

Novel Temperature-Sensitive Liposomes with Prolonged Circulation Time

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ABSTRACT

Hyperthermia increases the efficiency of various chemotherapeutic drugs and is administered as an adjunct to chemotherapy for the treatment of cancer patients. The temperature-dependent effect can be strongly increased by the use of temperature-sensitive liposomes in combination with regional hyperthermia, which specifically releases the entrapped drug in the heated tumor tissue. The novel lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglyceroglycerol (DPPGOG), which is closely related to the naturally occurring 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol, in combination with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-distearoyl-*sn*-glycero-3-phosphocholine provides long-circulating temperature-sensitive liposomes with favorable properties under mildly hyperthermic conditions (41–42°C). DPPGOG facilitates temperature-triggered drug release from these liposomes (diameter, 175 nm) and leads to a substantially prolonged plasma half-life for the encapsulated drug with $t_{1/2} = 9.6$ h in hamsters and $t_{1/2} = 5.0$ h in rats. Quantitative fluorescence microscopy of amelanotic melanoma grown in the transparent dorsal skin fold chamber of hamsters demonstrated a favorable drug accumulation in heated tissue after i.v. application of these liposomes (42°C for 1 h). The mean area under the curve for tissue drug concentration was increased by more than sixfold by application of the new liposomes compared with nonliposomal drug delivery. In summary, we present a new DPPGOG-based liposomal formulation enabling long circulation time combined with fast and efficient drug release under mild

hyperthermia. This adds positively to the results with lipid-grafted polyethylenglycol used thus far in temperature-sensitive liposomes and widens the possibilities for clinical applications.

INTRODUCTION

A major disadvantage of chemotherapy is the lack of tumor-selective drug delivery after systemic application. Only a small fraction of the administered drug usually reaches the tumor. However, the main part of the drug is distributed throughout the body, producing dose-limiting systemic side effects. One way of overcoming this problem is to use drugs encapsulated in a biocompatible material that can be administered i.v., ideally with exclusive delivery into the tumor. Liposomes theoretically fulfil the key requirements to retain, evade, target, and release (1) such carriers and have been evaluated clinically for drug delivery in a variety of diseases (2–4). Thus far, only retention of drugs and evasion from the immune system with “stealth” formulations, containing amphiphatic polyethylenglycol (PEG), have been established (5–7). Specific targeting and controlled release of drugs using liposomes is still a major problem because current liposomal formulations demonstrate reduced toxicity but not increased therapeutic efficacy (2, 8). However, a combination of regional hyperthermia (RHT), which is clinically established for multimodal treatment of certain solid tumors (*e.g.*, soft tissue sarcomas), with temperature-sensitive liposomes (TSLs) may bridge the gap (9–11).

Since the initial description of thermal control of drug delivery systems using TSL by Yatvin *et al.* (12) in 1978, many different liposomal formulations with temperature sensitivity mainly between 43–45°C have been reported (13, 14). Hyperthermia generally supports TSL via two routes. First, RHT facilitates extravasation of TSL into the heated tumor tissue; second, RHT induces controlled drug release of TSL in the heated region (13, 15, 16). TSLs have been prepared from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) because this lipid undergoes a gel-to-liquid phase transition in water at 41.5°C (T_m ; Refs. 14, 17, 18). In this transition state of the lipid bilayer, the passive permeability of ions is markedly increased (19). Drug release may occur principally below the main transition peak in the heating cycle (14). Therefore, DPPC often is mixed with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC; $T_m = 56.0^\circ\text{C}$) to increase transition temperature (14, 17, 18). Lipid-grafted PEG (14, 20) or ganglioside monosialoganglioside (18) was used to prevent TSL from rapid opsonization and uptake by the reticuloendothelial system (RES). This results in increased circulation times of hours or days. Several animal studies have demonstrated the effectiveness of TSL for antitumor drugs in combination with RHT either in enhanced tumor drug uptake or tumor growth inhibition (21). In traditional TSLs studied thus far, the temperature-triggered drug release was in the range of 43–45°C (13). Only a few TSLs have been de-

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Note: L. Lindner and M. Eichhorn contributed equally to this work.

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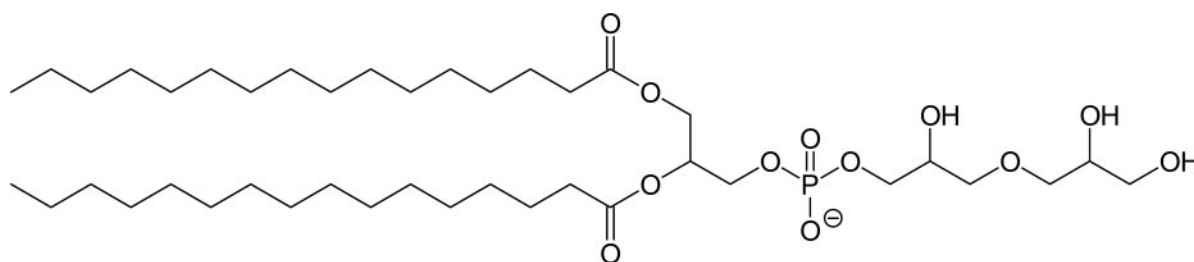


Fig. 1 Chemical structure of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPGOG).

scribed for temperatures $<43^{\circ}\text{C}$ (e.g., $39\text{--}40^{\circ}\text{C}$; Ref. 22). For clinical application, where intratumoral temperature does not exceed 42°C during the treatment time, the need for TSLs for mild hyperthermia in the range of $40\text{--}42^{\circ}\text{C}$ is obvious (11, 23–25). Time-averaged temperatures of 41°C can be achieved easily in 50% of all of the measured tumor sites in the clinical setting (11). Most current liposomal research involves the use of lipid-grafted PEG, which creates a steric barrier that inhibits RES uptake and increases the circulation time of liposomes (stealth liposomes; Ref. 26). Pegylated liposomal doxorubicin has proven to be effective for the management of AIDS-related Kaposi sarcoma and ovarian and breast cancer. However, despite the elimination of cardiotoxicity, new toxicities like desquamation of skin folds (palmar-plantar erythrodysesthesia) have been reported (27, 28). Palmar-plantar erythrodysesthesia and a report of acute hypersensitivity infusion reactions after application of PEG liposomes (29) and reduced circulation time after repeated injections (30, 31) make the development of alternative liposomal formulations desirable.

Therefore, the aim of the present study was to develop new temperature-sensitive and long-circulating liposomes for mild hyperthermia. We apply the substantially smaller synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPGOG; Fig. 1) with a molecular weight close to the natural 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), which surprisingly leads to a prolonged liposome circulation time. We describe the *in vitro* and *in vivo* characterization of these new temperature-sensitive liposomes (PGOG-LTSL). Encapsulated carboxyfluorescein (CF) was used to monitor potential drug release at chosen temperatures. For *in vivo* experiments, fluorescence microscopy of the amelanotic melanoma (A-Mel-3) grown in the dorsal skin fold chamber of Syrian golden hamsters under hyperthermic conditions was used.

MATERIALS AND METHODS

The phospholipids DPPC and DSPC were purchased from Sygena Ltd. (Liestal, Switzerland). The new lipid DPPGOG was designed and prepared in our laboratory (PCT/WO97/30058). The synthesis follows the preparation of DPPG as described previously (32). However, the protected diglycerol 2-*O*-benzylglycero-3,1-*O*-(isopropylidene)-glycerol was used instead of 1,2-(isopropylidene)-glycerol in the second phosphorylation step. 1,2-Dipalmitoyl-*sn*-glycerol was reacted with POCl_3 to form the respective monoester phosphodichloride. This product was converted to the diester by reaction with diglycerol 2-*O*-benzylglycero-3,1-*O*-(isopropylidene)-glycerol to generate

1,2-dipalmitoyl-*sn*-glycero-3-phospho-2-*O*-benzylglycero-3,1-(isopropylidene)-glycerol-monochloride. Hydrolysis of the phosphomonoester and removal of the protecting groups resulted in DPPGOG. The yield was $\sim 70\%$ with a purity of $>99\%$. CF was obtained from Fluka (Buchs, Switzerland) and purified by chromatography.

Preparation of Liposomes

Liposomes were prepared by the lipid film hydration and extrusion method (33). The molar ratio of different phospholipids is given in Table 1. Respective lipids were dissolved in chloroform in a round-bottomed flask, and the solvent was evaporated under vacuum in a rotary evaporator until a thin lipid film was formed. The film was dried for 12 h under vacuum. Hydration of the film was performed using 100 mM CF-solution of pH 7.2 at 60°C for 30 min. The resulting lipid concentration was 50 mM. Large unilamellar vesicles were obtained by extrusion through nanopore filters of 200-nm pore size (Avestin, Canada) using a thermobarrel extruder at 60°C (custom-made; MPI for Biophysical Chemistry, Goettingen, Germany). Free CF was removed from the liposome suspension by gel filtration through a Sephadex G-50 column (Pfizer, New York, NY). The size of the resultant large unilamellar vesicles was determined by photon correlation spectroscopy using a Zetasizer 3000 (Malvern Instruments, Worcestershire, United Kingdom). Zetasizer 3000 also was used for determination of the zeta-potential. Final phospholipid concentrations of the liposomes were determined by phosphate analysis (34). The efficiency of CF incorporation in different liposomal formulations varied between 4–8% (Table 1).

Temperature-Induced CF Release from Liposomes *in Vitro*

The quenching property of hydrophilic CF at concentrations of 100 mM was used to monitor temperature-triggered drug release from TSL. Encapsulated CF does not show fluorescence. A bright CF-fluorescence signal appears only after its release from liposomes and subsequent dilution by the surrounding medium. This behavior of CF was used as a test to study temperature-triggered CF release in TSL. For a more quantitative comparison of TSL, the amount of CF trapped inside the liposomes had to be determined. Therefore, 20- μl liposome suspensions with lipid concentrations of 1 mM were added to 200 μl of Triton X-100/ H_2O (2% v/v), vortexed, and heated at 55°C for 15 min. Twenty μl from this solution were adjusted to a total volume of 1 ml with Tris/NaCl 0.9% buffer [10 mM (pH 8.0)], giving a final lipid concentration of 1.8 μM . The analysis

Table 1 Sample characterization for different liposomal preparations

Liposome composition DPPC/DSPC/DPPGOG ^a (molar ratio)	Concentration of phospholipid determined by phosphate assay (mM)	Percentage capture of CF (%)	CF release after 60 min/ 14 h at 37°C in serum (%)	Average particle diameter (nm)	ζ Potential (mV)
Without DPPGOG					
10/0/0	49.3	2.9	40.7/73.7	68.0 ± 17.0	-0.3 ± 1.6
8/2/0	36.2	6.8	12.5/29.1	97.0 ± 24.7	-2.4 ± 21.1
10 mol% DPPGOG					
9/0/1	26.5	7.1	25.0/49.4	229.6 ± 26.7	-49.3 ± 1.6
8/1/1	28.5	8.1	23.1/36.1	209.2 ± 59.7	-53.2 ± 1.6
7/2/1	32.6	4.4	17.6/39.7	209.1 ± 48.0	-49.6 ± 1.6
6/3/1	28.3	4.2	11.6/38.8	207.5 ± 41.9	-49.2 ± 1.6
20 mol% DSPC					
6/2/2	29.5	5.6	5.4/27.8	250.1 ± 45.9	-70.4 ± 1.6
5/2/3	39.5	6.1	6.4/33.7	174.6 ± 8.9	-68.6 ± 1.6
4/2/4	38.5	7.4	10.2/62.4	212.5 ± 11.9	-67.9 ± 1.6
3/2/5	34.4	5.2	26.8/62.1	202.0 ± 48.5	-82.6 ± 1.6
2/2/6	33.0	10.6	26.2/86.9	224 ± 52.2	-71.0 ± 1.6
1/2/7	27.1	5.2	27.0/100.0	189.4 ± 49.2	-64.9 ± 1.6
0/2/8			no formation of liposomes		
30 mol% DPPGOG					
7/0/3	34.8	5.8	10.4/68.0	184.5 ± 50.3	-74.0 ± 1.6
6/1/3	33.9	7.8	5.7/54.5	214.3 ± 58.5	-72.1 ± 1.6
4/3/3	34.2	8.0	6.7/39.4	208.3 ± 49.5	-73.7 ± 1.6

^a DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DPPGOG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; CF, carboxy fluorescein.

was performed in a spectrofluorometer (RF-540; Shimadzu, Kyoto, Japan) using 493 nm as an excitation wavelength and 513 nm as an emission wavelength. Final CF concentration always was <10 μM, when CF is completely unquenched and quantitatively determined. The release of entrapped CF was measured at different temperatures between 37°C and 45°C after 5 and 60 min and after 14 h of incubation with fetal bovine serum (Invitrogen, Carlsbad, CA). Parallel to incubation with detergent, 20-μl liposome suspensions (1 mM lipids) were added to 200 μl of serum. Each sample was kept in a thermoshaker (Eppendorf, Hamburg, Germany) and incubated for the required period at a constant temperature. Twenty μl of the liposome suspensions again were diluted in Tris/NaCl 0.9% buffer [10 mM (pH 8.0)] to 1 ml total volume and assayed by fluorescence spectroscopy as described.

Animal Model

Studies were performed on male Syrian golden hamsters (Charles River, Sulzbach, Germany; body weight 50–60 g for intravital microscopy; body weight 100–120 g for plasma pharmacokinetics studies) and male Sprague Dawley rats (Charles River; body weight 600–700 g for pharmacokinetics studies) in accordance with the guide for the care and use of laboratory animals (35). The hamster dorsal skin fold chamber model with implanted A-Mel-3 was used to analyze the CF accumulation in heated tumor tissue after i.v. injection of PGOG-LTSL or nonliposomal CF by quantitative fluorescence microscopy (36). Animals were anesthetized i.p. with ketamine (Ketavet; Parke-Davis, Berlin, Germany; 100 mg/kg i.p.) and xylazine (Rompun; Bayer, Leverkusen, Germany; 10 mg/kg i.p.). The dorsal skin was shaved and chemically depilated (Pilcamed, Schwarzkopf, Germany). A transparent double-frame-type titanium chamber then was implanted in the dorsal skin fold. Following a 24-h

recovery period from anesthesia and microsurgery, chamber preparations were inoculated with 2 μl of dense tumor cell suspension (~2 × 10⁵ cells) of the A-Mel-3 of the hamster. Tumors were allowed to grow 7 days before experiments. Twenty-four h before injection of liposomes or nonliposomal CF, respectively, permanently indwelling fine polyethylene catheters (PE10; inner diameter, 0.28 mm) were implanted into the right jugular vein under general anesthesia as described previously.

Experimental Groups and Procedures

Intravital Microscopy in an A-Mel-3 Hamster Dorsal Skin Fold Chamber Model. In experiments investigating the uptake of CF in solid tumors and surrounding normal host tissue in relation to hyperthermia and encapsulation of CF in thermo-sensitive liposomes, 19 animals bearing a dorsal skin fold chamber with A-Mel-3 tumors growing for 7 days were assigned to three experimental groups. Animals of group 1 (PGOG³⁰-LTSL + RHT; *n* = 8) and group 2 (nonliposomal CF + RHT; *n* = 8) received i.v. application of PGOG³⁰-LTSL or nonliposomal CF in combination with RHT (42°C) for 60 min. In a control group, fluorescence patterns after injection of PGOG³⁰-LTSL (PGOG³⁰-LTSL – RHT; *n* = 3) were investigated at normothermia (37°C).

For performance of quantitative fluorescence microscopy, the awake animals were immobilized in a Perspex tube on a specially designed stage (Effenberger, Munich, Germany). The dorsal skin fold chamber was fixed on a specially designed heat supply warmed by a temperature-controlled water bath. In representative experiments, the tumor temperature was measured invasively using a microprobe (diameter, 0.25 mm) of a calibrated digital thermometer (Testo 922; Testo GmbH, Lenzkirch, Germany). The temperature of the water bath was adjusted to

achieve exactly 42°C or 37°C in the tumor. Before i.v. drug injection, the window chamber was allowed to reach steady-state temperature for 10 min. To enable subtraction of tissue autofluorescence, intravital microscopy was performed before i.v. injection of liposomes. At indicated time points after injection, the fluorescence signal was measured in the tumor and surrounding host tissue. Fluorescence was excited at 450–490 nm for 2 s with a power density of 200–300 $\mu\text{W}/\text{cm}^{-2}$ (100 W; HBO mercury lamp). Emission fluorescence was detected above 515 nm and acquired by a silicon-intensified target video camera (C2400–08; Hamamatsu, Herrsching, Germany). Fluorescence images were digitized on-line by an image analysis system (KS400; Zeiss, Oberkochen, Germany) and stored on a hard disk. Inhomogeneities of camera or illumination were compensated by shading correction. Densitometric measurement of fluorescence intensities was performed offline after subtraction of tissue autofluorescence. Fluorescence values are given as a percentage of a solid fluorescent reference signal (% standard) inserted into each chamber preparation (Impregum F; ESPE, Seefeld, Germany). The geometric resolution of all of the images was set to 512×512 pixels at a total densitometric resolution of 255 gray values. Regions of interest for fluorescence measurements in tumor and normal tissue were selected and set in the transillumination image of each chamber preparation acquired before injection of fluorescent liposomes.

Plasma Pharmacokinetics. Non-tumor-bearing hamsters equipped with titanium window chambers were anesthetized as described previously. Polyethylene catheters (Portex, Hythe, United Kingdom) were inserted into the right carotid artery for blood sample collection and into the right jugular vein for drug infusion. To determine the influence of RHT on the plasma concentration of fluorescent drugs, the window chamber was fixed on the heat supply as described previously. After i.v. application of CF liposomes (15.6 μmol lipid/kg body weight; 2.5 μmol CF/kg body weight; $n = 3$) or nonliposomal CF (2.5 μmol CF/kg body weight; $n = 3$), arterial blood samples (200 μl) were drawn from the arterial catheter using heparinized syringes at the indicated time points. In rats, anesthetization, catheterization, and blood sampling after injection of CF liposomes (25 μmol lipid/kg body weight) were performed in a similar way. Blood samples were centrifuged ($2500 \times g$ for 10 min at 4°C), and supernatant was stored at 4°C until analysis. CF quantification in plasma samples was done in a spectrofluorometer after liposome disruption with Triton X-100/ H_2O [20 μl plasma + 200 μl Triton X-100/ H_2O (2% v/v), 15 min at 55°C] and dilution in Tris/NaCl 0.9% buffer as described previously. For pharmacokinetic analysis, the TOPFIT, v2.0 (Gustav-Fischer Verlag, Stuttgart, Germany) pharmacokinetics program was used (37).

Statistics

Changes in fluorescence intensity in tumor or normal tissue were summarized by averaging data from individual animals in each group. Statistical significance between two experimental groups was determined using the Mann-Whitney nonparametric *U* test. Differences of $P < 0.05$ were considered significant.

RESULTS

Role of DPPGOG in DPPC/DSPC-Liposomes. TSLs have been prepared using DPPC and DSPC as matrix lipids. In addition, the negatively charged DPPGOG was chosen to facilitate formation of liposomes but particularly to increase circulation time.

At a constant 10 mol% DPPGOG with an increasing molar ratio of DSPC *versus* DPPC, the lowest temperature resulting in the highest CF release was shifted from 40°C (DPPC/DSPC/DPPGOG, 9:0:1) via 41°C (DPPC/DSPC/DPPGOG, 8:1:1), 42°C (DPPC/DSPC/DPPGOG, 7:2:1) to 43°C (DPPC/DSPC/DPPGOG, 6:3:1). The maximum amount of CF release after 5 min simultaneously decreased from 66% to 45% (Fig. 2A). Without DSPC, serum stability was low with already >40% CF being released after 60 min of incubation at 37°C (Table 1).

In the next set of experiments, the optimal DSPC content of 20% for release at 42°C was kept constant, and the molar ratio of DPPGOG was increased by up to 70% at the expense of DPPC. A DPPGOG content >20% resulted in increased CF release; molar concentrations of DPPGOG <20% resulted in relatively low CF release (<60%) after 5 min at 42°C. For formulations with 30–50% of DPPGOG, a markedly increased CF release of ~80–90% was observed. However, by far the best results with release rates of almost 100% were obtained by applying 60–70% DPPGOG. Unfortunately, these liposomes suffer from the fact that there is already substantial CF release at 38°C (Fig. 2B).

At a constant DPPGOG of 30%, DSPC was varied from 0–30% with the aim of increasing the slope of the temperature-triggered CF release. As observed previously, 20% DSPC was optimal in the formulation of DPPC/DSPC/DPPGOG = 5:2:3 (Fig. 2C). Optimal is characterized by low CF release between 37°C and 39°C and high CF release between 41°C and 42°C.

Results for 5 min of incubation at selected temperatures were predictive for longer incubation periods of 60 min or 14 h. In experiments with 20 mol% of DSPC and increasing amounts of DPPGOG, concentration-dependent CF release was almost detectable at 37°C. After 60 min of incubation, 10–27% CF release was obtained for 40–70 mol% DPPGOG. Less than 6% of CF was released for 20 and 30 mol% DPPGOG. After 14 h of incubation, CF release was 34% and 60–100% for 30% and 40–70% DPPGOG, respectively (Table 1).

Incubation of DPPC/DSPC/DPPGOG liposomes in a molar ratio of 5:2:3 at temperatures from 38°C to 42°C over 60 min again demonstrated favorable characteristics. After a 60-min incubation at 38°C, CF release was increased only slightly to 12.5% (6.4% at 37°C); after 60 min at 40°C, CF release was only ~30%. However, on reaching the critical temperature of 41°C, 65% of CF was released within 10 min (90% after 60 min), and for 42°C, >80% of CF was released within the first 3 min of exposure (Fig. 3).

The *in vitro* results of these liposomes indicate an advantage of the molar ratio of 5:2:3 over other combinations for further *in vivo* characterization. Therefore, in additional experiments these liposomes will be termed PGOG³⁰-LTSL.

Animal Model for Drug Delivery with PGOG³⁰-LTSL. To determine the decrease of PGOG³⁰-LTSL from blood circulation, liposomes labeled with CF were injected into non-tumor-

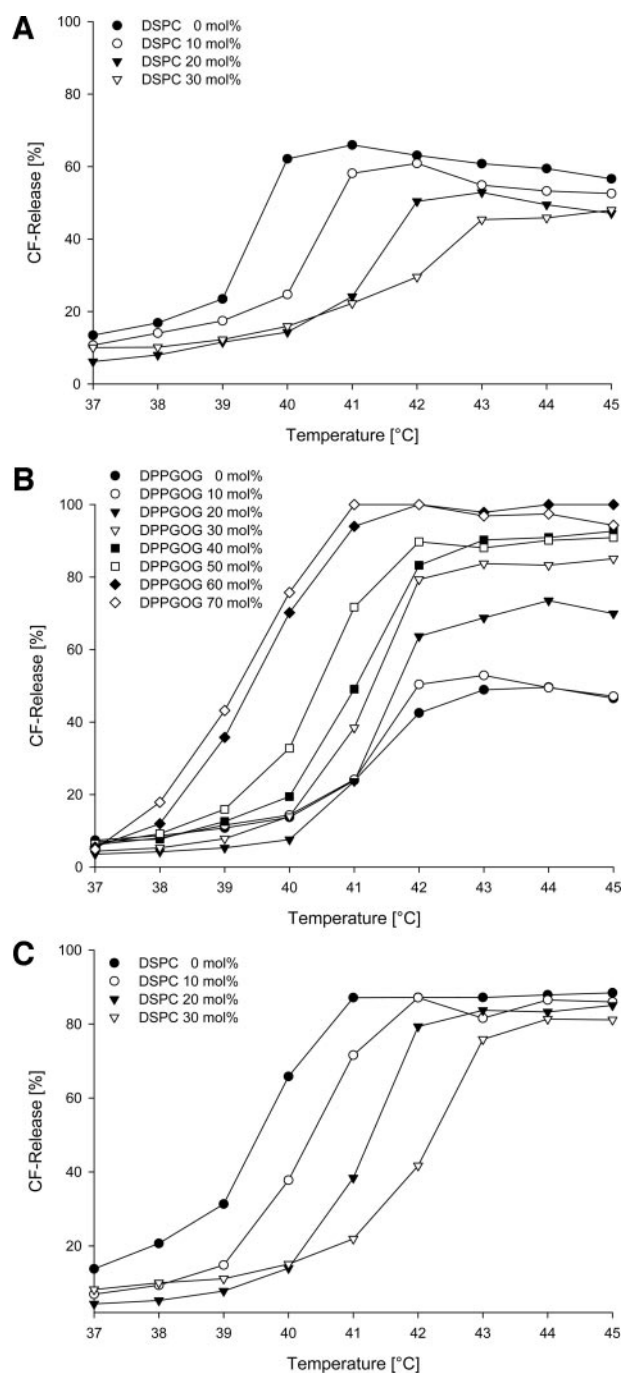


Fig. 2 Effect of various molar ratios of the three lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPGOG) on temperature-triggered carboxyfluorescein (CF) release of temperature-sensitive liposomes (TSLs). CF release from liposomes incubated with serum was measured on exposure to heating at different temperatures between 37°C and 45°C for 5 min as described in "Materials and Methods." A, effect of increasing amounts of DSPC in DPPC/DSPC/DPPGOG liposomes with constant 10 mol% DPPGOG and 90 mol% DPPC + DSPC. B, effect of increased amounts of DPPGOG in DPPC/DSPC/DPPGOG liposomes with constant 20 mol% DSPC. C, effect of increasing amounts of DSPC in DPPC/DSPC/DPPGOG liposomes with constant amount of 30 mol% DPPGOG. Results are given as mean of four measurements. SD was <5%.

bearing hamsters and rats with a dose of 15.6 μmol lipid/kg body weight or 25 μmol lipid/kg body weight, respectively. In both species, blood clearance was log-linear for the tested dose in the first 12 h after i.v. application (Fig. 4). For hamsters, the $t_{1/2}$ was determined to be 9.6 ± 1.3 h, and for rats it was 5.0 ± 0.9 h. More importantly, the blood content of PGOG³⁰-LTSL was >90% during the relevant period of 60 min for RHT, providing a continuous high blood pool for selective opening of the TSL in the tumor area. Application of PGOG³⁰-LTSL in tumor-bearing hamsters equipped with the titanium window chamber demonstrated a continuous increase of fluorescence

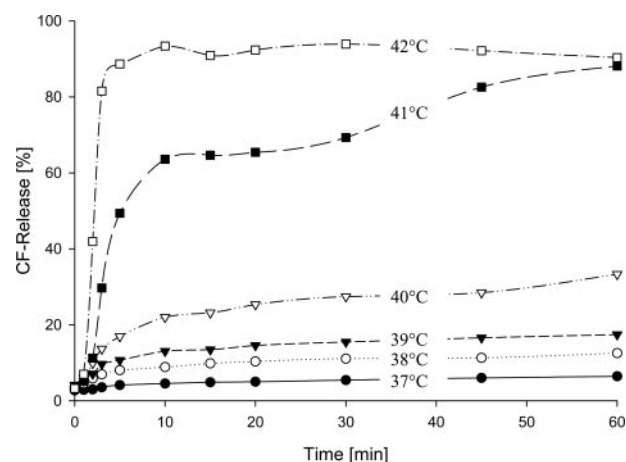


Fig. 3 Thermal properties of liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPGOG) at the molar ratio 5:2:3 (PGOG³⁰-LTSL) at different temperatures as a function of incubation time. Experiments were carried out in 90% bovine serum. Values are given as mean of four measurements. SD was <5%.

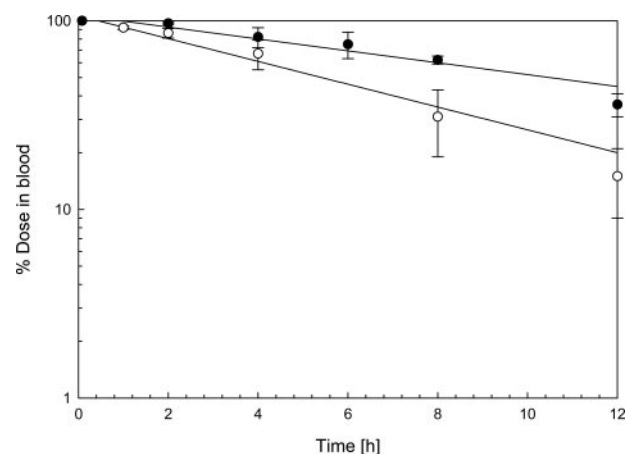


Fig. 4 Clearance of carboxyfluorescein entrapped in PGOG³⁰-LTSL from the blood circulation in hamsters (●) and rats (○) as a function of increasing time postinjection following i.v. injection. Circulation half-lives were determined as $t_{1/2} = 9.6 \pm 1.3$ h for hamsters (15.6 μmol lipid/kg body weight) and $t_{1/2} = 5.0 \pm 0.9$ h for rats (25 μmol lipid/kg body weight) as described in "Materials and Methods." Results are given as mean $\pm$ SD; $n = 3$.

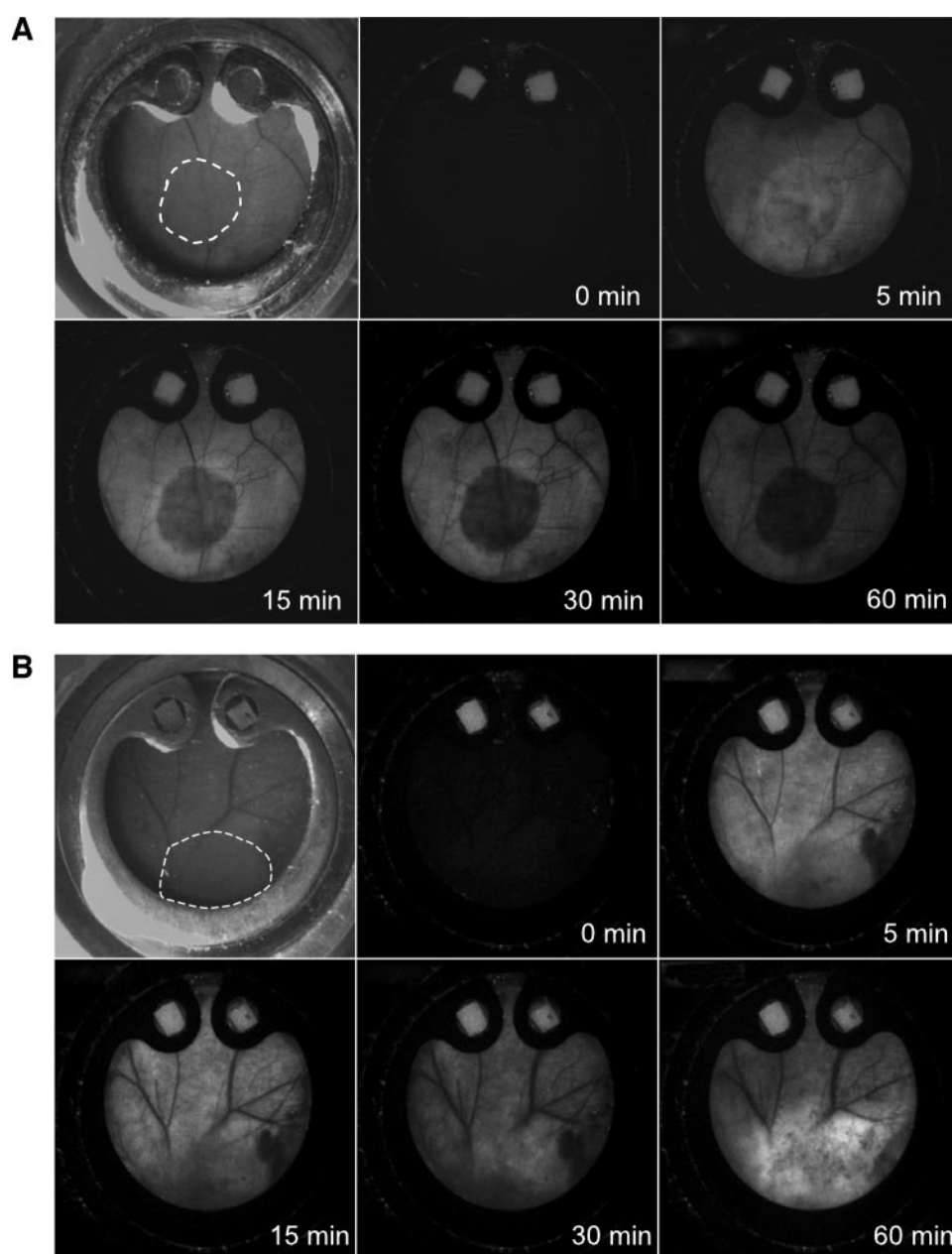


Fig. 5 Representative epifluorescent images of amelanotic melanoma and surrounding normal tissue in the dorsal skin fold chamber of hamsters after application of nonliposomal carboxyfluorescein (CF; **A**) or PGOG³⁰-LTSL with regional hyperthermia (**B**; CF 2.5 $\mu\text{mol/kg}$ body weight) under hyperthermic conditions. Encapsulated CF in PGOG³⁰-LTSL does not show fluorescence because of quenching concentrations (100 mM) and only becomes visible after its heat-induced release from liposomes and dilution in plasma. The temperature in the chamber was 42°C. Heating was started 10 min before drug injection and was continued up to 60 min. The first image of each panel shows an epi-illuminated picture of the chamber to localize the tumor (*dashed line*).

intensity in tumor tissue and in normal tissue during the entire heating process (Fig. 5 and Fig. 6). After 60 min, when heating was stopped, the maximum fluorescence intensity (I_{max}) in tumor ($51.8 \pm 8.5\%$ of standard) and normal tissue ($62.8 \pm 15.0\%$ of standard) was reached. In comparison, after i.v. injection of nonliposomal CF (2.5 $\mu\text{mol/kg}$) at 42°C, I_{max} in tumor ($29.4 \pm 6.3\%$ of standard) and normal tissue ($29.9 \pm 4.8\%$ of standard) was already observed 10 min after i.v. injection. Remarkably, after application of PGOG³⁰-LTSL, I_{max} in heated tumor and normal tissue was increased significantly by twofold compared with I_{max} after application of nonliposomal CF. After discontinuation of heating, the decrease of fluorescence inten-

sity in tumor and normal tissue was recorded for another period of 3 h. In normal tissue, $47.0 \pm 16.1\%$ of I_{max} was detectable 3 h after hyperthermia when PGOG³⁰-LTSL was injected. For nonliposomal CF, fluorescence intensity was already reduced to $25.2 \pm 12.1\%$ of I_{max} at the end of the heating period. At 3 h after hyperthermia, $<5\%$ of I_{max} was detectable. Hemorrhage of the fragile tumor vessels and petechial bleeding in the A-Mel-3 tumor were observed after 1 h of hyperthermia in the presence of PGOG³⁰-LTSL. After tumor bleeding, fluorescence measurements were impaired drastically by light absorption of hemoglobin (*dashed line*). Interestingly, this phenomenon was only observed after application of PGOG³⁰-LTSL under hyperther-

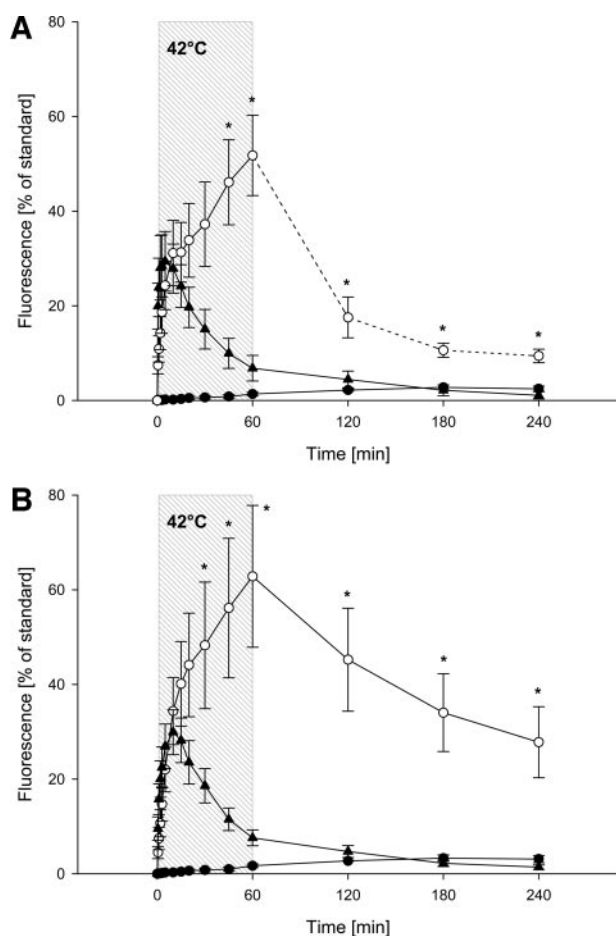


Fig. 6 Fluorescence intensity of tumor (A) and normal tissue (B) after i.v. injection of nonliposomal carboxyfluorescein (CF) $\blacktriangle$ (42°C ; $n = 8$), PGOG³⁰-LTSL with regional hyperthermia (RHT; 42°C ; $n = 8$) $\circ$, or PGOG³⁰-LTSL without RHT (37°C ; $n = 3$) $\bullet$. Regions of interest for quantitative fluorescence measurements were defined on epi-illuminated images and included the whole visible tumor and surrounding normal tissue. Fluorescence intensities are corrected for background fluorescence and are given as % of a solid fluorescence reference signal. *, $P < 0.05$ nonliposomal CF versus PGOG³⁰-LTSL. Dashed line indicates impaired fluorescence detection because of petechial hemorrhage in fragile tumor neovasculature occurring after 60 min of RHT in the presence of PGOG³⁰-LTSL.

mia (eight of eight), whereas application of PGOG³⁰-LTSL under normothermia (zero of three) or injection of nonliposomal CF (zero of eight) during hyperthermia did not result in petechial bleeding of tumor microvessels. Additionally, hemorrhage was only seen in tumor tissue and never observed in normal tissue.

Regarding the area under the curve (AUC) for the CF concentration in tumor and surrounding connective tissue (normal tissue), a 4–6-fold increase for liposomal CF compared with nonliposomal CF could be demonstrated (tumor tissue, $5,773 \pm 2,682$ versus $1,570 \pm 1,230$ AU, $P = 0.001$; normal tissue, $10,211 \pm 6,805$ versus $1,777 \pm 918$ AU, $P = 0.02$; Fig. 7).

To quantify the amount of heat-induced CF release from

PGOG³⁰-LTSL *in vivo*, blood was collected for 120 min after i.v. injection of liposomes at indicated time points. Because of the prolonged circulation time of PGOG³⁰-LTSL, $>80\%$ of the initial blood concentration (C_{max}) was detected after 60 min (Fig. 8). Hyperthermia at 42°C for 60 min in the chamber resulted in the expected reduction of liposomal concentration, which amounted to $\sim 50\%$ of C_{max} after 60 min. This indicates that $\sim 30\%$ of the injected liposomes were opened because of localized heating.

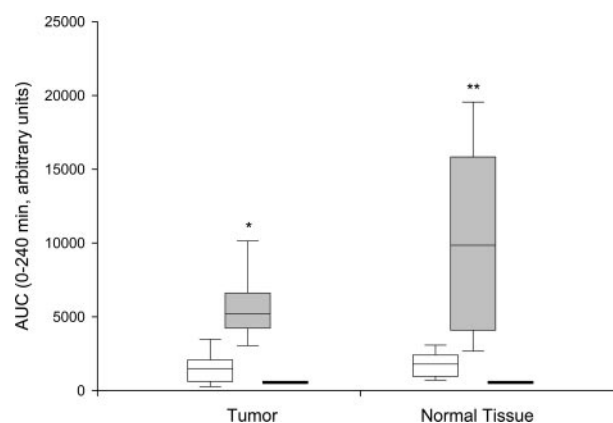


Fig. 7 Area under fluorescence time curves (AUC) in tumor and normal tissue after application of nonliposomal carboxyfluorescein (CF) with regional hyperthermia (RHT; 42°C ; $n = 8$) $\square$, PGOG³⁰-LTSL with RHT (42°C ; $n = 8$) $\square$, or PGOG³⁰-LTSL without RHT (37°C ; $n = 3$) $\blacksquare$. Because of hemorrhage of the fragile tumor vessels and thereby impaired fluorescence detection, which only occurred after application of PGOG³⁰-LTSL + RHT after 60 min, AUC in tumor tissue is lower than the AUC in normal tissue. *, $P = 0.001$ versus nonliposomal CF; **, $P < 0.02$ versus nonliposomal CF; Mann-Whitney nonparametric U test.

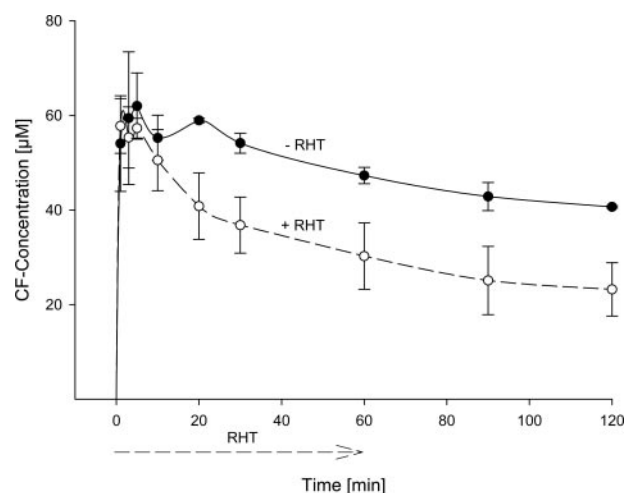


Fig. 8 Plasma levels of liposomal-entrapped carboxyfluorescein (CF) in PGOG³⁰-LTSL with regional hyperthermia (RHT; 42°C) $\circ$ and PGOG³⁰-LTSL without RHT (37°C) $\bullet$ as a function of time after i.v. injection of $2.5 \mu\text{mol/kg}$ total CF, respectively. CF in PGOG³⁰-LTSL was determined after opening of liposomes with detergent as described in "Materials and Methods." Values are given as mean $\pm$ SE; $n = 3$.

DISCUSSION

For clinical application, TSLs are expected to fulfill certain requirements such as serum stability at 37°C, prolonged circulation time to prevent rapid uptake by the RES, and drug release under mild clinically attainable hyperthermic conditions of temperatures between 41–42°C. Tumor blood flow and nanoparticle extravasation are selectively enhanced at this range of temperatures (15). Therefore, mild hyperthermic conditions are favorable for liposome delivery. Higher temperatures, which are needed to induce hyperthermic cell death (23), lead to hemorrhage and stasis in tumor vessels (15, 38) and thus are not useful regarding TSL.

Since the introduction of long-circulating TSL (LTSL) in 1993 by Maruyama *et al.* (18), a variety of LTSLs have been described in the literature (20, 39–41). Besides increased accumulation in implanted tumors (42–44), enhanced antitumor activity in combination with local hyperthermia in various animal models has been demonstrated (20, 22, 39, 45–47). Pegylated phospholipids frequently were used to increase circulation time. We present a different concept using new phosphatidyl-glyceroglycerol liposomes. In our formulation, DPPGOG leads to an increase in circulation time. It is chemically well defined with only a marginal increase in molecular weight by the addition of one more glycerol molecule to the original and natural phosphatidyl-glycerol structure; the result is that the DPPGOG used contains >90% (w/w) of the original phospholipid, and the added glycerol molecule is a natural subunit. The new chemical entity DPPGOG used in our liposomal formulations has different advantages in liposomal formulations. The calorimetric phase transition temperature is close to 40°C with a narrow transition range as in the case of the respective phosphatidylglycerol. This is different for pegylated lipids. DPPGOG can be prepared easily in a large scale to >99% purity from well-defined chemical subunits such as 1,2-dipalmitoyl-*sn*-glycerol and protected forms of glyceroglycerol. Compared with PEG liposomes, in which only ~10 mol% PEG lipids can be used in the liposomal formulation, DPPGOG with the favorable phase transition temperature of ~42°C can amount to 70 mol%, thereby enhancing release characteristics of TSL (Fig. 2B).

The adsorption of serum proteins to the liposome surface generally is believed to be the key step to induce phagocytosis in the liver and spleen. Therefore, suppression of liposome opsonization is an important factor for prolongation of liposome circulation time. For PEG-coated liposomes, it is assumed that the local surface concentration of highly hydrated groups sterically inhibits electrostatic and hydrophobic interactions with serum proteins (48). The novel DPPGOG containing free hydroxyl groups (Fig. 1) offers stronger hydrophilicity than PEG. By adding one glycerol unit via an ether bond to phosphatidylglycerol, the circulation time is increased dramatically. In small DPPC/CHOL (5:4) liposomes (diameter, 50 nm), almost 10 mol% of DPPGOG leads to an increased circulation half-life with $t_{1/2} = 17.6$ h in rats. These results were dose independent between doses of 25–100 μmol lipid/kg body weight (49). In our study, large cholesterol-free PGOG³⁰-LTSL (diameter, ~175 nm) also

provided remarkably long circulation half-lives, with at least $t_{1/2} = 9.6$ h in hamsters and $t_{1/2} = 5.0$ h in rats. In parallel to stealth liposomes, the decrease of blood content was log-linear in the measured time interval (Fig. 4). The linear nature of the data provides no evidence for a high affinity, low capacity uptake system for these liposomes similar to conventional liposomes (*e.g.*, PC/CHOL), which are eliminated from the circulation in two phases (biexponential process). The first process, which is thought to consist of recognition, binding, and uptake into the RES, mainly by the liver, is relatively rapid for these liposomes, with half-lives in the order of a few minutes, inversely related to liposome size. Negatively charged liposomes, with the exception of phosphatidylinositol (50), also are generally cleared more rapidly from the blood stream, presumably by interaction with distinct plasma proteins (51). Membrane fluidity also is of importance for RES uptake. By increasing the phase transition temperature of lipids or by incorporation of cholesterol into the membrane, uptake by the RES can be decreased. This effect is maximal when lipids with high transition temperatures, such as DPPC or DSPC, are mixed in equimolar ratios with cholesterol. For small unilamellar vesicles of this composition (diameter, 60 nm), half-lives of 16–20 h have been described in mice (52, 53). Therefore, to compare the $t_{1/2}$ of our new formulation with other formulations, only cholesterol-free liposomes composed of DPPC and DSPC could be chosen. Small unilamellar vesicles composed of DSPC alone (diameter, ~80 nm) showed $t_{1/2} = 0.47$ h in mice for lipid doses of 57 $\mu\text{mol}/\text{kg}$ body weight and $t_{1/2} = 8.4$ h if DSPE-PEG was added (9:1; Ref. 7). Large doxorubicin-filled DPPC/DSPC (9:1) liposomes (diameter, ~180 nm) were already cleared to <10% of injected dose (~30 μmol lipid/kg body weight) in the first 60 min in mice (39). Adding 6 mol% monosialoganglioside or 3 mol% DSPE-PEG1000–5000 to these liposomes only slightly increased the half-lives to ~3 h (18).

For evaluation of the pharmacokinetic parameters of liposomes, two different techniques are described in the literature. Either radioactive lipids (*e.g.*, [¹⁴C]DPPC and [³H]cholesteryl hexadecylether) have been incorporated into the lipid membrane or radioactive water-soluble model drugs (*e.g.*, [³H] inulin and ¹²⁵I-tyramininulin) have been encapsulated into the liposomes (39, 49, 54). In our experiments, we used encapsulated CF, which was analyzed after terminal lysis of liposomes. Because leakage of CF from cholesterol-free liposomes in blood circulation will occur to some degree, $t_{1/2}$ of liposomes is considered to be underestimated using this technique if compared with the membrane-labeling techniques. For our formulation, CF leakage was determined to be 6.4% in the first 60 min (37°C, 90% bovine serum; Table 1). However, sustained high blood concentrations of the water-soluble model drug CF >90% for at least 60 min in hamsters and mice are sufficient to provide optimal conditions for heat-induced drug release from TSL during RHT.

To provide a liposomal system with the thermal phase transition temperature between 40–42°C, cholesterol was removed from the liposomal system. However, the absence of cholesterol resulted in decreased liposomal serum stability (Table 1). This defect was repaired with DSPC as already

described by others (22, 39). In summary, we have prepared liposomes containing DPPC ($T_m = 41.5^\circ\text{C}$), DSPC ($T_m = 56.0^\circ\text{C}$), and DPPGOG ($T_m = 39.7^\circ\text{C}$) with a serum stability $<39^\circ\text{C}$ and an increased circulation time. For *in vitro* experiments, serum concentrations of 20% (39), 50% (18), and 90% (5) have been described in the literature. Serum facilitates drug release of TSL, probably by interaction of serum proteins with the liposomal membrane (21). Membrane access is especially provided when the lipid chains are in a fluid and thus relatively expanded state (55). To closely mimic the *in vivo* situation, the new liposomes were exposed to 90% serum in our experiments. Liposome size affects circulation time and temperature sensitivity. Optimal size with respect to a long circulation time is between 70–200 nm. Regarding temperature sensitivity, there is a clear relationship between size and amount of drug release with the higher release rate for larger liposomes (56). Thus, a liposome size of 200 nm was chosen for our experiments. A twofold increase in liposome diameter results in an eightfold increase in liposome volume available for drug incorporation. This is of particular importance with regard to clinical application.

We have shown in DPPC-based liposomes, that DSPC with the higher T_m functions as a membrane stabilizer below the expected release temperature of $41\text{--}42^\circ\text{C}$, demonstrating increased membrane stability with increased content of DSPC. It also can serve as a sensitive regulator of the liposomal release temperature, which can be altered between 40°C and 43°C using molar ratios of 0–30% of DSPC in the liposomal formulation (Fig. 2A). In contrast, DPPGOG with the lower T_m acts as a membrane destabilizer. With increasing amounts of DPPGOG, CF release could be increased from 60% to almost 100%. Unfortunately, TSLs with DPPGOG content >50 mol% suffer from low stability between $38\text{--}40^\circ\text{C}$ (Fig. 2B; Table 1). For clinical efficacy, a short time CF release is important. Compared with a traditional thermal sensitive lipid formulation (DPPC:HSPC:cholesterol:DSPE-PEG2000 = 100:50:30:6) with DX release of 40% after 30 min, our system containing DPPGOG is much more effective with a CF release of 80% within 3 min, each at 42°C (Fig. 3; Ref. 13). Only TSLs containing lysolipids demonstrate a faster release with a reported 45% release within the first 20 s of being exposed to 42°C (DPPC:MPPC:DSPE-PEG2000 = 90:10:4; Ref. 22).

Using the transparent dorsal skin fold chamber model of the hamster, we were able to compare accumulation of the model drug CF in tumor (A-Mel-3) and connective tissue for nonliposomal and liposomal i.v. CF application. As expected, the tissue concentration of the nonliposomal-delivered CF differed significantly from the liposomal-delivered CF. The peak concentration for nonliposomal-delivered CF is doubled by the use of PGOG³⁰-LTSL.

Regarding the mean AUC for the first 4-h interval, a more than sixfold increase could be achieved by application of the new liposomes combined with heat in contrast to the nonliposomal-delivered CF. Considering the fact that $>50\%$ of the liposomal-delivered drug is available 4 h after application compared with $<5\%$ of the nonliposomal-delivered drug, the real AUC difference is supposed to be even larger. A possible explanation for the longer-lasting tissue fluores-

cence after PGOG³⁰-LTSL administration could be a predominantly intracellular localization of CF. Otherwise the released CF should be redistributed as shown for nonliposomal CF. For adapting the data to the clinical situation, one has to consider that the selectivity between normal and tumor tissue could be more easily achieved because of more focused heating in a three-dimensional electromagnetic field.

Remarkably, synchronous administration of PGOG³⁰-LTSL and hyperthermia leads to hemorrhage in our A-Mel-3 tumor model after 60 min of heating. Therefore, tumor fluorescence after 60 min is decreased by hemoglobin interference. A fusion of activated liposomes at phase transition temperature with endothelial cells could be a possible mechanism. The rapidly and incompletely built tumor blood vessels seem to be more sensitive to this membrane attack because regular blood vessels outside the tumor do not show any damage under the same conditions. However, this vascular targeting effect could have great impact on the management of solid tumors using LTSL with RHT. Similar effects have only been described for multilamellar vesicles (57).

Hyperthermia has been introduced clinically for >20 years. However, besides positive results from preclinical studies, efficacy of hyperthermia in combination with chemotherapy in a clinical setting has to be proven. LTSL in combination with RHT could give us a powerful tool for the specific treatment of unresectable tumors. The novel liposomal system discussed is expected to be clinically valuable for the delivery of a wide range of chemotherapeutic agents for the management of solid tumors. Antitumor activity of doxorubicin encapsulated in PGOG³⁰-LTSL will be evaluated in future experiments.

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