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Controlled drug release from encapsulated nanometer liposomes using high intensity focused ultrasound

Junru Wu, and Di Chen

Physics Department and Materials Science Program, University of Vermont, Burlington VT 05405, USA

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ABSTRACT

A liposome with a diameter ranging from 150 to 200 nm has been considered to be one of the optimal vehicles for targeted drug delivery *in vivo* since it is able to encapsulate drug and also circulate in the blood stream stably. Its small size, however, makes controlled release of its encapsulated content difficult. A feasibility study for applications of high intensity focused ultrasound (HIFU) of the mega-hertz frequency to induce controlled release of its content was carried out. This study, using the dynamic light scattering and transmission electron microscopic observation, demonstrated 21.2% of encapsulated fluorescent materials (FITC) could be released from liposomes with an average diameter of 210 nm when exposed to continuous (cw) ultrasound at 1.1 MHz (I_{SPTA}= 900 W/cm2) for 10 s and the percentage release efficiency can reach to 70% after 60s' irradiation. This result also reveals that rupture of relatively large liposomes (>100nm) and generation of pore-like defects in the membrane of small liposomes (<100nm) due to HIFU excitation might be the main causes of the release; the inertial cavitation took place during the irradiation. The controlled drug release from liposomes by HIFU may be proven to be a potential useful modality for clinical applications.

INTRODUCTION

Since their discovery [1], liposomes have been explored to be carriers for potential applications in targeting and controlled drug delivery *in vivo*. Liposomes (or lipid vesicles) that encapsulate liquid or gas usually have spherical structures and are composed of curved and closed lipid bilayers. Their size ranges from 50 nm to several micrometers. The attractive features of the liposome for medical applications are: (1) ability to protect and carry hydrophilic or hydrophobic molecules encapsulated inside; (2) biocompatibility of their bilayers with cell membranes; (3) relatively low antigenicity; (4) nanometer small size allowing them to circulate in the blood stream stably, and (5) the relative ease of adding special ligands to their surface. Drug, DNA or other therapeutic agents can be either bound to the lipid or entrapped in the aqueous core of liposomes [2-4]. In general, a drug-loaded liposome remains stable and inactive physiologically until the physical integrity of the lipid membrane is disturbed. A desirable liposome drug delivery procedure should be as follows: First, liposomes are loaded with drug and circulate in the blood. The encapsulated drug is then released at a controlled rate upon an appropriate stimulus. Chemical and physical methods including pH value, temperature, electric fields, and ultrasound [5-8] for triggering drug release from liposomes have been explored. Among them, ultrasound (US) has a unique advantage as a localized stimulus tool since it can propagate into deep tissue and also can be focused specifically into the target [9]. Our previous study demonstrated that when exposed to 2.2 MHz 10% duty cycle pulsed US (*in situ* spatial pulse peak intensity, I_{SPPA} = 80W/cm^2), liposomes served as cavitation nuclei were able to promote acoustic cavitation during sonoporation process [10]; cavitation in turn enhanced the drug uptake by the cells. A study by Kopechek et al. [11] showed that 6-MHz ultrasound (continuous wave, 2-7 W/cm^2) could induce leakage from liposomes of mean diameter 780 nm. The percentage of calcein, the fluorescence marker encapsulated in the liposomes, released from liposomes reached 47.5% ± 33%. A similar release of 32% from liposomes of mean diameter 800 nm of encapsulated calcein was achieve by Huang and Macdonald [8] by excitation of 10s continuous wave ultrasound at 1 MHz frequency and 2 W/ cm^2 intensity.

To enhance the drug delivery efficiency, the size of liposomes plays a crucial role. It was demonstrated by Liu et al. [12] in a mouse experiment if the diameter of the liposome was greater than 200 nm, liposomes' uptake by the spleen increased and consequently the concentration of liposomes in the blood stream decreased quickly. They also showed if the diameter of liposomes was too small (less than 70 nm), 70% of the injected liposomes were taken up by the liver of a mouse. They concluded that optimal circulation activity of liposomes which correlated well with a relatively high uptake of liposomes by EMT6 tumor in a mouse could be reached when the diameter of liposomes was in a range between 70 to 200 nm. Another research group also showed intermediate sized liposomes (diameter, d ≅150-200 nm) circulated in the blood stream longest when three characteristic sizes (d>300 nm, d≅150-200 nm, and d<70 nm) of liposomes were injected in mice[13]; the large and small liposomes were easily accumulated in spleens and livers of mice.

It was also demonstrated that liposomes incorporated of lipids with a covalently-linked poly (ethylene glycol) (PEG) moiety increased their membrane permeability and became more sensitive to US excitations. The leakage and uptake process for the PEG-containing liposomes with mean diameters about 100 nm could be enhanced up to a 10-fold subjected to a 20 kHz ultrasound (2 W/cm^2) [14]. But whether this enhancement resulted from the membrane destabilization, generation of pores on the membranes, or both by US was not known at that time. Moreover, low-frequency ultrasound (20 kHz) is not attractive for clinical applications because it is difficult to be focused to a small target. To the best of our knowledge it has no literature reporting the use of mega-hertz US to trigger the release of encapsulated content from liposomes with a diameter less than 300 nm.

Motivated by those considerations, we have explored the responsiveness of PEG-containing nanometer-sized (diameter < 300nm) liposomes to mega-hertz therapeutic ultrasound generated by a single HIFU transducer. Our research procedure was to load fluorescent agent (FITC) into liposomes first. Then liposome solution was irradiated by HIFU ultrasound. To shed some light on the possible physical mechanisms of the content release, we analyzed the FITC release kinetics, the liposome size distribution change and physical integrity of the irradiated liposomes using a fluorescent microscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Liposome syntheses

1, 2-Diacyl-sn-glycero-3 phosphocholine (PC) (Sigma-Aldrich, St. Louis, MO, USA) and 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy (Polyethylene glycol)-2000] (PEG2000-DPPE) (Avanti Polar Lipidsm, Inc., Alabaster, AL, USA) were mixed to the mole percent ratio of 95:5 and dissolved in chloroform in a round-bottomed flask. The chloroform solvent was removed under N_2 using a rotary evaporator to generate a lipid film attached to the inner flash wall. Amount of 0.1 mg/mL FITC solution was made by dissolving the FITC powder (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) solution (pH 7.4). Appropriate amount of FITC solution (0.1 mg/mL) was added to the flask to rehydrate the lipid film to a concentration of 5 mg lipid/mL by continuous vortex-mixing of the flask for 3 hours in a 70ºC water bath. A milky solution of liposome was obtained subsequently. Some of the FITC solution was encapsulated inside the liposomes during the swelling of the lipid film. The liposome solution was distributed to plastic culture tubes (12 x 75mm, VWR, West Chester, PA,USA) by 1mL in each. The fluorescent light coming out from the test tube was too strong to be detected optically when the sample was observed by using a fluorescent microscope. To avoid optical saturation, we needed to reduce the fluorescent light intensity from the solution. The sample in the test tube was diluted two times. For each dilution, the sample solution was first made uniformly distributed in a test tube by a vortexer (model: G-560, VWR, West Chester, PA,USA) . Then we replaced the top half solution in each tube by 0.5 mL PBS. After two dilutions, the fluorescent light intensity reduced to about ¼ of the initial value. The concentration of liposomes in the diluted solution in the test tube did not change; it was checked out by using the dynamic light scattering method. These samples were ready to be sonicated. The average fluorescein (FITC) encapsulated in the liposome was much higher than that in the solution, the fluorescent intensity in the solution would increase if rupture of or leakage from liposomes was induced under the irradiation of US.

A group of control samples were made by the same procedures mentioned above except the lipid film was rehydrated by the PBS solution instead of FITC solution. A same amount of FITC powder was added to the solution after the liposomes had been formed to yield the same concentration of FITC in the solution (0.1 mg/mL). In other words, the liposomes in the control sample solution had no FITC solution encapsulated inside; the control sample was just a mixture of liposomes which encapsulated PBS and the FITC solution from which the other group of encapsulating FITC liposomes were made. The average fluorescent intensities (F_c) of the control samples (n = 4) should be equal to that of the encapsulating FITC liposome-solution after the 100% release of FITC from the liposomes.

Ultrasonic exposure and scanning system

The ultrasonic system is shown in Fig.1. The transducer used was a single-element focusing piezo-ceramic HIFU transducer (Sonic Concepts, Seattle, WA) operating at f_0=1.1 MHz (geometric focal length d = 62 mm, active radius, a = 32 mm). The transducer can be moved in 3 orthogonal directions by computer-driven stepper motors (AIMS-001, NTR Systems, Seattle, WA) with the smallest step of 0.1 mm. The transducer and test tube were mounted in a glass tank containing filtered distilled and degassed (d-d) water. Position of the transducer was carefully chosen in such a way that its focal point coincided with the center of the liposome suspension volume in the test tube. An arbitrary waveform function generator (33250A, Agilent, Santa Clara, CA), produces a continuous (cw) sinusoidal signal for various sonication durations as programmed and its output was applied to an input of a 55 dB Radio frequency (RF) power amplifier (ENI A300, Rochester, NY, USA) for amplification. The output of the power amplifier was used to drive the transducer.

To avoid the damage of the hydrophone, the transducer was first excited by a low voltage short electric pulse (3 cycles) with the center frequency equal to 1.1 MHz to generate a low acoustic signal in d-d water and the signal was measured using a needle pvdf broadband piezoelectric hydrophone (NP1000, NTR Systems, Seattle, WA) with an active sensing diameter of 0.2 mm. The hydrophone output voltage was digitized and displayed using a digital oscilloscope (model 54642A, Agilent, Santa Clara, CA) with a bandwidth of 500 MHz. The beam two-dimensional profile and the -6 dB beam-widths along the lateral (perpendicular to the US propagation direction) at the focal plane were determined to be approximately 2 mm.

The acoustic power was measured using the radiation force measurement method [15, 16]. The system included a laboratory balance with the accuracy of 1 mg (Model 403, Denver Instrument, Denver, Co.) and an ultrasound absorber made of a material has a negligible reflection coefficient. The total acoustic power W is calculated by $W=2cmg/\{1+\cos[\arctan(d/2a)]\}$, where m is reading from the balance in kg, c (speed of sound in water) = 1,500 *m/s, d* is the diameter of the transducer, a is the radius of the curvature of the transducer surface and g (gravitational acceleration) = 9.8 m/s^2. The spatial pulse peak average intensity I_{SPPA} in water (it is also equal to the spatial peak temporal average I_{SPTA} since it was cw) was estimated by using measured acoustic power divided by $\pi(d_{-6dB}/2)^2$. In this study, I_{SPPA}= I_{SPTA} = 900 W/cm^2, 1.1 MHz continuous wave (cw) was used.

Those sample tubes, after the two dilutions described above, were exposed to US for 10s, 20s, 30s, 40s, 50s and 60s. Ultrasound exposure was performed quickly after a sample was prepared. To sonicate the liposome suspension evenly, each test tube was rotated at 200 rpm by a DC motor throughout the exposure period. Immediately after the sonication, each test tube was measured for fluorescent light intensity. The same measurement was done to those control sample tubes.

Leakage measurements

Irradiation of ultrasound induced the rupture of the liposomes and/or leakage of encapsulated FITC to the ambient solution. The liposome solutions after exposed to various sonication periods were examined by an inverted fluorescence microscopy (Nikon, Garden city, NY) operating at an epifluorescent mode, the wavelengths of the excitation and fluorescence were about 440 nm and 530 nm respectively. The fluorescence 530 nm signal was captured using a pho-

tomultiplier tube (PMT) (Oriel 70680, ORIEL, Stratford, CT) equipped with a high-gain amplifier (Oriel 70701, ORIEL, Stratford, CT), and recorded as *F(t)*. The PMT was biased at 1100 V using a high-voltage power supply, (Oriel 70705, ORIEL, Stratford, CT). The ultrasound triggered liposome release was assessed as: *FITC release efficiency ={[F(t)-F_0]/[F_c-F_0]}100%,* where F_0 and F_c are fluorescent light intensity readings of the FITC encapsulated liposome suspension before US irradiation and the control suspensions respectively. If $F(t) = F_c$, FITC release efficiency = 100 %; complete encapsulated FITC release is achieved.

Liposome Size Distribution Analysis

Liposome size distributions before and after US irradiation and liposome concentration in solution were determined by the autocorrelation function of the dynamic light scattering (DLS) method [17] using a commercial particle sizer (BI-200SM, Brookhaven Instruments Co., Holtsville, NY).

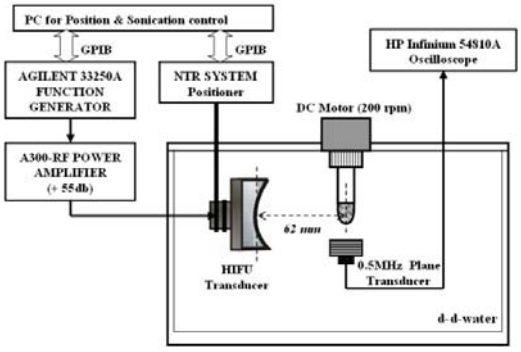

Figure 1. **Block diagram of the experimental setup**

Transmission electron microscope & the sample preparation

To observe the effects of US irrdiation on liposome membranes, such as rupture or leakage, liposomes before and after the sonication were imaged by a transmission electron microscopy (TEM) (JEM 1210, JEOL USA, Inc., Peabody, MA, USA). The liposome samples for TEM were prepared by a negative staining technique. In brief, the carbon coated grids were treated with 1% Alcian Blue in water to make them hydrophobic. 6 - 10 µl of liposome samples were placed on a grid. After 1 minute, seven drops of 1 or 2 % aqueous uranyl acetate were washed slowly over the sample side of the grid. The final drop of uranyl acetate was allowed to stay on the grid for 1 minute. It was then wicked off with a piece of filter paper. Grids were thoroughly air-dried and examined by TEM.

RESULTS AND DISCUSSIONS

All data points in this study are presented by the mean and its standard deviation of 4 measurements (mean ± div) unless specified otherwise. The liposome solution was diluted to 1/100 of its original concentration by the PBS before they were examined by the DLS. The mean diameter of the PC-PEG 2000 liposomes was measured to be 210.4 ± 2.4 nm using DLS. The mean diameter of the liposomes dropped to 179.2 ± 0.97 nm after they were exposed to the cw US for 120 s, as shown in Fig. 2. The 3 panels inserted in Fig. 2 are typical liposome size distributions of the samples that were sham-exposed (0 s), and sonicated for 30s and 120s, respectively. Panel A suggests that the diameters of liposomes synthesized by our method varied in a range from 130 nm to 300 nm. However, all the liposomes with diameters larger than 230 nm were probably ruptured after 30s of sonication since there was no light intensity contributed by them as indicated in panel B. Panel C, compared with panel B, suggests the size of liposomes was further decreased after 120 s' sonication.

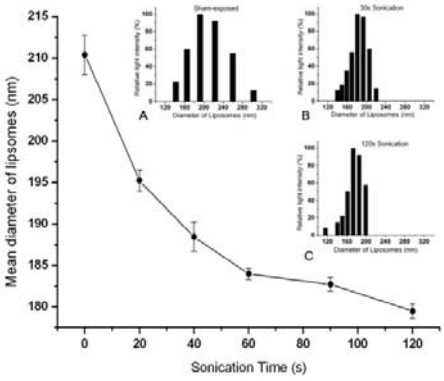

Figure 2. **The decreasing trend of liposome size with various duration of sonication. Panels A, B, and C are the typical liposome size distributions determined by the dynamic light scattering method for 0 s (sham-exposed), 30s and 120s sonicated liposome samples, respectively.**

Figure 3 shows the percentage release efficiency from the liposomes calculated using Eq. 1; all quantities in Eq. 1 were determined by the measurement of fluorescent light intensity described earlier (Eq. 1) after exposed to the US for 10s, 20s, 30s, 40s, 50s and 60s respectively. It can be seen that HIFU ultrasound (1.1 MHz, I_{SPTA}= 900 W/cm^2, cw) was able to release 21.2% ± 4.1% of encapsulated FITC from liposomes of 210 nm mean diameter within 10 s. The FITC release efficiency could reach as high as 70 % after 60s of sonication.

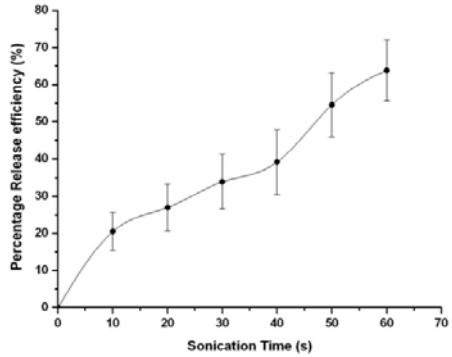

Figure 3. **Percentage FITC release from liposomes triggered by HIFU ultrasound (1.1 MHz, ISPTA= 900 W/cm2, cw) vs time**

In order to understand the physical mechanism of the release induced by HIFU further, a 0.5 MHz nonfocusing 1.2 cm diameter transducer as shown in Fig. 1 was used to detect ½ f_0 = 0.55 MHz (subharmonic) signals. The presence of the subharmonic frequency signal indicates acoustic cavitation may be involved [9]. The Fourier frequency spectra of acoustic pressure signals collected during the sonication process with the d-d water (top panel) and liposome solution (bottom panel) are separately shown in figure 4. Both samples were exposed to cw HIFU at f_0 =1.1 MHz (I_{SPTA}= 900 W/cm^2). As expected, when the test tube was filled with d-d water, no

other signal except the fundamental f_0 was received by the 0.5 MHz transducer. There is no sub-harmonic peak (in the vicinity of 0.55 MHz) in the spectrum shown in the top panel. The signal received by the 0.5 MHz plane transducer when test tube was filled with liposome solution has both fundamental (1.1 MHz) and sub-harmonic (0.55 MHz) components, which are 11 dB and 10 dB above the noise amplitude, respectively.

The structure of liposomes before and after exposure to HIFU was examined by transmission electron microscopy (TEM). Figure 5 (top left and top right) represents some typical liposomes before exposure to HIFU. Intact liposomes had circular shape and most of them had uni-lamellar structures. The liposome membranes were clearly visible as the inner aqueous compartments are slightly darker than the surrounding perimeters. The size of liposomes varied from less than 100 nm to 300 nm with an average diameter of 210 nm measured by the DLS technique. Structures of the liposomes after irradiated for 30 s by US of 1.1 MHz, I_{SPTA} =900 W/cm^2 are shown in Figure 5 bottom left and right panels. It is evident that HIFU induced fractures in liposomes of a wide range of sizes. Some Large liposomes (>100 nm) were broken in half or had significant portions of membranes missing. Some of the small liposomes had pores or openings on their surfaces. The number of smaller liposomes detected by DSL increased after the irradiation. Overall the color contrast between the inner core and the background solution outside of liposomes was reduced for the sonicated liposome solution, suggesting that the FITC leakage was induced from irradiated liposomes, there was probably no FITC left inside those liposomes though they still looked like circular in shape.

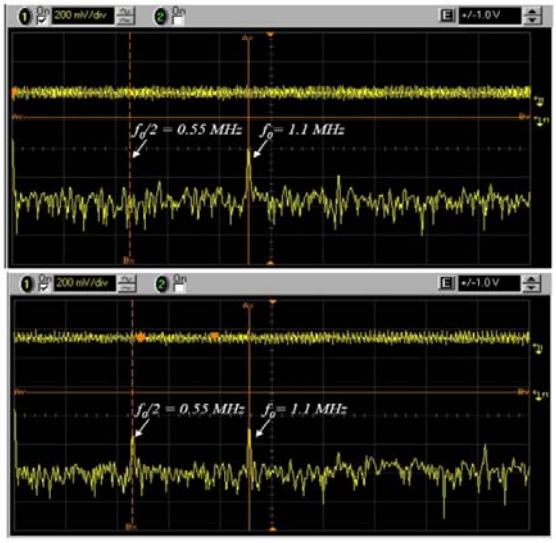

Figure 4. The frequency spectra of the signal captured by the 0.5 MHz plane transducer when sample tubes exposed to HIFU US irradiation (CW, 1.1 MHz, ISPTA= 900 W/cm2). (Top: test tube contained 1 ml distilled and degassed water. Bottom: test tube contained 1 ml liposome' solution)

CONCLUSIONS

This *in vitro* study demonstrated that HIFU is capable of effectively inducing leakage of contents from liposomes with an average diameter of 210 nm in a short period of irradiation. The release kinetics of the encapsulated fluorescent materials (FITC) suggests the content release efficiency up 70 % from liposomes in the suspension can be obtained after 60 s' sonication by US of I_{SPTA} = 900 W/cm^2. The TEM images of liposomes revealed the strong effect of HIFU on the liposome membrane as the main cause of the drug release. The destructive effect of the HIFU to the nanometer size liposomes and the presence of a strong subharmonic spectrum shown in the bottom panel of Fig. 4 suggest inertial cavitation might dominate in the sonication process and liposome might play a role as cavitation nuclei. Figure 5 suggests that fracture of relatively large liposomes (>100nm) and generation of pore-like defects in the membrane of small liposomes might be the predominant mechanisms of encapsulated content released from liposomes.

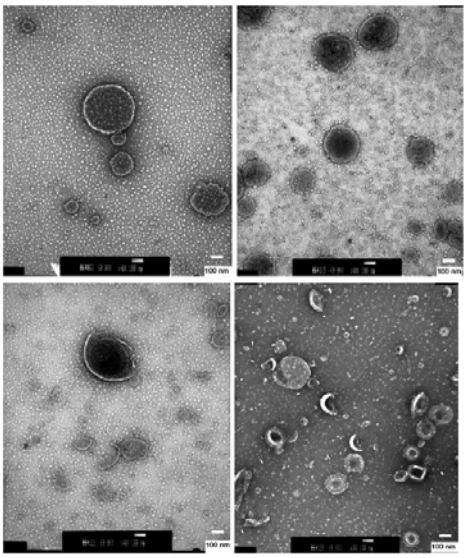

Figure 5. Transmission electron microscopy (TEM) images of liposomes before sonication (top left and right), liposomes after sonication (bottom left and right). US parameters: 1.1 MHz, ISPTA= 900 W/cm2, 30 s, CW. Scale bar = 100 nm.

The potential clinical applications of the targeting drug delivery *via* liposomes using HIFU may integrate with HIFU surgery [18] in treatment of cancers. The I_{SPTA} used here is much smaller for that required to cause tissue necrosis for cancer treatment for cancers [18]. Further *in vitro* and *in vivo* studies to address issues related to the integration of both treatment methods are needed.

REFERENCES

[1] A.D. Bangham,.; R. Flemans, D.H. Heard, R. FLEMANS & G. V. F. SEAMAN. An Apparatus for Microelectrophoresis of Small Particles. Nature 1958, 182, 642-644.

[2] T. Nii, F. Ishii, Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method, Int. J. Pharm. 298 (2005) 198–205.

[3] P.P. Karmali, A. Chaudhuri, Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises, Med. Res. Rev. 27 (2007) 696–722.

[4] S.D. Li, L. Huang, Gene therapy progress and prospects: Non-viral gene therapy by systemic delivery, Gene Ther. 13 (2006) 1313–1319.

[5] J.A. Reddy, P.S. Low. Enhanced folate receptor mediated gene therapy using a novel pH-sensitive lipid formulation. J Control Release 2000; 64:27–37.

[6] M.H. Gaber, K. Hong, S.K. Huang, D. Papahadjopoulos. Thermosensitive sterically stabilized liposomes: formulation and in vitro studies on mechanism of doxorubicin release by bovine serum and human plasma. Pharm Res 1995; 12:1407–1416.

[7] S. Murdan, Electro-responsive drug delivery from hydrogels, J. Control. Release 92 (2003) 1 – 17.

[8] S. Huang, R.C. MacDonald. Acoustically active liposomes for drug encapsulation and ultrasound-triggered release.Biochim Biophys Acta 2004; 1665:134–141.

[9] J. Wu, W. L. Nyborg, Ultrasound, cavitation bubbles and their interaction with cells. Advanced Drug Delivery Reviews, Volume 60, Issue 10, 30 June 2008, Pages 1103-1116

[10] J. Wu, D. Chen, J. Pepe, B. E. Himberg and M. Ricón, Application of liposomes to sonoporation, Ultrasound in Med. & Biol. (2006) 32 429-37.

[11] J.A. Kopechek, T.M. Abruzzo, B. Wang, S.M. Chrzanowski, D.A.B. Smith, P.H. Kee, S. Huang, J.H. Collier, D.D. McPherson and C.K. Holland, Ultrasound-Mediated Release of Hydrophilic and Lipophilic Agents From Echogenic Liposomes. (2008) J Ultrasound Med 27:1597-1606 • 0278-4297

[12] D.Liu, A. Mori, L. Huang. Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. (1992). Biochim Biophys Acta 1104:95–101.

[13] D.C. Litzinger, A.M.J. Buiting, N. Vanrooijen, L. Huang, Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene Glycol)-containing liposomes. Biochimica et Biophysica Acta-Biomembranes (1994)1190:99–107.

[14] H. Lin and James L. Thomas, PEG-Lipids and Oligo(ethylene glycol) Surfactants Enhance the Ultrasonic Permeabilizability of Liposomes. Langmuir, 2003, 19 (4), pp 1098–1105 DOI: 10.1021/la026604t.

[15] K. Beissner, Measurement techniques, in: M.C. Ziskin, P.A. Lewin (Eds.), Ultrasonic Exposimetry, CRC Press, Boca Raton, FL, 1993, pp. 127–142.

[16] J. Wu, Calculation of acoustics radiation force generated by focused beams

using the ray acoustics approach, J. Acoust. Soc. Am. 97 (1995) 2747–2750.

[17] J.B. Bruce and R. Pecora, Dynamic light scattering with applications to chemistry, biology, and physics. Mineola, NY: Dover Publications, Inc. 2000.

[18] ter Haar, G. Acoustic Surgery, Physics Today (2001) 29-34.