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Influence of cholesterol inclusion on the doxorubicin release characteristics of lysolipid-based thermosensitive liposomes

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ABSTRACT

Fast hyperthermia (i.e. 39–42 °C) triggered doxorubicin release from lysolipid-containing thermosensitive liposomes (LTSL) in the tumor vasculature has been demonstrated to result in considerable enhancement of bioavailable drug levels in heated tumor tissue in preclinical tumor models. However, there is also significant leakage of doxorubicin already at 37 °C in the bloodstream, making these LTSL less efficient and increasing the risk for systemic toxicity. In conventional liposomes, cholesterol is incorporated in the bilayer to increase the stability of the liposomes. Here, we investigate the effect of cholesterol inclusion on the doxorubicin release characteristics of LTSL at 37 °C and hyperthermic temperatures.

For this purpose, three LTSL formulations with 0, 5 and 10 mol% cholesterol were prepared. Inclusion of cholesterol reduced the undesired doxorubicin leakage at 37 °C in Hepes-buffered saline (HBS) as well as in fetal bovine serum (FBS). The incorporation of cholesterol in the LTSL bilayers did not influence the hyperthermia-triggered release property of the LTSL. These results were supported by DSC measurements.

Therefore, in conclusion, our data indicate that cholesterol inclusion in LTSL offers a simple solution to the problem of significant leakage of doxorubicin from LTSL already at 37 °C in the bloodstream.

1. Introduction

The efficacy of many traditional chemotherapeutic anticancer agents is limited by systemic toxicity and poor accumulation at the tumor site. Over the last decades many studies have demonstrated that encapsulating chemotherapeutics agents in long-circulating liposomes (LCL, e.g. PEGylated liposomes) enables the preferential delivery of such drugs to the tumor site (Torchilin, 2005; Woodle and Lasic, 1992). LCL accumulate preferentially in tumor tissue by virtue of the enhanced permeability and retention (EPR) effect, enabled by tumor vasculature with more permeable vessel walls as compared to those in other tissues (Dvorak et al., 1995; Maeda et al., 2000). Many studies with LCL formulations of doxorubicin and cisplatin, amongst others, have shown to indeed result in higher levels of drug accumulation in preclinical tumor models and less systemic toxicity (Newman et al., 1999; Papahadjopoulos et al., 1991). However, the few literature reports available show that these preclinical findings did not translate into patient survival (Gordon et al., 2001; O'Brien et al., 2004). In addition to the clinical notion that the EPR-effect is highly variable (Hansen et al., 2015; Lammers et al., 2012), also inefficient release of drugs from the liposomes extravasated into the tumor tissue may be an obstacle keeping bioavailable drug levels in the tumor tissue low.

To overcome the problem of low bioavailable drug levels in tumor tissue due to insufficient drug release, liposomal systems that respond to external stimuli were introduced (Torchilin, 2009), such as temperature-sensitive liposomes (TSL) (Landon et al., 2011). The most well-known TSL is ThermoDox® (Celsion Corporation, Columbia, MD, USA), doxorubicin encapsulated in TSL, which is currently being tested in several clinical trials (Wood et al., 2012; Zagar et al., 2014). Preclinical studies have shown that the combination of doxorubicin-loaded TSL and local hyperthermia results in considerable improvements of tumor drug levels and anti-tumor efficacy compared to conventional liposomal doxorubicin formulations (i.e. Doxil®) (Kong et al., 2000; Yarmolenko et al., 2010).

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Drug release may be triggered from TSL which have entered the tumor area, but even in situations where EPR-mediated tumor localization is low or absent, this drug targeting approach is highly useful. Fast triggered drug release can also be induced in the tumor vasculature, which results in elevated intravascular drug concentrations for as long as the tumor is heated and drug-loaded TSL pass the area. Subsequently, the high drug concentration gradient drives diffusion of the drug from the vascular compartment into the tumor interstitial space and cells, yielding much higher bioavailable tumor drug levels (Manzoor et al., 2012).

The fast release characteristics of ThermoDox at mild hyperthermic conditions (i.e. 80% doxorubicin release in 20 s at 42 °C) were obtained by the incorporation of the lysolipid monostearoylphosphatidylcholine (MSPC) in the bilayer (Needham et al., 2000). Inclusion of ~10 mol% lysolipid in the bilayer greatly enhances the membrane permeability around the phase transition temperature (T_m) (Needham et al., 1997). However, there is also significant leakage of doxorubicin already at 37 °C in the bloodstream (i.e. ~1%/min), making this triggered release system less efficient and increasing the risk for systemic toxicity. According to Banno et al., the undesired doxorubicin leakage in the bloodstream from lysolipid containing temperature-sensitive liposomes (LTSL) is caused by the slow dissociation of lysolipids from the bilayer, most likely due to interactions with plasma proteins (Banno et al., 2010).

Considerable efforts have been made to improve the formulation of TSL by reducing spontaneous leakage in the blood at 37 °C while maintaining the burst release properties at slightly elevated temperatures. For example Lindner et al. have developed a TSL that incorporates a synthetic phospholipid (i.e. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglyceroglycerol) which shows better retention of the drug in the circulation, while maintaining the triggered release property at hyperthermic temperatures (Hossann et al., 2007).

In case of conventional liposomes cholesterol is usually incorporated in the bilayer to increase the in vitro and in vivo stability (Lian and Ho, 2001). Cholesterol influences the fluidity of phospholipid bilayers and thereby reduces their permeability for entrapped drugs (Liu et al., 2000; Socaciu et al., 2000). Above a threshold concentration cholesterol also reduces the enthalpy change associated with the membrane's phase transition, causing the liposomes to be less thermoresponsive (Mcmullen and Mcelhaney, 1995; Papahadjopoulos et al., 1973). In this study we report our interim results on the effect of the bilayer inclusion of low amounts of cholesterol on the doxorubicin release characteristics of LTSL at 37 °C and hyperthermic temperatures.

2. Materials and methods

2.1. Chemicals

The phospholipids 1,2-dipalmityol-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG2000 (DSPE-PEG2000) were provided by Lipoid (Ludwigshafen, Germany). Monostearoylphosphatidylcholine (MSPC) was purchased from Avanti Polar Inc (Alabaster, AL, USA). Doxorubicin was purchased via the hospital pharmacy from Accord Healthcare BV (Utrecht, The Netherlands)Other chemical reagents were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands) unless otherwise specified.

2.2. Liposome preparation

Lysolipid containing temperature sensitive liposomes (LTSL) were prepared by the lipid film hydration and extrusion method (Hope et al., 1985). The compositions of the different LTSL formulations that were used in this study are given in Table 1 . The respective lipids were dissolved in chloroform: methanol (9:1 v/v) solution in the correct molar ratio and the solvent was evaporated under vacuum in a rotary evaporator until a thin and homogeneous lipid film was formed. The film was hydrated at 60 °C with a 240 mM ammonium sulfate buffer (pH 5.5). Liposomes were obtained by extruding the mixture successively through filters of decreasing pore size. Doxorubicin was loaded into the liposomes using the pH gradient-driven loading protocol as described by Mayer et al. (Hope et al., 1985). The initial drug:lipid ratio was 1:20 with 0.5 mg/mL of total lipids. Un-encapsulated drug was removed by size exclusion chromatography using a PD-10 desalting column (GE Healthcare, Europe GmbH) equilibrated with Hepes Buffered Saline (HBS) pH 7.4. The resulting liposomes were suspended in HBS pH 7.4 and stored at 4 °C until further use.

Table 1
Molar ratios of lipids for LTLS used in study.

Lipids	LTSL	LTSL-Chol5%	LTSL-Chol10%
DPPC	86	81.7	77.4
MSPC	10	9.5	9
DSPE-PEG2000	4	3.8	3.6
Chol	–	5	10

2.3. Liposome characterization

The mean size and polydispersity of the liposomes were determined by dynamic light scattering (DLS) (ALV CGS-3 platform, Langen, Germany) at 25 ± 1 °C. The intensity of the laser light scattered by the samples were detected at an angle of 90 °C with a photomultiplier.

The zeta potential of the liposomes was determined in Hepes buffer (10 mM; pH 7.4) at 25 °C with the Laser Doppler Micro-Electrophoresis method using a Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, United Kingdom).

The phase transition temperatures (T_m) of the lipid membranes were measured with differential scanning calorimetry (DSC; Discovery, TA Instruments, USA). Liposomes were diluted in HBS 20 mM pH 7.4 or 90% fetal bovine serum (FBS), transferred into T zero hermetic aluminum pans sealed with lids. The appropriate reference solutions were HBS 20 mM, pH 7.4 or FBS. Samples were thermally scanned from 37 °C to 45 °C at 0.5 °C/min heating rate. DSC data was analyzed using TRIOS v.4.2.1 software.

The encapsulation efficiency (EE) was determined by calculating the ratio of total doxorubicin concentration before and after free (i.e. unencapsulated) drug removal by PD-10 desalting column:

$$EE\ (\%) = (C_{after} / C_{before})*100$$

where C is the doxorubicin concentration, determined by UPLC as described below, of the liposome suspension after liposome lysis with acetonitrile.

All measurements were at least performed in triplicate.

2.4. Doxorubicin quantification

The concentration of doxorubicin was determined by using a Waters ACQUITY UPLC system consisting of a binary solvent manager, a sample manager and a fluorescence detector (λ_{ex}: 480 nm and λ_{em}: 565 nm). The runs were carried out with an ACQUITY BEH C18 column (1.7 µm, 2.1 × 50 mm). The mobile phase was composed of acetonitrile/water/perchloric acid (25:75:0.1, v/v). 7.5 µL per sample was injected and eluted with a flow rate of 0.9 mL/min, the column was maintained at 50 °C. The calibration curve (1–10 µg/mL) was prepared from doxorubicin stock solution diluted in 25% acetonitrile (ACN) and 75% water.

2.5. Release experiments

The time dependent release of doxorubicin from the different types of liposomes was determined after incubation of the dispersions (in

HBS) in a water bath (Memmert, GmbH, Germany). Incubation periods were 5, 15, 30, 60 and 120 min for 37 °C and 1, 2, 5, and 10 min for 42 °C. Similarly, doxorubicin release was measured after 5 min of incubation at temperatures in the range of 37–45 °C. Briefly, 8 µL of the LTSL suspensions were added to an Eppendorf containing 72 µL 20 mM HBS (pH 7.4) or FBS preheated at the desired temperature and incubated in the water bath for the desired duration. Next, the sample was put on ice and 420 µl HBS of 4 °C was added.

To separate the free doxorubicin fraction from the liposome-encapsulated doxorubicin fraction a reversed-phase solid phase extraction (SPE) technique was used. Briefly, a SupelTM-Select HLB column (Sigma Aldehich, 54181-U, 30 mg) was conditioned with 6 × 500 µL of methanol followed by 6 × 500 µL HBS 20 mM pH 7.4 (flow rate of 0.5 mL/min). Subsequently, the column was saturated with phospholipids using 10 mg of empty LTSL. The column sorbent was maintained in a wet state at all times. Following the column conditioning 500 µL of cooled down sample containing the liposomal and/or free doxorubicin was added to the top of the column. First, HBS and/or FBS components passing through the column (i.e. liposome containing fraction) was collected. Subsequently, the column was further washed with 2 × 500 µL of HBS. Finally, the column was eluted with 2 × 500 µL methanol to remove the free doxorubicin that was retained by the column matrix material.

In order to quantify the drug amount in the free doxorubicin fraction with UPLC, as described above, the methanol was evaporated using a rotational vacuum concentrator and subsequently doxorubicin powder was reconstituted in 25:75 v/v ACN:HBS. The percentage of doxorubicin release was calculated as:

$$\text{DOX release } \% = (C_s - C_0)/ (C_{tot} - C_0) \times 100\%$$

where C_s is the free doxorubicin concentration of a sample after incubation in the water bath, C_0 is the concentration of free doxorubicin present in the liposome suspension before incubation and C_{tot} is the total amount of doxorubicin present in the liposome suspension, which was determined by adding 8 µL liposomes suspension to 250 µL of acetonitrile and 742 µL HBS. All release experiments were performed in triplicate.

3. Results & discussion

3.1. Preparation and characterization of LTSL

The characteristics of all LTSL formulations are summarized in Table 2 . DLS measurements showed that the incorporation of 5 and 10 mol% cholesterol in the bilayer of LTSL had no significant effect on the mean diameter. The polydispersity and zeta potential were also comparable. The addition of cholesterol to the LTSL bilayers did not influence the loading efficiency, which remained above 90%.

3.2. Release profiles at 37 °C

The rate and extent of doxorubicin leakage was measured as function of incubation time in Hepes buffered saline (HBS) and Fetal bovine serum (FBS) at 37 °C (Fig. 1). The conventional LTSL showed a maximum leakage of 22% after 2 h incubation in HBS. The addition of 5 and 10 mol% cholesterol decreased the maximum release to 8% and 5% after 2 h incubation in HBS, respectively. The leakage of doxorubicin from the conventional LTSL was significantly increased when incubated in FBS and reached a maximum at about 35%. The decreased stability of the conventional LTSL formulation was also observed by other groups and is most likely due to the extraction of the lysolipid by plasma proteins (Banno et al., 2010). Importantly, the LTSL formulations containing 5 and 10 mol% cholesterol remained much more stable when incubated in FBS with leakage not exceeding 13%. This reduced drug leakage from cholesterol-containing LTSL in serum is beneficial for clinical applications since it will reduce premature release in the bloodstream and therefore the amount of non-liposomal doxorubicin in the circulation and its related risk of occurrence of toxicity.

3.3. Release profiles at 42 °C

Fig. 2 shows that all formulations showed a (nearly) complete drug release within 2 min incubation at 42 °C. Apparently, LTSL kept their fast release kinetics in the presence of cholesterol in the bilayer, which makes also these formulations suitable for the intravascular triggered release approach. Other studies came to a different conclusion in case of so-called traditional TSL, which lack lysolipids in their bilayers (Maruyama et al., 1993; Unezaki et al., 1994). Apparently, incorporation of lysolipids not only enhances the doxorubicin release rate from TSL, but also tolerates the incorporation of low amounts of cholesterol in the bilayers without negative effect on the triggered release property.

3.4. Temperature triggered release

To further investigate the temperature-dependent doxorubicin release properties of all LTSL formulations, drug release as well as the bilayer melting behavior were measured in HBS and FBS for the temperature range 37–45 °C (Fig. 3). The T_m in HBS, measured by DSC, of the LTSL in absence of cholesterol in the bilayer is 40.9 °C. The addition of 5 or 10 mol% cholesterol resulted in slightly lower phase transition temperatures of 40.1 °C and 40.0 °C, respectively (Table 3). In parallel to the phase transition temperature also the onset temperature slightly shifts to lower temperatures for the formulations containing cholesterol. Also the total enthalpy change is affected by cholesterol: it decreases with the increasing cholesterol content. These results correspond with other studies that also observed a decrease of T_m, T_{onset} and total enthalpy changewhen increasing cholesterol content in a DPPC bilayer (Matsingou and Demetzos, 2007; Mcmullen and Mcelhaney, 1995). The presence of FBS lowered the onset temperature of the LTSL formulations, but did not influence the effect of increasing cholesterol content on T_m, T_{onset} and total enthalpy change.

Fig. 3 shows the DSC melting curves along with temperature-dependent drug release results. For the latter purpose, the LTSL formulations were incubated during 5 min in HBS or FBS at different temperatures (range 37–45 °C). All formulations remained stable up to a temperature of 39 °C. Subsequently, a steep increase in doxorubicin release was observed with increasing the temperature. At 40 °C all formulations reached their maximum degree of release, which was equal to (nearly) complete drug release. The decrease of total enthalpy change when increasing cholesterol content corresponds with an intensification of the release kinetics.

Table 2
Physicochemical properties of LTSL formulations used in this study.

Formulation	Mean diameter (nm) ± SD[a]	Polydispersity index ± SD[a]	Zeta-potential (mV) ± SD[a]	Encapsulation efficiency (%)
LTSL	153 ± 3	0.12 ± 0.04	-13.4 ± 0.6	95
LTSL-Chol5%	143 ± 8	0.12 ± 0.01	-10.7 ± 0.3	95
LTSL-Chol10%	148 ± 8	0.19 ± 0.03	-9.8 ± 0.2	93

[a] Mean ± Standard Deviation (SD); n = 3.

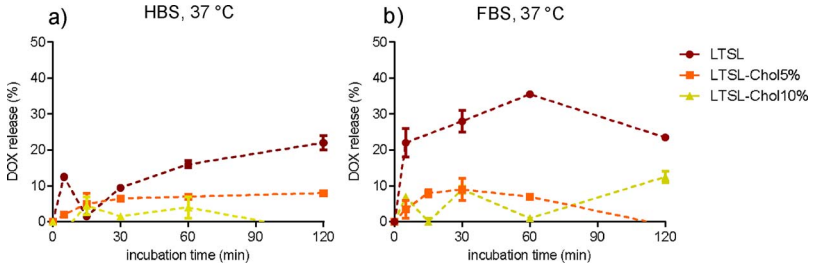

Fig. 1. Time-dependent doxorubicin release from LTSL formulations at 37 °C in HBS (a) and FBS (b).

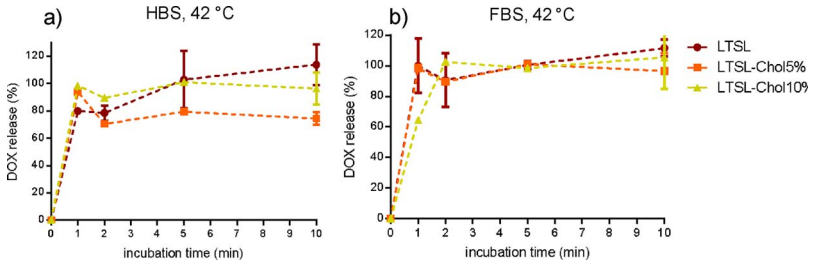

Fig. 2. Time-dependent doxorubicin release from LTSL formulations at 42 °C in HBS (a) and FBS (b).

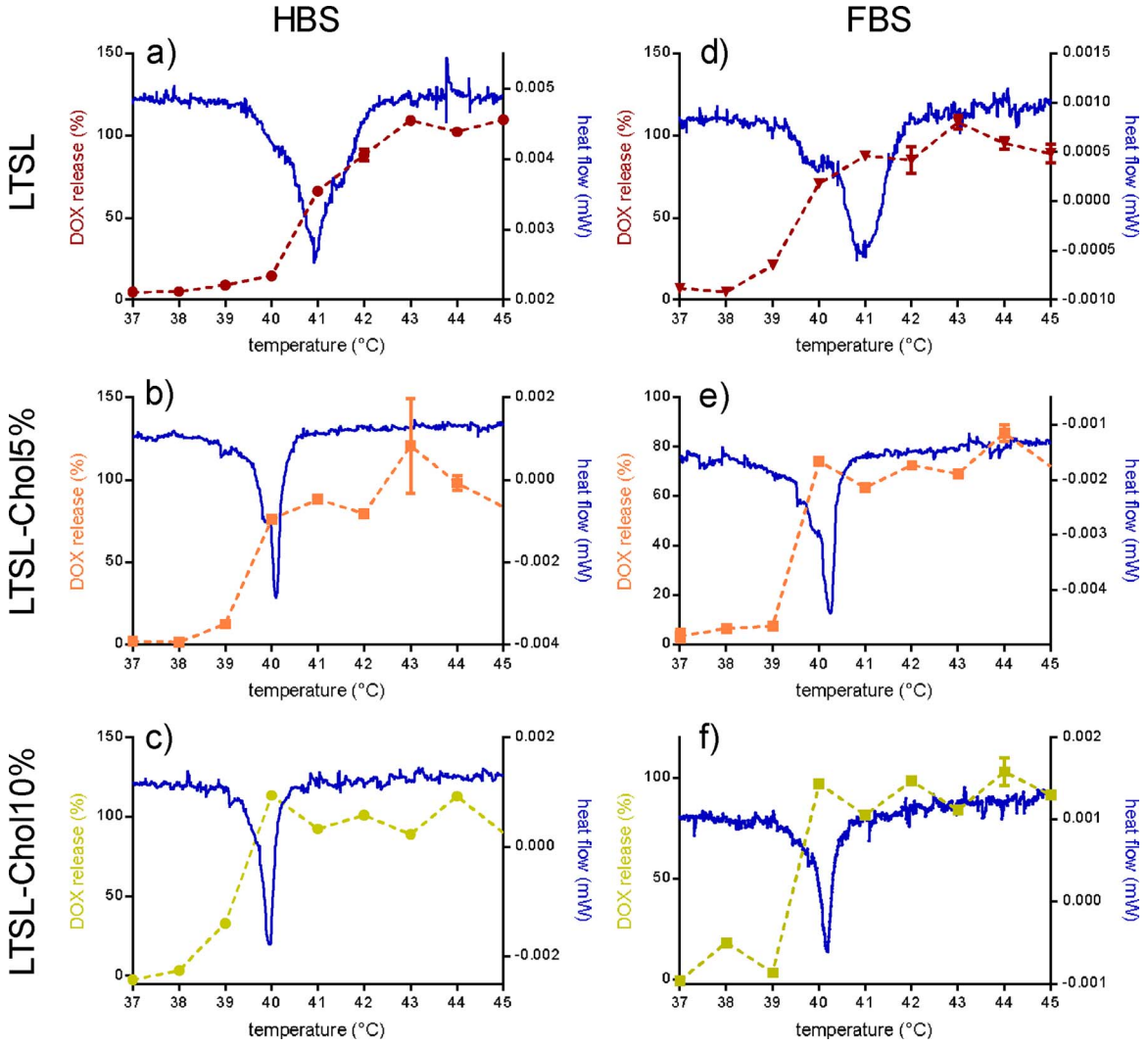

Fig. 3. Temperature-dependent release profiles of doxorubicin from LTSL formulations and DSC curves in HBS (a–c) and FBS (d–f). Conventional LTSL (a and b), LTSL-Chol5% (c and d) and LTSL-Chol10% (e and f) were incubated during 5 min at different temperatures in HBS and FBS and doxorubicin release was measured using HPLC.

Table 3
Overview of characteristic thermodynamic parameters of a DSC curve for different LTSL formulations. .

	Formulation	Total enthalpy change (normalized) (J/g)	Onset temperature (°C)	Phase transition temperature (°C)
HBS	LTSL	0.28	40.7	40.9
	LTSL-Chol5%	0.21	39.9	40.1
	LTSL-Chol10%	0.14	39.7	40.0
FBS	LTSL	0.19	40.2	40.8
	LTSL-Chol5%	0.19	39.7	40.2
	LTSL-Chol10%	0.10	39.9	40.2

4. Conclusion

Doxorubicin-containing LTSL formulations with different amounts of bilayer cholesterol were investigated for doxorubicin release properties at physiological and mild hyperthermia temperatures in HBS and FBS. Incorporation of 5 and 10 mol% cholesterol in the LTSL reduced the undesired leakage of doxorubicin at physiological temperature, but did not negatively affect the fast release kinetics property at elevated hyperthermia temperatures. Therefore, the use of cholesterol-containing LTSL may offer a considerable advantage over the conventional LTSL formulation in an in vivo situation. It is known already for a long time that inclusion of cholesterol in liposomal bilayers can influence blood clearance (Kirby et al., 1980) and uptake of liposomes by the reticuloendothelial system (Patel et al., 1983). Further in vivo experiments are warranted and should address the effects of the incorporation of cholesterol in LTSL on circulation kinetics and tumor accumulation.

Conflict of interest

None.

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