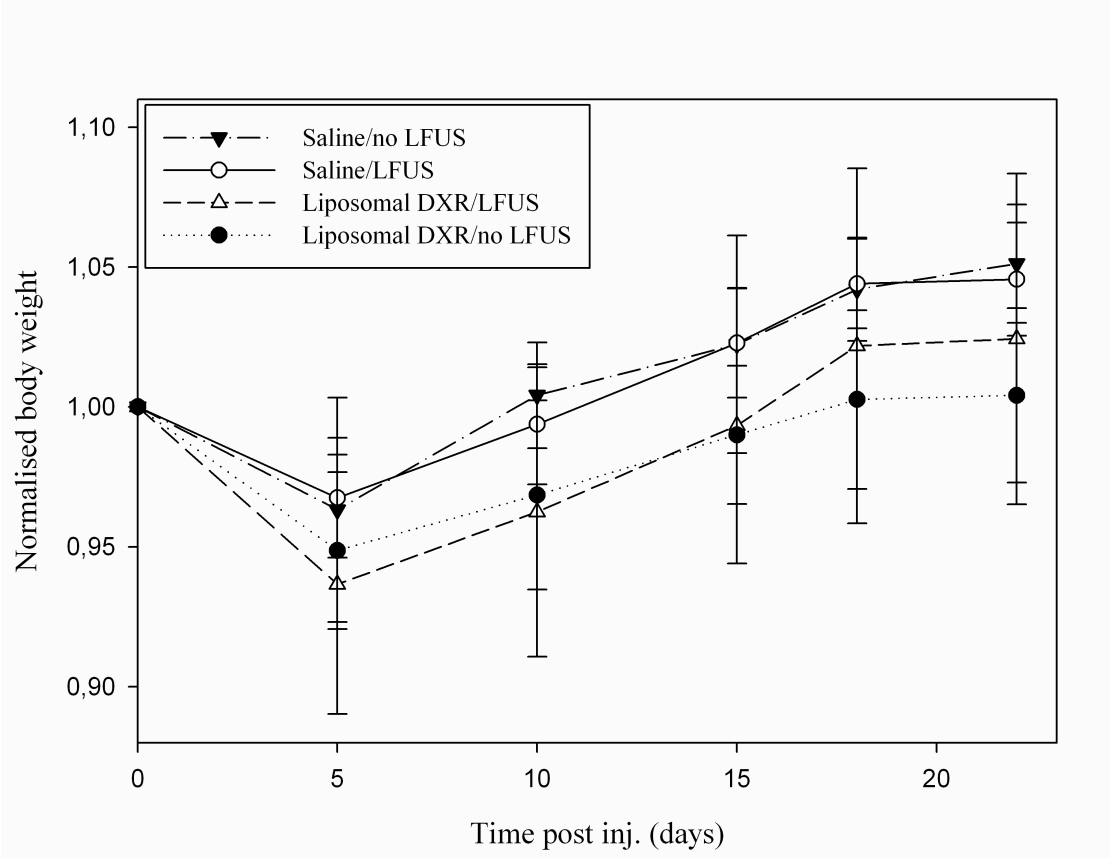


Figure 4.16 *Normalised body weight time evolution of mice receiving different treatments. The injected liposomal DXR dose was 3.5 mg/kg. (The Figure is reprinted from paper II).*

Peak levels of DXR in spleen and liver at 24 h post-injection represented 8 and 35% of the injected dose, respectively (paper II). Compared to other studies with standard liposomal DXR[18] and DOPE-containing liposomes (paper V), these higher concentrations indicate increased DXR uptake by the MPS for DSPE-based liposomes. Hence, liposome uptake or accumulation in organs such as liver, spleen and skin might account for the slightly higher weight loss of the group receiving liposomal DXR.

The therapy results indicate proof of principle and are promising for further development and improvement of the technology. Therapy studies with optimized DOPE-containing liposomes at clinically relevant doses in combination with optimized US treatment (confocal high frequency US, 1.13 MHz) are ongoing to investigate whether the therapeutic efficacy may be further improved.

5. CONCLUSIONS

Inclusion of the non-bilayer forming lipids DSPE and DOPE, respectively, in traditional liposome membranes containing DSPC, DSPE-PEG 2000 and cholesterol, significantly enhanced *in vitro* sonosensitivity. Variations in lipidbilayer composition influenced on sonosensitivity of both DSPE- and DOPE-based liposomes. DOPE, defined by a more pronounced inverted cone shaped structure than DSPE, was the most potent lipid modulator of sonosensitivity where only limited amounts (25 mol%) were required to obtain sufficient sonosensitivity. US release experiments in the presence of serum showed reduced sonosensitivity of DSPE-based liposomes, whilst sonosensitivity of DOPE-based liposomes was essentially maintained.

CryoTEM and PCS studies indicate different US-mediated release mechanisms for DOPE-based liposomes vs. traditional PC-based liposomes (standard liposomal DXR). Standard liposomal DXR remained mainly as intact, empty vesicles after US treatment, indicating US-induced pore-formation in the liposome membrane as the main cause of drug release. In contrast, the DOPE-based liposomes were mostly irreversibly disrupted after US treatment.

DOPE-containing liposomes showed generally improved stability in 20% serum in terms of drug retention compared to DSPE-based liposomes. Despite acceptable stability *in vitro*, DOPE-rich liposomes (52-62 mol% DOPE) displayed a relatively fast blood clearance compared to both DSPE-based liposomes and standard liposomal DXR. However, by reducing the DOPE level in liposomes to 25-32 mol%, a prolonged blood circulation time was obtained. The optimized DOPE-containing liposomes, comprising 32 mol% DOPE, 20 mol% DSPC, 8 mol% DSPE-PEG 2000 and 40 mol% cholesterol, showed similar blood clearance profile as standard liposomal DXR but a 8-fold improvement *in vitro* sonosensitivity in 20% serum. The DOPE-containing liposomes were the most promising product candidates as they fulfilled all the formulation criteria, including high sonosensitivity in 20% serum and long blood circulation time in mice.

An enhanced tumour regression of combining low frequency US and prototype DSPE-based liposomal DXR was demonstrated in tumoured mice. The data supports the concept that US mediated drug release provides a clinical benefit. Further optimization studies remain to investigate whether the DOPE-containing liposomes in combination with US treatment may further improve the therapeutic index of DXR.

6. GENERAL REMARKS AND FUTURE PERSPECTIVES

Studies are ongoing to further evaluate the novel sonosensitive DOPE-containing liposomes in several *in vivo* animal models. A brief note of the ongoing studies and future perspectives is given in this section.

Visualizing US-mediated release from liposomes in vivo by optical imaging.

To investigate US-mediated release *in vivo* liposomes containing the fluorescent porfyrin Phthalocyanine chloride tetrasulfonic acid (AlS4) were prepared. AlS4 has an excitation wavelength in the near infrared region and can therefore be detected *in vivo* by optical imaging [78]. Encapsulation of AlS4 into liposomes leads to quenching and signal loss, which is recovered when the AlS4 is released from the liposome.

AlS4-containing DOPE-liposomes (DOPE:DSPC:DSPE-PEG 2000:Cholesterol 25:27:8:40 mol%) and AlS4-containing HSPC-liposomes (HSPC:DSPE-PEG 2000:Cholesterol 57:5:38 mol%) (mean liposome size diameters of 98 ± 1 nm and 101±3 nm, respectively), were prepared by the thin-film hydration and sequential extrusion technique as previously described. AlS4 was passively entrapped in the liposomes by hydration of the dry lipid films with an isotonic PBS solution containing 5 mM AlS4 (pH 7).

100 μl of the AlS4-containing liposomes were directly injected into tumourvasculature of 22RV1 tumour-bearing athymic male nude mice. Whole body images of the mice were taken before and after exposing tumours to 1 min of 1.13 MHz US, using a confocal US set-up developed at INSERM, Lyon, France. The cavitation zone was positioned between the skin surface and the centre of the tumour.

As seen in Figure 6.1 AlS4-containing DOPE-liposomes showed considerable US-mediated release of AlS4 in tumours as evidenced by a significant increase in fluorescence signal intensity of factor 2.1 ± 0.7 ($p<0.05$, $n=4$). No significant increased fluorescence intensity in tumours injected with AlS4-containing HSPC-liposomes was observed after US treatment ($n=4$) (Figure 6.2). The results were consistently seen in all mice although there was a large variation between animals. Negative control animals, receiving no US, showed no increased fluorescence intensity, indicating that release was a result of US and not other factors such as passive leakage of AlS4 or destabilization of the liposomes due to for example tumour-associated macrophages. The results indicate that the DOPE-liposomes maintained sonosensitivity also *in vivo*.

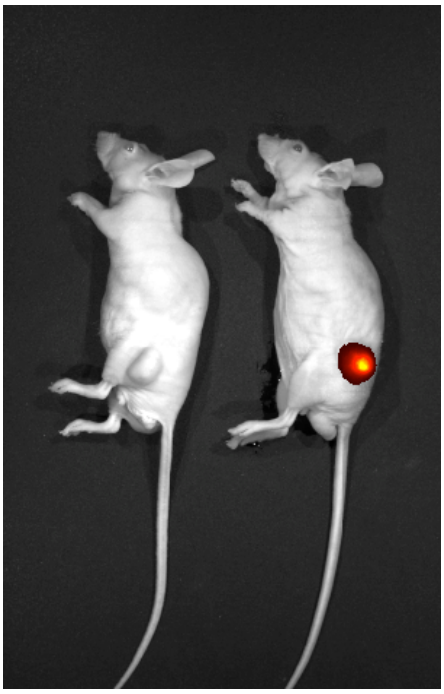
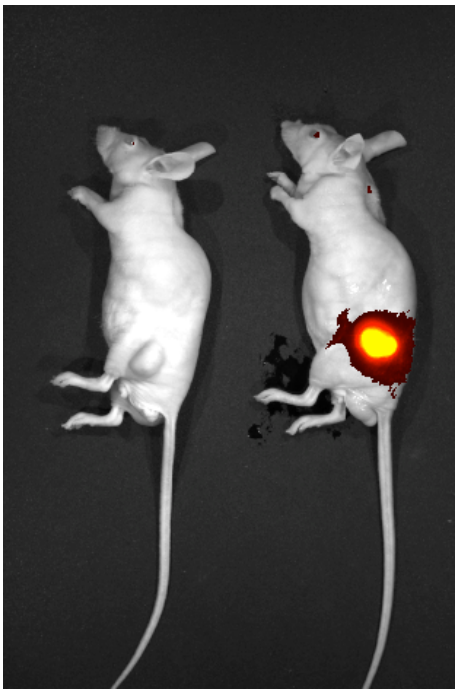
A  B 

Figure 6.1 *Mice receiving intratumoural injection of AlS4-containing DOPE-liposomes A) before US treatment and B) after US treatment. The mouse to the left in both images is an untreated control.*

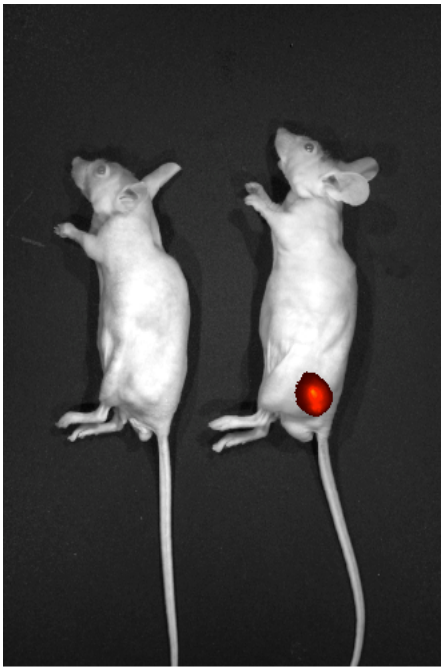
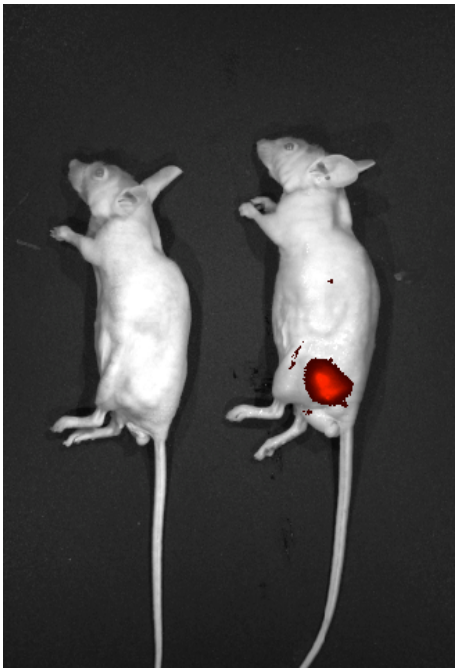
C  D 

Figure 6.2 *Mice receiving intratumoural injection of AlS4-containing HSPC-liposomes C) before US treatment and D) after US treatment. The mouse to the left in both images is an untreated control.*

The variation in data between the different mice indicated large tumour biological differences such as vascularisation and/or tumour permeability as well as presence of necrotic tissue. These factors may both affect tumour distribution of the liposomes as well as the extent

of cavitation produced in the tumour tissue. Improved monitoring of US dosimetry should be accomplished to better control the cavitation produced in different tumours.

It should be added that i.v. administration of the AlS4-containing DOPE-liposomes followed by US exposure of tumours at 5 and 24 h post-injection, respectively, did not increase the fluorescence intensity. Whether this was due to premature leakage of liposomal AlS4 prior to or upon accumulation in the tumour, poor assay sensitivity for low concentrations of AlS4 in the tumour tissue, lack of US-mediated release or other factors needs further investigation.

Therapy studies in tumoured rats

Tumour regression studies in rats receiving DXR-containing DOPE-liposomes and high frequency US are ongoing to further confirm the concept of US mediated drug release. Higher US frequencies (500 kHz-1.13 MHz) would give a more focused US beam, localizing cavitation only in the tumour tissue. Preliminary toxicology of both liposomes and US treatments and the combination of these are currently being investigated.

Loading of cytotoxic drugs in sonosensitive liposomes

Other relevant chemotherapeutic drugs than DXR as well as genetic material such as siRNA is under consideration for loading in DOPE-containing liposomes for improved targeted delivery of cytotoxic drugs to cancer.

7. REFERENCES

1. Bangham AD, Horne RW 1964. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J Mol Biol 8:660-668.

2. Lasic DD. 1993. General introduction to liposomes. Liposomes from Physics to Applications ed.: Elsevier p1-173.

3. Israelachvili J. 1991. Aggregation of amphiphilic molecules into micelles, bilayers, vesicles and biological membranes. Intermolecular and Surface Forces, ed., London: Academic press. p 366-395.

4. Holland JW, Cullis PR, Madden TD 1996. Poly(ethylene glycol)-lipid conjugates promote bilayer formation in mixtures of non-bilayer-forming lipids. Biochemistry 35:2610-2617.

5. Demel RA, De kruyff B 1976. The function of sterols in membranes. Biochimica et Biophysica Acta 457(2):109-132.

6. Damen J, Regts J, Scherphof G 1981. Transfer and exchange of phospholipid between small unilamellar liposomes and rat plasma high density lipoproteins. Dependence on cholesterol content and phospholipid composition. Biochim Biophys Acta 665(3):538-545.

7. 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 sciences 30:2123-2136.

8. Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D 1999. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumours. Pharmacological Reviews 51(4):691-743.

9. Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D 1994. Microvascular permeability and interstital penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. Cancer Research 54(13):3352-3356.

10. Huang SK, Stauffer PR, Hong K, Guo JWH, Philips TL, Huang A, Papahadjopoulos D 1994. Liposomes and hyperthermia in mice: Increased tumour uptake and therapeutic efficacy of doxorubicin in sterically stabilized liposomes. Cancer research 54:2186-2191.

11. Papahadjopoulos D 1996. Fate of liposomes in vivo: A brief introductory review. Journal of liposome research 1:3-17.

12. Wu NZ, Da D, Rudoll TL 1993. Increased microvascular permeability contributes to preferential accumulation of stealth liposomes in tumour tissue. Cancer research 53:3765-3770.

13. Matsumura Y, Maeda H 1986. A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANSC. Cancer research 46:6387-6392.

14. Huang SK, Mayhew E, Gilani S, Lasic DD, Martin FJ, Papahadjopoulos D 1992. Pharmacokinetics and Therapeutics of Sterically Stabilized Liposomes in Mice Bearing C-26 Colon Carcinoma. Cancer Research 52:6774-6781.

15. Woodle MC, Lasic DD 1992. Sterically stabilized liposomes. Biochim Biophys Acta 1113:171-199.

16. Zalipsky S. 1995. Polyethylene glycol-lipid conjugates. In Lasic D, Martin F, editors. Stealth liposomes, ed.: Boca Raton: CRS Press. p 93-102.

17. Haran G, Cohen R, Bar LK, Barenholz Y 1993. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta 1151(2):201-215.

18. Gabizon A, Scmeeda H, Barenholz Y 2003. Pharmacokinetics of pegylated liposomal doxorubicin. Review of animal and human studies. Clin Pharmacokinet 42(5):419-436.

19. Horowitz AT, Barenholz Y, Gabizon AA 1992. In vitro cytotoxicity of liposome-encapsulated doxorubicin: dependence on liposome composition and drug release. Biochimica et Biophysica Acta 1109(2):203-209.

20. Colbern G, Vaage J, Donovan D, Uster P, Working P 2000. Tumor uptake and therapeutic effects of drugs encapsulated in long-circulating pegylated stealth liposomes. Journal of liposome research 10(1):81-92.

21. Andresen TJ, Jensen SS, Kaasgaard T, Jørgensen K 2005. Triggered activation and release of liposomal prodrugs and drugs in cancer tissue by secretory phospholipase A2. Curr Drug Deliv 4(2):353-362.

22. Løkling K-E, Fossheim SL, Klavenes J, Skurtveit R 2004. Biodistribution of pH-responsive liposomes for MRI and a novel approach to improve the pH-responsiveness. Journal of controlled release 98(1):87-95.

23. Kono K, Takagishi T 2004. Temperature-sensitive liposomes. Methods Enzymology 387:73-82.

24. Needham D, Dewhirst MW 2001. The development of and testing of a new temperature-sensitive drug delivery system for the treatment of solid tumours. Adv Drug Deliv Rev 53(3):285-305.

25. Spratt T, Bondurant B, O`Brien DF 2003. Rapid release of liposomal contents upon photoinitiated destabilization with UV exposure. Biochim Biophys Acta 1611(1-2):35-43.

26. Kong G, Dewhirst MW 1999. Hyperthermia and liposomes. Int J Hyperthermia 5:345-370.

27. de Smet M, Heijman E, Langereis S, Hijnen N, Grull H 2010. Mangnetic resonance imaging of high intensity focused ultrasound mediated drug delivery from temperature-sensitive liposomes: An in vivo proof-of-concept study. Journal of Controlled Release.

28. Lasic DD. 1993. Liposomes as drug delivery systems. Liposomes from Physics to Applications, First ed.: Elsevier p265-321.

29. Schroeder A, Kost J, Barenholz Y 2009. Ultrasound, liposomes, and drug delivery: principles for using ultrasound to control the release of drugs from liposomes. Chemistry and Physics of Lipids 162:1-16.

30. Frenkel V 2008. Ultrasound mediated delivery of drugs and genes to solid tumors. Adv Drug Deliv Rev 60:1193-1208.

31. Steward EA, Gedroyc WM, Tempany CM, Quade BJ, Inbar Y, Ehrenstein T, Shushan A, Hindley JT, Goldin RD, David M, Sklair M, Rabinovici J 2003. Focused ultrasound treatment of uterine fibroid tumors: safety and feasibility of a noninvasive thermoablative technique. Am J Obstet Gynecol 189(1):48-54.

32. Thuroff S, Chaussy C, Vallancien G, Wieland W, Kiel HJ, Le Duc A, Desgrandchamps F, De La Rosette JJ, Gelet A 2003. High-intensity focused ultrasound and localized prostate cancer: efficacy results from the European multicentric study J Endourol 17(8):673-677.

33. Pitt WG, Husseini GA, Staples BJ 2004. Ultrasonic Drug Delivery- A General Review. Expert Opin Drug Deliv 1(1):37-56.

34. Ferrara K 2008. Driving delivery vehicles with ultrasound. Adv Drug Deliv Rev 60(10):1097-1102.

35. Huang S-L, McPherson DD, MacDonald RC 2008. A method to co-encapsulate gas and drugs in liposomes for ultrasound-controlled drug delivery. Ultrasound in medicine and biology. 34:1272-1280.

36. Unger E, C, McCreery T, P, Sweitzer R, H, Caldwell V, E 1998. Acoustically active lipospheres containing paclitaxel: A new therapeutic ultrasound contrast agent. Investigative radiology 33(12):887-892.

37. Huang S-L, MacDonald RC 2004. Acoustically active liposomes for drug encapsulation and ultrasound-triggered release. Biochim Biophys Acta 1665(1-2):134-141.

38. Lin H-Y, Thomas JL 2003. PEG-lipids and oligo(ethylene glycol) surfactants enhance the ultrasonic permeabilizability of liposomes. Langmuir 19:1098-1105.

39. Lin H-Y, Thomas JL 2004. Factors affecting responsitivity of unilamellar liposomes to 20 kHz ultrasound. Langmuir 20:6100-6106.

40. Pong M, Umchid S, Guarino AJ, Lewin PA, Litniewski J, Nowichi A, Wrenn SP 2006. In vitro ultrasound- mediated leakage from phospholipid vesicles. Ultrasonics 45:133-145.

41. New RRC. 1990. Preparation of liposomes. Liposomes a practical approach, ed.: Oxford University Press. p 33-103.

42. Hope MJ, Nayar R, Mayer LD, Cullis PR. 1993. Reduction of liposome size and preparation of unilamellar vesicles by extrusion techniques. Liposome Technology, ed.: Gregoriadis, G, CSC Press. p 124-139.

43. Düzgünes N, Bagatolli LA, Meers P, Oh Y-K, Straubinger RM. 2003. Fluorescence methods in liposome research. In Torchilin VP, Weissig V, editors. Liposomes, second ed.: Oxford university press. p 118-119.

44. Somaglino L, Bouchoux G, Mestas J-L, Lafon C 2010. Validation of an acoustic cavitation dose with hydroxyl radical production generated by inertial cavitation in pulsed mode- Application to in vitro drug release from liposomes. Ultrasonics Sonochemistry 2:577-588.

45. Rank A, Hauschild S, Førster S, Schubert R 2009. Preparation of monodisperse block copolymer vesicles via a thermotropic cylinder-vesicle transition. Langmuir 25:1337-1344.

46. Talmon Y 1996. Transmission electron microscopy of complex fluids: The state of the art. Berichte der bunsengesellschaft fur physikalische chemie 100(3):364-372.

47. Allen TM, Cleland LG 1980. Serum-induced leakage of liposome contents. Biochim Biophys Acta 597:418-426.

48. Gabizon A, Shiota R, Papahadjopoulos D 1989. Pharmacokinetics and tissue distribution of doxorubicin encapsulated in stable liposomes with long circulation times. J Natl Cancer Inst 81:1484-1488.

49. Favier J, Lapointe S, Maliba R, Sirois MG 2007. HIF2 alpha reduces growth rate but promotes angiogenesis in a mouse model of neuroblastoma. BMC Cancer 7(139).

50. Cohen J, Cohen P, West SG, Aiken LS editors. 2003. Applied multiple regression/correlation analysis for the behavioral sciences 3rd ed.: Mahwah, N.J: Erlbaum.

51. Pearson ES, Hartley HOE. 1976. Biometrica Tables for Statisticans. ed.: Biometrica Trust, University College London.

52. Marsh D, Seddon JM 1982. Gel-to-inverted hexagonal (Lbeta-HII) phase transitions in phosphatidylethanolamines and fatty acid-phosphatidylcholine mixtures, demonstrated by 31P-NMR spectroscopy and x-ray diffraction. Biochim Biophys Acta 690(1):117-123.

53. Kusube M, Matsuki H, Kaneshina S 2005. Thermotropic and barotropic phase transitions of N-methylated dipalmitoylphosphatidylethanolamine bilayers. Biochim Biophys Acta 1668:25-32.

54. Seddon JM, Cevc G, Marsh D 1983. Calorimetric studies of the gel-fluid (L beta-L alpha) and lamellar-inverted hexagonal (L alpha-HII) phase transitions in dialkyl- and diacylphosphatidylethanolamines. Biochemistry 22(1):1280-1289.

55. Lewis RN, Mannock DA, McElhaney RN, Turner DC, Gruner SM 1989. Effect of fatty acyl chain length and structure on the lamellar gel to liquid-crystalline and lamellar to reversed hexagonal phase transitions of aqueous phosphatidylethanolamine dispersions. Biochemistry 28:541-548.

56. Johnsson M, Edwards K 2001. Phase Behavior and Aggregate Structure in Mixtures of Dioleoylphosphatidylethanolamine and Poly(Ethylene Glycol)-Lipids. Biophysical Journal 80:313-323.

57. Cullis PR, Tilcock CP, Hope MJ. 1991. Lipid polymorphism In Wilschut J, Hoekstra D, editors. Membrane fusion, ed., New York. p 35-64.

58. Cavalcanti LP, Tho I, Konovalov O, Fossheim S, Brandl M 2011. Compressibility study of quaternary phospholipid blend monolayers. Colloids and surfaces B In press.

59. Lauten CL, Rognvaldsson KS, Fossheim S, Nilssen EA, Evjen TJ. 2009. Acoustically sensitive drug delivery particles comprising non-lamellar lipids. Publication No WO2010143972.

60. Lauten CL, Røgnvaldsson S, Fossheim S, Nilssen EA, Evjen TJ. 2010. Acoustically sensitive drug delivery particles comprising non-lamellar forming phosphatidylcholine. Publication No WO2010143970.

61. Lauten CL, Røgnvaldsson S, Fossheim S, Nilssen EA, Evjen TJ. 2010. Acoustically sensitive drug delivery particles comprising non-lamellar forming phosphatidylethanolamine. Publication No WO2010143969.

62. Schroeder A, Avnir Y, Weisman S, Najajreh Y, Gabizon A, Talmon Y, Kost J, Barenholz Y 2007. Controlling liposomal drug release with low frequency ultrasound: mechanism and feasibility. Langmuir 23(7):4019-4025.

63. Ingebrigtsen L, Brandl M 2002. Determination of the size distribution of liposomes by SEC fractionation, and PCS analysis and enzymatic assay of lipid content. AAPS PharmSciTech 2:9-15.

64. Marsh D. 1990. Handbook of Lipid Bilayers. ed.: CRC Press.

65. Liu D, Huang L 1990. Interactions of serum proteins with small unilamellar liposomes composed of dioleoylphosphatidylethanolamine and oleic acid: High-density lipoprotein, apolipoprotein A1, and amphipathic peptides stabilize liposomes. Biochemistry 29:3637-3643.

66. Charrois GJR, Allen TM 2004. Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of pegylated liposomal doxorubicin formulations in murine breast cancer. Biochim Biophys Acta 1663:167-177.

67. Lasic DD. 1993. Liposomes from Physics to Applications. ed., Amsterdam: Elsvier Science Publishers B.V.

68. Gill PS, Espina BM, Muggia F, Cabriales S, Tulpule A, Esplin JA, Liebman HA, Forssen E, Ross ME, Levine AM 1995. Phase I/II clinical and pharmacokinetic evaluation of liposomal daunorubicin. J Clin Oncol 13(4):996-1003.

69. Cui J, Li C, Guo W, Li Y, Wang C, Zhang L 2007. Direct comparison of two pegylated liposomal doxorubicin formulations: is AUC predictive for toxicity and efficacy? Journal of Controlled Release 118:204-215.

70. Gabizon A, Goren D, Horowitz AT, Tzemach D, Lossos A, Siegal T 1997. Long-circulating liposomes for drug delivery in cancer therapy: a review of biodistribution studies in tumor-bearing animals. . Advanced Drug Delivery Reviews 24:337-344.

71. Gabizon A, Barenholz Y, Bialer M 1993. Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid: pharmakokinetic studies in rodents and dogs. Pharmaceutical Research 10(5):703-708.

72. Ishida T, Okada Y, Kobayashi T, Kiwada H 2006. Development of pH-sensitive liposomes that efficiently retain encapsulated doxorubicin (DXR) in blood. International Journal of Pharmaceutics 309:94-100.

73. Gregoriadis G. 1988. Fate of injected liposomes: observations on entrapped solute retention, vesicle clearance and tissue distribution in vivo. Liposomes as drug carriers, ed., Chichester: John Wiley & sons Ltd. p 3-18.

74. Gabizon A, Papahadjopoulos D 1988. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumours. Proceedings of the National Academy of Sciences of the United States of America 85(18):6949-6953.

75. Myhr G, Moan J 2006. Synergistic and tumour selective effects of chemotherapy and ultrasound treatment. Cancer letters 232(2):206-213.

76. Schroeder A, Honen R, Turjeman K, Gabizon A, Kost J, Barenholz Y 2009. Ultrasound triggered release of cisplatin from liposomes in murine tumors. Journal of controlled release 137(1):63-68.

77. Working P, K, Dayan A, D 1996. Pharmacological-toxicological expert report. CAELYX. (Stealth liposomal doxorubicin HCl). Hum Exp Toxicol 15:751-785.

78. Noiseux I, Mermut O, Bouchard J-P, Cormier J-F, Desroches P, Fortin M, Gallant P, Leclair S, Vernon ML 2008. Effect of liposomal confinement on photochemical properties of photosensitizers with varying hydrophilizity. Journal of Biomedical Optics 13:041313.

POPULÆRVITENSKAPELIG SAMMENDRAG

Begrensingen med dagens kjemoterapi er uspesifikk distribusjon av cellegift i kroppen, noe som gir systemiske bivirkninger og redusert effekt. En ny strategi for å øke målstyring av cellegift til tumorer og kreftceller er å kombinere ultralydresponsive (sonosensitive) cellegift-bærende liposomer med ultralyd. Liposomer er nanovesikler som kan frakte cellegift til tumor via blodbanen. Ved å applisere ultralyd direkte mot tumor vil en kunne oppnå selektiv frigivelse av cellegiften fra liposomene samt øke opptak i kreftcellene. Dette forutsetter imidlertid at liposomene er sonosensitive, dvs. liposomene gir tilstrekkelig frisetting av cellegiften ved eksponering av ultralyd.

I denne studien har vi utviklet nye sonosensitive liposomer som inneholder cellegiften doxorubicin. Resultatene har gitt innsikt i hvilke membranparametere som innvirker på sonosensitivitet av liposomer. Liposomer basert på såkalte ikke-lamellære lipider (som lipidet DOPE) viste en signifikant forbedret sonosensitivitet sammenlignet med tradisjonelle liposomer, Caelyx®. Mekanismen bak frisetting av doxorubicin fra DOPE-baserte liposomer så ut til å være irreversibel ødeleggelse av liposom membranen under ultralydbehandling. Denne mekanismen så ikke ut for å være gjeldende for tradisjonelle liposomer.

Liposomer med både høy sonosensitivitet og lang sirkuleringstid i blodbanen ble utviklet ved å optimalisere membrankomposisjonen. Lang sirkuleringstid i blod er ønskelig for å oppnå mest mulig akkumulering av liposomene i tumorvev. Ved å kombinere prototype sonosensitive liposomer og ultralyd i tumor-bærende mus viste vi en signifikant redusert tumor vekst sammenlignet med kontroll grupper (mus som enten fikk liposomer eller ultralyd). Resultatene er lovende for videre utprøvning av sonosensitive liposomer for ultralydmediert målstyring av cellegift.

APPENDIX

Liposome membrane composition	Non-bilayer lipid	EE %	Mean size, nm (P.I)	% US-mediated release in buffered sucrose (40 kHz US)*		
X:DSPC:DSPE-PEG 2000:cholesterol	X			2 min	4 min	6 min
62:10:8:20	DOPE	94	86 (0.14)	69±3	87±2	95±2
62:10:8:20	SOPE	97	86 (0.06)	24±3	48±6	70±6
62:10:8:20	DSPE	99	85 (0.08)	46±3	59±3	64±3
62:10:8:20	DPPE	98	88 (0.07)	9±2	14±2	18±3
52:5:8:35	DNPC	93	82 (0.08)	55±4	78±4	87±4
52:5:8:35	DEPC	97	87 (0.10)	44±2	67±3	76±3
25:27:8:40	DOPC	95	88 (0.07)	22±2	36±4	45±4
25:27:8:40	Oleic acid	96	84 (0.14)	20±2	30±2	35±1
25:27:8:40	Stearic acid	96	86 (0.07)	4±1	14±1	25±1
25:27:8:40	Lyso PC	100	88 (0.07)	7±1	13±3	18±3

*The mean of triplicate US measurements of one liposome batch is given.

PAPER I

PAPER II

PAPER III

PAPER IV

PAPER V