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● *Original Contribution*

IN VITRO LOCALIZED RELEASE OF THERMOSENSITIVE LIPOSOMES WITH ULTRASOUND-INDUCED HYPERTHERMIA

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Abstract—Localized drug delivery with ultrasound-induced hyperthermia can enhance the therapeutic index of chemotherapeutic drugs by improving efficacy and reducing systemic toxicity. A novel *in vitro* method for the activation of drug-loaded thermosensitive liposomes is described. In particular, a dual-compartment, acoustically transparent container is used in which thermosensitive liposomes suspended in cell culture medium are immersed in a thermally absorptive medium, glycerol. Hyperthermia is induced with ultrasound in the glycerol, which in turn heats the culture medium by thermal conduction. The method approximately mimics the *in vivo* scenario of thermosensitive liposomes collected in the interstitial spaces of tumors, where ultrasound induces hyperthermia in the tumor tissue, which in turn heats the thermosensitive liposomes by conduction and induces release of the encapsulated drug. The acoustic conditions for the desired hyperthermia are derived theoretically and validated experimentally. Eighty percent release of doxorubicin from thermosensitive liposomes is achieved. (E-mail: maverk@ucy.ac.cy) © **2013 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Thermosensitive liposomes, Hyperthermia, *In vitro*, Localized drug release.**

INTRODUCTION

Increasing the therapeutic index (the ratio of the average toxic dose to the average therapeutic dose) of anti-cancer pharmaceuticals is one of the main challenges in cancer therapy today. Conventional cancer treatment procedures using chemotherapy drugs, such as the widely used anthracycline doxorubicin, are limited mainly because of the severe and undesired side effects. Liposomes were initially designed as possible drug carriers for reduced systemic exposure. Today, liposomal formulations of doxorubicin (Doxil, Caelyx) are used in the clinic and have been found to have the ability to reduce cytotoxicity in healthy tissue as compared with their unencapsulated counterpart (Northfelt et al. 1998; O'Brien et al. 2004). In addition, increased accumulation of drug-loaded liposomes, mainly as a result of the enhanced permeability and retention (EPR) effect, has been observed in tumors (Drummond et al. 1999; Gabizon 1992; Gabizon et al. 1994; Northfelt et al. 1996, 1998). A major drawback, however, was the limited bioavailability of the drug within the tumor. Despite the higher concentrations of liposomal drug present, passive drug release from the liposomes is slow, resulting in reduced drug effectiveness (Bandak et al. 1999; Kong et al. 2000a).

Over the past 30 years, technology advances have resulted in the development of a variety of liposomes that can be triggered to release their payload in response to an external stimulus (Torchilin 2005). The use of mild hyperthermia as a stimulus for the activation of thermosensitive liposomes (TSL) was first proposed by Yatvin et al. (1978) and has gained significant momentum over the last decade (Ponce et al. 2006). Furthermore, hyperthermia has also been known to increase liposome accumulation in tumors. In their review, Kong and Dewhirst (1999) identify more than 100 reports of hyperthermia-treated tumors exhibiting increased drug accumulation, as well as improved efficacy, for a variety of drugs. Finally, pre-clinical studies have indicated that in addition to its direct cytotoxic effect (Dewhirst et al. 1997), hyperthermia may augment the potency of several chemotherapeutic agents (Hahn et al. 1975; Herman 1983).

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Thermosensitive liposomes remain stable for a long time in circulation at physiologic temperatures and are able to quickly release their encapsulated content (*e.g.,* doxorubicin) when heated near their phase transition temperature (T_m), at which the liposome membrane changes from the gel to liquid crystalline phase (Gaber et al. 1996; Kong and Dewhirst 1999; Kong et al. 2000a; Lindner et al. 2004; Mills and Needham 2004; Needham and Dewhirst 2001; Papahadjopoulos et al. 1973; Yatvin et al. 1978). Several sources of hyperthermia have been considered over the years, including superficial heating with water baths (Chen et al. 2008; Gaber et al. 1996; Kong et al. 2000a, 2000b, 2001; Needham and Dewhirst 2001; Needham et al. 2000) microwave radiation (Hauck et al. 2006; Huang et al. 1994; Khoobehi et al. 1989), infra-red radiation (Salomir et al. 2005) and heating with invasive catheters (Ahmed and Goldberg 2004; Ponce et al. 2007; Viglianti et al. 2006). Lately, the use of ultrasound as an external energy source to trigger drug release from thermosensitive liposomes is becoming more popular (Dromi et al. 2007; O'Neill and Li 2008; Patel et al. 2008). Briefly, focused ultrasound is used to non-invasively induce local hyperthermia with millimeter accuracy in a selected region of interest. Recent advances in electronic and mechanical ultrasound beam steering have enabled the uniform heating of larger targets, such as a tumor deep inside the human body, while maintaining physiologic temperatures in the surrounding tissue and along the path of ultrasound propagation (Diederich and Hynynen 1999; Enholm et al. 2010; Harari et al. 1991; Hynynen et al. 1987; Köhler et al. 2009; Mougenot et al. 2011; Stauffer 2005). The combined benefits of ultrasound, hyperthermia and drug-loaded thermosensitive liposomes pose a very attractive localized drug delivery approach and have great potential in increasing the therapeutic index in cancer treatment.

Whereas more and more reports of *in vivo* studies with ultrasound as a source of hyperthermia are being published (de Smet et al. 2011; Dromi et al. 2007; Grull and Langereis 2012; Ng and Liu 2002; Sanches et al. 2011; Staruch et al. 2011; Yudina et al. 2011), reports on *in vitro* work are scarce. *In vitro* work is a logical precursor to *in vivo* work because it allows simplifications of the system under study, offers a more predictable and controlled environment and is usually both time and cost efficient, compared with *in vivo* work. To our knowledge, there have been no *in vitro* drug delivery studies on the release of drug-loaded thermosensitive liposomes in which ultrasound was used as the source of hyperthermia. One of the main obstacles to using ultrasound-induced hyperthermia for drug delivery is finding an appropriate medium in which the experiments can be carried out. An ideal medium should be biocompatible, it should not interact with liposomes in any way that may potentially compromise their stability and thermal properties, and it should have a high ultrasound thermoviscous absorption coefficient so that it may be heated with ultrasound energy. Cell culture media satisfy nearly all of these criteria, but have very low thermoviscous absorption coefficients and are thus unable to be heated with ultrasound. Several other media (*e.g.,* blood, serum, oils, glycerol, gels) have been tested by the authors, but finding a biocompatible, inert and high-absorption-coefficient medium has not been possible thus far.

In this work, an *in vitro* method for the controlled release of thermosensitive liposomes with ultrasound-induced hyperthermia is described. The need for an "ideal" medium is overcome by the design of a dual-compartment holder in which the sample (suspension of TSL in cell culture medium) is placed in an acoustically transparent holder that is immersed in glycerol. Hyperthermia is induced in the glycerol by a focused transducer, and then heat is transferred to the sample *via* thermal conduction, thus activating the drug-loaded TSL. The proposed method resembles the *in vivo* scenario, where ultrasound induces hyperthermia in the tumor tissue and the TSL (accumulated in the tumor interstitial spaces) are heated by thermal conduction. Theoretical predictions with the modified Pennes (1948) bioheat equation were first used to calculate the acoustic conditions needed for the desired temperature elevation. The theoretical predictions were then validated with measurements of the induced temperature elevation with a fine-wire thermocouple in glycerol. In vitro activation of TSL and doxorubicin release under the derived ultrasound conditions were evaluated.

METHODS

Experimental setup

Figure 1 is a schematic of the experimental setup designed to implement the thermal conduction method. A 10 × 10 × 30-mm plastic cuvette (Fisher Scientific SAS, Illkirch, France) was filled with 99% glycerol (Sigma-Aldrich, Munich, Germany), which has a high thermoviscous absorption coefficient, $\alpha_0 = 3.6$ Np/m at 1.1 MHz, where the frequency dependence of absorption is described by

$$A = a_0 * f^2 \quad (1)$$

Acoustically transparent Mylar windows on all four cuvette walls allowed free propagation of ultrasound and avoided heating of the plastic walls. The TSL were suspended in a cell culture medium (OptiMEM, Gibco-Invitrogen, Carlsbad, CA, USA) and placed in a cylindrical holder (outer diameter = 3 mm) made

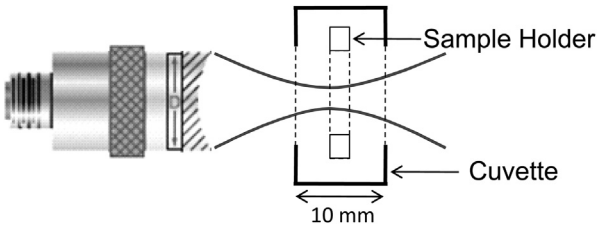

Fig. 1. Ultrasound exposure setup with focused transducer and double compartment, the outer filled with glycerol and the inner filled with cell culture medium (OptiMEM). *Dotted lines* represent Mylar acoustic windows on the cuvette (outer compartment with dimensions 10 × 10 × 30 mm) and sample holder (3-mm-diameter inner compartment) walls. The sample holder is placed at the focus of the transducer.

entirely of the same Mylar sheet. The sample holder was then immersed in the glycerol, and a custom-made single-element focused transducer with 1.1-MHz center frequency, 51-mm diameter and 52-mm focus (radius of curvature) was used to induce hyperthermia in the glycerol. The transducer was placed in such a way that the maximum pressure (1.7 mm before the focus [see Fig. 2a]) was exactly halfway between the sample holder (with the OptiMEM and TSL) and the cuvette wall (filled with glycerol). This provided the maximum heating effect. A focused transducer was used to ensure that a high enough pressure would result for the required temperature elevation. The TSL solution reached the desired temperature *via* thermal conduction, which occurred fairly quickly (2–3 min) because of the small volume of the sample holder (>0.5 mL). All experiments were carried out in a 37°C water bath.

Before the experiments, detailed characterization of the ultrasound field produced by the transducer was carried out. Propagation curves (variation of the acoustic pressure along the source axis) and beam patterns (distribution of the acoustic pressure on the transverse axis) at various fixed positions from the face of the source were measured using a calibrated 0.4-mm membrane hydrophone (Precision Acoustics, Dorchester, UK). These were compared with the theoretical predictions (linear theory with Rayleigh integral). A motorized three-axis micro-positioning system (Newport, Irvine, CA, USA) was used to control the movement of the receiver along the linear (*x, y, z*) axis, and a manual 360° rotation stage controlled the motion along the rotation axis, θ. The pressure at the focus, as a function of input voltage, was also measured using the calibrated membrane hydrophone.

Numerical simulation of temperature elevation

Numerical predictions of the ultrasound-induced temperature elevation were performed using a modified Pennes (1948) bio-heat transfer equation. The bioheat equation calculates the temperature distribution in tissue produced by ultrasound exposure taking into consideration perfusion and metabolic heat sources. During *in vitro* work, metabolic activity and perfusion are not present, and therefore, the corresponding terms may be neglected. The pressure field of the transducer was calculated with the Rayleigh integral

$$p(x,y,z;t) = \frac{jk\rho_0 c_0 u_0 e^{j\omega t}}{2\pi}\int_s \frac{e^{-jkR}}{R}dS, \qquad (2)$$

where

$$R = \sqrt{(x-x')^2+(y-y')^2+z^2}$$

for a planar source. For a circular focused source, spherical coordinates are used and the surface R is transformed into a curved interface R_f according to the radius of curvature.

The 3-D volume pressure data were then imported into the bioheat equation, and the predicted temperature distribution with respect to time and space was calculated. For the simulations, we assumed propagation in glycerol with density of 1258 kg m^{-3}, specific heat capacity of 2381 J kg^{-1} K^{-1}, thermal diffusivity of 0.95 e^{-7} m^2 s^{-1}, thermoviscous absorption coefficient of 3.6 Np m^{-1} MHz^{-2} and speed of sound of 1935 m s^{-1}. Cooling due to streaming was not considered.

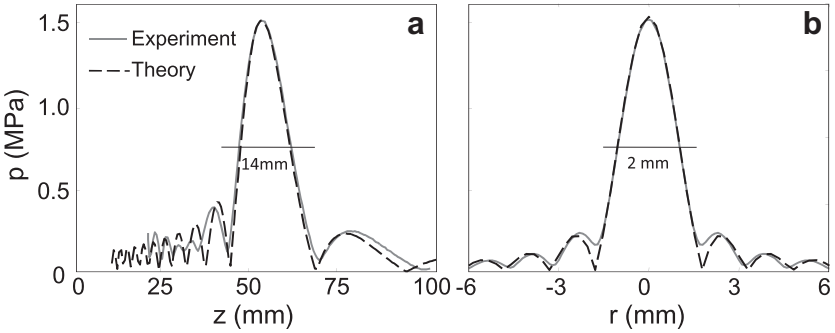

Fig. 2. Acoustic pressure field of the transducer used for ultrasound-induced hyperthermia: (a) axial, (b) transverse. *Solid lines* represent measurements, and *dashed lines* represent theoretical predictions based on the Rayleigh integral.

The objective of this work was to develop an *in vitro* method for the activation of thermosensitive liposomes using ultrasound-induced hyperthermia and not to experimentally verify the bioheat equation for the current setup. Temperature predictions were used to provide an estimate of the ultrasound conditions needed to produce the desired temperature elevation. Increased heating caused by non-linear propagation (Bacon and Carstensen 1990; Haar and Coussios 2007) was also neglected, as the acoustic amplitude used for a temperature elevation of 5°C–8°C is relatively small. According to Bessanova et al. (2009, 2010), who investigated the influence of the non-linear and diffraction effects on the amplification factors of focused ultrasound systems and the resulting effect on wave intensity and heat deposition, neglecting non-linearity for the ultrasound settings used in our experiments was a valid assumption.

Temperature measurements

Temperature measurements were made with a 50-μm K-type fine-wire thermocouple. The transducer-thermocouple setup was immersed in a glycerol bath in the absence of the dual-compartment setup, and the thermocouple junction was first aligned with the focus of the transducer by maximizing the reflected echo in a pulse-echo system using a Ritec diplexer (RDX-6, Warwick, RI, USA). The final alignment was done by scanning the x-, y- and z-planes for the point of maximum temperature elevation for a given ultrasound setting (0.5 MPa, 500 cycles, 45% duty cycle for 4–5 s). Temperature readings were taken every 0.1 mm in the radial direction and 0.2 mm in the axial direction. For each step, the temperature was allowed to return to its ambient value before the next reading was taken. Once the alignment was complete, the temperature elevation, as a function of pressure, was captured with the fine-wire thermocouple and recorded using a digital thermometer data logger (TEC-PEL-DTM 318, Taipei, Taiwan). The ultrasound parameters used were 1.1-MHz frequency and 45% duty cycle, and the heating time was 30 s, with temperature recordings taken at the rate of 1 Hz.

The small diameter of the thermocouple (compared with the wavelength) allows for more accurate temperature readings; however, it is still susceptible to the thermoviscous artifact (Fry and Fry 1954a, 1954b). Although this artifact is lessened when bare fine-wire thermocouples are used (Hynynen et al. 1983), several authors have reported that it is still significant (Huang et al. 2004; Hynynen and Edwards 1989; Morris et al. 2008).

Temperature measurements, as a function of time inside the sample holder, were recorded to determine which ultrasound parameters are needed to induce a temperature elevation of 5°C–8°C, as well as the rate of heating of the samples from thermal conduction. To avoid any temperature measurement errors from the thermoviscous artifact, the thermocouple was fixed in the sample holder as far from the ultrasound field as possible (about 6 mm from the focus, beam width = 2 mm) (see Fig. 2b). The acoustic streaming produced was effectively stirring the mixture during the 15-min ultrasound application to produce a spatially uniform temperature.

Preparation of doxorubicin-loaded thermosensitive liposomes

Thermosensitive liposomes are prepared *via* the lipid film re-hydration method. Briefly, dipalmitoylphosphatidylcholine (DPPC), hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (Chol) and distearylphosphatidylethanolamine-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were solubilized in chloroform in the molar ratio 100:33:27:7 (DPPC:HSPC:Chol:DSPE-PEG2000). The solvent was then evaporated under a nitrogen flow. The lipid film was hydrated at 55°C with 5 mL of 300 mM ammonium hydrogen phosphate at pH 5 (pH was adjusted with HCl), so that the final lipid concentration was 20 mM. Six freeze-thaw cycles were achieved by successively plunging the sample into liquid nitrogen and into a water bath regulated at 55°C. Then, at 55°C, liposomes were extruded 5 times through a 0.45-μm polyvinylidene fluoride (PVDF) membrane filter and 10 times through a 0.22-μm PVDF filter.

The liposome size distribution was measured by dynamic light scattering (Zetasizer Nano ZS, Malvern) at 190 nm with a dispersity index of 0.17. The external buffer was then exchanged by dialysis in Hepes-buffered saline (Hepes 25 mM, NaCl 140 mM, pH 7.4) with a 50-kDa dialysis membrane cutoff and a dialysis factor of 400 * 400 * 400. Doxorubicin was added to the liposome solutions at a drug:lipid ratio of 0.1:1 (w/w) and incubated overnight at 37°C (Abraham et al. 2005; Fritze et al. 2006; Zucker et al. 2009). Non-encapsulated doxorubicin was eliminated by size-exclusion chromatography on a Sephadex G50 column. The doxorubicin concentration of the purified doxorubicin-loaded liposomes was assayed by UV-visible spectroscopy (Cary 100 Scan, Varian) against a calibration curve and was measured to be 194 μg/mL. The absorbance of doxorubicin was read at 485 nm.

Activation protocol

The TSL were diluted in fresh OptiMEM medium to give a final doxorubicin concentration of 3 μg/mL. For each experiment, 0.5 mL of the TSL solution was used, and each experiment was repeated three times. To ensure maximum drug release from the TSL (and after release

mechanism experiments not shown here), the duration of the experiment was 15 min. The experiments were divided into seven groups:

1. OptiMEM alone [*No TSL*]. This group is included to show the fluorescence of the cell medium itself. (The text in parentheses denotes the label used in Figures 6 and 7, and this applies to all groups described here.)
2. OptiMEM exposed to ultrasound [*No TSL + US(Q)*]. This group was included to check if ultrasound exposure alters the parameters of the cell medium compared with group 1. The notation *US(Q)* denotes that the ultrasound applied is depositing heat and causing the desired hyperthermia.
3. TSL alone [*TSL*]. This is the negative control group.
4. TSL exposed to ultrasound but without heating [*TSL + US(noQ)*]. Replacing the glycerol in the cuvette with water allows the same ultrasound exposure to be applied to the samples but without induction of heating because water has very low absorption. This is done to check if ultrasound alone (as mechanical energy) induces any release from the TSL. The notation *US(noQ)* denotes that the ultrasound applied is not depositing any heat.
5. TSL exposed to ultrasound and causing hyperthermia [*TSL + US(Q)*]. This is the group in which the TSL are activated by ultrasound-induced hyperthermia under the following acoustic conditions: 1.1 MHz, 500 cycles, 45% duty cycle, 1.6 MPa.
6. TSL exposed to ultrasound and causing hyperthermia at higher pressure than in group 5 [*TSL + US*(Q)*]. This group is very similar to group 5, except that a higher acoustic pressure, 2.6 MPa, was used to observe the effect of increased hyperthermia on TSL activation.
7. TSL in Nonidet P-40 (Sigma-Aldrich) solution [*TSL + Nonidet*]. This group is the positive control because Nonidet P-40 detergent dissolves the liposomes, causing 100% release.

Release of doxorubicin from thermosensitive liposomes

Doxorubicin is a naturally fluorescent molecule whose fluorescence is quenched when in the liposomal form. Therefore, the fluorescence intensity of a doxorubicin-containing medium is proportional to the extraliposomal doxorubicin or, in this case, the doxorubicin released from the TSL (de Smet et al. 2010). After the activation protocol, 150 μL of solution was transferred to polystyrene 96-well plates (Iwaki, Tokyo, Japan), and fluorescence intensity was measured in a VICTOR X4 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA) at room temperature. The excitation, λ_{ex}, and emission, λ_{em}, wavelengths were 485 and 580 nm, respectively. Each measurement was repeated three times, and the results are expressed as the mean ± standard error. The percentage release of doxorubicin from the liposomes was evaluated according to the equation (de Smet et al. 2010).

$$\%\ \text{Release} = \frac{I_{exp} - I_{neg}}{I_{pos} - I_{neg}}, \tag{3}$$

where I_{exp} is the intensity of the fluorescence after the application of hyperthermia, I_{neg} is the intensity of the fluorescence of the negative control and I_{pos} is the intensity of the fluorescence after the addition of Nonidet P-40 (positive control).

RESULTS

Focused ultrasound field

Measurements of the acoustic pressure of the ultrasound transducer are illustrated in Figure 2 (*solid line*). The acoustic measurements were compared with theoretical predictions based on the Rayleigh integral (*dashed lines* in Fig. 2). The propagation curve (pressure as a function of axial distance) is provided in Figure 2a, and the beam pattern (pressure as a function of radial distance) at the focus of the transducer (z = 52 mm), in Figure 2b. Good agreement was found between theory and experiment, suggesting that our transducer was behaving like a typical piston source. This enables us to use the theoretical prediction for calculation of the full 3-D field instead of taking acoustic measurements. In addition, the focal dimensions (–6 dB down points) were calculated at 14 mm in the axial and 2 mm in the transverse direction.

Numerical simulations and measurements of ultrasound-induced temperature elevation

The pressure distribution field of our transducer calculated with the Rayleigh integral is illustrated in Figure 3a. The acoustic pressure at the focus was 1.6 MPa, the frequency was 1.1 MHz and the duty cycle was 45% at a heating time of 20 s. Figure 3b, c provides the predicted temperature distributions for water and glycerol, respectively (plotted in logarithmic scale). The predictions presented here are for free field (not for the dual-compartment setup). It is evident from our results that water (or water-based media such as OptiMEM), which has a low absorption coefficient, does not heat up with ultrasound, whereas media with higher absorption coefficients, such as glycerol, may reach the desired temperature elevation with the given ultrasound parameters. As expected, the profile of the heated area closely resembles the profile of the pressure field, with the higher temperatures achieved where the higher pressures are applied. In Figure 4, the predicted temperature elevation, as a function of pressure (at the focus), is illustrated for

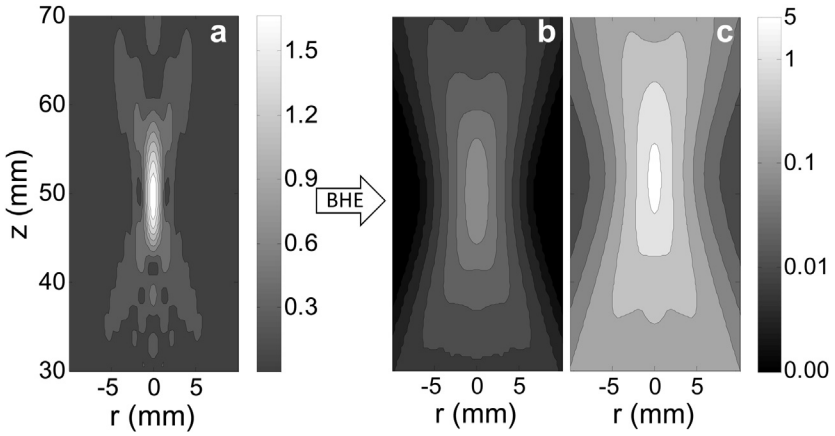

Fig. 3. (a) Contour plot of the axial pressure distribution [plane (r = 0, z)]. (b, c) Predicted temperature elevation after 20 s of ultrasound exposure in water (b) and glycerol(c).

various media. The predictions for water are represented by the *dash-dot line with solid squares*, those for olive oil by the *dashed line with open circles* and those for 99% glycerol by the *dotted line with solid triangles*. Heating of water using ultrasound was negligible, whereas high-absorption media (olive oil, glycerol) were successfully heated. According to these predictions, 1.6 MPa pressure at the focus is required to induce a 5°C temperature rise in glycerol, whereas 2.0 MPa would induce an 8°C temperature elevation.

The theoretical predictions were compared with experimental measurements of ultrasound-induced hyperthermia (after 20 s exposure, 1.1-MHz frequency, 500 cycles, and 45% duty cycle) obtained with a 50-μm fine-wire thermocouple also shown in Figure 4 (*solid line with solid-circle markers*). To compare the results with theoretical predictions, the experiments were carried out in a glycerol bath and the thermocouple was placed exactly at the focus of the transducer. At the lower acoustic pressures, the measured temperature was higher than the predicted value, and this may be attributed to the thermoviscous heating artifact causing the thermocouple to record a higher value. At higher pressures (and overall intensities), there was increased acoustic streaming, which causes cooling and reduction of the measured temperature. As a result, the predicted values were higher than the measured values (streaming was not taken into consideration in our heat transfer model). From Figure 4, we confirm that a pressure of 1.6 MPa at the focus is adequate to raise the temperature in glycerol from 37°C to the 43°C required for thermal activation of the TSL.

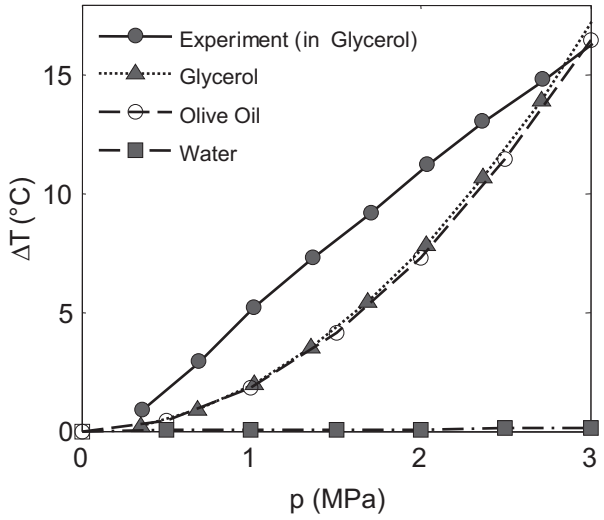

Fig. 4. Predictions of temperature elevation as a function of pressure at focus for different media and comparison with measured values in glycerol. Ultrasound conditions: 1.1 MHz, 500 cycles, 45% duty cycle, 20-s duration.

Temperature elevation as a function of time inside the sample holder, using the above ultrasound settings (1.6 MPa, 45% duty cycle), is illustrated in Figure 5. Although the target temperature was successfully reached, the rate of heating was slow; 2–3 min was required to reach 43°C (ΔT = 6°C), and about 6 min passed before equilibrium at 45°C (ΔT = 8°C) was reached.

Doxorubicin release from thermosensitive liposomes

Thermosensitive liposome activation (mean fluorescence intensity) in the different experiment groups is illustrated in Figure 6. Ultrasound alone does not affect the fluorescence of the medium because the natural fluorescence intensity of the medium remained the same before and after sonication [*No TSL* ≈ *No TSL + US(Q)*]. Here we note that the background fluorescence of other media such as olive oil, when insonified, changed, and thus, it was not possible to quantify drug release on the basis of fluorescence alone.

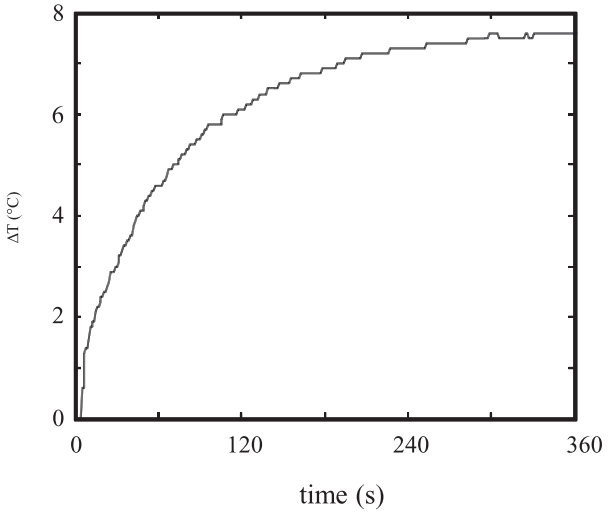

Fig. 5. Temperature elevation of OptiMEM in the sample holder caused by thermal conduction from the glycerol. Ultrasound conditions: p = 1.6 MPa, f = 1.1 MHz, 500 cycles, 45% duty cycle.

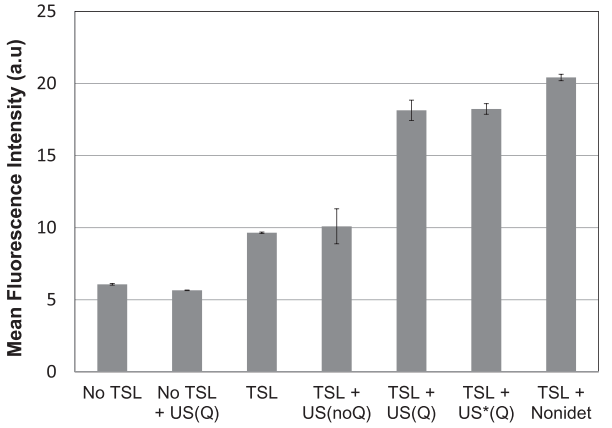

Fig. 6. Mean fluorescence intensity (in arbitrary units) measurements of the different groups.

Furthermore, ultrasound alone without hyperthermia did not influence TSL or cause any release [*TSL* ≈ *TSL* + *US(noQ)*]. The higher intensity observed compared with the *No TSL* group was due to the quenched fluorescence of the liposomal doxorubicin.

When hyperthermia was induced with ultrasound [*TSL* + *US(Q)*], a significant amount of TSL activation was observed (about 80% of that of the positive control group [*TSL* + *Nonidet*]) (Fig. 7). Further increasing the pressure to 2.6 MPa [*TSL* + *US*(Q)*], which corresponded to temperatures above 50°C, did not yield any increase in fluorescence.

DISCUSSION

An *in vitro* method for the controlled release of thermosensitive liposomes using ultrasound-induced temperature elevation has been described. The heating-by-conduction approach mimics the *in vivo* scenario of TSL in the interstitial tumor space, where ultrasound induces hyperthermia in the tumor tissue, and then, by conduction, the TSL are heated and activated to release their payload. Furthermore, it offers an *in vitro* method of TSL activation that eliminates the need for an ideal medium (biocompatible, inert with liposomes, high absorption coefficient), which is required for a single-compartment *in vitro* setup and which has not been found yet. The proposed setup may be used to study cell drug uptake by suspending cells together with the TSL in the culture medium. The secondary heating approach is also applicable to setups of seeded cells (*e.g.,* heating in OptiCell chambers immersed in glycerol). Experimental setups with TSL suspended tissue-mimicking gel phantoms have also been used to study drug release (de Smet et al. 2011). However, the preparation of the gels, as well as the analysis and quantification of the results, is time demanding. In addition, accuracy may be compromised as cells and TSL must be incorporated into the liquid gel before it solidifies. This, of course, may lead to cell death and premature activation of the TSL, which will affect the results.

The focused transducers were chosen to ensure the acoustic pressures required for 5°C–8°C temperature elevation in the absence of a very strong power amplifier. However, in retrospect, we realize that we could have reached these temperatures even with unfocused transducers. The one clear advantage of focused transducers is that the heated area is small and can easily be controlled and directed to a specific area. In addition, with our setup and specific parameters, there is streaming that actually mixes the fluid and causes a more spatially uniform temperature.

Significant release of doxorubicin from the TSL was achieved with the proposed method. In Figure 7, the percentage doxorubicin release is illustrated for four of the groups: *TSL* + *US(noQ)*, *TSL* + *US(Q)*, *TSL* + *US*(Q)*, *TSL* + *Nonidet*. It is evident that the mechanical action of ultrasound alone did not trigger doxorubicin release. However, additional improvements in the setup and conditions may further increase drug release.

Figure 5 gives the temperature evolution of the OptiMEM in the sample holder with the proposed method. About 300 s was required for the desired temperature to be reached, whereas it was much faster when a heated water bath was used (data not shown here). One reason for the longer heating time is that focused transducers heat only a small area (see Fig. 3), and it took much longer for the whole volume to heat up. It is possible to produce larger and more uniform heating

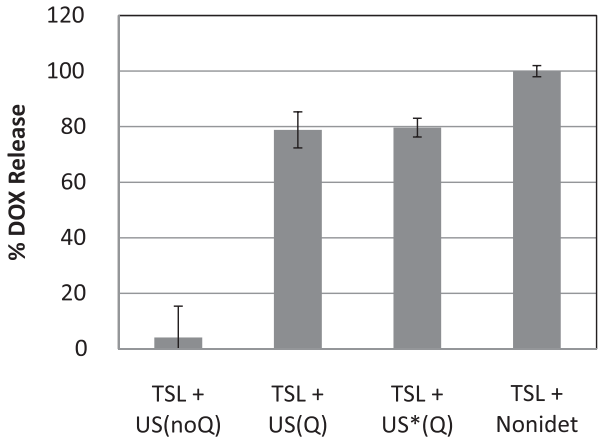

Fig. 7. Percentage induced doxorubicin (DOX) release from thermosensitive liposomes. Values were calculated with eqn (3).

areas with the use of flat or lightly focused transducers (currently under investigation) or even the near field of a focused transducer (Rahim et al. 2006).

Methods of efficient mixing of the heated glycerol may also improve the uniformity of the heating. In the present work, mixing was achieved by acoustic streaming (Starritt et al. 1989; Tjotta 1967). Acoustic streaming has been shown to efficiently mix solutions in containers (Suri et al. 2002). The ultrasound parameters considered here induce acoustic streaming (Mannaris and Averkiou 2012) in both the glycerol and the cell medium. However, because both the cuvette and the inner cylindrical holder are small, the induced streaming causes a spatially uniform temperature elevation.

Often in ultrasound-induced thermal ablation applications (high-intensity focused ultrasound [HIFU]), nonlinear sound propagation plays an important role (Haar and Coussios 2007). Bessanova et al. (2009, 2010) published an in-depth investigation on the influence of non-linear and diffraction effects on amplification factors of focused ultrasound systems, comparing analytical solutions and experimental data. According to these publications, the non-linear wave propagation and the resulting effect on wave intensity and heat deposition for the parameters used in this work (1.1 MHz, 1.6 MPa, non-linearity parameter $N \approx 0.03$), as well as for the geometry of our transducer (focusing gain = 29), was small and thus excluded from our model. The same applies for the higher pressure used in this work (3 MPa, $N \approx 0.06$).

CONCLUSIONS

A method for *in vitro* activation of thermosensitive liposomes with ultrasound-induced hyperthermia has been developed. Ultrasound-induced hyperthermia offers the advantage of activation of localized delivery of drugs from thermosensitive liposomes. The method overcomes the inability of cell culture media such as OptiMEM to be heated by ultrasound. This is achieved by using another thermally absorptive medium such as glycerol to heat by ultrasound, which in turn heats the cell culture medium by conduction. The acoustic conditions for ultrasound-induced hyperthermia and activation of thermosensitive liposomes were first derived from theoretical models and then confirmed with pressure and temperature measurements. With the proposed method, 80% of the thermosensitive liposomes were activated with 15 min exposure to 1.1-MHz, 1.6-MPa, 500-cycle, 45% duty cycle ultrasound.

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