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Sequential HIFU heating and nanobubble encapsulation provide efficient drug penetration from stealth and temperature sensitive liposomes in colon cancer

Joshua VanOsdol*, Kalyani Ektate*, Selvarani Ramasami, Danny Maples, Willie Collins, Jerry Malayer, and Ashish Ranjan

Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma

Abstract

Mild hyperthermia generated using high intensity focused ultrasound (HIFU) and microbubbles (MBs) can improve tumor drug delivery from non-thermosensitive liposomes (NTSLs) and low temperature sensitive liposomes (LTSLs). However, MB and HIFU are limited by the half-life of the contrast agent and challenges in accurate control of large volume tumor hyperthermia for longer duration (>30min.). The objectives of this study were to: 1) synthesize and characterized long-circulating echogenic nanobubble encapsulated LTSLs (ELTSLs) and NTSLs (ENTSLs), 2) evaluate in vivo drug release following short duration (~20 min each) HIFU treatments administered sequentially over an hour in a large volume of mouse xenograft colon tumor, and 3) determine the impact of the HIFU/nanobubble combination on intratumoral drug distribution. LTSLs and NTSLs containing doxorubicin (Dox) were co-loaded with a nanobubble contrast agent (perfluoropentane, PFP) using a one-step sonoporation method to create ELTSLs and ENTSLS, which then were characterized for size, release in a physiological buffer, and ability to encapsulate PFP. For the HIFU group, mild hyperthermia (40–42 °C) was completed within 90 min after liposome infusion administered sequentially in three regions of the tumor. Fluorescence microscopy and high performance liquid chromatography analysis were performed to determine the spatial distribution and concentration of Dox in the treated regions. PFP encapsulation within ELTSLs and ENTSLS did not impact size or cause premature drug release in physiological buffer. As time progressed, the delivery of Dox decreased in HIFU-treated tumors with ELTSLs, but this phenomenon was absent in the LTSL, NTSL, and ENTSLS groups. Most importantly, PFP encapsulation improved Dox penetration in the tumor periphery and core and did not impact the distribution of Dox in non-tumor organs/tissues. Data from this study suggest that short duration and sequential HIFU treatment could have significant benefits and that its action can be potentiated by nanobubble agents to result in improved drug penetration.

Correspondence: Dr. Ashish Ranjan, B.V.Sc., Ph.D., Associate Professor & Kerr Endowed Chair, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma-74074, Phone: 4057446292; Fax: 4057448263, ashish.ranjan@okstate.edu.

*Both authors contributed equally to this study

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Graphical abstract

Schematic illustration of Doxorubicin (dox) delivery regulated by zonal administration of High Intensity Focused Ultrasound (HIFU) across mouse colon tumor

Keywords

HIFU; echogenic liposomes; drug penetration; stealth liposomes; temperature sensitive liposomes

1. Introduction

To improve cancer chemotherapy delivery and survival outcomes, particularly in difficult cancers (e.g. ovarian, pancreas, primary liver tumor etc.), a key current direction is use of nanomedicine such as liposomes. These range in type from long-circulating non-thermosensitive liposomes (NTSL; e.g. Doxil, Onivyde, etc.) and low temperature sensitive liposomes (LTSL) that release drug above 40°C (e.g. Thermodox [1–5]). These liposomes are also being combined with high intensity focused ultrasound (HIFU) and microbubbles (MBs) to leverage precisely selected and dynamic modulation of biological, physiological, and mass transport properties of the tumor microenvironment [6–10]. These are promising advancements, but they are associated with several limitations. For example, MBs have short half-lives (within a few minutes) and limited drug payload capacity [11], and additional innovations are needed in formulation approaches to improve their use for image guided drug delivery (IGDD) [12]. Administering mild hyperthermia (40–45 °C) with HIFU to the entire volume of a deep seated tumor for long duration (~30min.–1 h) is associated with technical challenges related to spatiotemporal control, thereby reducing its feasibility for clinical use [13]. Thus, developing new approaches for administration of HIFU treatment for desired drug release from liposomes in tumors is needed.

Theoretically, liposomes offer the key advantage of ferrying both imaging and therapeutic agents that can potentially be utilized to measure/monitor the temporal and spatial patterns of solid tumor IGDD. However, such capability is limited by multiple factors, including target tissue movement, low spatial resolution (in positron emission tomography (PET), off-target radiation exposure (in PET or computed tomography (CT)), and inability to accurately define regions of interest (ROIs) at certain tissue depths (in fluorescence and luminescence modalities). Unlike these IGDD modalities, US imaging is safe, portable, widely-accessible and provides unlimited field of view at large distances from the body surface for routine clinical use. In prior research, the encapsulation of a perfluoropentane (PFP)-based nanobubble contrast agent in enabled it to stay in liquid form in the liposome core because

of Laplace pressure, thereby allowing PFP to attain gas bubble state and echogenicity slowly at body temperature [14]. This phenomenon resulted in longer circulatory life and stable ultrasound imageability [15]. A variety of methodologies have been reported for PFP encapsulation in liposomes. Recently, we adapted the PFP emulsification encapsulation technology reported previously by Ibsen et al. and others [16–18] to synthesize nanobubble encapsulated echogenic LTSLs (ELTSLs) and ENTSLs. Our central hypothesis is that interaction of long circulating echogenic liposomes with HIFU hyperthermia ($\sim 40\text{--}42^\circ\text{C}$) can acoustically modulate the tumor microenvironment to result in improved drug penetration relative to liposomes alone. The motivation to this idea stems from our previous studies where increased liposomal drug penetration by reducing interstitial fluid pressure [7, 19] and improving tumor perfusion was noted [20, 21]. We believe that by incorporating PFP in liposomes and its combination with short bursts of HIFU treatment, the drug release and liposome transport in tumor blood vessels within the first hour of injection can be further improved drastically, followed by transport of doxorubicin across the endothelial barrier and cellular uptake in tumor by bubble mediated sonoporation [20, 21].

The current approach in liposome mediated drug delivery is to apply HIFU homogeneously in a small volume of target region for $\sim 30\text{--}60\text{min}$. This is promising, but for effective therapeutic outcome and realize the full potential of liposome and HIFU combination in clinic, a large volume of tumor must be treated within the first hour of injection. In this regard, we hypothesized that in contrast to focal heating of small volumes for long duration, zonal heating of a tumor covering the entire volume for shorter durations upon echogenic liposome injection can be an elegant way to increase extravasation of the released drug from NTSLs or LTSLs (see schematic illustration), however this has not heretofore been adapted to cancer drug delivery. The objective of this study was to apply ELTSLs and ENTSLs in a mouse model and determine their impact on drug delivery following zonal hyperthermia of a large volume of colon tumor. Additionally, the influence of tumor transit time of LTSLs/NTSLs and the synergistic benefits of thermally induced drug release as opposed to ultrasound induced inertial cavitation for modulation of the tumor microenvironment and extracellular matrix for tumor drug penetration was assessed.

2. Materials and Methods

2.1 Materials

PFP (99%, Exflur Research Corporation, Round Rock, TX, USA) was used as the US contrast agent. Monostearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (Polyethylene glycol)2000] (DSPE-mPEG2000) were obtained from Corden Pharma Corporation (Boulder, CO, USA). 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (LissamineTM Rhodamine B) was obtained from Thermo Fisher Scientific Inc., and PKH67 Green Fluorescent Cell Linker was obtained from Sigma Aldrich (Milwaukee WI, USA). Dox was obtained from LC Laboratory (Woburn, MA, USA). Acetonitrile (high performance liquid chromatography (HPLC) grade) was obtained from Pharmco-AAPER (Brookfield, CT, USA). Ethylene glycol (99%, spectrophotometric grade), phenylboronic acid (98%), and 2,2-

dimethoxypropane (98%) were purchased from Alpha Aesar (Ward Hill, MA, USA). The PD-10 column was obtained from GE Healthcare Life Sciences, (Buckinghamshire, United Kingdom, UK). C26 cells were kindly provided by the National Cancer Institute.

2.2 Synthesis of ELTSLs and ENTSLs

LTSLs (lipid composition: DPPC, MSPC, and DSPE-mPEG2000 molar ratio of 85.3:9.7:5.0) and NTSLs (DPPC, cholesterol, and DSPE-mPEG2000 in the molar ratio of 58.1:36.8:5.07) were prepared by hydration of a lipid film followed by the extrusion method as described previously [22–24]. Briefly, lipid mixtures were dissolved in chloroform. The solvent was evaporated and the resulting lipid film was hydrated in citrate buffer (pH 4.0) mixed with 1,3-propanediol (1, 3-PD) (0.65 M, for PFP emulsification) at 55 °C for 30 min and extruded five times through double stacked 200 nm polycarbonate filters to yield a final lipid concentration of 50 mg lipid/ml (80.8 mM for LTSLs and 70.3 mM NTSLs). A PD-10 size-exclusion column equilibrated with 5–10 column volumes of 1x phosphate buffered saline (PBS) was used to remove free 1,3-PD from the outside of the liposomes.

Encapsulation of Dox into the LTSLs and NTSLs was carried out using the pH-gradient loading protocol described by Mayer et al. [21]. In general, the outside of the ELTSLs was adjusted (by column) to about pH 7.4 using PBS, whereas the inside remained acidic at pH 4. Dox was loaded at 2 mg/100 mg lipid concentration at 37 °C for 1 h. PFP-loaded ELTSLs and ENTSLs were prepared using a one-step sonoporation method. Briefly, 2 mL of the liposomal formulations were incubated under continuous sonication (~20 khz) in 3 mL vials along with PFP (boiling point 30 °C; 20 µL/100 mg lipid) for 1–2 min. PFP and LTSLs were kept cold prior to being combined, and the sonication bath was kept at 4 °C to minimize PFP vaporization. This method was repeated at least in triplicate (n = 3) for evaluation. Free Dox and PFP were removed using a PD-10 column. For all in vitro characterizations, LTSLs were used as a positive control. LTSL synthesis was carried out using our previously published procedure [23].

2.3 Confirmation of PFP encapsulation in ELTSLs using a fluorescence technique

To confirm PFP loading in LTSLs, a fluorescent methodology was developed. Briefly, a thin film of LTSLs (lipid composition: DPPC, MSPC, DSPE-mPEG2000, and LissamineTM Rhodamine B at a molar ratio of 84.3:9.7:5:1) was prepared as described in section 2.2 and hydrated with 1 mL of water for 15–30 min at 55 °C. Next, 9 µL of PFP and 1 µL of PKH67 were transferred in a 0.5 mL tube and sonicated for 5 min at 4 °C, and 5 µL of PKH-67 labeled PFP then were sonicated with 1 ml of LTSLs and directly imaged on a mattek well plate under an Olympus IX81 confocal microscope using a 2 ms exposure time with the Tetramethylrhodamine (TRITC, ex/em of 545/600 filter) and a 100 ms exposure time with the green fluorescent protein (GFP, ex/em of 480/530) filter at 60x APO.

To measure PFP stability as a function of temperature (25–42°C), ELTSLs (50 mg lipid/ml) were diluted 300 fold in PBS, and 3 mL of sample were placed in a quartz cuvette equipped with a stopper and magnetic stirrer. Fluorescence of the released PKH67 labelled PFP was recorded at an excitation wavelength of 490 nm and fluorescence emission was measured at 502 nm using a Cary Eclipse Fluorescence Spectrometer (Agilent Technologies, Santa Clara,

CA, USA) equipped with an inbuilt temperature control system. The temperature was set to steadily increase from 25.5 to 42.5°C. A fluorescent reading was recorded at every 0.5°C increment. 10x triton was used to lyse the ELTSLs after the temperature increase to allow complete release of PFP. Empty LTSL signal was used as a blank.

2.4 Characterization of ELTSLs and ENTSLs

ELTSLs and ENTSLs were characterized for size (z-average) using dynamic light scattering (DLS) with a 90 plus PALS Nanobrook device (Brookhaven Instruments, Holtsville, NY, USA). Briefly, 10–20 µL of ELTSLs or ENTSLs were added to 2 ml of PBS in a cuvette, and DLS measurements were recorded at room temperature. For each liposomal formulation an average of five measurements were taken, and the mean size and standard deviation were calculated for the ELTSL and ENTSL samples. Zeta potentials were measured using water rather than PBS with the same equipment and lipids.

2.5 Dox release from ELTSLs and ENTSLs

Stability was assessed by measuring release of encapsulated Dox from ELTSLs and ENTSLs as a function of temperature (25–42°C) in PBS. ELTSLs (50 mg lipid/ml) and ENTSLs (50 mg lipid/ml) were diluted 300 fold in PBS, and 3 mL of sample were placed in a quartz cuvette equipped with a stopper and magnetic stirrer. Fluorescence of the released Dox was recorded at an excitation wavelength of 480 nm and fluorescence emission was measured at 590 nm using a Cary Eclipse Fluorescence Spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an inbuilt temperature control system. The temperature was set to steadily increase from 25.5 to 42.5°C. A fluorescent reading was recorded at every 0.5°C increment.

Drug release based on fluorescence quantification as a function of change in temperature was determined using the following equation:

$$\% \text{ Dox release} = [(I_t - I_o) / (I_m - I_o)] \times 100$$

where I_o represents the initial fluorescence intensity of ELTSLs and ENTSLs in suspension at 25°C, I_t is the intensity of the same sample at a predetermined temperature, and I_m represents is the fluorescence intensity of completely released Dox from ELTSLs at 45 °C or from ENTSLs post-breaking of the liposomes with 10x triton.

2.6 In vivo drug delivery study

2.6.1 Study design—Athymic nude mice bearing C26 mouse adenocarcinoma cell tumors were divided into nine treatment groups designed using a combination of four different liposome formulations with or without HIFU: NTSLs +/- HIFU, ENTSLs +/- HIFU, LTSLs +/- HIFU, ELTSLs +/- HIFU, and free Dox only. In all groups (n = 5–6 mice), 5 mg Dox/kg body weight were administered intravenously.

2.6.2 Establishment of mouse model of colon cancer—All animal-related procedures were approved and carried out under the regulations and guidelines of the Oklahoma State University Animal Care and Use Committee. C26 cells were grown as a

monolayer to 80–90% confluence in RPMI supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v streptomycin/penicillin. Confluent cells were harvested, washed, and diluted with sterile cold PBS to generate 0.5×10^5 cells/50 μ l. Next, 50 μ l of cell inoculum was injected per mouse in the thigh region of the mouse hind leg using a 25-gauge needle (BD, Franklin Lakes, NJ, USA). Mice were monitored and tumor growth was measured by serial caliper measurements (General Tools Fraction+™, New York, NY, USA). Tumor volumes were calculated using the formula $(\text{length} \times \text{width}^2)/2$, where length is the largest dimension and width is the smallest dimension perpendicular to length. Tumors were allowed to grow to a volume of 300–400 mm³ prior to initiating studies, and treated at 5mg Dox/Kg Body weight.

2.6.3 Segmental hyperthermia generation in mouse tumors—An integrated ultrasound-HIFU alpinion system was used for tumor identification, sonication, and treatment characterization. The HIFU transducer has 1.0 MHz central frequency, 45 mm radius, and 64 mm aperture diameter with a central opening 40 mm in diameter. For HIFU treatments, each tumor was divided into two halves (top and bottom). The top half was further divided into three fragments along the y-axis: F1, F2, and F3, where F1 was on top, F2 in the middle, and F3 on the bottom (Fig 1a). A 3×3 raster pattern of focal points (A, B, C) along the x axis was used to generate a mild-hyperthermia gradient of 40–42°C for ~20min in each fragment (Fig. 1b). Each focal point (~5.23 mm³) with dimensions of 1×1×10mm in x, y and z, axis, respectively was heated sequentially for 7min. Thus, this methodology covered ~ 25mm³ of each fragment for a 20min. hyperthermia session (Fig. 1b). Since the transducer footprint (10mm) was aligned along the z axis, adjacent muscle and bone tissue were also heated during sequential heating.

2.6.4 HIFU treatment set-up and methodology—Mice were anesthetized with 2–5% isoflurane and restrained in custom built mouse holders attached to a 3D positioning stage. The tumor bearing flank region was dipped in degassed water maintained at 37 °C for coupling with the HIFU transducer. Using real time ultrasound guidance, the tumors were positioned so that the target was in the center of the focal zone of the transducer. Prior to actual drug delivery studies, we calibrated the instrument to a temperature of 42 °C in tumor bearing mice by optimizing the HIFU parameters (duty cycle, pulse repetitive frequency, total acoustic power, and time) as described previously [25, 26]. Furthermore, a temperature gradient was established by inserting a thermocouple inside of the mouse tumor and taking readings at and up to 2 mm away from the focus. This was done to ascertain the effective diameter over which sufficient heating for Dox release could be achieved (Fig. 1b). Tumors were allowed to equilibrate for 3 min before commencing the treatment. For hyperthermia treatment, the center of the tumor was aligned with the HIFU focus at a fixed focal depth for efficient coverage, and VIFU-2000 software was used to define the target boundary and slice distance in X, Y, and Z directions for automatic rastering of the transducer. HIFU treatment parameters used were as follows: HIFU treatment parameters used were as follows: 5 Hz frequency, 50% duty cycle, 10 W acoustic power, and a peak positive/negative pressure of 6.15/–4.41 MPa to achieve a mean target temperature of 40–42.5 °C at the focus.

2.6.5 Post HIFU treatment tissue harvesting procedures—Upon completion of treatment, mice were euthanized. To clear the vasculature of drug/liposomes, transcardiac perfusion was performed immediately by opening the chest cavity and intracardially injecting 10 mL of PBS. Tumor and tissue samples from liver, spleen, lung, heart, kidney, skin, and muscle both adjacent and contralateral to the heated tumor were excised, weighed, snap-frozen over liquid nitrogen, and stored at -80°C until Dox analysis.

2.7 Quantification of Dox by HPLC

Tissue homogenization and sample preparation for HPLC were carried out using previously published methods [7, 22]. Briefly, samples were homogenized, and Dox was extracted and quantified with HPLC using daunorubicin as the internal standard. The HPLC detection system consisted of fluorescence detectors, and detection was performed at ex/em of 498/593 nm. Data were acquired using Shimadzu LC solution software. Concentrations of the analyte in tissues were determined using peak-area ratios of the sample analyte to the internal standard from the calibration curve.

2.8 Histology

A single whole tumor tissue sample from each HIFU group as well as the free drug group was selected prior to homogenization to be saved for histology. Histological analysis of NTSL and LTSL alone treated tumors were not performed since they do not demonstrate Dox penetration in the absence of HIFU. To analyze Dox distribution, 8 μm sections of frozen tumors were prepared, and two serial sections were mounted per slide. Fluorescently-labeled tissues were mounted with mounting medium containing DAPI (Vector Laboratories) to visualize cell nuclei at an exposure time of 10 ms (ex/em of 365/440), and Dox was imaged at an exposure 100 ms (ex/em of 480/590). Image acquisition and display parameters were constant for different treatments to allow for qualitative comparison. Whole-section digital histological scans were acquired using a 10X objective on an Olympus ZDC2 IX81 fluorescence microscope equipped with a color CCD camera, cooled monochrome CCD camera, motorized scanning stage, and Metamorph mosaic stitching software. Cell density, and cavitation were assessed in hematoxylin- and eosin- (H&E) stained sections using an Aperio ScanScope at 20x.

2.9 Statistical analysis

Treatment groups were compared for differences in mean tumor Dox concentration using analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. All analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc.). All p-values were two-sided, and $p < 0.05$ was taken to indicate statistical significance. Values were reported as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1 Fluorescence analysis of PFP encapsulation in liposomes

Confocal microscopy confirmed the presence of the PKH-labeled PFP emulsion within the liposome aqueous core at 25°C (Fig. 2a–c). Its presence was indicated by distinct yellow-to-orange spots formed by co-localization of red LTSLs with green PFP following sonication.

PFP remained stable inside the ELTSL from 25–43°C, and a more intense signal indicative of release was noticed following triton (Fig. 2d).

3.2 Characterization of ELTSLs and ENTSLs

Active loading of Dox by transmembrane pH gradient yielded an encapsulation efficiency of ~95% and ~65% for ELTSLs and ENTSLs, respectively. Following PFP loading by one-step sonoporation, ELTSLs and ENTSLs retained about 70–80% of the encapsulated drug. Table 1 shows the hydrodynamic diameter, polydispersity index, and zeta potential values of ELTSLs and ENTSLs at room temperature (25 °C), and these were fairly similar among various groups following PFP loading.

3.3 Dox release from ELTSLs and ENTSLs in physiological buffer

Percent Dox release from ELTSL and LTSL alike in PBS by its fluorescence dequenching was minimal (<5%) at 25–39°C (Fig. 3); was followed by a more gradual release at 40°C (~20%), and was rapid and complete (>95%) near the temperature giving maximum release rate (~41–42°C). In 10% FBS, Dox release from ELTSL and LTSL was ~20% at 37°C, and ~90% at 40°C. Greater than 95% release was attained at 41–42°C. In contrast, release from NTSL and ENTSL was alike in serum/PBS, and was less than 5% at all the temperatures tested (Fig. 3).

3.4 Biodistribution of Dox

3.4.1 Whole tumor—Tumor Dox concentrations were 1.2 ± 0.2 , 1.6 ± 0.1 , 0.9 ± 0.1 , 2.8 ± 0.7 , 1.6 ± 0.1 , 2.1 ± 0.3 , 5.2 ± 0.5 , and 3.8 ± 0.1 µg Dox/g tissue for free Dox, NTSLs, LTSLs, ELTSLs, ENTSLs, ENTSLs + HIFU, ELTSLs + HIFU, and LTSLs + HIFU, respectively. Drug delivery to both heated LTSL and ELTSL treated tumors exceeded free drug delivery by ~4- and ~5-fold, respectively, and was significantly higher than all other treatment groups. Compared to the LTSLs + HIFU treatment, heated tumors treated with ELTSLs achieved ~1.5-fold greater drug delivery. In addition, the unheated ELTSLs had twice the Dox concentration compared to LTSL (Fig. 4 and Table 2).

3.4.2 Tumor fragments—Dox delivery in the ELTSLs + HIFU treated tumor showed a general trend of decreasing concentration starting from the F1 fragment to the bottom (Fig. 5 and Table 3). ELTSL HIFU tumor fragment doxorubicin concentrations were 6.4 ± 1.3 , 5.4 ± 0.5 , 4.7 ± 0.9 and 2.8 ± 0.4 for F1, F2, F3, and bottom respectively. In contrast, F1, F2, and F3 demonstrated similar drug delivery for LTSLs, NTSLs, and or ENTSLs in all heated and unheated fragments.

3.4.3 Organ biodistribution—Treatment with NTSLs and ENTSLs alone resulted in significantly higher Dox concentrations in spleen than the other groups. Adding HIFU to LTSL and ELTSL treatment resulted in significantly greater Dox concentrations in heated muscle compared to the Dox group. Dox levels in, the heart, the kidney, and liver were comparable between various groups (Fig. 6, Table 2).

3.4.4 Ratio of Dox in heated tumor adjoining muscle and unheated contralateral muscle—Because HIFU treatment also heated the muscles adjoining the

tumor, the Dox concentration was significantly greater relative to contralateral unheated muscle by 4- to 5-fold for ELTSL and LTSL (Table 2). Additionally, ELTSLs achieved ~1.2-fold greater drug uptake compared to LTSLs alone in the heated muscle relative to contralateral unheated muscle (Fig. 7). Drug delivery attained by NTSL and ENTSL were similar in heated muscle compared to the unheated contralateral muscles with slightly greater uptake of Dox for NTSLs.

3.5 Histological examination of whole tumors

H&E images did not reveal any cavitation-like effect following HIFU and ELTSL treatment. Moderate to high Dox delivery along the periphery of the tumor was evident in all HIFU treated tumors. In the ELTSL and ENTSL treated tumors and to a lesser extent the LTSL treated tumors, drug penetration into the tumor core was more evident (Fig. 8). The LTSL treatment resulted in some brighter areas along the periphery to the core on the bottom and top of tumor, whereas both the ENTSL and ELTSL groups had a much more uniform distribution of Dox throughout the whole tumor. Unlike HPLC data, the fluorescence images for ELTSLs + HIFU treatment did not demonstrate an apparent drug gradient decrease from top to bottom of the tumor.

4. Discussion

Improving the ability to target liposomes and release drugs in real time at sufficient levels with enhanced penetration in a solid tumor is an important clinical need. The objective of this study was to understand the mechanisms of focal drug delivery from liposomes and HIFU in a mouse model and ways we can optimize this technology for clinical use.

Previously, MBs encapsulated in a solid lipid or polymeric shell have been utilized for controlled drug delivery in tumors [27, 28]. Such treatments are limited by the short half-life of MBs, which is caused by quick destruction by the reticulo-endothelial system due to their large size, and instability upon systemic administration. In contrast to MBs, nanosized echogenic liposomes encapsulating PFP theoretically should have increased stability and half-life [29]. To confirm that bubble encapsulation inside ELTSLs is indeed possible, we loaded optically labeled PFP into LTSLs. Confocal microscopy clearly showed that the process of PFP emulsification achieved effective encapsulation in liposomes, rather simply forming an admixture with the lipids (Fig. 2). To rule out alteration in liposome integrity and to investigate the role of PFP permeability through the liposomal membrane, the thermal stability of ELTSL and ENTSLs was explored in vitro (Fig. 3). Our data suggest that PFP encapsulation has no impact on Dox release in physiological buffer. Additional investigations in mouse colon tumors indicated that both LTSL + HIFU and ELTSL + HIFU treatments resulted in 4- to 5-fold greater Dox delivery at 42 °C than the LTSL and Dox groups at body temperature. Also, drug delivery attained by ELTSLs was slightly greater (~1.5-fold) than that of LTSLs, which suggests that bubble formation during HIFU treatment may provide an additive response (Fig. 4). As in tumors, in adjoining heated muscle tissues we observed relatively higher Dox concentration for the ELTSLs + HIFU treatment, which reinforces the role of bubble agents in improved cellular uptake (Fig. 5). Recently, Deese et al. reported that sonoporation using MBs enhanced the extravasation and penetration of

relatively large liposomal nanocarriers out of the blood vessels into the tumor interstitium by a variety of mechanisms, including perfusion modulation, mechanical disruption, and pore formation in tumor vessels [30]. Whether a similar mechanisms drive drug delivery and uptake in presence of nanobubbles is currently not known. Although the data for thermally sensitive ELTSLs are in line with Deese et al studies, we observed that NTSLs and ENTSLs were relatively less effective in achieving such outcomes. We believe that the presence of liposomal components (cholesterol, PEG) in NTSLs that resist triggered release of a drug at hyperthermic temperatures and support liposome accumulation in tumors relative to non-tumor tissue through the enhanced permeation and retention effect could be responsible for slow stromal transport of chemotherapeutic agents [31]. Thus, we propose that enhanced uptake of chemotherapeutics in a tumor from thermally and non-thermally sensitive carriers in presence of nanobubbles is an interplay among a rapid drug release rate, enhanced diffusion from blood vessels, and reduced resistance to drug transport upon nanobubble and hyperthermia treatment. For NTSLs and ENTSLs, the apparent lack of such a phenomenon suggests that the lipid components are the rate limiting factors. Future studies, especially those focused on the roles of intravascular versus interstitial transport of released drug from NTSLs + HIFU, would shed more light on these processes.

In a previous study, the plasma pharmacokinetics of Dox in a LTSL formulation was evaluated after intravenous administration. In contrast to Dox, which demonstrates a rapid initial clearance of > 95% in ~5 min, the LTSL formulation decreased ~90% over a 4 h time period [7]. Thus, currently HIFU treatments are generally performed over the first hour so that the maximal amount of drug is released at the peak plasma LTSL concentration. However, achieving spatially and temporally controlled homogeneous heating of deep seated tumors for long durations (30min to an hour) is not always easy with HIFU due to target motion and if the treatment cell area (focal spot) has a large volume [13]. Thus, a gap between LTSL injection and HIFU hyperthermia treatment can impact drug delivery, which highlights the importance of mean transit time and quick intravascular heating of tumors [32, 33]. To assess such impacts and devise alternative methodologies of tumor heating following liposome injection, we chose three regions in our tumor model and sequentially heated them for durations of 21 min each. Our data suggest that segmental heating of tumors does not impact LTSL drug delivery when the plasma concentration of LTSLs is > 75% of C_{max} over the first hour, and it achieves a similar level of drug in the chosen regions even when heated for shorter duration. However, relative to LTSLs, the sustained presence of PFP nanobubbles in the tumor microenvironment from ELTSLs has a positive influence on maintaining high concentrations of Dox in the tumor. In case of MBs, ultrasound contrast agents acoustically shield the beam from the underlying tissue resulting in temperatures higher than the target temperature[34]. However, encapsulated PFC emulsions in echogenic liposomes tend to be incompressible in liquid state[35], and produce poor oscillation and backscatter in the US, and thus instead of generating unusually high temperature, they most likely cause sonoporation and reduced resistance to drug transport upon combination with mild hyperthermia. Thus, we propose that zonal hyperthermia using HIFU for shorter durations (15–20min) with nanobubble liposome provides larger drug coverage when administered sequentially, without adversely impacting net drug delivery compared to single point larger

duration heating (~1hr), and thus this approach may be useful for treating deep seated tumors, and should be an investigation of future studies.

Successfully implementing a drug delivery system that permits selective anatomic delivery from passively accumulated NTSLs in tumors faces additional challenges, such as lack of enhanced intratumoral drug distribution [36]. Previously, the MB and HIFU combination has been shown to modulate microvascular permeability within a tumor [37]. In other studies, NTSLs that contained echo-contrast PFP gas enhanced gene and siRNA delivery in vitro and in vivo [38–40]. For MBs, the mechanism governing enhanced penetration was reported to be impulsive pressures exerted by the collapse of gaseous agents and formation of subsequent cavitation bubbles, resulting in improved transmembrane permeability [41]. Although a direct cavitation response was not detected in our H&E images following nanobubble treatment, ENTSLs positively impacted the intratumoral penetration of Dox. Fluorescence analysis of ELTSLs + HIFU treated tumors also revealed significantly improved intratumoral Dox delivery compared to treatments with Dox or NTSLs (Fig. 8). Prior research has shown that the extravascular transport distances of 70-nm nanoparticles were 40 and 10 μm in tumors with high and low permeability, respectively[42]. Thus, if acoustic pressure, pulse resonance frequency, and sonication rates are synergistically optimized with nanobubbles, our data suggest that this can improve intratumoral penetration relative to nanoparticle or hyperthermia alone. While the degree of permeability produced by HIFU hyperthermia or bubbles needs to be studied in greater detail, our initial data suggest that nanobubble action is potentiated in the presence of hyperthermia to cause greater transport of drug to the tumor core, thus they may serve as a suitable alternative to MBs.

In conclusion, the results of our study suggest that zonal administration of HIFU sequentially for short duration is a promising method for improving drug delivery into solid tumors from LTSLs. Intravenous administration of PFP encapsulated in LTSLs or NTSLs allowed Dox to reach the tumor core, and the mechanisms governing such transport involve the interplay of hyperthermia and reduced tissue barriers. This approach might prevent tumor recurrence in deep seated tumors and should be the subject of future investigations.

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References

1. Soloman R, Gabizon AA. Clinical pharmacology of liposomal anthracyclines: focus on pegylated liposomal Doxorubicin. Clin Lymphoma Myeloma. 2008; 8:21–32. [PubMed: 18501085]
2. Sha X, Guo J, Chen Y, Fang X. Effect of phospholipid composition on pharmacokinetics and biodistribution of epirubicin liposomes. J Liposome Res. 2012; 22:80–88. [PubMed: 22022836]
3. Park SM, Kim MS, Park SJ, Park ES, Choi KS, Kim YS, Kim HR. Novel temperature-triggered liposome with high stability: formulation, in vitro evaluation, and in vivo study combined with high-intensity focused ultrasound (HIFU). J Control Release. 2013; 170:373–379. [PubMed: 23770213]

4. Grull H, Langereis S. Hyperthermia-triggered drug delivery from temperature-sensitive liposomes using MRI-guided high intensity focused ultrasound. *J Control Release*. 2012; 161:317–327. [PubMed: 22565055]
5. Fernando R, Downs J, Maples D, Ranjan A. MRI-guided monitoring of thermal dose and targeted drug delivery for cancer therapy. *Pharmaceutical research*. 2013; 30:2709–2717. [PubMed: 23780716]
6. Frenkel V, Etherington A, Greene M, Quijano J, Xie J, Hunter F, Dromi S, Li KC. Delivery of liposomal doxorubicin (Doxil) in a breast cancer tumor model: investigation of potential enhancement by pulsed-high intensity focused ultrasound exposure. *Academic radiology*. 2006; 13:469–479. [PubMed: 16554227]
7. Ranjan A, Jacobs GC, Woods DL, Negussie AH, Partanen A, Yarmolenko PS, Gacchina CE, Sharma KV, Frenkel V, Wood BJ, Dreher MR. Image-guided drug delivery with magnetic resonance guided high intensity focused ultrasound and temperature sensitive liposomes in a rabbit Vx2 tumor model. *J Control Release*. 2012; 158:487–494. [PubMed: 22210162]
8. Staruch R, Chopra R, Hynynen K. Localised drug release using MRI-controlled focused ultrasound hyperthermia. *Int J Hyperthermia*. 2011; 27:156–171. [PubMed: 21158487]
9. de Smet M, Heijman E, Langereis S, Hijnen NM, Grull H. Magnetic resonance imaging of high intensity focused ultrasound mediated drug delivery from temperature-sensitive liposomes: an in vivo proof-of-concept study. *J Control Release*. 2011; 150:102–110. [PubMed: 21059375]
10. Chen H, Hwang JH. Ultrasound-targeted microbubble destruction for chemotherapeutic drug delivery to solid tumors. *Journal of therapeutic ultrasound*. 2013; 1:10. [PubMed: 25512858]
11. Ibsen S, Schutt CE, Esener S. Microbubble-mediated ultrasound therapy: a review of its potential in cancer treatment. *Drug design, development and therapy*. 2013; 7:375–388.
12. Lin PS, Ho KC, Sung SJ, Gladding J. Effect of tumour necrosis factor, heat, and radiation on the viability and microfilament organization in cultured endothelial cells. *Int J Hyperthermia*. 1992; 8:667–677. [PubMed: 1383359]
13. Tillander M, Hokland S, Koskela J, Dam H, Andersen NP, Pedersen M, Tanderup K, Ylihautala M, Kohler M. High intensity focused ultrasound induced in vivo large volume hyperthermia under 3D MRI temperature control. *Medical physics*. 2016; 43:1539–1549. [PubMed: 26936737]
14. Rapoport N, Nam KH, Gupta R, Gao Z, Mohan P, Payne A, Todd N, Liu X, Kim T, Shea J, Scaife C, Parker DL, Jeong EK, Kennedy AM. Ultrasound-mediated tumor imaging and nanotherapy using drug loaded, block copolymer stabilized perfluorocarbon nanoemulsions. *J Control Release*. 2011; 153:4–15. [PubMed: 21277919]
15. Lin CY, Pitt WG. Acoustic droplet vaporization in biology and medicine. *BioMed research international*. 2013; 2013:404361. [PubMed: 24350267]
16. Ibsen S, Benchimol M, Simberg D, Schutt C, Steiner J, Esener S. A novel nested liposome drug delivery vehicle capable of ultrasound triggered release of its payload. *J Control Release*. 2011; 155:358–366. [PubMed: 21745505]
17. Chen CC, Borden MA. The role of poly(ethylene glycol) brush architecture in complement activation on targeted microbubble surfaces. *Biomaterials*. 2011; 32:6579–6587. [PubMed: 21683439]
18. Radhakrishnan K, Haworth KJ, Huang SL, Klegerman ME, McPherson DD, Holland CK. Stability of echogenic liposomes as a blood pool ultrasound contrast agent in a physiologic flow phantom. *Ultrasound Med Biol*. 2012; 38:1970–1981. [PubMed: 22929652]
19. Sen A, Capitano ML, Spornyak JA, Schueckler JT, Thomas S, Singh AK, Evans SS, Hylander BL, Repasky EA. Mild elevation of body temperature reduces tumor interstitial fluid pressure and hypoxia and enhances efficacy of radiotherapy in murine tumor models. *Cancer research*. 2011; 71:3872–3880. [PubMed: 21512134]
20. Chen Q, Tong S, Dewhirst MW, Yuan F. Targeting tumor microvessels using doxorubicin encapsulated in a novel thermosensitive liposome. *Mol Cancer Ther*. 2004; 3:1311–1317. [PubMed: 15486198]
21. Gasselhuber A, Dreher MR, Negussie A, Wood BJ, Rattay F, Haemmerich D. Mathematical spatio-temporal model of drug delivery from low temperature sensitive liposomes during radiofrequency tumour ablation. *Int J Hyperthermia*. 2010; 26:499–513. [PubMed: 20377363]

22. Maples D, McLean K, Sahoo K, Newhardt R, Venkatesan P, Wood B, Ranjan A. Synthesis and characterisation of ultrasound imageable heat-sensitive liposomes for HIFU therapy. *Int J Hyperthermia*. 2015; 31:674–685. [PubMed: 26185910]
23. Senavirathna LK, Fernando R, Maples D, Zheng Y, Polf JC, Ranjan A. Tumor Spheroids as an in vitro model for determining the therapeutic response to proton beam radiotherapy and thermally sensitive nanocarriers. *Theranostics*. 2013; 3:687–691. [PubMed: 24019853]
24. Fernando R, Maples D, Senavirathna LK, Zheng Y, Polf JC, Benton ER, Bartels KE, Piao D, Ranjan A. Hyperthermia sensitization and proton beam triggered liposomal drug release for targeted tumor therapy. *Pharmaceutical research*. 2014; 31:3120–3126. [PubMed: 24852892]
25. Dromi S, Frenkel V, Luk A, Traugbber B, Angstadt M, Bur M, Poff J, Xie J, Libutti SK, Li KC, Wood BJ. Pulsed-high intensity focused ultrasound and low temperature-sensitive liposomes for enhanced targeted drug delivery and antitumor effect. *Clin Cancer Res*. 2007; 13:2722–2727. [PubMed: 17473205]
26. Yuh EL, Shulman SG, Mehta SA, Xie JW, Chen LL, Frenkel V, Bednarski MD, Li KCP. Delivery of systemic chemotherapeutic agent to tumors by using focused ultrasound: Study in a murine model. *Radiology*. 2005; 234:431–437. [PubMed: 15671000]
27. Hernot S, Klivanov AL. Microbubbles in ultrasound-triggered drug and gene delivery. *Advanced drug delivery reviews*. 2008; 60:1153–1166. [PubMed: 18486268]
28. Fokong S, Theek B, Wu Z, Koczera P, Appold L, Jorge S, Resch-Genger U, van Zandvoort M, Storm G, Kiessling F, Lammers T. Image-guided, targeted and triggered drug delivery to tumors using polymer-based microbubbles. *J Control Release*. 2012; 163:75–81. [PubMed: 22580225]
29. Javadi M, Pitt WG, Belnap DM, Tsosie NH, Hartley JM. Encapsulating nanoemulsions inside eLiposomes for ultrasonic drug delivery. *Langmuir: the ACS journal of surfaces and colloids*. 2012; 28:14720–14729. [PubMed: 22989347]
30. Theek B, Baues M, Ojha T, Mockel D, Veettil SK, Steitz J, van Bloois L, Storm G, Kiessling F, Lammers T. Sonoporation enhances liposome accumulation and penetration in tumors with low EPR. *J Control Release*. 2016; 231:77–85. [PubMed: 26878973]
31. Koning GA, Eggermont AM, Lindner LH, ten Hagen TL. Hyperthermia and thermosensitive liposomes for improved delivery of chemotherapeutic drugs to solid tumors. *Pharmaceutical research*. 2010; 27:1750–1754. [PubMed: 20424894]
32. J-YP, Landon, Chelsea D., Needham, David, Dewhirst, Mark W. Drug Delivery and Hyperthermia: The Materials Design and Preclinical and Clinical Testing of Low Temperature-Sensitive Liposomes Used in Combination with Mild Hyperthermia in the treatment of Local. *The open Nanomedicine Journal*. 2011; 3:38–64. [PubMed: 23807899]
33. Zhu M, Jiang L, Fabiilli ML, Zhang A, Fowlkes JB, Xu LX. Treatment of murine tumors using acoustic droplet vaporization-enhanced high intensity focused ultrasound. *Physics in medicine and biology*. 2013; 58:6179–6191. [PubMed: 23948709]
34. Giustetto P, Bisazza A, Biagioni A, Alippi A, Bettucci A, Cavalli R, Guiot C. Heat enhances gas delivery and acoustic attenuation in CO(2) filled microbubbles. *Conference proceedings: ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Annual Conference*. 2008; 2008:2306–2309.
35. Ektate K, Kapoor A, Maples D, Tuysuzoglu A, VanOsdol J, Ramasami S, Ranjan A. Motion Compensated Ultrasound Imaging Allows Thermometry and Image Guided Drug Delivery Monitoring from Echogenic Liposomes. *Theranostics*. 2016; 6:1963–1974. [PubMed: 27570563]
36. Eetezadi S, De Souza R, Vythilingam M, Lessa Cataldi R, Allen C. Effects of Doxorubicin Delivery Systems and Mild Hyperthermia on Tissue Penetration in 3D Cell Culture Models of Ovarian Cancer Residual Disease. *Molecular pharmaceutics*. 2015; 12:3973–3985. [PubMed: 26394060]
37. Liu HL, Hsieh HY, Lu LA, Kang CW, Wu MF, Lin CY. Low-pressure pulsed focused ultrasound with microbubbles promotes an anticancer immunological response. *Journal of translational medicine*. 2012; 10:221. [PubMed: 23140567]
38. Endo-Takahashi Y, Negishi Y, Nakamura A, Suzuki D, Ukai S, Sugimoto K, Moriyasu F, Takagi N, Suzuki R, Maruyama K, Aramaki Y. pDNA-loaded Bubble liposomes as potential ultrasound imaging and gene delivery agents. *Biomaterials*. 2013; 34:2807–2813. [PubMed: 23343634]

39. Endo-Takahashi Y, Negishi Y, Nakamura A, Ukai S, Ooaku K, Oda Y, Sugimoto K, Moriyasu F, Takagi N, Suzuki R, Maruyama K, Aramaki Y. Systemic delivery of miR-126 by miRNA-loaded Bubble liposomes for the treatment of hindlimb ischemia. *Scientific reports*. 2014; 4:3883. [PubMed: 24457599]
40. Suzuki R, Takizawa T, Negishi Y, Utoguchi N, Maruyama K. Effective gene delivery with liposomal bubbles and ultrasound as novel non-viral system. *Journal of drug targeting*. 2007; 15:531–537. [PubMed: 17671899]
41. Kato S, Mori S, Kodama T. A Novel Treatment Method for Lymph Node Metastasis Using a Lymphatic Drug Delivery System with Nano/Microbubbles and Ultrasound. *Journal of Cancer*. 2015; 6:1282–1294. [PubMed: 26640589]
42. Ho YJ, Chang YC, Yeh CK. Improving Nanoparticle Penetration in Tumors by Vascular Disruption with Acoustic Droplet Vaporization. *Theranostics*. 2016; 6:392–403. [PubMed: 26909113]

Fig. 1.

a) The tumor was divided into a top half consisting of three sequentially heated fragments and a bottom unheated half. Before injection, fragment 1 (F1) was heated for 3 min. During injection, starting at F1, a 1 min heating regime was applied to point A, followed by B, and finally C. This process was repeated seven times to ensure that F1 was heated between 40–42°C for a total of 21 minutes. F2 and F3 were heated in the same way to achieve a cumulative heating of ~60min.; b) Illustration of the thermal gradient in the mouse tumor model up to 2 mm away from the HIFU focus. Temperature was measured throughout the tumor by moving the HIFU focus over a thermocouple inserted into the tumor. Any area within the $1 \times 1 \times 10$ mm focus volume and up to 3 mm radius could be heated for 40–42°C.

Fig. 2.

Confocal microscopy showing PFP (green) encapsulated in the lipid bilayer (red) at 25 °C;.

d) Thermal scan for ELTSL loaded with PKH67 labelled PFP. Increase in fluorescence signal is observed post lysis of ELTSL with triton, due to de-quenching of PKH67 PFP from the liposomes. LTSL were used as control for the baseline fluorescence from liposomes (data not shown).

Fig. 3. Percent release of doxorubicin (Dox) in PBS and FBS between 25–42.5°C for NTSLs, ENTSLs, LTSLs, and ELTSLs.

Fig. 4.

Doxorubicin (Dox) concentrations in tumors at body temperature (37 °C) as well as HIFU treated tumors for free Dox, NTSLs, ENTSLs, LTSLs, and ELTSLs for all liposome formulations following a 5mg/kg Dox injection. Data are shown as mean Dox concentration $\pm$ standard error of mean ($n = 3-6$), * $p < 0.05$ (Tukey's multiple comparison); *Indicate significant differences from the unheated internal control.

Fig. 5. Doxorubicin (Dox) concentrations in HIFU treated tumor fragments (F1, F2, and F3) for NTSLs, ENTSLs, LTSLs, and ELTSLs following a 5mg/kg Dox injection. Data are shown as mean Dox concentration $\pm$ standard error of mean (n: 3–6). ELTSL F1* is significantly different from unheated bottom fragment ($p < 0.05$, pair wise comparison).

Fig. 6.

Doxorubicin (Dox) concentrations in $\mu\text{g Dox/g}$ of tissue for organs and muscle distal to tumors in a 37°C water bath for Dox, NTSLs, ENTSLs, LTSLs, and ELTSLs as well as HIFU treated tumors for all liposome formulations following a 5mg/kg Dox injection. Data are shown as mean Dox concentration $\pm$ standard error of mean ($n = 5-6$); *Indicate significant differences with Dox

Fig 7.

Ratio of doxorubicin (Dox) in tumor adjoining muscle heated with HIFU and unheated contralateral muscle for NTSLs, ENTSLs, LTSLs, and ELTSLs, Data are shown as mean concentration of Dox in tissue $\pm$ standard error of mean (n= 4–6).

Fig. 8. H&E (left column) and fluorescent (right column) images of HIFU treated tumors. All tumors are oriented with the F1 fragment on top when appropriate.

Table 1
Size, polydispersity and zeta potential of NTSL, ENTSL, LTSL and ELTSL at 25°C

Liposome	Size(nm)		Polydispersity		Zeta potential	
	mean	SEM	mean	SEM	mean	SEM
NTSL	190.1	1.9	0.10	0.01	-26.7	3.6
ENTSL	193.8	1.2	0.11	0.04	-43.8	0.6
LTSL	176.8	0.5	0.11	0.01	-48.0	1.7
ELTSL	171.6	0.5	0.12	0.01	-28.3	1.1

Average Dox delivery in various organs with and without HIFU for various liposome groups. (Data are expressed as $\mu\text{g Dox/gram of tissue}$; (mean $\pm\text{SEM}$))

Table 2

Organs	Dox	LTSL	LTSL HIFU	NTSL	NTSL HIFU	ELTSL	ELTSL HIFU	ENTSL	ENTSL HIFU
Spleen	7.2 (1.9)	7.8 (1.0)	7.3 (0.7)	27 (1.7)	16 (3.9)	8.0 (0.8)	6.5 (0.9)	29 (3.9)	16.3 (2.7)
Heart	4.1 (0.7)	3.9 (0.5)	3.8 (0.3)	2.4 (0.4)	1.2 (0.1)	3.4 (0.3)	5.1 (1.2)	2.0 (0.3)	1.1 (0.1)
Kidney	15.1 (0.5)	22.5 (4.5)	10.5 (1.5)	6.3 (0.6)	2.3 (0.5)	23.6 (3.6)	12 (2.0)	6.0 (1.2)	1.6 (0.1)
Lung	1.7 (0.1)	3.0 (0.5)	2.6 (0.5)	1.6 (0.4)	2.4 (0.6)	1.1 (0.3)	3.5 (0.2)	1.3 (0.1)	1.0 (0.2)
Liver	18.7 (1.3)	14 (1.1)	11 (2.7)	4.9 (0.9)	4.2 (1.6)	15.8 (0.9)	12 (2.1)	7.2 (0.9)	6.0 (1.2)
Muscle	2.5 (0.1)	1.9 (0.2)	8.1 (1.3)	0.9 (0.2)	1.4 (0.2)	1.5 (0.1)	9.9 (0.4)	0.9 (0.1)	1.1 (0.2)
Tumor	1.2 (0.2)	0.9 (0.1)	3.8 (0.1)	1.6 (0.1)	1.9 (0.1)	2.1 (0.4)	5.1 (0.4)	1.6 (0.1)	2.1 (0.3)

Table 3

Average Dox delivery in heated fragments (F1, F2 and F3), and unheated fraction of colon tumor, (data are expressed as μg Dox/gram of tissue; (mean $\pm$ SEM))

Liposome	F1	F2	F3	Unheated fraction
NTSL/HIFU	2.3 (0.3)	2.2 (0.1)	2.5 (0.4)	1.6 (0.1)
ENTSL HIFU	1.7 (0.4)	2.4 (0.5)	2.0 (0.5)	2.0 (0.2)
LTSL/HIFU	4.2 (0.8)	3.4 (0.6)	3.8 (1.1)	3.4 (0.6)
ELTSL/HIFU	6.4 (1.3)	5.4 (0.5)	4.7 (0.9)	2.8 (0.4)