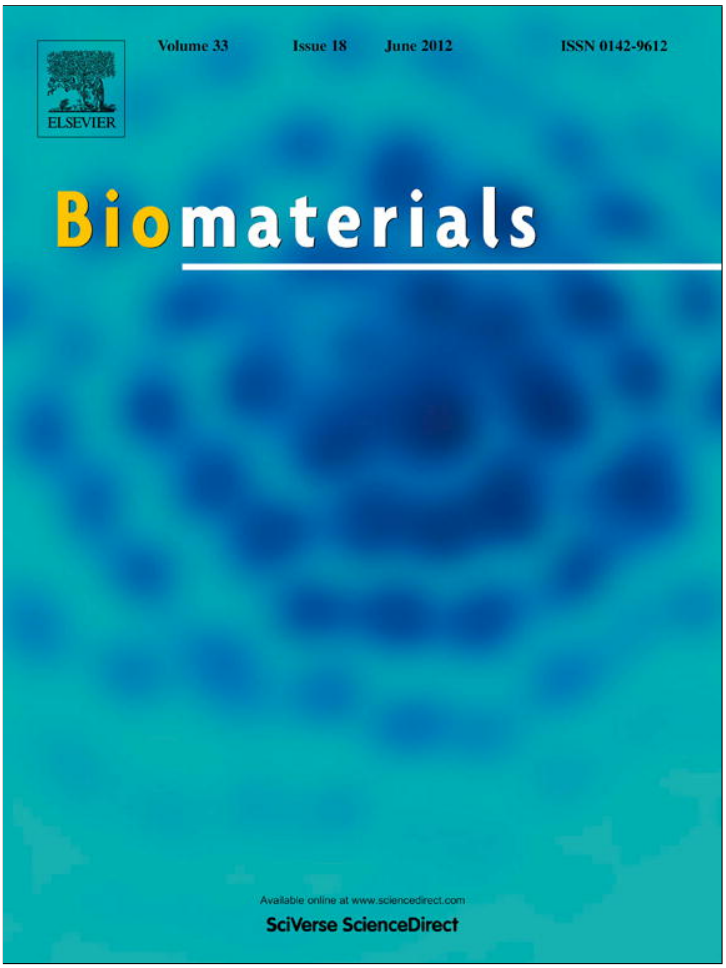

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Pharmacokinetics & tissue distribution of temperature-sensitive liposomal doxorubicin in tumor-bearing mice triggered with mild hyperthermia

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ABSTRACT

Drug-loaded temperature-sensitive liposomes (TSL) in combination with hyperthermia (HT) have attracted considerable attention for cancer treatment. Different TSL systems have been designed with wide variations in their temperature sensitivity and drug release profile. Low temperature-sensitive liposomes (LTSL) with the capacity for ultrafast drug release, traditional temperature-sensitive (TTSL) with intermediate drug release properties and non-temperature-sensitive liposomes (NTSL) (no drug release) were dual-labeled with ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) lipid and loaded with ^{14}C-doxorubicin (^{14}C-Dox). Their blood profile, serum stability, tissue distribution and tumor localization (B16F10 melanoma) were studied after intravenous administration and mild HT treatment. LTSL showed higher affinity for the liver compared to TTSL and NTSL which were uptaken mainly by spleen. Under normal conditions (no HT) Dox leakage from liposomes was expected, higher for LTSL, less for TTSL and minimal for NTSL. Localized HT did not affect the overall blood circulation or organ accumulation for all TSL studied. Since LTSL showed ultrafast Dox release kinetics at 42 °C, the highest drug accumulation in tumors was observed using this system immediately after HT, however decreased significantly after 24 h. In contrast, TTSL and NTSL showed 2-3 fold increase in both liposome and Dox levels that indicated enhanced tumor extravasation of intact Dox-loaded liposomes during the 60 min HT applications. More interestingly, high levels of drug tumor accumulation were achieved 24 h post-HT. This study offers further understanding on how the mechanisms of drug release from temperature-sensitive liposomes affect their pharmacological profile under mild hyperthermia.

1. Introduction

Liposomes are spherical lipid-based delivery systems that dramatically improve the pharmacokinetics and biodistribution of chemotherapeutics after systemic administration. Liposomes encapsulating doxorubicin (Dox), such as Doxil®, have been superior to free drug primarily due to their reduced cardiotoxicity. Despite their preferential accumulation at the tumor site, the therapeutic efficacy of such liposomal drug formulations has been limited due to the low drug bioavailability within the tumor interstitium [1]. That is one of the key reasons that has held the clinical translation of more advanced (e.g. targeted) liposomal drugs. A strategy to improve that involves the combination of liposome drug delivery systems with externally triggered and spatiotemporally selective drug release to enhance drug efficacy without increasing the side effects on healthy tissue.

Clinically, local hyperthermia (HT) has been used to augment radiotherapy and chemotherapy [2–4] by enhanced blood perfusion, extravasation and increased cell sensitization to the treatment [5–8]. Previous preclinical studies have used localized HT combined with long-circulating liposomes [9] and temperature-sensitive liposomes (TSL) [10]. These studies generally report increased therapeutic efficacy for TSL that carry encapsulated anticancer agents in combination with mild local (tumor) HT, due to enhanced liposome extravastion and drug release within the tumor mass [11–15].

TSL were first described by Yatvin et al. using HT to increase encapsulated neomycin release leading to enhanced *Escherichia coli* elimination *in vitro* [16]. Since then, different TSL have been described and by careful selection of the lipid composition their temperature sensitivity can be successfully tuned between 41 and 42 °C [10,17–21] that is clinically preferable. In addition, long-circulating TSL introduced by Maruyama et al. have been shown to extravasate and passively target the tumor following systemic administration [17,22]. Despite their high *in vivo* stability and

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temperature sensitivity these 'traditional' temperature-sensitive liposomes (TTSL) exhibited slow kinetics of drug release [11,16,23]. Improve on this, Needham et al. proposed the incorporation of lysolipids in the lipid composition of the liposome bilayer that could induce burst-release kinetics of the encapsulated drug [14]. These lysolipid-containing liposomes could release 100% of their doxorubicin content under mild HT achievable in the clinic (41–42 °C), compared to TTSL that required temperatures higher than 42 °C. Since then, more TSL formulations have been developed by many groups using synthetic lipids that improve the stability of such liposomes without affecting their temperature sensitivity [18–20,24]. The most clinically advanced TSL system, known as ThermoDox®, is composed of DPPC: MSPC:DSPE-PEG_{2000} (86:10:4 M ratio) described as lysolipid-temperature-sensitive liposomes or low-temperature-sensitive liposomes (LTSL), and is currently in Phase III clinical trials against liver cancer and Phase II against breast cancer recurrence at the chest wall [25].

The need for detailed and systematic pharmacological understanding of TSL has emerged as several TSL formulations have been studied in combination with localized HT. Moreover, selection of the optimum HT protocol for these different TSL systems is based on more knowledge regarding the pharmacokinetics, biodistribution, *in vivo* stability and tumor accumulation of the TSL-drug carrying systems. Doxil® is a non-temperature-sensitive liposome (NTSL), serum-stable and long-circulating that shows marked accumulation at the tumor site due to the enhanced permeation and retention (EPR) effect [26] while limited understanding of the pharmacokinetics of TSL systems is available [24,27,28]. Most of the studies using TSL triggered by HT have reported the tumor uptake of doxorubicin (Dox) in the presence of HT without studying the pharmacological behavior of the TSL carriers In addition, no studies have investigated thoroughly the effect of local HT on blood circulation, organ uptake and drug leakage from TSL *in vivo*. Since mild HT interventions may become clinically widespread as a means to externally trigger drug release, more thorough understanding of drug carrier biodistribution and pharmacokinetics from such protocols are greatly needed.

This study aimed to better understand the effect of drug release properties that different temperature-sensitive liposome carriers exhibit and their reflection on their systemic pharmacokinetic and biodistribution profiles *in vivo*. In order to achieve this aim we studied systematically and quantitatively three different liposomal doxorubicin formulations that varied in their temperature responsiveness: LTSL with the capacity for ultrafast drug release, TTSL with intermediate drug release properties and lastly NTSL that are non-temperature-sensitive liposomes. All three systems were radiolabeled with ^{3}H-CHE lipid and loaded with ^{14}C-Dox to allow simultaneous tracking of both the liposome and the drug component. Their biodistribution and *in vivo* stability were studied in tumor-bearing mice following systemic administration, in the presence and absence of local HT. Liposome (via the ^{3}H-lipid) and drug (via the ^{14}C-Dox) uptake in murine B16F10 melanoma tumors and other healthy tissues were studied up to 24 h post-HT treatment.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC); monostearoyl phosphatidylcholine (MSPC); hydrogenated soy phosphatidylcholine (HSPC); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}) were kind gifts from Lipoid GmbH (Germany). Cholesterol, isopropanol, chloroform, methanol, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and doxorubicin hydrochloride (Dox) were purchased from Sigma (UK). [3H] Cholesteryl hexadecyl ether (CHE) and [14C] Doxorubicin hydrochloride (^{14}C-Dox) were purchased from PerkinElmer (UK). All chemical substances and solvents were used without further purification.

2.2. Liposome preparation and Dox encapsulation

Phospholipids were dissolved in chloroform: methanol (4:1 v/v) mixture. Multilamellar vesicles (MLV) were prepared by evaporating the organic solvent under vacuum for 60 min at 40 °C using a rotovaporator (BÜCHI, Switzerland), then flushed with N2 stream to remove any residual traces of organic solvent. The dried lipid films of LTSL [DPPC:MSPC:DSPE-PEG_{2000} (86:10:4)]; TTSL [DPPC:HSPC:Chol :DSPE-PEG_{2000}(50:25:15:3)]; and NTSL [HSPC:Chol:DSPE-PEG_{2000} (75:50:3)] were hydrated with ammonium sulfate buffer pH 5.4 (240 mM $(NH4)_2SO_4$) to achieve a final lipid concentration of 5 mM (Dox release studies) and 25 mM (DSC and *in vivo* studies). All ratios between lipids were molar ratios. Small unilamellar vesicles (SUV) were prepared by extrusion at 60 °C through 800 nm, 200 nm, and 100 nm polycarbonate filters 5 times each, followed by 10 times extrusion through 80 nm membranes using a mini-Extruder (Avanti Polar Lipids, USA), then flushed with N_2 and stored in the fridge to anneal overnight. Liposome size and surface charge were measured by using Zetasizer Nano ZS (Malvern, UK, He-Ne laser). Dox was loaded into liposomes using the pH-gradient method as described elsewhere [29]. Briefly, liposome external buffer was first exchanged using Sepharose CL-2B column (15 cm * 1.5 cm) (Sigma, UK) equilibrated with HBS pH 7.4 (20 mM HEPES, 150 mM NaCl) then liposomes were incubated with doxorubicin hydrochloride (5 mg/mL) at 20:1 lipid:Dox mass ratio in respect to the original total lipid concentration. Subsequently, samples were incubated for 90 min at 37 °C in the case of LTSL, 1 h at 60 °C for NTSL and overnight at 39 °C for TTSL. Following the incubation Free Dox was removed using Sepharose CL-2B column as described above. Liposomes were reconcentrated for *in vivo* experiments using Vivaspin 100,000 MWCO (Vivascience, Sartorus, Germany). Dox encapsulation efficiency (EE) was calculated by comparing the total fluorescence intensity of Dox before and after purification.

% EE = I(t) after purification/I(t) before purification * 100, where I(t) is the fluorescence intensity liposome suspension after liposome lysis with 1% Triton X-100. Liposomes prepared at high concentration were diluted in 200 μl isopropyl alcohol since NTSL at high lipid concentration could not be solubilized with 1% Triton X-100. Dox liposomes were diluted before and after purification in isopropyl alcohol to be able to compare their florescence intensities under same conditions to assess %EE.

2.3. Differential scanning calorimetry measurements (DSC)

In order to determine the phase transition temperatures of the liposomes, 20 μL samples of MLV suspensions were placed in T zero hermetic aluminum pans sealed with lids. Samples were then thermally scanned from 30 °C to 60 °C at 1 °C/min heating rate using differential scanning calorimetry (Q2000 differential scanning calorimeter, TA Instruments, USA).

2.4. Serum stability and temperature sensitivity of liposomes

The release experiments were performed at 37 °C and 42 °C in 50% CD-1 mouse serum (Sera Laboratories International, UK). At different time points 50 μL samples were withdrawn and further diluted to 200 μL with HBS (pH 7.4) and measured at 480 nm excitation wavelength and 595 nm emission wavelength (slit 15/20 nm) in a quartz cuvette using PerkinElmer Luminescence Fluorimeter (LS50B). The intensity of the fluorescence signals was then normalized and the % of Dox release was calculated as; Dox release % = $[I_{(s)} - I_{(0)}]/[I_{(t)} - I_{(0)}]$, where $I_{(s)}$ is the fluorescence intensity of individual samples at different time points, $I_{(0)}$ is the background fluorescence intensity of liposome samples after purification and $I_{(t)}$ is the fluorescence intensity of liposomes suspension after lysis with 1% Triton X-100.

2.5. Tumor model

5-6 week-old female C57BL6 mice (15–20 g) were purchased from Harlan (UK Limited, U.K). Animal procedures were performed in compliance with the UK Home Office Code of Practice for the Housing and Care of Animals used in Scientific Procedures. Mice were housed in groups of 5 with free access to water and kept at a temperature of 19–22 °C and relative humidity of 45–65%. Before performing the procedures animals where acclimatized to the environment for at least 7 days. B16F10 melanoma (ATCC, USA) was established by subcutaneous injection of 2.5×10^5 B16F10 melanoma cells in a volume of 20 μL of PBS into the right lower leg using 26G needles. The tumor volume was estimated by measuring three orthogonal diameters (a, b, and c) with calipers; the volume was calculated as $(a \times b \times c) \times 0.5$ mm^3. The experiments were performed when the tumor volume reached 200–400 mm^3.

2.6. Hyperthermia and biodistribution of dual-labeled liposomes in vivo

In order to study liposome biodistribution and *in vivo* stability, all liposomes were dual radiolabeled as described elsewhere [30]. Briefly, lipid bilayers were radiolabeled with ^{3}H-CHE lipid marker during the lipid film formation, then extruded liposomes were loaded with Dox solution spiked with ^{14}C Dox to maintain the lipid:Dox ratio 20:1 w/w. Unencapsulated Dox and ^{14}C-Dox were removed as described previously. C57BL6 Mice ($n = 4$) were anesthetized by inhalation of isoflurane and injected via the tail vein with 200 μL of the liposomes suspension

(equivalent to 1.2μCi/0.2 μCi $^{3}H/^{14}C$, 2.5 μmol of lipids/200 μL, Dox 5 mg/kg) in HBS. For HT treatment, local HT was applied immediately after liposome injection and maintained for 60 min. The tumor-bearing leg was supported on a wooden splint to extend the leg without affecting the blood flow then wrapped with a polythene bag, to insulate the leg from water and avoid edema formation [13,31]. Anaesthetized mice were then placed on a Polyvinyl chloride (PVC) stage containing holes, which was placed over water bath set up at 43.5 °C (Grant, Germany) so the tumor-bearing leg was immersed completely in 42.5 °C water bath (water temperature as measured by a thermocouple). Tumor temperature during hyperthermia was measured using hypodermic thermocouple (Omega Supplies UK Limited, UK) and tumor temperature reached 40–41 °C between 2 and 5 min (SI Fig. 2). During HT animals were anesthetized by inhalation of isoflurane and the body temperature of the mice was monitored with a rectal thermocouple. A fan and a heating pad were used to maintain the body temperature at 36–37 °C [15]. At different time points, the mice were bled by tail vein puncture and 35 μL of blood was collected using heparinized capillary tubes. Blood withdrawn did not exceed 10% of the mouse blood volume per day. Mice were killed after 1 h and 24 h post-injection by cervical dislocation, and organs and tumors were excised. During injection and bleeding mouse body temperature was carefully monitored to not exceed 38 °C. Total radioactivity in the blood was calculated based on the assumption that the total blood volume is accounting for 7.3% of the total body weight [32]. The results were represented as the percentage of the injected dose (%ID) per organ or gram tissue.

2.7. Blood and organ radioactivity measurements

Radioactivity measurements were performed as previously described [33]. Blood and whole organs (except liver ~100–200 mg) and tumor samples were transferred to 20 mL scintillation vials and solubilized with 1 mL of Soluene-350 tissue solubilizer (PerkinElmer, UK), and shaken overnight at 55 °C. Before adding the scintillation cocktail samples were decolorized by adding 0.3 mL of 30% H_2O_2 and isopropanol as an antifoaming agent. Samples were shaken at 55 °C for 1–3 h to expel H_2O_2 before adding the scintillation cocktail. Samples were then mixed with 20 mL of Optiphase "Safe" scintillation cocktail (Fisher Scientific, UK) acidified with 0.7% (v/v) glacial acetic acid to eliminate any chemiluminescene. ^{3}H and ^{14}C radioactivity of both isotopes was quantified simultaneously for each sample using LS6500 multipurpose scintillation counter (Beckman, USA).

2.8. Statistical analysis

Data were analyzed using two sample, single sided, student's *t*-test. Differences were considered significant at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Preparation and characterization of TSL

Two types of TSL systems have been extensively reported in the literature today in combination with localized mild HT. The low-temperature-sensitive liposomes (LTSL) (Fig. 1, top) exhibit low phase transition temperature with ultrafast release kinetics due to the insertion of lysolipids in the bilayer, thought to result in the stabilization of long lasting pores at the boundaries between solid and liquid crystalline phase [21]. Traditional-temperature-sensitive liposomes (TTSL) (Fig. 1, middle) release the encapsulated material at 42 °C or above at slower rates, dependent on the lipid bilayer transition from the gel to a fluid phase. Non-temperature-sensitive liposomes (NTSL) (Fig. 1, bottom) were included as a negative control throughout this study, since they do not show any temperature sensitivity. Doxorubicin (Dox)–loaded liposomes were prepared following lipid film hydration and extrusion and loaded with Dox at 20:1 lipid:drug weight ratio. Table 1 summarizes the liposome composition, size, surface charge and Dox encapsulation efficiency of Dox-loaded liposomes used in this study (LTSL-Dox, TTSL-Dox, NTSL-Dox). All liposome systems were between 100 and 120 nm in diameter with slight negative surface charge and high Dox loading efficiency (>90%).

3.2. Differential scanning calorimetry measurements and Dox release in vitro

Differential scanning calorimetry (DSC) and Dox release studies were performed to compare the temperature sensitivity and serum stability of these Dox-loaded liposomes. The DSC thermogram of LTSL showed a lower transition temperature compared to TTSL with the main phase transition peaks at 41.5 °C and 44.2 °C, respectively. In contrast, no phase transition peak was detected with NTSL (Fig. 2A), in agreement with previous reports [21,34]. The lower transition temperature observed with LTSL is due to the presence of MSPC lysolipid in the lipid bilayer, which leads to a slightly less ordered phospholipid molecule arrangement in the gel phase [14]. On the other hand, the disappearance of NTSL transition temperature is due to the high cholesterol content in the liposome formulation [23].

In order to evaluate the stability of these TSL, Dox leakage was measured over time in 50% CD-1 mouse serum, to simulate the *in vivo* conditions. At 37 °C LTSL-Dox released 30% of encapsulated Dox after 30 min of incubation and complete Dox release (100%) was observed after 120 min (Fig. 2B and C; triangles), similar to results published by other groups [24,35]. Under the same conditions, TTSL-Dox showed higher stability since 10% and 60% of Dox was released after 30 min and 1440 min (24 h), respectively (Fig. 2B and C; circles). No Dox leakage was observed at 37 °C in HBS with all formulations (SI Fig. 1). After determination of the stability in serum at 37 °C, Dox leakage from the liposomes was studied at 42 °C. LTSL-Dox released 100% of their Dox content at 42 °C within 1 min (Fig. 2D; triangles), while TTSL-Dox released 70% in the first 5 min of incubation and reached 100% after 15 min of incubation (Fig. 2D; circles). Under all conditions studied, NTSL-Dox were stable showing no Dox release within 24 h (Fig. 2C and 2D; squares).

3.3. Blood circulation and in vivo stability of dual-labeled TSL

In order to understand the behavior of different TSL and compare their *in vivo* stability, LTSL-Dox and TTSL-Dox were radiolabeled with ^{3}H-CHE, non-exchangeable and non-metabolizeable lipid, during liposome preparation, then loaded with radiolabeled ^{14}C-Dox. Dual-labeling of liposomes was used to enable simultaneous tracking of both the lipid vesicles and the encapsulated drug *in vivo*. NTSL-Dox were included in our study for comparison since they exhibit long blood circulation and high serum stability, resulting in passive tumor accumulation as reported [36]. Dual-labeled LTSL-Dox, TTSL-Dox and NTSL-Dox were injected intravenously at 5 mg Dox/kg in tumor (B16F10)-bearing mice (C57BL6) and both liposome and Dox levels were measured in the blood at different time points using beta counting. The effect of localized HT on liposomes and Dox biodistribution was also assessed by immersing the tumor-bearing leg in a 42.5 °C water bath for 60 min, immediately after liposome administration. Particular attention has been placed (since this can be a source of serious experimental errors) in monitoring the animal body temperature during the application of HT using a rectal thermocouple and a heating pad (SI Fig. 2). The body temperature of all animals was maintained between 36 and 37.5 °C throughout these studies, in particular during application of HT.

Fig. 3 summarizes the liposome (Fig. 3A and B) levels in blood in the absence and presence of HT. By tracking the ^{3}H-labeled lipid marker, we observed that all Dox-loaded liposomes were circulating during the first hour. LTSL-Dox decreased to around 20% after 6 h of injection (Fig. 3A, triangles), while TTSL-Dox showed longer blood circulation, similar to NTSL-Dox, with almost 40%ID and 15% ID of the injected liposome dose detected in the blood after 6 h and 24 h, respectively (Fig. 3A, circles & squares). No significant difference was observed in blood circulation of all liposome systems in the presence of HT (Fig. 3B).

The shorter blood circulation detected for NTSL-Dox compared to previous reports was thought to be due to a larger mean liposome diameter compared to the clinically used Doxil®(80–90 nm)

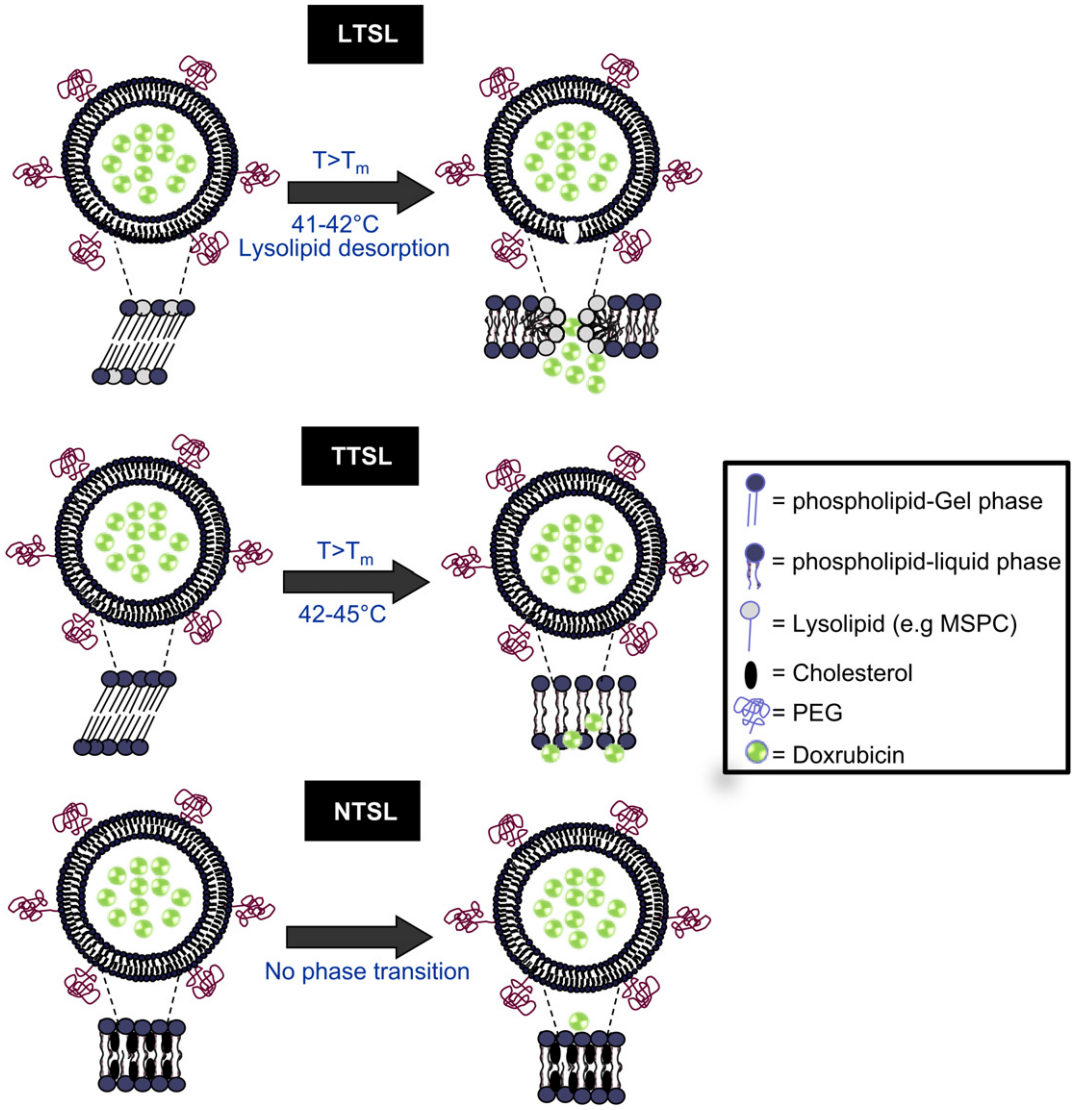

Fig. 1. Schematic representation of TSL (LTSL-Dox, TTSL-Dox) and NTSL-Dox composition, their bilayer changes above their phase transition temperature (T_m) and the potential mechanism of encapsulated Dox release.

[37,38]. To further investigate the effect of mean liposome diameter, two additional sizes (90 nm and 150–175 nm) of LTSL-Dox and NTSL-Dox were radiolabeled with ^{14}C-lipid and studied *in vivo* (SI Fig. 4A). Small NTSL-Dox (90 nm) showed longer blood circulation up to 24 h compared to larger liposomes (175 nm) and 30%ID and 5%ID, respectively, were detected in the blood after 24 h post-injection (SI Fig. 4B, right). The shorter blood circulation of the larger liposomes was attributed to higher spleen uptake (SI Fig. 4C and 4D, right) [39]. Longer blood circulation of liposomes smaller than 100 nm has been previously reported by others [40]. In contrast, reducing the size of LTSL-Dox to 90 nm slightly increased the liposome blood circulation only at the early time points (up to 2 h; SI Fig. 4B left, insert). No difference in the blood level was observed with LTSL-Dox of different sizes between 2 h and 24 h (SI Fig. 4B). These findings highlighted that mean liposome diameter has as impact on blood circulation for the robust and circulating NTSL systems, but is of less importance for LTSL. The effect of Dox content was also studied and did not show any effect on NTSL pharmacokinetics (SI Fig. 5). To evaluate the drug leakage from these TSL and to assess whether the decrease in Dox blood levels

Table 1
Lipid composition, size, surface charge and Dox encapsulation efficiency of temperature-sensitive liposomes used in this study. The mean average diameter (nm), polydispersity index and surface charge of LTSL (Low Temperature-sensitive Liposomes), TTSL (Traditional Temperature-sensitive liposomes) and NTSL (Non-Temperature-sensitive Liposomes) were obtained using the Nanosizer ZS (Malvern, UK). Dox encapsulation efficiency was measured using spectrofluorometer.

Liposome	Lipid composition (molar ratio)	Mean diameter (nm) ±S.D[a]	Polydispersity index ±S.D[a]	Surface charge (mV) ±S.D[a]	Encapsulation efficiency of Dox (%)
LTSL-Dox	DPPC:MSPC:DSPE-PEG$_{2000}$(86:10:4)	101.5 ± 0.9	0.06 ± 0.01	−9.1 ± 0.4	100
TTSL-Dox	DPPC:HSPC:Chol:DSPE-PEG$_{2000}$(50:25:15:3)	97.5 ± 1.6	0.10 ± 0.05	−8.1 ± 0.3	97
NTSL-Dox	HSPC:Chol:DSPE-PEG$_{2000}$(75:50:3)	119.6 ± 1.2	0.05 ± 0.01	−15.9 ± 1.0	93

[a] Mean ± standard deviation; $n = 3$.

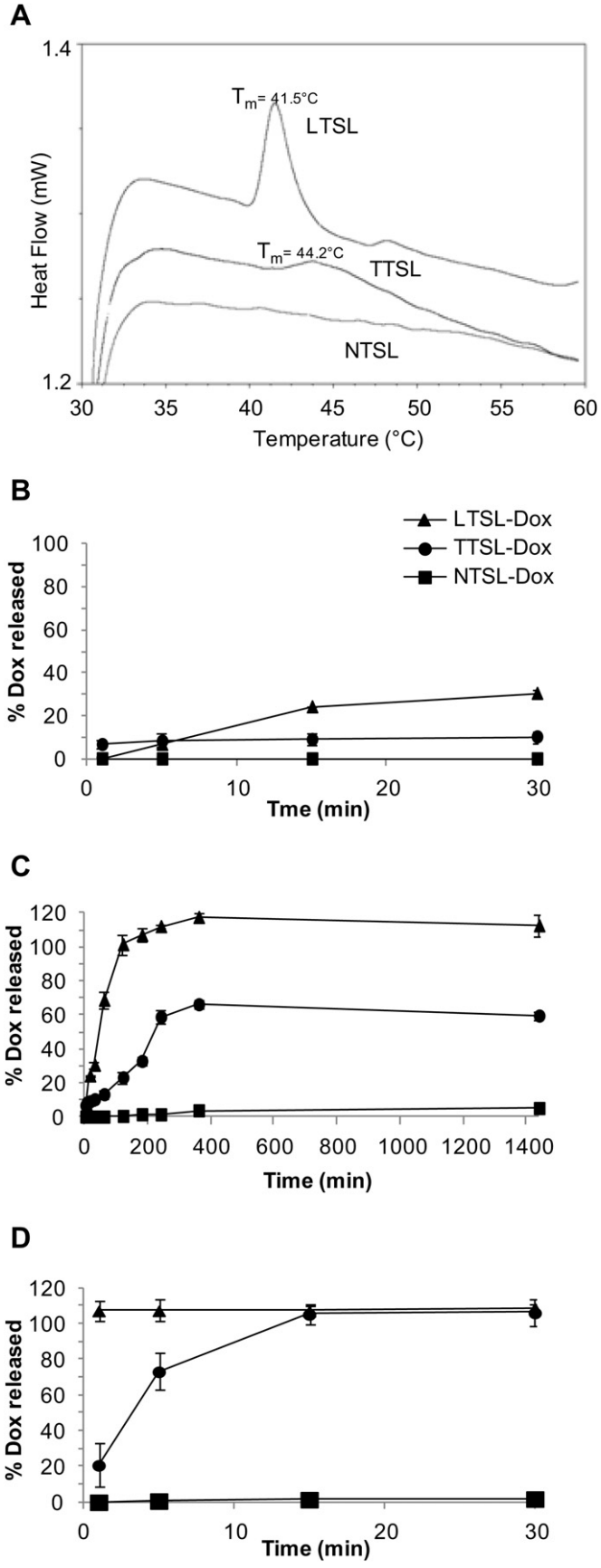

Fig. 2. Temperature sensitivity of Dox-LTLS, TTSL-Dox and NTSL-Dox liposomes in 50% mouse serum. (A) Differential scanning calorimetry (DSC) graph of LTSL-Dox, TTSL-Dox and NTSL-Dox. Release profile of encapsulated Dox from the three liposomal systems incubated in 50% mouse serum at 37 °C for (B) 30 min and (C) 24 h and (D) 42 °C for 30 min ($n = 3 \pm$ S.D).

was due to Dox leakage from liposomes and clearance, or due to the clearance of the intact lipsomes from the blood stream, the *in vivo* stability of dual labeled LTSL-Dox, TTSL-Dox and NTSL-Dox was measured by calculating the normalized Dox:phospholipid ($^{14}C/^{3}H$) ratio at different time points and compared to the ratio at zero time point [30]. In the absence of HT, the LTSL-Dox ratio decreased from 1 to 0.7 after 0.16 h (10 min) from injection, before further decreases to 0.4 after 1 h (Fig. 3C, triangles), indicating Dox clearance from the blood following release from LTSL. Chiu et al. and Tagami et al. recently reported 50–60% Dox release *in vivo* determined by high performance liquid chromatography (HPLC) after 60 min of intravenous administration [24,28]. The Dox:phospholipid ratio decreased further during HT (10–60 min) which indicates that Dox was released from LTSL in a temperature-sensitive manner at the heated tissues (Fig. 3D, triangles). TTSL-Dox showed unreported higher *in vivo* serum stability since its Dox:phospholipid ratio remained around 1 for the first hour post-injection, before gradual decrease to 0.67 and 0.51 after 6 h and 24 h, respectively. HT had a minimum effect on the *in vivo* stability of TTSL (Fig. 3D, circles). NTSL-Dox maintained the Dox:phospholipid ratio at 1 up to 24 h under all conditions (Fig. 3C and 3D, squares), which agrees with Cheng et al. who previously reported the clearance of intact Doxil liposomes from the blood stream [30]. The lower Dox levels with LTSL during HT were not due to systemic Dox release, since the mouse body temperature was controlled and did not exceed 37.5 °C for all injected groups (SI Fig. 2). The mouse body temperature was monitored carefully to make sure it was maintained between 36 and 37.5 °C, since rapid Dox release in blood circulation, especially in the case of LTSL-Dox, was observed if the body temperature increased to 39–42 °C (SI Fig. 3).

3.4. Tissue distribution of dual-labeled TSL

Organ uptake of ^{3}H-labeled liposomes and ^{14}C-Dox after 1 h (Fig. 4) and 24 h (Fig. 5) from injection in the presence and absence of HT were also obtained. All liposome systems in this study showed 5-6%ID liver accumulation 1 h post-injection (Fig. 4A), due to the presence of PEG_{2000} polymer at the liposome surface that reduces liposome opsonization with blood proteins [41]. Higher spleen accumulation (2–3 fold) was observed with NTSL (2.1%ID) and TTSL (1.4%ID) compared to LTSL (0.68%ID) (Fig. 4A). Negligible liposome uptake was detected in lung, kidney and heart. There was no marked difference in the tissue concentration of liposomes between groups with and without HT (Fig. 4B). A similar observation regarding the minimum effect of localized HT on organ uptake was previously reported with the delivery of monoclonal antibody fragments to subcutaneous human glioma xenografts in mice [42]. Organ-associated Dox correlated with liposome distribution in tissues 1 h post-injection, except with LTSL, where higher Dox compared to liposomes, was detected in the liver and kidney (16.2%ID & 8.7%ID, respectively) (Fig. 4C, black bars). Lower serum stability of LTSL-Dox could explain the higher level of Dox in liver and kidney. HT did not dramatically change Dox organ uptake 1 h after the injection, except lower Dox was present in the kidney with LTSL-Dox group treated with HT compared with LTSL-Dox without HT (Fig. 4D), that can be due to the rapid clearance of Dox from the kidney to the bladder.

24 h post-injection liver uptake of liposomes was significantly higher with all liposomes (Fig. 5A) compared to 1 h (Fig. 4A). Significant liver and spleen accumulation could account for the reduction in blood circulation shown in Fig. 3A and B. LTSL-Dox

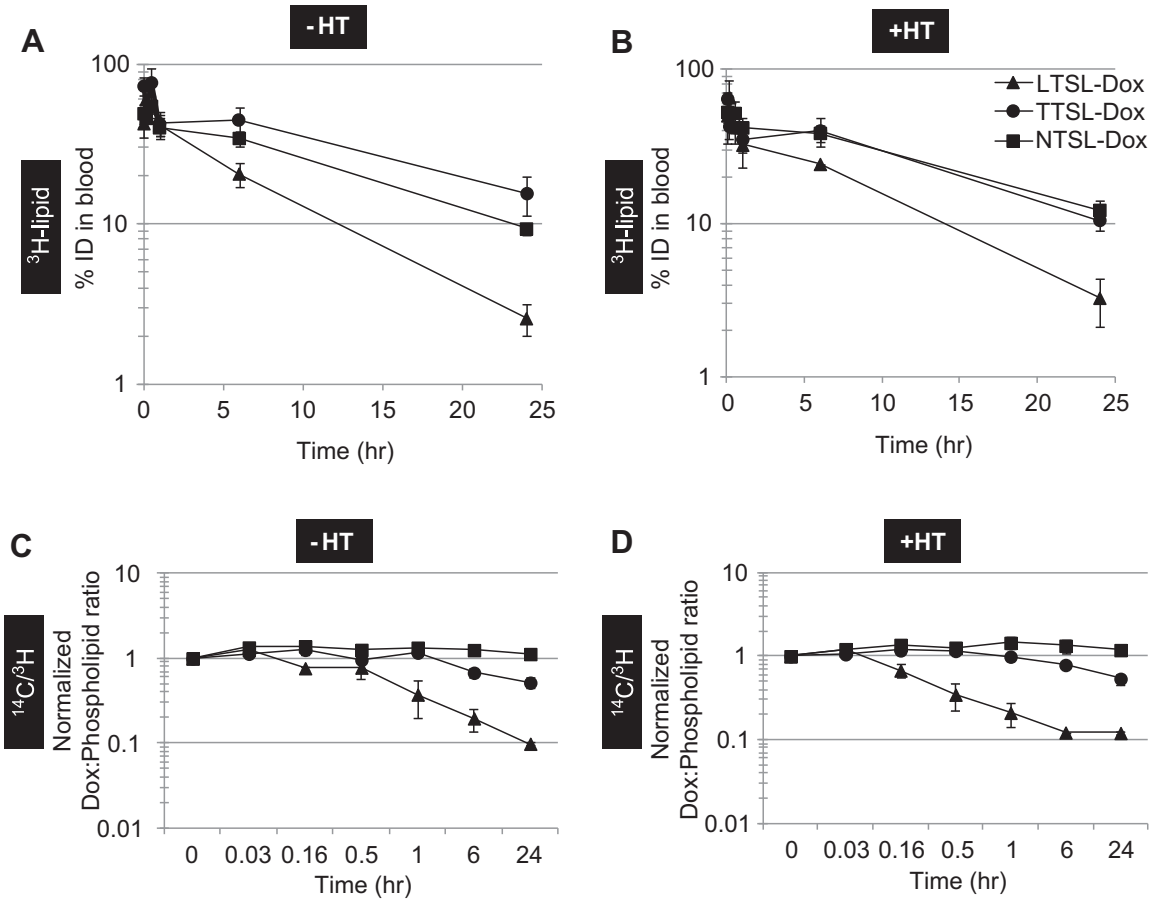

Fig. 3. Time-course blood residence of dual labeled (^{3}H/^{14}C) LTSL-Dox, TTSL-Dox, and NTSL-Dox liposomes in B16F10 tumor-bearing C57BL6 mice in the absence and presence of HT. Blood clearance profile of ^{3}H-labeled liposomes in the absence (A) and presence (B) of HT. I*n vivo* stability of dual labeled lipsomes was calculated by measuring normalized Dox:phospholipid ratio without (C) and with (D) HT. Data are expressed as mean ± S.D (n = 3–4).

showed slightly higher liver uptake (23.8%ID) compared to TTSL-Dox (15.3%ID) and NTSL-Dox (13.2%ID). On the other hand, NTSL-Dox and TTSL-Dox (to a lesser extent) showed higher spleen uptake (5.3 & 3.8%ID, respectively) compared to LTSL-Dox (1.03% ID). Within 24 h Dox was almost completely cleared from all organs in animals injected with LTSL-Dox. The highest Dox levels were obtained for NTSL-Dox in liver and spleen, reflecting the high liposome stability and drug retention of these liposomes [36].

3.5. Tumor uptake of dual-labeled TSL

The effect of localized, mild HT on liposome and Dox tumor accumulation is shown in Fig. 6. There was no significant difference in the tumor accumulation of ^{3}H-labeled liposomes 1 h after injection (Fig. 6A, left). However, significantly higher levels of liposome accumulation within the tumor tissue were observed after 24 h due to the enhanced permeation and retention effect (Fig. 6A, right) [26]. Highest tumor accumulation was obtained for the long blood circulating TTSL-Dox and NTSL-Dox. Good correlation in the tumor accumulation was obtained between ^{3}H-labled liposomes (Fig. 6A) and ^{14}C-Dox (Fig. 6C) at 1 h and 24 h after injection, except with LTSL-Dox, for which only moderate increase in liposome tumor accumulation was seen after 24 h (Fig. 6A, black bars) that did not result in higher Dox in the tumor (Fig. 6C, black bars). This finding was thought to be due to the lower serum stability of these liposomes compared to TTSL and NTSL (Fig. 3C and D).

Mild, localized HT increased liposome accumulation in the tumor 1 h post-injection by 1.5–3 fold, presumably due to increased blood perfusion and extravasation of liposomes (Fig. 6B, left). More importantly, such 'vasodilation effect' persisted even after the application of HT was terminated, similar to previous reports using both nanoparticles and Doxil-like liposomes [6,9]. At 24 h post-injection and HT, TTSL-Dox and NTSL-Dox showed a 10-fold increase in liposome accumulation (Fig. 6B, right) compared to that after 1 h of HT (Fig. 6B, left). In contrast, no significant increase in tumor accumulation of LTSL-Dox was observed 24 h post-HT.

In terms of drug (Dox) tumor uptake, immediately after HT (1 h) LTSL-Dox achieved the highest Dox accumulation (7.6%ID/g) (Fig. 6D, left) presumably due to rapid intravascular release of the drug in the heated tumor, as predicted from their release profile at 42 °C (Fig. 2D). Animals treated with TTSL-Dox or NTSL-Dox and HT, showed a 2-fold increase in Dox accumulation in tumors after 1 h, consistent with the HT-mediated 'vasodilation effect' observed when liposomes were tracked (Fig. 6B, left) [37]. Dox levels in the tumor tissue from LTSL-Dox at 24 h post-injection and HT was reduced to 2.7%ID/g. This was considered an indication that the drug was burst-released from these liposomes at the tumor vasculature and only a fraction of the Dox molecules were uptaken by tumor cells. A similar reduction in Dox levels has been observed after triggering the release of Dox from LTSL using high intensity focused ultrasound (HIFU) in murine adenocarcinoma tumors [43]. Tumor localization of Dox after 24 h post-injection and HT with TTSL-Dox and NTSL-Dox (13.5% and 31.4%ID/g, respectively) was found dramatically enhanced (Fig. 6D, right). This finding correlated with the high serum stability of these liposomes (Fig. 3C and D) confirming that prolonged tumor extravasation of these long-circulating liposomes (Fig. 3A) was occurring.

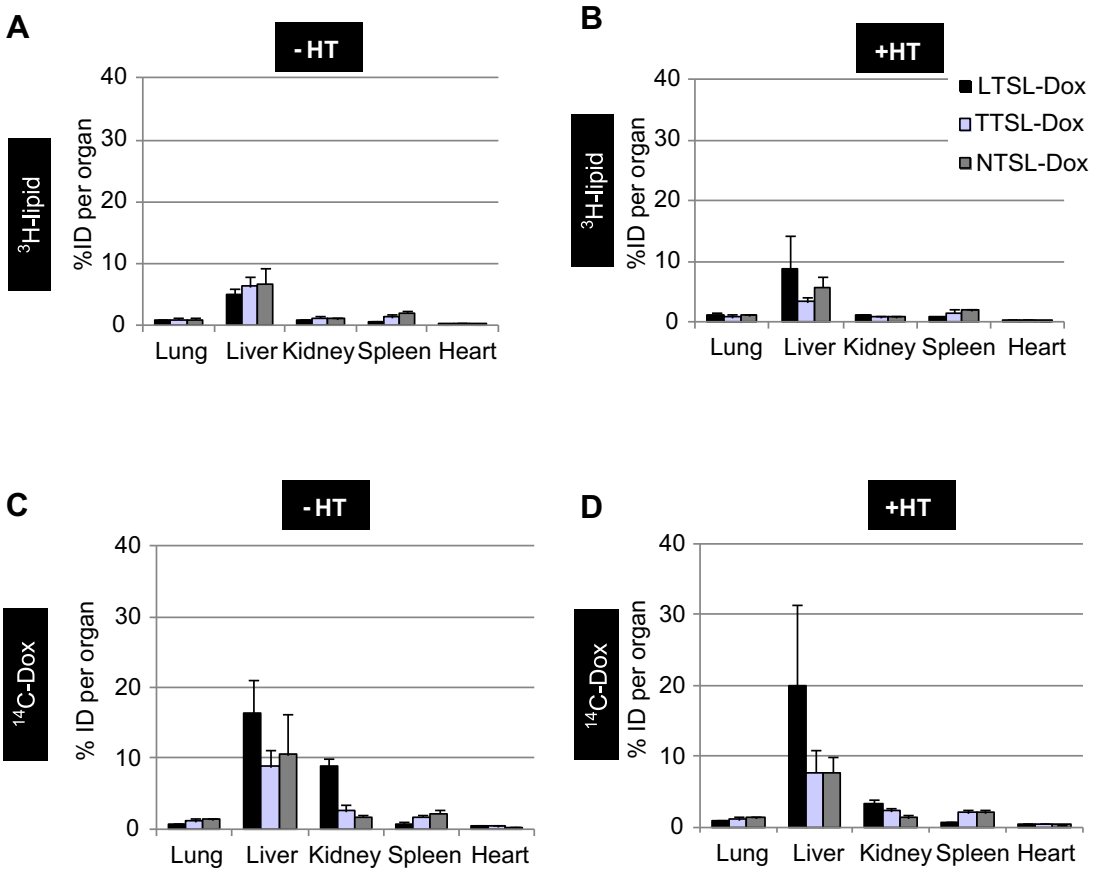

Fig. 4. Organ biodistribution of dual-labeled (^{3}H/^{14}C) LTSL-Dox, TTSL-Dox, and NTSL-Dox liposomes in tumor-bearing C57BL6 mice 1 h post intravenous administration without or with hyperthermia. Organ uptake of ^{3}H-labeled liposomes (LTSL-Dox, TTSL-Dox and NTSL-Dox) in the absence (A) and presence (B) of HT. Organ uptake of ^{14}C-Dox loaded liposomes in the absence (C) and presence (D) of HT. Data are expressed as mean ± S.D ($n = 3–4$).

4. Discussion

Drug-loaded temperature-sensitive liposomes (TSL) in combination with HT have attracted considerable attention for cancer treatment both clinically [25] and pre-clinically [44–46]. This combination is designed to selectively increase the local drug concentration in tumors and lead to a significant improvement in therapeutic efficacy. Different TSL systems have been designed in the last two decades with wide variations in their serum stability and temperature sensitivity [11,16,18–20,23]. In principle, liposome systems that retain the encapsulated drug while in the blood stream before releasing their load upon HT-mediated triggering at the target tissue is a prerequisite for clinical applications. Despite the extensive study and analysis of tissue distribution and the sophisticated pharmacokinetic modeling for NTSL, there is little such knowledge available about TSL. The existing biodistribution studies of different TSL types are based on the determination of drug (mostly doxorubicin) levels in the blood and tumors and, occasionally, in some normal organs. For example, Banno et al. studied the functional role of PEG_{2000} and lysolipid content on the drug release from LTSL following systemic administration *in vivo* [27]. The same group previously reported improved Dox loading into LTSL via complexation with transition metals (manganese) without affecting the LTSL *in vivo* profile [28]. These two LTSL biodistribution studies were performed in non-tumor bearing mice, in the absence of HT application and Dox levels were assessed only in the blood. Their results indicated that Dox leakage was highly dependent on the content of lysolipid in the formulation. Inclusion of 10% lysolipid molecules in the composition of the liposomes showed 50% Dox leakage at 60 min post-injection which was similar to our findings (Fig. 3C). No quantitative organ biodistribution data was reported with LTSL until the recent study by Tagami et al. where Dox levels in different organs were compared to free Dox or Dox-loaded Brij78-containing liposomes following HT treatment [24]. In the present work we attempted to offer a comprehensive study of the pharmacokinetics and organ uptake of doxorubicin-loaded TSL by simultaneous and quantitative tracking of both Dox and the liposome carriers that has been lacking. Furthermore, this study explored the immediate and delayed (up to 24 h) effects of mild, localized HT on the pharmacokinetics and biodistribution of these liposomal systems in tumor-bearing mice.

NTSL, comparable to the clinically used Doxil®, were extremely stable *in vitro* and *in vivo* under physiological conditions as well as at 42 °C (Figs. 2 and 3). Two types of TSL of different composition and thermo-responsiveness showed different kinetics in serum and under HT conditions (Figs. 2 and 3). LTSL showed lower serum stability in the blood stream, primarily due to the presence of lysolipid in the lipid bilayer that can exchange and translocate with various blood components as described [27]. Such translocation can lead to a thinning in the liposomes membrane, forming pore defects [47] and drug leakage following burst-release kinetics. TTSL is an alternative thermosensitive system with intermediate drug release kinetics that showed significantly higher stability in serum and under HT.

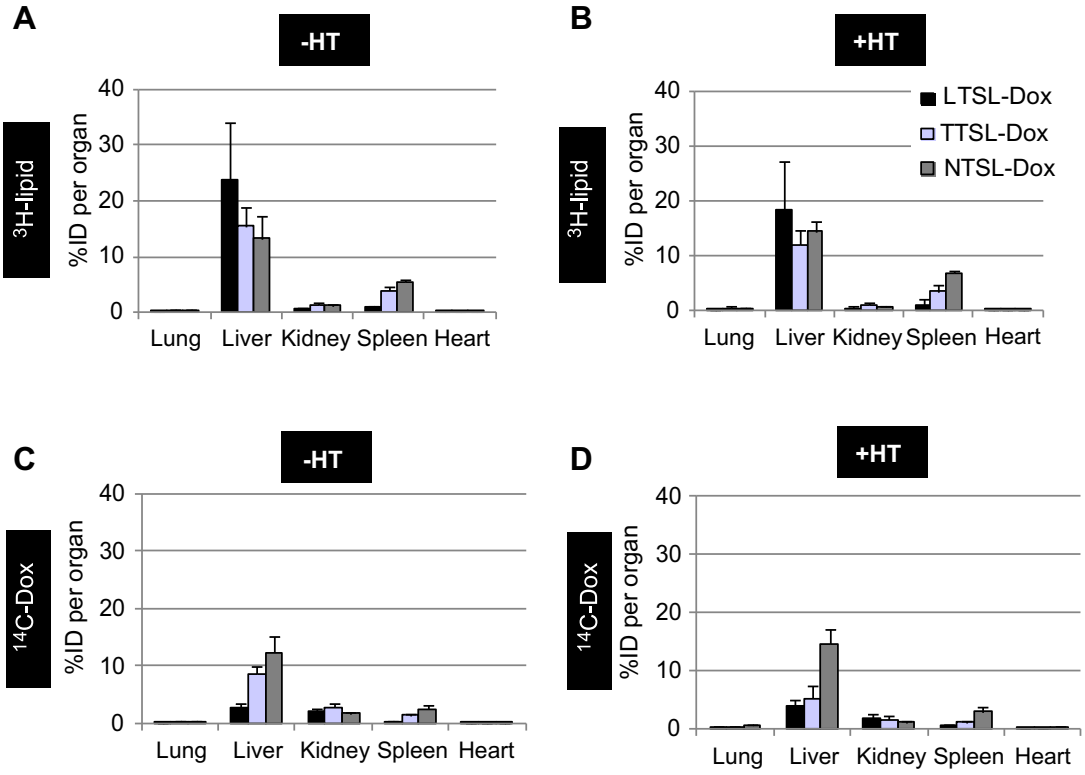

Fig. 5. Organ biodistribution of dual-labeled (^{3}H/^{14}C) LTSL-Dox, TTSL-Dox, and NTSL-Dox liposomes in tumor-bearing C57BL6 mice 24 h post intravenous administration without or with hyperthermia. Organ uptake of ^{3}H-labeled liposomes (LTSL-Dox, TTSL-Dox and NTSL-Dox) in the absence (A) and presence (B) of HT. Organ uptake of ^{14}C-Dox loaded liposomes in the absence (C) and presence (D) of HT. Data are expressed as mean ± S.D ($n = 3$–4).

Moreover, all the critical parameters that are well-described to affect the pharmacological behavior of liposomes, such as diameter and lipid composition have been largely ignored for TSL systems [40,48]. The results here showed that liposome size and composition dramatically affected TSL blood circulation and organ uptake. The large-sized NTSL exhibited shorter blood circulation times with significantly higher spleen uptake, while large-sized LTSL showed only marginally faster blood clearance (compared to small LTSL) and the same level of liver and spleen uptake irrespective of mean diameter (Fig. 5). These observations may indicate the involvement of serum protein interactions specifically with the lysolipid molecules contained in LTSL bilayers. Banno et al. recently showed lysolipid desorption of LTSL *in vivo* [27] and further work is needed to further understand how such interactions may lead to LTSL liver uptake. In general, determination of TSL organ uptake is also critical to identify any potential tissue toxicity following their administration and accumulation in organs. For example, LTSL showed higher liver uptake 24 h post-injection compared to TTSL and NTSL, while the latter showed preferential splenic uptake (Fig. 5). Such differences in tissue accumulation may allow careful monitoring of the tissues under higher toxicological risk, especially in the case of the clinically developed lysolipid-containing liposomes. More studies need to be conducted to assess the fate of lysolipids incorporated into LTSL to address any concern regarding the *in vivo* toxicity of these molecules [49–51]. In terms of the clinical context of the present study, determination of the *in vivo* stability of TSL (Fig. 3) can offer better understanding of the current clinical protocols used in treatments with LTSL-type systems (such as ThermoDox®). In the current clinical protocols, Dox-loaded LTSL are administered as an infusion over 30 min starting 15 min before HT application [52,53]. Dox leakage from LTSL *in vivo* (Fig. 3) can justify the need of LTSL infusion to maintain high blood levels of the LTSL-Dox once HT is applied [52,53]. This study also indicated that alternative TSL systems with higher serum stability and longer blood circulation may allow enhanced Dox-liposome tumor extravasation after application of HT (Fig. 6).

Localized (tumor tissue only), mild HT did not affect the liposome uptake in normal tissues, however had a dramatic effect on tumor uptake (Fig. 6). Localized mild HT had an effect on all liposome types studied, with tumor uptake increases ranging between 1.5 and 3 fold within the first hour (Fig. 6). Most interestingly though was the dramatically enhanced liposome tumor extravasation observed up to 24 h post-injection, even though the tissue was mildly heated only during the first hour, as observed in the case of the longer-circulating TTSL and NTSL. Such impact from HT treatments had been previously described by Dewhirst and colleagues, mainly in relation to tumor vasculature and an endothelium shrinkage in response to HT, leading to larger pore fenestrations in tumor blood vessels persistent for up to 6 h post-HT treatment [5,6]. This effect, in combination with accelerated blood flow and the stability of TTSL and NTS can explain the marked increase in liposome and Dox accumulation in tumors after 24 h (Fig. 6). Increased Dox accumulation 24 h post-HT treatment was previously described only for NTSL in a C-26 tumor model. No such report for any type of TSL has been published before, since most studies determined Dox levels within tumors only after 1 h [13,15,24,54]. Such unreported observations can be of direct relevance to clinical practice of HT procedures suggesting the benefit of multiple mild HT sessions to release drugs from TSL after the significant tumor extravasation observed. Further work needs to be undertaken to achieve adequate Dox release from tumor-residing TSL systems using different HT regimens that can lead to enhanced therapeutic efficacy.

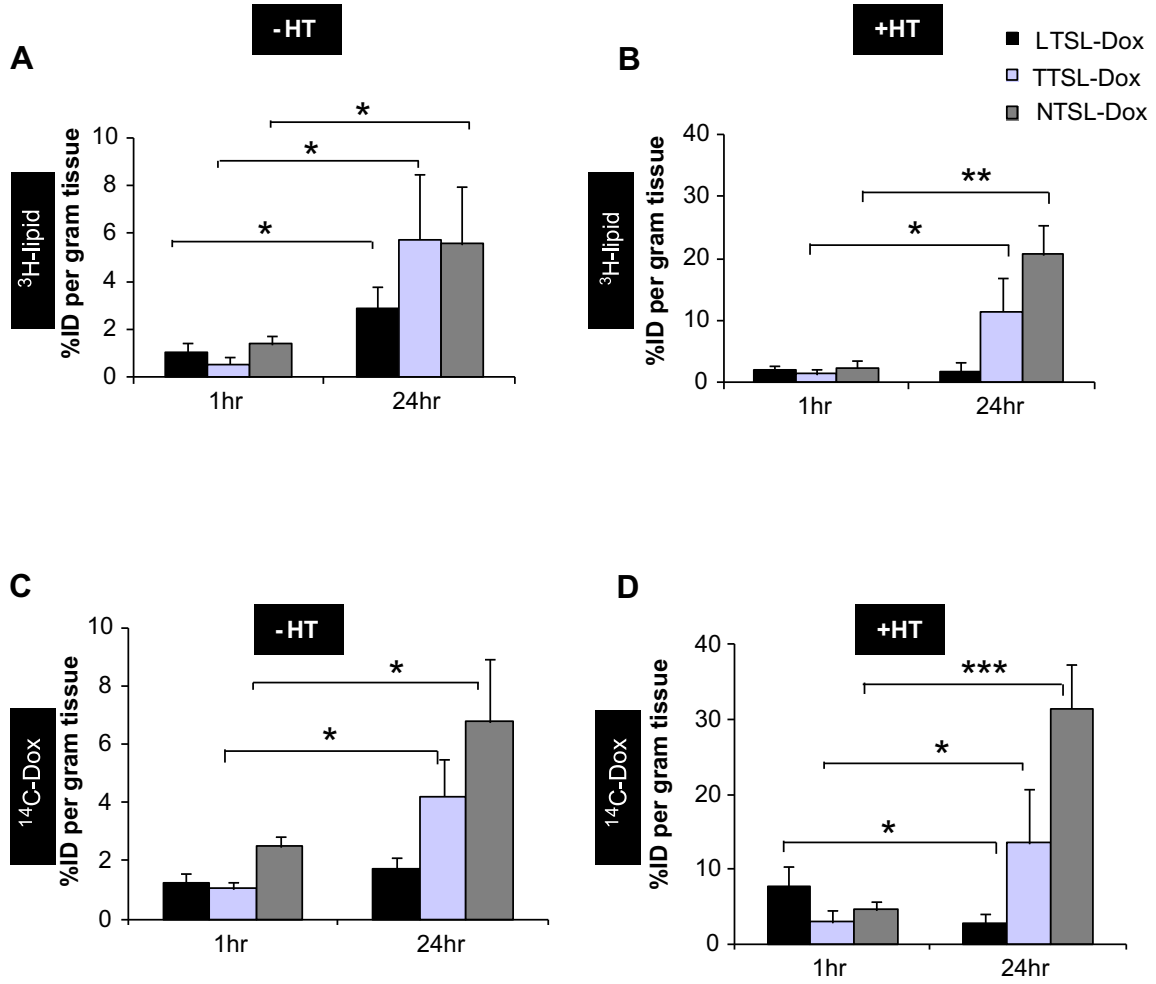

Fig. 6. Time-course of tumor accumulation of dual labeled (^{3}H/^{14}C) LTSL-Dox, TTSL-Dox, and NTSL-Dox liposomes in B16F10 tumor-bearing C57BL6 mice without or with HT. Tumor uptake of ^{3}H-labeled liposomes after 1hr and 24hr following intravenous administration in the absence (A) and presence (B) of HT. Tumor uptake of ^{14}C-Dox loaded liposomes after 1 h and 24 h following intravenous administration in the absence (C) and presence (D) of HT. Data are expressed as mean ± S.D ($n = 3–4$). Differences were considered significant at $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

5. Conclusion

The present study offers a pharmacological framework of understanding based on systematic and quantitative experimental data of drug release kinetics from doxorubicin-loaded TSL under mild HT treatment. The balance between *in vivo* stability, blood circulation and tissue distribution of TSL systems in the presence and absence of localized (tumor tissue only), mild HT within 24 h determine the levels of drug accumulation that can be achieved in tumors. These results can also contribute in the optimization of clinically-relevant HT protocols to best match the type of TSL in use to maximize therapeutic efficacy.

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Appendix A. Supporting information

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2012.03.018.

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