Journal of Controlled Release 178 (2014) 101–107

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

# Inertial cavitation to non-invasively trigger and monitor intratumoral release of drug from intravenously delivered liposomes

Susan M. Graham [a], Robert Carlisle [a,*], James J. Choi [a], Mark Stevenson [c], Apurva R. Shah [c], Rachel S. Myers [a], Kerry Fisher [c], Miriam-Bazan Peregrino [b], Len Seymour [c], Constantin C. Coussios [a]

[a] Institute of Biomedical Engineering, Department of Engineering Science, University of Oxford, Old Road Campus Research Building, Oxford, OX3 7DQ, UK
[b] Institut d'Investigacio Biomedica de Bellvitge, Gran Via 199, L'Hospitalet de Llobregat, 08908 Barcelona, Spain
[c] Department of Oncology, University of Oxford, Old Road Campus Research Building, Oxford, OX3 7DQ, UK

ARTICLE INFO

*Article history:*
Received 18 July 2013
Accepted 16 December 2013
Available online 22 December 2013

*Keywords:*
Liposomes
Ultrasound
Inertial cavitation
Drug delivery
Cancer

ABSTRACT

The encapsulation of cytotoxic drugs within liposomes enhances pharmacokinetics and allows passive accumulation within tumors. However, liposomes designed to achieve good stability during the delivery phase often have compromised activity at the target site. This problem of inefficient and unpredictable drug release is compounded by the present lack of low-cost, non-invasive methods to measure such release. Here we show that focused ultrasound, used at pressures similar to those applied during diagnostic ultrasound scanning, can be utilised to both trigger and monitor release of payload from liposomes. Notably, drug release was influenced by liposome composition and the presence of SonoVue® microbubbles, which provided the nuclei for the initiation of an event known as inertial cavitation. *In vitro* studies demonstrated that liposomes formulated with a high proportion of 1,2 distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) released up to 30% of payload following ultrasound exposure in the presence of SonoVue®, provided that the exposure created sufficient inertial cavitation events, as characterised by violent bubble collapse and the generation of broadband acoustic emissions. In contrast a 'Doxil'-like liposome formulation gave no such triggered release. In pre-clinical studies, ultrasound was used as a non-invasive, targeted stimulus to trigger a 16-fold increase in the level of payload release within tumors following intravenous delivery. The inertial cavitation events driving this release could be measured remotely in real-time and were a reliable predictor of drug release.



## 1. Introduction

The powerful cytotoxicity of low molecular weight chemotherapeutics could provide far greater anticancer efficacy if dosing was not restricted by severe adverse effects in non-target tissues. In response, blood stable liposomal delivery systems have been developed to extend the circulation of these drugs and to allow their selective passive accumulation into tumors via the enhanced permeability and retention effect (EPR) [1]. However, although formulations such as Doxil®, an HSPC-based liposome containing doxorubicin, have provided clinical benefit in the treatment of cancers such as Kaposi's sarcoma [2,3], the more widespread use of such agents has been restricted. In particular, their clinical efficacy is limited by two main factors: poor penetration into non-vascularised tumor regions and poor release of payload within the tumor [4,5].

Although it is clear that the EPR effect increases accumulation of liposomes in the tumor, this accumulation is predominantly around the perivascular space, usually in the periphery of the tumor [4,6]. To achieve effective treatment of the whole tumor such limited penetration needs to be overcome. Furthermore, depending on the formulation used, the release of free drug may only occur over extended periods, and so the required therapeutic concentration and distribution of free drug may never be achieved [5]. It is therefore apparent that although the stability provided by liposomal formulations is desirable during the intravenous delivery phase, following arrival within the tumor, drug in its free form is required to provide higher and more widespread bio-availability. In response, several strategies have been developed to instigate the tumor-specific release of payload from liposomes. These have included alterations to liposome formulation to achieve sensitivity to the phenotypically distinct tumor environment [7,8] or to externally applied stimuli such as heat or ultrasound [9–13].

Ultrasound (US) can initiate the release of drugs from liposomes via an event called inertial cavitation, whereby the rarefactional phase of an ultrasound wave causes the expansion of a gas bubble followed by a violent collapse due to the inertia of the surrounding media. This collapse creates shock waves which can disrupt the stability of co-localised liposomal drug carriers. To date, studies have concentrated on the use of

* Corresponding author.
*E-mail address:* robert.carlisle@eng.ox.ac.uk (R. Carlisle).
*URL:* http://www.ibme.ox.ac.uk/bubbl (R. Carlisle).

0168-3659 
http://dx.doi.org/10.1016/j.jconrel.2013.12.016

low frequency or high intensity US to generate gas bubbles *in situ*, and most recently such parameters have been used to achieve a variable level of triggered drug release following an intratumoral injection of liposomes [14]. However, concerns persist over the damage to non-target tissue that such US exposure parameters may cause and whether ultimately they will be widely clinically applicable. An alternative strategy is to utilise high-frequency US pulses at pressures in the diagnostic range in the presence of pre-existing gas bubbles. This provides an inertial cavitation stimulus for drug release using safe, clinically achievable US exposure conditions and approved US contrast agents [15]. Indeed, in the context of improving the delivery of therapeutics such as oncolytic viruses, this approach has already shown great promise [16]. A further advantage of this approach is that US-induced cavitation events produce distinct acoustic emissions that can be recorded and characterised providing non-invasive feedback, a feature which has proven useful in ablative US applications [17–19].

Here we demonstrate that liposomes can be formulated to achieve sensitivity to cavitation events created by the application of focused US in the presence of a gas filled microbubble formulation SonoVue® (SV). We show that such cavitation events can be triggered using US parameters similar to those used in diagnostic US scanning and can be recorded in real time. These studies may ultimately provide new methods by which the efficacy and monitoring of intravenous drug delivery to tumors can be improved in the clinical setting.

## 2. Materials and methods

### 2.1. Chemicals and cells

Hydrogenated soy phosphocholine (HSPC, 840058), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE, 850715) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG 880120) were purchased from Avanti Polar Lipids Inc (Ala, USA). Cholesterol (C8667) was purchased from Sigma (UK). D-Luciferin potassium salt was purchased from Gold Biotechnology, MO, USA, and luciferase from *Photinus pyralis* (firefly) was from Sigma (L9506). B16-F10-luciferase cells were purchased from Caliper life sciences (USA) and maintained as recommended by the manufacturer.

### 2.2. Liposome formulation

Liposomes were formulated by mixing HSPC, cholesterol and DSPE-PEG at ratios of 56:39:5 (HSPC liposomes) or DSPE, cholesterol, DSPC and DSPE-PEG at ratios of 65:25:3:7 (DSPE liposomes) in 2 chloroform: 1 methanol, in a round bottomed flask. To confer triggered destabilisation upon liposomes, the lipid DSPE was incorporated. This lipid has a packing parameter above 1 and a propensity to form inverted hexagonal structures at pH <9 when the head phosphate group and amine group carry opposing charges. A 3% DSPC was included to enhance stability. After complete dissolution, rotary evaporation for 30 min at 55 °C achieved a thin film, which after drying under high vacuum overnight was rehydrated in 300 mM Tris pH 10 for 60 min. Subsequent purification, sizing and achievement and assessment of luciferin loading was performed as described by Carlisle et al. [20], except that a multiwell plate reader (BMG, Fluorstar Optima) was used. Size and polydispersity were measured using a Zetasizer Nano ZS (laser wavelength 633 nm; Malvern Instruments, UK). The general purpose analysis mode; 3 runs per measurement; and backscatter angle of 173 degrees were used.

### 2.3. Stability and blood component interactions

Liposomes were mixed with freshly isolated, complement active human plasma for 5, 10, 15, 20, 25 or 30 min at 37 °C and the release of payload measured. Sample was heated to 100 °C or not and then assayed for free luciferin as described by Carlisle et al. [20]. The amount of free or still encapsulated luciferin could then be calculated as a percentage of the total.

Stability *in vivo* was tested using a luciferase reporter mouse system developed from that previously described [21]. HDLacOLuc transgenic mice were generated by isolating the transgenic construct from pHDLacOluc via *Bgl*II/*Bam*HI digestion followed by *Pvu*I digestion to leave a 2.7-kb isolated fragment, which was purified and diluted to 5 ng/μl in microinjection buffer (10 mM Tris–HCl pH 7.4, 0.1 mM EDTA). Three-week-old female B6CBAF1 mice were superovulated and mated. The resulting fertilised oocytes were collected from the oviduct of plugged female mice and cultured until two clear pronuclei were visible. The purified HDLacOLuc transgenic constructs were microinjected into one of the pronuclei and injected embryos were cultivated overnight to the two-cell stage. Two-cell embryos were then re-implanted into the oviduct of pseudo pregnant CD1 foster mothers at 0.5 days post-corium. Between 18 and 19 days later, pups were born from the foster mothers. Ear biopsies from these pups were taken, DNA extracted and PCR performed to identify transgenic founder mice. Founder mice were mated with C57Bl/6 mice and the resultant pups genotyped to identify transgenics. These F1 transgenics were mated with C57Bl/6 mice to produce an F2 generation and establish the line [22]. An intravenous injection of 50 μg of free luciferin and imaging 30 min post-injection using an IVIS 100 system (Caliper Life Sciences, USA) confirmed the ubiquitous expression of the luciferase reporter gene throughout the mice. HSPC or DSPE liposomes encapsulating the same 50 μg quantity of luciferin were then injected intravenously and luminescence measured after 30 min. Images were all identically scaled (lower limit $1 \times 10^5$, upper limit $1 \times 10^8$ photons/second/cm$^2$).

### 2.4. Ultrasound generation and cavitation detection

A 0.5-MHz spherically focused, single-element ultrasound transducer (H-107D SN13; Sonic Concepts, Bothell, Washington, USA) was used as the driving transducer to insonate samples (setup shown in supplementary Fig. 1). The elliptical focal zone at −6 dB measured (4 mm laterally × 37 mm axially). Custom-made software using LabView 8.5 (National Instruments) was used to control and collect ultrasound signals. Voltage pulses from a function generator (33250A; Agilent Technologies, Santa Clara, CA, USA) were amplified using a 55-dB, RF power amplifier (A300 Power Amplifier, 017098, Electronics and Innovation; Rochester, NY, USA) and passed through its matching network to the driving transducer. An absolute pressure calibration and beam profile characterisation was performed using a 0.4-mm diameter needle hydrophone (ONDA 1056; Onda Corporation, Sunnyvale, CA, USA). All pressures reported throughout the present study are peak rarefactional focal pressures (PRFPs). Acoustic emissions were detected passively using a 7.5-MHz single-element focused transducer (SN 671678, Panametrics, Waltham, MA, USA) referred to as the passive cavitation detector (PCD). The PCD was inserted through a central opening in the driving transducer and was confocally and coaxially aligned with the driving transducer as in [23]. Voltage signals were filtered using a 2-MHz analogue high pass filter (FILT-HP2-A; Allen Avionics Inc., NY, USA) to exclude reflections of the driving frequency, amplified by a factor of 5 (SR445A; Stanford Research Systems, Sunnyvale, CA, USA) and then acquired using a DAQ card and saved on a computer.

### 2.5. In vitro experiments

A Delrin® cylindrical phantom (volume = 6.3 ml) with acoustically transparent Mylar® sheets on the front and back face was used to hold each sample. Liposomal samples (50 μl) were diluted in PBS and mixed with or without SV (300 μl) in the phantom. A 3D positioning system was used to immerse the phantom in the water tank, aligned with the transducer focus. A magnetic PTFE stir bar with a stir plate (Variomag Compact, Thermo Electron Corporation) was used to mix samples for 15 s, 1 min before ultrasound exposure. Samples were insonated at

PRFPs of 0.14, 0.5, 0.8, 1.2 and 1.5 MPa with corresponding intensities shown in supplementary Table 1. A pulse length of 100 ms and pulse repetition period of 2 s (i.e., 5% duty cycle) was used to insonate each sample for 30 s. Data post processing was carried out using custom-designed software in Matlab® (MathWorks). A fast Fourier transform (FFT) was conducted, and all data were displayed as power spectral densities. Electrical energy was calculated as the integral across all frequencies between 0 and 15 MHz and across time points over 30 s for each power spectral density. The influence of ultrasound exposure on temperature increase was assessed by placing a thermocouple (Type T, Omega Thermocouple) connected to a thermocouple reader (TC-08, Pico Technology Ltd) within the phantom during exposure.

### 2.6. In vivo experiments

C57BL6 mice were implanted with B16-F10-luciferase tumors as described by Carlisle et al. [20], and the ultrasound system and exposure conditions were as described by Bazan-Peregrino et al. [24], except that total exposure time was 12 min, with 25 μL of SV injected every 90 s and repositioning of the focal volume every 3 min. A total of 100 μL of liposome sample (158 μg luciferin) was injected. Imaging was performed using an IVIS 100 system 100 (Caliper Life Sciences, USA). Ultrasound exposure was performed immediately after injection, i.e., when bloodstream concentrations of liposomes and SV was highest, to ensure the best chance of co-localisation of these two agents within tumor vasculature. *In vitro* assay of liver, tumor and blood luciferin content was performed as described by Carlisle et al. [20]. Energy from cavitation events were represented as normalised variance as described by Bazan-Peregrino et al. [24].

## 3. Results

### 3.1. Loading and in vitro and in vivo stability of DSPE or HSPC liposomes

Dynamic light scattering confirmed the formation of stable liposomes of similar size (diameter less than 150 nm) and polydispersity (PDI approximately 0.1) to liposomes produced using HSPC (Fig. 1A). Although this diameter is greater than that of formulations currently used in the clinics, this size was chosen to exceed the cutoff for the endothelial fenestrae of murine hepatocytes and thereby provide more extended circulation in our pre-clinical studies [25]. Stability experiments verified that the inclusion of DSPE within the liposomes did not increase their payload release upon exposure to body temperature or incubation in fresh human plasma (Fig. 1B). After 30 min incubation at 37 °C, both HSPC and DSPE liposomes maintained almost complete encapsulation of their payload (Fig. 1B), with no more than 5% of the input dose being detected as free agent at any time point. In mice engineered to have endogenous expression of luciferase in all tissues, the injection of free luciferin produced luminescent signal throughout (Fig. 1C). In contrast, when the same amount of luciferin was delivered encapsulated within either 'Doxil'-like HSPC or our DSPE liposomes negligible luminescence was detected, demonstrating that these two formulations achieved equivalent stability during this 30-min time frame. Indeed, the bladder of mice was the only site where any luciferin signal was detected, suggesting release was not achieved until the degradation and excretion of the liposome constructs. In subsequent pre-clinical studies of cavitation-triggered payload release from DSPE liposomes, the measurement of tumor luminescence was taken 30 min after intravenous injection.

### 3.2. In vitro analysis of sensitivity to ultrasound-induced inertial cavitation events

*In vitro* testing of the cavitation-induced release of payload from liposomes was performed using a 0.5-MHz transducer focused on a 6.3-cm$^3$ cylindrical phantom containing degassed phosphate-buffered saline (PBS) with or without liposome test sample and with or without

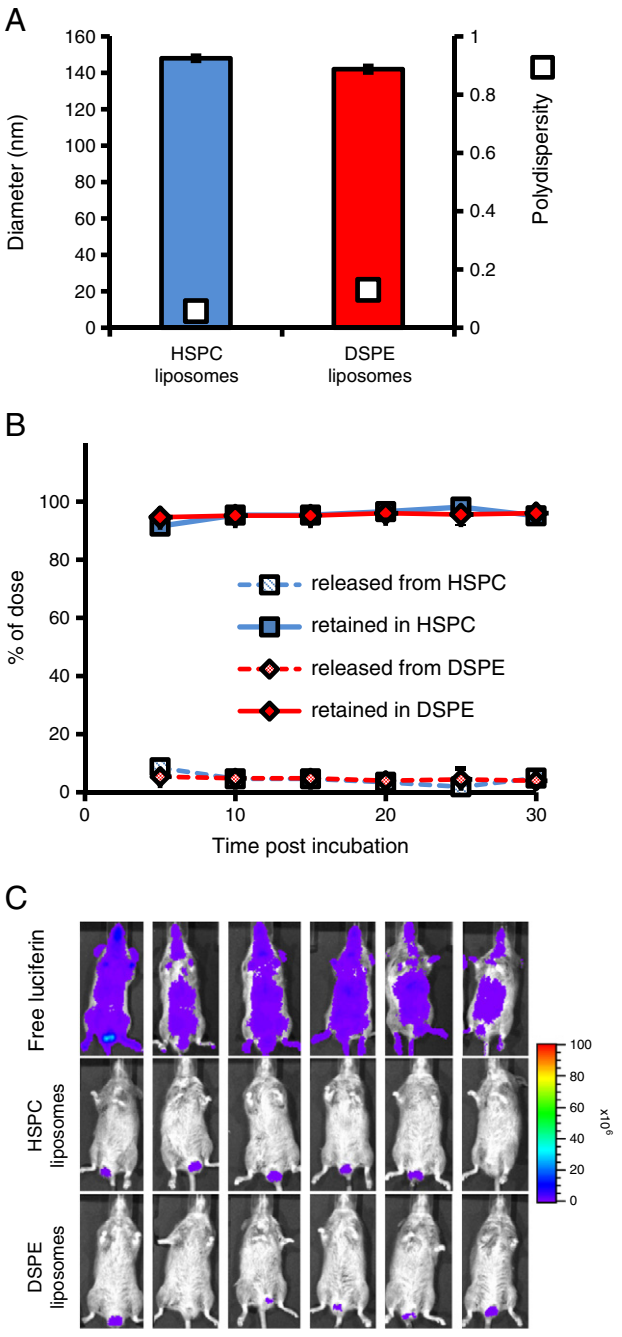
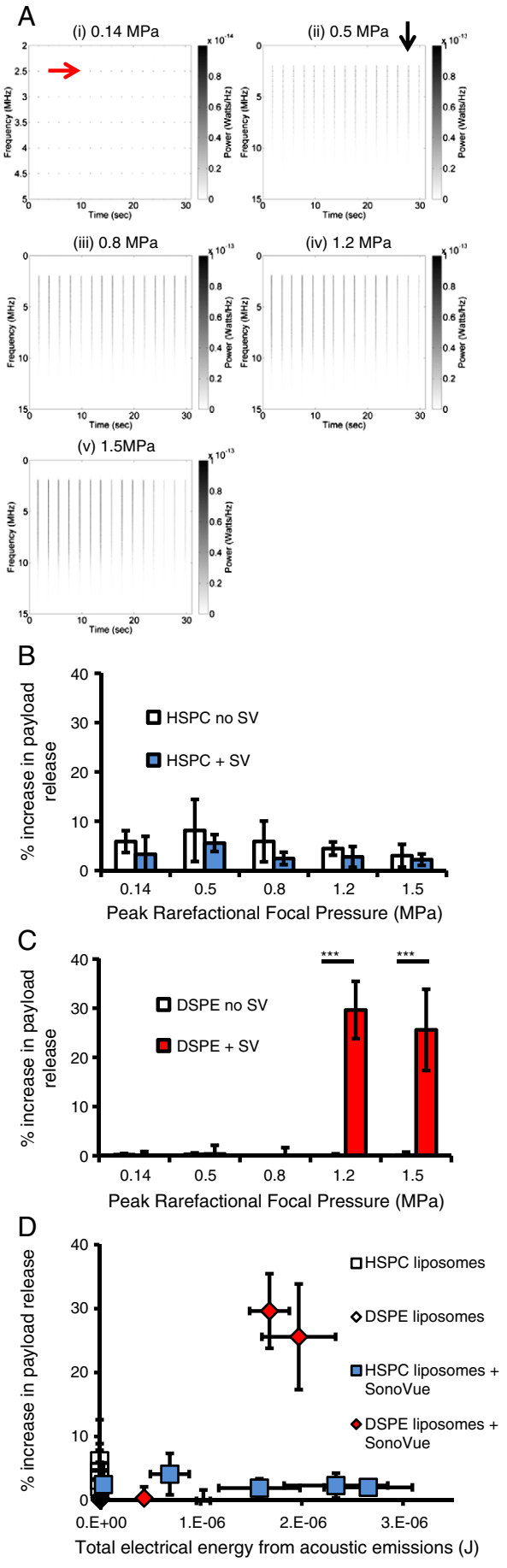



**Fig. 1.** Assessment of size and stability of liposomes to temperature and plasma. The hydrodynamic diameter and polydispersity of liposomes were measured as described in methods, $n = 3$, ±standard deviation. For stability studies, liposomes were incubated with neat human plasma and luciferin release assayed and plotted as percentage of total dose. $N = 3$, standard deviation shown. *In vivo* stability was assessed using a reporter mouse model with body-wide endogenous expression of luciferase (see methods). Free luciferin, HSPC liposomes or DSPE liposomes (all 50 μg luciferin) were injected intravenously and luminescence analysed at 30 min.

SonoVue® (SV) (see methods). The setup is demonstrated diagrammatically in Supplementary Fig. 1. The US exposure conditions were tailored to the eventual studies in murine models by giving consideration to the parameters that are achievable and necessary *in vivo*. Notably, the pulse repetition frequency (PRF) was designed to mimic the need for replenishment of SV cavitation nuclei into the focus of the transducer in a flow scenario such as the vessels within a tumor [26]. Exposure pressures were varied to achieve a range of acoustic responses from low energy, stable cavitation to high-energy inertial cavitation as characterised by the power spectra of acoustic emissions [12,15].



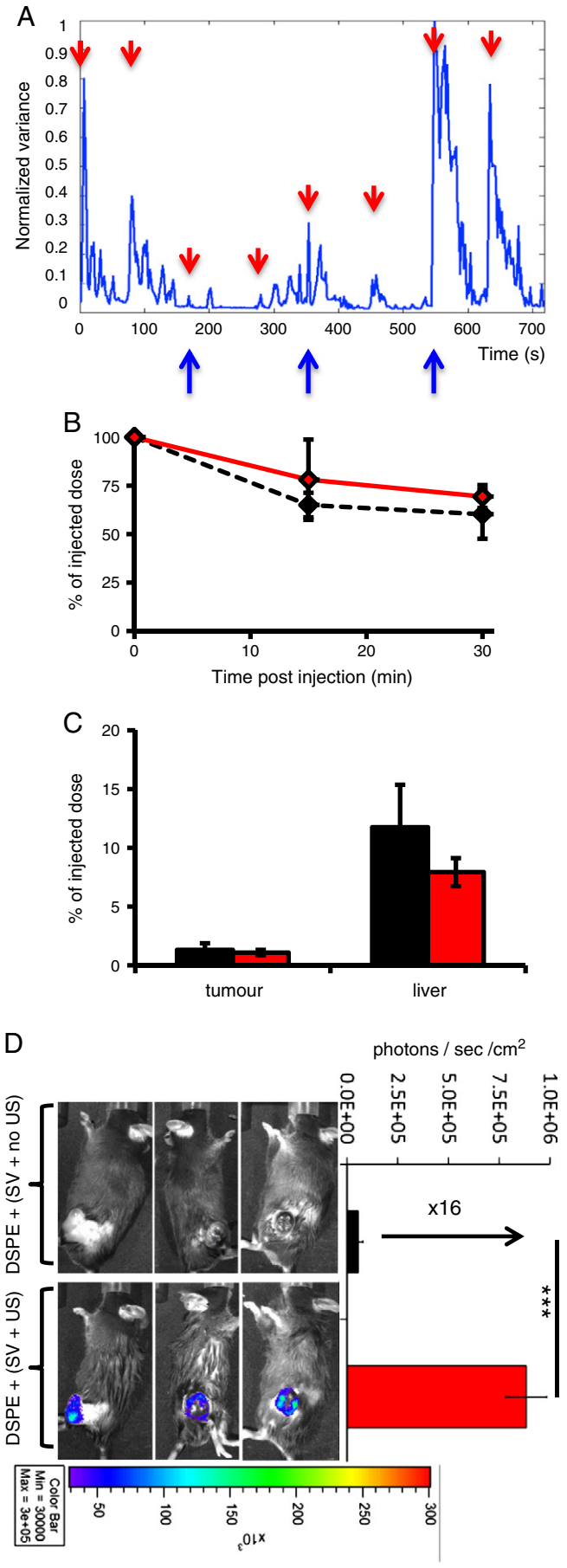

In the absence of SV, no acoustic emissions were observed at any exposure pressure, indicating that such pressures were insufficient to instigate the creation of gas bubbles in the test media (data not show). In contrast, Fig. 2A shows that in the presence of SV acoustic emissions could be detected and characterised across a range of exposure pressures. At 0.14 MPa (panel i), low-energy spectra with fixed frequencies were detected and plotted as multiple horizontal lines on the power spectral density plot (for example, at about 2.5 MHz, see red arrow). Such repeated signal at the same frequency is indicative of stable cavitation taking place with bubbles oscillating to produce harmonics of the 0.5-MHz driving frequency. The energy of emissions increased as exposure pressure increased to 0.5 MPa or 0.8 MPa (panel ii and iii). In addition to harmonic emissions, a pattern of vertical lines (black arrows) was observed showing that for each US exposure an acoustic response comprised of a wide range of frequencies (2.5 MHz–10 MHz) was also detected. Such broadband emissions are characteristic of the shock waves created when unstable bubble oscillation leads to inertial cavitation. These emissions persist throughout the 30-s exposure, indicating that the amount of viable SV is not exhausted within this timeframe. At 1.2 MPa or 1.5 MPa (panels iv and v), high-energy broadband emissions dominated the signal as seen previously [15]; however, there is a decay in the signal at the end of the 30 s. This is due to the more rapid destruction of SV bubbles at these pressures, as also evidenced by a decrease in the opacity within the phantom during exposure. The presence of liposomes did not influence the spectra produced with SonoVue present, and liposomes alone showed no cavitation signals on the power spectra (Supplementary Fig. 2B). When HSPC liposomes were tested in the absence of SV, the application of pressures from 0.14 MPa up to 1.5 MPa failed to trigger the release of payload (Fig. 2B). When SV was included, regardless of the acoustic emissions detected, i.e., low-energy stable cavitation at 0.14 MPa or high-energy inertial cavitation at 1.5 MPa, there was still no enhancement in the release of luciferin payload. This indicates that under these exposure conditions these control HSPC 'Doxil'-like formulation did not give the triggered release of its payload.

Notably, thermocouple measurements demonstrated that no increase in temperature was detected within the phantom at any of the exposure conditions tested (Supplementary Fig. 3A). Furthermore, stability tests performed by heating DSPE sample to 43 °C showed no increase in luciferin payload release (Supplementary Fig. 3B).

When DSPE liposomes were reformulated to contain doxorubicin rather than luciferin, the release of this drug could be triggered using the same inertial cavitation, instigating conditions (Supplementary Fig. 4A) that provided triggered release in Fig. 2C. Specifically, only PRFP values of 1.2 MPa or 1.5 MPa gave substantial and significant ($p < 0.05$) increases in release and this was only in the presence of SonoVue. This ultimately led to enhanced kill when these samples were added to B16-F10-luc cancer cells *in vitro* (Supplementary Fig. 4B). Indeed, the level of cell kill was equivalent to that achieved with the same amount of free drug and 2-fold higher than that achieved with samples where no enhanced doxorubicin release was detected.

Similarly, when DSPE liposomes were tested in the absence of SV, no release of luciferin payload was observed following exposure to pressures ranging from 0.14 MPa to 1.5 MPa. Upon the inclusion of SV, the application of 0.14 MPa, 0.5 MPa or 0.8 MPa and the associated

**Fig. 2.** *In vitro* assessment of inertial cavitation induced drug release. The 0.5-MHz ultrasound was used to expose samples at 5% duty cycle with 50,000 cycle pulses with a pulse repetition frequency of 0.5 Hz for 30 s. (A) Representative power spectral densities of DSPE liposomes in the presence of SV at (i) 0.14 MPa, (ii) 0.5 MPa, (iii) 0.8 MPa, (iv) 1.2 MPa and (v) 1.5 MPa. Drug release from HSPC liposomes (B) and DSPE liposomes (C) is shown as percentage increase from background (non-exposed) samples over the pressure range tested. Two-way ANOVA for significance, $^{***}p < 0.001$, $n = 3$, standard deviation shown. (D) Correlation between energy of acoustic emissions and liposomal release is shown for each sample set of HSPC liposomes and DSPE liposomes in the absence and presence of SV. $n = 3$, standard deviation for both energy and release is shown.




low-energy stable cavitation and mixed stable and inertial cavitation that resulted did not cause luciferin release. However, when a pressure of 1.2 MPa or 1.5 MPa was applied and high-energy inertial cavitation was instigated, broadband energy dominated the power spectrum and significant release of luciferin (30%, $p < 0.001$, ANOVA) payload from the liposomes was observed (Fig. 2C). It is important to emphasise that this triggered release was not instigated by US exposure *per se* but by specific inertial cavitation events. It is notable that such events can be tightly regulated and monitored using the systems described here. This adds to the potential clinical utility of the approach we have described.

The energy of acoustic emissions associated with the chaotic bubble oscillation increased almost linearly with applied ultrasound pressure (Supplementary Fig. 2). A correlation between percentage release and electrical energy from acoustic emissions can be seen in Fig. 2D, where below 1.68 μJ no significant release is seen from either HSPC liposomes or DSPE liposomes. Above 1.68 μJ, an increase in release from DSPE liposomes is seen whereas HSPC liposomes remain stable.

### 3.3. In vivo analysis of focused ultrasound mediated payload release

Having established the benefit of using DSPE rather than HSPC liposomes and identified the US parameters capable of producing optimal triggered release, experiments were performed in a murine preclinical model using the setup represented by Bazan-Peregrino et al. [24]. The circulation kinetics and organ distribution of DSPE liposomes loaded with luciferin were tested in C57Bl6 mice bearing B16F10-luciferase tumors. Inertial cavitation taking place within the focal volume was detected in real-time throughout the 12-min exposure (representative trace shown in Fig. 3A). Multiple re-dosing with SV (at each red arrow) was required to provide a constantly replenished source of cavitation nuclei [27], whilst repositioning of the US focal volume (blue arrows) ensured the maximum possible volume of tumor was exposed. Notably, distinct cavitation events were detected upon administration of SV, but the magnitude of the events was dramatically different at different sites within the same tumor. This emphasises the substantial inter-regional differences in perfusion and, therefore, SV content throughout the tumor [28] and highlights the importance of achieving good US exposure over the whole tumor volume. It is also noteworthy that the exposure parameters used in these studies caused no damage to the skin of the mice, in contrast to studies that have used higher pressures [29], emphasising the improved safety profile of our approach.

Pharmacokinetic and biodistribution studies (Figs. 3B and 3C) demonstrated that there was no significant change in liposomal clearance profile or tumor accumulation in mice treated with liposomes + SV + US compared to those treated with only liposomes + SV. In both cases, approximately 75% of the injected dose was present in the circulation at 30 min and just 1% was present in tumors. These figures are a measure of sum total of free and encapsulated luciferin present. However, the combination of US + SV did provide a substantial and significant increase in the level of luciferin released within tumors. When imaged directly after treatment a 16-fold ($p < 0.001$) increase in photons/second/cm$^2$ was detected in the

**Fig. 3.** Assessment of the pharmacokinetics, biodistribution and drug release profile of DSPE liposomes *in vivo*. C57BL6 mice bearing B16F10-luciferase tumor were intravenously dosed and bloods collected, US exposures performed and organs imaged and harvested and described in methods. (A) Representative normalised variance (blue line) calculated from the acoustic emissions detected by the passive cavitation detector over the 12-min US exposure regime; red arrows denote times of SV administration, and blue arrows denote the repositioning of US focal volume with respect to tumor volume. (B) Clearance profile of liposomes from the blood of mice with US-treated (red diamond) and non-US-treated (black diamond, dashed line) tumors. (C) Tumor and liver accumulation of liposomes in mice with US-treated (red bar) or non-US-treated (black bar) tumors shown as a percentage of the injected dose. (D) IVIS imaging of luminescence in mice and mean levels plotted for US-treated (red bar) or non-US-treated (black bar) tumors. In all experiments, $n = 3$, standard deviation shown, Student's *t*-test for significance, $^{***}p < 0.001$.

tumors of liposome + US + SV-treated mice compared to those, which received liposomes + SV alone, demonstrating a substantial and significant enhancement of the delivery of luciferin to the luciferase expressing tumor cells (Fig. 3D).

## 4. Discussion

Ultrasound (US) is emerging as a powerful therapeutic modality [30,31], particularly in the context of improving the delivery of liposomal drug carriers to target tumors [32]. Strategies are now being developed so that the release of payload from liposomes can also be triggered using US. In accordance with recent publications, we have demonstrated that US can be used as a trigger to disrupt blood-stable liposome formulations and cause such drug release [11,14]. Our *in vitro* studies demonstrate that release is dependent on liposome composition, with Doxil-like phosphocholine liposomes proving non-US-responsive, whereas phosphoethanolamine liposomes with the same heat and plasma stability showed up to 30% release upon US exposure. This may be a consequence of reported differences in packing parameter and the consequent propensity of phosphoethanolamine lipids to destabilise and form inverted hexagonal structures [33,11]. The analysis of the acoustic emissions resulting from US exposure enabled us to prove that inertial cavitation, as characterised by broadband emissions [15], was the phenomenon required to drive this payload release. The co-delivery of SV provided the nuclei for the initiation of such phenomenon and in its absence no inertial cavitation or drug release was observed. The presence of SV allows inertial cavitation events to be created at exposure frequencies and pressures that are within the range of current diagnostic US scanners. The mechanical index (MI) used in diagnostic imaging usually ranges from 0.04 to 1.7 [34]. Even though the MI is not directly applicable for ultrasound pulses longer than a single cycle, the lowest pressure where release was seen (1.2 MPa) would have a corresponding MI of 1.697. By co-injecting SonoVue, we were able to trigger payload release at a spatial peak average pulse intensity (ISPPA) of 0.0486 kW/cm$^2$ compared to the reported 10.5 kW/cm$^2$ required to achieve cavitation and payload release without pre-existing bubbles [14]. These factors combine to enhance the safety and clinical applicability of the approach we have taken. However, it is notable that at the highest pressure of exposure 1.5 MPa, there was a loss in both the proportionality of the total energy produced by acoustic emissions over the exposure period and the amount of payload release achieved. This meant that 1.5 MPa exposure gave no significant increase in either of these facets compared to the use of 1.2 MPa, which is probably the result of the exhaustion of the supply of SV inertial cavitation nuclei within the 30-s exposure. This serves to emphasise the importance of maintaining a constant supply of cavitation nuclei throughout the US exposure. This factor was considered when designing the pulse repetition frequency of our US pulses to align with what was achievable and useful *in vivo* [26].

Our *in vivo* studies were performed by intravenous injection of inertial cavitation sensitive DPSE liposomes into a murine model. Delivery and acoustic parameters were optimised to achieve a constant supply of SV and insonation of a large tumor area. Notably, dramatic differences in acoustic response were observed in different regions of the tumor. This serves to both emphasise the importance of good tumor perfusion in order to allow a constant supply of SV and highlight the value of being able to obtain real-time feedback on the occurrence of inertial cavitation as delivery is taking place. Inertial cavitation was shown to be a necessary requirement for luciferin release from liposomes. The detection of inertial cavitation can therefore be used as a non-invasive, reliable and accurate proxy for the measurement of the release of payload from liposomes. This has important beneficial ramifications for the clinical utility of this ultrasound technology, potentially providing clinicians with low-cost, real-time, non-invasive feedback on the success of drug delivery, something which no other imaging modality can currently provide. Such measurement of the acoustic output from cavitation events and its correlation with drug release offers substantial benefit over the approach of using acoustic input as a proxy for release [35] because of the stochastic nature of the creation of inertial cavitation events, especially in complex biological environments. However, the variability in the levels of inertial cavitation we observe between different tumor regions highlights a weakness of our approach in that the SV delivered to provide cavitation nuclei may not achieve sufficient and liposome-coincident intratumoral distribution. This is due to the uniquely deregulated state of the vascularisation found within tumors [36]. A further limitation of our approach is the requirement to replenish SV because it is destroyed in the act of generating cavitation events. To address these limitations, particles designed to provide more sustainable and reliable cavitation nuclei will need to be developed.

The fact that SV delivery provided variable intratumoral inertial cavitation levels may explain why no measurable diminishment in the circulating liposome dose or increase in total luciferin level within tumors was observed. Indeed, this is in accordance with similar studies we have performed using SV with oncolytic virus. However, it contrasts markedly with our studies using high intensity focused ultrasound to instigate heat mediated liposome destabilisation, where complete drug release can be achieved [37]. In our studies, triggered release from cavitation sensitive DSPE formulations is still sub-optimal and some way behind the release achieved from heat-sensitive formulations [9].

However, despite the variability in intratumoral inertial cavitation levels and location, sufficient events were generated to instigate substantial and highly significant increases in payload release. This demonstrates that although increases in the total amount of luciferin reaching the tumor could not be achieved, US + SV did instigate an increase in the amount of luciferin released, thereby providing an 16-fold increase in the bio-available dose within the tumor. This is the first demonstration that such clinically applicable US parameters can be used to improve intratumoral payload release from an intravenously injected liposomal delivery agent.

## Acknowledgements

Research was supported by Wellcome Trust & EPSRC under grant number WT088877/Z/09/Z (R.C.C., L.W.S. and C.C.C.), the Clarendon Fund and RCUK (Digital Economy Programme grant number EP/G036861/1 Oxford Centre for Doctoral Training in Healthcare Innovation) (S.G.), the Engineering and Physical Sciences Research Council (EPSRC) of the United Kingdom (C.C.C.) under Challenging Engineering award EP/F011547/1, Cancer Research UK (L.W.S.) and the Frederick V. Hunt Postdoctoral Research Fellowship from the Acoustical Society of America (J.C.). The authors would like to thank Mr. James Fisk for manufacturing the various holders, transducer cones and tanks used in this work.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.12.016.

## References

[1] H. Maeda, The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting, Adv. Enzyme Regul. 41 (2001) 189–207.
[2] Y. Barenholz, Doxil(R)–the first FDA-approved nano-drug: lessons learned, J. Control. Release 160 (2012) 117–134.
[3] A.J. Coukell, C.M. Spencer, Polyethylene glycol-liposomal doxorubicin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in the management of AIDS-related Kaposi's sarcoma, Drugs 53 (1997) 520–538.
[4] T.D. Tailor, G. Hanna, P.S. Yarmolenko, M.R. Dreher, A.S. Betof, A.B. Nixon, I. Spasojevic, M.W. Dewhirst, Effect of pazopanib on tumor microenvironment and liposome delivery, Mol. Cancer Ther. 9 (2010) 1798–1808.
[5] K.M. Laginha, S. Verwoert, G.J. Charrois, T.M. Allen, Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors, Clin. Cancer Res. 11 (2005) 6944–6949.

[6] F. Yuan, M. Dellian, D. Fukumura, M. Leunig, D.A. Berk, V.P. Torchilin, R.K. Jain, Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size, Cancer Res. 55 (1995) 3752–3756.
[7] H. Karanth, R.S. Murthy, pH-sensitive liposomes–principle and application in cancer therapy, J. Pharm. Pharmacol. 59 (2007) 469–483.
[8] B. Goldenbogen, N. Brodersen, A. Gramatica, M. Loew, J. Liebscher, A. Herrmann, H. Egger, B. Budde, A. Arbuzova, Reduction-sensitive liposomes from a multifunctional lipid conjugate and natural phospholipids: reduction and release kinetics and cellular uptake, Langmuir 27 (2011) 10820–10829.
[9] S. Dromi, V. Frenkel, A. Luk, B. Traughber, M. Angstadt, M. Bur, J. Poff, J. Xie, S.K. Libutti, K.C. Li, B.J. Wood, Pulsed-high intensity focused ultrasound and low temperature-sensitive liposomes for enhanced targeted drug delivery and antitumor effect, Clin. Cancer Res. 13 (2007) 2722–2727.
[10] A. Kheirolomoom, D.E. Kruse, S. Qin, K.E. Watson, C.Y. Lai, L.J. Young, R.D. Cardiff, K.W. Ferrara, Enhanced *in vivo* bioluminescence imaging using liposomal luciferin delivery system, J. Control. Release 141 (2010) 128–136.
[11] T.J. Evjen, E.A. Nilssen, S. Rognvaldsson, M. Brandl, S.L. Fossheim, Distearoylphosphatidylethanolamine-based liposomes for ultrasound-mediated drug delivery, Eur. J. Pharm. Biopharm. 75 (2010) 327–333.
[12] S. Mo, C.C. Coussios, L. Seymour, R. Carlisle, Ultrasound-enhanced drug delivery for cancer, Expert Opin. Drug Deliv. 9 (2012) 1525–1538.
[13] P. Giustetto, D.D. Castelli, C. Boffa, S. Rizzitelli, D. Durando, J.C. Cutrin, S. Aime, E. Terreno, Release of a paramagnetic magnetic resonance imaging agent from liposomes triggered by low intensity non-focused ultrasound, J. Med. Imaging Health Inform. 3 (2013) 356–366.
[14] T.J. Evjen, E. Hagtvet, A. Moussatov, S. Rognvaldsson, J.L. Mestas, R.A. Fowler, C. Lafon, E.A. Nilssen, *In vivo* monitoring of liposomal release in tumours following ultrasound stimulation, Eur. J. Pharm. Biopharm. 84 (2013) 526–531.
[15] C.D. Arvanitis, M. Bazan-Peregrino, B. Rifai, L.W. Seymour, C.C. Coussios, Cavitation-enhanced extravasation for drug delivery, Ultrasound Med. Biol. 37 (2011) 1838–1852.
[16] M. Bazan-Peregrino, B. Rifai, R.C. Carlisle, J. Choi, C.D. Arvanitis, L.W. Seymour, C.C. Coussios, Cavitation-enhanced delivery of a replicating oncolytic adenovirus to tumors using focused ultrasound, J. Control. Release 169 (2013) 40–47.
[17] C.H. Farny, R.G. Holt, R.A. Roy, Temporal and spatial detection of HIFU-induced inertial and hot-vapor cavitation with a diagnostic ultrasound system, Ultrasound Med. Biol. 35 (2009) 603–615.
[18] M. Gyongy, C.C. Coussios, Passive spatial mapping of inertial cavitation during HIFU exposure, IEEE Trans. Biomed. Eng. 57 (2010) 48–56.
[19] J.J. Choi, C.C. Coussios, Spatiotemporal evolution of cavitation dynamics exhibited by flowing microbubbles during ultrasound exposure, J. Acoust. Soc. Am. 132 (2012) 3538–3549.
[20] R. Carlisle, L.W. Seymour, C.C. Coussios, Targeting of Liposomes via PSGL1 for Enhanced Tumor Accumulation, Pharm. Res. 30 (2013) 352–361.
[21] A. Ryan, H. Scrable, Visualization of the dynamics of gene expression in the living mouse, Mol. Imaging 3 (2004) 33–42.
[22] M. Stevenson, R. Carlisle, B. Davies, C. Preece, M. Hammett, W.L. Liu, K.D. Fisher, A. Ryan, H. Scrable, L.W. Seymour, Development of a positive-readout mouse model of siRNA pharmacodynamics, Mol. Ther. Nucleic Acids 2 (2013) e133.
[23] N. Hockham, C.C. Coussios, M. Arora, A real-time controller for sustaining thermally relevant acoustic cavitation during ultrasound therapy, IEEE Trans. Ultrason. Ferroelectr. Freq. Control 57 (2010) 2685–2694.
[24] M. Bazan-Peregrino, B. Rifai, R.C. Carlisle, J. Choi, C.D. Arvanitis, L.W. Seymour, C.C. Coussios, Cavitation-enhanced delivery of a replicating oncolytic adenovirus to tumors using focused ultrasound, J. Control. Release 169 (2013) 40–47.
[25] E. Wisse, F. Jacobs, B. Topal, P. Frederik, B. De Geest, The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer, Gene Ther. 15 (2008) 1193–1199.
[26] J.J. Choi, K. Selert, F. Vlachos, A. Wong, E.E. Konofagou, Noninvasive and localized neuronal delivery using short ultrasonic pulses and microbubbles, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 16539–16544.
[27] M. Bazan-Peregrino, C.D. Arvanitis, B. Rifai, L.W. Seymour, C.C. Coussios, Ultrasound-induced cavitation enhances the delivery and therapeutic efficacy of an oncolytic virus in an *in vitro* model, J. Control. Release 157 (2012) 235–242.
[28] R.K. Jain, Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy, Science 307 (2005) 58–62.
[29] D.L. Miller, J. Song, Tumor growth reduction and DNA transfer by cavitation-enhanced high-intensity focused ultrasound *in vivo*, Ultrasound Med. Biol. 29 (2003) 887–893.
[30] S. Mitragotri, Healing sound: the use of ultrasound in drug delivery and other therapeutic applications, Nat. Rev. Drug Discov. 4 (2005) 255–260.
[31] R. Carlisle, J. Choi, M. Bazan-Peregrino, R. Laga, V. Subr, L. Kostka, K. Ulbrich, C.C. Coussios, L.W. Seymour, Enhanced Tumor Uptake and Penetration of Virotherapy Using Polymer Stealthing and Focused Ultrasound, J. Natl. Cancer Inst. 105 (2013) 1701–1710.
[32] L.H. Treat, N. McDannold, Y. Zhang, N. Vykhodtseva, K. Hynynen, Improved anti-tumor effect of liposomal doxorubicin after targeted blood–brain barrier disruption by MRI-guided focused ultrasound in rat glioma, Ultrasound Med. Biol. 38 (2012) 1716–1725.
[33] D. Marsh, J.M. Seddon, Gel-to-inverted hexagonal (L beta-HII) phase transitions in phosphatidylethanolamines and fatty acid-phosphatidylcholine mixtures, demonstrated by $^{31}$P NMR spectroscopy and x-ray diffraction, Biochim. Biophys. Acta 690 (1982) 117–123.
[34] R.S. Cobbold, Foundations of biomedical ultrasound, Oxford University Press, New York, 2007.
[35] T.J. Evjen, S. Hupfeld, S. Barnert, S. Fossheim, R. Schubert, M. Brandl, Physicochemical characterization of liposomes after ultrasound exposure—mechanisms of drug release, J. Pharm. Biomed. Anal. 78–79 (2013) 118–122.
[36] L.T. Baxter, R.K. Jain, Transport of fluid and macromolecules in tumors. II. Role of heterogeneous perfusion and lymphatics, Microvasc. Res. 40 (1990) 246–263.
[37] E. Mylonopoulou, M. Bazan-Peregrino, C.D. Arvanitis, C.C. Coussios, A non-exothermic cell-embedding tissue-mimicking material for studies of ultrasound-induced hyperthermia and drug release, Int. J. Hyperthermia 29 (2013) 133–144.