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In vitro stability and content release properties of phosphatidylglyceroglycerol containing thermosensitive liposomes

Martin Hossann [a], Michael Wiggenhorn [b], Alenka Schwerdt [c], Kirsten Wachholz [a], Nicole Teichert [a], Hansjörg Eibl [d], Rolf D. Issels [a,e], Lars H. Lindner [a,e,*]

[a] Department of Internal Medicine III, Klinikum Grosshadern Medical Center (KGMC), Ludwig-Maximilians University, Munich, Germany
[b] Pharmaceutical Technology and Biopharmaceutics, Department for Pharmacy, Ludwig-Maximilians University, Munich, Germany
[c] Pharmaceutical Biology-Biotechnology, Department for Pharmacy, Ludwig-Maximilians University, Munich, Germany
[d] Max Planck Institute for Biophysical Chemistry, Goettingen, Germany
[e] CCG Hyperthermia/GSF, National Research Center for Environment and Health, Munich, Germany

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Abstract

Recently, we reported that 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglyceroglycerol (DPPGOG) prolongs the circulation time of thermosensitive liposomes (TSL). Since the only TSL formulation in clinical trials applies DSPE-PEG2000 and lysophosphatidylcholine (P-lyso-PC), the objective of this study was to compare the influence of these lipids with DPPGOG on in vitro stability and heat-induced drug release properties of TSL. The content release rate was significantly increased by incorporating DPPGOG or P-lyso-PC in TSL formulations. DPPC/DSPC/DPPGOG 50:20:30 (m/m) and DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) did not differ significantly in their release rate of carboxyfluorescein with >70% being released within the first 10s at their phase transition temperature. Furthermore, DPPC/DSPC/DPPGOG showed an improved stability at 37 °C in serum compared to the PEGylated TSL. The in vitro properties of DPPGOG-containing TSL remained unchanged when encapsulating doxorubicin instead of carboxyfluorescein. The TSL retained 89.1±4.0% of doxorubicin over 3 h at 37 °C in the presence of serum. The drug was almost completely released within 120s at 42 °C. In conclusion, DPPGOG improves the in vitro properties in TSL formulations compared to DSPE-PEG2000, since it not only increases the in vivo half-life, it even increases the content release rate without negative effect on TSL stability at 37 °C which has been seen for DSPE-PEG2000/P-lyso-PC containing TSL.

Keywords: Thermosensitive liposomes; Content release rate; Hyperthermia; Poly(ethylene glycol); Phosphatidylglyceroglycerol; Doxorubicin

1. Introduction

Liposomes are composed of lipid bilayers entrapping an internal aqueous space [1]. To avoid rapid opsonization and subsequent uptake of liposomes by the reticuloendothelial system (RES), the surface of liposomes is often modified with Poly(ethylene glycol) (PEG), e.g. by using 1,2-distearoyl-*sn*-glycero- 3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) (Fig. 1). This PEGylation strongly increases the circulation half-life of cholesterol-containing [2–4] and cholesterol-free liposomes [5] after intravenous application. The escape mechanism of liposomes from the RES remains enigmatic [5].

Side effects of cytotoxic drugs like doxorubicin (DOX) or amphotericin B (Ambisome®) are significantly reduced by liposomal encapsulation. This has been demonstrated in various clinical studies using PEGylated-liposomal DOX (Caelyx®, Doxil®) and non-PEGylated DOX (Myocet™) with regard to cardiotoxicity [6,7]. Ambisome® shows less infusion-related toxicity and less nephrotoxicity in comparison to conventional amphotericin B [8]. However, compared to free drug, no improved effectiveness of these clinically approved liposomal formulations has been reported [8,9].

In 1978 Yatvin and colleagues introduced liposomes that release neomycin at specific temperatures and inhibit bacteria

* Corresponding author. Ludwig-Maximilians Universität, Medizinische Klinik III, Klinikum Grosshadern, Marchioninistr. 15, D-81377 Munich, Germany. Tel.: +49 89 7095 3147; fax: +49 89 7095 6137.
E-mail address: Lars.Lindner@med.uni-muenchen.de (L.H. Lindner).

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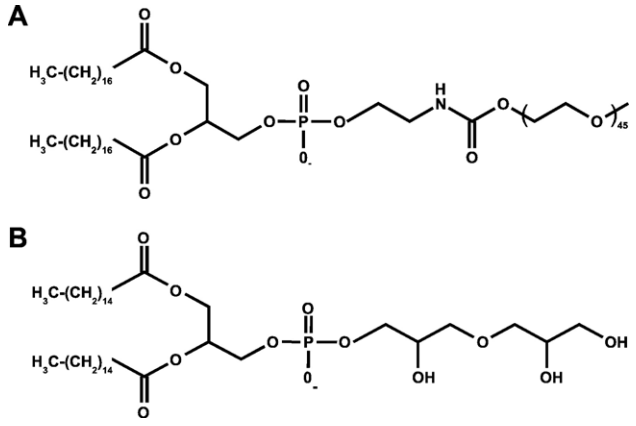

Fig. 1. Chemical structure of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[Methoxy(Polyethylene glycol)-2000] (DSPE-PEG2000) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglyceroglycerol (DPPGOG).

protein synthesis in vitro [10]. These so-called thermosensitive liposomes (TSL) were further developed over the last decades [11–15]. Generally, at their phase transition temperature (T_m) lipids change from the "solid" gel to the "fluid" liquid crystalline state [16]. A conformational change of C–C single bonds in the alkyl chains of the lipids leads to an increase in the total volume occupied by the hydrocarbon chains in the membrane, and therefore increases the permeability of the bilayer membrane. At T_m the permeability is additionally increased as a result of the coexistence of membrane areas in both phases [17]. Moreover, with the incorporation of lysophosphatidylcholines (e.g. 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, P-lyso-PC) into the liposomal membrane it is possible to further accelerate the content release rate at T_m [18,19]. Since P-lyso-PC has a conical molecular shape with the tendency to form spherical micelles in solution, the formation of membrane pores is discussed as a mechanism for increased permeability [15]. The combination of miscible lipids with different T_m results in membranes with a T_m in between those of each lipid [16]. The original formulation based on DPPC (T_m=4 °C [20]) and DSPC (T_m=54.9 °C [20]) with a 3:1 molar ratio released its content between 42.5 and 44.5 °C [10].

Hauck and colleagues reported recently the first phase I trial with TSL in dogs [21]. They treated spontaneous canine tumours with DOX encapsulated in DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) and local hyperthermia. Remarkably, the maximum tolerable dose was slightly lower than known for free DOX or Caelyx®/Doxil® in dogs and the pharmacokinetic profile was also more comparable to free than to liposomally encapsulated drug. The authors attributed this to the fact that DOX was released so rapidly in the heated tumour. However, the average area under the curve as well as the intratumoural drug levels were significantly higher when compared to free DOX.

Recently, we reported a novel formulation of TSL with a prolonged half-life in vivo without the use of PEGylated lipids [14]. This was achieved by the synthetic lipid dipalmitoyl-*sn*-glycero-3-phospho-glyceroglycerol (DPPGOG) in the formulation DPPC/DSPC/DPPGOG 50:20:30 (m/m). The structure of DPPGOG is based on the natural lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol and contains an additional glycerol molecule bound to the glycerol head group via an ether bond (Fig. 1).

The objective of this study was to compare the influence of DPPGOG, DSPE-PEG2000 and/or P-lyso-PC on the in vitro stability and drug release properties of TSL formulations. The DPPGOG-containing formulation DPPC/DSPC/DPPGOG 50:20:30 (m/m) was compared to DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) and PEGylated TSL without P-lyso-PC in presence of fetal calf serum (FCS) to simulate in vivo conditions. The amount of encapsulated carboxyfluorescein (CF) was used to reveal the influence of the different lipids on the TSL properties. Encapsulation of CF is a high-precise method that allows the artefact-free detection of even low marker concentrations in serum. Whereas the fluorescence of the encapsulated CF dye is low due to self-quenching at a concentration of 100 mM, an intense signal appears after CF-release from TSL as a result of liberation and dilution in the outer medium [22]. Furthermore, DOX was encapsulated in DPPC/DSPC/DPPGOG 50:20:30 (m/m) to obtain a therapeutically relevant formulation. The release and stability properties of this formulation were compared to the CF containing formulation.

2. Materials and methods

2.1. Chemicals

The phospholipids DPPC, DSPC and P-lyso-PC were purchased from Genzyme Pharmaceuticals (Sygena Ltd., Liestal, Switzerland). DSPE-PEG2000 was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). The lipid DPPGOG was synthesized (PCT/WO97/30058) as described previously [23]. Carboxyfluorescein (CF) was purchased from Fluka (Buchs, Switzerland) and additionally purified by recrystallization. Doxorubicin was obtained from Sigma Aldrich (München, Germany) and Caelyx® from Essex Pharma GmbH (München, Germany). For high-performance thin layer chromatography (HPTLC) 0.22 mm thick, chemically unmodified silica gel 60 coated on glass plates (Macherey-Nagel, Düren, Germany) was used.

2.2. Preparation of TSL with encapsulated CF

Liposomes were prepared by the lipid film hydration and extrusion method [24]. The composition of the different formulations used in this study are given in Table 1. Respective lipids were dissolved in chloroform using a round-bottomed flask, and the solvent was evaporated under vacuum in a rotary evaporator until a thin and homogeneous lipid film was formed. Hydration of the film was performed using 100 mM CF solution, pH 7.2 at 60 °C for 30 min. The resulting lipid concentration was 50 mM. Unilamellar vesicles were obtained by seventeen times extrusion through two polycarbonate nanopore filters of 200 nm pore size (Avestin, Canada) using a thermobarrel extruder at 60 °C (custom-made; MPI for Biophysical Chemistry, Goettingen, Germany). Unencapsulated CF was removed from the liposome suspension by gel filtration through a Sephadex G-50 column (Pfizer, New York, NY), eluting in 0.9% NaCl solution.

2.3. Preparation of TSL with encapsulated DOX

For the encapsulation of DOX a pH-gradient-driven loading method was carried out [25], with minor modifications. In brief, the TSL were prepared as described above but 300 mM citrate, pH 4 was used for the hydration process instead of CF. Unencapsulated citrate was removed from the liposome suspension by gel filtration through a Sephadex G-50 column equilibrated with 20 mM HEPES, 150 mM NaCl, pH 7.4 (HN buffer). TSL and DOX were

Table 1
Characterization of the CF-TSL (*n* represents the number of independent preparations)

Liposome	c (lipid) (mM)	ζ-potential (mV)	Size (*z* average) (nm)	T_m (°C)	Polydispersity index	CF:lipid (m:m)	head group modified lipid (%)	CF-leakage 1 h/37 °C in FCS (%)
DPPC/DSPC/DPPGOG 50:20:30 (m/m)								
(*n*=7)	41.5 (±9.6)	−22.5 (±9.2)	168 (±8)	42.4 (±0.1)	0.05 (±0.02)	0.19 (±0.03)	27.2 (±3.3)	3.9 (±1.1)
DPPC/DSPC/DSPE-PEG2000 80:20-x:x (m/m)								
x=0 (*n*=3)	37.8 (±11.1)	0.1 (±0.3)	170 (±12)	43.1 (±1.5)	0.06 (±0.04)	0.17 (±0.03)	–	2.1 (±1.1)
x= 1 (*n*=2)	40.0 (±5.4)	1.3 (±1.1)	153 (±47)	43.6 (±0.3)	0.31 (±0.12)	0.21 (±0.08)	1.0 (±0.0)	1.7 (±0.8)
x= 5 (*n*=3)	35.9 (±7.1)	1.2 (±1.7)	141 (±37)	43.7 (±0.2)	0.10 (±0.05)	0.21 (±0.05)	4.4 (±0.4)	2.4 (±2.2)
x=10 (*n*=2)	40.8 (±1.8)	−2.7 (±0.9)	144 (±11)	43.5 (±0.2)	0.09 (±0.01)	0.12 (±0.02)	10.6 (±1.6)	3.6 (±4.2)
DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m)								
(*n*=3)	47.3 (±5.3)	−2.0 (±2.0)	141 (±3)	40.9 (±0.1)	0.05 (±0.02)	0.20 (±0.02)	3.3 (±0.2)	2.9 (±0.6)

The standard deviations are given in parenthesis.

diluted with HN buffer to obtain a final drug:lipid ratio of 0.10 (m/m) and incubated at 38 °C for DOX loading.

2.4. Thin layer chromatography

The phospholipid composition of liposomes was analyzed by thin layer chromatography. Therefore 1 μmol TSL was diluted with water to obtain a total volume of 0.5 mL. Subsequently, 0.5 mL 20% NaCl and 2 mL chloroform: methanol 1:1 (v/v) were added. The solution was mixed and centrifuged to separate the phases. The organic phase was removed and collected. The aqueous phase was reextracted with chloroform:methanol 3:1 (v/v). The combined chloroform extracts were freed from solvent at 40 °C under a steady stream of nitrogen. For TLC analysis 20 mM solutions of lipids were prepared in chloroform:methanol 9:1 (v/v). After application of the samples on silica gel 60 HPTLC plates, the lipids were developed with chloroform:methanol:ammonia (5%) 60:40:1 (v/v) as solution. The poor separation of DPPC and DSPC did not allow the calculation of the DPPC:DSPC ratio. For DSPE-PEG2000 chloroform:methanol 9:1 (v/v) was used as a solution system, where DSPE-PEG2000 formed well-defined spots. Selective staining of phospholipids was performed using the phosphate molybdenum spray of Dittmer and Lester [26].

2.5. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to study the phase transition temperature of the unilamellar vesicles with different lipid compositions. The liposome suspensions were transferred into 40 μL aluminium crucibles without pin (ME 26763) sealed and analyzed using a Mettler Toledo DSC 822e (Giessen, Germany). The samples were scanned from 20 to 60 °C at an average heating rate of 1 °C/min.

2.6. Photon correlation spectroscopy

The size and ζ-potential of the resultant unilamellar vesicles were determined by photon correlation spectroscopy (PCS) in a Zetasizer 3000 (Malvern Instruments, Worcestershire, United Kingdom). For the size measurements the TSL were diluted in water, while the ζ-potential was obtained in 0.9% NaCl.

2.7. Determination of TSL concentration

The overall concentration of phospholipids in the TSL was determined by phosphate analysis as described previously [27].

2.8. Determination of the doxorubicin concentration

The concentration of DOX in the TSL preparations was measured using a Waters HPLC system, with 510 HPLC pumps, 717plus autosampler and the 470 fluorescence detector with an excitation wavelength of 480 nm and an emission wavelength of 560 nm. As described previously [28], the measurement was performed with minor modifications. The runs were carried out on a C_{18} column (250 mm × 4.6 mm internal diameter, 5 μm particle size; 125 Å pore size) from Phenomenex Ltd., Germany. The samples were prepared by an extraction protocol as follows: TSL were diluted to 1 mL using phosphate buffered saline. To each sample, 100 μL internal standard (daunorubicin, 10 μg/mL) and 10 mL dichlormethane/isopropanole 9:1 (v/v) was added. After vigorous mixing, the sample was centrifuged and the obtained organic phase was transferred to a fresh tube. Under a steady stream of nitrogen at 40 °C the extract was freed from solvent. The sediment was dissolved in 1 mL 80 mM potassium dihydrogenphosphate/acetonitrile 73:27 (v/v), also serving as eluent. After centrifugation, the solvent was transferred to injection tubes. 50 μL per sample was injected and eluted with a flow of 1 mL/min. A commercially available 2 mg/mL doxorubicin solution for injection in humans (Adriblastin, Pfizer AG, Germany) was used as standard.

2.9. Temperature dependent CF release

For quantification of entrapped CF, an aliquot of 20 μL liposome suspension (1 mM) was added to 200 μL of 2% Triton X-100 in water. After vigorous mixing and heating at 45 °C for 15 min, 20 μL of this solution was adjusted to a total volume of 1 mL with Tris/NaCl 0.9% buffer [10 mM (pH 8.0)] and the fluorescence (Ex. 493 nm/Em. 513 nm) was measured in a spectrofluorometer (RF-540 from Shimadzu, Kyoto, Japan). The obtained value was taken as 100% release (I_∞). Parallel to the incubation with detergent, 20 μL liposome suspension was added to 200 μL of fetal calf serum (FCS, Invitrogen, Carlsbad, CA). Each sample was kept in a thermoshaker (Eppendorf, Hamburg, Germany) and incubated for the required time period at constant temperature (for detailed parameters see Results). After incubation, 980 μL Tris/NaCl 0.9% buffer [10 mM (pH 8.0)] was added and the amount of free CF was determined as described above. CF release was calculated as:

$$\text{CF release } (\%) = (I(t) - I_0)/(I_\infty - I_0)*100 \quad (1)$$

where $I(t)$ is the fluorescence intensity after incubation of TSL for the time period t and I_0 is the fluorescence baseline.

2.10. Time dependent CF release

The time dependent CF-release of the different TSL formulations was studied at their T_m to allow the comparison of the different formulations. Three mL of FCS were incubated at the desired temperature in a Cary Eclipse fluorescence spectrometer (Varian Inc., Palo Alto, CA, USA) using the implemented heating device, until thermal equilibrium was reached. For the final measurement, 10 μL liposome suspension was diluted with 490 μL 0.9% NaCl. Twenty μL of this solution was given to the heated FCS under stirring and the increase in fluorescence over time ($I(t)$) was measured at an excitation wavelength of 493 nm and an emission wavelength of 513 nm. With the addition of a small sample volume of TSL the formulations immediately reached their T_m at time point t=0 s. For quantification of liposomal entrapped CF, 20 μL of 10%

Triton X-100 in water was added an the resulting fluorescence was taken as 100% release (I_∞). In the measured concentration range, the fluorescence of a CF standard solution in FCS displayed a linear correlation with the concentration of the dye (R=0.9992), indicating no interfering quenching effects. The CF release was calculated using Eq. (1).

The leakage rate of liposomal content follows first order kinetics [29]. Komatsu and Okada used this assumption to calculate the rate constant k of CF-leakage from liposomes treated with ethanol [30], with Eq.(2).

$$\ln(1 - I(t)/I\infty) = A - kt \quad (2)$$

A is expressed as $\ln(1-I_0/I_\infty)$, with I_0 as the initial fluorescence intensity. While plotting $\ln(1-I(t)/I_\infty)$ against the incubation time t, k could be calculated using linear regression. The first 20 s of incubation at T_m were used for calculation of k.

2.11. Time dependent DOX release

All DOX release measurements were carried out using a Varian Cary Eclipse fluorescence spectrometer. It was possible to measure a single sample over the whole time period as the implemented xenon flash lamp is only active when a data point is acquired. Hence, constant excitation of DOX was avoided, so the sample showed no photobleaching. Measurements were accomplished as described above for the time dependent CF release with minor modifications. Undiluted samples were added directly to the preheated FCS or HN buffer (37 to 45 °C, 1 °C steps) over a time period of up to 180 min. In the measured concentration range, the fluorescence of a DOX standard solution in FCS or HN buffer was linear with the concentration of the dye (R>0.99) indicating no interfering quenching effects. The obtained equation from linear regression could be used to quantify the DOX concentration in the samples. Percentage DOX-release was calculated according to Eq. (1). For calculation of k_{DOX} with Eq. (2), the first 120 s of incubation at desired temperatures was used. It was necessary to measure at high amplification, so the fluorescence of FCS had to been taken into account. With the Varian Cary Eclipse fluorescence spectrometer, it was possible to measure up to 4 samples simultaneously. Therefore, FCS reference samples were analyzed in all measurements with DOX containing samples to obtain and subtract the FCS background fluorescence.

2.12. Statistical analysis

The data are expressed as the mean±standard deviation (S.D.) of triplicate samples of at least three independent measurements.

3. Results

3.1. Preparation and characterization of TSL with encapsulated CF

To compare the influence of DPPGOG and DSPE-PEG2000 on the in vitro properties of DPPC/DSPC-based TSL, the formulations DPPC/DSPC/DSPE-PEG2000 80:20-x:x (m/m) (x=1, 5, 10) and DPPC/DSPC/DPPGOG 50:20:30 (m/m) were prepared besides DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m). Thus, the ratio of palmitic- and stearic acid esters in the TSL was kept constant with 80/20 mol% and a direct comparison between DSPE-PEG2000 and DPPGOG-containing formulations was made feasible.

The TSL with encapsulated CF (CF-TSL) were assayed and their properties compared with the analytical protocol, based on phospholipid concentration, size, ζ-potential, CF to lipid ratio and T_m (Table 1). HPTLC was used to assay the phospholipid composition quantitatively. The correlation between targeted composition and real lipid ratio measured densitometrically is given for all formulations (Table 1 and data not shown). No signs of decomposition have been observed.

Although DPPGOG and DSPE-PEG2000 are both negatively charged lipids, only the CF-TSL composed of DPPGOG exhibited a negative ζ-potential. CF-TSL with DSPE-PEG2000 showed a ζ-potential around zero, which could be explained by the shielding effect of the PEG moiety [31]. The size of all TSL varied between 141 and 170 nm. The obtained size of 141±3 nm for the DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) formulation was consistent with the observation by Needham et al. [19]. The observation that increasing DSPE-PEG2000 content decreases the size of the TSL was not statistically significant. The size distribution obtained was dependent on the formulation used. Incorporation of 1 and 5 mol% DSPE-PEG2000 yielded a broad size distribution with a polydispersity index (PD) of 0.31±0.12 and 0.10±0.05, respectively. All other formulations resulted in preparations with narrow size distributions (PD 0.06±0.02).

All formulations showed a comparable CF to lipid ratio around 0.2, indicating almost the same amount of CF encapsulated into the different formulations. The variance in CF to lipid ratio for the 1 and 5 mol% DSPE-PEG2000 preparations can be explained by the greater variance in size compared to the other preparations. Moreover, for DPPC/DSPC/DSPE-PEG2000 80:10:10 (m/m) a significant decrease in CF to lipid ratio was observed. This might be a result of the formation of micelles and membrane discs for formulations with high DSPE-PEG content [32,33], so this formulation was excluded from the study. In practice, it is not necessary to choose a PEG2000-content greater than 5 mol% which is sufficient to cover the whole surface of the liposomes and avoid elimination by the RES [5].

3.2. Temperature dependent CF release

The phase transition temperatures of the non P-lyso-PC containing formulations varied between 41.4 and 44.1 °C (Table 1). The

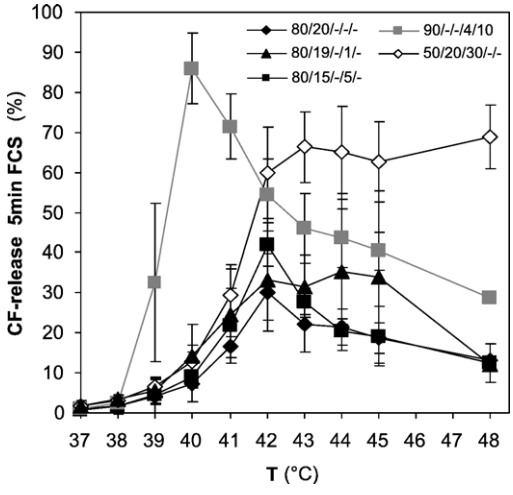

Fig. 2. Temperature dependent CF-release profile of different TSL. After diluting the liposomes with FCS to an appropriate concentration the samples were incubated at the desired temperature for 5 min. The CF release was measured from 37 to 48 °C for each formulation. Whereas the DPPC/DSPC/DSPE-PEG2000 and the DPPGOG-containing TSL released CF around 42 °C, DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) started the CF release between 39 and 40 °C. The formulations are given as DPPC/DSPC/DPPGOG/DSPE-PEG2000/P-lyso-PC (m/m).

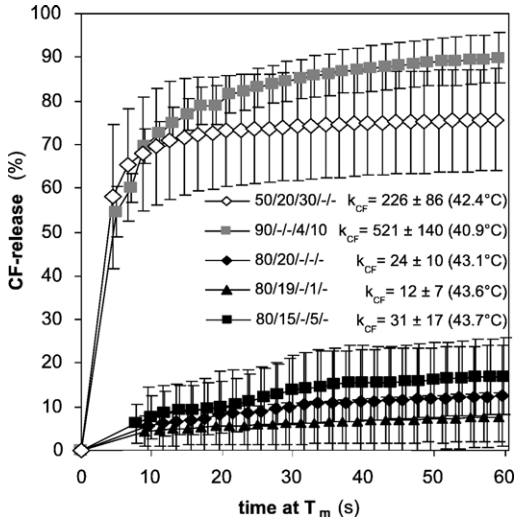

Fig. 3. Influence of the lipid composition on the time-dependent CF-release rate of TSL at T_m in FCS. DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) showed the fastest content release, followed by the DPPGOG-containing formulation. The content release properties were also influenced by DSPE-PEG2000. For 5 mol% DSPE-PEG2000 the CF-release rate was increased, whereas for 1 mol% DSPE-PEG2000 the CF-release rate was decreased compared to DPPC/DSPC 80:20 (m/m). The formulations are given as DPPC/DSPC/DPPGOG/DSPE-PEG2000/P-lyso-PC (m/m). The calculated rate constants k_{CF} are presented as 10^{-4} s^{-1}. For every formulation the individual T_m is listed in parenthesis.

main phase transition temperature of 43.1±1.5 °C for DPPC/DSPC 80:20 (m/m) was not significantly altered by the addition of 1 and 5 mol% DSPE-PEG2000 or 30 mol% DPPGOG. As described [13,19], the addition of P-lyso-PC to TSL lowers T_m, so DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) exhibited a phase transition temperature of 40.9±0.1 °C. The DSC measurements were in agreement with the temperature dependent content release assay (Fig. 2) in which all formulations showed a maximum content release at temperatures approximately one degree below T_m. The onset of the temperature dependent content release was consistent with the beginning of the phase transition peak observed with DSC (data not shown). Further on, the temperature release profile measured for DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) with encapsulated CF was similar in the temperature range from 37 to 41 °C as published for the DOX containing formulation [19]. Interestingly, DPPC/DSPC/DPPGOG 50:20:30 (m/m) behave differently as the other formulations tested at temperatures above T_m. In contrast to the non-DPPGOG-containing TSL, where the amount of CF released at temperatures above T_m was decreased, the DPPGOG-containing formulation still released more than 60% CF.

To evaluate the effect of the distinct phospholipids on the content release rate, the CF-release was measured over time at T_m for the individual formulation (Fig. 3). DPPC/DSPC/DSPE-PEG2000 80:20-x:x (x = 0, 1, 5) released less than 20% of CF during the first 60 s. The content release rate was significantly increased by incorporating DPPGOG or P-lyso-PC in the formulation. At the beginning of heating DPPC/DSPC/DPPGOG 50:20:30 (m/m) and DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) exhibited a fast release of CF with >70% within the first 10 s. Afterwards the CF-release of the P-lyso-PC-TSL increased further up to 88.1±5.3% after 60 s whereas the CF-release of the DPPGOG-TSL remained almost constant with 75.7±11.6%. The calculated rate constants k_{CF} are also presented in Fig. 3. However, the rate constant for DPPC/DSPC/DPPGOG 50:20:30 (m/m) was underestimated due to the fact, that the CF-release did not follow first order kinetics in the analyzed time period (0 to 20 s). Compared to DPPC/DSPC 80:20 (m/m) the DPPGOG containing formulation released CF nine times faster, whereas the incorporation of P-lyso-PC raised the content release rate by a factor of 22. For DPPC/DSPC/DSPE-PEG2000 80:20-x:x (m/m), a concentration dependent effect of DSPE-PEG2000 on the rate constant was found. Incorporation of 1 mol% PEG2000 decreased the content release, whereas 5 mol% DSPE-PEG2000 increased the CF-release at T_m, compared to DPPC/DSPC 80:20 (m/m). The decrease in membrane permeability for low PEG concentrations is consistent with the observations by Nikolova et al. [34]. The maximum permeability of DPPE-PEG2000 liposomes containing DPPC and PI was previously shown by Nicholas et al. for a 4 mol% PEG2000 formulation [35], a concentration where the PEG moiety changes from the mushroom to the brush chain conformation [32].

For optimal TSL-based drug delivery it is important that the vesicles retain their content when travelling the blood vessels before reaching the heated tumour. To analyze the effect of the different lipids on vesicle stability, in vivo conditions were simulated by performing the experiments in the presence of FCS at 37 °C. In Table 1 the CF-leakage during 1 h of incubation is presented. All formulations showed a slow leakage below 4%. Furthermore, the stability was studied over a time period of 24 h (Fig. 4). While DPPC/DSPC 80:20 (m/m) was stable up to 12 h with less then 15% leakage, the 5 mol% PEG2000 formulation became unstable after 8 h with more than 15% leakage. The influence of DPPGOG on the TSL stability was less pronounced

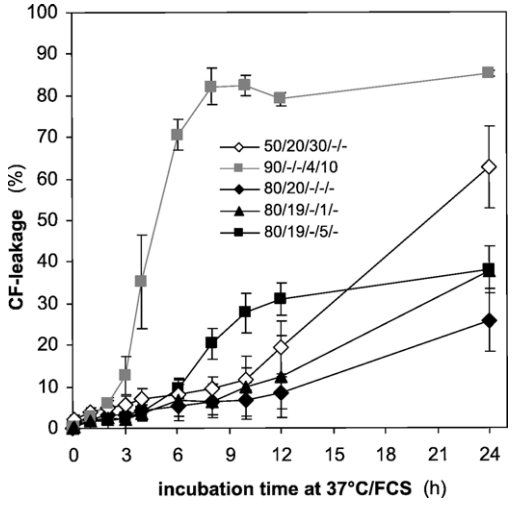

Fig. 4. Influence of the lipid composition on the in vitro stability of TSL at 37 °C in FCS. The TSL were diluted with FCS to an appropriate concentration and incubated at 37 °C using a thermomixer. Samples were collected after defined time points and the CF leakage was measured fluorometrically. The formulations are given as DPPC/DSPC/DPPGOG/DSPE-PEG2000/P-lyso-PC (m/m).

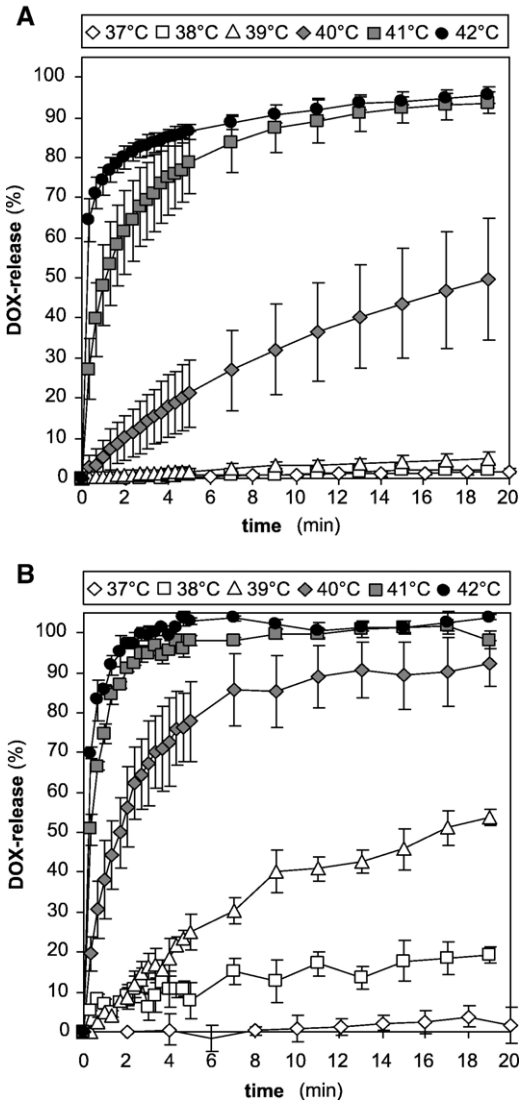

Fig. 5. Temperature dependent content release profile of DOX encapsulated in DPPC/DSPC/DPPGOG 50:20:30 (m/m) TSL. The temperature profile was measured over 20 min at temperatures from 37 °C to 42 °C. (A) DOX-TSL measured in HN buffer, pH 7.4. (B) DOX-TSL measured in FCS.

compared to DSPE-PEG2000. Even with 30 mol% DPPGOG CF-leakage was less than 10% during 8 h, comparable with DPPC/DSPC/DSPE-PEG2000 80:19:1 (m/m). DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) showed a sustained CF-leakage with up to 15% during the first 3 h. Thereafter, the formulation became highly unstable, releasing more than 80% CF in 8 h.

3.3. Preparation and characterization of DPPGOG-containing TSL with encapsulated DOX

For the preparation of DPPC/DSPC/DPPGOG 50:20:30 (m/m) with encapsulated DOX (DOX-TSL), a pH gradient driven loading method was applied [25]. For all loading experiments, a TSL concentration of 2.5 mM was chosen. The samples were incubated at 38 °C. The method yielded an almost complete trapping of DOX inside DPPC/DSPC/DPPGOG 50:20:30 (m/m), as only traces of free DOX were detectable during subsequent size exclusion chromatography. The resultant TSL had a lipid concentration of 2.1±0.1 mM. The DOX concentration was 0.25±0.02 mM and 0.24±0.01 mM, measured by HPLC and fluorescence spectroscopy, respectively. Hence, the final drug: lipid ratio of the preparations was 0.12±0.01 (m/m).

The acidic buffer inside the TSL before the DOX loading procedure is unfavourable for phospholipids [36]. Storing TSL with encapsulated 300 mM citrate, pH 4 at 4 °C for weeks yielded in hydrolysis of the phospholipids and the generation of lysolipids (data not shown). Hence, we examined the lipid composition of all DOX-TSL preparations with TLC. The amount of DPPGOG was 27.8±1.0%. No signs of decomposition was observed, so the DOX-TSL did not contain detectable concentrations of lyso-lipids.

3.4. Temperature dependent DOX-release

As mentioned above, drug retention at physiological temperatures in the presence of serum is crucial regarding a potential clinical application of TSL. DOX-TSL were incubated for up to 3 h in FCS at 37 °C and the DOX-leakage was followed by fluorescence spectroscopy. DPPC/DSPC/DPPGOG 50:20:30 (m/m) retained 96.4±1.1% DOX during 1 h of incubation, which was comparable to the clinically approved non-thermosensitive liposome Caelyx®, which retained 99.3±1.1% DOX in this assay. After 3 h the thermosensitive formulation still retained 89.1±4.0% DOX, whereas Caelyx® showed 94.9±2.1% DOX retention.

Furthermore, the temperature dependent DOX-release profile for the temperature range 37 to 42 °C was measured. First, the DOX-TSL were incubated in HN buffer (Fig. 5A). The vesicles remained stable at temperatures up to 39 °C over a time period of 20 min. DOX-release started at 40 °C and the release rate increased with increasing temperatures. Incubation at 42 °C yielded in the fastest DOX-release with 79.9±2.9% in the first 2 min of incubation. For temperatures from 43 to 45 °C the release profile was not significantly different from the profile measured at 42 °C (data not shown). Secondly, the temperature dependent DOX-release profile was measured in FCS (Fig. 5B). In contrast to the incubation in HN buffer, DOX-release started already at temperatures ≥38 °C. At 42 °C 97.3±1.4% DOX was released in the first 2 min of incubation. Again, for temperatures from 43 to 45 °C the release profile was not

Table 2
DOX release rate constants for DPPC/DSPC/DPPGOG 50:20:30 (m/m) TSL calculated from the temperature dependent release profile presented in Fig. 5

T (°C)	k_{DOX}	
	HN buffer (10^{-4} s^{-1})	FCS (10^{-4} s^{-1})
38	–	2 (±2)
39	1 (±0)	9 (±2)
40	8 (±3)	61 (±17)
41	64 (±15)	168 (±8)
42	55 (±5)	263 (±102)
43	38 (±15)	236 (±39)

Standard deviations are given in parenthesis.

significantly different from the profile measured at 42 °C (data not shown). To quantify the serum effect, the rate constants k_{DOX} were calculated (Table 2). In the presence of serum k_{DOX} was increased 4.8 and 6.2 fold at 42 and 43 °C, respectively. As expected, the non-thermosensitive liposome Caelyx® did not show a temperature dependent DOX-release in this assay (data not shown).

4. Discussion

Although highly clinical relevant for targeted tumour therapy, there are no data published concerning a successful clinical application of DOX-TSL in humans to date. The only in vivo data so far have been accomplished in mice [19] and dogs [21] and show quite promising results for this treatment modality. However, the dog study has been hampered by severe anaphylactic side effects most probably induced by the lipid formulation used which was based on DSPE-PEG2000 and P-lyso-PC (DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m)). Moreover, the pharmacokinetic profile was closer to free than to liposomal encapsulated drug, indicating an ultra-fast release due to heating or an insufficient stability of the vesicles in the bloodstream.

The reason for our study was to test a novel lipid formulation based on phosphatidyloligoglycerol (DPPGOG) for DOX-TSL in vitro which might offer an alternative clinical approach to the ones used to date, especially P-lyso-PC, in the future. Therefore we compared the influence of DPPGOG, DSPE-PEG2000 and/or P-lyso-PC on the in vitro stability and drug release properties of different TSL formulations with encapsulated CF (Table 1).

One prerequisite for clinical application of TSL is a sufficient high content release rate to achieve therapeutical concentrations in the heated tumour. The CF-release rate constant k_{CF} at T_m was notably influenced by the phospholipids used (Fig. 3). Compared to DPPC/DSPC 80:20 (m/m) incorporation of 30 mol% DPPGOG increased k_{CF} 9.4 fold. The effect of DSPE-PEG2000 was significantly less pronounced, yielding only a 1.3 fold increase in k_{CF} when incorporated with 5 mol%. Hence, in contrast to DSPE-PEG2000, DPPGOG is not only able to prolong the in vivo half-life of TSL [14], it also markedly increases the permeability of TSL around the phase transition. DPPC/DSPC/DPPGOG 50:20:30 (m/m) and DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) exhibited a comparable fast CF-release with >70% within the first 10 s. However, DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) showed the fastest CF release rate, with nearly 90% released in the first 60 s of incubation at 40.9 °C. The curve in Fig. 3 fits well to the observations presented by Mills and Needham (see Fig. 9 in [37]).

Further on, stability of TSL preparations was also influenced by distinct phospholipids (Fig. 4). Despite the higher content of head group modified phospholipid, DPPGOG containing TSL showed a higher in vitro stability in comparison to the PEGylated TSL. DPPC/DSPC/DPPGOG 50:20:30 (m/m) retained CF up to 10 h at 37 °C in serum, whereas PEGylated TSL became unstable after 6 h. The size of the head group modification (74 Da versus approximately 2000 Da) is the main difference between DPPGOG and DSPE-PEG2000 and may be responsible for this observation. The coupling of the huge PEG2000 moiety to phosphatidylethanolamine changes the physical and chemical properties of the phospholipid. The volume of the polar head group is dramatically increased, influencing e.g. T_m [16] or the ability to form additional structures in aqueous solutions like micelles [5,32] or membrane discs [33]. In DPPGOG, there is no need to reduce the repulsive steric pressure between the lipid head groups, as necessary for PEGylated lipids [32]. In contrast to DPPC/DSPC/DPPGOG 50:20:30 (m/m), the fast-release formulation DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) became highly unstable after 3 h at 37 °C in serum.

According to the presented data of CF-TSL, DPPGOG has an advantage over DSPE-PEG2000 in TSL formulations. DPPGOG not only increases the in vivo half-life, it even on increases the content release rate without the negative effect observed for DSPE-PEG2000/P-lyso-PC containing TSL on the stability at 37 °C.

For DPPC/P-Lyso-PC/DSPE-PEG2000 90:10:4 (m/m), Mills and Needham demonstrated, that DOX was released much quicker than CF at T_m [15,37]. To examine this for DPPC/DSPC/DPPGOG 50:20:30 (m/m) and to obtain a clinical relevant DPPGOG-containing formulation, DOX was successfully encapsulated with a drug:lipid ratio of 0.12±0.01 (m/m). The main difference between CF and DOX release from DPPC/DSPC/DPPGOG 50:20:30 (m/m) was that DOX was released completely when incubated 3 min at temperatures ≥42 °C in FCS (compare Figs. 3 and 5B). The DPPGOG-containing formulation released 69.7±1.4% DOX and 72.8±1.8% during 18 s at 42 °C and 43 °C, respectively. Nevertheless, the formulation DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) released DOX quicker and at 1.5 °C lower temperatures than DPPC/DSPC/DPPGOG 50:20:30 (m/m). At 41.3 °C, a burst release with 100% of DOX released in seconds was observed [15]. However, the lower permeability at temperatures ≤41 °C of DPPC/DSPC/DPPGOG 50:20:30 (m/m) had the same positive influence on the stability at body temperature as observed for the CF-TSL. In stability experiments, the formulation exhibited only a minor increase in DOX-leakage compared to the non-thermosensitive formulation Caelyx® when incubated over 3 h at 37 °C in serum.

Taking into account the two formulations discussed above, two different strategies for clinical application of drug loaded TSL are achievable. First, TSL like DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) release the drug with a burst release at mild hyperthermic temperatures and damage the tumour vasculature as a result of high local DOX concentrations [19,21]. This might lead to a complete shutdown of the tumour blood flow [38]. Secondly, exploiting the enhanced permeability and retention effect (EPR) liposomes passively accumulate in the tumour tissue [39,40] after intravenous application Thereafter, hyperthermia could be used to release the drug in therapeutically efficient concentrations. TSL like DPPC/DSPC/DPPGOG 50:20:30 (m/m) may be more favourable for the EPR-strategy due to the improved stability at body temperature.

In summary, the choice of the accurate lipid composition for TSL is important to obtain a formulation that fits all necessary criteria for clinical application. All tested TSL showed great

differences in stability and content release properties. The content release rate was significantly increased by incorporating DPPGOG or P-lyso-PC in TSL formulations. DPPC/DSPC/DPPGOG 50:20:30 (m/m) and DPPC/P-lyso-PC/DSPE-PEG2000 90:10:4 (m/m) exhibited a comparable fast release of CF with >70% within the first 10 s at T_m. Furthermore, DPPC/DSPC/DPPGOG 50:20:30 (m/m) exhibited an improved stability at 37 °C in serum compared to the PEGylated TSL. The in vitro properties of DPPGOG-containing TSL were not significantly changed when encapsulating DOX instead of CF. In conclusion, DPPGOG has an advantage over DSPE-PEG2000/P-lyso-PC in TSL formulations, since it not only increases the in vivo half-life, it even on increases the content release rate without the negative effect on the stability at 37 °C observed for DSPE-PEG2000/P-lyso-PC containing TSL. This might be a novel option for the design of TSL in the clinical setting. However, further in vivo evaluation of this new formulation is warranted.

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