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Investigation of lipophilic chemotherapeutics and high-molecular weight compounds for the local delivery by phosphatidyldiglycerol-based thermosensitive liposomes

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Erklärung

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Eidesstattliche Versicherung

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For Lisa and My Parents

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Publications arising from this thesis

[1] W.J.M. Lokerse, B. Kneidl, **A. Rysin**, M. Petrini, L.H. Lindner, CHAPTER 6 Liposomes for Hyperthermia Triggered Drug Release, Theranostics and Image Guided Drug Delivery, The Royal Society of Chemistry, 2018, pp. 137-163.

The manuscripts [2-4] are ready for submission and will be published after the final decision on patenting.

[2] Alexander **Rysin**, Michael Paal, Wouter J. M. Lokerse, Barbara Wedmann, Martin Hossann, Michael Vogeser, Gerhard Winter, Lars H. Lindner, **Evaluation of stability, release and pharmacokinetics of hexadecylphosphocholine (miltefosine) in phosphatidyldiglycerol-based thermosensitive liposomes**

[3] Alexander **Rysin**, Wouter J. M. Lokerse, Michael Paal, Katharina Habler, Barbara Wedmann, Gerhard Winter, Lars H. Lindner, **Usage of suitable prodrugs or excipients enables heat-triggered release of dexamethasone from thermosensitive liposomes**

[4] Alexander **Rysin**, Wouter J. M. Lokerse, Agnieszka Mach, Martin Hossann, Barbara Wedmann, Gerhard Winter, Lars H. Lindner, **Serum components and osmotic stress are crucial for release of macromolecules from phosphatidyldiglycerol-based thermosensitive liposomes**

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CHAPTER 1: General Introduction and Aim of the Thesis

1. Current nanomedicines in cancer: possibilities and limitations

Nanotherapeutics emerged as an essential part of modern chemotherapy [1, 2]. Since the approval of liposomal doxorubicin (DOX, Doxil®) in 1995, many other cytotoxic drugs followed, including liposomal cytarabine (DepoCyte®), albumin-bound paclitaxel (Abraxane®) and more recently liposomal irinotecan (Onivyde®). These nanoparticulate drug delivery systems were capable to reduce or alter the side effect spectrum of chemotherapeutics contributing to an improved quality of life for patients [3, 4]. However, only modest improvement of therapeutic response was at best achievable for the approved nanomedicines [5-7]. A complex tumor physiology with strong heterogeneity between cancer types and individual patients is hereby considered as a determinant factor for the poor therapeutic outcomes [8]. The Enhanced Permeability and Retention (EPR) effect is the underlying mechanism for accumulation of common nanoparticles within solid tumors [9, 10]. However, the EPR extent is highly variable and it is not ubiquitous in all tumor types [11, 12]. Moreover, EPR-mediated extravasation into the tumor interstitium is generally not efficient for nanoparticles due to their large size in comparison to free drugs, hindrance by perivascular cell layers and distinct features of the tumor-specific microenvironment such as dense extracellular matrix and elevated interstitial fluid pressure [13, 14]. Besides a low delivery efficiency [15], the drug's bioavailability in the tumor might be further reduced by a slow release from the accumulated carrier and the inability to reach therapeutically relevant concentrations [16, 17]. Current research aims to improve the therapeutic outcome of nanomedicines by a regular implementation of theranostics to allow a patient pre-selection with a sufficiently high nanoparticle accumulation in tumors [18]. Moreover, pharmacological and physical approaches are applied to enhance the EPR-mediated extravasation [19, 20]. Alternatively, nanomedicines are in development which do not necessarily require a passive accumulation by EPR and enable a spatiotemporal drug release by internal or external triggers [21].

2. Thermosensitive liposomes (TSL) as delivery vehicles for chemotherapeutics

2.1 Composition-related achievements of TSL formulations

Thermosensitive liposomes (TSL) represent stimuli-responsive drug delivery systems which release encapsulated compounds when exposed to elevated temperatures in the mild hyperthermia (HT) range (41-43°C) [22]. To trigger drug release from TSL *in vivo*, various external heating sources are applied ranging from water bath or lamp heating of tumor tissue

in rodents [23] to sophisticated systems applying the radiofrequency ablation or focused ultrasound in humans [24, 25]. TSL were originally introduced as heat-triggered drug delivery systems by Yatvin et al. and were composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) lipids [26]. DPPC is the main lipid used in all kinds of TSL due to the transition temperature (T_m) of 41°C [22]. DSPC (T_m=54°C) can be added at a certain ratio to shift the T_m of TSL to higher values [26] and was later found to increase drug release at T_m [27] and improve drug retention in circulation [28]. Although the molecular mechanism might slightly differ between TSL formulations, the HT-induced drug release is assumed to take place at boundary regions between solid and liquid crystalline phases which are formed in the TSL bilayer when heating to T_m [29, 30]. Further developments in this field involved PEGylation to prolong circulation time of TSL [31, 32] and incorporation of certain additives to accelerate the release of encapsulated material at T_m [33]. The latter strategy is considered to be beneficial in view of a rapid blood passage through the heated tumor vessels [34]. The release-enhancing components can be lysolipids as applied in the low temperature-sensitive liposome (LTSL) currently tested in the phase III clinical study for DOX (ThermoDOX®) [25, 35, 36] or Brij78, a PEGylated single acyl chain, in the Hyperthermia-activated cytoToxic TSL (HaT-TSL) [37]. Both components are assumed to form nanopores at T_m leading to enhanced drug release upon HT [38, 39]. However, the therapeutic benefit of these TSL modifications is up for debate since lysolipids and Brij78 can be transferred from TSL to suitable biological acceptors such as (bio)-membranes and serum proteins [40-44]. Moreover, the unwanted lysolipid extraction might be a reason for increased leakage of DOX from LTSL at body temperature [43, 45].

2.2 Phosphatidyldiglycerol-based TSL

1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol-containing TSL ($DPPG_2$-TSL) are distinct from other TSL since PEGylation is not applied to achieve a prolonged circulation time. However, an extended *in-vivo* half-life is achieved by the $DPPG_2$ phospholipid [46, 47] and a rapid drug release at T_m is enabled upon interaction of $DPPG_2$-TSL with distinct serum components [48, 49]. A schematic representation of HT-induced drug release from $DPPG_2$-TSL is shown in Figure 1. Pre-clinically, $DPPG_2$-TSL in combination with local HT demonstrated promising results for DOX leading to a strongly increased drug accumulation in comparison to non-heated tumors [23] and to a positive therapeutic response in feline soft tissue sarcoma [50]. In addition, gemcitabine-containing $DPPG_2$-TSL were capable to

significantly delay the tumor growth in rats with soft tissue sarcoma in comparison to the free drug and $DPPG_2$-TSL without applied HT [47].

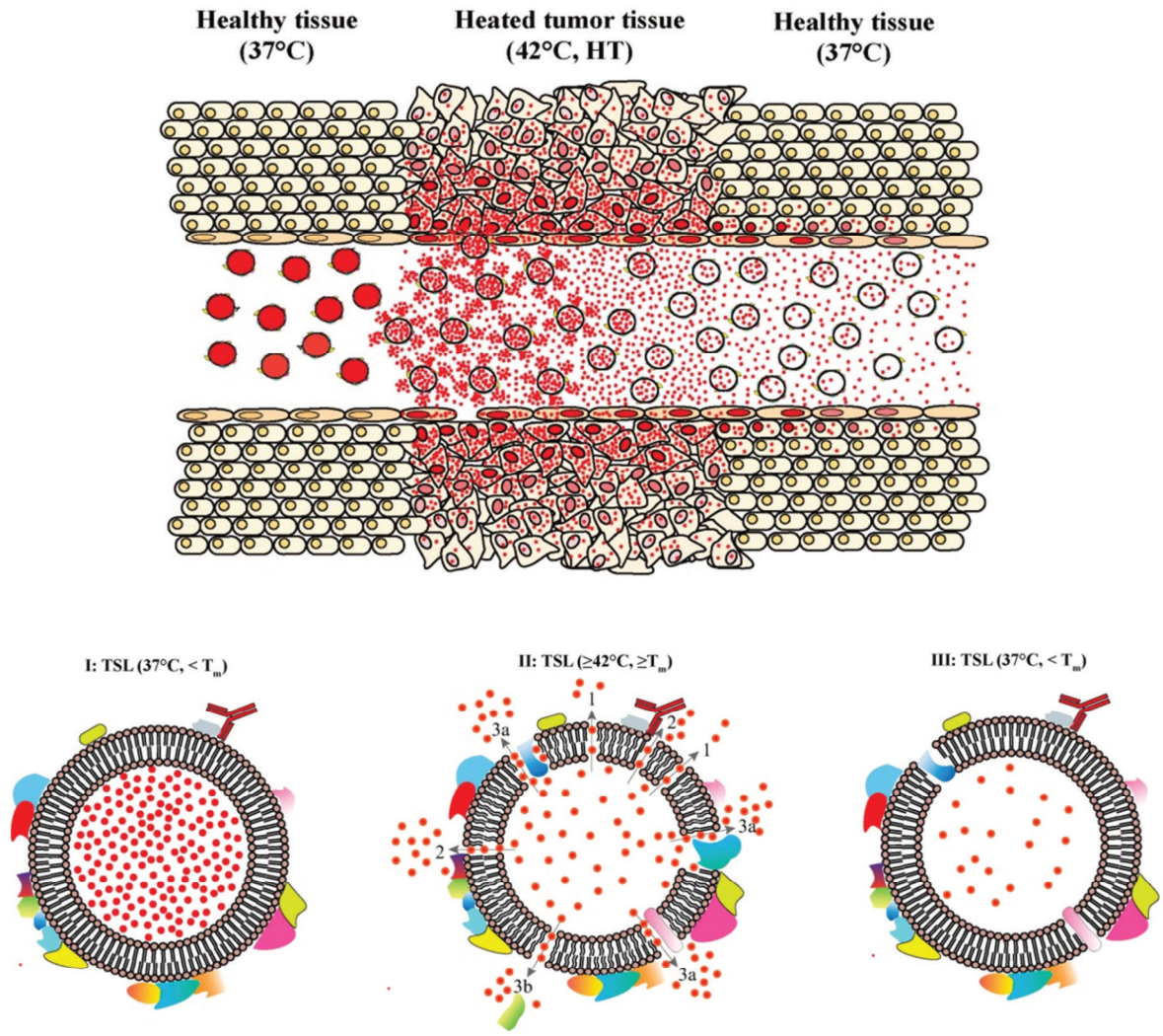

Figure 1: Schematic representation of hyperthermia (HT-) mediated intravascular drug release from $DPPG_2$-TSL. I: TSL stably encapsulate the drug at body temperature (37°C) and form a distinct protein corona (PC) on their surface after intravenous application. **II**: When TSL pass the blood vessels in the heated tumor area (II), the drug is released by a combined action of different hypothetical mechanisms including the increased permeability at boundaries between solid and fluid-crystalline phases at T_m (1), formation of structural defects due to (serum) protein-induced lipid immobilization (2), facilitated protein penetration into the bilayer in the liquid-disordered phase (3a) and leaving of vacant spaces in the bilayer after protein dissociation (3b). Subsequently, the released drug extravasates from vessels into the tumor interstitium, whereas a certain fraction might be washed out by the blood flow. **III:** In case of an incomplete drug release after the first passage through the heated vessels, TSL can potentially release the drug again during subsequent passages due to a prolonged and continuous application of HT (typically 60 minutes). The HT-induced release of doxorubicin from TSL and subsequent extravasation into the tumor tissue was visualized *in vivo* by intravital microscopy [51]. Adapted and modified with permission from the reference [22].

2.3 TSL: can we extend the portfolio of therapeutics?

Extensive investigation of TSL over the last years yielded a detailed characterization of this nanocarrier in terms of lipid composition, release mechanism and heating methods [22, 52]. So far, a lot of research was dedicated to development of an optimal TSL formulation for DOX. The majority of other chemotherapeutics reported in TSL mainly included compounds which can be encapsulated within the aqueous core of TSL [53]. This was achieved either as a consequence of drug's high aqueous solubility or by an active loading via pH/ion gradient resulting in formation of charged and membrane-impermeable species within TSL [22].

However, many anti-cancer drugs are lipophilic and at the same time do not possess functional groups which render them suitable for the active loading approach. Without any further modification, these drugs are expected to be associated with the TSL bilayer and might demonstrate a release behavior different from hydrophilic compounds. Furthermore, they can potentially influence the T_m of TSL. However, our group previously reported that lipophilic hexadecylphosphocholine (HePC, Miltefosine) did not negatively affect the T_m of $DPPG_2$-TSL [54]. Intriguingly, HePC also exhibited a HT-mediated transfer from $DPPG_2$-TSL to cancer cells leading to increased cytotoxicity [54]. More recently, taxanes were described as suitable candidates for a local delivery by lysolipid-containing TSL [55-57].

Besides lipophilic anti-cancer drugs, there is a second less investigated group of therapeutics in TSL which is respresented by (bio)-macromolecules. Currently, these agents mainly include peptide- and protein-based drugs [58]. They are susceptible to enzymatic degradation resulting in short *in vivo* half-lives and low bioavailability [59]. Furthermore, they can have immunogenic side effects upon systemic exposure [60]. Encapsulation in TSL might potentially improve the pharmacokinetics of these agents and increase therapeutic concentrations at pharmacologically relevant sites limiting off-target effects. However, it is not obvious whether TSL are permeable to these drugs at HT due to their high molecular weight and size in comparison to small molecules. Intriguingly, recent *in vitro* studies showed that therapeutically active biomacromolecules including thrombolytics [61] and a cytotoxic protein [62] are released from LTSL in combination with HT, albeit less effectively than small molecules.

3. Aim of the thesis

The focus of this thesis was to study the potential of $DPPG_2$-TSL for the local delivery of lipophilic anti-cancer chemotherapeutics and high molecular weight compounds.

Chapter 2: This chapter follows on the promising results obtained by the group in cancer cell studies with HePC-containing $DPPG_2$-TSL [54]. In particular, stability and temperature-dependent transfer of HePC (Miltefosine) from $DPPG_2$-TSL is investigated in presence of various biological media (serum, full blood) and multilamellar vesicles. The obtained *in vitro* results are confirmed by a pharmacokinetic study of $DPPG_2$-TSL-HePC in rats. Ultimately, a hypothetical mechanism is postulated for the temperature-dependent behavior of HePC in $DPPG_2$-TSL.

Chapter 3: This chapter investigates *in vitro* the potential of $DPPG_2$-TSL and LTSL for a local delivery of Dexamethasone (DXM), approved DXM prodrugs and DXM solubilised by a cyclodextrin (CD). Cyclodextrins (CD) are studied for their potential use in TSL due to the enormous solubilizing potential for many lipophilic compounds. Notably, DXM complexed by a CD reveals a greatly improved performance in terms of loading capacity, stability and thermosensitive release from TSL. The results of this study demonstrate that formation of drug:CD complexes might be a general strategy to extend the use of TSL to poor water-soluble drugs.

Chapter 4: Motivated by recent studies [55, 56], this chapter compares the potential of $DPPG_2$-TSL and LTSL for encapsulation and thermosensitive release of Docetaxel (DTX). A prodrug of DTX provided by a cooperation partner is investigated for a suitable active loading strategy in $DPPG_2$-TSL and potential release upon HT.

Chapter 5: This chapter investigates the feasibility of (bio)-macromolecule release from $DPPG_2$-TSL using dextrans ($\leq$70 kDa) and bovine serum albumin as model compounds. The study is of particular interest since $DPPG_2$-TSL does not contain pore-forming lipids in contrast to LTSL. Intra-liposomal osmotic imbalance in combination with destabilizing effects of intrinsically present serum components is identified to be a promising strategy for a HT-induced release of macromolecules from $DPPG_2$-TSL

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CHAPTER 2: Evaluation of stability, release and pharmacokinetics of hexadecylphosphocholine (miltefosine) in phosphatidyldiglycerol-based thermosensitive liposomes

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Note:

The manuscript was written by Alexander Rysin. The manuscript is ready for submission and will be published after the final decision on patenting.

All experiments presented here were designed and performed by Alexander Rysin. Michael Paal was responsible for the development of the mass spectrometry based detection method and measurement of the samples from the pharmacokinetik study. Wouter Lokerse is thanked for the help with the animal work, his scientific input into this project and first correction of the manuscript. Barbara Wedmann is thanked for the help with lipid quantification. The project would not have been possible without a scientific guidance of Lars Lindner, Gerhard Winter, Michael Vogeser and Wouter Lokerse. All the authors is additionally thanked for the critical review of the manuscript.

1. Abstract

Hexadecylphosphocholine (HePC, Miltefosine) is a drug from the class of alkylphosphocholines with an antineoplastic and antiprotozoal activity. We previously reported that HePC uptake from thermosensitive liposomes (TSL) containing 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol ($DPPG_2$) into cancer cells is accelerated at mild hyperthermia (HT) resulting in increased cytotoxicity. In this study, we compared HePC release of different TSL formulations in serum. Independently of the TSL formulation, HePC showed rapid but incomplete release below the transition temperature (T_m) in serum. Short heating to $\geq T_m$ increased HePC release from $DPPG_2$-TSL by a factor of two. Bovine serum albumin (BSA) induced HePC release from $DPPG_2$-TSL comparable to serum. Furthermore, multilamellar vesicles (MLV) were capable to extract HePC from $DPPG_2$-TSL in a concentration- and temperature-dependent manner. Repetitive exposure of $DPPG_2$-TSL to MLV at 37°C led to a fast initial release of HePC which slowed down after subsequent extraction cycles finally reaching approx. 50% HePC release. A pharmacokinetic study in rats revealed a biphasic pattern with an immediate clearance of approx. 50% HePC whereas the remaining 50% HePC showed a prolonged circulation time. We speculate that HePC located in the external leaflet of $DPPG_2$-TSL is rapidly released upon contact with suitable biological acceptors. As demonstrated by MLV transfer experiments, asymmetric incorporation of HePC into the internal leaflet of $DPPG_2$-TSL might improve HePC retention in presence of complex biological media and still give rise to HT-induced HePC release.

2. Introduction

Liposomes are biocompatible phospholipid vesicles which are extensively used as carriers in drug delivery (1). Forming a closed bilayer system, liposomes are versatile in their ability to encapsulate a wide range of therapeutics. Hydrophilic molecules are entrapped in the internal aqueous compartment whereas lipophilic compounds are located in the lipid bilayer (2, 3). Over the last decades, liposomes demonstrated their potential as drug carriers resulting in a wide range of clinically approved products (4) which are used in treatments of cancer, rheumatoid arthritis (5) or fungal infections (6). However, the clinical success observed in treatment of cancer patients was mainly due to reduced toxicity in comparison to free drug without a significantly improved therapeutic outcome (7, 8). The lack of the therapeutic efficacy was mostly attributed to a suboptimal site-specific drug delivery and bioavailability of a drug by common nanomedicines (9-11). Many strategies were proposed to enhance a local drug delivery including physico-chemical modification of physiological barriers (12, 13), patient pre-selection (14) or the use of stimuli-responsive systems (15, 16).

Thermosensitive liposomes (TSL) belong to a class of stimuli-responsive nanocarriers which can release encapsulated compounds at temperatures in the mild hyperthermia (HT) range (41°-43°C) (17, 18). Although the release mechanism might slightly differ between TSL formulations, it is generally assumed that drug release predominantly occurs at boundary regions between solid-gel and liquid-crystalline phases which are formed in the TSL bilayer upon heating to T_m (19-21). This provides a possibility for the intravascular, HT-induced drug release from TSL resulting in improved tumor accumulation of the bioavailable drug (22, 23). In contrast to TSL, many nanoparticles have to rely on a passive tumor accumulation by the enhanced permeability and retention (EPR) effect which varies greatly between tumor types (24) and is in general more pronounced in fast-growing tumors in rodents (25, 26). Additionally, the drug's bioavailability might be limited due to a slow or incomplete release from the accumulated nanoparticles (27).

Despite an extensive investigation of TSL over the last years, only a few compounds were described and successfully formulated in TSL, including mainly fluorescent dyes, contrast agents as well as a few anti-cancer drugs (doxorubicin, cisplatin) (28). Moreover, these substances allowed an encapsulation inside the aqueous core of TSL either due to sufficient water solubility or presence of functional groups suitable for active loading. However, many new drug candidates in pharmaceutical development including anti-cancer drugs are lipophilic and poor water-soluble (29, 30), leading to their preferred incorporation in liposomal bilayers.

It is conceivable that these compounds require a detailed investigation in TSL formulations and might finally benefit from a local delivery at the disease site.

Alkylphosphocholines represent a class of synthetic alkylphospholipids with antiproliferative and antimicrobial properties. By targeting cancer cell membranes, they activate several signaling pathways leading to apoptosis (31-33). Hexadecylphosphocholine (HePC) represents a lead compound from the group of alkyphosphocholines (34) which is approved for a topical treatment of metastasized breast cancer (35, 36). Preclinically, HePC showed a promising anti-cancer activity on many tumor cell lines *in-vitro* as well as in animal tumor models *in vivo* (37, 38) but failed later to progress in clinical studies on patients due to a dose-limiting gastrointestinal toxicity following the oral administration (39-41). Parenteral application of the drug is not possible since HePC has hemolytic effects on blood cells (42). Liposomes were found to be a suitable vehicle for the intravenous application of HePC resulting in reduction of side effects known from oral administration as well as hemolysis (43, 44). However, the anti-tumor activity of liposomal HePC was observed only in a small number of tumor models, mainly mammary carcinomas (43-45). Although the reasons for the insufficient anti-tumor activity are not fully understood yet, TSL might improve the therapeutic outcome of HePC by a local delivery of the bioavailable drug to the tumor.

Our group showed that incorporation of the anionic lipid 1,2-dipalmitoyl-sn-glycero-3-phospho-1‘-rac-diglycerol ($DPPG_2$) into a TSL formulation prolongs the circulation time and increases drug release at T_m (46-49). Preclinically, $DPPG_2$-TSL increased DOX accumulation in a heated tumor and resulted in a significant tumor growth delay for both DOX (50, 51) and gemcitabine (47) in comparison to the free drugs. We reported previously that HePC-containing $DPPG_2$-TSL show a HT-induced toxicity on tumor cells *in vitro* (52). Correspondingly, an increased uptake of HePC was detected in cancer cells after treatment with $DPPG_2$-TSL at 42°C (HT) in comparison to 37°C. The objective of this study was to further evaluate the potential of HePC-containing $DPPG_2$-TSL for a future pre-clinical *in-vivo* study. Therefore, we identified factors affecting the release of this drug from TSL. We extended our *in-vitro* results by pharmacokinetic measurements in rats tracking HePC and the liposomal carrier simultaneously by a mass spectrometry-based detection method.

3. Materials and Methods

3.1 Chemicals

DPPC and DSPC were purchased from Corden Pharma (Switzerland). $DPPG_2$ was kindly provided by Thermosome GmbH (Munich, Germany). HePC, DSPE-PEG_{2000} and $DHPG_1$ were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Deuterated HePC ($^2H_4$-HePC) was obtained from Alsachim (France). Cholesterol and bovine serum albumin (BSA) were purchased from Sigma Aldrich GmbH (Munich, Germany). Fetal calf serum (FCS) was from Biochrom AG (Berlin, Germany). Sepharose CL-4B was obtained from GE-Healthcare (Chicago, USA). The phosphate standard solution (1000 mg/ml) was from Merck KGaA (Darmstadt, Germany). Aluminium pans (standard crucible with lid, 40 µl) were from IVA Analysentechnik GmbH & Co. KG (Germany). All other chemicals were either from Carl Roth GmbH (Karlsruhe, Germany) or Sigma Aldrich GmbH (Munich, Germany).

3.2 Preparation of thermosensitive liposomes (TSL)

TSL were prepared by the lipid hydration and extrusion method as described previously (52). Corresponding amounts of lipids including HePC (Table 1) were dissolved separately in chloroform:methanol (9:1) and combined in a round-bottomed flask. The lipid film was formed under reduced pressure using a rotary evaporator. The lipid film was hydrated with HEPES-buffered saline (HBS) (20 mM HEPES, 150 mM NaCl, pH=7.4) at 60°C for 30 min to obtain multilamellar vesicles (MLV) at 50 mM lipid concentration. MLV were extruded 10 times through two 200 nm pore size membranes (Whatman, Nuclepore Track-Etch Membrane) using a thermobarrel extruder at 60 °C (Northern Lipids, Vancouver, Canada).

3.3 Preparation of multilamellar vesicles (MLV)

MLV composed of DSPC and Cholesterol (55:45 mol:mol) were prepared as described elsewhere (53). Corresponding amounts of lipids were dissolved in chloroform:methanol (9:1, vol:vol) and combined in a round-bottomed flask. The lipid film was formed under reduced pressure using a rotary evaporator. The lipid film was hydrated with 300 mM sucrose to yield MLV at 100 mM lipid concentration. Subsequently, the mixture was centrifuged for 10 min (25°C, 1600xg). After centrifugation, MLV were concentrated as a layer in the upper phase. The bottom phase containing sucrose was removed using a 21G needle syringe. Afterwards, MLV were resuspended in HBS and centrifuged again for 10 min (25°C, 1600xg) forming a MLV pellet. Supernatant was discarded and MLV resuspended in HBS. The washing procedure of MLV with HBS was repeated three times. MLV were finally resuspended in a certain volume of HBS resulting in 200 mM MLV.

3.4 Dynamic light scattering (DLS)

Z-average (nm), ζ-potential (mV) and polydispersity index of the final TSL formulations were measured by DLS using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). TSL (0.5 mM) were diluted in physiological saline prior to DLS measurements.

3.5 Differential scanning calorimetry (DSC)

The transition temperature (T_m) of TSL formulations was determined by DSC. Liposome suspensions (20 µl, 40 mM) were transferred into aluminium pans and measured on a Mettler Toledo DSC 821e (Mettler Toledo, Giessen, Germany). The samples were scanned from 20°C to 60°C at a heating rate of 2 K/min.

3.6 Measurement of lipid concentration (phosphate assay)

The lipid concentration was determined by the phosphate assay as described in detail elsewhere (54). TSL formulations were first diluted with distilled water followed by addition of sulfuric- and perchloric acid containing solutions. The samples were heated for 2 hours at 300°C to form inorganic phosphate. Subsequently, a solution containing ammonium heptamolybdate and Triton X-100 was added and the turbidity of the samples resulting from a formed complex was measured at 660 nm in a spectrophotometer (Beckmann DU 640, Beckman Coulter GmbH, Krefeld, Germany). The quantification was performed based on the standard line obtained from 1 g/l phosphate solution that was treated the same way like TSL samples.

3.7 Temperature-dependent HePC retention in TSL

HePC-containing TSL were diluted to 4.0 mM lipid with either HBS, fetal calf serum (FCS) or bovine serum albumin (BSA) solution in physiological saline (16 mg/ml), respectively. 120 µl samples were distributed in Eppendorf tubes and incubated at a certain temperature in a thermomixer (750 rpm) for either 5 or 60 min. Immediately after the incubation, the samples were put on ice for 5 min. To remove released (micellar or protein-bound) HePC, TSL samples were purified by size-exclusion with manually prepared spin columns filled with Sepharose CL-4B. Before use, the columns were washed three times and pre-equilibrated with HBS. In detail, 100 µl TSL sample was transferred on a CL-4B mini spin column and centrifuged for 2 min (25°C, 2000xg) resulting in elution of 100 µl TSL. 900 µl methanol were added, the mixture strongly vortexed and incubated in a thermomixer for 20 min (25°C, 1200 rpm). After the incubation, samples were centrifuged for 10 min (25°C, 16000xg) to

remove precipitated proteins. The supernatant was transferred into HPLC vials and the lipids quantified by HPLC-CAD as described in section 3.10.

3.8 Temperature-dependent HePC transfer to MLV

The assay was performed as previously described elsewhere (53). HePC-containing $DPPG_2$-TSL were incubated in presence of MLV in HBS in a thermomixer (1000 rpm) at a certain temperature and time. Subsequently, $DPPG_2$-TSL were separated from MLV by centrifugation at 1600xg (RT, 10 min). In case of repetitive extraction, HePC-containing $DPPG_2$-TSL were exposed to MLV at 37°C for 10 min four times in a row. After each exposure step, MLV were removed by centrifugation at 1600xg (room temperature, 10 min) and the supernatant containing solely $DPPG_2$-TSL-HePC transferred to a new Eppendorf tube. Subsequently, fresh MLV from a concentrated stock were spiked to the supernatant. Small aliquots were taken after each MLV extraction step from the supernatant containing $DPPG_2$-TSL-HePC for lipid quantification. After the fourth MLV extraction, one part of $DPPG_2$-TSL-HePC supernatant was exposed either again to fresh MLV at 37°C for 5 min and another part to fresh MLV at 42°C for 5 min. Samples containing $DPPG_2$-TSL-HePC were diluted with methanol (1:10), the mixture strongly vortexed and incubated in a thermomixer for 20 min (25°C, 1200 rpm). The lipids were quantified by HPLC-CAD as described in section 3.10

3.9 Stability of $DPPG_2$-TSL-HePC in human blood

HePC-containing $DPPG_2$-TSL were diluted to 2.0 mM lipid (1:20) with fresh, heparinized human blood from healthy volunteers. The samples were incubated at 37°C for 5, 30, 60 and 120 min in a thermomixer (1000 rpm). Immediately after the incubation, the samples were centrifuged for 10 min (25°C, 2000xg) to pellet down blood cells. To remove protein-bound HePC, 100 µl of supernatant was transferred on CL-4B mini spin column and centrifuged for 2 min (25°C, 2000xg) resulting in elution of 100 µl TSL. 900 µl methanol was added and the mixture strongly vortexed and incubated in a thermomixer for 20 min (25°C, 1200 rpm). After the incubation, samples were centrifuged for 10 min (25°C, 16000xg) to remove precipitated proteins. The supernatant was transferred into HPLC vials and the lipids quantified by HPLC-CAD as described in section 3.10.

3.10 Quantification of lipids by HPLC-CAD

Samples were analysed for lipid concentration using a Thermo Fisher Scientific Ultimate 3000 HPLC System equipped with a charged aerosol detector (CAD corona Veo). Chromatographic separation was achieved with a XBridge® Phenyl Column (150 mm x 2.1

mm, 3.5 µm, 130 Å) using a gradient elution with increasing amount of organic solvent. Seven calibration standards containing HePC, DPPC and $DPPG_2$ were prepared in methanol yielding a calibration range of 6-375 µg/ml lipid. The injection volume was 10 µl. Linear response was obtained over the whole calibration range with $R^2 > 0.97$ for HePC, $DPPG_2$ and DPPC.

3.11 Assessment of hemolysis

The hematocrit and total hemoglobin of whole blood from a healthy volunteer were determined with a clinical laboratory hematology analyzer (XN-1000™, Sysmex, Kōbe, Japan). Micellar HePC in HBS and $DPPG_2$-TSL-HePC were diluted (1:20) with fresh, heparinized human whole blood to 0.2 mM HePC. HePC-free $DPPG_2$-TSL and physiological saline were used as negative controls. The samples were incubated at 37°C for 5 and 15 min in a thermomixer (600 rpm). Immediately after the incubation, intact blood cells were removed by centrifugation for 10 min (25°C, 2000xg). Cell-free hemoglobin in the supernatant was quantified with a standard spectrophotometer (U-1900, Hitachi High-Technologies, Tokyo, Japan) and a previously described second-derivative fitting method (55). The hemolysis rate was calculated using the whole-blood hematocrit (hct), total hemoglobin (HbT) concentration and cell-free hemoglobin (fHb) according to the following equation (1):

$$hemolysis\ (\%) = \frac{[(100 - hct\ (\%)] * fHb\ (g/L)}{HbT\ (g/L)} \qquad (1)$$

3.12 Pharmacokinetik study (PK) of $DPPG_2$-TSL-HePC

The animal experiments were performed according to protocols approved by the responsible authority (Regierung of Oberbayern, Az. ROB-55.2-2532.Vet_02-18-61). $DPPG_2$-TSL-HePC were intravenously injected at a HePC dose of 10 mg/kg into Male Brown Norway rats (~230 g). Blood samples were collected at different time points in lithium heparin microcuvettes and immediately centrifuged for 10 min at 2000xg. Plasma samples were stored at -20°C until further quantification by LC-MS/MS. HePC and $DPPG_2$ were analysed by LC-MS/MS from the same plasma samples tracking simultaneously HePC (drug) and $DPPG_2$ (liposomal carrier). The value for 100% of the injected dose (ID) was obtained for HePC and $DPPG_2$ based on the calculation of a (weight-based) plasma volume of the rat as described elsewhere (56). Subsequently, $DPPG_2$-TSL-HePC was spiked into rat plasma *ex vivo* at the same concentration as applied in the animal and analysed by LC-MS/MS as PK samples.

To investigate the distribution of HePC in plasma (liposomal HePC versus serum protein-bound HePC), PK samples obtained at 30 min and 60 min time points were measured by LC-MS/MS before and after purification with manually prepared CL-4B columns according to the procedure described in previous sections.

The plasma concentrations of HePC and $DPPG_2$ were fitted using the mono-exponential function (2):

$$c(t) = c(0) * e^{-kt} \quad (2)$$

where $c(t)$ is the HePC and $DPPG_2$ concentrations at time t (min) after i.v. administration and k is the rate constant of elimination. The area under the curve (AUC) was obtained by integration of the equation (1) from 0 min to 120 min.

The half-life t was calculated using the following equation (3):

$$t = \frac{\ln(2)}{k} \quad (3)$$

3.13 Quantification of HePC and $DPPG_2$ from the PK study by LC-MS/MS

For pharmacokinetic studies, HePC and $DPPG_2$ were quantified in rat plasma with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) in electrospray positive mode (ESI+) using deuterated HePC-D4 and 1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) $DHPG_1$ as internal standards, respectively. Calibration standards and quality controls (QC) were prepared in rat plasma by spiking yielding a calibration range of 10 – 500 µg/ml for HePC and 50 – 2500 µg/ml for $DPPG_2$. For cleanup, 20 µl sample (calibrators, QCs, pharmacokinetic samples) were directly pipetted into 980 µl absolute methanol including 0.5 µg/ml HePC-D4 and 8 µg/ml $DHPG_1$ and incubated for 5 min at room temperature on a vortex shaker (Eppendorf, Hamburg, Germany). After centrifugation (20.000xg, 10 min, 4°C), the supernatant was diluted 1:10 with absolute methanol and then loaded to a glass autosampler vial with an insert. Sample analysis was performed with a Waters 2795 Alliance HPLC system that was coupled to a Waters Micromass Quattro Ultima Pt atmospheric pressure ionization tandem Quadrupole system (Waters, Milford, Massachusetts, USA). Analytes were chromatographically separated within 10 minutes on a 60°C preheated Wates C18 SunFire column (100 mm x 2.1 mm, 3.5 µm particle size) using mobile phase A with 10 % methanol and mobile phase B with 2 mM ammonium acetate in 98 % methanol. The injection volume was 15 µl. Starting with 20 % mobile phase B for 0.3 min with a flow rate of 0.5 mL/min (0 – 0.3 min), the amount of mobile phase B was increased to 95 % for the next 3.7 min with a flow rate of 0.6 mL/min (0.3 – 4.0 min) and kept for another 3 min (4.0 – 7.0 min). Finally the column was re-

equilibrated to starting conditons for 3 min with 20% mobile phase B at a flow rate of 0.5 mL/min (7.0 – 10.0 min). Analytes were monitored using the following mass transitions (*m/z*): 408.2 > 124.9 for HePC, 412.3 > 129.0 for HePC-D4, 797.6 > 551.2 for $DPPG_2$ and 751.5 > 579.3 for $DHPG_1$. Linear response was obtained over the whole calibration range with R^2 > 0.99 for both analytes. Intra- and inter-day inaccuracy and imprecision never exceeded 9.1 % for quality control samples with a concentration of 20 and 100 µg/ml HePC and 100 and 1000 µg/ml $DPPG_2$, respectively. Carry-over was negligible with a carry-over rate consistently < 5 % when compared to the lowest calibrator.

4. Results

4.1 Characterization of liposome formulations

Table 1: Characterization of liposome formulations. Values are given as mean of three independently prepared liposome batches.

Liposome	Lipid composition (mol:mol)	z-average (nm)	PDI	ζ-potential (mV)	T_m (°C)
$DPPG_2$-TSL	DPPC:DSPC:$DPPG_2$ (65:5:30)	145 (±2)	0.12 (±0.03)	-25.6 (±2.6)	41.2±0.1
PEG-TSL	DPPC:DSPE-PEG_{2000} (95:5)	132 (±1)	0.06 (±0.01)	-1.4 (±2.5)	41.6±0.1
$DPPG_2$-TSL-HePC	DPPC:DSPC:$DPPG_2$:HePC (55:5:30:10)	138 (±2)	0.10 (±0.01)	-26.7 (±2.3)	40.8±0.1
PEG-TSL-HePC	DPPC:DSPE-PEG_{2000}:HePC (85:5:10)	129 (±1)	0.08 (±0.02)	-2.5 (±0.8)	41.5±0.1
DPPC-TSL-HePC	DPPC:HePC (90:10)	195 (±18)	0.28 (±0.09)	-2.1 (±0.8)	40.4±0.1
DSPC-TSL-HePC	DSPC:HePC (90:10)	260 (±57)	0.49 (±0.15)	-1.2 (±1.7)	52.7±0.1
MLV	DSPC:Cholesterol (55:45)	2865 (±643)	0.74 (±0.21)	-2.7 (±1.5)	not detectable

TSL: thermosensitive liposome, MLV: multilamellar vesicles, PDI: polydispersity index, T_m: transition temperature.

Thermosensitive liposome (TSL) formulations containing $DPPG_2$ and DSPE-PEG_{2000} had a comparable size (z-average) between 130 and 150 nm and narrow size distributions with a polydispersity index (PDI) of approx. 0.1 (Table 1). $DPPG_2$-TSL had a negative ζ-potential due to incorporation of the anionic $DPPG_2$ at a molar ratio of 30%. Removal of surface modification ($DPPG_2$, DSPE-PEG_{2000}) facilitated liposome agglomeration resulting in an increase of z-average and PDI. Transition temperatures (T_m) for all TSL were in the expected size range (~41°C) whereas a higher value was found for DSPC-TSL-HePC (52.7±0.1°C) since it was mainly composed of DSPC (T_m=55°C). Multilamellar vesicles (MLV) had an average size in the µm range and a relatively high PDI since these were not subjected to extrusion. As expected, incorporation of 45 mol% cholesterol abolished the T_m of MLV.

4.2 Temperature-dependent HePC retention in TSL

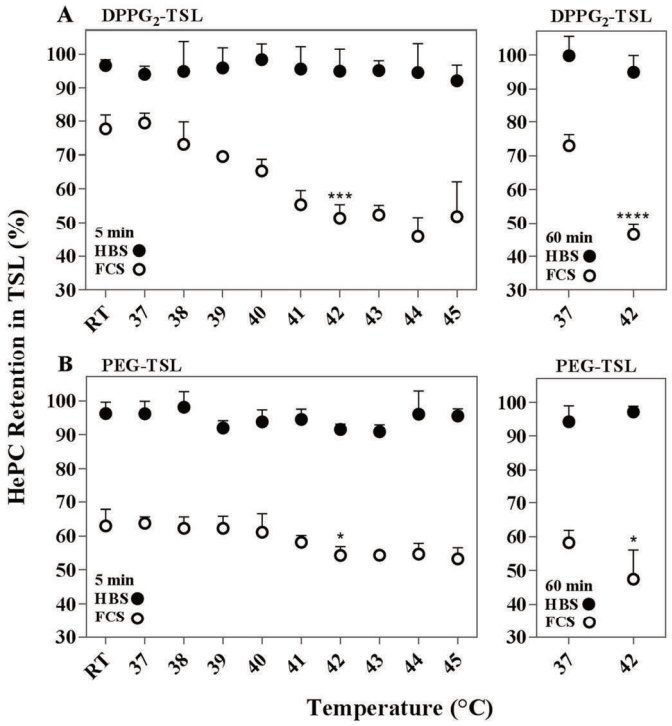

Figure 1. Temperature-dependent HePC retention in $DPPG_2$-TSL and PEG-TSL. $DPPG_2$-TSL-HePC (A) and PEG-TSL-HePC (B) were incubated either for 5 or 60 min at corresponding temperatures in HBS or FCS. Values are given as mean of three independently prepared liposome batches. Values obtained at 37°C and 42°C were compared to RT and analysed using one way ANOVA followed by Dunnet's post-hoc test. *= $p<0.05$, ****= $p<0.0001$.

In all cases, conclusions on the temperature-dependent HePC release were made indirectly by measuring the HePC retention in TSL. Due to various molecular structures formed by the released HePC depending on the incubation medium (e.g. protein-bound, micelles) as well as strong binding to filter materials, common techniques including dialysis and ultrafiltration were not successful in reproducible separation of non-liposomal HePC (data not shown). Centrifugation could not be applied since TSL did not completely sedimentate, even after a prolonged time in a high-speed centrifuge (75000xg, 2 hours). Therefore, we developed a fast method based on size-exclusion with CL-4B mini spin columns which resulted in reproducible elution of liposomal HePC. However, released (non-liposomal) HePC was effectively retained on the CL-4B mini column due to a smaller size of micellar or protein-bound HePC in comparison to TSL.

Both $DPPG_2$-TSL-HePC and PEG-TSL-HePC stably incorporated HePC which was not released in HBS at investigated dilution (1:10) and temperatures ranging from 37-45°C (Figure 1). However, HePC retention decreased immediately at the same dilution in FCS at room temperature (RT) with a significant difference between $DPPG_2$-TSL (78±4%) and PEG-TSL (63±5%). Temperature increase to 37°C did not significantly change the retention values

observed at RT for both formulations. However, HePC retention in $DPPG_2$-TSL was significantly reduced in the mild HT range (42°C) in comparison to 37°C reaching 51±4%. In case of PEG-TSL, the observed decrease in HePC retention at 42°C was less pronounced with 54±3%. Incubation for 60 min barely caused any further decrease in HePC retention for both formulations.

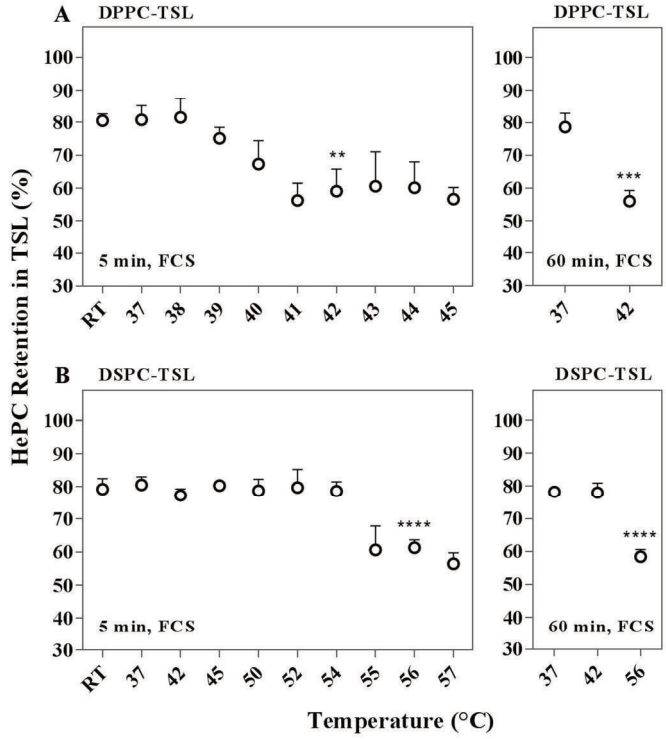

Figure 2: Temperature-dependent HePC retention of DPPC-TSL and DSPC-TSL in FCS. DPPC-TSL-HePC (A) and DSPC-TSL-HePC (B) were incubated either for 5 or 60 min at corresponding temperatures in FCS. Values are given as mean of three independently prepared liposome batches. Values obtained at 37°C, 42°C and 56°C were compared to RT and analysed using one way ANOVA followed by Dunnet's post-hoc test. **= $p<0.005$, ***= $p<0.001$, ****= $p<0.0001$.

To investigate the influence of TSL surface modification ($DPPG_2$ or DSPE-PEG_{2000}), DPPC-TSL were analysed yielding a HePC retention profile similar to $DPPG_2$-TSL. After 5 min in FCS, comparable values were obtained at RT (81±2%) and 37°C (81±4) whereas a stronger decrease in HePC retention followed at 42°C (59±7%) (Figure 2, A). DSPC-TSL were also chosen for the study to confirm the correlation of increased HePC release with T_m (Figure 2, B). In contrast to other TSL formulations, HePC retention did not decrease at 42°C (77±2%) in comparison to RT (79±3%) and 37°C (81±2%). Due to a shift of T_m to higher temperatures, a decrease in HePC retention was observed starting from 55°C (61±7%). Similar to other TSL formulations, incubation for 60 min did not cause a further decrease in HePC retention.

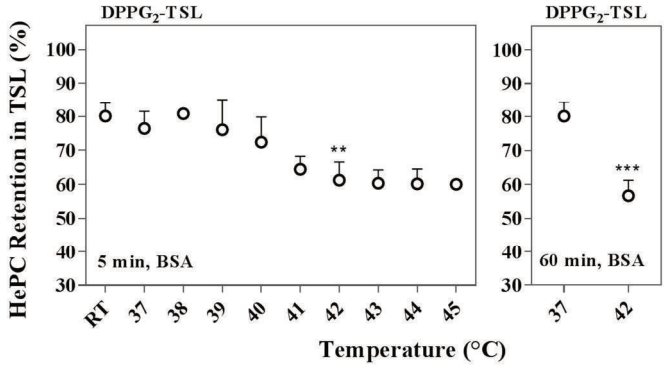

Figure 3: Temperature-dependent HePC retention of $DPPG_2$-TSL in BSA. $DPPG_2$-TSL-HePC were incubated either for 5 or 60 min at corresponding temperatures in BSA (16 mg/ml). Values are given as mean of three independently prepared liposome batches. Values obtained at 37°C and 42°C were compared to RT and analysed using one way ANOVA followed by Dunnet's post-hoc test. ***= p<0.001, **=p<0.005

Due to the most promising HePC release profile (Figure 1, A) and colloidal stability upon storage (Table 1), $DPPG_2$-TSL were chosen for further investigation of HePC release in presence of different media. HePC retention in presence of BSA (16 mg/ml) was comparable to FCS at RT (80±4%), 37°C (76±5%) and 42°C (61±5%) after 5 min (Figure 3). The BSA concentration was chosen based on the albumin concentration reported in the FCS certificate of analysis.

4.3 Temperature-dependent HePC transfer to MLV

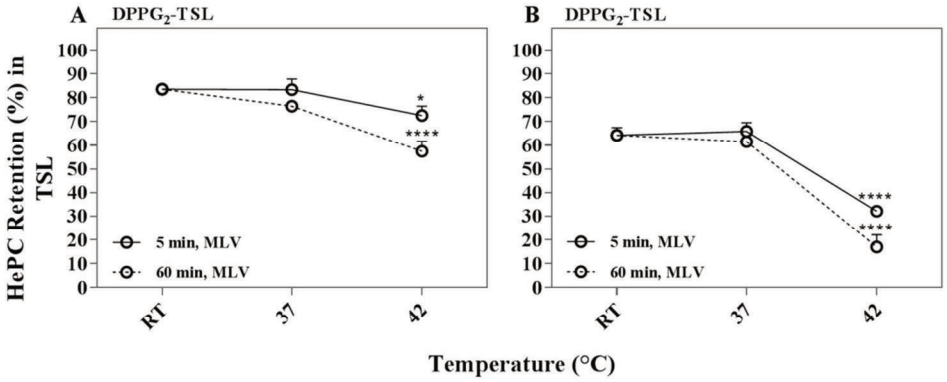

Figure 4: Temperature-dependent HePC retention in $DPPG_2$-TSL in presence of MLV. $DPPG_2$-TSL-HePC (4.0 mM lipid) were incubated either for 5 or 60 min at corresponding temperatures in presence of MLV at 40.0 mM (A) and 200.0 mM (B) lipid concentration. The values were measured on three independently prepared batches. Values obtained at 37°C and 42°C were compared to RT (5 min) and analysed using one way ANOVA followed by Dunnet's post-hoc test. **** = p<0.0001, *=p<0.05.

Multilamellar vesicles (MLV) were previously reported as an accurate *in vitro* assay to predict liposomal retention of lipophilic compounds *in vivo* (53). Therefore, HePC release from $DPPG_2$-TSL was also tested in presence of MLV at two different concentrations (Figure 4). At a molar TSL:MLV lipid ratio of 1:10, HePC retention was comparable between RT (84±2%) and 37°C (83±4%) and slightly decreased at 42°C (73±4%) after 5 min (Figure 4 A).

Prolonged incubation for 60 min caused a further decrease in HePC retention at 37°C (76±2%) and 42°C (57±4%). At a higher molar TSL:MLV lipid ratio of 1:50, HePC retention decreased for all temperatures (Figure 4 B). However, a significant difference between RT (64±3%) and 37°C (66±4%) in comparison to 42°C (32±1%) was maintained after 5 min and became even more pronounced between 37°C (62±1%) and 42°C (17±5%) after 60 min.

4.4 HePC retention and hemolysis in human blood

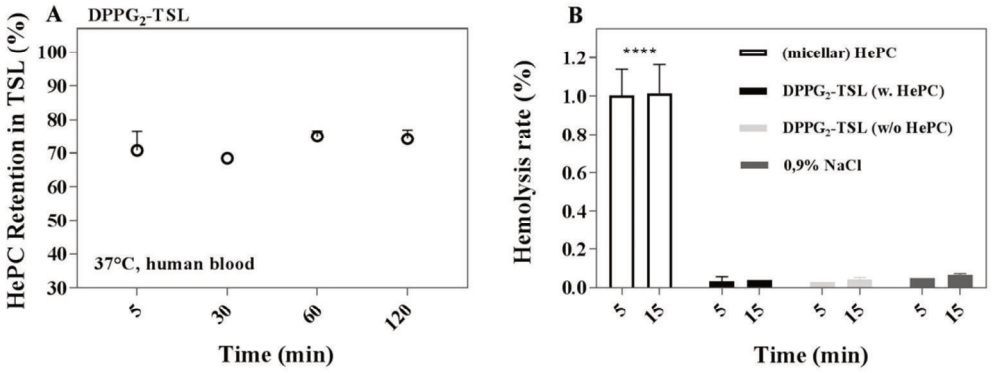

Figure 5: HePC retention and effect on hemolysis in human blood. A: $DPPG_2$-TSL-HePC (2.0 mM) were incubated for 5, 30, 60 and 120 min at 37°C in human blood. Values obtained at 30, 60 and 120 min were compared to 5 min and analysed using one way ANOVA followed by Dunnet's post-hoc test. B: Hemolysis rate (%) was measured for $DPPG_2$-TSL-HePC and (micellar) HePC based at a fixed HePC dosage (0.2 mM) as well as for HePC-free $DPPG_2$-TSL and physiological saline (0.9% NaCl). Values are given as mean of three independent preparations. Values obtained for free (micellar) HePC as well as $DPPG_2$-TSL with and without HePC were compared to 0.9% NaCl and analysed using one way ANOVA followed by Dunnet's post-hoc test. ****= $p<0.0001$.

HePC retention of $DPPG_2$-TSL was investigated at 37°C in heparinized human whole blood for a time period of 2 hours (Figure 5 A). There was no significant decrease in HePC retention (71±6%) after 5 min upon prolonged incubation suggesting a fast initial release of HePC. Interestingly, the hemolysis rate in human blood was not increased after incubation with HePC-containing $DPPG_2$-TSL in comparison to empty $DPPG_2$-TSL or physiological saline (0.9% NaCl) as a background (Figure 5 B). However, non-liposomal (micellar) HePC induced a strong and rapid hemolysis. Hemolysis measurement was performed for 15 min only since a prolonged shaking of human blood resulted in false positive results, probably due to the hemolysis induced by the shear stress upon shaking.

4.5 Pharmacokinetik study (PK) of $DPPG_2$-TSL-HePC

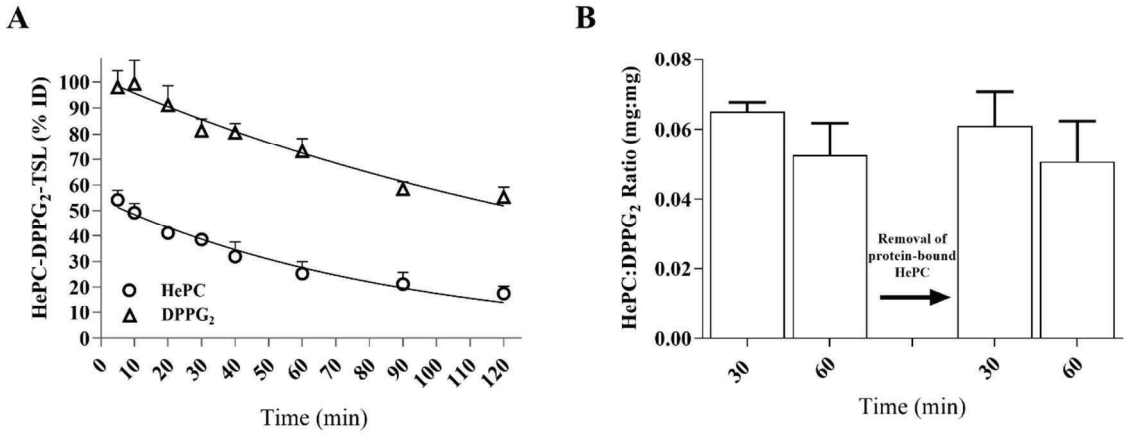

Figure 6: Pharmacokinetic (PK) study of $DPPG_2$-TSL-HePC in Brown Norway rats (n=3). A: $DPPG_2$-TSL-HePC were injected intravenously at a HePC dose of 10 mg/kg. HePC and $DPPG_2$ were quantified from obtained plasma by LC-MS/MS. The values were plotted as percentage of the injected dose (ID) where 100% ID correponds to the expected and *ex vivo* determined concentration of HePC (241.8±5.6 µg/ml) and $DPPG_2$ (1723.9±109.3 µg/ml) in plasma at t=0 (min). $DPPG_2$ and HePC were fitted by mono-exponential functions. B: PK samples obtained at 30 and 60 min were purified with CL-4B columns to remove protein-bound HePC and a HePC:$DPPG_2$ ratio (mg:mg) was obtained before and after purification.

PK analysis of $DPPG_2$-TSL-HePC was performed in Brown Norway rats (Figure 6). HePC in its micellar form was excluded from this investigation since it induced strong hemolysis *in vitro* (Figure 5, B). Approx. 50% of the injected liposomal HePC was cleared within the first 5 min whereas the remaining HePC showed a prolonged circulation time and kinetics comparable to $DPPG_2$ (Figure 6 A, Table 2). Stable association of long-circulating HePC with $DPPG_2$-TSL was confirmed by purification of plasma samples obtained at 30 and 60 min timepoints with CL-4B columns which could efficiently separate protein-bound HePC from liposomal HePC. Interestingly, there was no significant difference before and after plasma purification for the obtained HePC:$DPPG_2$ ratio (mg:mg) indicating that the main HePC fraction in plasma was still incorporated inside $DPPG_2$-TSL whereas a possible serum-protein bound fraction of HePC was not detectable (Figure 6 B).

Table 2: Pharmacokinetic parameters of HePC encapulated in $DPPG_2$-TSL. HePC was fitted using a mono-exponential function. C_0: initial plasma concentration of HePC; t: half-life; R^2: coefficient of determination; AUC: area under the curve.

TSL formulation	C_0(µg/ml)	$AUC_{0\text{-}2h}$ (µg*h/ml)	t(min)	R^2
$DPPG_2$-TSL-**HePC**	131.4	66.6	61.2	0.9128

4.6 Simulation of the PK study by repetitive exposure of $DPPG_2$-TSL-HePC to MLV

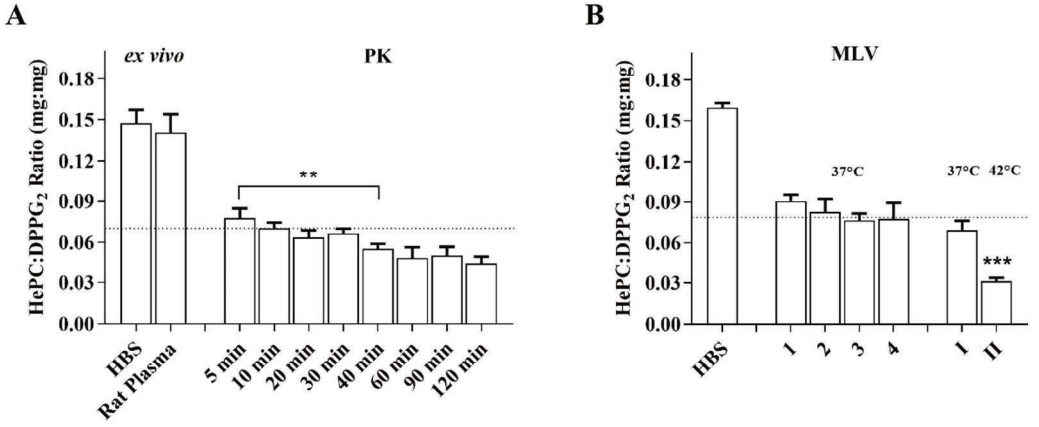

Figure 7: *In vitro* simulation of the PK study by the repetitive exposure of $DPPG_2$-TSL-HePC to MLV. A: Alternative representation of the results from the PK study, shown as a HePC:$DPPG_2$ ratio (mg:mg) B: $DPPG_2$-TSL-HePC (1.0 mM lipid) were repeatedly (4x) incubated with MLV (50.0 mM lipid) at 37°C for 10 min each. For the subsequent 5th incubation, $DPPG_2$-TSL-HePC were exposed either to MLV again at 37° (I) or 42°C (II) for 5 min each. The dashed line is shown for visual purpose to indicate the HePC:$DPPG_2$ ratio, corresponding to 50% of liposomal HePC. The values were analysed using one way ANOVA followed by Dunnet's post-hoc test. **= $p<0.005$, ***= $p<0.0005$.

To simulate HePC retention in $DPPG_2$-TSL observed in the PK study (Figure 6-7, A), we exposed $DPPG_2$-TSL-HePC to subsequent extraction cycles with MLV at 37°C for 10 min each (Figure 7, B). By these means, released HePC is removed with MLV after each extraction cycle simulating the sink conditions as known from the situation *in vivo* where an infinite amount of biological acceptors is present and released drug is constantly cleared from the blood stream. After the first MLV extraction, HePC retention was reduced immediately to approx. 50% of liposomal HePC and barely decreased after subsequent three extractions (Figure 7, B). In agreement with previous results obtained in FCS and in presence of BSA, heating to 42°C in the fifth extraction step resulted again in significantly reduced HePC retention in contrast to 37°C after 5 min (Figure 7, B). It is noteworthy that HePC release does not stop at 37°C after the extraction of the first 50% HePC but rather drastically slows down as can be observed for a slow decrease of HePC:$DPPG_2$ ratio (5 min versus ≥ 40 min) in the PK study (Figure 7, A).

5. Discussion

We previously reported that $DPPG_2$-TSL showed a hyperthermia- (HT-) induced transfer of HePC into two different cancer cell lines, thereby having a substantial cytotoxic effect (52). The aim of this study was to gain understanding in the release mechanism of HePC from TSL and to investigate the potential for future preclinical drug delivery studies.

HePC release from TSL was only possible in presence of serum, bovine serum albumin (BSA) and multilamellar vesicles (MLV) acting as suitable acceptors. BSA was equally

effective as fetal calf serum (FCS) in releasing HePC, confirming a possible role of this protein for HePC extraction and transport *in vivo*. HePC as well as structurally similar lysolipids are known to bind and to be transported by albumin *in vivo* (42, 57-59). However, we showed previously that human serum albumin (HSA) has a weak binding to $DPPG_2$-TSL *in vitro* and is rapidly detached from the liposomal surface upon addition of FCS (60). Therefore, we can not exclude that the interaction between $DPPG_2$-TSL and albumin (HSA or BSA) is rather artificial and does only take place when other serum components are not present. Interestingly, BSA increased HePC release around T_m of $DPPG_2$-TSL. Previously, we observed that HSA is capable to increase the permeability of $DPPG_2$-TSL around T_m and induce the release of hydrophilic compounds, suggesting a possible interaction with the liquid-disordered lipid phase of $DPPG_2$-TSL (49).

MLV were proposed to more accurately predict retention of bilayer-deposited liposomal drugs *in vivo* (53). Therefore, we also investigated the release of HePC in presence of MLV, composed of DSPC and Cholesterol. Although this lipid composition can not fully represent a biomembrane, we could confirm a temperature-dependent transfer of HePC from $DPPG_2$-TSL to MLV. On average, HePC release from $DPPG_2$-TSL increased by a factor of two upon heating to T_m (42°C) in presence of MLV, FCS or BSA. This is in agreement with our previous results where also a two times higher HePC uptake was detected in BFS-1 fibrosarcoma and C6 glioma cells *in vitro* after incubation with $DPPG_2$-TSL at T_m (42°C) in comparison to 37°C (52).

To further investigate the potential of $DPPG_2$-TSL-HePC as a carrier for the local drug delivery, we measured HePC retention in $DPPG_2$-TSL at 37°C in (human) blood *in vitro* and determined the hemolysis rate. The concentration of $DPPG_2$-TSL-HePC was the same as used in a later pharmacokinetic experiment. Although full blood contains both serum proteins and blood cells as possible HePC acceptors, the release of HePC at 37°C stayed comparable to FCS, BSA or in presence of MLV. Surprisingly, we did not observe an increase in hemolysis rate in comparison to background (physiological saline) or HePC-free $DPPG_2$-TSL. If the extent of HePC release is in the first place mediated by serum proteins, than released HePC is probably strongly serum protein-bound and subsequently not or only slowly transferred to blood cells. This is supported by a comparable HePC release in FCS at 37°C which only slightly increased in full blood despite the presence of blood cells. However, free (micellar) HePC at the same dosage resulted in strong hemolysis. High HePC concentrations at the injection site could saturate the local binding capacity of serum proteins, leaving the unbound

HePC available for blood cell interaction. This could lead to an uptake of a certain amount of HePC by red blood cells and consequently to hemolysis.

An improved safety profile with regards to reduction in hemolysis provided a possibility for *in vivo* study of $DPPG_2$-TSL-HePC. The observed instability of liposomal HePC at 37°C *in vitro* was confirmed *in vivo* with the pharmacokinetics (PK) of $DPPG_2$-TSL-HePC. Within 5 min after injection, nearly 50% HePC was extracted from TSL and cleared from circulation. Interestingly, the remaining 50% HePC showed a prolonged circulation time following a comparable kinetics to $DPPG_2$-TSL. We speculated that the remaining 50% of HePC is retained with the $DPPG_2$-TSL and is slowly cleared within the liposomes by liver and spleen. The *in vitro* simulation of the PK experiment was carried out by subsequent extraction cycles with MLV at 37°C confirming the observation that HePC extraction from $DPPG_2$-TSL seems to stop or significantly slow down after the release of approx. 50% HePC.

The most likely explanation for the rapid initial clearance of 50% HePC is the location in the liposome bilayer. Based on the preparation method of $DPPG_2$-TSL-HePC and the size of approx. 140 nm, we would expect an equal distribution of HePC between the two leaflets of $DPPG_2$-TSL. In this case, it would mean that the rapidly extracted HePC fraction *in vivo* is likely the one located in the external leaflet of $DPPG_2$-TSL, whereas the long-circulating HePC fraction located in the internal leaflet is stably retained inside the liposome.

Previously, it was shown that detergents can exhibit either a fast or slow trans-bilayer movement (flip-flop) in lipid bilayers (61-63). The difference in flip flop kinetics between surfactants was largely attributed to the molecular structure. The detergents with a large polar (dodecylmaltoside) or (zwitter-)-ionic headgroup (lysophosphocholines (LPC), sodium dodecyl sulfate) show a slow flip-flop whereas those with a relatively small or uncharged headgroup can equilibrate rapidly between the lipid leaflets (Triton X-100) (61-64).

Surprisingly, no data are available for the flip-flop rates of HePC (or other alkylphosphocholines) despite their biological importance with cell membranes as a proposed pharmacological target. However, a careful look on the structure of HePC reveals a strong similarity to the better-investigated LPC (Figure 8).

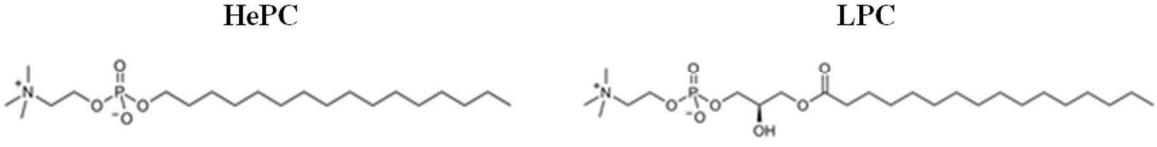

Figure 8: Structures of HePC (Miltefosine) and a lysophosphocholine (LPC).

Previously, 16:0 and 18:0 LPC were shown to be extracted by albumin or MLV only from the outer leaflet of liposomes (65, 66). This was explained by a slow flip-flop which was estimated by nuclear magnetic resonance (NMR) to have a half-life of more than 12 hours for 16:0 PC (65). Supported by the MLV transfer experiments and PK results in this study as well as findings reported for structurally similar LPC, we speculate that HePC also shows a slow flip-flop in a $DPPG_2$-TSL bilayer at 37°C. Therefore, after the initial rapid release of HePC from the external leaflet of $DPPG_2$-TSL, the release will become rate limited by the trans-bilayer movement of HePC from the internal to the external leaflet (Figure 9). However, we could demonstrate that the remaining HePC fraction located in the internal leaflet of $DPPG_2$-TSL can be still released and rapidly transferred to MLV at 42°C. This suggests a drastically accelerated flip-flop at $\geq T_m$ in contrast to 37°C. Previously, several groups reported a strongly increased flip-flop of DPPC and DMPC lipids at the T_m of liposomes (67, 68).

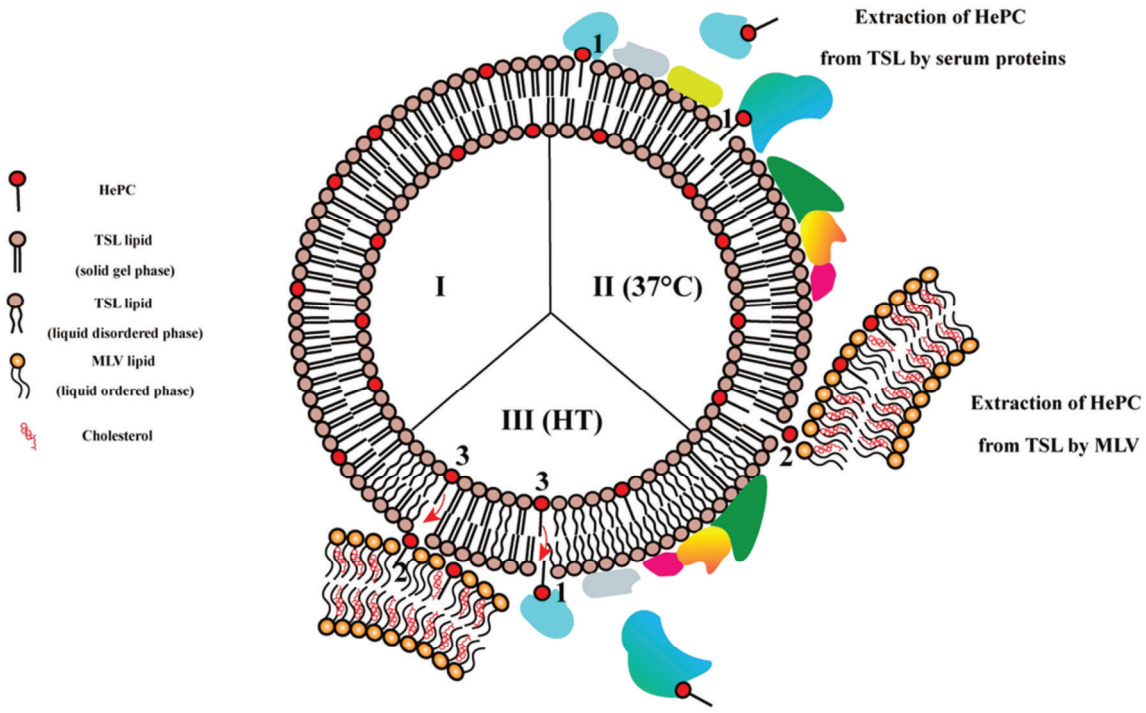

Figure 9: Schematic representation of hypothetical release mechanism of HePC from $DPPG_2$-TSL. I: HePC is distributed equally between both leaflets due to the preparation method and size of $DPPG_2$-TSL-HePC (~ 140 nm). II: In presence of sufficient amount of biological acceptors (serum proteins, MLV), HePC is rapidly extracted below T_m (37°C) from the outer leaflet of $DPPG_2$-TSL. On the contrary, HePC in the inner leaflet is retained for a prolonged time period (hours) due to a slow flip-flop from the inner to the outer leaflet. III: Upon heating to T_m (HT), structural defects are formed between solid and liquid phases which facilitate a trans-bilayer movement of HePC from the inner to the outer leaflet leading to the additional HePC release at HT.

In conclusion, we could demonstrate that HePC release from ($DPPG_2$)-TSL is mediated by various acceptors like serum proteins or (lipid) membranes. We could confirm an increased release of HePC at T_m, previously reported in studies with cancer cell lines *in vitro* (52).

However, we also observed a rapid initial loss of maximally 50% HePC from $DPPG_2$-TSL in circulation or in presence of MLV. In future, we want to investigate if ($DPPG_2$)-TSL with HePC located only in the internal leaflet will improve the drug retention in presence of biological media. However, the success will strongly depend on how fast HePC will re-equilibrate between both leaflets and if storage conditions can be found that drastically reduce the flip-flop. Despite an initial HePC loss from $DPPG_2$-TSL *in vivo*, there is a chance for a local intravascular and HT-induced release of the remaining, long-circulating HePC from the internal leaflet of $DPPG_2$-TSL. However, a subsequent HePC accumulation inside a solid tumor might be reduced by a strong binding of a serum protein (e.g. albumin) and fast wash-out of the protein-bound HePC from the heated vessels. Surface-modified or locally injected TSL designed to bind selectively tumor or endothelial cells could provide a direct transfer of HePC upon HT trigger hereby avoding the undesirable serum protein binding.

6. Acknowledgment

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7. Conflict of interest

Lars H. Lindner and Martin Hossann hold shares of Thermosome GmbH, Planegg/Martinsried, Germany. All other authors declare no conflict of interest.

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CHAPTER 3: Usage of suitable prodrugs or excipients enables heat-triggered release of dexamethasone from thermosensitive liposomes

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Note:

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1. Abstract

Dexamethasone (DXM) is a potent glucocorticoid with an anti-inflammatory and anti-angiogenic activity which is clinically used for treatment of rheumatoid arthritis (RA) and shows potential as a tumor growth inhibitor. Severe side effects limit the long-term use of DXM in patients requiring formulations which deliver and selectively release the drug at pathological sites. This *in vitro* study compares the suitability of DXM and commonly used prodrugs dexamethasone-21-phosphate (DXMP) and dexamethasone-21-palmitate (DP) as well as DXM complexed by 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD) for the use in thermosensitive liposomes (TSL). DXM showed a poor retention and a low final drug:lipid ratio in a 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol-based TSL ($DPPG_2$-TSL) and a low-temperature sensitive liposome (LTSL). In contrast to DXM, DXMP and DP were stably retained at 37°C in TSL in serum and could be encapsulated with high drug:lipid ratios in $DPPG_2$-TSL and LTSL. DXMP showed a rapid release at mild hyperthermia (HT) from both TSL in serum, whereas DP remained incorporated in the TSL bilayer. Increasing DP:lipid ratio lowered the transition temperature (T_m) of both TSL formulations. According to release experiments with carboxyfluorescein (CF), HP-γ-CD and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) are suitable vehicles for the loading of DXM into $DPPG_2$-TSL and LTSL. Complexation of DXM with HP-γ-CD increased the aqueous solubility of the drug leading to approx. ten times higher DXM:lipid ratio in $DPPG_2$-TSL and LTSL in comparison to un-complexed DXM. Both DXM and HP-γ-CD showed increased release at HT in comparison to 37°C in serum. In conclusion, DXMP and DXM complexed by HP-γ-CD represent promising candidates for TSL delivery.

2. Introduction

Liposomes are currently the most successful nano-scale drug delivery systems which have been translated into several clinically-used products [1]. As spherical bilayers composed of phospholipids, liposomes are biocompatible and are capable to encapsulate a variety of compounds including small and macromolecule pharmaceutics [2]. Consequently, liposomal drugs benefit from improved aqueous solubility, prolonged pharmacokinetics and reduced systemic toxicity [3]. The enhanced permeability and retention (EPR) effect facilitates accumulation of liposomes in tumor and inflamed tissues which are characterized by the leaky vasculature [4, 5]. However, drug release does not take place until the extravasated liposomes are disintegrated in the target tissue [6, 7]. In particular cases, the liposomal drug escape was shown to be slow leading to the low bioavailability and compromising the therapeutic efficacy [8, 9]. Additionally, an efficient extravasation of nanoparticles into the tumor interstitium is reduced with increasing particle size leading to penetration of only perivascular tumor areas [10, 11]. Furthermore, the EPR effect is in general not as pronounced in humans as in fast-growing tumors in rodents [12, 13].

Therefore, nanoparticles which can ensure a triggered drug release specifically at the disease site hold great potential to circumvent the above mentioned limitations of common nanomedicines [14]. Thermosensitive liposomes (TSL) show intravascular drug release triggered by mild hyperthermia (HT) (41-43°C) [15]. In comparison to free drugs and non–thermosensitive liposomes, TSL increased the bioavailability and penetration depth of drugs within solid tumors, ultimately leading to an improved therapeutic efficacy [16-19]. So far, the TSL concept has been mainly investigated for drugs which can be encapsulated in the aqueous core of liposomes [20]. However, a few studies also reported a successful incorporation of lipophilic drugs in a TSL bilayer without compromising the thermosensitivity of the formulation [21-24]. An extensive investigation of TSL is particularly important for the poor water-soluble drugs as their number has strongly increased in recent years leading to serious challenges in formulation development.

Glucocorticoids (GC) are potent medicines with anti-inflammatory and immunosuppressive properties [25-27]. GC are clinically used for the treatment of several inflammatory disorders including rheumatoid arthritis (RA). However, GC have a number of severe systemic side effects, including diabetes, hypertension and osteoporosis limiting their long-term application [28, 29]. Liposomal encapsulation of GC decreased the systemic toxicity and significantly prolonged the pharmakokinetics resulting in a passive accumulation of liposomes in inflamed

joints [30]. Consequently, liposomal GC demonstrated a strong anti-arthritic effect at a drastically lower dose in comparison to free drugs [31, 32]. Interestingly, liposomal GC were also shown to be beneficial in treatment of solid tumors leading to a tumor growth delay, presumambly by a downregulation of the angiogenesis [33, 34]. However, a passive accumulation of liposomes via EPR usually leads to a low delivery efficiency in target tissue with a considerable amount of the administered dose ultimately taken up by the reticulo-endothelial system (RES) [35, 36]. To increase a local concentration of GC in inflamed joints, the intra-articular injection (IAI) is applied, but it bears the risk of skin atrophy and infection [37, 38]. We believe that GC delivery via TSL might be a potential alternative to IAI and would increase the intra-articular drug concentrations in comparison to non-thermosensitive liposomes. Similarly, a HT-mediated intravascular release of GC close to a solid tumor could potentially lead to a facilitated drug uptake in tumor tissue, enhanced bioavailability and ultimately improved therapeutic response in comparison to EPR-based delivery systems.

The objective of this study was to develop a TSL formulation which can stably retain a GC at body temperature but rapidly release the drug at elevated temperatures in the HT range (41-43°C). Therefore, we selected dexamethasone (DXM) as a clinically used GC and analysed its stability and release behavior in a low temperature-sensitive liposome (LTSL) [39, 40] and 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol ($DPPG_2$)-based TSL [41-43]. We extended our study to the commonly-used prodrugs of DXM: a hydrophilic dexamethasone-21-phosphate (DXMP) [33] as well as a lipophilic dexamethasone-21-palmitate (DP) [44]. Alternatively to the prodrug concept, we investigated if complexation of DXM by a cyclodextrin (CD), generally known as drug-in-cyclodextrin-in-liposome (DCL) concept [45, 46], can be also successfully applied in TSL formulations. By these means, we identified the most promising strategies for DXM delivery in TSL and laid the foundations for future pre-clinical studies.

3. Materials and Methods

3.1 Chemicals

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC) were purchased from Corden Pharma (Liestal, Switzerland). $DPPG_2$ was provided by Thermosome (Munich, Germany). The ammonium salt of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG_{2000}) was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Dexamethasone (DXM) and

dexamethasone-21-phosphate disodium salt (DXMP) were purchased from TCI Chemicals (Japan) and dexamethasone-21-palmitate (DP) from Carbosynth (Oxford, UK). Prednisolone (PRN), 5(6)-carboxyfluorescein (CF), methyl-β-cyclodextrin (Me-β-CD) and 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD) were purchased from Sigma Aldrich GmbH (Munich, Germany). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) was from AppliChem (Darmstadt, Germany). Fetal calf serum (FCS) was from Biochrom AG (Berlin, Germany). Sepharose CL-4B and Sephadex G-25 M (PD-10 columns) were obtained from GE-Healthcare (Chicago, USA). TLC Silica gel 60 glass plates (10x20 cm) and the phosphate standard solution (1000 mg/ml) were from Merck KGaA (Darmstadt, Germany). Aluminium pans (standard crucible with lid, 40 µl) were from IVA Analysentechnik GmbH & Co. KG (Germany). The Optimole osmolarity standards (100, 290 and 1000 mmol/kg) were obtained from Wescor Inc. (Utah, USA). All other chemicals were either from Carl Roth GmbH (Karlsruhe, Germany) or Sigma Aldrich GmbH (Munich, Germany).

3.2 Preparation of TSL

TSL were prepared by the lipid hydration and extrusion method. TSL were composed either of DPPC:DSPC:$DPPG_2$ ($DPPG_2$-TSL) or DPPC:MSPC:DSPE-PEG_{2000} (LTSL) in a molar ratio of 50:20:30 or 90:10:4, respectively.

3.2.1 Dexamethasone (DXM) and dexamethasone-21-palmitate (DP)

Lipids and drugs (DXM or DP) were dissolved separately in chloroform:methanol (9:1, vol:vol) and methanol, respectively. Drug- and lipid-containing solutions were combined in a round-bottomed flask in a molar drug:lipid ratio of 0.05 (DXM) and 0.05 or 0.10 (DP), respectively. The drug-containing lipid film was formed under reduced pressure using a rotary evaporator. The lipid film was hydrated for 30 min at 60°C with HEPES-buffered saline (HBS) (20 mM HEPES, 150 mM NaCl, pH=7.4) to obtain multilamellar vesicles (MLV) at 50 mM lipid concentration. MLV were extruded 10 times through two 200 nm pore size membranes (Whatman, Nuclepore Track-Etch Membrane) using a thermobarrel extruder at 60 °C (Northern Lipids, Vancouver, Canada). Non-encapsulated drugs were removed by size-exclusion with Sephadex G-25 (DXM) or CL-4B Sepharose columns (DP), pre-equilibrated with HBS.

3.2.2 Dexamethasone-21-phosphate disodium salt (DXMP) and sodium carboxyfluorescein (CF)

Lipids were dissolved separately in chloroform:methanol (9:1, vol:vol) and combined in a round-bottomed flask. The lipid film was formed under reduced pressure using a rotary evaporator. CF and DXMP were passively loaded inside TSL during lipid film hydration. For CF-TSL, the lipid film (LTSL or $DPPG_2$-TSL) was hydrated with an aqueous solution of CF (100 mM, pH=7.4). For DXMP-TSL, the lipid film was hydrated with an aqueous solution of DXMP (100 mM, pH=7.4) for $DPPG_2$-TSL whereas an aqueous solution of DXMP (20 mM, pH=7.4), adjusted to physiological osmolarity with NaCl, was used for LTSL. The DXMP concentration was reduced to 20 mM in case of LTSL since no liposome formation was detected after extrusion in presence of 100 mM DXMP in contrast to $DPPG_2$-TSL. In all cases, the lipid films were hydrated for 30 min at 60°C to obtain MLV at 50 mM lipid concentration. DXMP or CF-containing MLV were extruded and purified from non-encapsulated DXMP or CF as described in the previous section for DXM.

3.2.3 DXM complexed by 2-hydroxypropyl-γ-cyclodextrin (DXM:HP-γ-CD)

DXM was complexed by HP-γ-CD in a similar procedure as described for Betamethasone elsewhere [47]. DXM (98 mM) was dissolved in aqueous solution of 2-Hydroxypropyl-γ-cyclodextrin (HP-γ-CD) (226 mM) under strong vortexing and subsequent shaking in a thermomixer for 2 hours (25°C, 1000 rpm). If required, the obtained solution can be centrifuged for 10 min (16000xg, 25°C) to remove the insoluble and non-complexed DXM. However, DXM was completely dissolved in the aqueous HP-γ-CD solution at used concentrations. Immediately after preparation, the water-soluble DXM:HP-γ-CD complex was passively loaded inside TSL during lipid film hydration. Therefore, the lipid film (LTSL or $DPPG_2$-TSL) was hydrated with an aqueous solution of DXM:HP-γ-CD (98:226 mM) at 60°C for 30 min to obtain MLV at 50 mM lipid concentration. Subsequently, MLV were extruded and the non-encapsulated DXM:HP-γ-CD was removed from TSL in a two step purification. The first step involved a purification with Sephadex G-25 columns as described in the previous section for DXM. The second step was performed by centrifugation in a high-speed centrifuge (60 min, 70000xg, 15°C) after dilution of 1.0 ml of DXM:HP-γ-CD-TSL (approx. 30-35 mM lipid) to 50 ml with HBS. The supernatant containing a non-encapsulated DXM:HP-y-CD complex was discarded and the liposomal pellet resuspended in 0.5 ml HBS under shaking in a thermomixer (20 min, 25°C, 450 rpm).

3.2.4 Preparation of multilamellar vesicles (MLV)

MLV composed of DSPC and Cholesterol (55:45 mol:mol) were prepared as described elsewhere [48]. Corresponding amounts of lipids were dissolved in chloroform:methanol (9:1, vol:vol) and combined in a round-bottomed flask. The lipid film was formed under reduced pressure using a rotary evaporator and hydrated with 300 mM sucrose to yield MLV at 100 mM lipid concentration. Subsequently, the mixture was centrifuged for 10 min (25°C, 1600xg). After centrifugation, MLV were concentrated as a layer in the upper phase. The bottom phase containing sucrose was removed using a 21G needle syringe. Afterwards, MLV were resuspended in HBS and centrifuged again for 10 min (25°C, 1600xg) forming a MLV pellet. The supernatant was discarded and MLV resuspended again in HBS. The washing procedure of MLV with HBS was repeated three times. MLV were finally resuspended in a certain volume of HBS resulting in 200 mM MLV.

3.3 Osmolarity measurement

To avoid any osmotic-driven effects on drug release, all drug-containing solutions used for lipid film hydrations as well as release media (HBS, physiological saline, HP-β-CD and HP-γ-CD containing solutions) were adjusted to physiological osmolarity with NaCl. Osmolarity was measured in a vapor pressure osmometer (Vapro 5600). Before each measurement, the osmometer was calibrated with three standard solutions with osmolarities of 100, 290 and 1000 mOsm/L.

3.4 Dynamic light scattering (DLS)

Z-average, polydispersity index (PDI) and ζ-potential of the final TSL formulations were measured by DLS using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). TSL (0.5 mM) were diluted in physiological saline prior to DLS measurements.

3.5 Differential scanning calorimetry (DSC)

The transition temperature of TSL formulations was determined by DSC. Liposome suspensions (20 µl, 30 mM) were transfered into aluminium pans, closed and measured on Mettler Toledo DSC 821e (Mettler Toledo, Giessen, Germany). The samples were scanned from 20°C to 60°C at a heating rate of 2K/min.

3.6 Measurement of lipid concentration (phosphate assay)

The lipid concentration was determined by the phosphate assay as described in detail elsewhere [49]. In brief, TSL formulations were diluted with distilled water, sulfuric- and perchloric acid containing solutions were added and the samples heated for 2 hours at 300°C.

Subsequently, ammonium heptamolybdate was added and the formed complex measured at 660 nm in a spectrophotometer (Beckmann DU 640, Beckman Coulter GmbH, Krefeld, Germany). The quantification was performed based on the standard line obtained from 1 g/l phosphate solution that was treated the same way like TSL samples.

3.7 Measurement of lipid composition (thin layer chromatography)

Thin layer chromatograpy (TLC) was used to confirm the lipid composition of the final TSL formulations. In brief, TSL (1500 nmol) were diluted in 1 ml physiological saline and 2 ml chloroform:methanol (1:1, vol:vol) was added. After short vortexing, the mixture was centrifuged for 10 min (25°C, 3200xg). The chloroform phase containing the lipids was transferred to a new tube and dried under nitrogen stream (40°C). The dried lipids were redissolved in 100 µl of chloroform:methanol (9:1 vol:vol) and 1.5 µl were spotted on a TLC plate. The mobile phase was composed of chlorofom/methanol/acetic acid/H_2O (100:60:10:5, vol:vol). A standard solution with corresponding lipids was used for the lipid spot identification. After the run, the lipids (MSPC, DPPC/DSPC, $DPPG_2$ and DSPE-PEG_{2000}) were visualized as separate blue spots after staining with molybdenum spray of Dittmer and Lester [50]. The intensity of the lipid spots represented the relative lipid composition of each formulation and was analyzed densitometrically with ImageJ.

3.8 Temperature-dependent drug retention in TSL

Drug-containing TSL were diluted to 3.0 mM lipid (1:10) with either HBS or fetal calf serum (FCS). 120 µl samples were distributed in Eppendorf tubes and incubated at a certain temperature in a thermomixer (750 rpm). Immediately after the incubation, the samples were put on ice for 5 min. To remove the released (free or protein-bound) drug, TSL samples were purified by size-exclusion with manually prepared spin columns filled with Sepharose CL-4B. Before use, the columns were washed three-times with HBS. 100 µl TSL sample was transferred on CL-4B mini spin column and centrifuged for 2 min (25°C, 2000xg) resulting in elution of 100 µl (drug-containing) TSL. Drug retention in TSL was quantified either by HPLC-UV (for DXM and DXMP) or HPLC-CAD (for DP) after extraction as in detail described in the next sections (3.9-3.11).

For DP, the temperature dependent retention in TSL was also investigated in presence of multilamellar vesicles (MLV). $DPPG_2$-TSL-DP and LTSL-DP (3.0 mM lipid) were incubated in presence of MLV (150 mM lipid) for 60 min in HBS in a thermomixer (1000 rpm) at a certain temperature. Subsequently, TSL-DP were separated from MLV by centrifugation at

1600xg (RT, 10 min). DP retention in TSL was quantified by HPLC-CAD after extraction as in detail described in section 3.10.

3.9 Extraction and quantification of DXM by HPLC-UV

a). $DPPG_2$-TSL and LTSL containing DXM

Prednisolone (PRN) was used as an internal standard (IS) for DXM quantification. Seven calibration (CAL) standards (100 µl) were prepared in HBS or FCS yielding a calibration range of 4-250 µg/ml for DXM or PRN. The IS-solution with a concentration of 300 µg/ml PRN was prepared in methanol. TSL samples (100 µl) purified by CL-4B mini columns were spiked with 10 µl of IS-solution (300 µg/ml PRN in methanol). Subsequently, 900 µl methanol was added to CAL and TSL samples, the mixtures strongly vortexed and incubated in a thermomixer for 20 min (25°C, 1200 rpm). After centrifugation for 10 min (RT, 16000xg), supernatants were transferred to glass tubes to be dried under nitrogen stream at 60°C. Dried samples (containing lipids and drugs) were first redissolved under strong vortexing in 100 µl methanol. Afterwards, 900 µl of mobile phase composed of acetonitril/H_2O (25:75 v:v) was added, samples again vortexed and transferred to Eppendorf tubes. After centrifugation for 10 min (RT, 16000xg), supernatants were transferred to vials and analysed by HPLC equipped with UV detector (242 nm). The injection volume was 200 µl. Linear response was obtained over the whole calibration range with $R^2 > 0.99$ for both DXM and PRN. The run was performed isocratically on a Kinetex C18 column (2.6 µm, 100Å, 100x3 mm) operated at 30°C with 0.5 ml/min flow. The retention times for PRN and DXM were 6 and 10 min, respectively.

b). $DPPG_2$-TSL and LTSL containing DXM complexed by HP-γ-CD

Sample extraction and DXM quantification by HPLC-UV was performed the same way as described before except the concentration changes resulting from approx. 10x higher encapsulation of DXM in HP-γ-CD-containing TSL. Seven calibration (CAL) standards (100 µl) were prepared in methanol or FCS yielding a calibration range of 15-1000 µg/ml for DXM or PRN. TSL samples (100 µl) purified by CL-4B mini columns were spiked with 10 µl of IS-solution (2500 µg/ml PRN in methanol). The injection volume was 50 µl. Linear response was obtained over the whole calibration range with $R^2 > 0.99$ for both DXM and PRN.

3.10 Extraction and quantification of DP by HPLC-CAD

DP was quantified in relation to the DPPC lipid, already present in both $DPPG_2$-TSL and LTSL formulations. Seven calibration (CAL) standards (100 µl) were prepared in methanol

yielding a calibration range of 20-1000 µg/ml for DP or 60-3750 µg/ml DPPC. 1900 µl methanol to DP-containing TSL samples (100 µl), the mixtures strongly vortexed and incubated in a thermomixer for 20 min (25°C, 1200 rpm). After centrifugation for 10 min (RT, 16000xg), supernatants were transferred to glass tubes to be dried under nitrogen stream at 60°C. Dried samples were redissolved under strong vortexing in 500 µl methanol and transferred to eppendorf tubes. After centrifugation for 10 min (RT, 16000xg), supernatants were analysed by HPLC equipped with a charged aerosol detector (CAD corona Veo). Chromatographic separation of DP and DPPC was achieved with a XBridge® Phenyl Column (150 mm x 2.1 mm, 3.5 µm, 130 Å) using a gradient elution with increasing amount of organic solvent. The injection volume was 10 µl. Retention times for DP and DPPC were 17.8 and 19.9 min, respectively. Linear response was obtained over the whole calibration range with $R^2 > 0.99$ for both DP and DPPC.

3.11 Extraction and quantification of DXMP by HPLC-UV

Six calibration (CAL) standards (100 µl) were prepared in HBS or FCS yielding a calibration range of 15-500 µg/ml for DXMP. CAL and TSL samples (100 µl) purified by CL-4B mini columns were combined with 900 µl methanol, the mixtures strongly vortexed and incubated in a thermomixer for 20 min (25°C, 1200 rpm). After centrifugation for 10 min (RT, 16000xg), supernatants were transferred to glass tubes to be dried under nitrogen stream at 60°C. Dried samples were redissolved under strong vortexing in 1 ml (DXMP-$DPPG_2$-TSL) or 200 µl (DXMP-LTSL) of mobile phase composed of phosphate-citrate buffer (McIlvaine-Buffer, 100 mM, pH=2.8):methanol (70:30 vol:vol) and transferred to Eppendorf tubes. After centrifugation for 10 min (RT, 16000xg), supernatants were transferred to vials and analysed by HPLC equipped with UV detector (242 nm). The run was performed isocratically on a Partisil 10 SAX column (Whatman, 4.6x250 mm) operated at 30°C with 1,5 ml/min flow. The injection volume was 100 µl. The retention time for DXMP was 7 min. Linear response was obtained over the whole calibration range with $R^2 > 0.99$.

3.12 Extraction and quantification of HP-γ-CD by UHPLC-MS/MS

HP-γ-CD was quantified by ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) in electrospray positive mode (ESI+) using methyl-β-cyclodextrin (Me-ß-CD) as internal standard (IS). Stock solutions of HP-γ-CD (10 mg/ml) and Me-ß-CD (1 mg/ml) were prepared in distilled water and methanol-water solution (50:50, v/v), respectively. For each analytic run, HP-γ-CD calibration standards and quality controls (QC) were freshly prepared in distilled water from stock solution, yielding seven calibrators

(CAL) covering the concentration from 0.1 – 10 mg/mL and QCs with 0.2 and 7.5 mg/mL HP-γ-CD, respectively. The IS-solution with a concentration 10 µg/mL Me-ß-CD was prepared from the corresponding stock by dilution in methanol-water (50:50, v/v). For analysis, 100 µl IS-solution was added to 100 µl sample (CAL, QCs, TSL samples) and briefly vortexed. After addition of 800 µl methanol and strong vortexing, the suspension was centrifuged for 10 min (RT, 16000xg). The supernatants were diluted in glass vials 1:50 with methanol-water (50:50, v/v) and subsequently placed into the autosampler for injection. The injection volume was 10 µl. Sample analysis was performed with a Waters Aquity UPLC system that was coupled to a Waters Xevo TQ-S micro tandem Quadrupole system (Waters, Milford, Massachusetts, USA). Separation was performed with an Aquitiy UPLC BEH C18 column (2.1x 100 mm, 1.7 µm, Waters) at 40°C. HP-γ-CD and Me-ß-CD were eluted with a 4 min gradient at a flow rate of 0.5 ml/min using 5 mM ammonium formate in water as mobile phase A and acetonitrile as mobile phase B. The linear gradient was as follows: 0-0.5 min 10 % B, 0.5 – 2.0 min 10-70% B; 2.0 – 2.1 min 70-100 % B; 2.1 – 2.8 min 100% B; 2.8 – 2.9 min 100–10% B; 2.9 – 4.0 min 10% B. Since HP-γ-CD and Me-ß-CD are heterogeneous mixtures of homologs with variable number of either 2-hydroxypropyl- or methyl- groups substituted at different positions of the sugar moieties, we used the mass transitions of one specific homolog as probe for the quantification of all homologs [51]. Both HP-γ-CD and Me-ß-CD were monitored in multiple reaction monitoring using the following mass transitions (*m/z*) for corresponding ammonium-adduct ions: 1721.0 → 221.2 for HP-γ-CD and 1320.7 → 155.3 for Me-ß-CD, respectively (Supplemental Figure 1). Linear response was obtained over the whole calibration range with $R^2 > 0.99$. Intra- and inter-day inaccuracy and imprecision were ≤ 8.5 % and ≤ 11.5 % for QC samples, respectively. Carry-over was negligible with a carry-over rate consistently < 1 % when compared to the lowest calibrator.

3.13 Temperature-dependent destabilization of CF-containing $DPPG_2$-TSL and LTSL in presence of HP-γ-CD and HP-β-CD

Temperature-dependent destabilization of TSL by cyclodextrins (CD) was analysed by quantification of released CF based on de-quenching. $DPPG_2$-TSL or LTSL (20 µl, 0.1 mM lipid) containing CF at a self-quenched concentration (100 mM) were incubated at different temperatures for 5 and 60 min in physiological saline, FCS, as well as in HP-β-CD (226.0 mM, 10.0 mM, 1.0 mM) or HP-γ-CD (226.0 mM, 10.0 mM, 1.0 mM). As stated previously, CD-containing solutions (10.0 mM and 1.0 mM) were adjusted to the physiological osmolarity with NaCl to avoid any osmotic-dependent effects on CF release from TSL. After

incubation, the samples were immediately cooled down on ice for 5 min and afterwards 1000 µL of NaCl/Tris solution (pH=8.0) was added. A 100%-value for CF release was obtained after lysis of TSL (20 µl, 0.1 mM lipid) with 10% Triton X-100 (20 µl) and incubation at 45°C for 15 min. For Triton samples, 980 µl of NaCl/Tris solution (pH=8.0) was added. All samples were analyzed for CF release by fluorescence measurement at Ex = 493 nm / Em = 513 nm. The CF fluorescence was not influenced in presence of CD as confirmed by standard lines of free CF in CD-containing solutions leading to comparable values obtained in physiological saline.

3.14 Determination of encapsulation efficacy (EE)

Encapsulation efficacy (EE) of investigated compounds (drugs) was calculated according to the following formula:

$$EE\ (\%) = \frac{C_e(drug) * C_i(lipid)}{C_e(lipid) * C_i(drug)} * 100$$

$C_e(drug)$ = drug concentration (mmol/L) in the final TSL formulation

$C_e(lipid)$ = lipid concentration (mmol/L) in the final TSL formulation

$C_i(drug)$ = drug concentration (mmol/L) used initially for TSL preparation

$C_i(lipid)$ = lipid concentration (mmol/L) used initially for TSL preparation

As described in previous sections, the lipid and drug concentrations were quantified by the phosphate assay and HPLC, respectively.

4. Results

4.1 Characterization of TSL

$DPPG_2$-TSL and LTSL had a narrow size distribution with a polydispersity index (PDI) of approx. 0.1, independently of the encapsulated compound (Table 1). $DPPG_2$-TSL had a negative ζ-potential due to incorporation of the negatively charged $DPPG_2$ at a molar ratio of 30%. The expected lipid composition was confirmed for all TSL formulations by TLC (data not shown). No lysolipid formation was detected in $DPPG_2$-TSL formulations whereas LTSL contained MSPC lysolipid (10% mol) (data not shown). DXM could be incorporated within $DPPG_2$-TSL and LTSL with a high initial encapsulation efficacy (EE). However, the final DXM:lipid ratio could not be further increased in presence of higher DXM concentrations during TSL preparation (initial DXM:lipid 0.05 vs. 0.1). In contrast to DXM, DP was fully encapsulated in both $DPPG_2$-TSL and LTSL leading to higher final drug:lipid ratios. In contrast to other compounds, DP strongly influenced the transition temperature (T_m) of $DPPG_2$-TSL and LTSL (Figure 1). Increasing amount of DP (5 mol% vs. 10 mol%) led to

broadening and shifting of the transition peak to lower temperature values in comparison to empty TSL.

The size of $DPPG_2$-TSL was significantly decreased after DXMP encapsulation in contrast to other compounds used in this study. In case of LTSL, DXMP interfered with a formation of unilamellar vesicles. Consequently, the formation of intact LTSL could not be detected by DLS after extrusion and the drug was subsequently removed during the batch purification by size-exclusion (data not shown). To obtain intact LTSL, the DXMP concentration was reduced by a factor of five during passive loading leading to a drastically lower final DXMP:lipid ratio in LTSL in contrast to $DPPG_2$-TSL.

The aqueous solubility of DXM with a reported value of approx. 0.1 mg/ml was increased to 36.3±0.7 mg/ml after complexation by HP-γ-CD at a molar ratio of DXM:HP-γ-CD (1.0:2.3). Passive loading of the water-soluble DXM:HP-γ-CD complex in LTSL and $DPPG_2$-TSL yielded an approx. 10x higher final DXM:lipid ratio in contrast to DXM-TSL.

CF-containing $DPPG_2$-TSL and LTSL were used in this study to investigate the interaction and bilayer stability in presence of HP-γ-CD and HP-β-CD. These had comparable characteristics as CF-TSL described by our group elsewhere [41, 42].

Table 1: Characterization of TSL formulations. The values are given as mean of three independently prepared liposome batches.

TSL	z-average (nm)	PDI	ζ-potential (mV)	T_m (°C)	drug/lipid (mol/mol)	EE (%)
$DPPG_2$-TSL	150 (±3)	0.10 (±0.01)	-26.7 (±2.0)	42.1 (±0.1)	no drug used	no drug used
$DPPG_2$-TSL-DXM	152 (±3)	0.08 (±0.02)	-27.3 (±2.4)	42.1 (±0.1)	0.032 (±0.002)	60.8±3.8
$DPPG_2$-TSL-DP:						
-5 mol% DP	151 (±5)	0.10 (±0.01)	-25.3 (±3.5)	41.4 (±0.1)	0.046 (±0.002)	92.6±4.4
-10 mol% DP	157 (±6)	0.08 (±0.02)	-25.7 (±5.2)	39.5 (±0.2)	0.095 (±0.004)	95.6±4,2
$DPPG_2$-TSL-DXMP	123 (±3)	0.09 (±0.01)	-26.1 (±2.1)	42.8 (±0.1)	0.193 (±0.008)	10.0±0.4
$DPPG_2$-TSL-DXM-CD	157 (±4)	0.05 (±0.01)	-28.8 (±1.0)	42.4 (±0.2)	0.301 (±0.004)	15.7±0.4
LTSL	129 (±9)	0.08 (±0.02)	-3.3 (±0.8)	42.0 (±0.2)	no drug used	no drug used
LTSL-DXM	124 (±10)	0.09 (±0.01)	-1.6 (±1.4)	41.9 (±0.1)	0.027 (±0.002)	51.3±3.8
LTSL-DP:						
-5 mol% DP	138 (±5)	0.09 (±0.01)	-1.8 (±2.3)	40.6 (±0.1)	0.047 (±0.002)	94.1±4.2
-10 mol% DP	135 (±8)	0.09 (±0.01)	-1.2 (±3.1)	38.4 (±0.2)	0.097 (±0.003)	97.3±3.2
LTSL-DXMP	128 (±4)	0.09 (±0.02)	-1.8 (±3.1)	42.0 (±0.1)	0.014 (±0.010)	3.7±2.5
LTSL-DXM-CD	146 (±3)	0.07 (±0.02)	-2.2 (±2.1)	41.4 (±0.1)	0.291 (±0.053)	13.3±0.7
$DPPG_2$-TSL-CF	150 (±1)	0.07 (±0.01)	-25.7 (±1.3)	42.2 (±0.1)	0.136 (±0.024)	6.8±1.2
LTSL-CF	127 (±2)	0.06 (±0.02)	-1.7 (±0.9)	41.8 (±0.1)	0.130 (±0.037)	6.5±1.9

TSL: Thermosensitive liposome, PDI: polydispersity index, EE: encapsulation efficacy, DXM: dexamethasone, DP: dexamethasone-21-palmitate DXMP: dexamethasone-21-phosphate disodium salt, CD: 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD), CF: carboxyfluorescein.

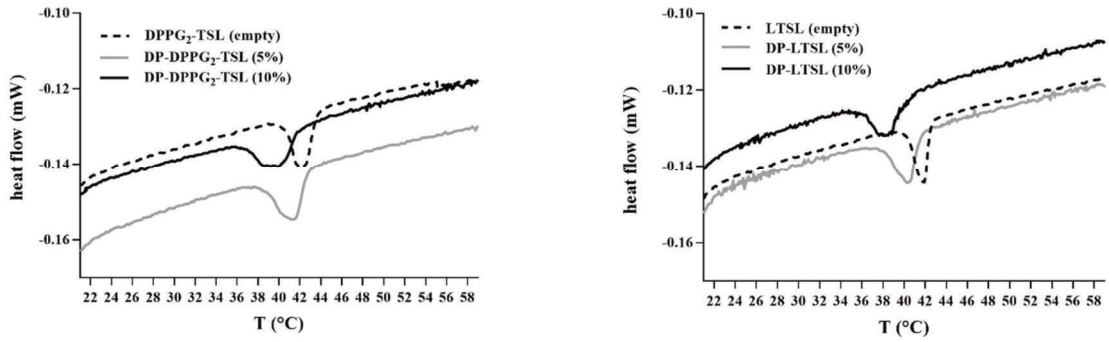

Figure 1: Differential scanning calorimetry (DSC) of DP-containing $DPPG_2$-TSL (A) and LTSL (B). DP was incorporated in $DPPG_2$-TSL and LTSL with either 5% or 10% molar ratio.

4.2 Temperature-dependent drug retention in TSL

A temperature-dependent retention of DXM, the prodrugs DP and DXMP as well as the cyclodextrin HP-γ-CD was analysed before and after incubation in HEPES-buffered saline (HBS, pH=7.4) and fetal calf serum (FCS) at distinct temperatures. Although hydrophilic small-molecules (e.g. DXMP or HP-γ-CD) can be easily separated from TSL by ultrafiltration and quantified as released compounds, the same procedure is more challenging for lipophilic drugs (e.g. DXM or DP) due to possible binding to (large) serum proteins. Consequently, at least two fractions (free drug and protein-bound drug) can be present in serum after release of lipophilic drugs from TSL. Binding of DXM to albumin has been previously reported in several studies [52, 53]. Although filters with a high molecular weight cut-off (MWCO) of 300 KDa are suitable for an efficient ultrafiltration of albumin (66.5 kDa) [54], the flow-through containing the released (protein-bound) drug was in our hands contaminated by a certain fraction of TSL, probably due to centrifugal forces or irregular pore size distribution in the filters. Besides, lipophilic drugs including glucocorticoids as well as proteins tend to bind to filter materials [55] requiring a specific pre-treatment (e.g. passivation, use of blocking agents). Centrifugation as an alternative separation method could not be applied since both $DPPG_2$-TSL as well as LTSL did not completely sediment, even after a prolonged centrifugation time (75000xg, 120 min). So far, only dialysis was reported as a method to investigate the release of lipophilic drugs from TSL under serum-free conditions [23, 24]. However, dialysis usually shows a rate-limiting diffusion of drugs through the dialysis membrane and requires relatively high amounts of TSL. Furthermore, it is challenging to perform dialysis in presence of serum. Therefore, we developed a fast method based on size-exclusion with CL-4B mini spin columns which reproducibly eluted TSL but effectively retained both released free and protein-bound drugs. For comparison, we finally kept the

separation method the same for each drug modification since it was equally suitable for hydrophilic and lipophilic compounds in this study.

4.2.1 Dexamethasone (DXM) and dexamethasone-21-palmitate (DP)

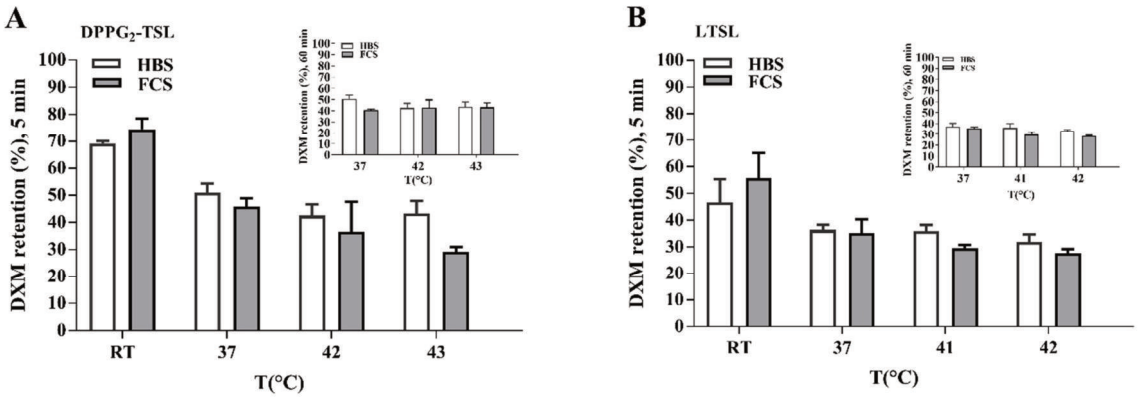

Figure 2: Temperature-dependent retention of DXM in $DPPG_2$-TSL (A) and LTSL (B). $DPPG_2$-TSL-DXM (A) and LTSL-DXM (B) were incubated for 5 min or 60 min (inlet) at corresponding temperatures in HBS or FCS. The values are given as mean of three independently prepared liposome batches. The statistical significance of hypethermia (HT) values (41-42°C for LTSL and 42-43°C for $DPPG_2$-TSL) versus body temperature (37°C) in the corresponding medium (HBS or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test.

DXM showed a poor retention in both $DPPG_2$-TSL and LTSL resulting in a strong leakage at room temperature (RT) after dilution (Figure 2). Although, the retention was further reduced at higher temperatures, there was no significant difference between 37°C and temperatures in the hyperthermia (HT) range (41-43°C). The decrease in retention was already observed in HBS and was comparable to FCS. This is not entirely surprising since the final concentration of DXM in the TSL dispersion was approx. 0.4-0.5 mg/ml due to a low DXM:lipid ratio. After dilution in the release medium, the resulting DXM concentration was below the reported aqueous solubility of DXM (0.1 mg/ml) [56, 57].

Previously, lipophilic esters of glucocorticoids (e.g. DP) were proposed as prodrugs of glucocorticoids (GC) with an improved retention in liposomes [58, 59]. However, DP did not show any detectable release from TSL formulations in HBS, FCS or in presence of multilamellar vesicles (MLV) within 60 min (Supplemental Figure 2).

4.2.2 Dexamethasone-21-phosphate (DXMP)

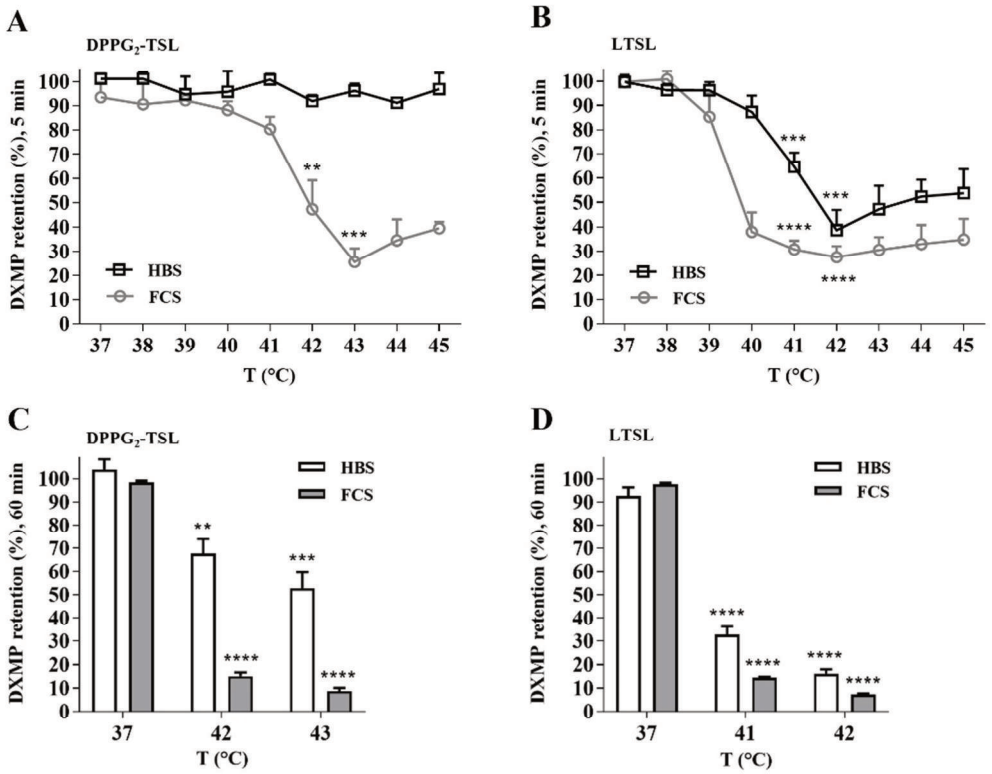

Figure 3: Temperature-dependent retention of DXMP in $DPPG_2$-TSL (A, C) and LTSL (B, D). $DPPG_2$-TSL-DXMP and LTSL-DXMP were incubated for 5 min (A, B) or 60 min (C, D) at corresponding temperatures in HBS or FCS. The values are given as mean of three independently prepared liposome batches. The statistical significance of HT values (41-42°C for LTSL and 42-43°C for $DPPG_2$-TSL) versus 37°C in the corresponding medium (HBS or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, **=p< 0.01, ***=p<0.005, ****=p<0.0001.

The hydrophilic DXMP is a sodium salt of the 21-phosphate ester of DXM with an improved liposomal retention in comparison to DXM [33]. Similar to the lipophilic DP, DXMP was stably retained in both TSL formulations at 37°C within 60 min in HBS and FCS (Figure 3 C, D). $DPPG_2$-TSL showed a comparably slow decrease in retention after 60 min at 42°C (67.6±6.3) and 43°C (52.8±6.9) in HBS (Figure 3 C). On the contrary, LTSL showed a fast decrease in DXMP retention at 42°C (38.6±8.3) in HBS (Figure 3 B). In FCS, both formulations showed an equally fast decline in DXMP retention at T_m with minimal retention values observed at 43°C for $DPPG_2$-TSL after 5 min (25.6±5.6%) and 60 min (8.8±1.4%) and at 42°C for LTSL after 5 min (27.5±4.6%) and 60 min (7.2±1.6) (Figure 3).

4.2.3 Investigation of $DPPG_2$-TSL and LTSL for the loading of DXM complexed by cyclodextrins (CD)

The cyclodextrins HP-β-CD and HP-γ-CD which have both a high aqueous solubility (>500 mg/ml) [60] and are capable to form a complex with GC including DXM [47, 61-63], were pre-selected as potential excipients for TSL encapsulation. However, CD's are also known to

complex lipids and by these means destabilize liposomes [64, 65]. Therefore, we first tested the influence of different HP-β-CD and HP-γ-CD concentrations on the stability and thermosensitivity of $DPPG_2$-TSL and LTSL. Similar to the procedure previously described for non-thermosensitive liposomes [66-68], this can be achieved by analyzing the release of hydrophilic fluorescent dyes (e.g. CF, calcein) in CD-containing solutions in comparison to physiological saline (Figure 4-5),

4.2.3.1 Temperature-dependent destabilization of CF-containing $DPPG_2$-TSL and LTSL in presence of HP-γ-CD and HP-β-CD

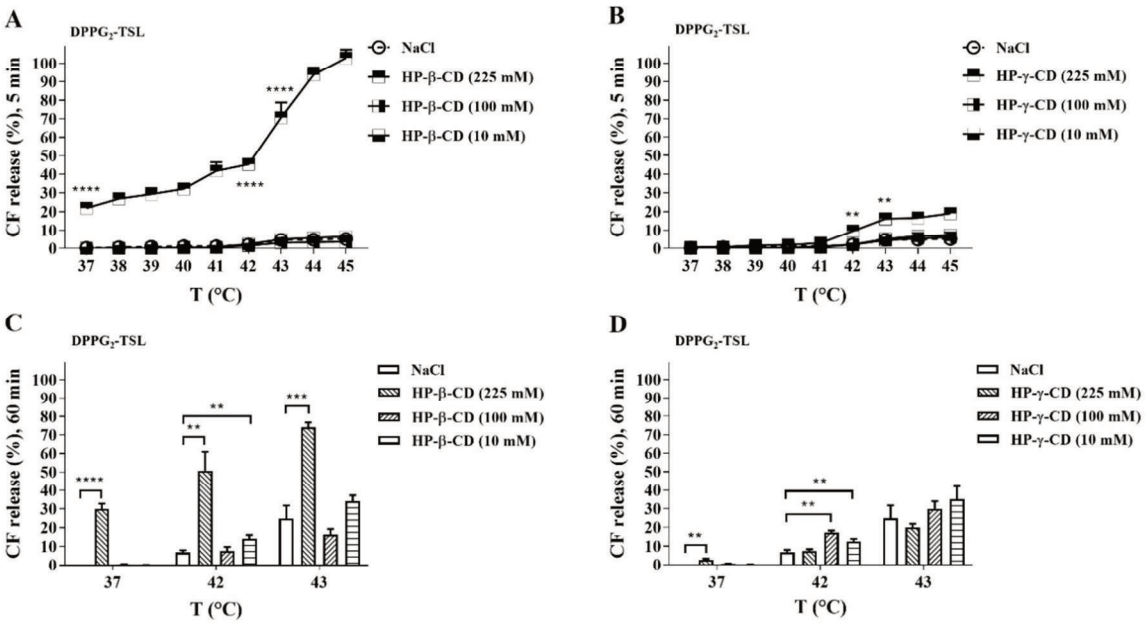

Figure 4: Temperature-dependent release of CF from $DPPG_2$-TSL in presence of HP-β-CD (A, C) and HP-γ-CD (B, D). $DPPG_2$-TSL-CF (0.1 mM lipid) were incubated for 5 min or 60 min at corresponding temperatures in physiological saline or different concentrations (10 mM, 100 mM, 225 mM) of HP-β-CD (A, C) and HP-γ-CD (B, D). The values are given as mean of three independently prepared liposome batches. The statistical significance of values obtained for 37°C, 42°C and 43°C in physiological saline versus CD-containing solutions was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, **= p<0.01, ***=p<0.005, ****=p<0.0001.

Under protein-free conditions, $DPPG_2$-TSL stably encapsulate passively loaded, hydrophilic small molecules including CF at < T_m whereas a slow release takes place at ≥T_m. Therefore, the release of CF was detectable only after 60 min at 42°C (6.8±1.2%) and 43°C (24.9±7.0%) (Figure 4 C, D). However, HP-β-CD at the highest concentration (225 mM) destabilized $DPPG_2$-TSL leading to increased CF release at 37°C (21.9±2.3% after 5 min) and (30.0±2.9% after 60 min) (Figure 4 A, C). The CF release further increased at 43°C (70.6±8.2% after 5 min and (74.2±2.5% after 60 min). However, lower concentrations of HP-β-CD (100 mM, 10 mM) did not induce any further CF release in comparison to saline. In contrast to HP-β-CD (225 mM), HP-γ-CD (225 mM) minimally increased CF release only at ≥T_m after 5 min

(Figure 4 B, D). At lower concentrations of HP-γ-CD (100 mM, 10 mM), no deviation in CF release was observed in comparison to saline (Figure 4 B, D).

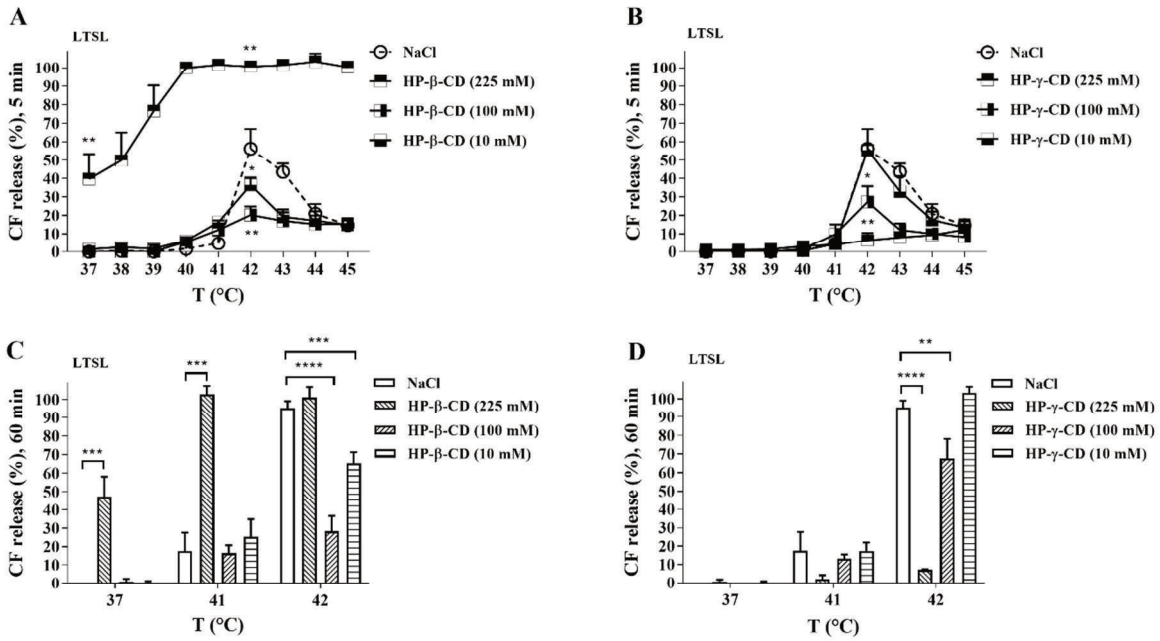

Figure 5: Temperature-dependent release of CF from LTSL in presence of HP-β-CD (A, C) and HP-γ-CD (B, D). LTSL-CF (0.1 mM lipid) were incubated for 5 min or 60 min at corresponding temperatures in physiological saline or different concentrations (10 mM, 100 mM, 225 mM) of HP-β-CD (A, C) and HP-γ-CD (B, D). The values are given as mean of three independently prepared liposome batches. The statistical significance of values obtained for 37°C, 41°C and 42°C in physiological saline versus CD-containing solutions was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p< 0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001.

In contrast to $DPPG_2$-TSL, LTSL are capable to rapidly release CF at $\geq T_m$ under serum protein-free conditions due to the lysolipid incorporation (56.4±10.5% after 5 min and 95.2±3.8% after 60 min) (Figure 5 A, C). HP-β-CD (225 mM) induced a partial CF release at 37°C (39.7±13.7% after 5 min) which increased to 100% starting from 40°C (Figure 5 A). Although lower concentrations of HP-β-CD (100 mM, 10 mM) did not significantly alter the release profile below T_m, a decrease in CF release was observed at 42°C after 5 and 60 min in comparison to saline (Figure 5 A, B). Interestingly, HP-γ-CD (225 mM) drastically decreased the CF release at 42°C after 5 min (6.4±4.0%) and 60 min (7.2±0.3%). This finding was confirmed by a purification of LTSL from released CF by two independent separation methods (size-exclusion with CL-4B or centrifugation) and subsequent analysis of CF:lipid ratio (data not shown). HP-γ-CD (225 mM) strongly reduced the CF release from LTSL in contrast to $DPPG_2$-TSL where it was higher (20.0±2.0% after 60 min) and comparable to saline (24.9±7.0 after 60 min). However, lower concentrations of HP-γ-CD (100 mM, 10 mM) had less effect on the thermosensitivity of LTSL with HP-γ-CD (10 mM) not causing any significant differences in comparison to saline after 5 and 60 min (Figure 5 B, D).

To investigate the possible long-term effect of HP-β-CD and HP-γ-CD on destabilization of $DPPG_2$-TSL and LTSL, the CF-containing formulations were incubated at 4°C for 7 days in presence of HP-γ-CD and HP-β-CD at the same lipid:CD ratios as shown in Figure 4-5. However, no additional CF release was observed during this time frame (data not shown).

4.2.3.2 DXM complexed by HP-γ-CD in $DPPG_2$-TSL and LTSL

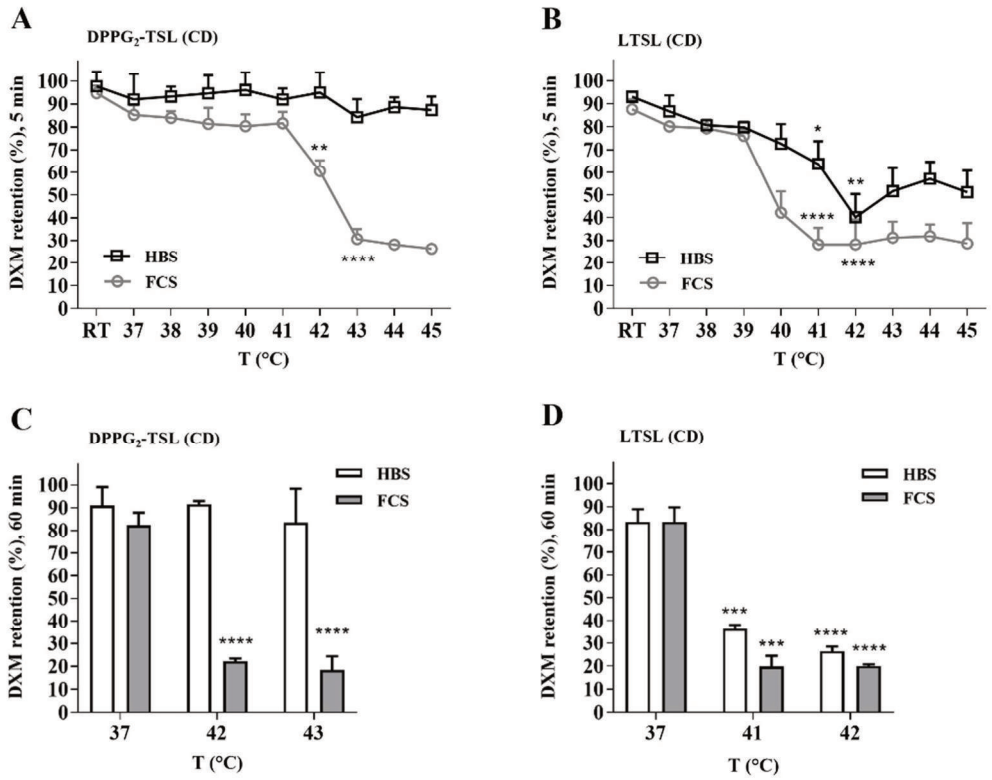

Figure 6: Temperature-dependent retention of DXM in HP-γ-CD-containing $DPPG_2$-TSL (A, C) and LTSL (B, D). $DPPG_2$-TSL-DXM-HP-γ-CD-and LTSL-DXM-HP-γ-CD were incubated for 5 min (A, B) or 60 min (C, D) at corresponding temperatures in HBS or FCS. The values are given as mean of three independently prepared liposome batches. The statistical significance of HT values (41-42°C for LTSL and 42-43°C for $DPPG_2$-TSL) versus 37°C in the corresponding medium (HBS or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001.

Based on the CF release data, it is not possible to predict if HP-β-CD or HP-γ-CD is better suitable for DXM encapsulation in TSL since both CD's barely influenced the liposomal integrity and thermosensitivity at the molar CD:lipid ratio ≤10 (Figure 4, 5). However, we chose HP-γ-CD for further investigation since HP-γ-CD showed on average a three times higher solubilisation efficacy of DXM in comparison to HP-β-CD (data not shown). Consequently, the aqueous DXM:HP-γ-CD complex was encapsulated in $DPPG_2$-TSL and LTSL whereby the CD:lipid ratio was approx. 5 during passive loading.

DXM retention in HP-γ-CD-containing $DPPG_2$-TSL and LTSL (Figure 6) showed a temperature-dependent profile comparable to the prodrug DXMP (Figure 3). LTSL showed a

significantly decreased retention at 42°C (40.2±10.1% after 5 min) and (26.5±2.0% after 60 min) in comparison to 37°C (86.7±7.0% after 5 min) and (83.4±5.5% after 60 min) in HBS (Figure 6 B, D). On the contrary, $DPPG_2$-TSL strongly retained DXM at all investigated temperatures after 5 and 60 min in HBS (Figure 6 A, C). In FCS, both LTSL and $DPPG_2$-TSL showed a fast decrease in DXM retention at $\geq T_m$ within 5 min (28.2±11.0% at 42°C for LTSL) and (30.7±4.3% at 43°C for $DPPG_2$-TSL). However, an unwanted decrease in DXM retention was also observed in FCS at 37°C after 5 min for $DPPG_2$-TSL (85.2±4.3%) and LTSL (80.2±1.6%).

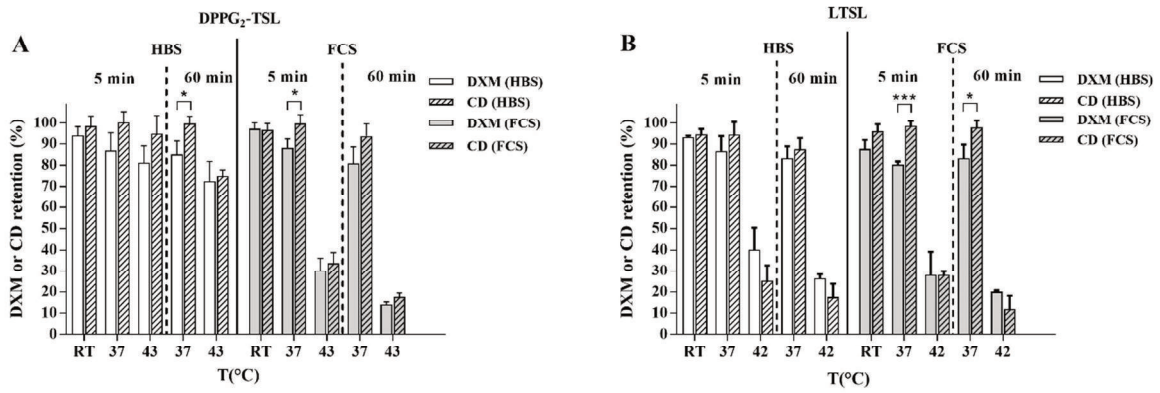

Figure 7: Comparison of temperature-dependent retentions of DXM and HP-γ-CD in $DPPG_2$-TSL (A) and LTSL (B). $DPPG_2$-TSL-DXM-HP-γ-CD (A) and LTSL-DXM-HP-γ-CD (B) were incubated for 5 min or 60 min (C, D) at corresponding temperatures in HBS or FCS. The values are given as mean of three independently prepared liposome batches. The statistical significance of values obtained at corresponding temperatures for DXM and HP-γ-CD was analysed by the unpaired t-test. *= $p<0.05$, ***=$p<0.005$.

The complex formation between drugs and CD's is generally described as dynamic with constant association and dissociation [69]. Furthermore, drug:CD complexes are easily destroyed upon dilution and competitive replacement with serum components [70]. To obtain a correlation between a temperature-dependent retention of DXM and HP-γ-CD, both compounds were quantified as separate entities (Figure 7). The temperature-dependent retention of HP-γ-CD was overall comparable to DXM for both $DPPG_2$-TSL and LTSL (Figure 7). However, a slightly stronger retention of HP-γ-CD in comparison to DXM was observed at $\leq T_m$ (RT and 37°) in HBS and FCS whereas no specific trend was detected at $\geq T_m$.

5. Discussion

This study investigated dexamethasone (DXM), the prodrugs dexamethasone-21-phosphate (DXMP) and dexamethasone-21-palmitate (DP) as well as DXM complexed by 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD) for potential use in TSL. Although several TSL

formulations have been described in literature [15], we selected a low-temperature sensitive liposome (LTSL) and a 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol-based TSL ($DPPG_2$-TSL) due to their clinical relevance [71-73]. LTSL is a PEGylated TSL which contains a lysolipid to accelerate the drug release at hyperthermia (HT) [39, 40]. $DPPG_2$-TSL is a non-PEGylated and lysolipid-free TSL that makes use of the $DPPG_2$ phospholipid to achieve prolonged circulation time *in-vivo* and accelerated drug release at HT in presence of serum [74, 75]. Despite a considerable difference in lipid composition and possible mechanism behind a HT-induced drug release, the performance of both TSL was comparable in this study.

$DPPG_2$-TSL and LTSL demonstrated a poor retention and a low loading capacity for DXM. The retention did not show any significant difference between buffer and serum. However, the final concentration of liposomal DXM was below the reported aqueous solubility (~0.1 mg/ml at 25°C) of DXM [56, 57] after TSL dilution in the release medium. Therefore, the presence of biological acceptors (e.g. serum proteins) was not necessarily required to induce DXM release from a TSL bilayer, presumambly due to the lack of sufficiently strong interactions between the drug and the lipids. Although the retention of DXM in TSL slightly decreased with higher temperature (room temperature versus 37°C or HT), this effect is possibly due to an improved aqueous solubility of DXM with increasing temperature of the release medium. Overall, the findings are in line with previous studies where a high burst release of DXM as well as comparably low drug to lipid ratios were observed in unilamellar liposomes [76, 77].

Esterification of glucocorticoids (GC) with alkyl chains of a suitable length was proposed as a general solution to improve the liposomal retention of this drug class. This synthetic drug modification leads to an increased lipophilicity and favors the orientation of GC within liposomal bilayers [44, 58, 59, 78]. We previously reported that the lipophilic hexadecylphosphocholine (HePC, Miltefosine) shows increased transfer from $DPPG_2$-TSL into cancer cell lines at HT in comparison to 37°C [21]. Therefore, we considered DP and HePC as structurally similar compounds with regards to the alkyl chain composed of 16 carbon atoms and assumed a possible HT-mediated transfer of DP from TSL to suitable biological acceptors. However, DP was stably retained within the TSL membrane in serum or in presence of multilamellar vesicles in this study. Extensive investigation of DP in non-thermosensitive liposomes showed that DP release rather correlates with the esterase activity and the drug is released in the DXM form after cleavage of the ester bond [79]. If the same is

true for TSL, DP is not a promising candidate for further investigation since DXM release was not increased by HT. However, we can not fully rule out the possibility that DP is released as an intact prodrug in a heat-inducible way in presence of cells or other biological membranes, similar to HePC. Additionally, it has to be taken into account that increasing liposomal amount of DP lowered the transition temperature (T_m) of TSL. Recently, DP was shown to lower the T_m of DPPC-containing particles [80]. Therefore, further adjustment of the TSL lipid composition might be required to shift the T_m back to the mild HT range (41-43°C).

Esterification of GC to phosphate derivatives drastically increases their aqueous solubility leading to a preferential drug encapsulation within an aqueous core of liposomes and consequently improved liposomal retention [33]. In $DPPG_2$-TSL and LTSL, DXMP showed a drastically decreased retention selectively at HT, whereas no substantial leakage could be detected at 37°C in serum. Since DXMP is a negatively charged molecule, it can not cross a TSL bilayer in the solid-gel phase ($<T_m$) as easily as the uncharged and hydrophobic DXM. However, structural packing defects formed at $\geq T_m$ between the lipid domains in the solid-gel phase and liquid-crystalline phase of TSL enable a fast DXMP release.

The prodrug concept is a widely applied method to tailor the physico-chemical properties of drugs and by these means improve the retention and release behavior from liposomes [81-83]. However, it involves a chemical modification of drugs and can consequently alter the pharmacological activity [84]. Furthermore, a majority of lipophilic drugs (e.g. DP) might be less suitable for the incorporation in TSL, either due to considerable effects on the T_m or strong interaction with the lipids leading to no substantial release at HT. Cyclodextrins (CD) represent a promising strategy to increase the aqueous solubility of lipophilic drugs without any chemical modifications and by these means shift their preferential location from a bilayer to the aqueous core of liposomes. This concept, known as “drug-in-cyclodextrin-in-liposome” (DCL), was extensively investigated for non-thermosensitive liposomes leading to increased loading capacities of lipophilic drugs, prolonged pharmacokinetics and reduced toxicity [45, 46, 85]. However, a drawback associated with the DCL approach is a possible complexation of cholesterol and lipids by certain CD types resulting in liposomal destabilization [64-67].
To the best of our knowledge, there are currently no reports available that describe the use of drugs complexed by CD in TSL. We pre-selected 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD) since they are suitable for the DXM

complexation [47, 61-63]. Additionally, HP-β-CD and HP-γ-CD are both well water-soluble (>500 mg/ml) to prepare aqueous solutions with high DXM concentrations. This was required to obtain therapeutically relevant liposomal concentrations of DXM since passive loading usually leads to a low encapsulation efficacy of drugs in liposomes. Both HP-β-CD and HP-γ-CD were first tested for their ability to affect the integrity and thermosensitivity of TSL based on the release of fluorescent dyes described for non-thermosensitive liposomes elsewhere [66-68]. Although HP-β-CD and HP-γ-CD affected the thermosensitivity and destabilized $DPPG_2$-TSL and LTSL in a concentration-dependent manner, the effect was comparably low for both CD at the molar CD:lipid ratio of ≤10. Finally, we selected HP-γ-CD since it solubilized the same amount of DXM at a three times lower concentration in comparison to HP-β-CD ensuring a CD:lipid ratio <10 during passive loading.

The HP-γ-CD:DXM complex was successfully encapsulated within $DPPG_2$-TSL and LTSL leading to a ten fold increase in DXM:lipid ratio in comparison to non-complexed DXM. Furthermore, DXM and HP-γ-CD showed a strongly improved release from TSL in serum at HT in comparison to 37°C. It is noteworthy that the temperature-dependent retention profile of HP-γ-CD:DXM was comparable to the hydrophilic prodrug DXMP. Although DXM might be complexed by CD during release at HT, the drug:CD complexes are short-lived in presence of serum components [70]. Therefore, we expect that DXM would immediately become bioavailable *in vivo*, independently of the CD presence. In contrast to HP-γ-CD, a certain fraction of DXM was also released at 37°C. This indicates a possible CD-independent and likely bilayer-deposited DXM fraction. This theory is further supported by a comparably high encapsulation efficacy (EE) in case of approx. 12-15% for DXM when complexed by HP-γ-CD. In our experience, the EE is expected to be ≤10% in case of (hydrophilic) drugs loaded exclusively within the aqueous core of TSL of this size. Since the interaction of drugs and CD does not involve any covalent forces [69], DXM can potentially dissociate from HP-γ-CD during TSL preparation leading to formation of a double-loaded TSL with two DXM fractions. The main fraction of DXM is complexed by HP-γ-CD and therefore solubilised in the aqueous core of TSL. However, another (minor) fraction of DXM is located in the TSL bilayer and is therefore released already at $<T_m$.

In conclusion, this study showed the limitations and possibilities for DXM delivery by TSL. DXM encapsulation in TSL was only achieved with a low DXM:lipid ratio. Additionally, DXM showed notable leakage from TSL below T_m with no significant difference in release at

37°C and HT. The retention in TSL and final drug:lipid ratios could be significantly improved after encapsulation of prodrugs (DP and DXMP) or complexation of DXM with a CD. Although both the hydrophilic DXMP and the lipophilic DP showed a stable retention in TSL below T_m, only DXMP was released from TSL at HT. Intriguingly, CD complexation of DXM did not only increase the final DXM:lipid ratios but also provided a possibility to release DXM in a heat-inducible way from TSL. Based on our *in vitro* results, DXMP and DXM:CD represent promising candidates for further investigation in pre-clinical animal models. Future biodistribution studies with tumor-bearing rats will reveal if DXMP and DXM:CD will be efficiently delivered by TSL to solid tumors in combination with regional HT.

6. Acknowledgment

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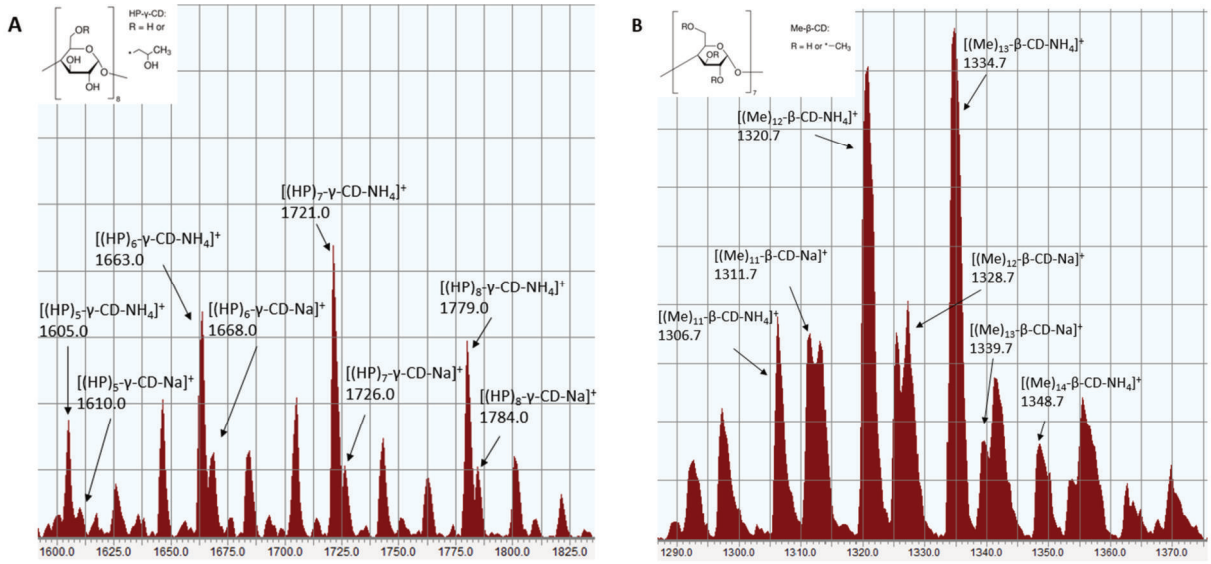
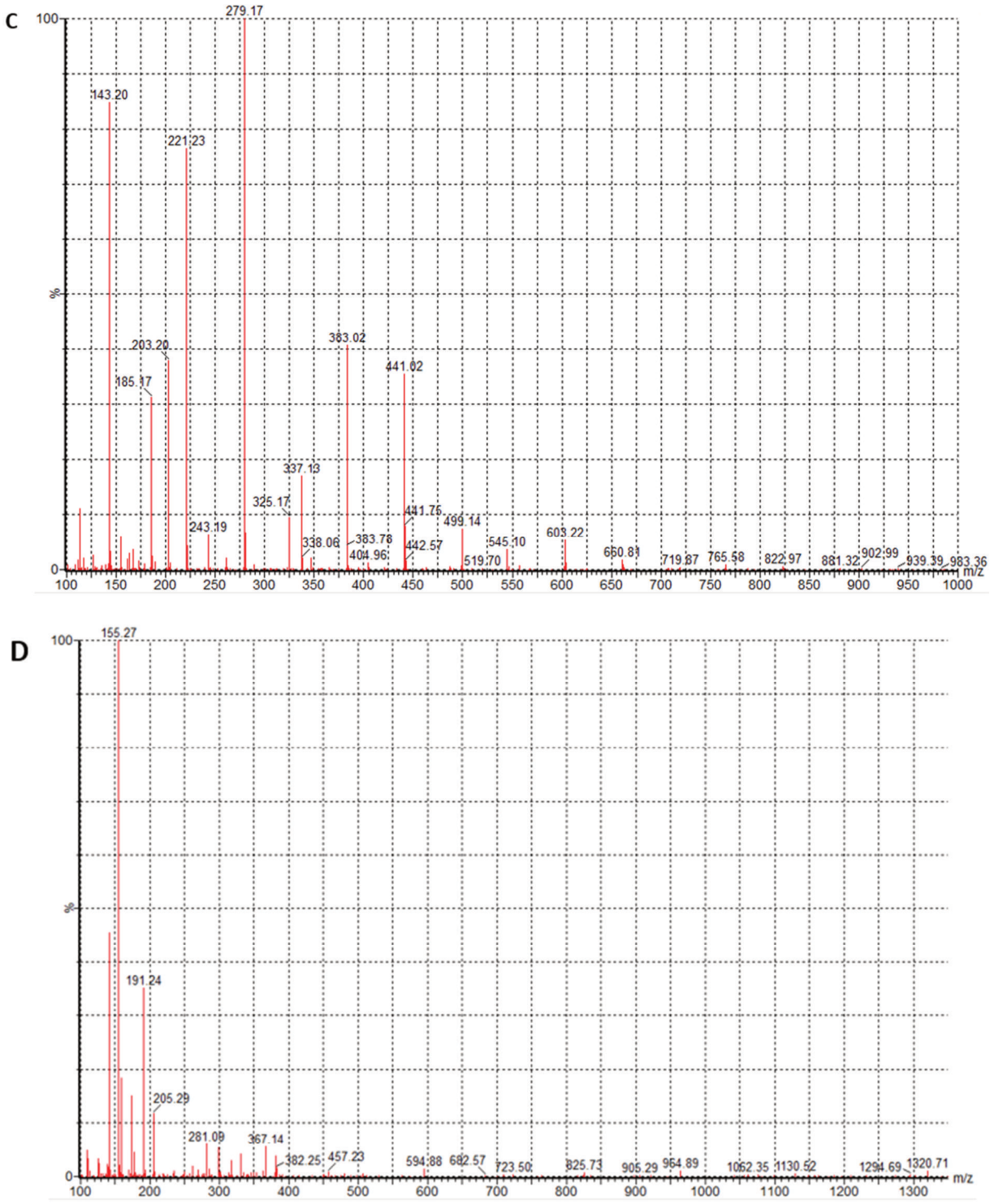
7. Conflict of interest

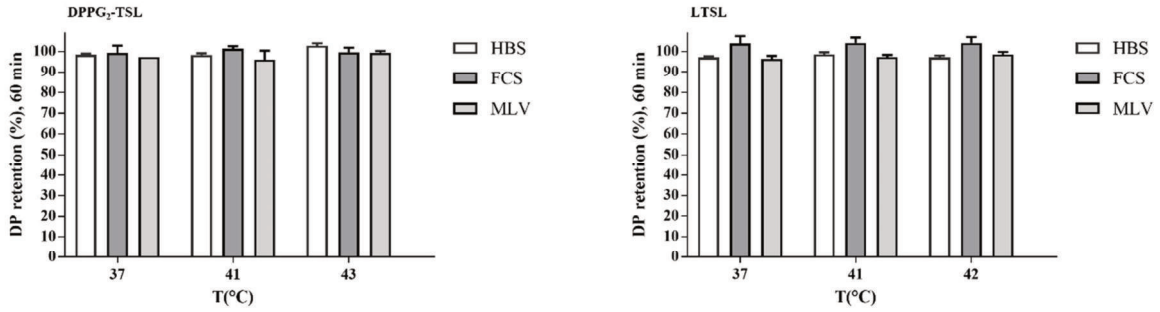
Lars H. Lindner hold shares of Thermosome GmbH, Planegg/Martinsried, Germany. All other authors declare no conflict of interest.

8. Supplemental material





Supplemental Figure 1: Molecular structures and ESI+ ion mode mass spectra of HP-γ-CD (A) and Me-ß-CD (B) in which $[M+NH_4]^+$ adducts dominate compared to $[M+Na]^+$ ions. Tandem mass transitions (*m/z*) 1721.0 -> 221.2 and 1320.7 -> 155.3 were used for detection of HP-γ-CD (C) and Me-ß-CD (D), respectively.



Supplemental Figure 2: Temperature-dependent retention of dexamethasone-21-palmitate (DP) in $DPPG_2$-TSL (A) and LTSL (B). $DPPG_2$-TSL-DP and LTSL-DP were incubated for 60 min at corresponding temperatures in HBS, FCS or in presence of MLV. The values are given as mean of two independently prepared liposome batches. The statistical significance of HT values (41-43°C)) versus 37°C in the corresponding medium (HBS, FCS, MLV) was analysed by one way ANOVA followed by Dunnet's post-hoc test.

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10. Pharmacokinetics (PK) of non-liposomal DXMP versus $DPPG_2$-TSL-DXMP (not part of the manuscript)

These data are not included in the manuscript (Chapter 3). They are part of an independent *in vivo* study comparing the pharmacokinetics, biodistribution and therapeutic efficacy of the two most promising $DPPG_2$-TSL formulations containing the prodrug DXMP and DXM complexed by a cyclodextrin (CD). The first results of the DXMP-containing $DPPG_2$-TSL are already included in this thesis.

10.1 Materials and Methods

a). Pharmacokinetic (PK) study of DXMP versus $DPPG_2$-TSL-DXMP

The animal experiments were performed according to protocols approved by the responsible authority (Regierung of Oberbayern, Az. ROB-55.2-2532.Vet_02-18-61). $DPPG_2$-TSL-DXMP in HBS and free DXMP in HBS were intravenously injected at the same DXMP dose (10 mg/kg) and the same injection volume into Male Brown Norway rats (~230 g). Blood samples were collected at different time points in lithium heparin microcuvettes and immediately centrifuged for 10 min at 2000xg. Additionally, $DPPG_2$-TSL-DXMP and free DXMP in HBS were spiked into rat plasma *ex vivo* at the same concentration and dilution as applied in the PK study to obtain a theroretical value representing 100% of the injected dose (ID). Plasma samples were stored at -20°C until further quantification by HPLC-UV.

b). Extraction and quantification of DXMP in the PK study

Prednisolone-21-phosphate (PRNP) was used as an internal standard for DXMP quantification in rat plasma. Seven calibration standards (CAL, 20 µl) containing DXMP and PRNP were freshly prepared in rat plasma with a calibration range of 6-400 µg/ml. 980 µl methanol was added to the PK samples (20 µl) and calibration standards (CAL, 20 µl) and the mixtures incubated in a thermomixer for 20 min (RT, 1000 rpm). After subsequent centrifugation for 10 min (RT, 16000xg), the supernatant (950 µl) was transferred to a glass tube to be dried in a heat-block under nitrogen flow (20 min, 40°C). Subsequently, 300 µl of ammonium acetate in H_2O (10 mM, pH=4.5):acetonitrile (80:20, vol:vol) was added to dried samples. After strong vortexing, the mixture was transferred to Eppendorf tubes and centrifuged for 10 min (RT, 16000xg). The supernatant was transferred to HPLC vials. DXMP and PRN were quantified by HPLC-UV (230 nm). An Atlantis T3 (3µm, 4.6x100 mm) column was operated at 30°C with a flow of 1.0 ml/min using mobile phase A containing acetonitrile and mobile phase B containing 10 mM ammonium acetate in H_2O (10

mM, pH=4.5). The gradient applied was 2 min at 20% (A), 0.5 min 20%-30% A, 2.5-7.0 min at 30% A, 1 min back to 20% A with a total run time of 13 min. The elution times of PRNP and DXMP were 5.7 and 6.7 min, respectively. The injection volume was 50 µl. A linear response was obtained over the whole calibration range with $R^2 > 0.99$.

10.2 Results

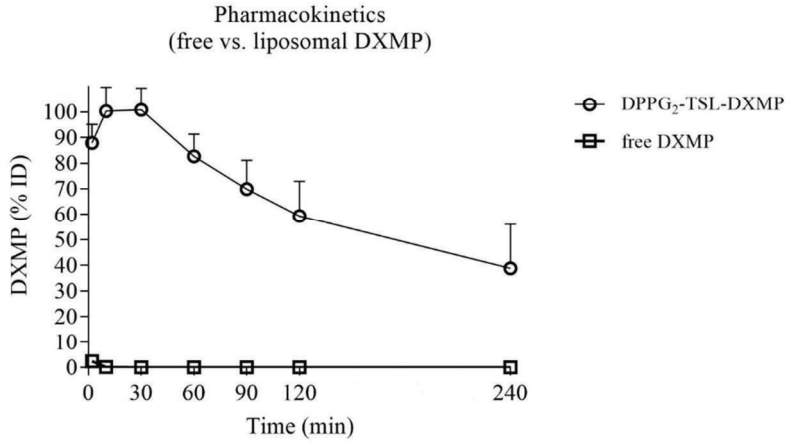

Figure 1: Pharmacokinetic (PK) study of DXMP and $DPPG_2$-TSL-DXMP in Brown Norway rats (n=3). DXMP and $DPPG_2$-TSL-DXMP were injected intravenously at a DXMP dose of 10 mg/kg. The values were plotted as percentage of the injected dose (ID) where 100% ID correponds to the theoretical and *ex vivo* determined concentration of DXMP (248±23 µg/ml) in plasma at t=0 (min).

Encapsulation in $DPPG_2$-TSL drastically prolonged the circulation time of DXMP *in vivo* in comparison to the non-liposomal drug (Figure 1). For $DPPG_2$-TSL-DXMP, ~100% of the injected dose (ID) was detected in circulation after complete distribution in the blood volume (10-30 min). On the contrary, non-liposomal DXMP was rapidly cleared from circulation leading to only ~5% ID after 5 min and no detectable drug concentrations in plasma after 10 min. The circulation time of the liposomal DXMP (Figure 1) and the $DPPG_2$-TSL carrier (Chapter 2, Figure 6: PK of $DPPG_2$-TSL-HePC) are comparable indicating that DXMP is stably encapsulated in $DPPG_2$-TSL *in vivo* and is likely cleared with the liposomes. The PK data also confirm the *in vitro* results from the temperature-dependent retention profile of DXMP with no detectable leakage at 37°C in FCS (Chapter 3, Figure 3).

CHAPTER 4: *In vitro* study on the release of docetaxel and its prodrug from the phosphatidyldiglycerol-based thermosensitive liposomes

1. Introduction

Taxanes are potent chemotherapeutics which are approved for the treatment of several solid tumor types [1]. The extremely low aqueous solubility of these compounds required the use of solubilising excipients in commercial formulations. A mixture of Cremophor EL® (CrEL) and ethanol as well as Tween 80 and ethanol is used in approved products for paclitaxel (PTX, Taxol®) and docetaxel (DTX, Taxotere®), respectively [2]. However, CrEL and Tween 80 are assumed to cause or contribute to frequent hypersensitivity reactions in cancer patients [3, 4]. A novel approved formulation of PTX (Abraxane®) is based on the drug solubilisation by albumin nanoparticles and is therefore absent of CrEL reducing the risk of hypersensitivity reactions [5]. Although Abraxane had an improved toxicity profile in comparison to Taxol allowing a higher maximum tolerated dose, it showed at best only modest benefits in therapeutic outcomes for cancer patients in comparison to Taxol [5-7].

Several nano-based delivery systems including liposomes and micelles are currently investigated for taxanes in clinical phases [6-8]. Liposomes as delivery vehicles represent a logical choice for many lipophilic drugs due to their solubilisation potency and biocompatibility [9]. However, recent surveys came to the conclusion that approved liposomal products and those in clinical phases do not represent stable carriers for taxanes *in vivo* with pharmacokinetics comparable to Abraxane or Taxol [6, 7, 10, 11]. Therefore, these products are not expected to lead to superior therapeutic efficacy in patients [2, 6].

A suitable chemical modification of a drug to various prodrugs can improve the poor retention and loading efficacy of poor water-soluble and uncharged drugs in liposomes. This can be achieved by a drug conjugation with a lipid chain of a suitable length forming a lipid-prodrug which has a strong interaction with lipids in the bilayer [12-14]. A second common strategy involves an introduction of functional groups which are charged in a pH range suitable for liposomes. By these means, either a passive or active loading by a pH or ion gradient can be applied for a drug loading into the aqueous core of liposomes [15, 16]. Recently, a docetaxel prodrug (DTX-P) was developed with a functional group suitable for the ammonia-based pH-gradient loading method [17]. The prodrug demonstrated a superior retention and loading efficacy in liposomes in comparison to unmodified DTX [17]. DTX-P was also described in the low temperature-sensitive liposome (LTSL) which was capable to release the prodrug at

temperatures in the mild hyperthermia (HT) range (41-43°C) and led to a prolonged pharmacokinetics in comparison to the free drug [18]. On the contrary, recent studies also showed that both PTX and DTX have a HT-enhanced release *in vitro* from LTSL without any chemical drug modification [19, 20]. However, there was no significant difference in intra-tumoral drug concentrations for DTX-containing LTSL with and without application of HT *in vivo* [21].

The objective of this study is to investigate if DTX will show an accelerated release from $DPPG_2$-TSL upon HT. Additionally, the release behaviour of the prodrug DTX-P is evaluated for $DPPG_2$-TSL.

2. Materials and Methods

2.1 Chemicals

Docetaxel (DTX) and paclitaxel (PTX) were purchased from TCI Chemicals (Japan). 2′-O-(N-methyl-piperazinyl butanoyl) docetaxel (DTX-P) is commercially not available and was kindly provided to our group by Prof. Holger Grüll (UKK Cologne). Trifluoroacetic acid (TFA, HPLC grade) was obtained from Sigma Aldrich GmbH (Munich, Germany). All other chemicals used in this study are from the same commercial sources as described in previous chapters.

2.2 Preparation of TSL

2.2.1 Docetaxel (DTX)

TSL were prepared by the lipid hydration and extrusion method. TSL were composed either of DPPC:DSPC:$DPPG_2$ ($DPPG_2$-TSL) or DPPC:MSPC:DSPE-PEG_{2000} (LTSL) in a molar ratio of 50:20:30 or 90:10:4, respectively. DTX-containing $DPPG_2$-TSL and LTSL were prepared and purified from non-encapsulated DTX exactly the same way as described for Dexamethasone (DXM) (Chapter 3). The initial molar DTX:lipid ratio during formation of the DTX-containing lipid film was 0.05 for both TSL.

2.2.2 Docetaxel prodrug (DTX-P)

Lipids ($DPPG_2$-TSL) were dissolved separately in chloroform:methanol (9:1, vol:vol) and combined in a round-bottomed flask. The lipid film was formed under reduced pressure using a rotary evaporator. The lipid film was hydrated with an aqueous solution of 240 mM $(NH_4)_2SO_4$ (pH=4) for 30 min at 60°C to obtain MLV at 50 mM lipid concentration. MLV were extruded 10 times through two 200 nm pore size membranes (Whatman, Nuclepore

Track-Etch Membrane) using a thermobarrel extruder at 60 °C (Northern Lipids, Vancouver, Canada). Subsequently, $DPPG_2$-TSL were cooled at 4°C for at least 10 min. A subsequent size-exclusion with a PD10 column, pre-equilibrated with HEPES-buffered saline (HBS, pH=7.4), was performed to exchange the extra-liposomal phase to HBS. The active loading of DTX-P into $DPPG_2$-TSL was performed as described in [18] with slight modifications. In detail, DTX-P (10 mM in absolute ethanol) and pre-formed $DPPG_2$-TSL (35-40 mM lipid) were spiked into pre-heated HBS (37°C) to yield the final $DPPG_2$-TSL concentration of 3 mM lipid with the final molar DTX-P:lipid ratio of 0.1. The loading mixture (12 ml) was incubated in a 15 ml falcon placed in a thermoshaker for 60 min (750 rpm, 39-40°C) whereby the desired temperature (37°C) of the loading solution was controlled by a temperature probe. Afterwards, the loading solution (12 ml) was transferred to a 50 ml centrifugal tube and diluted to 50 ml with cold HBS (4°C). After subsequent centrifugation for 60 min (75000xg, 15°C) in a high-speed centrifuge, the supernatant containing the non-encapsulated drug was discarded and the precipitated $DPPG_2$-TSL was resuspended in 0.5 ml HBS to yield DTX-P loaded $DPPG_2$-TSL (25-30 mM lipid). The resuspended $DPPG_2$-TSL-DTX-P were additionally purified from non-encapsulated DTX-P by size-exclusion with PD10 column, pre-equilibrated with HBS.

2.3 Analytical characterization of TSL

Dynamic light scattering (DLS), quantification of the lipid concentration during and after TSL preparation by the phosphate assay and determination of the lipid composition in final TSL formulations by the thin layer chromatography (TLC) were performed as previously described (Chapter 2, 3).

2.4 Temperature-dependent DTX and DTX-P retention in TSL

DTX- or DTX-P containing TSL were diluted to 3.0 mM lipid (1:10) with either HBS or fetal calf serum (FCS). 120 µl samples were distributed in Eppendorf tubes and incubated at a certain temperature in a thermomixer (750 rpm). Immediately after the incubation (without cooling), TSL samples were purified by size-exclusion with manually prepared spin columns filled with Sepharose CL-4B to remove the released (free or protein-bound) drug. Before use, the columns were washed three-times with HBS. 100 µl TSL sample was transferred on CL-4B mini spin column and centrifuged for 2 min (25°C, 2000xg) resulting in elution of 100 µl (drug-containing) TSL. Drug retention in TSL was quantified by HPLC-UV after extraction from TSL as described in the sections 2.5-2.6.

2.5 Extraction and quantification of DTX by HPLC-UV

For sample extraction, TSL samples after CL-4B purification (100 µl) were spiked with 10 µl of PTX as an internal standard (5 mg/ml in methanol). Subsequently, 190 µl of methanol was added and the mixture incubated in a thermomixer for 20 min (RT, 1200 rpm) to allow the full extraction of DTX and PTX from TSL and precipitated proteins. Afterwards, the samples were centrifuged for 10 min (RT, 16000xg) and the supernatants transferred to HPLC vials. DTX and PTX were quantified by HPLC-UV (230 nm). An Aqua 5u C18 (125 Å, 250 x 4.60 mm) column was operated at 40°C with a flow of 0.4 ml/min and a mobile phase composed of methanol:H_2O (70:30, vol:vol). The retention times for PTX and DTX were 26 and 29 min, respectively. The injection volume was 100 µl. Six calibration (CAL) standards (95 µl) were prepared in HBS or FCS yielding a calibration range of 4-250 µg/ml for DTX or PRN. CAL samples were exposed to the same extraction procedure as TSL samples. Linear response was obtained over the whole calibration range with $R^2 > 0.99$.

2.6 Extraction and quantification of DTX-P by HPLC-UV

For sample extraction, 900 µl methanol was added to the TSL samples (100 µl) after CL-4B purification and the mixture incubated in a thermomixer for 20 min (RT, 1200 rpm) to allow the full extraction of DTX-P from TSL and precipitated proteins. The samples were subsequently centrifuged for 10 min (RT, 16000xg) and the supernatant (900 µl) was transferred to a glass tube to be dried at 60°C under nitrogen stream (typically 20 min). The pellet containing TSL lipids and DTX-P was resuspended under strong vortexing with 300 µl of acetonitrile:H_2O (50:50, vol:vol). The mixture was transferred to an Eppendorf tube and centrifuged for 10 min (RT, 16000xg). The supernatant containing DTX-P was transferred to an HPLC vial. DTX-P was quantified by HPLC-UV (230 nm) as described in [18] with slight modifications. A µBondapak C18 (125 Å, 3.9x300 mm) was operated at 20°C with a flow of 1.5 ml/min using mobile phase A containing acetonitrile with 0.01% TFA (vol:vol) and mobile phase B containing water with 0.01% TFA (vol:vol). The gradient applied was 2 min at 38% (A), 30 seconds 38%-65% A, 2.5 min at 65% A, 30 seconds back to 38% A with a total run time of 11 min. The elution time of DTX-P was 5.9 min. The injection volume was 150 µl. Since a suitable internal standard for DTX-P was not available, the quantification of DTX-P was performed based on a standard line with six CAL standards (100 µl) yielding a calibration range of 4-250 µg/ml. CAL samples were exposed to the same extraction procedure as TSL samples. Linear response was obtained over the whole calibration range with $R^2 > 0.99$.

2.7 Determination of encapsulation efficacy (EE)

EE of DTX and DTX-P in TSL was calculated according to the following formula:

$$EE\ (\%) = \frac{C_e(drug) * C_i(lipid)}{C_e(lipid) * C_i(drug)} * 100$$

$C_e(drug)$ = drug concentration (mmol/L) in the final TSL formulation

$C_e(lipid)$ = lipid concentration (mmol/L) in the final TSL formulation

$C_i(drug)$ = drug concentration (mmol/L) used initially for TSL preparation

$C_i(lipid)$ = lipid concentration (mmol/L) used initially for TSL preparation

As described in previous sections, the lipid and drug concentrations were quantified by the phosphate assay and HPLC-UV, respectively.

3. Results

3.1 Characterization of TSL formulations

Table 2: Characterization of TSL formulations. The values are given as mean of three independently prepared TSL batches.

TSL	z-average (nm)	PDI	ζ-potential (mV)	T_m (°C)	drug/lipid (mol/mol)	EE (%)
$DPPG_2$-TSL-DTX	162 (±2)	0.09 (±0.03)	-25.3 (±2.4)	42.2 (±0.2)	0.038 (±0.010)	75.3 (±20.8)
LTSL-DTX	137 (±3)	0.08 (±0.02)	-1.9 (±0.5)	41.8 (±0.1)	0.034 (±0.007)	67.3 (±14.7)
$DPPG_2$-TSL-DTX-P	196 (±8)	0.12 (±0.05)	-26.4 (±1.5)	not measured	0.045 (±0.005)	44.7 (±4.6)

TSL: thermosensitive liposome, PDI: polydispersity index, EE: encapsulation efficacy, T_m: transition temperature, DTX: Docetaxel, DTX-P: Docetxal prodrug DP.

All TSL had a narrow size distribution with a polydispersity index (PDI) of ~0.1 (Table 1). $DPPG_2$-TSL had a negative ζ-potential due to incorporation of the negatively charged $DPPG_2$ at a molar ratio of 30%. The expected lipid composition was confirmed for all TSL formulations by TLC (data not shown). No lysolipid formation was detected in $DPPG_2$-TSL formulations whereas LTSL contained MSPC lysolipid (10% mol) (data not shown). Passive loading of DTX into $DPPG_2$-TSL and LTSL by adding it to the lipid film led to a comparable and high encapsulation efficacy (EE) in final TSL. In contrast to DTX, the prodrug DTX-P was actively loaded into the aqueous core of $DPPG_2$-TSL. The transition temperature (T_m) was not influenced after incorporation of DTX in comparison to empty TSL (T_m of empty $DPPG_2$-TSL and LTSL are given in Chapter 3 and Chapter 5). The T_m of $DPPG_2$-TSL containing DTX-P could not be measured due to a very limited amount of DTX-P available.

3.2 Temperature-dependent DTX retention

$DPPG_2$-TSL and LTSL showed a decreased retention of DTX at 37°C and hyperthermia (HT, 42-43°C) in comparison to room temperature (RT) after 5 or 60 min in the corresponding release medium (HBS or FCS) (Figure 1). However, no significant difference in DTX retention could be detected at 37°C and HT.

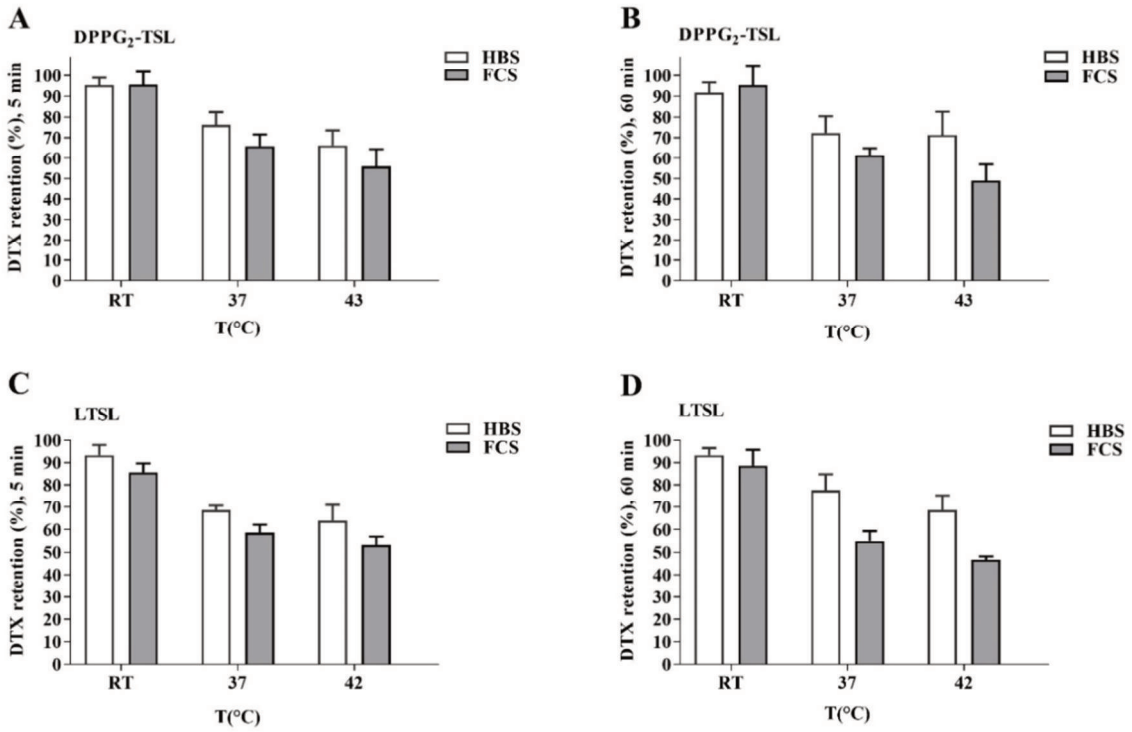

Figure 1: Temperature-dependent retention of DTX in $DPPG_2$-TSL (A, B) and LTSL (C,D). $DPPG_2$-TSL-DTX and LTSL-DTX were incubated for 5 min or 60 min at corresponding temperatures in HEPES-buffered saline (HBS, pH=7.4) or fetal calf serum (FCS). The values are given as mean of three independently prepared liposome batches. The statistical significance of hypethermia (HT) values (42 or 43°C) versus body temperature (37°C) in the corresponding medium (FCS or HBS) was analysed by the unpaired t-test.

3.3 Temperature-dependent DTX-P retention

In contrast to DTX, DTX-P showed a significantly decreased retention in $DPPG_2$-TSL at HT in comparison to 37°C (Figure 2). In HBS, DTX-P retention was 68.3±9.9% at 42°C and 62.0±9.2% at 43°C in comparison to 93.0±9.2% at 37°C after 5 min. In FCS, the difference became even more pronounced with DTX-P retention values of 41.3±5.9% at 42°C, 41.0±3.0% at 43°C and 97.0±8.5% at 37°C after 5 min. The incubation for 60 min did not significantly change the retention values at 37°C but reduced the HT values even further in presence of FCS.

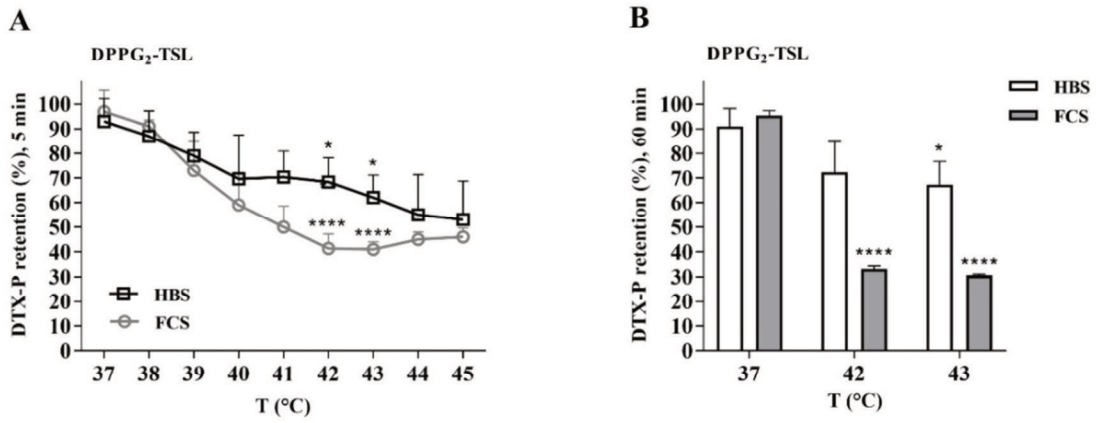

Figure 2: Temperature-dependent retention of DTX-P in $DPPG_2$-TSL (A, B). $DPPG_2$-TSL-DTX-P were incubated for 5 min or 60 min at corresponding temperatures in HBS or FCS. The values are given as mean of three independently prepared liposome batches. The statistical significance of HT values (42-43°C) versus body temperature (37°C) in the corresponding medium (HBS or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, ****=p< 0.0001

4. Discussion

The objective of this study was to investigate if docetaxel (DTX) shows an enhanced release from $DPPG_2$-TSL at hyperthermia (HT). Furthermore, a thermosensitive release of a DTX prodrug (DTX-P) which is suitable for the active loading, was studied from $DPPG_2$-TSL.

DTX did not show a significantly higher release from $DPPG_2$-TSL and LTSL at HT in comparison to 37°C. As a lipophilic compound it is incorporated in the TSL bilayer. The TSL bilayer is in the solid-gel phase below the transition temperature ($<T_m$) and undergoes a transition to a liquid-disordered state at HT ($\geq T_m$) [22]. However, the significant difference in DTX release was only observed between room temperature (RT) and 37°C when the TSL bilayer is still in the solid-gel phase at both temperatures. A shift of T_m to lower temperatures after incorporation of DTX was not observed for $DPPG_2$-TSL and LTSL as confirmed by differential scanning calorimetry (DSC). Therefore, the release of DTX from both TSL was presumably due to increasing aqueous solubility of DTX at a higher temperature of the release medium. Indeed, a decrease of lipid solubility in unilamellar liposomes (by four times) was observed for a structurally similar paclitaxel (PTX) with increasing temperature (25°C vs 35°C) [23].

These results are in line with observations for dexamethasone (DXM) where a significant difference in release was also observed between RT and 37°C but not 37°C and HT (Chapter 3). On the contrary, hexadecylphosphocholine (HePC) did show a HT-enhanced release from $DPPG_2$-TSL (Chapter 2). However, the temperature-dependent release behaviour of HePC is unique among the investigated lipophilic compounds in this thesis. We assume that it exhibits

a trans-bilayer movement (flip-flop) from the inner leaflet to the outer leaflet of TSL through the packing defects at boundaries between solid and liquid phases at T_m and/or a generally increased flip-flop in a liquid-disordered bilayer at $\geq T_m$ (Chapter 2). Besides, HePC has a charged headgroup which makes the trans-bilayer movement of HePC energetically unfavorable. In contrast to HePC, DTX and DXM are not charged and can exchange between leaflets. Ultimately, HePC is a lipid which has a strong interaction with host lipids of TSL. Therefore, HePC release was not observed under serum-free conditions and a presence of biological acceptors was required. Although DXM and DTX are lipophilic, they were released upon dilution in buffer (HBS) since they do not have structural elements which would favor their interaction with host lipids of TSL as HePC. The important contribution of the lipid:lipid interaction was confirmed for dexamethasone-21-palmitate (DP) which demonstrated a stable retention at all investigated temperatures in TSL, independently of the release medium (Chapter 3).

Although PTX and DTX were proposed as suitable candidates for delivery by LTSL (by the same group) [19, 20], it is to our knowledge the only report about a HT-accelerated release of a bilayer-deposited lipophilic drug from TSL. Moreover, the practical significance of these results is arguable with regards to the *in vivo* applications since the release was investigated in a highly artificial setting by dialysis, under serum-free conditions and in presence of hydrotropic agents. However, the diffusion rate of a drug through a dialysis membrane and interaction of LTSL with hydrotropic agents might depend on temperature. Moreover, LTSL is disintegrated to open liposomes and bilayer discs upon prolonged heating at T_m [24]. It is not stated by the authors if these effects were ruled out in this study. Finally, LTSL also released a considerable amount of drug at 37°C, albeit at a slower rate than at 42°C.

In contrast to DTX, the prodrug DTX-P demonstrated a stable retention in $DPPG_2$-TSL at 37°C within 60 min in presence of serum and a rapid release at HT. Indeed, DTX-P was developed in view of the poor taxane retention in liposomes in circulation [6, 10, 11, 17] and demonstrated a drastically prolonged pharmacokinetics in comparison to Taxol [17]. Since DTX-P has an amino group, it is suitable for the active loading by ammonia-based pH gradient into the interior of the liposomes (pH=4) forming a positively charged and therefore bilayer-impermeable drug [17]. However, DTX-P can rapidly escape from TSL through the packing defects formed in the bilayer at HT [25]. Moreover, DTX-P was shown to be rapidly converted to active DTX in presence of plasma by esterases [17, 18].

In conclusion, we could not detect a HT-increased release of DTX from $DPPG_2$-TSL and LTSL. In agreement with studies on non-thermosensitive liposomes, a poor retention of DTX was observed at 37°C. DTX-P represents a prodrug of DTX which is stably retained at 37°C in $DPPG_2$-TSL in serum but is rapidly released at HT. Considering the results obtained for DXM (Chapter 3), DTX is a potential candidate for cyclodextrin (CD) mediated loading which can lead to improved retention and at the same time HT-induced release from TSL.

5. References

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CHAPTER 5: Serum components and osmotic stress are crucial for release of macromolecules from phosphatidyldiglycerol-based thermosensitive liposomes

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Note:

The manuscript was written by Alexander Rysin. The manuscript is ready for submission and will be published after the final decision on patenting.

All experiments presented here were designed and performed by Alexander Rysin. Wouter Lokerse developed the purification method for TSL preparation with a hard protein corona. Agnieszka Mach is thanked for technical assistance with release experiments. The project would not have been possible without a scientific guidance of Lars Lindner, Gerhard Winter and Wouter Lokerse. All the authors is thanked for a critical review of the manuscript.

1. Abstract

Thermosensitive liposomes (TSL) are increasingly recognized as suitable systems for a local delivery of (bio)-macromolecules in combination with mild hyperthermia (HT). A low-temperature sensitive liposome (LTSL) is hereby the most investigated TSL formulation due to the postulated release mechanism involving a lysolipid-mediated formation of nanopores at transition temperature (T_m). Here, we considered the potential of a lysolipid-free TSL containing 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol ($DPPG_2$) for the delivery of macromolecular agents. Carboxyfluorescein (CF), fluorescently conjugated dextrans (FD) and bovine serum albumin (F-BSA) were used as model compounds for estimation of $DPPG_2$-TSL permeability at T_m in physiological saline and full serum. Equilibrium loading revealed an increased bilayer permeability of $DPPG_2$-TSL to CF at T_m but in contrast to LTSL not to FD with a molecular weight of 4 kDa. $DPPG_2$-TSL and LTSL demonstrated a comparable release of FD 10 kDa (~25%) and FD 70 kDa (~10-15%) after 5 min at T_m which only marginally increased after 60 min and was only observed in presence of serum. Serum components strongly associated with $DPPG_2$-TSL were found to induce CF and FD-10 release equivalent to full serum. Intra-liposomal hypo- and hyper-osmolarity could increase the release of FD-70 (~40-50% after 5 min) from $DPPG_2$-TSL at T_m in serum but not in physiological saline. In contrast to CF and FD, F-BSA showed an unspecific release from $DPPG_2$-TSL below T_m which remained unchanged at HT under iso-osmotic conditions. On the contrary, intra-liposomal osmotic imbalance could significantly increase the release of F-BSA at T_m from $DPPG_2$-TSL. Osmotic stress and permeabilizing effects of serum components at T_m are determinants of the macromolecule release from $DPPG_2$-TSL.

2. Introduction

Macromolecular therapeutics emerged as promising medications for a variety of severe clinical indications including oncologic, immunological or genetic disorders [1, 2]. They mainly include biopharmaceuticals such as peptides, recombinant proteins or nucleic acid analogs which fulfill highly complex functions as enzymes, hormones, cytokines or act on a genetic level [3, 4]. However, an efficient pharmaceutical delivery of these agents remains challenging due to their high molecular weight, susceptibility to chemical and enzymatic degradation and poor permeation through biological membranes [5]. Consequently, short *in vivo* half-lives, low (intracellular) delivery efficiencies and immunogenicity are common drawbacks associated with a parenteral administration of biopharmaceuticals [6]. Several strategies including chemical modifications or use of colloidal delivery systems have been realised to improve safety, biodistribution and bioavailability of biopharmaceuticals [7, 8].

Liposomes are vesicles composed of lipid bilayers which demonstrated their potential as delivery vehicles for different classes of therapeutic agents [9]. For biomacromolecules, liposomal encapsulation led to reduced degradation, prolonged circulation time, improved toxicity and therapeutic efficacy [10-13]. However, traditional liposome formulations can barely offer control over a spatial and temporal release of therapeutic agents in target cells or diseased tissue. Stimuli-responsive nanocarriers provide an opportunity for a controlled release of drugs as successfully demonstrated for thermosensitive liposomes (TSL) in treatment of solid tumors [14-17]. TSL are capable to release their cargo in the mild hyperthermia (HT) range (41-43°C) and proved to be suitable delivery systems for several small molecules [18, 19].

Moreover, TSL are gaining increasing interest as a potential delivery system for macromolecular therapeutics. A low temperature-sensitive liposome (LTSL) is hereby the most investigated TSL formulation which was shown to release various types of macromolecules including dextranes, enzymes and proteins [20-23]. The composition of LTSL is distinct from other TSL formulations since it contains a combination of a lysolipid and DSPE-PEG_{2000} which are postulated to form nanopores (~10 nm) at HT enabling a release of high-molecular weight compounds [24].

The objective of this study was to investigate the potential of a lysolipid- and DSPE-PEG-free TSL formulation for the release of macromolecular agents. We used a 1,2-dipalmitoyl-sn-

glycero-3-phospho-1‘-rac-diglycerol ($DPPG_2$)-containing TSL which preclinically demonstrated promising results for several (cytotoxic) small-molecules [16, 25, 26]. Previously, we reported that $DPPG_2$-TSL shows a drastically accelerated cargo release in presence of serum proteins [27, 28]. We sought to determine if serum components might also contribute to the release of high molecular weight compounds from $DPPG_2$-TSL. Therefore, we investigated the permeability of $DPPG_2$-TSL in serum in comparison to physiological saline using fluorescent markers including carboxyfluorescein (CF), fluorescein isothiocyanate (FITC) conjugated dextrans (FD) of different molecular weight as well as FITC-bovine serum albumin (F-BSA). Previous studies showed that CF release from $DPPG_2$-TSL was not affected by the osmotic pressure of the release medium (100-600 mOsm/L) [27]. Here, we examined the influence of the intra-liposomal hypo- and hyperosmolarity on the release of FD and F-BSA from $DPPG_2$-TSL. Based on the results in this study, we believe that lysolipid-free TSL might be also effective for a local delivery of (bio)-macromolecules.

3. Materials and Methods

3.1 Chemicals

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC) were purchased from Corden Pharma (Switzerland). $DPPG_2$ was provided by Thermosome (Munich, Germany). The ammonium salt of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG_{2000}) was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). 5(6)-Carboxyfluorescein (CF), fluorescein isothiocyanate conjugated dextrans with molecular weights of 4, 10 and 70 kDa (FD4 , FD10, FD70) and fluorescein isothiocyanate conjugated bovine serum albumin (F-BSA) were purchased from Sigma Aldrich GmbH (Munich, Germany). Fetal calf serum (FCS) was from Biochrom AG (Berlin, Germany). Sepharose CL-4B and Sephadex G-25 M (PD-10 columns) were obtained from GE-Healthcare (Chicago, USA). TLC Silica gel 60 glass plates (10x20 cm) and the phosphate standard solution (1000 mg/ml) were from Merck KGaA (Darmstadt, Germany). Aluminium pans (standard crucible with lid, 40 µl) were from IVA Analysentechnik GmbH & Co. KG (Germany). The Detergent-Compatible (DC) Protein Assay Kit was obtained from Bio-Rad Laboratories (Germany). All other chemicals were either from Carl Roth GmbH (Karlsruhe, Germany) or Sigma Aldrich GmbH (Munich, Germany).

3.2 Preparation of TSL

TSL were prepared by the lipid hydration and extrusion method. TSL were composed either of DPPC:DSPC:$DPPG_2$ ($DPPG_2$-TSL) or DPPC:MSPC:DSPE-PEG_{2000} (LTSL) in a molar ratio of 50:20:30 or 90:10:4, respectively. Lipids were dissolved separately in chloroform:methanol (9:1, vol:vol) and combined in a round-bottomed flask. The lipid film was formed under reduced pressure using a rotary evaporator. The hydration of the lipid was performed either with an aqueous solution of the sodium salt of carboxyfluorescein (CF, 100 mM, pH=7.4) or HEPES-buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH=7.4) containing either CF (5 mM), FITC-dextran 10 kDa (FD10, 50 mg/ml, 5 mM), FITC-dextran 70 kDa (FD70, 50 mg/ml, 0.7 mM) or FITC-bovine serum albumin (F-BSA, 20 mg/ml, 0.3 mM). Prior to hydration, the physiological osmolarity of the above mentioned solutions was confirmed by the osmolarity measurement. For TSL with hypo-osmotic or hyper-osmotic internal aqueous compartments, FD70 (50 mg/ml) and F-BSA (20 mg/ml) were dissolved either in distilled water or a hyper-osmolar HBS (20 mM HEPES, 450 mM NaCl, pH=7.4), respectively. In all cases, the lipid films were hydrated for 30 min at 60°C to obtain MLV at 50 mM lipid concentration. MLV were extruded 10 times through two 200 nm pore size membranes (Whatman, Nuclepore Track-Etch Membrane) using a thermobarrel extruder at 60 °C (Northern Lipids, Vancouver, Canada). TSL were purified from non-encapsulated CF with Sephadex G-25 columns whereas CL-4B resin was used for other fluorescent markers. The size-exclusion columns were in all cases pre-equilibrated with HBS at physiological osmolarity.

3.3 Osmolarity Measurement

Osmolarity was measured in a vapor pressure osmometer (Vapro 5600, Wescor Inc., Logan, Utah, USA). Before each measurement series, the osmometer was calibrated with three standard solutions with osmolarities of 100 mOsm/L, 290 mOsm/L and 1000 mOsm/L (Optimole, Wescor Inc., Logan, Utah, USA).

3.4 Dynamic light scattering (DLS)

Z-average, polydispersity index (PDI) and ζ-potential of the final TSL formulations were measured by DLS using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). TSL (0.5 mM) were diluted in physiological saline prior to DLS measurements.

3.5 Differential scanning calorimetry (DSC)

The transition temperature of TSL formulations was determined by DSC. Liposome suspensions (20 µl, 30 mM) were transfered into aluminium pans, closed and measured on Mettler Toledo DSC 821e (Mettler Toledo, Giessen, Germany). The samples were scanned from 20°C to 60°C at a heating rate of 2K/min.

3.6 Measurement of lipid concentration (phosphate assay)

The lipid concentration was determined by the phosphate assay as described in detail elsewhere [29]. TSL formulations were diluted with distilled water, sulfuric- and perchloric acid containing solutions were added and the samples heated for 2 hours at 300°C. Subsequently, ammonium heptamolybdate was added and the formed complex measured at 660 nm in a spectrophotometer (Beckmann DU 640, Beckman Coulter GmbH, Krefeld, Germany). The quantification was performed based on calibration samples obtained from 1 g/l phosphate solution treated the same way like TSL samples.

3.7 Measurement of lipid composition (thin layer chromatography)

Thin layer chromatograpy (TLC) was used to confirm the lipid composition of the final TSL formulations. TSL (1500 nmol) were diluted in 1 ml physiological saline and 2 ml chloroform:methanol (1:1 vol:vol) was added. After short vortexing, the mixture was centrifuged for 10 min (25°C, 3200xg). The chloroform phase containing the lipids was tranferred to a new tube and dried under nitrogen stream (40°C). The dried lipids were redissolved in 100 µl of chloroform:methanol (9:1 vol:vol) and 1.5 µl were spotted on a TLC plate. The mobile phase was composed of chlorofom/methanol/acetic acid/H_2O (100:60:10:5 v:v). A standard solution with corresponding lipids was used for the lipid spot identification. After the run, the lipids (MSPC, DPPC/DSPC, $DPPG_2$ and DSPE-PEG_{2000}) were visualized as separate blue spots after staining with molybdenum spray of Dittmer and Lester [30]. The intensity of the lipid spots represented the relative lipid composition of each formulation and was analyzed densitometrically with ImageJ.

3.8 Quantification of temperature-dependent carboxyfluorescein (CF) release from TSL

The quantification of CF release was performed as described by our group elsewhere [27]. $DPPG_2$-TSL or LTSL (20 µl, 0.1 mM lipid) containing CF at a self-quenched concentration (100 mM) were diluted in physiological saline or fetal calf serum (FCS) and incubated in a pre-heated thermomixer at different temperatures for 5 and 60 min. Immediately after the incubation, the samples were cooled down on ice for 5 min and afterwards 1000 µL of TRIS

buffer (10 mM TRIS in 0.9% NaCl, pH=8.0) was added. The (low) fluorescence value obtained for TSL incubated at room temperature (RT) was used as background. The 100%-value for CF release was obtained after lysis of TSL (20 µl, 0.1 mM lipid) with 10% Triton X-100 (20 µl) and incubation at 45°C for 15 min. For Triton samples, 980 µl of the TRIS buffer was added. All samples were analyzed for CF release by fluorescence measurement at Ex = 493 nm / Em = 513 nm using a Cary Eclipse fluorescence spectrometer (Varian Inc., Palo Alto, CA, USA).

3.9 Quantification of temperature-dependent retention of fluorescent markers in TSL

Since FITC-dextrans (FD10, FD70), FITC-albumin (F-BSA) and CF (5 mM) were not fully quenched in both $DPPG_2$-TSL and LTSL at encapsulated concentrations, the quantification of released fluorescent markers as described in section 3.8 was not possible. Therefore, we quantified the retention of fluorescent markers in TSL after removal of the released compound using a purification with manually prepared CL-4B mini spin columns. $DPPG_2$-TSL or LTSL (20 µl, 1.0 mM lipid for CF and FD10 and 2.0 mM lipid for FD70 and F-BSA) were first diluted in physiological saline or FCS and subsequently incubated in a pre-heated thermomixer at different temperatures for 5 and 60 min. Immediately after the incubation, the samples were put on ice for 5 min and afterwards 100 µl of physiological saline was added. To remove the released fluorescent marker, TSL samples were purified using CL-4B mini spin columns, pre-washed three-times with physiological saline. In detail, 100 µl TSL sample was transferred on a column and centrifuged for 2 min (25°C, 2000xg) resulting in elution of 100 µl TSL. To disrupt TSL and liberate the retained fluorescent marker, 20 µl of 10% Triton X-100 was added to all samples which were subsequently incubated at 45°C for 15 min. Afterwards, 1000 µL of the TRIS buffer was added. The fluorescence value obtained for TSL incubated at room temperature (RT) was used as 100% retention. All samples were analyzed by fluorescence measurement at Ex= 493 nm / Em= 513 nm for CF, Ex = 490 nm / Em = 520 nm for FD10 and FD70 and Ex = 495 nm / Em = 520 nm for F-BSA using a Cary Eclipse fluorescence spectrometer (Varian Inc., Palo Alto, CA, USA).

3.10 Quantification of TSL permeability by equilibrium loading

The investigation of TSL permeability by equlibrium loading was performed as briefly described for LTSL elsewhere [24]. Empty TSL were combined with either CF or FITC-dextran 4 kDa (FD4) in HBS yielding a final TSL concentration of 17.5 mM lipid and 12.5 mM of CF or FD4 in a final volume of 100 µl. Subsequent sample incubation at room temperature (RT), 37°C and HT (42°C for LTSL and 43°C for $DPPG_2$-TSL) was carried out

under mixing (750 rpm) in a pre-heated thermomixer for 60 min. Immediately after the incubation, the samples were placed for 30 min at 4°C. Non-encapsulated CF and FD4 were removed by several subsequent purification cycles with manually prepared CL-4B mini spin columns as described in 2.9. The liposomal content of CF and FD4 was quantified via fluorescence after disruption of TSL with 10% Triton X-100 as described in 3.9. Calibration samples with seven different concentrations of CF or FD4 were used for quantification yielding a linear response with $R^2 > 0.99$ for both analytes. The lipid concentration was determined via phosphate assay as described in 3.6. The values were finally shown as CF or FD4:lipid ratios (µmol:mmol).

3.11 Preparation of $DPPG_2$-TSL with a hard protein corona

CF- or FD10-containing $DPPG_2$-TSL (100 µl, 25.0 mM lipid) were diluted with 900 µl FCS and incubated for 30 min at 37°C in a pre-heated thermomixer (400 rpm). After the incubation, TSL mixture (1 ml) was purified from unbound serum proteins using a column filled with CL-4B. Previously, the efficient separation of unbound serum proteins from $DPPG_2$-TSL was validated by collection of 40 eluate fractions (1 ml each) and subsequent analysis of lipid and protein concentration in each fraction. The 1 ml fractions (11-15) containing $DPPG_2$-TSL with associated serum proteins were combined and concentrated by ultra-filtration with centrifugal Amicon Ultra 15 10K units (60 min, 20°C, 5000 rpm). Afterwards, the concentrated TSL (200 µl) were tranferred to VivaSpin 500 filter units with molecular cut-off (MWCO) of 1000 kDa and further purified from weakly bound proteins by repeated (4x) centrifugation (40 min, 20°C, 8600 x g) and washing (4x) with 100 µl physiological saline. The final (purified) $DPPG_2$-TSL with a hard protein corona were analysed by DLS, phosphate assay, TLC as well as for the liposomal content of CF and FD10, as described in previous sections. The temperature-dependent release of CF and temperature-dependent retention of FD10 were analysed by spiking $DPPG_2$-TSL with the hard protein corona (0.1 mM lipid for CF and 1 mM lipid for FD10) in physiological saline as described in section 3.8 and 3.9, respectively. Additionally, pure FCS (without added $DPPG_2$-TSL) was purified according to the described procedure. This control experiment is supposed to verify if the serum proteins or serum components which are responsible for the enhanced release from $DPPG_2$-TSL at T_m, are removed by the described purification procedure. Consequently, the release (CF) and retention (FD10) were investigated for $DPPG_2$-TSL (without a hard corona) in the solution obtained from FCS purification as the release medium.

3.12 Protein quantification

The protein content of samples containing either $DPPG_2$-TSL in FCS or $DPPG_2$-TSL with a hard protein corona was determined using a commercial DC™ (detergent compatible) Protein Assay Kit. The dilution and analysis of the samples was performed according to the manual instructions. Six calibration samples (CAL) with bovine serum albumin (BSA) as a standard were used for protein quantification yielding a linear response ($R^2 > 0.99$) in the 5-250 µg/ml protein range. Prior to analysis, TSL and CAL samples were heated at 99°C for 5 min in a 10% sodium dodecyl sulfate (SDS) solution to achieve a TSL solubilisation. Un-solubilised TSL were found to influence the absorbance at 750 nm. 10% SDS is compatible with the assay as stated by the manufatcturer and confirmed by our measurements. The absorbance at 750 nm was analysed with a spectrophotometer (Beckmann DU 640, Beckman Coulter GmbH, Krefeld, Germany).

3.13 Determination of encapsulation efficacy (EE)

Encapsulation efficacy (EE) of investigated fluorescent markers in TSL was calculated according to the following formula:

$$EE\ (\%) = \frac{C_e(marker) * C_i(lipid)}{C_e(lipid) * C_i(marker)} * 100$$

$C_e(marker)$ = drug concentration (mmol/L) in the final TSL formulation

$C_e(lipid)$ = lipid concentration (mmol/L) in the final TSL formulation

$C_i(marker)$ = drug concentration (mmol/L) used initially for TSL preparation

$C_i(lipid)$ = lipid concentration (mmol/L) used initially for TSL preparation

As described in previous sections, the lipid and marker concentrations were quantified by the phosphate assay (section 3.6) and fluorescence measurements (section 3.9), respectively.

4. Results

4.1 Characterization of TSL with carboxyfluorescein (CF) and FITC-dextrans (FD)

$DPPG_2$-TSL and LTSL had a narrow size distribution with a polydispersity index (PDI) of approx. 0.1 (Table 1). The size (nm) increased slightly with the molecular weight of the encapsulated fluorescent marker for both TSL. The ζ-potential of $DPPG_2$-TSL was negative due to incorporation of the anionic lipid $DPPG_2$ at 30 mol%. CF, FD10 and FD70 were encapsulated in TSL by the passive loading during liposome formation. The encapsulation efficacy (EE) was lower for $DPPG_2$-TSL in comparison to LTSL and decreased significantly in case of FD70 for both TSL. The transition temperature (T_m) was not affected by encapsulated compounds and was slightly higher (42-43°C) for $DPPG_2$-TSL than for LTSL (41-42°C).

Table 1: Characterization of TSL formulations. The values are given as mean (±SD) of three independently prepared liposome batches.

TSL	z-average (nm)	PDI	ζ-potential (mV)	T_m (°C)	drug/lipid (µmol/mmol)	EE (%)
$DPPG_2$-TSL	150 (±3)	0.10 (±0.01)	-26.7 (±2.0)	42.1 (±0.1)	no drug used	no drug used
$DPPG_2$-TSL-CF						
-100 mM CF	163 (±1)	0.10 (±0.02)	-25.5 (±0.3)	42.2 (±0.1)	170.0 (±9.7)	8.55 (±0.49)
-5 mM CF	155 (±2)	0.10 (±0.01)	-27.8 (±0.6)	42.5 (±0.1)	7.8 (±0.8)	7.85 (±0.75)
$DPPG_2$-TSL-FD10	158 (±9)	0.10 (±0.01)	-26.1 (±2.8)	42.9 (±0.3)	6.2 (±0.9)	6.21 (±0.87)
$DPPG_2$-TSL-FD70	170 (±1)	0.07 (±0.01)	-28.1 (±2.8)	42.0 (±0.2)	0.55 (±0.04)	3.84 (±0.25)
LTSL	129 (±9)	0.08 (±0.02)	-3.3 (±0.8)	41.9 (±0.3)	no drug used	no drug used
LTSL-CF						
-100 mM CF	127 (±2)	0.06 (±0.01)	-1.7 (±0.9)	41.8 (±0.1)	220.1 (±9.7)	11.01 (±0.48)
-5 mM CF	134 (±1)	0.08 (±0.01)	-2.2 (±1.3)	41.8 (±0.1)	10.9 (±0.4)	10.89 (±0.44)
LTSL-FD10	143 (±2)	0.07 (±0.01)	-1.5 (±1.8)	42.0 (±0.1)	10.2 (±1.2)	10.23 (±1.24)
LTSL-FD70	150 (±5)	0.08 (±0.02)	-2.9 (±2.8)	41.8 (±0.1)	1.12 (±0.04)	7.83 (±0.30)

TSL: thermosensitive liposome, PDI: polydispersity index, EE: encapsulation efficacy, T_m: transition temperature, CF: carboxyfluorescein, FD10 or FD70: FITC-Dextran 10 or 70 kDa, FITC: fluorescein isothiocyanate.

4.2 Investigation of $DPPG_2$-TSL and LTSL permeability by equilibrium loading

Equilibrium loading of fluorescently labeled dextrans into pre-formed, empty liposomes was previously used by the inventors of LTSL as a method to estimate the size of nanopores formed at T_m [24]. Using this method, we tested the permeability of $DPPG_2$-TSL and LTSL for CF and FD4. Both $DPPG_2$-TSL and LTSL led to a significantly higher encapsulation of CF at T_m (42°C for LTSL and 43°C for $DPPG_2$-TSL) in comparison to 37°C (Figure 1). The loading efficacy at T_m decreased for FD4 in comparison to CF for both $DPPG_2$-TSL and LTSL. Furthermore, only LTSL demonstrated a significantly higher FD4 amount loaded at T_m in comparison to 37°C.

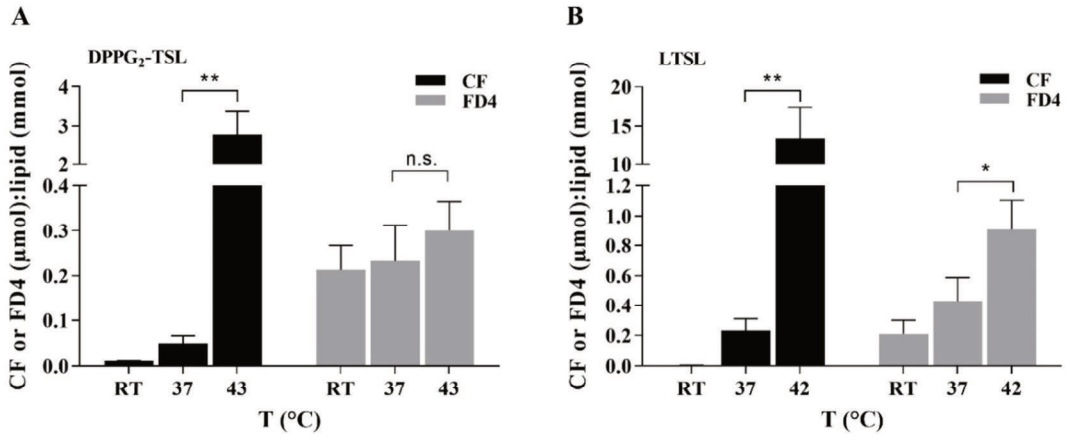

Figure 1: Equlibrium loading of CF and FITC-Dextran 4 kDa (FD4) in $DPPG_2$-TSL (A) and LTSL (B) at corresponding temperatures. Empty, pre-formed TSL were incubated in presence of CF or FD4 in HEPES-buffered saline (HBS) (pH=7.4) at RT, 37°C and T_m (42°C or 43°C) for 30 min. CF or FD4:lipid (µmol:mmol) ratio was determined after removal of non-encapsulated dye by size-exclusion chromatography. The values are given as mean of three independently prepared liposome batches. The statistical significance of hyperthermia (HT) values (41-43°C) versus body temperature (37°C) was analysed by the unpaired t-test. *=p< 0.05, **=p<0.01

4.3 Comparison of temperature-dependent release and liposomal retention for $DPPG_2$-TSL and LTSL using CF

A quenched (low) fluorescence signal is obtained after CF encapsulation in liposomes at high concentrations, e.g. using a hydration solution containing 100 mM CF for the passive loading of TSL [31, 32]. If CF is released from liposomes and sufficiently diluted, the fluorescence intensity increases due to de-quenching and correlates with the released CF amount. Consequently, the released CF can be quantified without a separation from the liposomal (quenched) CF fraction. However, this method is not suitable if fluorescent compounds (e.g. FD) are not (fully) quenched within liposomes. In that case, a separation of released and liposomal compounds is required for an accurate quantification.

We compared the outcome of both assays (release vs. retention) on $DPPG_2$-TSL and LTSL using CF encapsulated at a quenched concentration (100 mM) (Figure 2). A careful examination of the release profile (Figure 2 A, B) and retention profile (Figure 2 C, D) reveals that both assays lead to comparable results. For each temperature and time point, the sum of the corresponding release and retention values results in approx. 100% CF representing a suitable separation and quantification of both free and liposomal CF. In saline, CF release from $DPPG_2$-TSL was 3.0±0.7% and 13.6±1.4% after 5 and 60 min at 43°C whereas for LTSL 56.4±10.5% and 95.2±3.8% after 5 and 60 min were observed at 42°C (Figure 2 A, B). The corresponding retention values were 95.7±1.5% and 87.0±4.4% for $DPPG_2$-TSL as well as 56.7±6.7% and 25.3±3.2% for LTSL (Figure 2 C, D). In fetal calf serum (FCS), the difference in CF release from $DPPG_2$-TSL (67.4±3.2% and 88.2±2.0% after 5 and 60 min at 43°C) and LTSL (87.7±6.4% and 96.8±6.3% after 5 and 60 min min at 42°C) was much less pronounced in comparison to saline (Figure 2 A, B). The corresponding retention values were 46.3±3.1% and 25.0±2.0% for $DPPG_2$-TSL as well as 22.3±4.0% and 19.3±3.5% for LTSL (Figure 2 C, D).

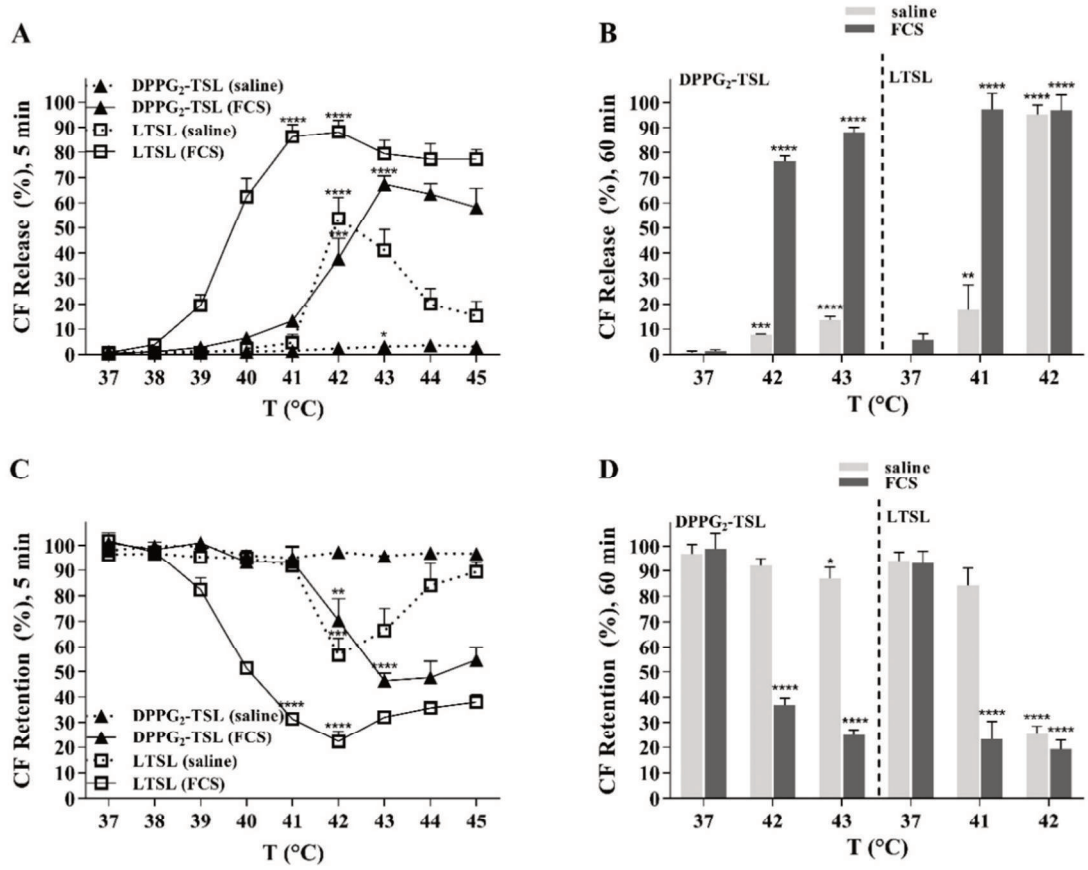

Figure 2: Comparison of temperature-dependent release (A, B) and liposomal retention (C, D) of CF (100 mM) in $DPPG_2$-TSL and LTSL. $DPPG_2$-TSL-CF (100) and LTSL-CF were incubated for 5 min (A, C) or 60 min (B, D) at corresponding temperatures in physiological saline or fetal calf serum (FCS). The values are given as mean of three independently prepared liposome batches. The statistical significance of HT values (41-42°C for LTSL and 42-43°C for $DPPG_2$-TSL) versus body temperature (37°C) in the corresponding medium (saline or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001

4.4 Temperature-dependent retention of fluorescent markers in $DPPG_2$-TSL and LTSL

In our hands, CF (at 5 mM) as well as FD10 and FD70 were quenched to less than 10% in both TSL requiring a separation procedure for an accurate quantification. Therefore, we used the retention assay described in the previous section. To obtain a comparable final marker:lipid ratio in TSL, CF (2 mg/ml) and FD10 (50 mg/ml) were passively loaded using the same molar concentration (5.0 mM) in the hydration solution (Table 1). For FD70, the molar concentration in the hydration solution (50 mg/ml, 0.7 mM) was lower than for CF and FD10 as a consequence of the higher molecular weight. In saline, $DPPG_2$-TSL were permeable to CF leading to a decreased retention at 43°C (85.3±3.2% and 33.3±7.0% after 5 and 60 min) whereas no significant release was observed for FD10 and FD70 (Figure 3 A, B). However, a significant decrease in retention was observed for all compounds at 43°C in FCS (54.3+4.2% for CF, 75.0±1.7% for FD10 and 87.0±2.6% for FD70 after 5 min) which did not significantly change upon prolonged incubation for 60 min.

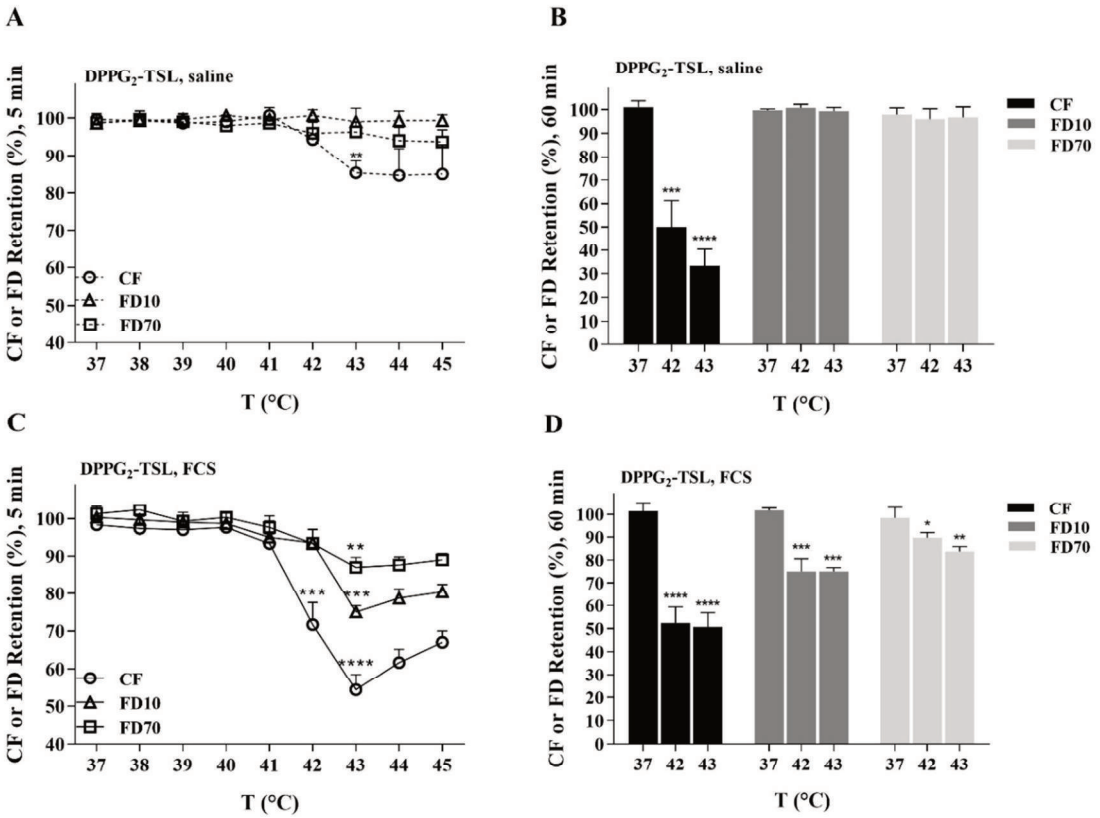

Figure 3: Temperature-dependent retention of CF (5.0 mM), FD10 (5.0 mM) and FD70 (0.7 mM) in $DPPG_2$-TSL in presence of physiological saline (A, B) or FCS (C, D). The values are given as mean of three independently prepared liposome batches. The statistical significance of HT values (41-42°C for LTSL and 42-43°C for $DPPG_2$-TSL) versus body temperature (37°C) in the corresponding medium (saline or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001

Similar to $DPPG_2$-TSL, LTSL were permeable in saline to CF at 42°C (83.0±3.5% and 36.3±3.8% retention after 5 and 60 min), whereas no release of FD10 and FD70 was detected even after a prolonged incubation for 60 min (Figure 4 A, B). However, the retention decreased in FCS at 42°C within 5 min for all markers (36.7±3.2 for CF, 74.7±4.7% for FD10 and 79.7±5.5% for FD70). After 60 min in FCS, the retention further decreased significantly for CF (24.0±1.7%) and FD10 (61.5±2.1%) but stayed nearly unchanged for FD70 (70.3±6.5%).

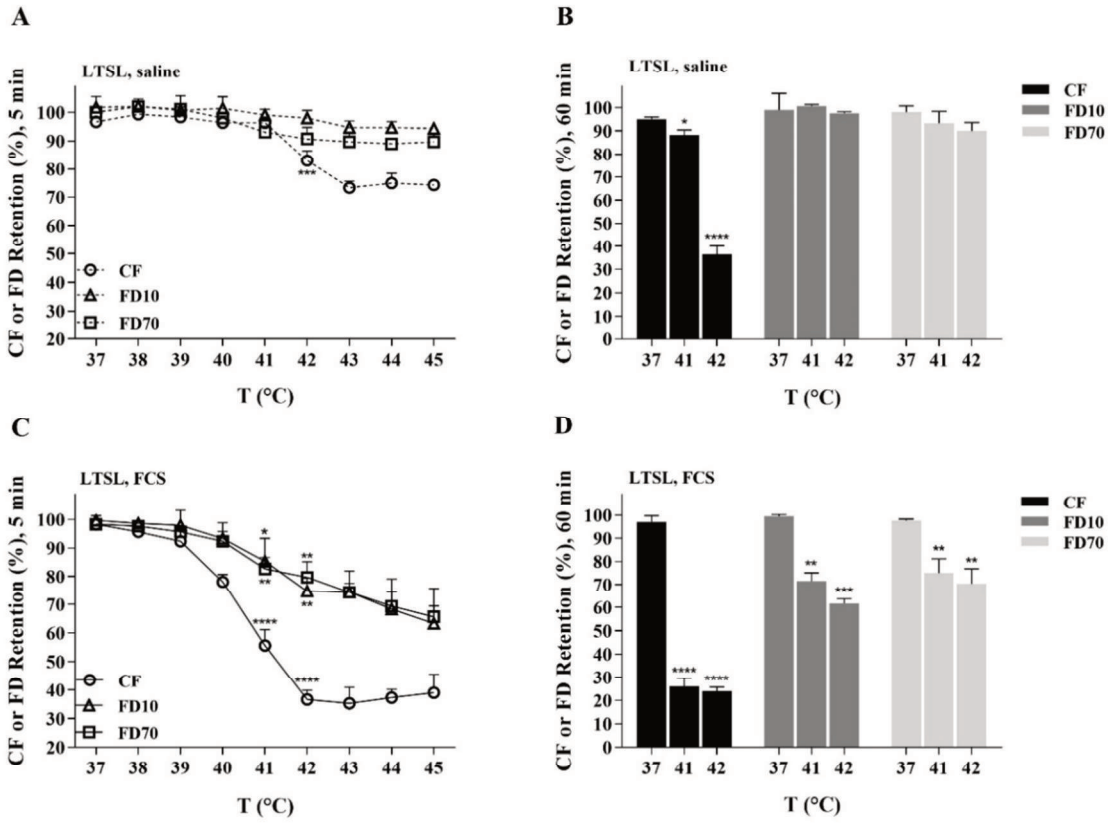

Figure 4: Temperature-dependent retention of CF (5.0 mM), FD10 (5.0 mM) and FD70 (0.7 mM) in LTSL in presence of physiological saline (A, B) and FCS (C, D). The values are given as mean of three independently prepared liposome batches. The statistical significance of HT values (41-42°C for LTSL and 42-43°C for $DPPG_2$-TSL) versus body temperature (37°C) in the corresponding medium (saline or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001.

4.5 Influence of a hard protein corona on the HT-induced release from $DPPG_2$-TSL

We previously reported that serum components (SC) e.g. serum proteins accelerate the release of small molecules from TSL [27, 28]. As demonstrated in previous sections, SC might also contribute to the release of high molecular weight compounds from TSL. We were interested if this permeabilizing effect is already achieved by formation of a hard protein corona during TSL incubation in FCS at 37°C. We selected $DPPG_2$-TSL for this investigation since the release is not biased by the presence of a lysolipid as it is the case for LTSL. $DPPG_2$-TSL with a hard protein corona were obtained according to the purification method developed in our group which involves size-exclusion and ultra-filtration procedures to remove un-bound serum proteins. Purified $DPPG_2$-TSL samples with a hard pr□tein c□r□na (~0.1 mg/ml pr□tein) contained below 1% of the initial pr□tein am□unt (~40 mg/ml protein) as observed for un-purified $DPPG_2$-TSL in FCS. Since we can not rule out the possibility that besides proteins also other serum molecules (e.g. fatty acids) are incorporated in $DPPG_2$-TSL and subsequently not removed by the purification procedure, we use the broad term SC which includes the hard protein corona on the surface of $DPPG_2$-TSL. The size (nm), ζ-potential

(mV) and encapsulated amount of CF and FD10 were not influenced by the purification procedure and presence of the hard protein corona (Table 2). The lipid composition of $DPPG_2$-TSL before and after purification stayed unchanged and no incorporation of lysolipids intrinsically present in FCS was detectable in $DPPG_2$-TSL as analyzed by TLC (data not shown).

Table 2: Characterization of $DPPG_2$-TSL formulations with and w/o hard protein corona. The values are given as mean of three independently prepared liposome batches.

TSL	z-average (nm)	PDI	ζ-potential (mV)	drug/lipid (µmol/mmol)	protein/lipid (mg/mg)
$DPPG_2$-TSL-CF	153 (±1)	0.07 (±0.02)	-27.4 (±1.1)	156.9 (±0.9)	-
$DPPG_2$-TSL-CF-SC	151 (±2)	0.09 (±0.02)	-21.5 (±3.0)	153.6 (±8.8)	0.021 (±0.001)
$DPPG_2$-TSL-FD10	154 (±2)	0.09 (±0.01)	-24.9 (±1.1)	7.1 (±1.5)	-
$DPPG_2$-TSL-FD10-SC	152 (±1)	0.10 (±0.02)	-24.0 (±2.6)	6.9 (±0.8)	0.020 (±0.001)

TSL: thermosensitive liposome, PDI: polydispersity index, CF: Carboxyfluorescein, FD10: FITC-Dextran 10 kDa, FITC: fluorescein isothiocyanate, SC: serum components associated with $DPPG_2$-TSL including the hard protein corona

$DPPG_2$-TSL with a hard protein corona ($DPPG_2$-TSL-SC) and CF encapsulated at a self-quenched concentration (100 mM) were analysed for CF release in physiological saline (Figure 5). Surprisingly, the formation of the hard protein corona (and/or binding of other unknown SC) was sufficient to induce CF release comparable to full FCS (37-43°C after 5 min). Moreover, the release increased even further at 44°C and 45°C. In comparison, $DPPG_2$-TSL without a protein corona barely released any CF in saline within 5 min. In addition, we analysed the CF release in FCS which was purified exactly as $DPPG_2$-TSL-SC. In this case, the release was as slow as in physiological saline (data not shown) indicating that the SC specifically bound or enriched by $DPPG_2$-TSL are responsible for the permeabilizing effect at HT.

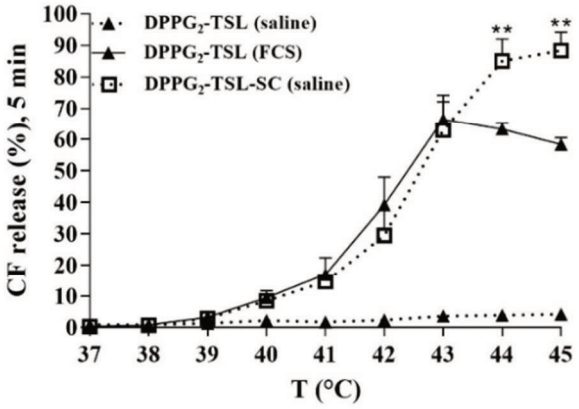

Figure 5: Influence of serum components (SC) bound to $DPPG_2$-TSL on the temperature-dependent release of CF (100 mM). The values are given as mean of three independently prepared liposome batches. The statistical significance of temperature values between $DPPG_2$-TSL (FCS) and $DPPG_2$-TSL-SC (saline) groups was analysed by the unpaired t-test. *=$p<0.05$, **= $p<0.01$

We extended the investigation of SC effects analysing the temperature-dependent retention of FD10 in $DPPG_2$-TSL (Figure 6). Similar to CF, the presence of SC bound to $DPPG_2$-TSL was sufficient to decrease the retention of FD10 at T_m and increase the release of FD10 even more at 44°C and 45 °C within 5 min.

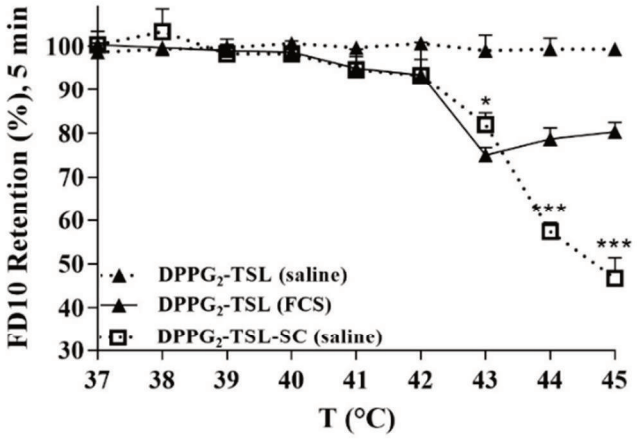

Figure 6: Influence of serum components (SC) bound to $DPPG_2$-TSL on the temperature-dependent retention of FD10. The values are given as mean of three independently prepared liposome batches. The statistical significance of temperature values between $DPPG_2$-TSL (FCS) and $DPPG_2$-TSL-SC (saline) groups was analysed by the unpaired t-test. *=p<0.05, **=p<0.01

4.6 Influence of the osmotic stress on the HT-induced release of (bio)-macromolecules from $DPPG_2$-TSL

Osmolarity differences between the intra- and extra-liposomal compartments can influence the release behaviour of encapsulated compounds from TSL [27, 33]. However, we previously reported that the release of CF, loaded at physiological osmolarity in $DPPG_2$-TSL, was not changed in a serum-free release medium in the osmolarity range of 100-600 mosm/L [27]. Here, we investigate the influence of both hypo- and hyper-osmolarity in the internal aqueous compartment of $DPPG_2$-TSL on the release of FD70 and F-BSA in physiological saline and FCS. To prepare $DPPG_2$-TSL with a different intra-liposomal osmolarity, we used the hydration solutions supplemented with saline and HEPES for the TSL formation and passive loading of FD70 and F-BSA (Table 3). For FD70, the size of $DPPG_2$-TSL increased with higher intra-liposomal osmolarity, whereas for F-BSA this trend was less pronounced (Table 3). The encapsulation efficacy (EE) was approx. 2x higher for FD70 and F-BSA in case of distilled water as a hydration medium in comparison to HBS solutions. Overall, a significantly higher EE was observed for F-BSA in comparison to FD-70 under the same TSL preparation conditions.

Table 3: Characterization of FD70- and F-BSA-containing $DPPG_2$-TSL with different intra-liposomal osmolarities. The values are given as mean of three independently prepared liposome batches.

TSL	Hydration Solution (Composition)	Hydration Solution (Osmolarity, mosm/L)	z-average (nm)	PDI	ζ-potential (mV)	T_m (°C)	drug/lipid (µmol/mmol)	EE (%)
$DPPG_2$-TSL-FD70 (iso-osmotic)	FD70 (50 mg/ml) in HBS (pH=7.4) iso-osmotic	307 (±8)	170 (±1)	0.07 (±0.01)	-28.1 (±2.8)	42.0 (±0.2)	0.55 (±0.04)	3.84 (±0.25)
$DPPG_2$-TSL-FD70 (hypo-osmotic)	FD70 (50 mg/ml) in distilled water hypo-osmotic	68 (±9)	145 (±1)	0.08 (±0.01)	-25.7 (±2.6)	42.2 (±0.1)	1.01 (±0.08)	7.10 (±0.58)
$DPPG_2$-TSL-FD70 (hyper-osmotic)	FD70 (50 mg/ml) in HBS (pH=7.4) hyper-osmotic	928 (±5)	182 (±4)	0.09 (±0.01)	-29.5 (±2.4)	42.7 (±0.2)	0.57 (±0.01)	4.02 (±0.10)
$DPPG_2$-TSL-F-BSA (iso-osmotic)	F-BSA (20 mg/ml) in HBS (pH=7.4) iso-osmotic	306 (±5)	162 (±2)	0.10 (±0.02)	-25.5 (±2.1)	42.1 (±0.1)	0.42 (±0.03)	6.95 (±0.42)
$DPPG_2$-TSL-F-BSA (hypo-osmotic)	F-BSA (20 mg/ml) in distilled water hypo-osmotic	43 (±11)	159 (±8)	0.16 (±0.01)	-22,3 (±1.0)	42.0 (±0.2)	0.69 (±0.01)	11.33 (±0.20)
$DPPG_2$-TSL-F-BSA (hyper-osmotic)	F-BSA (20 mg/ml) in HBS (pH=7.4) hyper-osmotic	875 (±23)	172 (±1)	0.10 (±0.01)	-28.4 (±2.2)	42.8 (±0.1)	0.45 (±0.04)	7.48 (±0.51)

TSL: thermosensitive liposome, PDI: polydispersity index, FITC: fluorescein isothiocyanate

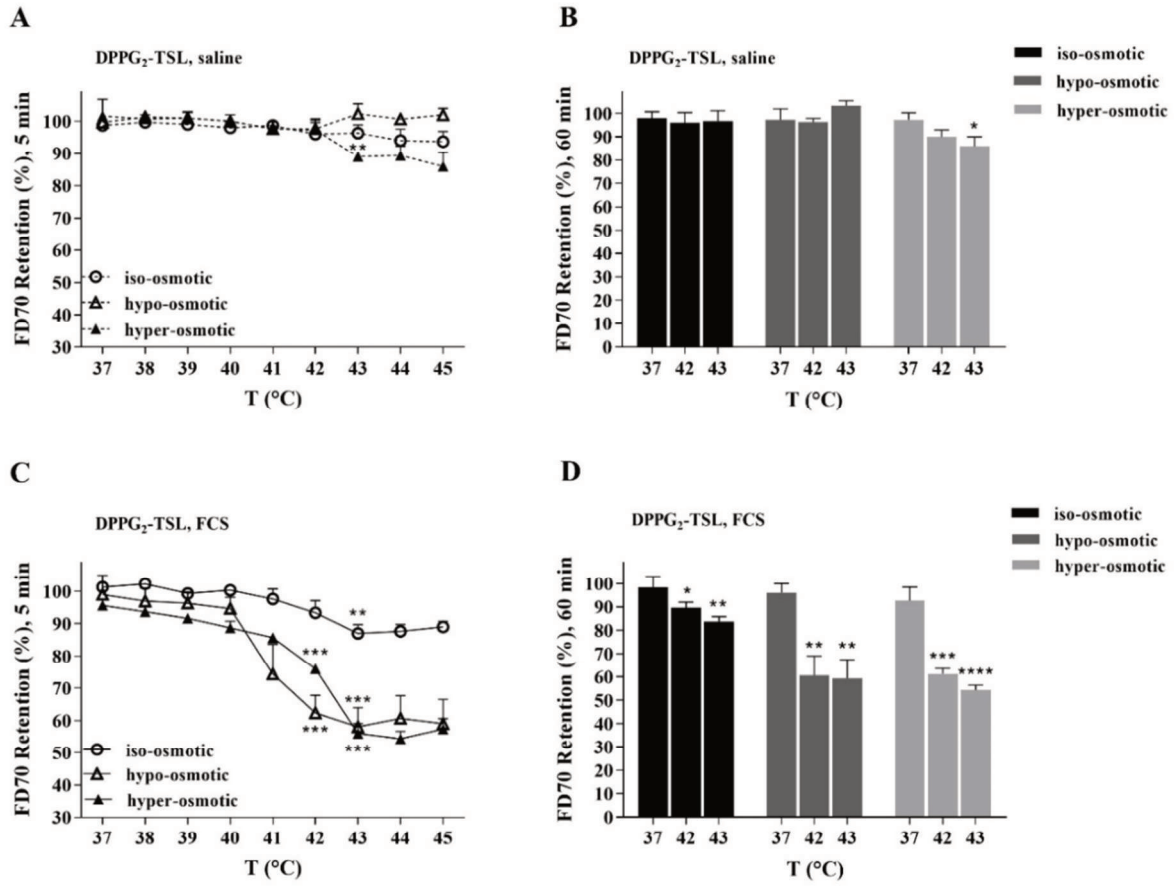

Figure 7: Temperature-dependent retention of FD70 loaded under different osmotic conditions in $DPPG_2$-TSL in presence of physiological saline (A, B) or FCS (C, D). The values are given as mean of three independently prepared liposome batches. The statistical significance of HT values (42-43°C) versus body temperature (37°C) in the corresponding medium (saline or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001.

In saline, a slight decrease of FD70 retention at 43°C was only observed for $DPPG_2$-TSL with the intra-liposomal hyper-osmolarity (Figure 7 A, B). However, the FD70 retention decreased to approx. 50-60% after 5 min at 43°C in FCS for $DPPG_2$-TSL with both intra-liposomal hyper- and hypo-osmolarity which was significantly lower than observed under iso-osmotic conditions (Figure 7 C, D).

In contrast to CF and FD, F-BSA showed unspecific release of approx. 10-20% after dilution in both saline and FCS (Figure 8). For $DPPG_2$-TSL with the physiological intra-liposomal osmolarity, no significant difference in F-BSA retention was observed between 37°C and HT in saline or FCS. For $DPPG_2$-TSL with the intra-liposomal hypo-osmolarity, the F-BSA retention decreased in FCS to 52±1% at 42°C and 43±1% at 43°C in contrast to 77±2% at 37°C after 5 min. For $DPPG_2$-TSL with the intra-liposomal hyper-osmolarity, the difference between HT (71±2% at 42°C and 61±6% at 43°C after 5 min) and 37°C (82±7% after 5 min) became less pronounced. In saline, no statistically significant release differences between 37°C and HT could be detected, independently of the $DPPG_2$-TSL formulation.

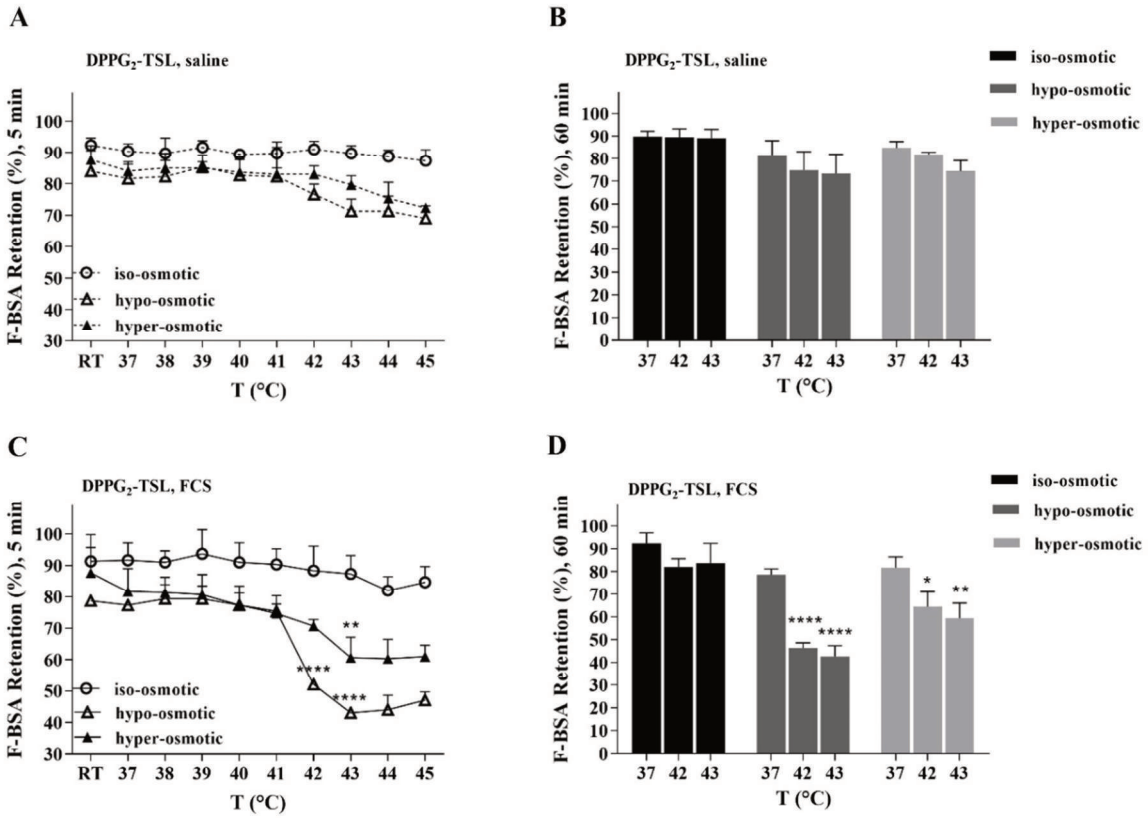

Figure 8: Temperature-dependent retention of F-BSA loaded under different osmotic conditions in $DPPG_2$-TSL in presence of physiological saline (A, B) or FCS (C, D). The values are given as mean of three independently prepared liposome batches. The statistical significance of HT values (42-43°C) versus body temperature (37°C) in the corresponding medium (saline or FCS) was analysed by one way ANOVA followed by Dunnet's post-hoc test. *=p<0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001.

5. Discussion

This study investigated the potential of a 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol-based TSL ($DPPG_2$-TSL) for the release of high molecular weight compounds (≥ 10 kDa). We used a low temperature-sensitive TSL (LTSL) as a reference formulation since it contains the lysolipid MSPC and DSPE-PEG_{2000} which in combination form sterically-stabilized nanopores at the transition temperature (T_m) [24]. Based on the equilibrium loading, Needham et al. concluded that the permeability threshold is achieved for a fluorescent dextran (FD) with a molecular weight of 10 kDa (FD-10) as a largest dextran successfully loaded inside LTSL through nanopores at T_m [24]. Using the same methodology, we could demonstrate that $DPPG_2$-TSL is permeable at T_m to carboxyfluorescein (CF) but not to a FD with a molecular weight of 4 kDa (FD-4). On the contrary, LTSL allowed the passage of FD-4 through the permeabilized bilayer at T_m.

Since the equilibrium loading procedure does not take into account a possible release-enhancing effect of serum components (e.g. serum proteins) [27, 28, 34], we extended our investigation analyzing the release of fluorescent markers. Therefore, we developed a method based on size-exclusion (SE) with CL-4B mini columns that showed an efficient separation of liposomal and released small- and macromolecules. The separation was required since FD were not fully quenched inside both TSL formulations. Indeed, an ultrafiltration and a similar separation procedure based on SE with CL-4B were described recently by other groups investigating the release of macromolecules from TSL [22, 23]. In our hands, $DPPG_2$-TSL and LTSL were both not capable to release FD-10 and FD-70 in saline at T_m, even after a prolonged heating time of 60 min. According to a recent study, FD-10 was released from LTSL to approx. 20% after 15 min under serum-free conditions [23]. The observed difference might be due to a different composition of the LTSL formulation containing the lysolipid MPPC. However, both $DPPG_2$-TSL and LTSL showed a rapid but incomplete release of FD-10 (~25%) and FD-70 (~10-15%) after 5 min at T_m in FCS. Interestingly, the release was comparable for both TSL formulations in FCS and the presence of pore-forming lipids (lysolipid, DSPE-PEG) was not required for $DPPG_2$-TSL. Although the molecular mechanism for serum-mediated release from TSL is not fully understood yet, various serum components were shown to accelerate cargo release at T_m [27, 28, 32, 35]. Surprisingly, the resulting structural defects induced by serum components in the $DPPG_2$-TSL bilayer at T_m seem to be large enough to enable the release of macromolecules.

To gain a further insight into the permeabilizing effects of serum, we analysed the release of CF and FD-10 from $DPPG_2$-TSL containing the hard protein corona. This is of particular importance *in vivo* since TSL circulate a certain amount of time at body temperature before they reach heated vessels and release their cargo. PEGylated liposomes are known to rapidly form a protein corona in circulation [36]. More recently, the protein corona formation was also found to affect the release doxorubicin (DOX) from LTSL [37]. We were curious if the formation of hard protein corona can explain a strongly accelerated release of encapsulated compounds from $DPPG_2$-TSL in serum. Surprisingly, $DPPG_2$-TSL with a hard protein corona demonstrated a CF a FD-10 release in saline comparable to full serum. However, we can not fully rule out the liposomal binding of unknown and potentially permeabilizing serum components other than proteins.

The osmotic imbalance between intra- and extraliposomal compartments can affect the release from TSL. Indeed, intra-liposomal hyper-osmolarity up to three times of physiological osmolarity was used as a strategy to increase the release of macromolecules from lysolipid-free TSL [33, 38]. We previously reported that CF release from $DPPG_2$-TSL was unaffected by the extra-liposomal osmotic stress (100-600 mosm/L) without a presence of serum components [27]. Here, we investigated if hyper- and hypo-osmolarity within $DPPG_2$-TSL might have an effect on the release of macromolecules in physiological solutions as saline and FCS. We selected FD-70 for this study since it showed the lowest release (~10%) in FCS at T_m if encapsulated at physiological osmolarity in $DPPG_2$-TSL. Both hypo- and hyper-osmolarity rendered the $DPPG_2$-TSL to be more permeable at T_m leading to an approx. 4-5 higher release of FD-70 in FCS after 5 min without any significant destabilization at 37°C. Interestingly, the osmotic stress alone barely increased the release of FD-70 from $DPPG_2$-TSL and the presence of serum was still required.

Dextrans represent widely used markers for the estimation of permeability in various biological systems [39, 40]. However, dextrans are poly-disperse representing a distribution of polymers with an average molecular weight rather than a single defined species and show a molecular weight depedent change in shape [41, 42]. Therefore, we chose a fluorescently conjugated bovine serum albumin (F-BSA) to confirm the results obtained with FD. BSA respresents a globular protein with a molecular weight of 66 kDa [43] and was extensively used in its fluorescent from (as F-BSA) in release studies with LTSL [20, 22, 23]. In contrast to FD-10 and FD-70, F-BSA could not be released from $DPPG_2$-TSL at T_m in saline or FCS when encapsulated at physiological intra-liposomal osmolarity. However, a rapid F-BSA

release (40-60% after 5 min) was observed from $DPPG_2$-TSL with the intra-liposomal osmotic imbalance in serum as previously seen for FD-70. In contrast to hydrophilic dextrans, BSA has several hydrophobic domains and can adsorb to a liposome surface [44, 45]. This is supported by a higher encapsulation efficacy of F-BSA in $DPPG_2$-TSL in contrast to FD-70 as well as by an unwanted release below T_m. We previously reported that human serum albumin (HSA) has a weak binding to $DPPG_2$-TSL and is easily replaced by other serum proteins [28]. Although BSA and HSA are not identical, we speculate that the leakage of BSA below T_m could be a result of a competitive replacement by other serum components. However, the leakage was also detected in saline indicating a dilution-mediated release of BSA from the surface of $DPPG_2$-TSL. According to literature, the release behaviour of F-BSA in LTSL strongly differed between studies reaching release values at T_m in the range from approx. 10% [23] to 30% [22] or even 80% [20] after 10 min under serum-free conditions. This inconsistency might be explained by different LTSL compositions including either MPPC or MSPC as the lysolipid, different quantification protocols for the released F-BSA involving dequenching [20] or separation of released F-BSA by various methods [22, 23]. Additionally, a significant bilayer-associated fraction of F-BSA was also reported [20, 22] complicating the conclusion if released F-BSA was indeed capable to pass through the nanopores of LTSL at T_m or rather showed a temperature dependent dissociation from the LTSL bilayer. The latter might happen as a result of significant structural changes in LTSL at T_m including a formation of open liposomes as well as membrane discs [46].

Our *in vitro* study indicates that $DPPG_2$-TSL is a promising system for a local delivery of (bio)-macromolecules. Serum components were found crucial in mediating release of macromolecules from $DPPG_2$-TSL, presumambly by creating large packing defects at T_m. Osmotic stress in combination with the permeabilizing effect of serum components was effective in releasing large polymers (dextran with 70 kDa) as well as bovine serum albumin. It is remarkable that $DPPG_2$-TSL and LTSL performed comparable in terms of dextran release in serum questioning the necessity of a lysolipid for macromolecule release from TSL. Moreover, lysolipids are rapidly transferred from TSL to biological acceptors such as serum proteins [47] or biomembranes [48, 49]. Importantly, the rapid lysolipid extraction was also confirmed *in vivo* leading to approx. 60% of lysolipid loss from LTSL after 10 min in circulation [50]. Future studies are expected to provide more insight into therapeutic potential of TSL for local delivery of macromolecules and lead to better understanding of molecular mechanisms of TSL interaction with serum components.

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7. Conflict of interest

Lars H. Lindner and Martin Hossann hold shares of Thermosome GmbH, Planegg/Martinsried, Germany. All other authors declare no conflict of interest.

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CHAPTER 6: Summary

Thermosensitive liposomes (TSL) are promising delivery vehicles for chemotherapeutics which are able to selectively deliver a drug to solid tumors in combination with regional hyperthermia (HT). In comparison to the free drugs and non-thermosensitive liposomes, TSL showed improved therapeutic outcomes in various pre-clinical tumor models. The clinical relevance of the TSL approach is currently investigated for a doxorubicin (DOX) containing TSL (ThermoDOX®) in the Phase III study for the treatment of hepatocellular carcinoma. Although TSL were introduced back in the late 1970s, the portfolio of compounds for TSL delivery remains low with the main research efforts dedicated to DOX and several hydrophilic anti-cancer drugs.

The main part of this thesis is dedicated to feasibility studies of TSL for lipophilic chemotherapeutics (Chapter 2-4). Due to a rising clinical relevance of biopharmaceuticals, we also studied the potential of TSL for delivery of high molecular weight compounds (Chapter 5). In cooperation with Thermosome GmbH, we focused on a phosphatidyldiglycerol-based TSL ($DPPG_2$-TSL) which already demonstrated promising pre-clinical results for several chemotherapeutics (Chapter 1).

Hexadecylphosphocholine (HePC, Chapter 2), dexamethasone (DXM, Chapter 3) and docetaxel (DTX, Chapter 4) were selected as lipophilic small molecules. HePC is a cytotoxic lipid which forms micelles in aqueous solutions. Remarkably, HePC-containing $DPPG_2$-TSL previously demonstrated a HT-induced toxicity in two cancer cell lines *in vitro*. DXM is a steroid and resembles cholesterol which is regularly used in traditional liposomes. DTX is a taxane with an extremely low aqueous solubility.

The incorporation of HePC, DXM and DTX in $DPPG_2$-TSL was straightforward since these drugs are solubilized in the lipid bilayer during the lipid film hydration. The main difficulty was to develop a release assay which is suitable for lipophilic drugs and can simulate the TSL behavior *in vivo*. Up to date, only two studies are available on this topic and both make use of dialysis. However, we should consider that TSL might rapidly pass through (heated) tumor vessels, presumably in the range of seconds to a few minutes and the drug is ideally released in this time frame and selectively at HT. Therefore, a release assay was required which allows an accurate temperature control and a rapid separation of released drug from TSL. Although dialysis is generally used to assess the release of poor water-soluble drugs from various formulation types, it has a very limited predictability for TSL. In a common dialysis setting, released compounds can only be quantified after diffusion through a semi-permeable

membrane which represents a rate-limiting step. Moreover, drug release from TSL (and liposomes in general) is highly affected by serum components such as proteins. The most abundant protein in serum is albumin (SA) which is also known to bind and transport HePC, DXM and DTX *in vivo*. Since SA is a large protein (66 kDa), the diffusion of a SA-bound (lipophilic) drug through a dialysis membrane is not easily achieved. However, full serum as release medium was a requirement for TSL. Centrifugation was investigated as an alternative method to dialysis which is usually suitable for a separation of released free and protein-bound drugs (supernatant) from liposomes (pellet). However, $DPPG_2$-TSL did not completely sedimentate, even after a prolonged centrifugation time in a high-speed centrifuge (75000xg, 2 hours). Moreover, $DPPG_2$-TSL also showed an increased sedimentation after incubation in serum at or above the transition temperature ($\geq T_m$) with up to 5x higher lipid concentrations in the pellet in contrast to $<T_m$. At this moment, we can only speculate about the reasons for this unexpected finding. Although an increased binding and/or penetration of serum proteins into the $DPPG_2$-TSL bilayer at $\geq T_m$ was a possible explanation, the protein:lipid ratio obtained in the liposomal pellet was comparable after the incubation in serum in the 37-45°C temperature range. Ultrafiltration is commonly applied in our lab for a separation of released hydrophilic drugs from TSL. However, it was not suitable for lipophilic compounds due to their strong binding to filter materials. Moreover, a high molecular weight cut-off (MWCO, ≥300 kDa) required for an efficient filtration of SA also led to a cross-contamination of the flow-through by a certain fraction of $DPPG_2$-TSL. Finally, we identified a fast and reproducible separation method which is suitable for serum samples and requires low amounts of TSL. It is based on the principle of mini spin columns and achieves a separation by size-exclusion. The CL-4B sepharose was hereby identified as a suitable material for separation since it effectively retained released unbound and protein-bound drug but reproducibly eluted TSL by centrifugation within 2 min.

For HePC (Chapter 2), we could detect a 2x higher release from $DPPG_2$-TSL at HT in serum, SA solution or in presence of multilamellar vesicles (MLV). This is in agreement with previous studies where a 2x higher uptake of HePC from $DPPG_2$-TSL was shown in cancer cells. A pharmacokinetic study in rats was performed which allowed a simultaneous tracking of HePC and $DPPG_2$-TSL carrier by mass spectrometry. 50% of HePC was immediately extracted from $DPPG_2$-TSL after intravenous injection whereas the remaining 50% were stably retained in $DPPG_2$-TSL and had a prolonged circulation time. The results of the PK study were confirmed by repetitive exposure cycles to MLV at 37°C. 50% of HePC was rapidly transferred to MLV after the first exposure but no significant transfer was detected

anymore in the subsequent exposure cycles. However, short heating to T_m could again increase the transfer of HePC to MLV. Based on the results from this study, we hypothesized that HePC was extracted selectively from the outer leaflet of the $DPPG_2$-TSL bilayer at 37°C *in vitro* and *in vivo* but stably retained in the inner leaflet due to a slow trans-bilayer movement (flip-flop). However, the flip-flop of HePC from the inner to the outer leaflet might be enabled through packing defects in the lipid bilayer at T_m after application of HT. This can potentially lead to the enhanced intravascular release of HePC from $DPPG_2$-TSL upon HT *in vivo* and consequently to increased concentrations of this drug in solid tumors. Biodistribution studies in tumor-bearing rats are planned to reveal the therapeutic relevance of this finding.

For DXM (Chapter 3) and DTX (Chapter 4), the release from $DPPG_2$-TSL increased with temperature but in contrast to HePC did not lead to a significant difference between 37°C and HT. However, a clinically used dexamethasone-21-phosphate (DXMP) as well as a DTX prodrug (DTX-P) synthesized by our cooperation partner demonstrated a HT-induced release from $DPPG_2$-TSL and at the same time a stable encapsulation at 37°C. In contrast to DXM and DTX, the prodrugs DXMP and DTX-P were encapsulated as charged and membrane-impermeable species within the aqueous core of $DPPG_2$-TSL. Upon HT, they were rapidly released from $DPPG_2$-TSL through bilayer defects at T_m. A stable encapsulation of DXMP in $DPPG_2$-TSL *in vivo* was confirmed by a PK study in rats. We extended our investigation to a lipophilic prodrug dexamethasone-21-palmitate (DP) which is structurally similar to HePC due to a presence of the alkyl chain. However, the behavior of HePC and DP was different in $DPPG_2$-TSL. Whereas HePC exchanged in a temperature-dependent manner between $DPPG_2$-TSL and serum proteins or MLV, DP was stably retained in the bilayer under the same experimental conditions and furthermore decreased the T_m of $DPPG_2$-TSL. The prodrug concept is frequently applied as a strategy to modify the physico-chemical properties of lipophilic drugs to achieve improved release and retention properties in liposomes. However, this approach involves synthetic modifications which can alter the pharmacological activity so that a conversion to an active metabolite will be required in target tissue.

We hypothesized that solubilizing excipients might represent suitable vehicles for the loading of lipophilic drugs into the aqueous core of TSL and might be an alternative to the prodrug strategy. Cyclodextrins (CD) are well-known for their potential to increase the aqueous solubility of poor water-soluble drugs due to formation of water-soluble drug:CD complexes. Drug:CD complexes were extensively investigated in traditional liposomes where several limitations were discovered including the undesired complexation of cholesterol and lipids

from the bilayer leading to liposomal destabilization. To the best of our knowledge, the use of CD for TSL has not been described so far. According to literature, we pre-selected two CD's for the study based on a high aqueous solubility and solubilisation efficacy for DXM. In addition to $DPPG_2$-TSL, we also included the low temperature-sensitive liposome (LTSL) to get general conclusions on the stability and thermosensitivity of TSL in presence of different CD concentrations. According to the release studies with carboxyfluorescein (CF), both CD's were capable to destabilize $DPPG_2$-TSL and LTSL. However, this effect was only observed at very high molar CD to lipid ratios (≥100). Motivated by the promising results indicating sufficient stability of TSL in presence of CD's, we passively loaded a DXM:CD complex into $DPPG_2$-TSL and LTSL. We could achieve a 10x higher loading capacity for DXM:CD in comparison to the non-complexed DXM in both TSL. As confirmed by differential scanning calorimetry (DSC), the T_m was not affected after the encapsulation of DXM:CD. Intriguingly, we could show that DXM and CD are both rapidly released from TSL in serum at HT. DXM release was hereby comparable to the hydrophilic prodrug DXMP. Although DXM might be released as a complex with CD, drug:CD's are rapidly disintegrated by competitive reactions with serum components. Therefore, we expect that DXM will immediately become bioavailable *in vivo*. In contrast to DXMP, a minor fraction of DXM was also released at 37°C. Since the release at 37°C was not observed for the CD, we speculate that a CD-independent and likely bilayer-associated fraction of DXM is present in both $DPPG_2$-TSL and LTSL.

The second part of the thesis studied the potential of $DPPG_2$-TSL in comparison to LTSL for the delivery of high molecular weight compounds (Chapter 5). Previously, our group showed that the release of hydrophilic small molecules is strongly accelerated from $DPPG_2$-TSL selectively at HT in presence of serum proteins. Our hypothesis was that serum components might contribute to the large structural defects at T_m of $DPPG_2$-TSL enabling a release of macromolecules. To address this question, we selected fluorescently conjugated dextrans of 4, 10 and 70 kDa (FD4, FD10, FD70) and bovine serum albumin (F-BSA) as a model protein and performed comparative release studies in buffer and full serum. In contrast to LTSL, $DPPG_2$-TSL does not contain pore-forming lipids and was consequently only permeable to CF at T_m in buffer. On the contrary, LTSL additionally allowed the passage of FD4, presumably through stabilized nanopores. However, both TSL were not anymore permeable under serum-free conditions at T_m to FD10. In serum, LTSL and $DPPG_2$-TSL indeed showed a HT-induced release of FD10 and FD70 which was comparable between both formulations and as expected significantly lower than for CF. Additional analysis revealed that solely a

formation of a hard protein corona on the surface of $DPPG_2$-TSL is sufficient to induce the release of CF and FD10 observed in full serum. The intra-liposomal osmotic imbalance (hypo- or hyper-osmolarity) in comparison to the physiological osmolarity could drastically increase the HT-induced release of FD70 from $DPPG_2$-TSL in serum without any observed leakage at 37°C within 60 min. The results obtained for FD70 could be finally translated to F-BSA which demonstrated a comparable HT-enhanced release in serum from $DPPG_2$-TSL with the osmotic imbalance.

In conclusion, we could demonstrate that lipophilic chemotherapeutics as well as high molecular weight compounds can be loaded and released upon HT from $DPPG_2$-TSL. However, only HePC showed promising results without any further optimization. For the other lipophilic drugs, we had to identify a suitable prodrug or take advantage of the solubilizing action of CD. Since the CD approach does not require chemical modifications of parent drugs, it is particularly interesting and requires further investigation for other lipophilic chemotherapeutics such as taxanes. (Bio)-macromolecules also represent a promising class of therapeutics for future studies with $DPPG_2$-TSL. The destabilizing action of intrinsically present serum components at T_m of $DPPG_2$-TSL can already induce a release of macromolecules. An additional contribution of the intra-liposomal osmotic imbalance has to be taken into account as a possible strategy for the release optimization at HT. However, a vulnerable nature of many biopharmaceuticals would require a development of loading methods for TSL which do not rely on heat and lead to higher encapsulation efficacies than achievable by a passive loading. It is of particular importance to emphasize that TSL might be therapeutically beneficial for clinical disorders other than cancer. We proposed that DXM-containing TSL developed in this thesis should be evaluated for a local drug delivery to inflamed joints in rheumatoid arthritis. Meanwhile, TSL are gaining increasing interest in theranostics. We believe that TSL as a delivery platform will significantly advance in the near future and holds a great potential for a variety of therapeutics and clinical applications.