Applications of Acoustic Techniques to Targeting Drug Delivery and Dust Removal Relevant to NASA Projects

Di Chen
University of Vermont

Follow this and additional works at: https://scholarworks.uvm.edu/graddis

Recommended Citation
Chen, Di, "Applications of Acoustic Techniques to Targeting Drug Delivery and Dust Removal Relevant to NASA Projects" (2010). Graduate College Dissertations and Theses. 44.
https://scholarworks.uvm.edu/graddis/44
APPLICATIONS OF ACOUSTIC TECHNIQUES TO TARGETING DRUG DELIVERY AND DUST REMOVAL RELEVANT TO NASA PROJECTS

A Dissertation Presented

by

Di Chen

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy
Specializing in Materials Science

October, 2010
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Materials Science.

Dissertation Examination Committee:

Advisor

Junguo Wu, Ph.D.

Jie Yang, Ph.D.

Randall L. Headrick

Randall L. Headrick, Ph.D.

Chairperson

Jianke Yang, Ph.D.

Dean, Graduate College

Domenico Grasso, Ph.D.

Date: September 1st, 2010
ABSTRACT

Sonoporation, enhanced by ultrasound contrast agents has been explored as a promising non-viral technique to achieve gene transfection and targeting drug delivery in recent years. However, the short lifespan of traditional ultrasound contrast agents like Optison® microbubbles under moderate intensity ultrasound exposure limits their application. Liposomes, as drug carriers consisting of curved spherical closed phospholipid bilayer shells, have the following characteristics: 1) The ability to encapsulate and carry hydrophilic or hydrophobic molecules. 2) The biocompatibility with cell membranes. 3) The nanometer size and the relative ease of adding special ligands to their surface to target a specific disease site. 4) The stability in the blood stream. 5) Targeted ultrasound irradiation can induce rupture of liposomes letting the drug encapsulated in them leak out to achieve controlled release of the therapeutic agents at a certain concentration and a delivery rate.

In this thesis, several liposome synthesis methods are presented. Liposomes synthesized in our laboratory were characterized acoustically and optically. Anti rabbit IgG conjugated with Alexafluor 647 was delivered into Jurkat cells in a suspension containing liposomes by 10 % duty cycle ultrasound tonebursts of 2.2 MHz (the in situ spatially averaged and temporally averaged intensity, ISATA = 80W/cm²) with an efficiency of 13 %. It has been experimentally shown that liposomes may be an alternative stable agent to Optison® to cause sonoporation. Furthermore, a type of nanometer-sized liposome (<300nm) was synthesized to explore the feasibility of ultrasound-triggered release from drug encapsulated liposomes. It has been demonstrated encapsulated fluorescence materials (FITC) can be released from liposomes with an average diameter of 210 nm when exposed to high intensity focused ultrasound (HIFU) at 1.142MHz (ISPTA= 900 W/cm²). Rupture of relatively large liposomes (>100nm) and porelike defects in the membrane of small liposomes due to the excitation of HIFU were the main causes of the content release. The great enhancement of HIFU-mediated release in the nanometer-sized liposomes may prove useful for clinical applications.

The presence of fine particles in Martian and lunar soil poses a significant threat to NASA's viable long-term exploration and habitation of either the moon or Mars. It has been experimentally shown that the acoustic levitating radiation force produced by a 13.8 kHz 128 dB sound-level standing wave between a 3 cm-aperture acoustic tweeter and a reflector separated by 9 cm is strong enough to overcome the van der Waals adhesive force between the dust-particles and the reflector-surface. The majority of fine particles (> 2μm diameter) on a reflector surface can be dislodged and removed by a technique combining acoustic levitation and airflow methods. This dust removal technique may be used in space-stations or other enclosures for habitation.
CITATIONS

Material from this dissertation has been published in the following form:


ACKNOWLEDGEMENTS

First, I would like to express my immense gratitude to my advisor, Professor Junru Wu, not only for his tremendous knowledge and expertise in this field, but also for his extraordinary support for my study and life since the first day I came to the University of Vermont. This thesis would not have been possible without his continuous encouragement, patience and guidance throughout my years of study.

I would like to thank Professors Meiyin Wu, Dong Zhang, Ning Gu, Helene Langevin, Jeff Marshall, Darren L. Hitt and Jeff Frolik for their constructive suggestions and sharing of their research equipments and resources in our collaborating projects.

I would like to sincerely thank our technicians Mr. Michael Hamblin and Mr. David Hammond who have made great contributions to our lab facilities with their talented skills and experience. I also extend my sincere thanks to Ms. Marilyn Eldred and Ms. Denise Fontaine, who offered me countless help. I thank all the faculty members of the Physics department for their instruction and advice during my study and research.

Special thanks to my doctoral committee members, Professors Jie Yang, Randy Headrick, Jianke Yang for sharing their knowledge, as well as reading my thesis and serving on my proposal and defense.

Finally, I would like to thank my parents who brought me up with their love; thank my wife, Qin, for her love and selfless dedication to our family; thank my little daughter Mia who has given me great happiness since she was born; thank all my relatives in China for their love and support. Thanks to all my friends: Hua, Huining, Wenbo, Binran,
Shan, Liya, Fred, Bing, Minghao, Qin, Jian, Yanting, Xiaoxi, Tingbo, Bonnie, Changfei, Xiayun, Wenjie, Chong, Jingjun, Zhenwen, Lan and those I might have forgotten to mention. There are also many other people I need to thank, such as my teachers, colleagues and friends all over the world.

In the end, I would like to thank the research grant from the NASA under Cooperative Agreement No. NNX08AZ07A, research grant from Environmental Protection Agency (EPA) via a Contract 06-00326804 and teaching assistantships from the Physics Department.
TABLE OF CONTENTS

CITATIONS .................................................................................................................................................. ii

ACKNOWLEDGEMENTS ............................................................................................................................... iii

LIST OF TABLES ............................................................................................................................................... vii

LIST OF FIGURES .......................................................................................................................................... viii

CHAPTER 1  COMPREHENSIVE LITERATURE REVIEW .............................................................................. 1

1.1 Organization of dissertation and introduction ...................................................................................... 1

1.2 Sonoporation, liposome and targeting drug delivery ............................................................................ 3

1.2.1 Sonoporation, gene transfection and targeting drug delivery using ultrasound ......................... 3

1.2.2 Application of liposomes to sonoporation in targeting drug delivery ........................................ 18

1.2.3 Ultrasound-mediated drug release from nanometer liposomes ............................................ 28

1.3 Dust removal from a surface by acoustic technique .......................................................................... 31

1.3.1 Background of NASA dust mitigation project ........................................................................ 31

1.3.2 Acoustic levitation and its application in dust removal from a surface .................................. 32

CHAPTER 2  APPLICATION OF LIPOSOMES TO SONOPORATION ...................................................... 36

2.1 Introduction .......................................................................................................................................... 38

2.2 Materials and methods ...................................................................................................................... 40

2.2.1 Liposome and its Characterization ............................................................................................ 40

2.2.2 Sonoporation Assisted by Liposomes ..................................................................................... 44

2.3 Results and discussion ..................................................................................................................... 48

CHAPTER 3  AN IN VITRO FEASIBILITY STUDY OF CONTROLLED DRUG REALEASE FROM ENCAPSULATED NANOMETER LIPOSOMES USING HIGH INTENSITY FOCUSED ULTRASOUND .......................................................... 62

3.1 Introduction ......................................................................................................................................... 64

3.2 Materials and methods ..................................................................................................................... 67
LIST OF TABLES

Table 1. 1. Comparison of transfection and viability of PBMC between electroporation and sonoporation. ................................................................. 8

Table 1. 2. Antibody Delivery Efficiency and Cell Viability for Jurkat Lymphocytes..............................................................................................................11

Table 1. 3. Antibody Delivery Efficiency and Cell Viability for PBMC ..........11

Table 1. 4. Liposome and lipid-based products approved for clinical use in the United States Adapted from Lian, J Pharm Sci. Volume 90(6): 667–80. 2001 ... 23
LIST OF FIGURES

Figure 1. 1. Diagram of gene therapy using an adenovirus vector from Roberts et al. 2009 .............................................................................................................................. 6

Figure 1. 2. Scanning electron microscopic images of HL-60 cells. Irradiated with ultrasound in the presence of MC 540 (A to D), untreated intact cells (E), cells irradiated with ultrasound alone (F). Adapted from Tachibana et al.1999.. 8

Figure 1. 3. Pictures taken from a confocal microscope (objective x 60) (Bio Rad, MRC 1024 ES CLSM, Hercules, CA) show that anti mouse IgD conjugated with fluorescein isothyocyanate-dextran (FITC) was delivered into Jurkat lymphocytes. A picture on the left is for a control case (sham-exposure) and that on the right is for a case after sonoporation........................................................... 9

Figure 1. 4. Pictures taken from a confocal microscope (objective x 60) (Bio Rad, MRC 1024 ES CLSM, Hercules, CA) show that anti mouse IgD conjugated with fluorescein isothyocyanate-dextran (FITC) was delivered into PBMC. A picture on the left is for a control case (sham-exposure) and that on the right is for a case after sonoporation.............................................................................................. 9

Figure 1. 5. Destruction of microbubbles by ultrasound resulting in increased membrane permeability by shear stress, temperature rise and activation of reactive oxygen species. Drug delivery by microbubbles by: (a) transient holes induced by shear stress; (b) increase in membrane fluidity; (c) endocytosis of microbubbles; (d) fusion of the microbubble membrane with the cell membrane. Adopted from Dijkmans et al. 2004........................................................................ 12

Figure 1. 6. Morphology of cells exposed to 20-cycle US pulses at 1 MHz, with varied (a), acoustic pressure (P−), (b) US treatment time, and (c) PRF. The white arrows point out some sonoporation pores. Adopted from Qiu et al.2010 ......... 15

Figure 1. 7. The influences of US parameters on sonoporation pore size. (a) The sonoporation pore sizes measured according to SEM images are plotted as a function of P−, with PRF=250 Hz, treatment time=20 s; (b) pore size vs. treatment time, with P−=2.2 MPa, PRF=250 Hz; and (c) the relationship between pore size and PRF, with P−=2.2 MPa, treatment time=20 s. Adopted from Qiu et al.2010 .............................................................. 16

Figure 1. 8. Assessment of DNA transfection efficiency for the cells with (1) naked DNA, no US, (2) naked DNA and UCA, exposed to US, (3) PEI:DNA, no US, (4) PEI:DNA, exposed to US, and (5) PEI:DNA and UCA, exposed to US. US conditions: P−=2.2 MPa, 250-Hz PRF, and 20-s treatment time. Adopted
Figure 1. 9. Schematic representation of the ultrasound technique called sonoporation that will be researched by Philips and GlyGenix Therapeutics for the targeted delivery of genes. Sonoporation involves the use of microbubbles that are co-injected into the bloodstream along with the therapeutic genes. When they arrive at the target organ, the microbubbles are subjected to high-intensity focused ultrasound causing them to rupture. This increases the permeability of the blood vessel wall and cell walls in the underlying tissue and facilitates the local uptake of the therapeutic genes. Adapted from Philips Research Press Release (2009).

Figure 1. 10. Left: A phospholipid with a hydrophilic head and hydrophobic tails. Right: A lipid bilayer. Adapted from http://www.bioteach.ubc.ca/.

Figure 1. 11. A schematic of a liposome. Adapted from Encyclopædia Britannica.

Figure 1. 12. The structure of liposomal delivery systems. (a) Drug-containing liposomes in the absence of a polyethylene glycol (PEG) coating. Hydrophobic drug is present in the liposome membrane and hydrophilic drug is present in the liposome aqueous interior. Protein opsonins are shown absorbing to the ‘naked’ liposome surface. (b) PEG-coated liposome with entrapped drug in the liposome interior. Protein opsonins have significantly less absorption to the liposome surface. Adopted from Allen 1997.

Figure 1. 13. Several mechanisms to attach drugs to microbubbles. a: Incorporation in the bubble; b: incorporation in the bubble membrane; c: attachment to the membrane; d: attachment to a ligand; e: incorporation in multilayer microbubble. Adopted from Dijkmans et al. 2004.

Figure 1. 14. Schematic of Acoustic levitation. Small objects are pushed to the velocity antinode by acoustic pressure.

Figure 1. 15. Typical acoustic effect of a standing wave on a suspension consisting of small Pyrex glass spheres with diameter 100 micron in water.

Figure 2. 1. Illustration and blockdiagram of ultrasound spectroscopy technique used to measure the phase velocity and attenuation of the liposome solution. The measurement was performed immediately after the sample was stirred. The water tank was filled with distilled water and temperature was measured to be 22 °C.

Figure 2. 2. Terason 2000 ultrasound imaging system was used to study the
stability of the liposome solution. The measurement was performed immediately after the sample was stirred. The water tank was filled with distilled water and temperature was measured to be 22 °C. .......................................................... 44

Figure 2. 3. Ultrasound exposure setup for sonoporation experiments. The transducer used was a 2.5 cm diameter planar non-focusing transducer (Model: A 304 S, Panametrics, MA, USA). Acoustic parameters used are: 2.2 MHz, 10 % duty cycle, ISATA = 80 W/cm². The water was filled with distilled water and temperature was measured to be 22 °C. .......................................................... 46

Figure 2. 4. Relative scattered light intensity histogram of the liposome sample ................................................................................................................. 49

Figure 2. 5. Phase velocity and attenuation of the liposome sample. Solid lines are for phase velocity and dotted lines are for attenuation. ................................. 50

Figure 2. 6. (a) Stability test results obtained for the Optison® sample. (b) Stability test results obtained for the liposome sample. (Please read the text for the detail.) .......................................................... 52

Figure 2. 7. Sonoporation result comparison when Optison® and liposome were used.................................................................................................................. 55

Figure 2. 8. Illustration of a passive cavitation detector. The transducer (Model: A 304 S, Panametrics, MA, USA) was mounted in such a way to make sure that ultrasound transmitted by the transducer would reach the sample but not be received directly by the needle hydrophone. The hydrophone could only receive the scattered signal from Optison® spheres or liposomes. The acoustic parameters used were the same as those shown in Fig. 3........................................ 56

Figure 3. 1. Block diagram of the experimental setup........................................ 70

Figure 3. 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........................................ 73

Figure 3. 3. Percentage FITC release from liposomes triggered by HIFU ultrasound (1.1 MHz, ISPTA = 900 W/cm², cw) vs time......................................................... 74

Figure 3. 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)......................................................... 75
Figure 3. 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, $I_{SPTA}= 900 \text{ W/cm}^2$, 30 s, CW. Scale bar = 100 nm.

Figure 4. 1. Illustration of experimental setup (not scaled).

Figure 4. 2. Microphone measurements. Left: Acoustic pressure amplitude between the tweeter without cylindrical waveguide and silicon wafer. Right: Acoustic pressure amplitude is significantly improved by using a cylindrical waveguide coupled to the tweeter.

Figure 4. 3. Photos of four reflector surfaces of 9 cm x 9 cm size made of different materials: silicon wafer, commercial solar panel, synthetic leather and Teflon.

Figure 4. 4. Mars dust simulants lodged on the silicon wafer were removed by airflow after they were levitated in a standing acoustic field. The airflow had no effect on the dust-particles without the acoustic field as shown in image 1.

Figure 4. 5. Typical microscopic images of (A) Mars and (B) lunar dust simulants accompanied with the percent-number size-distribution histograms shown on their right.

Figure 4. 6. Dust-particle removal efficiencies for the four target-materials (silicon wafer, commercial solar panel, synthetic leather and Teflon).

Figure 4. 7. Solar panel voltage output vs time. At $t = 25$ s, the Mar dust simulants were spreaded on the solar panel and output voltage dropped from 3.0 V to 0.4 V. At $t = 90$ s, the tweeter and the airflow were tuned on. As the dust particles were removed, the output voltage gradually recovered to 2.95 V. With the air flow alone, no effect was observed.

Figure B. 1. Left: Constructive Interference; Right: Destructive Interference. Adapted from http://www.chemeurope.com.

Figure B. 2. Typical intensity fluctuations for large and small particles adapted from http://en.wikipedia.org/wiki/Dynamic_light_scattering.

Figure B. 3. Determination of the size information of particles in solution by DLS technique.
CHAPTER 1

COMPREHENSIVE LITERATURE REVIEW

1.1 Organization of dissertation and introduction

This dissertation is composed of five chapters.

In chapter 1, a brief review of the current techniques of viral and non-viral gene transfection and targeting drug delivery is presented. Sonoporation, a promising non-viral technique using ultrasound to achieve gene transfection and targeting drug delivery with spatial and temporal specificity is introduced. Possible mechanisms and in vitro applications of sonoporation in DNA, anticancer drug and antibody deliveries are discussed. The structure and characteristics of liposomes have been reviewed. The motivation of applying liposomes to sonoporation is illustrated. In addition to the biomedical applications of acoustics, concept of acoustic levitation and its potential application to NASA’s dust removal project are presented.

Chapter 2 is a paper that describes an experiment of application of liposomes to sonoporation. A method of liposome production and the characterization of the liposome products are introduced. It has been experimentally shown that liposomes synthesized in our laboratory were quite stable under the sonication and liposomes like Optison® (GE Healthcare, Princeton, NJ, USA) were able to assist ultrasound to deliver antibodies into Jurkart cells. This paper suggests that liposomes may be an alternative stable agent to Optison® to cause sonoporation.
Chapter 3 is a journal paper to explore the feasibility of ultrasound-triggered release from drug encapsulated liposomes. A type of nanometer-sized liposome (<300nm) was synthesized. Liposomes loaded with a fluorescence agent (FITC) were irradiated by high intensity focused ultrasound (HIFU) ultrasound in a well defined manner. The kinetics of release, the size distribution change and physical integrity of the irradiated liposomes were analyzed by using dynamic light scattering technique and transmission electron microscopy (TEM). It has been demonstrated encapsulated fluorescence materials (FITC) can be released from liposomes with an average diameter of 210 nm when exposed to US at 1.142MHz (the spatial peak and temporal average intensity, \( I_{SPTA}= 900 \, \text{W/cm}^2 \)). Rupture of relatively large liposomes (>100nm) and porelike defects in the membrane of small liposomes due to the excitation of HIFU were the main causes of content release. The great enhancement of HIFU-mediated release in the nanometer-sized liposomes may prove useful for clinical applications.

In Chapter 4, a feasibility study to develop a dust removal technique, which may be used in space-stations or other enclosures for NASA’s long-term project (i.e., exploration and habitation of the moon and Mars) is reported. It is shown experimentally that the acoustic levitating radiation force produced by a 13.8 kHz 128 dB sound-level standing wave between a 3 cm-aperture tweeter and a reflector separated by 9 cm is strong enough to overcome the van der Waals adhesive force between the dust-particles and the reflector-surface. Thus the majority of fine particles (> 2 \( \mu \text{m} \) diameter) on a reflector surface can be dislodged and removed by a technique combining acoustic levitation and airflow methods. The removal efficiency deteriorates for particles of less than 2 \( \mu \text{m} \) in
size.

Summary and conclusions are made in the last chapter.

1.2 Sonoporation, liposome and targeting drug delivery

1.2.1 Sonoporation, gene transfection and targeting drug delivery using ultrasound

1.2.1.1 Gene transfection and targeting drug delivery

Targeting drug and DNA delivery technique is potentially an important technologies in modern medicine. An ideal in vivo technique should be able to deliver macromolecules, including pharmaceutical drugs, antibodies or DNA to their specific site of action. For pharmaceutical drug and antibodies, the dosage delivered should be at therapeutically relevant levels. Targeting delivery offers enormous advantages compared with conventional deliveries. For example, since conventional methods lack target specificity, some highly toxic anticancer drugs delivered conventionally may severely damage healthy tissues and organs undesirably. Targeting delivery not only increases drug efficacy but also minimizes any possible side effect of the conventional delivery method.

Transfection, a process of introducing recombinant DNA into eukaryotic cells (eukaryotic cells have chromosomes with nucleosomal structure) and subsequently integrating the DNA into the recipient cell’s chromosomal DNA (Anderson 1992), is the necessary step in all forms of genetic manipulation. Meanwhile, transfection is also a major limiting step because DNA is a large, highly charged molecule that cannot diffuse through the cell membrane effectively. The barriers from the cell membranes, as well as
from nuclear membranes for DNA, and also for antibodies and drugs if their targets are located in the cell nuclei are the major difficulties to targeting gene transfection and drug delivery. Current transfection protocols can be divided into two broad categories—viral and nonviral. The viral transfection techniques, as the name suggests, utilize viruses as vectors such as retroviruses and adenoviruses to achieve the goal as shown in Figure 1.1 (Roberts et al. 2009). In order to replace a dysfunctional abnormal targeted protein, a gene transfer vector such as a disabled adenovirus is modified so that it contains the gene that encodes for this protein. Once the modified vector is delivered to the affected cells, the gene transfer vector then transfers the therapeutic gene to the cell, and ideally the cell’s own machinery turns the therapeutic gene into correct version of the protein, which in essence fixes the dysfunctional cell. However, viral transfection techniques have drawbacks including its lack of spatially targeting nature (no control over where the gene becomes transfected) and possible adverse side effects such as resistance to metabolic degradation and attack by the immune system. It is desirable to develop a controllable nonviral transfection technique to deliver macromolecules, including DNA, drug and other therapeutic agents, safely and efficiently into specific target sites in vivo as well in vitro.

Forms of nonviral transfection are represented by lipofection, electroporation and particle bombardment. Lipofection or liposome transfection is one of the most widely used nonviral methods of transferring genetic material to living cells. Essentially, liposomes or phospholipid vesicles encapsulate the negatively charged DNA and facilitate transfer of the gene through the cell membrane (Gershon et al. 1993)
The application of liposome has shown great potential in gene transfection (Lasic 1993) because of its properties, and biocompatibility with cell membranes and the possible addition of special ligands on their surface. The lipofection method allows high transfection rates with minimal cellular toxicity, but as with the viral vectors, it does not allow control of spatial or temporal specificity of delivery. Electroporation uses high-voltage electrical pulses to make cell membranes transiently permeable permitting cellular uptake of foreign macromolecules (Chang et al. 1990). This method allows some spatial targeting, but requires electrode placement, which can be invasive. Particle bombardment represents yet another way of “injecting” foreign DNA into cells by coupling the gene to projectiles that are made to penetrate the membrane at high speed (Daniell 1993). This method also allows accurate placement of DNA delivery, but appears to be limited to surface applications.

It has been demonstrated (Bao et al. 1997; Greenleaf et al., 1998; Lawrie et al. 2000; Lu et al. 2003; Miller et al. 1999; 2003; Ward et al. 1999; 2000; Wu et al. 2002; Wu et al. 2006a) that ultrasound (US) assisted by encapsulated microbubbles (EMB) could make cell membranes temporarily open and deliver DNA and other macromolecules into cell nuclei and make affected cells transfected. Most EMB used in these applications belongs to ultrasound imaging contrast agents used clinically. The ultrasound technique associated with this process is called sonoporation.
As introduced above, sonoporation is a technique that utilizes ultrasound to enhance the permeability of the cell plasma membrane. With the presence of macromolecules, ultrasonically induced multiple surface pores on a cell (Figure 1.2) were first observed by Tachibana et al. (1999). The rapid process of cell membrane porosity during the sonication may even induce cell killing.

On the other hand, delivery of both DNA and other macromolecules into affected cells under ultrasound excitation has been demonstrated by several investigators (Kim et al. 1996; Bao et al. 1997; Tata et al. 1997; Greeleaf et al. 1998; Mukherjee 2000). After
the identification of cavitation as the probable mechanism behind the increased cell permeability, and the demonstration of further enhancement of transfection efficiency by using cavitation nuclei, such as encapsulated microbubbles (Bao et al. 1997; Greeleaf et al. 1998; Porter et al. 1996; Lawrie et al. 2000), sonoporation has gained considerable researchers’ attention as a promising technique for targeting gene transfer and drug delivery.

There was an experimental study performed to compare cell viability and transfection efficiency between sonoporation and electroporation for cell suspensions (Pepe et al. 2004). The electroporation was performed using a Gene Pulser® II Apparatus (BIO-RAD, Hercules, CA, USA) with optimum voltage of 250 V, and the sonoporation was achieved by using 2 MHz pulsed ultrasound exposure (the spatial average and temporal average intensity, $I_{SATA} = 80 \text{ W/cm}^2$) assisted with Optison®, a type of encapsulated bubbles commercially obtained (GE Healthcare, Princeton, NJ, USA). It was shown that significantly more human peripheral blood mononuclear cells (PBMC) (primary cells) survived after sonoporation (64.8% ± 1.51%) for “PULSE US, GFP (GFP is a green fluorescent protein used as a marker to detect fluorescent cells.)” than electroporation (53.7% ± 1.53%); significantly ($P < 0.015$) more PBMC showed fluorescence (successful transfection) after sonoporation (2.73% ± 0.21%) than electroporation (0.43% ± 0.06%) (Table 1.1). Considering that the transfection efficiency, which may be defined as the number of cells transfected divided by the number of cells surviving the treatment, the transfection efficiency of sonoporation in regard to the initial number of cells is even higher than that of electroporation.
Table 1. Comparison of transfection and viability of PBMC between electroporation and sonoporation.

<table>
<thead>
<tr>
<th></th>
<th>PULSE US, No GFP</th>
<th>PULSE US, GFP</th>
<th>Electrical, GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent (%)</td>
<td>0.13 ± 0.06</td>
<td>2.73 ± 0.21</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>63.0 ± 0.84</td>
<td>64.8 ± 1.51</td>
<td>53.7 ± 1.53</td>
</tr>
</tbody>
</table>

Figure 1. Scanning electron microscopic images of HL-60 cells. Irradiated with ultrasound in the presence of MC 540 (A to D), untreated intact cells (E), cells irradiated with ultrasound alone (F). Adapted from Tachibana et al. 1999
Figure 1. 3. Pictures taken from a confocal microscope (objective x 60) (Bio Rad, MRC 1024 ES CLSM, Hercules, CA) show that anti mouse IgD conjugated with fluorescein isothiocyanate-dextran (FITC) was delivered into Jurkat lymphocytes. A picture on the left is for a control case (sham-exposure) and that on the right is for a case after sonoporation.

Figure 1. 4. Pictures taken from a confocal microscope (objective x 60) (Bio Rad, MRC 1024 ES CLSM, Hercules, CA) show that anti mouse IgD conjugated with fluorescein isothiocyanate-dextran (FITC) was delivered into PBMC. A picture on the left is for a control case (sham-exposure) and that on the right is for a case after sonoporation.
Furthermore, instead of delivery of plasmid DNA into cell nuclei, antibodies such as anti-rabbit IgG conjugated with fluorophore (Alexafluor 647, Molecular Probes, Eugene, Oregon), anti-mouse IgD conjugated with fluorescein isothiocyanate-dextran (FITC, Pharmingen, San Diego, CA) were delivered into the cytoplasm of PBMC and Jurkat lymphocytes in a following study (Wu et al. 2006a). Fluorophore (Alexafluor 647) and FITC were used as markers for detection of the success of the antibody delivery. Pictures taken from a confocal microscope show that anti mouse IgD conjugated with fluorescein isothiocyanate-dextran (FITC) was successfully delivered into Jurkat lymphocytes (Figure 1.3) and PBMC (Figure 1.4) cells. The cell viability and delivery efficiency obtained using a flow cytometer are presented in Table 1.2 and 1.3. It has been shown that the sonoporation with the presence of Optison® dramatically increased the delivery efficiency while maintained excellent cell viability.

Antibodies usually are hydrophobic and large in size; it has been a challenge to send them into the cytoplasm of a cell because of the protection of the cell membrane. For example IgG, the major immunoglobulin in human serum, has a molecular weight of 150,000 Da and another immunoglobulin in the blood serum, IgD, has a molecular weight of 180,000 Da. Antibodies play an essential role in immunity. Antibodies are often used as clinical therapy to block the interaction of soluble molecules to their receptors and prevent the initiation of the signaling pathways triggered by the receptor. Antibodies can also be used to block intracellular pathways when they recognize proteins involved in signaling pathways, transcription factors, and cytoskeleton etc. The successful delivery of antibodies into cells achieved in this study suggests that
sonoporation may be a promising, non-invasive technique in delivering macromolecular antibodies and anticancer drugs into cells.

### Table 1. 2. Antibody Delivery Efficiency and Cell Viability for Jurkat Lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>NO US, Goat Anti Rabbit IgG</th>
<th>US, Goat Anti Rabbit IgG</th>
<th>NO US, Anti Mouse IgD</th>
<th>US, Anti Mouse IgD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Efficiency (%)</strong></td>
<td>1.60 ± 0.00</td>
<td>78.60 ± 3.60</td>
<td>1.33 ± 0.31</td>
<td>34.9 ± 1.80</td>
</tr>
<tr>
<td><strong>Cell Viability (%)</strong></td>
<td>87.2 ± 0.62</td>
<td>81.1 ± 0.44</td>
<td>92.0 ± 0.42</td>
<td>65.7 ± 2.21</td>
</tr>
</tbody>
</table>

### Table 1. 3. Antibody Delivery Efficiency and Cell Viability for PBMC

<table>
<thead>
<tr>
<th></th>
<th>NO US, Goat Anti Rabbit IgG</th>
<th>US, Goat Anti Rabbit IgG</th>
<th>NO US, Anti Mouse IgD</th>
<th>US, Anti Mouse IgD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Efficiency (%)</strong></td>
<td>11.3 ± 0.81</td>
<td>57.5 ± 4.23</td>
<td>1.66 ± 0.06</td>
<td>32.5 ± 4.36</td>
</tr>
<tr>
<td><strong>Cell Viability (%)</strong></td>
<td>81.1 ± 2.32</td>
<td>61.1 ± 4.23</td>
<td>67.9 ± 4.30</td>
<td>61.8 ± 3.89</td>
</tr>
</tbody>
</table>

Acoustically induced transfection via sonoporation in vitro uses ultrasonic waves of megahertz frequency to excite encapsulated microbubbles (EMB) such as Optison® microbubbles mixed with cells in a medium. EMB could oscillate moderately under the ultrasonic agitation (it is called non-inertial or stable cavitation), generating a shear stress on the cell membrane of a nearby cell. Microstreaming (a DC flow) as a result of noninertial cavitation may be established around an oscillating EMB (Gormley and Wu 1998) facilitating the entrance of DNA into a cell (Wu et al. 2002). EMB may also oscillate violently and collapse, experiencing inertial cavitation or transient cavitation (NCRP 2002). In both cases, as shown in Figure 1.5 (a) and (b), cell membranes of those cells in a medium may “open” for short time allowing foreign molecules or DNA to enter the cells (Prentice et al. 2005; Dijkmans et al. 2004).
The use of non-inertial cavitation is much more controllable than the inertial cavitation and generates less non-reparable sonoporated cells. Particularly, in targeting drug and gene delivery in vitro and in vivo experiments, it has been demonstrated that
EMB excited by moderate intensity US can increase permeability of cell membranes allowing gene, therapeutic drugs and antibodies to enter cells (Unger et al. 2001; 2002; Miller 2006; Tachibana & Tachibana 2006; Wu 2006) with the affected cells remaining viable. This process is now called reparable sonoporation. Tran et al. (2007) have performed an in vitro experiment using the ruptured-patch-clamp whole-cell technique. They demonstrated the hyperpolarization of the membrane of a marked cell (mammary breast cancer cell line MDA-MB-231) during sonoporation (1 MHz, 0.15 MPa negative peak US; Sono Vue microbubbles were used). The hyperpolarization of a cell means an above-normal increase in the trans-membrane voltage. They concluded that ultrasound activated oscillations of EMB modify the electro-physiologic cell activities by their “cellular massage” action and thus enhance the cell’s permeability toward macroparticle uptake. “Cellular massage” here may mean the actions on a cell through the moderate shear stress generated by nearby oscillating bubbles.

Another recent study applied the scanning electron microscopy (SEM) to investigate the sonoporation effects on the cell membrane (Qiu et al. 2010). It was found that with the increasing of ultrasound parameters, or inertial cavitation dose (higher acoustic peak negative pressure amplitude (P−), longer treatment time, or faster pulse-repetition-frequency (PRF)), larger sonoporation pores and more rough regions were generated on the cell membranes as shown by SEM images (Figure 1.6). The sonoporation pore sizes measured according to SEM images as a function of acoustic peak negative pressure amplitude are plotted in Figure 1.7. It can be noticed that the sonoporation pore size increases with the increasing of P−, treatment time and PRF. They
also observed that ultrasound mediated DNA transfection efficiency with the presence of ultrasound contrast agents was dramatically enhanced as shown in Figure 1.8. Their results suggested sonoporation pores on the cell membrane could be highly correlated with inertial cavitation dose and inertial cavitation activities accumulated during ultrasound exposure should play an important role in the ultrasound-mediated DNA transfection through sonoporation. Moreover, quantified inertial cavitation could be used as an effective tool to monitor and control the ultrasound-mediated gene or drug delivery effect.

These findings opened tremendous opportunities for application of sonoporation in the field of targeting drug delivery and gene transfer. Conceptually, DNA, pharmaceutical drugs or other macromolecules mixed with ultrasound contrast agents could be injected intravenously, and targeting delivery of drug could be achieved by selective insonation of a predefined area as demonstrated in Figure 1.9. Sonoporation, though it is a relative newcomer in gene transfer and drug delivery, has been a promising modality that combines the capability of enhancing delivery efficiency with the possibility to allow control of spatial and temporal specificity of drug delivery.
Figure 1.6. Morphology of cells exposed to 20-cycle US pulses at 1 MHz, with varied (a), acoustic pressure ($P$), (b) US treatment time, and (c) PRF. The white arrows point out some sonoporation pores. Adapted from Qiu et al. 2010
Figure 1. 7. The influences of US parameters on sonoporation pore size. (a) The sonoporation pore sizes measured according to SEM images are plotted as a function of $P_\text{ac}$, with PRF=250 Hz, treatment time=20 s; (b) pore size vs. treatment time, with $P_\text{ac}=2.2$ MPa, PRF=250 Hz; and (c) the relationship between pore size and PRF, with $P_\text{ac}=2.2$ MPa, treatment time=20 s. Adapted from Qiu et al. 2010
Figure 1. 8. Assessment of DNA transfection efficiency for the cells with (1) naked DNA, no US, (2) naked DNA and UCA, exposed to US, (3) PEI:DNA, no US, (4) PEI:DNA, exposed to US, and (5) PEI:DNA and UCA, exposed to US. US conditions: $P \approx 2.2$ MPa, 250-Hz PRF, and 20-s treatment time. Adapted from Qiu et al. 2010

Figure 1. 9. Schematic representation of the ultrasound technique called sonoporation that will be researched by Philips and GlyGenix Therapeutics for the targeting delivery of genes. Sonoporation involves the use of microbubbles that are co-injected into the bloodstream along with the therapeutic genes. When they arrive at the target organ, the microbubbles are subjected to high-intensity focused ultrasound causing them to rupture. This increases the permeability of the blood vessel wall and cell walls in the underlying tissue and facilitates the local uptake of the therapeutic genes. Adapted from Philips Research Press Release (2009).
1.2.2 Application of liposomes to sonoporation in targeting drug delivery

1.2.2.1 Liposome structure and characteristics.

A liposome or phospholipid vesicle basically is a tiny bubble consisting of an aqueous core enclosed in one or more phospholipid layers. Liposomes were first described in 1965 by Bangham when he discovered that phospholipids in water form closed vesicles (Bangham 1965). They were initially used in the study of cellular membranes, as they are made from neutral phospholipids such as lecithin and cholesterol, the same materials to construct a cell membrane. Such lipids are amphipathic, meaning they have a head group and a tail group (Figure 1.10). The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water. In nature, phospholipids are found in stable membranes composed of a bilayer as shown in Figure 1.10. When placed in an appropriate environment and at specific concentrations, these membrane phospholipids can reassemble themselves into a spherical structure. The sphere is a closed bilayered vesicle as shown in Figure 1.11. The hydrophilic polar groups face the aqueous interior core, while the hydrophobic nonpolar portions are tucked into the interior of the membrane bilayer. This effectively results in two potential compartments to entrap a substance. The hydrophilic aqueous core can trap a hydrophilic substance and the hydrophobic interior of the membrane can trap lipid soluble substances.
Figure 1. 10. Left: A phospholipid with a hydrophilic head and hydrophobic tails. Right: A lipid bilayer. Adapted from http://www.bioteach.ubc.ca/

Figure 1. 11. A schematic of a liposome. Adapted from Encyclopædia Britannica
Liposomes can be manufactured with a diameter ranging from several nanometers to several micrometers. The size of liposomes varies according to their intended use. Liposomes in most current drug delivery applications have a diameter in the submicron range. This range is a compromise between the efficiency of loading, liposome stability and the ability of the liposome to extravasate from the vasculature. For example, large liposomes can be loaded relatively easily but tend to remain in blood vessels.

1.2.2.2 Liposomes as a drug delivery system

Another emerging non-viral delivery technique is to use a liposome as a carrier of DNA or drugs. For an optimal drug-carrier delivery system, the drug should be retained within a stable carrier, the carrier should be “invisible” to the reticuloendothelial system, and there should be a built in or controllable release mechanism for the drug. Ideally, the drug would localize directly and specifically at the intended targets, resulting in excellent therapeutic efficacy, with few or no adverse effects.

Liposomes have been extensively investigated for their promise of being an ideal drug delivery system (Lasic et al. 1995). Conventional liposomes were composed of egg phosphatidylcholine and cholesterol (Lasic et al. 1995). When injected intravenously, plasma opsonins and lipoproteins coat the outer surface of these conventional liposomes. Subsequently, reticuloendothelial cells quickly phagocytize and remove these liposomes from circulation. The widespread enthusiasm for the use of liposomes as drug delivery systems were demonstrated after the discovery of a polyethylene glycol (PEG) derivative as a liposome coating. The new formulation of PEG-coated liposomes greatly extended liposome circulation times. These liposomes have been termed second-generation or
sterically stabilized liposomes because of their ability to evade phagocytosis by the reticuloendothelial system (Lasic et al. 1995). Sterically stabilized liposomes have the ability to remain in circulation up to 100x longer than conventional liposomes (Papahadjopoulos 1991). This is due to the ability of the PEG coating to form a steric barrier by attracting water to the liposome surface (Figure 1.12). The liposome surface becomes hydrophobic, resulting in enhanced stability by inhibiting interactions with plasma proteins such as opsonins and lipoproteins. Thus, PEG coated liposomes have a decreased affinity for the reticuloendothelial system. The ability of PEG coated liposomes to avoid rapid reticuloendothelial system uptake has led to their use in drug delivery systems for sustained drug release and selective delivery of encapsulated substances to specific target sites.

In addition to the success and extensive use of PEGylation for surface modification of liposomes, research development is ongoing using different copolymers, crosslinking or polymerization of lipid bilayers, and molecular manipulation of the liposome surface to alter its behavior after injection. The most important biological consequence of these different surface modification substances and techniques is a significant increase in liposome circulation time and stability through the reticuloendothelial system. Hence, several liposomal formulations have been approved by the FDA as listed in Table 1.4 (Lian et al. 2001).
Figure 1.2. The structure of liposomal delivery systems. (a) Drug-containing liposomes in the absence of a polyethylene glycol (PEG) coating. Hydrophobic drug is present in the liposome membrane and hydrophilic drug is present in the liposome aqueous interior. Protein opsonins are shown absorbing to the ‘naked’ liposome surface. (b) PEG-coated liposome with entrapped drug in the liposome interior. Protein opsonins have significantly less absorption to the liposome surface. Adapted from Allen 1997.
Table 1. 4. Liposome and lipid-based products approved for clinical use in the United States Adapted from Lian, J Pharm Sci. Volume 90(6): 667–80. 2001

<table>
<thead>
<tr>
<th>Product</th>
<th>Drug (lipid:drug ratio)</th>
<th>Lipid formulation</th>
<th>Marketed by</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil™, Caelyx™</td>
<td>Doxorubicin (8:1)</td>
<td>PEG-DSPE:HSPC:Chol (5:56:39)</td>
<td>Alza Corporation (formerly Sequus)</td>
<td>Kaposi sarcoma in AIDS; Refractory refractory ovarian cancer</td>
</tr>
<tr>
<td>DaunoXome™</td>
<td>Daunorubincin citrate (15:1)</td>
<td>DSPC:Chol (2:1)</td>
<td>Gilead Sciences (formerly NeXstar)</td>
<td>Kaposi sarcoma in AIDS</td>
</tr>
<tr>
<td>Ambisome™</td>
<td>Amphotericin B (3.8:0.4)</td>
<td>HSPC:DSPG:Chol (2:0.8:1)</td>
<td>Gilead Sciences (formerly NeXstar)</td>
<td>Serous fungal infections; Cryptococcal meningitis in patients HIV</td>
</tr>
<tr>
<td>Amphotec™</td>
<td>Amphotericin B (1:1)</td>
<td>Cholesteryl sulfate</td>
<td>Alza Corporation (formerly Sequus)</td>
<td>Serious fungal infections</td>
</tr>
<tr>
<td>Abelect™</td>
<td>Amphotericin B (1:1)</td>
<td>DMPC:DMPG (7:3)</td>
<td>Elan Corporation (formerly, The Liposome Company)</td>
<td>Serious fungal infections</td>
</tr>
</tbody>
</table>

The unique structural properties of liposomes also allow different methods to be used to entrap substances or drugs. Depending on the substance used, it will either be trapped while liposomes form spontaneously or the substance can be loaded into preformed liposomes. Substances with intermediate solubility can be loaded into the aqueous interior of existing liposomes by the use of ionic gradients or molecular complexes. It has been shown that water-soluble and water-insoluble materials can be encapsulated together in a liposome (Nii et al. 2005). Water-soluble materials are entrapped in the aqueous core, while water-insoluble and oil-soluble hydrophobic drugs reside within the lipid bilayer. The entrapped drug is protected from metabolism within the liposome interior and cannot be active or metabolized until released. The extended
circulation times of liposome may favorably alter the ultimate rate and pathway of drug metabolism. Several other ways of attachment of drugs to a liposome or a lipid coating microbubble have become available as described by Dijkmans et al. (2004) (Figure 1.13).

Figure 1.13. Several mechanisms to attach drugs to microbubbles. a: Incorporation in the bubble; b: incorporation in the bubble membrane; c: attachment to the membrane; d: attachment to a ligand; e: incorporation in multilayer microbubble. Adapted from Dijkmans et al. 2004.
Another interesting property of liposomes is their natural ability to target cancer cells. The endothelial wall of all healthy human blood vessels are encapsulated by endothelial cells that are bound together by tight junctions. These tight junctions stop any large particle in the bloodstream from leaking out of the vessel. Regions of solid tumor growth, infection and inflammation usually have capillaries with increased permeability (Gerlowski et al. 1986).

Liposomes of certain sizes, typically less than 400nm, encapsulated with drugs can rapidly enter the interstitium of tumors from the blood, but are kept in the bloodstream by the endothelial wall in healthy tissue vasculature. Once in the tumor interstitial space, the liposomes are heterogeneously distributed throughout the tumor by diffusion and/or hydraulic convection (Gerlowski et al. 1986). It has been shown that not only does the PEG coating of liposomes increase circulation times but also allows greater extravasation than conventional liposomes, which enhances the accumulation of drug in diseased tissue by several fold compared to normal tissue.

As for hematogenous tumors or metastatic cells in the blood or lymph where passive targeting cannot be done, liposomes coated with surface ligands would be especially useful (Lasic 1998; Allen 1994a; Lasic et al. 1995). Techniques used to attach the ligand to sterically stabilized liposomes include covalent attachment of antibody to the liposome surface, covalent attachment of antibody to liposome surface by end-functionalized PEG, and noncovalent binding of biotinylated antibody using an avidin linker (Allen 1994a). By using antibodies attached to sterically stabilized liposomes, it has been possible to specifically target cancers, both in vitro and in vivo (Allen 1994b; Ahmad et al. 1992;
Ahmad et al. 1993; Pagnan et al. 2000; Park et al. 2002; Sapra et al. 2003).

1.2.2.3 Sonoporation in drug delivery by using liposomes

As indicated in the previous section, sonoporation might be a promising non-viral modality to fulfill gene transfection and drug delivery with spatial and temporal specificity. Sonoporation process is primarily caused by acoustic cavitation of encapsulated microbubbles, a mainly mechanical process, and can be non-lethal to cells.

Delivery of macromolecules including DNA into cells by using ultrasound assisted by EMB such as Albunex® and Optison® has been demonstrated (Bao et al. 1997; Greenleaf et al. 1998; Miller et al. 1999; Ward et al. 1999; Lawrie et al. 2000; Ward et al. 2000; Wu 2002; Lu et al. 2003; Miller et al. 2003). The Optison® spheres have been successfully used in therapeutic applications in addition to be a contrast agent. With the presence of Optison®, sonoporation effect has also been demonstrated for monolayer cells by a 3.5 MHz diagnostic ultrasound (Miller 2000). As mentioned previously, sonoporation assisted by Optison® is also effective to deliver antibodies and drugs into cells (Wu 2006).

However, one shortcoming of Optison® is its fragility behavior under exposure of 1-2 MHz ultrasound of a modest intensity (Wu et al. 1998). Optison® microspheres can be easily broken under exposure to ultrasound whose amplitude is as low as few hundred kilo-Pascals (Miller et al. 1999).

As discussed previously, liposomes have been studied extensively for their promise of being an ideal drug delivery system. Used as a drug carrier, liposomes have the
following characteristics:

1) The ability to encapsulate, protect and carry hydrophilic or hydrophobic molecules.

2) The biocompatibility with cell membranes and biodegradability.

3) The nanometer size and the relative ease of adding special ligands to their surface to target a specific disease site.

4) The stability in the blood stream.

5) Targeted ultrasound irradiation can induce rupture of liposomes letting drug encapsulated in liposomes leaking out from liposomes, to achieve controlled release of the therapeutic agents at a certain concentration and a delivery rate.

Based on the unique properties of liposomes, we have considered them as a potential alternative to traditional ultrasound contrast agents like Optison® in the sonoporation applications. Our long-term goal is to use liposomes to enclose macromolecules such as drugs, DNA or antibodies and, under the irradiation of ultrasound, enhancing cell membrane permeability transiently allowing liposomes to enter cells. Under appropriate stimuli, the macromolecules can be released in a controllable manner to achieve targeting drug delivery into cells.

In the work presented in Chapter 2, a method to synthesize liposome was introduced. Our liposome products were characterized acoustically and optically by using ultrasonic spectropscopy (Wu 1996) and dynamic light scattering techniques. Anti rabbit IgG conjugated with Alexafluor 647 was delivered into Jurkat cells in suspension using the liposomes composed of phosphatidylcholine, stearylamine, and cholesterol, by 10 % duty
cycle ultrasound tonebursts of 2.2 MHz (the in situ spatial average temporal average = 80W/cm^2) with an efficiency of 13%. It has been experimentally shown that liposomes may be an alternative stable agent to Optison® for delivering macromolecules into cells.

1.2.3 Ultrasound-mediated drug release from nanometer liposomes

Used as non-toxic, biodegradable and non-immunogenic drug delivery vehicles, ideally, liposomes should be able to carry an appropriate drug dose, remain stable in circulation (Bakker-Woudenberg 2002) and then release their drug contents such that the local concentration is high enough to mediate an effective therapeutic effect at the target site. Currently in cancer chemotherapy, liposomes loaded with cytotoxic drugs are used since they can take advantage of the higher permeability of tumor vasculature to extravasate at the desired site of action. The drug loaded liposomes appear to remain at the desired site long enough that the entrapped drug is able to leak from them and effect the destruction of tumor cells.

To deliver a high local dose, it is desirable to release the therapeutic agents of a liposome more rapidly than passive transmembrane diffusion will allow. In the best case, rapid release triggered by a local stimulus could be an effective method of targeting a drug to a desired location. Although a number of methodologies for both drug encapsulation and prolonging the circulation of liposomes have been developed over past 20 years (Kono 2001; Allen et al. 1991), few procedures are available for triggered drug release. Strategies for triggering drug release from liposomes have been proposed, including chemical approaches (Guo et al. 2003), biological approaches (Meers 2001),
and methods based on physical phenomena such as electric fields (Murdan 2003), magnetic fields (Babinova et al. 2002), temperature (Kono et al. 1999), visible light (Anderson et al. 1992; Mueller et al. 2000), pH (Drummond et al. 2000), and ultrasound (Marin et al. 2002). Ultrasound is of special interest in controlled release applications because ultrasound is non-invasive and yet is able to penetrate into the interior of the body. In addition, ultrasound can be focused on targeted sites and moreover has been shown to increase the permeability of blood–tissue barriers and cell membranes (Price et al. 1998).

To enhance the drug delivery efficiency, the size of liposomes also plays a crucial role. It was demonstrated by Liu et al. (1992) 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 into mice (Litzinger 1994); 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 PEG moiety increased their membrane permeability and became more sensitive to ultrasound 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/cm²) (Lin et al. 2003). But whether this enhancement resulted from the membrane destabilization, generation of pores on the membranes, or both by ultrasound 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 ultrasound 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 in the work presented in chapter 3. Our research procedure was to load fluorescent agent (FITC) into liposomes and the FITC-encapsulated liposomes 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).
1.3 Dust removal from a surface by acoustic technique

1.3.1 Background of NASA dust mitigation project

Exploration and habitation of the moon and Mars have become a long-term project for NASA (Calle et al. 2008). The presence of fine particles in Martian and lunar soil poses a significant threat to the success of the project. Since there is no water or other liquids in either environment, the mundane operation of washing away the fine particulates from any surface becomes impractical. The majority of particulates existing on the moon and Mars are silicon dioxides (SiO$_2$). The particle-size range is broad; it can be as large as sub-millimeter and as small as 10 nm (Greeley et al. 1991; Hecht et al. 1999; Marshall et al. 1999). It has been reported that transient dust clouds exists above the lunar surface even the atmosphere (mixture of H, He, Ar, Na and K) pressure is very low on the moon surface (Stern 1999). It was suggested that the possible physical principle may be as follows: The dust particles on the moon may be electrically charged by charged particles from the solar wind as well as UV radiation (Horanyi 1998). Consequently, levitation and migration of dust particles may take place near lunar surface. The solar panels brought to the moon during exploration by the astronauts, from which the electricity would be generated, may be quickly covered by dust particles making the electrical equipment nonoperational. Dust particles can also carry bacterial spores (Lin et al. 2007), as well as organic chemicals, which if transmitted out of the habitation can contaminate the surrounding lunar or Martian environment (Horneck 2003). Furthermore, the fact that dust can penetrate through seals on space suits, hatches and vehicle wheels, etc. may also become troublesome (Ferguson 1998; Galer 2007; Marshall 1999a;
Marshall 1999b). The lunar and Martian particles are also hazardous to health by causing respiratory disease if they are inhaled (Schrunk et al. 1999); removal of dust particles from these surfaces should be important.

Development of robust dust mitigation technology is important for the viable long-term exploration and habitation of either the moon or Mars. Such technology should be effective for dislodging particles of diameter ranging from 0.01-100 μm from a surface of a solar panel or space suits, etc. Existing dust mitigation technology, such as electrostatic precipitators and mechanical filters, either use large concentration of ions or require frequent filter changes or manual cleaning. Moreover, such technologies are designed more for cleaning a flowing gas stream, rather than for the problem of dust mitigation from a surface, such as a solar panel, spacesuit, hatch, heat exchanger tube, etc. Thus, a technique to effectively remove fine dust particles from the surface in such a way that utilizes minimal energy and manpower as well as minimizes the surface abrasion involved in many manual cleaning processes is urgently needed.

1.3.2 Acoustic levitation and its application in dust removal from a surface

Sound is a disturbance or vibration that moves through a medium (air, liquids or solids) in the form of waves. The source of sound is any object that moves or rapidly changes shape which causes the medium around the object to vibrate. Acoustic levitation is a technique using acoustic standing waves that counteracts the pull of gravity and makes an object float in a medium.
Figure 1. 14. Schematic of Acoustic levitation. Small objects are pushed to the velocity antinode by acoustic pressure.

Figure 1. 15. Typical acoustic effect of a standing wave on a suspension consisting of small Pyrex glass spheres with diameter 100 micron in water.
An acoustic levitator has two parts - a transducer or sound source and a reflector which bounces the sound wave back. As shown in Figure 1.14, a one-dimensional incident traveling plane-wave with frequency \( f \) and wavelength \( \lambda \) propagating along \( x \) direction can be represented by \( u(x, t) = u_0 \cos(kx - \omega t) \), where \( k = \frac{2\pi}{\lambda}, \ \omega = 2\pi f, \ u(x, t) \) and \( u_0 \) are instantaneous particle velocity and particle velocity amplitude respectively.

Now when we place a perfect plane reflector at \( x = L \), perpendicular to the wave propagation direction and ignore the attenuation, letting the incident wave be superposed on another traveling wave traveling in the “-x” direction (reflected plane-wave), \( u_0 \cos(kx + \omega t) \), we obtain a standing wave given by \( u = 2u_0 \cos kx \cos \omega t \).

The boundary condition at the reflector (assuming the reflector is rigid) requires: \( kL = (n + \frac{1}{2})\pi \), i.e. \( L = (n + 1/2) \frac{\lambda}{2} \). Particle velocity antinodes (maxima) or pressure nodes are found when \( \cos kx = \pm 1 \), \( x = 0, \lambda/2, \lambda, 3\lambda/2, \ldots \). Likewise, pressure antinodes (maxima) or velocity nodes are found where \( \cos kx = 0 \) at positions \( x = \lambda/4, 3\lambda/4, 5\lambda/4, \ldots \). One of the acoustic pressure antinodes should locate at \( x = L = \frac{\lambda(2n+1)}{4} \), where \( n \) are integers. The spacing between nearest pressure nodes or pressure antinodes is \( \lambda/2 \) (wavelength/2). In the standing wave field, the small particles or small objects will be levitated and driven to acoustic pressure nodes or velocity antinodes by acoustic radiation force. Then the particles trapped within the planes migrate closer together due to the interparticle forces, whereby coagulation and even coalescence may be triggered.
Figure 1.15 shows the typical acoustic effect of a standing wave (670 kHz) on a suspension consisting of small Pyrex glass spheres with diameter 100 micron in water. At the beginning of irradiation, almost instantaneously the spheres are driven towards the acoustic pressure nodes and form a series of bright clusters, whereby the average distance between the particles considerably diminishes. The phenomenon is applicable to all kinds of dispersions. The particles can be gaseous, liquid, solid, or even biological cells. The dispersion fluid can be gaseous or liquid.

The application of acoustic levitation method may provide a feasible surface cleaning technique that would significantly reduce astronaut labor, as well as avoid the surface abrasion involved in many manual cleaning processes.
CHAPTER 2

APPLICATION OF LIPOSOMES TO SONOPORATION

This chapter contains the following publication:

APPLICATION OF LIPOSOMES TO SONOPORATION

Junru Wu, Di Chen, Jason Pepe, Benjamin E. Himberg and Mercedes Ricón*

Department of Physics, * Department of Medicine,
University of Vermont, Burlington, VT 05405

Abstract

A method to prepare liposomes is presented. Liposomes made in our laboratory were characterized acoustically and optically. The phase velocity and attenuation of liposomes in suspension (concentration = $10^9$/mL) were measured ranging from 2 to 14 MHz using ultrasound spectroscopy. Anti rabbit IgG conjugated with Alexafluor 647 was delivered into Jurkat cells in suspension using the liposomes, by 10 % duty cycle ultrasound tonebursts of 2.2 MHz (the in situ spatial average temporal average = 80W/cm²) with an efficiency of 13 %. It has been experimentally shown that liposomes may be an alternative stable agent to Optison® for delivering macromolecules into cells.

Key Words: Ultrasound, Liposomes, Drug delivery, In vitro cells
INTRODUCTION

Liposomes or lipid vesicles are spherical structures composed of curved closed lipid bilayers that encapsulate liquid or gas. Their size ranges from nanometers to microns and the thickness of the membrane is in the order of nanometers. The most obvious features of the liposome that make it attractive to medicine (pharmacology in particular) include its ability to dissolve, protect and carry hydrophilic or hydrophobic molecules, its biocompatibility with cell membranes, its low antigenicity, its nanometer size allowing it entering organs such as the lung, spleen and liver through circulation and the relative ease of adding special ligands to their surface. Current research on the possible uses of liposomes in medicine include therapeutic (Torchilin 1985; Gabizon 1995; Gupta et al. 2005) as well as diagnostic (Alkan-Onyuksel et al. 1996; Huang et al. 2001; Huang et al. 2002) applications.

Optison® (GE Healthcare, Princeton, NJ, USA), an FDA approved ultrasound imaging contrast agent, contains micron-size denatured albumin microspheres filled with octafluoropropane (an inert gas). The microbubble concentration is $5 \times 8 \times 10^{8}/mL$ and the mean measured radius of the microbubbles is 1 to 2.25 $\mu m$, according to the specification provided by the manufacturer. Optison® spheres have been successfully used in therapeutic applications in addition to be a contrast agent. It has been demonstrated (Bao et al. 1997; Greenleaf et al. 1998; Miller et al. 1999; Ward et al. 1999; Lawrie et al. 2000; Ward et al. 2000; Wu 2002; Lu et al. 2003; Miller et al. 2003) that ultrasound assisted by encapsulated microbubbles (EMB) such as Optison® can
transiently enhance the permeability of the cell membranes and deliver macromolecules including DNA into cells. This process is primarily caused by acoustic cavitation of EMB, a mainly mechanical process, and can be non-lethal to cells; this ultrasound technique is now called “sonoporation”. Recent studies have also shown (Wu 2005) that sonoporation assisted by Optison® can also be used as a method to deliver antibodies and drugs into cells.

One shortcoming of Optison® is its fragility behavior under exposure of 1-2 MHz ultrasound (Wu et al. 1998) of a modest intensity. Optison® micro-spheres can be easily broken under exposure to ultrasound whose amplitude is as low as few hundred kilo-pascals (Miller et al. 1999).

It has been reported that liposomes can be stable over periods of days to weeks (Lasic 1993). Furthermore, liposomes that encapsulate non-gaseous materials often do not respond to megahertz ultrasound as vigorously as Optison®; they should be much more stable under ultrasound exposure of therapeutic intensity (Coussios et al., 2004). Our long-term goal is to use liposomes to enclose macromolecules such as drugs, DNA or antibodies and, under the irradiation of ultrasound, enhancing cell membrane permeability transiently allowing liposomes to enter cells. Under chemical stimuli, the macromolecules can be released from liposomes through channels existing in the vesicle membranes to achieve targeting drug delivery into cells (Langer 1998).

Nowadays, various methods for the preparation of liposomes are available; they were reviewed in a technical book written by Lasic (Lasic 1993). The liposomes applied for this research were prepared via a cast-film method using the lipids
Phosphatidylcholine (PC), stearylamine(SA), cholesterol (CH) mixed in the mole percent ratio of 65:5:30. This method was originally developed by Gupta et al. (2005). We have modified the molar percent ratio in preparation to maximize the effect sought by us.

In this paper, we will introduce a method of liposome production and the way our liposome products were characterized acoustically using the ultrasonic spectroscopy (Wu 1996) and acoustic imaging. We will also briefly mention how Liposome size distribution was determined optically using a dynamic light scattering method. Lastly, we will report how liposomes were used to deliver antibodies into Jurkat cells, and compare its delivery efficiency with that of Optison®. Our results are preliminary and further improvement is needed.

MATERIALS AND METHODS

I. Liposome and its Characterization

1. Preparation of Liposomes

Phosphatidylcholine (PC), stearylamine(SA), and cholesterol (CH) were purchased from Sigma Chemicals (Sigma Chemical Co., St. Luis MO). Routine reagents and solvent were of analytical grade.

Component lipids of PC, SA and CH, totaling 350mg, were mixed in the mole percent ratio of 65:5:30 and dissolved in 10ml of chloroform in a round-bottomed flask. The chloroform solvent was removed by evaporation under reduced pressure using a rotary vacuum evaporator (Buchi Rotavapor R-200, BÜCHI Labortechnik AG Meierseggstrasse 40 Postfach CH-9230 Flawil 1 Switzerland). Lipid film was hydrated...
with 35 ml deionized water to yield a concentration of 10 mg lipid/mL by continuous
vortex-mixing of the flask. The rehydrated mixture in a plastic culture tube of 10 mm
diameter and 75 mm length (Kimble, Owens-Illinois, Toledo, OH) was sonicated with 20
kHz ultrasound for 2 minutes with a power of 12W (reading from the system) using the
microprobe of a sonicator (Misonix Sonicator 3000, Misonix, Inc. New Highway
Farmingdale, NY, USA).

2. Sample holder and Sample Preparation

Liposomes or Optison® in phosphate buffered saline (PBS) with a concentration of
about $10^9$/mL and $4.5 \times 10^7$/mL respectively was introduced into a metallic cylindrical
sample-holder with a wall thickness of 3 mm and a diameter of 58 mm. The cylinder uses
two latex membrane (60 μm) acoustic windows (32 mm diameter) centrally located at
both ends of the sample holder (Fig. 1). The membranes were also used to seal the sample
from leakage. Dimensions of the container that can be used to hold sample are 32 mm × 7
mm (diameter x width).

3. Liposome Sample Characterization using Ultrasonic Spectroscopy and the light
scattering

Ultrasonic spectroscopy was described in detail elsewhere (Wu 1996). It provides a
means of determining both attenuation and phase velocity simultaneously over a broad
range of frequency. In brief, a temporally narrow ultrasonic pulse generated by a
broadband polyvinylidene fluoride (PVDF) transducer (Ultrasonic Limited, Fleet,
Hapshire, England, GU13, 9RL) driven by a pulser/reciver (Model PR 5000, Matec
Instrument, Northborough, MA, USA), in a water tank is received by another similar
PVDF transducer, amplified and digitized using a digital HP 5810 A oscilloscope; then, the sample/sample holder is inserted between them and the waveform transmitted through the specimen is also digitized (Fig. 1). A complex spectrum of Fourier Transform is obtained from the waveform with the sample inserted divided by that without the sample inserted to calculate the attenuation and velocity of the sample. Data are corrected for acoustic impedance discontinuities at both membrane of the sample holder and water interfaces. The detail will be discussed in a section below.

Fig. 1. Illustration and block diagram of ultrasound spectroscopy technique used to measure the phase velocity and attenuation of the liposome solution. The measurement was performed immediately after the sample was stirred. The water tank was filled with distilled water and temperature was measured to be 22 °C.
Liposomes size distribution was determined by the autocorrelation function determined by the dynamic light scattering method (Bruce et al. 2000) using a commercial system (BI-200SM goniometer, Brookhaven Instruments Co. Holtsville, NY, USA).

4. Evaluation of the stability of Liposomes

The stabilities of Liposomes and Optison® under exposure of ultrasound were tested by using a portable ultrasonic imaging system (4V2, 5 MHz Array, Model 2000, Terason, Burlington, MA, USA). Liposomes or Optison® suspension was prepared as described above with a concentration $10^9$/mL and $4.5 \times 10^7$/mL respectively. Prepared suspension then was poured into the cylindrical sample-holders that were described in section I, 2. An ultrasound absorbing disk (SOAB, a product was manufactured a long time ago, the manufacturer is unknown) was glued to the back of the sample holder to minimize reflection of the tank wall. The sample holder was placed in the water tank as shown in Fig. 2. Ultrasonic images were captured using the Terason 2000 imaging system. An off-line pixel gray scale analysis of the sample image was then performed.

The same sample/sample holder was taken out of the water tank and exposed to ultrasound (US) for 20 s (exposure parameters will be described in section II, 3). Immediately after US exposure, the sample/sample holder was placed in the water tank again. Ultrasonic images were captured using the Terason 2000 imaging system and an off-line pixel gray scale analysis of the sample image was then performed again.
II. Sonoporation Assisted by Liposomes

1. Cell Preparation

Jurkat lymphocytes, human T-lymphocyte cells, widely used in biological research, were incubated in a humidified 37° C, 5% CO₂ atmosphere inside 75 cm² tissue culture flasks (Corning Incorporated, Corning, NY, USA) within a solution of RPMI (Medium 1640, liquid tissue culture medium containing a nutrient blend of amino acids, vitamins, carbohydrates, organic and inorganic supplements and salts) (GIBCO BRL, Grand Island,
NY, USA) supplemented with 5% fetal bovine serum (FBS).

2. Ultrasound Exposure Preparation

Antibodies or immunoglobulins (Ig) are a group of glycoproteins present in the blood serum and tissue fluids of mammals. One type of antibodies is IgG; most antibodies in serum are in the IgG class. Here we used anti rabbit IgG. Each sample consisted of 1ml of Jurkat cell suspension supplemented with 10μL anti rabbit IgG conjugated with Alexafluor 647 (Molecular Probes, Eugene, Oregon, USA) of a 2mg/mL concentration. Alexafluor 647, a fluorophore, was used as a marker for detection. The molecular weight of IgG is about 150,000 Da. Normally, it is not possible to introduce IgG into cells non-invasively. When fluorescent Jurkat cells were detected by a flow cytometer (see section II. 4 for detail), IgG conjugated with Alexafluor 647 was delivered into the cells.

Each sample was placed in a 12×75mm culture tube (VWR Scientific, West Chester, PA, USA) for ultrasound exposure or sham exposure with addition of either 1) Optison® or 2) liposomes. Optison®’s initial bubble concentration obtained from the commercial vial was 5.0 to 8.0×10⁸/mL according to the specification of the manufacturer. The final bubble concentration was 4.5×10⁷/mL. The initial concentration of liposomes was about 10¹⁰ (Coussios et al. 2004) and the final value was 10⁹/mL.

3. Ultrasound calibration and application

The ultrasonic system used was described elsewhere (Pepe et al. 2004). The transducer used was a 2.5 cm diameter planar non-focusing transducer (Model: A 304 S, Panametrics, Waltham, MA, USA), driven by a Hewlett Packard 3314A function
generator (Hewlett Packard, Palo Alto, CA, USA) and an ENI 3200L RF power amplifier (ENI, Rochester, NY, USA) as shown in Fig. 3. The measured -6dB beam-width \textit{at situ} was 2.5 cm. The center frequency was 2.2 MHz. The transducer was driven intermittently with a 10% duty cycle (on cycles/off cycles = 20/180, pulse repetition frequency = 10 kHz). The test tube was situated at 1 cm distance from the source transducer; the -6 dB beam-width is about 2.5 cm, much greater than the test tube diameter.

![Diagram](image)

\textbf{Fig. 3.} Ultrasound exposure setup for sonoporation experiments. The transducer used was a 2.5 cm diameter planar non-focusing transducer (Model: A 304 S, Panametrics, MA, USA). Acoustic parameters used are: 2.2 MHz, 10 % duty cycle, $I_{\text{SATA}} = 80 \text{ W/cm}^2$. The water was filled with distilled water and temperature was measured to be 22 °C.
Each tube sample was rotated at 200 rpm by a DC motor throughout the exposure period; the rotation helps to mix cells with Optison® bubbles (liposomes) evenly. The test tube sample was lowered into a glass tank filled with distilled water and aligned axially with the ultrasound transducer. The Optison® spheres and cell suspension (hereafter it is called Optison® sample) or the liposomes and cell suspension (hereafter it is called liposome sample) were mixed and then immediately exposed to ultrasound (US). The control samples (liposomes + antibody + cells) were sham-exposed to US.

The *in situ* spatial average temporal average (SATA) intensity for each case after attenuation correction was 80W/cm² and the value of $p / \sqrt{f}$, a quantity related to the mechanical index MI (NCRP, 2002), was calculated to be 1.1, where $p$ in MPa and $f$ in MHz are the rarefaction amplitude and frequency of ultrasound respectively. These parameters were measured by a needle pvdf (0.2 mm diameter) hydrophone (NTR Systems, Seattle, Washington, USA) that was calibrated by measuring the acoustic radiation force generated by the transducer using an electronic balance and scanning a two-dimensional sound field *in situ*. The attenuation of the test tube was found by measuring the short ultrasound pulse amplitude with/without placing the test tube before the hydrophone (note that the ultrasound was attenuated twice using this method and the standing wave effect is small as the reflection coefficient of acoustic pressure of the test tube was measured to be less than 0.1). Since the sample tube was placed in the near field, the nonlinear distortion of the waveform was not serious, and the peak positive and peak negative pressures were found to be equal.

Each sample was exposed to US in the following way: Samples with 100 μL of
either 1) Optison® or 2) liposomes added were exposed to ultrasound for 10 seconds.

Three trials were performed in the same fashion for each of the three cases: liposome sample sham-exposed, liposome sample ultrasound exposed and Optison® sample ultrasound exposed. A t-test statistical model (two-sample assuming unequal variances) provided by a software package SigmaStat® (SPSS Inc. Chicago, USA) was used to compare the delivery efficiency between liposome sample, sham-exposed and liposome sample US exposed and between liposome sample, sham-exposed and Optison® sample US exposed.

4. Evaluation of transfected cells - Flow Cytometer

Immediately after sonoporation experiments, the cells were washed twice, resuspended in 1 ml PBS and tested for viability and fluorescence (it is an indication of successful delivery) by using a flow cytometer (Coulter EPICS ELITE ESP, Beckman Coulter International, Inc., Miami, FL, USA) housed in the University Medical College.

RESULTS AND DISCUSSION

Figure 4 is a histogram of relative scattered light intensity vs liposome diameter. This figure suggests the diameters of liposomes were clustered into three different scale groups. The mean diameter was determined to be 110 nm by the light scattering method. According to the scattering $R^4$ law, where $R$ is the radius of a sphere (Marion et al. 1980), the large diameter liposome should scatter much more light than a small one. Thus, in terms of number density (numbers/volume), actually, number of small diameter liposomes (~ 50 nm) should dominate.
Fig. 4. Relative scattered light intensity histogram of the liposome sample

The phase velocity vs frequency and attenuation vs frequency for the liposome in PBS solution of the number concentration $10^9$ are shown in fig. 5. It is clear the attenuation of liposome sample is two orders of magnitude smaller than that of Optison® of $10^7$/mL concentration (the attenuation of the Optison® sample can be higher than 100 dB/cm) at 5 MHz (Marsh et al. 1997; Wu 1998). The phase velocity did not change significantly with the frequency and it was very close to that of blood (Duck 1990).
Fig. 5. Phase velocity and attenuation of the liposome sample. Solid lines are for phase velocity and dotted lines are for attenuation.

The first row of figure 6a contains 2 images of the Optison® sample/sample holder (from left to right) captured before sonification and after sonification (2.2 MHz and the in situ spatial average temporal average was 80W/cm², the detailed sonication parameter was described in section II, 3) by the Terason 2000 imaging system using a 4V2 (5 MHz) array. The image on the left panel shows a bright white-band located by the top arrow on the left in the figure. The similar but slightly less thick white-band occurred when Optison® spheres were replaced by pure water (not shown); it suggests that the white-band illustrated by the top arrow was generated by the front latex membrane window and Optison® spheres just under the membrane. Since attenuation of the
Optison® spheres of $10^7$/mL concentration can be higher than 100 dB/cm at 5 MHz (Marsh et al. 1997; Wu 1998), a strong shadowing effect has made Optison® spheres under the band, the back latex window of the container and the front SOAB surface, invisible as pointed by the bottom arrow on the left. On the other hand, the back window and the SOAB surface were visible from the right panel after 20 s ultrasound exposure. The 20 s ultrasound exposure made many Optison® spheres rupture and disappear and ultrasound could now reach as far as the front SOAB surface.

The two panels of the second row are enlarged images of the portions (between two arrows) of images of the top row respectively. The two bar figures of the third row show the gray scale histograms of the images of the second row. Each image shown in the second row was spatially uniformly divided into 8 sub-bands; each sub-band had 10 (row)×187 (column) pixels. The gray scale of each pixel was presented in a 8 bit integer. The gray scale of a sub-band shown in a bar figure of the third row was the sum of the gray scale of all pixels in the sub-band. The gray scales of the first three sub-bands of the left image were higher than those corresponding sub-bands of the right image. On the contrary, the gray scales of the last five sub-bands for the right image were higher. These observations were results of disruption of Optison® spheres under ultrasound exposure.
Fig. 6. (a) Stability test results obtained for the Optison® sample. (b) Stability test results obtained for the liposome sample. (Please read the text for the detail.)
Fig. 6. Continued.
Figure 6b is a similar plot when the sample holder was filled with liposomes of $10^9$/mL concentration. The first row has three images; they were captured by the same ultrasound system before, after 20 s and 40 s ultrasound exposure respectively. The common feature of these three images is that they all have three bright white bands. They were generated by the front, back acoustic windows and the front surface of the SOAB. Since the attenuation of liposomes at megahertz frequencies was rather low as shown in fig. 5, there was no shadowing effect observed. The three panels of the second row are enlarged images of the portions (between two arrows) of images of the top row respectively. The three bar figures of the third row show the gray scale histograms of the images of the second row. The first two sub-bands’ gray scales of the middle panel decreased slightly from the left panel’s. The other sub-bands’ gray scales were essentially unchanged. Those bar figures indicate that liposomes were quite stable under the sonification.

Figure 7 is a plot that shows the results of the antibody delivery experiment. Cell viability and fluorescence (it is an indication of successful delivery) for the controls (Sham exposure), Optison® samples and liposome samples detected by a flow cytometer are presented here. Each datum is presented as a mean of three trials of three samples prepared the same way ± standard deviation. The cell viabilities were 93.3 % ± 0.7 %, 89.2 % ± 4.8 %, 88.4 % ± 1.1% for the control, liposome sample and Optison® sample respectively. The antibody delivery efficiencies were 1.4 % ± 0.2 %, 13.1 % ± 2.6 %, and 35.1 % ± 0.4 % for the liposome sample sham-exposed, liposome sample and Optison® sample respectively. The delivery efficiencies of Optison® ($p < 3 \times 10^{-7}$) and liposome ($p$
< 0.008) were significantly higher than that of the sham-exposed when they were individually compared with the data of sham-exposed using the t-test statistical model. The cell viabilities were very close to each other for all three cases (sham-exposed, liposome and Optison®).

![Comparison Between Liposome and Optison Samples](image)

**Fig. 7. Sonoporation result comparison when Optison® and liposome were used.**

Our above preliminary experimental results have shown that liposomes like Optison® have assisted ultrasound to deliver antibodies into Jurkat cells. Although the delivery efficiency of our current technique is lower than that when Optison® was used, the improvement of liposome preparation method and optimizing acoustic parameters may enhance the efficiency in future.
In order to understand the physical mechanism of this technique that uses liposomes, a passive acoustic cavitation detector (Roy et al. 1990) as shown in fig. 8 was set up to detect if cavitation was involved. The ultrasound transducer and acoustic parameters used in fig. 3 were also used as the source transducer and the parameters in this experiment. The sample holder in fig. 8 was mounted horizontally instead of vertically. The reason for
doing that was since Optison® sphere usually floated to the top, thus, a needle pvdf (0.2 mm diameter) hydrophone (NTR Systems, Seattle, Washington, USA) used to detect the scattering signal could be mounted very close to Optison® sphere or liposomes. Thus, the hydrophone with a 30 dB amplifier was sensitive enough to detect the sub-harmonics component (according to the calibration of the hydrophone manufacturer, the sensitivity of the hydrophone at 1 MHz is similar to that of 2 MHz), a signature of acoustic cavitation (Leighton 1994). The transducer was mounted in such a way to make sure that ultrasound transmitted by the transducer would reach the sample but not be received directly by the needle hydrophone. Ultrasound reflected by the latex membrane window could not reach the hydrophone either. The hydrophone could only receive the scattered signal from Optison® spheres or liposomes. As expected, when the sample holder was filled with distilled water, no meaningful signal except noise was received by the hydrophone. The signal received by the hydrophone when the sample holder was filled with Optison® sample or liposome sample had fundamental (2.2 MHz) and sub-harmonic (1.1 MHz) components among other harmonic components. For the fundamental, they were 12 dB and 10 dB above the noise amplitude for Optison® sample and liposome sample respectively. For the sub-harmonic, they were 6 dB and 4 dB above the noise amplitude for Optison® sample and liposome sample respectively. These results seem to suggest acoustic cavitation did occur during sonoporation process with the presence of liposomes and liposomes might serve as cavitation nuclei. Further studies are certainly needed.
ACKNOWLEDGEMENT

We would like to thank Dr. Ludwig Weiman for useful instructions regarding to biochemistry aspect of liposomes and thank Dr. Dan Savin and Miss Kay Gebhardt for their help in the light scattering experiment.

REFERENCES


Gupta Y, Soni V, Chourasia MK, Hain A, Khare P, MPharm K and Jain SK. Targeted drug


Miller DL, Bao S and Morris JE. Sonoporation of cultured cells in the rotating tube


Wu J. Temperature rise generated by ultrasound in the presence of contrast agents.


CHAPTER 3

AN IN VITRO FEASIBILITY STUDY OF CONTROLLED DRUG REALEASE FROM ENCAPSULATED NANOMETER LIPOSOMES USING HIGH INTENSITY FOCUSED ULTRASOUND

This chapter contains the following publication:

An in vitro feasibility study of controlled drug release from encapsulated nanometer liposomes using high intensity focused ultrasound

Di Chen, and Junru Wu

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

Abstract

A liposome with a diameter ranging from 150 to 200 nm has been considered to be one of the optimal vehicles for targeting 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 (ISPTA = 900 W/cm²) for 10 s and the percentage release efficiency can reach to 70% after 60 s’ irradiation. This result also reveals that rupture of relatively large liposomes (>100 nm) and generation of pore-like defects in the membrane of small liposomes (<100 nm) 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.
1. 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

PASC: 43.35, 43.60

Key Words: High intensity focused ultrasound (HIFU), Liposomes, Inertial cavitation,
Targeting drug delivery
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 average temporal average intensity, \( I_{SATA} = 80\text{W/cm}^2 \)), 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 and 2 W/cm\(^2\).

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, \( d \approx 150-200 \text{ nm} \)) 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 into 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 $\text{W/cm}^2$ ) [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).
2. Materials and methods

2.1 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 N₂ using a rotary evaporator to generate a lipid film attached to the inner flask 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 70°C 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 (12×75mm, 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 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 (detailed description will follow in later section). 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.

2.2 Ultrasonic exposure and scanning system

The ultrasonic system was 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 peak pulse average intensity \( I_{\text{SPPA}} \) in water (it is also equal to the spatial peak temporal average \( I_{\text{SPTA}} \) since it was cw) was estimated by using measured acoustic power divided by \( \pi (d_{\text{dB}}/2)^2 \). In this study, \( I_{\text{SPPA}} = I_{\text{SPTA}} = 900 \text{ W/cm}^2 \), 1.1 MHz continuous wave (cw) was used.

\[ \text{Fig. 1. Block diagram of the experimental setup.} \]

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.

2.3 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 photomultiplier 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:

\[
\text{Percentage FITC release efficiency} = \left(1 - \frac{F(t)}{F_c}ight) \times 100\% \quad (1)
\]

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.

2.4 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).

2.5 Transmission electron microscope & the sample preparation

To observe the effects of US irradiation 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). 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.

3. Results and discussion

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 is 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.

Fig. 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.
Fig. 3. Percentage FITC release from liposomes triggered by HIFU ultrasound (1.1 MHz, $I_{SPTA}=900$ W/cm$^2$, cw) vs time.

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.
Fig. 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, $I_{SPTA}= 900 \text{ W/cm}^2$). (Top: test tube contained 1 ml distilled and degassed water. Bottom: test tube contained 1 ml liposome solution)

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 $\frac{1}{2}f_0 = 0.55 \text{ MHz}$ (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) were 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 was 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 had both fundamental (1.1 MHz) and sub-harmonic (0.55 MHz) components, which were 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 were 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.

Fig. 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, $I_{SPTA} = 900$ W/cm$^2$, 30 s, CW. Scale bar = 100 nm.
4. 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 $I_{SPTA} = 900 \text{ W/cm}^2$. 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 liposomes 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.

The potential clinical applications of the targeted drug delivery via liposomes using HIFU may integrate with HIFU surgery [18] in treatment of cancers. The $I_{SPTA}$ used here is lower than that ($I_{SPTA} \sim 1000$ to $4660 \text{ W/cm}^2$) used to cause tissue necrosis for cancer treatment for cancers [19]; it needs to be noted above-mentioned $I_{SPTA}$ was determined from data obtained from a water tank (free-field). Assuming that the same value $I_{SPTA}$ is needed in situ for *in vivo* applications, to get the in situ value of $I_{SPTA}$ equal to $900 \text{ W/cm}^2$, depending on the depth of the target in tissue, the free-field intensity should be much higher. Using the average intensity attenuation coefficient of tissue $\mu = 0.2 \text{ NP/cm}$ [18],
and the in situ intensity $I = I_0 e^{-\mu x}$, here $I$ is 900 W/cm$^2$; assuming the full US passage distance = the focal length of the transducer, i.e., $x = 6.2$ cm, it can be calculated $I_0 = 3110$ W/cm$^2$. If US passes water for a distance before it enters the tissue, thus the tissue part of the US propagation distance $x < 6.2$ cm, $I_0 < 3110$ W/cm$^2$. Further in vivo studies are needed to determine the in situ value of $I_{SPTA}$ required to release the content of liposomes in vivo.

References


CHAPTER 4

DISLODGEMENT AND REMOVAL OF DUST-PARTICLES FROM A SURFACE BY A TECHNIQUE COMBINING ACOUSTIC STANDING WAVE AND AIRFLOW

This chapter contains the following publication:

Dislodgement and Removal of Dust-particles from a Surface by a Technique Combining Acoustic Standing Wave and Airflow

Di Chen and Junru Wu
Physics Department and Materials Science Program
The University of Vermont
Burlington, VT 05405

Abstract

It is known that there are many fine particles on the moon and Mars. Their existence may cause risk for the success of a long-term project for NASA; i.e., exploration and habitation of the moon and Mars. These dust-particles might cover the solar panels making them fail to generate electricity, and they might also penetrate through seals on space suits, hatches and vehicle wheels causing many incidents. The particles would be hazardous to human health if they were inhaled. Development of robust dust mitigation technology is urgently needed for the viable long-term exploration and habitation of either the moon or Mars. We report here a feasibility study to develop a dust removal technique, which may be used in space-stations or other enclosures for habitation. It is shown experimentally that the acoustic levitating radiation force produced by a 13.8 kHz 128 dB sound-level standing wave between a 3 cm-aperture tweeter and a reflector separated by 9 cm is strong enough to overcome the van der Waals adhesive force
between the dust-particles and the reflector-surface. Thus the majority of fine particles (> 2 \(\mu\)m diameter) on a reflector surface can be dislodged and removed by a technique combining acoustic levitation and airflow methods. The removal efficiency deteriorates for particles of less than 2 \(\mu\)m in size.

PAC numbers: 43.25. Gf, 43.25. Uv

I. INTRODUCTION

Exploration and habitation of the moon and Mars have become a long-term project for NASA (Calle et al., 2008). The presence of fine particles in Martian and lunar soil poses a significant threat to the success of the project. Since there is no water or other liquids in either environment, the mundane operation of washing away the fine particulates from any surface becomes impractical. The majority of particulates existing on the moon and Mars are silicon dioxides (SiO\(_2\)). The particle-size range is broad; it can be as large as sub-millimeter and as small as 10 nm. It has been reported that transient dust clouds exists above the lunar surface even the atmosphere (mixture of H, He, Ar, Na and K) pressure is very low on the moon surface (Stern, 1999). It was suggested that the possible physical principle may be as follows: The dust particles on the moon may be electrically charged by charged particles from the solar wind as well as UV radiation (Horanyi, 1998). Consequently, levitation and migration of dust particles may take place near lunar surface. The solar panels brought to the moon during exploration by the astronauts, from which the electricity would be generated, may be quickly covered by dust particles making the electrical equipment nonoperational. Furthermore, the fact that
dust can penetrate through seals on space suits, hatches and vehicle wheels, etc. may also become troublesome. Furthermore, the lunar and Martian particles are hazardous to health by causing respiratory disease if they are inhaled (Schrunk et al., 1999); removal of dust particles from these surfaces should be important.

Development of robust dust mitigation technology is urgently needed for the viable long-term exploration and habitation of either the moon or Mars. Such technology should be effective for dislodging particles of diameter ranging from 0.01-100 μm from a surface of a solar panel or space suits, etc. Existing dust mitigation technology, such as electrostatic precipitators and mechanical filters, either use large concentration of ions or require frequent filter changes or manual cleaning.

It has been reported that lunar or Martian environment is very different from that on Earth. For example, more than 95% of Martian atmosphere is carbon dioxide. Mean surface level pressure is 600 Pa, only 0.6-0.7 % of the Earth atmosphere. The lunar atmosphere is even less significant in comparison with that of Mars and the Earth. Therefore, application of acoustic technique in an open space would be ineffective particularly for lunar applications. However, in a space station or an equivalent enclosure for exploration and habitation, the atmosphere condition should be very similar to that of the Earth. Thus the application of the acoustic method may provide an alternative surface cleaning technique.

This study is designed to investigate the feasibility of using acoustic levitation method in dislodging dust from a dust-covered-surface possibly related to exploration and habitation of the moon and Mars.
II. THEORY

A one-dimensional incident traveling plane-wave with frequency $f$ and wavelength $\lambda$ propagating along $x$ direction (vertical downward as shown in Fig. 1) can be represented by $u(x,t) = u_0 \cos(kx - \omega t)$, where $k = \frac{2\pi}{\lambda}$, $\omega = 2\pi f$, $u(x, t)$ and $u_0$ are instantaneous particle velocity and particle velocity amplitude respectively. Now when we place a perfect plane reflector at $x = L$, perpendicular to the wave propagation direction and ignore the attenuation, letting the incident wave be superposed on another traveling wave traveling in the “-x” direction (reflected plane-wave), $u_0 \cos(kx + \omega t)$, we obtain a standing wave given by $u = 2u_0 \cos kx \cos \omega t$. The boundary condition at the reflector (assuming the reflector is rigid) requires: $kL = (n + \frac{1}{2})\pi$, i.e. $L = (n + 1/2)\lambda/2$.

Particle velocity antinodes (maxima) or pressure nodes are found when $\cos kx = \pm 1$, $x = 0, \lambda/2, \lambda, 3\lambda/2, \ldots$. Likewise, pressure antinodes (maxima) or velocity nodes are found where $\cos kx = 0$ at positions $x = \lambda/4, 3\lambda/4, 5\lambda/4, \ldots$. One of the acoustic pressure antinodes should locate at $x = L = \frac{\lambda(2n+1)}{4}$, where $n$ are integers. The spacing between nearest pressure nodes or pressure antinodes is $\lambda/2$ (wavelength/2).

The second order nonlinear effect of a sound field can produce a force on a particle in a fluid exposed to the sound field. The basis for such forces was expressed by Gor’kov (1962) in an equation for the acoustic radiation force on a spherical particle of volume $v$ in a medium where the time averaged densities of the kinetic energy density $<E_k>$ and potential energy $<E_p>$ are known. Letting $\beta$ be the ratio of the compressibility of the
particle material and the surrounding medium and \( \rho_s \) and \( \rho_o \) are the densities for the particle and the surrounding medium respectively, Gor’kov’s equation, applied to the \( x \) component \( F_x \) of the force, is

\[
F_x = v D \frac{d\Phi}{dx},
\]

(1)

where \( D = 3 (\rho_s - \rho_o) / (2\rho_s + \rho_o) \); \( \Phi \) is called the acoustic force potential and is given by

\[
\Phi = <E_k> - (1 - \beta) <E_p>.
\]

(2)

Here the time-averaged kinetic energy \( <E_k> \) is proportional to the square of the particle velocity amplitude; since the velocity amplitude is just \( 2u_o \cos kx \) in the standing wave field, maxima of \( <E_k> \) occur one-half wavelength apart, i.e., when \( \cos kx \) is equal to \( \pm 1 \). Since the net energy transported in a perfect standing wave is zero, it follows that \( d<E_p>/dx \) is equal and opposite to \( d<E_k>/dx \). The dust particles are denser and less compressible than the surrounding fluid; for these, \( D > 1 \) and \( \beta < 1 \). Hence \( F_x \) is in the direction of increasing \( <E_k> \) or decreasing \( <E_p> \). In other words, theory predicts that when a plane standing sound wave is established in space, the dust-particles will migrate toward planes where acoustic pressure amplitude is minimum, i.e., toward planes separated by a distance of \( \lambda/2 \) (one-half wavelength).

We have used a circular rigid walled waveguide to couple with a sound source, a tweeter. For this type of waveguide of radius \( a \), to satisfy the normal particle velocity being zero at \( r = a \) the acoustic pressures of possible modes may be written as (Kinsler et al. 1982)

\[
p_{ml} = A_{ml} J_m(k_m r) \cos(m\theta)e^{(\omega t-kz)},
\]

(3)

here \( J_m \) is the \( m \)th-order Bessel function,
\[ k_z = \sqrt{\left( \frac{2\pi f}{c} \right)^2 - k_{ml}^2}, \quad (4) \]

and

\[ k_{ml} = j'_{ml} / a, \quad (5) \]

where \( j'_{ml} \) is the \( l \)th root of \( \frac{dJ_m(x)}{dx} = 0 \).

The possible modes which can propagate in the waveguide excited by a sound source with circular symmetry and maximum vibration velocity amplitude at the center may be limited to \( m = 0 \) modes. For \( m = 0 \) and \( n = 1 \), it is a plane wave which can always propagate in the waveguide with a phase velocity \( c = 343 \text{ m/s} \) in air at 20°C. In addition to this plane wave mode, it is possible to have the excitation frequency \( f \) of the acoustic source at \( z = 0 \), satisfying the following condition: \( ck_{02}/2\pi < f < ck_{03}/2\pi \) (Kinsler et al. 1982), the nonplanar (0, 2) mode may also propagate in the waveguide. Its mathematic expression is given by

\[ p_{02} = AJ_0(k_{02}r)e^{i(\omega t-k_zz)}, \quad (6) \]

where \( k_{02} = j'_{02} / a = 3.83 / a \). For our application, \( a = 1.7 \text{ cm}, c = 343 \text{ m/s} \) (Kinsler et al., 1982), \( k_{02} = 3.83 / 1.7 \text{ cm}, \quad k_{03} = 7.02 / 1.7 \text{ cm}, \quad f_{02} = ck_{02}/(2\pi) \approx 12.3 \text{ kHz}, \quad \text{and} \quad f_{03} = ck_{03}/(2\pi) \approx 22.5 \text{ kHz}. \) Since the sound source has the highest transmitting efficiency at \( f = 13.8 \text{ kHz}, \quad f = 13.8 \text{ kHz} \) was chosen for the excitation frequency to maximize the acoustic radiation force, which is between \( f_{02} \) and \( f_{03} \). The (0, 2) mode phase velocity \( c_{p,02} = c/\sqrt{1-(f_{02}/f)^2} = 756 \text{ m/s} \) and its wavelength along the propagation direction
\[ \lambda_{02} = \frac{c_{p,02}}{f} = 5.48 \text{ cm} \] (Kinsler et al., 1982). For the planar wave, \[ \lambda = \frac{c}{f} = 2.48 \text{ cm}. \]

### III. EXPERIMENTAL METHODS AND RESULTS

The main experimental setup (Fig 1.) consists a tweeter as a sound source (KSN-1188A, CTS, Elkhart, IN) coupled with a cylindrical waveguide, a plane reflector, loaded with Mars (JSC Mars-1A, Planet LLC, Madison, WI) or lunar (JSC-1A, Planet LLC, Madison, WI) dust simulants, installed perpendicular to the acoustic axis of the tweeter by keeping \( L = (9 \pm 0.1) \text{ cm} \), and a color CCD camera (STC-H630, SENTECH, Carrollton, Texas) used to monitor the movements of the dust simulants. The sinusoidal electric signal of 13.8 kHz generated by a function generator (HP 3325, Hewlett Packard, Palo Alto, CA) and amplified by an audio power amplifier (Sony STR-K660P amplifier, New York, NY) was applied to the tweeter to establish a standing wave field between the tweeter and reflector. A \( \frac{1}{4}'' \) microphone (model 4133, Brüel & Kjær, Denmark) coupled with a hollow 2mm diameter metallic tube (Fig. 1) and associated preamplifier was used to measure sound pressure amplitude as a function of axial distance from the reflector. The output of the preamplifier was connected to the input of a digital oscilloscope (54641A, Agilent Technology, Palo Alto, CA). Activities of the dust particles on the reflector were monitored through the CCD camera with a lens system (Vivitar Series 1, 70-210mm, Vivitar Co., Edison, NJ). A video capture card (Cx2388x, Harmonics, Newport Beach, CA) was used to capture the video signals which was then converted and stored in a digital format into a computer for further processing.
Fig. 1. Illustration of experimental setup (not scaled).

Fig. 2. Microphone measurements. Left: Acoustic pressure amplitude between the tweeter without cylindrical waveguide and silicon wafer. Right: Acoustic pressure amplitude is significantly improved by using a cylindrical waveguide coupled to the tweeter.
The acoustic pressure amplitude was measured spatially every 0.5cm step upwards from the reflector. The best-fit solid curve for the acoustic pressure amplitudes vs the distance between the reflector and tweeter was obtained using the “Spline” mode of a software (Origin 6.0, OriginLab Corporation, Northampton, MA) as shown in Fig. 2 (left panel); it suggests a standing wave acoustic field was established in space. To further enhance the acoustic field, a cylindrical lucite waveguide (8 cm long, 3.4 cm diameter) was then closely coupled to the tweeter. The axial acoustic pressure amplitude near the reflector surface was measured and was found to be significantly enhanced by about 6 fold or by 15.6 dB magnification as show in Fig. 2 (right panel). Due to the presence of the waveguide, the space limitation only allowed us to measure acoustic pressure amplitude in a small region near the reflector where the waveguide was terminated. We expected the similar enhancement would take place within the waveguide. The spatial averaged acoustic intensity over ¼” diameter cross-section was estimated by using a ¼” sound level meter with the linear mode setting (Model 215, Quest Electronics, Oconomowoc, Wisconsin). The spatial-averaged acoustic intensity generated by the tweeter with the cylindrical waveguide was 128 dB, 8 dB higher than that without the waveguide. The waveguide played a role of concentration of sound energy in space and also altered the wavelength as it left the waveguide and propagated into open-space as described by Eq. 4 and demonstrated by the right panel of Fig. 2. It was observed that the majority of dust particles were levitated in the acoustic standing wave field. It is suggested that the acoustic radiation force was strong enough to overcome gravity of the particle and the adhesive van der Waals force between the particles and reflector surface.
We then turned on the air flow generated by a small fan (diameter 13.5 cm). The dust particles were quickly blown away after they were levitated from the surface. Without the acoustic field, the airflow alone had no effect on the dust particles lodged on the reflector surface due to the strong van der Waals force.

![Silicon wafer](image1.png) ![Commercial solar panel](image2.png) ![Synthetic leather](image3.png) ![Teflon](image4.png)

**Fig. 3. Photos of four reflector surfaces of 9 cm x 9 cm size made of different materials: silicon wafer, commercial solar panel, synthetic leather and Teflon.**

We studied the particle remove efficiency on 4 different reflectors of 9×9 cm² size made of different materials: silicon wafer, commercial solar panel, synthetic leather and Teflon as shown by Fig. 3. All these 4 different reflectors were placed on top of an aluminum square block (10×10 cm²). All parameters including $L$, frequency and acoustic intensity were kept the same for all four materials. Each reflector was exposed to the acoustic excitation and air flow for 90 seconds during the removal operation. Size
distributions of the residual particles after removal on the reflectors were studied by direct counting using a microscope (Nikon LABOPHOT-2, Nikon Instruments Inc., Melville, N.Y.).

Figure 4 contains 6 images taken in sequence when silicon wafer was chosen as the reflector. Arrow represents the air flow direction. Air flow was continuously on for all 6 images were taken and acoustic signal was continuously turned on starting at images 2. There was no particles’ movement at the time when image 1 was taken when only air flow was on. Particles began to be removed as soon as the tweeter was turned on as shown in images 2-6. Mars dust simulants were removed effectively by the air flow after they were levitated by the standing wave acoustic field.

Fig. 4. Mars dust simulants lodged on the silicon wafer were removed by air flow after they were levitated in a standing acoustic field. The airflow had no effect on the dust-particles without the acoustic field as shown in image 1. (Color on line)
Fig. 5. Typical microscopic images of (A) Mars and (B) lunar dust simulants accompanied with the percent-number size-distribution histograms shown on their right.

Figure 5(a) and 5(b) are typical microscopy images of Mars and lunar dust simulants accompanied with the percent-number size-distribution histograms shown on the right. The histograms of Mars and lunar dust simulants indicate that they both have a large component of particles with diameter less than 6μm, specially in the 2-4μm range. The
percent-number of Mars dust stimulants for > 4μm is about 25 % and that for 2μm < particle-size < 4μm is about 45 %. The lunar dust simulant has a similar percent-number (12% vs 14%) as the Mars dust simulant in the size range >4μm, a bit lower percent-number (40% vs 45%) in the size range 2μm - 4μm, and it has more particles in the range < 2μm (38%) than Martian dust simulant has (29%). For further experimental tests, we decided that only Martian dust simulants would be used.

The dust-particle removal efficiencies for the four target-reflectors are illustrated by figure 6. There are 3 panels for each of Fig. 6(a)-6(d); the first two are photos of a reflector covered by Mars dust simulant taken under the microscope before and after the dust-simulants removal operation by air flow and acoustic standing wave, the third panel on the right is the size distribution histogram of the particles residual on the reflector after the removal procedure. Few particles larger than 4μm were left (from more than 25% before the operation to less than 9% after the operation) on the silicon wafer, solar panel and synthetic leather after the removing operation as shown in Fig. 6(a)-6(c).

Particle-number in the range of 2-4μm was also dramatically reduced from 46% to around 20% on silicon wafer and 15% on the solar-panel and leather surfaces. Small particles whose size was less than 2μm dominated in the residuals. When Teflon was used as the target-material, the particle-removal efficiency decreased significantly as shown in Fig. 6(d). However, we were still able to see Mars dust simulants bouncing around on the Teflon material under the excitation of acoustic standing wave. But due to the loose nature and surface roughness of the fabric texture a lot of large and small particles were trapped in the texture.
Fig. 6. Dust-particle removal efficiencies for the four target-materials (silicon wafer, commercial solar panel, synthetic leather and Teflon). (Color on line)
Fig. 7. Solar panel voltage output vs time. At $t = 25$ s, the Mars dust simulants were spreaded on the solar panel and output voltage dropped from 3.0 V to 0.4 V. At $t = 90$ s, the tweeter and the airflow were tuned on. As the dust particles were removed, the output voltage gradually recovered to 2.95 V. With the air flow alone, no effect was observed.

Figure 7 shows the solar panel output voltage measured by a Digital Multi-meter (Model 2000, Keithley Instruments, Inc., Cleveland, Ohio) as function of time. It reflects the voltage output changed with time as the Mars dust simulants were sprayed on the surface and gradually removed from it by the combination of acoustic standing wave effect and airflow method. There was a significant drop in solar panel output voltage (below 15% of its initial voltage) due to the deposition and coverage of Mars dust simulants. The output voltage remained constant in time until the air flow and acoustic
field were applied at $t = 90$ s. The output-voltage of the solar panel restored quickly to around 65% in the first 20 seconds, then increased gradually up to 98.4% after 4 minutes and no further improvement could be observed.

**IV. SUMMARY AND DISCUSSION**

Our feasibility study has shown that the majority particles of the dust simulants can be levitated from a surface by the radiation force due to a standing-wave acoustic field. It has been observed that significant reduction of Mars dust simulants lodged on the target surfaces of silicon wafer, solar panel and synthetic leather few minutes after using the acoustic technique incorporated with air flow. It has also been observed that the efficiency becomes lower for submicron size particles’ removal from a target. As shown in our previous work (Wu and Du, 1990), acoustic radiation force is proportional to the $a^3$ ($a$ is particle radius). For smaller particles, the acoustic radiation force becomes too weak compared to the strong adhesive van der Waals force between the particles and the target-surface.

Equation 1 suggests that the radiation force is proportional to the gradient of acoustic force potential $\Phi$. In order to increase the radiation force, it is advantageous to increase the frequency or decrease the wavelength. On the other hand, the acoustic attenuation increases with frequency. It was found experimentally the sound source (tweeter) has highest transmitting efficiency near 13.8 kHz. Thus, $f = 13.8$ kHz was chosen for the excitation frequency. Another advantage for the higher frequency (> 10 kHz) is that human ears are less sensitive above 10 kHz, therefore high amplitude ten
kHz sound wave would cause less annoyance than the same sound level of kHz sound waves.

As discussed earlier, the possible modes propagating in the waveguide which can be excited by a sound source with circular symmetry and maximum vibration velocity at the center may be limited to \( m = 0 \) modes. Since experimentally we found the sound source has the maximum transmitting efficiency at frequency of 13.8 kHz, we chose \( f = 13.8 \text{ kHz} \) for the excitation frequency, which is between \( f_{02} \) and \( f_{03} \). There should be a wave that is superposition of a planar \((0, 1)\) wave and a nonplanar \((0, 2)\) wave propagating in the waveguide. If it were a pure planar wave, \( \frac{\lambda}{2} = \frac{c}{2f} = \frac{34300}{(2 \times 13800)} = 1.24\text{ cm} \). For \((0, 2)\) mode, \( \lambda_{02} = \frac{c_{p,02}}{(2f)} = 2.74 \text{ cm} \). Experimentally, the location of the reflector \((L = 9 \text{ cm})\) was determined by maximizing levitation effect (maximizing the acoustic radiation force) during the experiment. According to the planar waveguide theory described earlier, the distance between the source and the pressure antinode \( L \) is given by \( L = \frac{\lambda(2n+1)}{4} \). Thus \( \frac{\lambda}{2} = \frac{L}{(n+1/2)} = 9 \text{ cm}/(7.5) = 1.20 \text{ cm} \) when \( n = 7 \). The fact that the error incurred for the measured wavelength compared with the pure planar wave being quite small, equal to \( (1.24-1.20)/1.24 \approx 3\% \), implies that the reflector’s location by maximizing the levitation force was nearly at one of the pressure antinodes of a standing wave generated by superposition of the pure planar wave and its reflection. It suggests that in the waveguide of our experiment, the planar wave may dominate. Strictly speaking, the velocity amplitude being \( 2u_o \cos kx \) in a standing wave field from Gor’kov’s equation was based on a plane wave assumption. Theoretical modification might be needed to the current
experimental situation since the wave was no longer a pure planar wave. On the other hand, as suggested by above argument, the planar wave might still dominate in our experiment, so it might still approximately be considered a planar wave.

As stated earlier, this study was performed under the Earth atmosphere condition. The technique may be limited to be applied in a space station or other enclosures for habitation. The condition on the moon and Mars are quite different. In order to use it in an open space, a gas tank is needed to be bought there and the tweeter-waveguide-reflector system needs to be sealed.

ACKNOWLEDGEMENTS

This work has been partially supported by NASA under cooperative agreement number NNX08AZ07A. Sincerely thanks to a reviewer for his careful review and constructive suggestions.
REFERENCES

Calle, C. I., McFall, J. L., Buhler, C. R., Snyder, S. J., Arens, E. E., Chen, A., Ritz, M. L.,
electrostatic and dielectrophoretic forces with applications to NASA exploration
missions,” Proc. ESA Annual Meeting on Electrostatics, Minneapolis, Minnesota,
USA 1-14.

Sciences XXIX, 1527.

ideal fluid,” Sov Phys Dokl 6, 773-775.


Elsevier Scientific Publishing Co. Amsterdam.

Schrunk, D. G., Sharpe, B. L., Cooper, B. L., Thangavelu, M., (1999) “The moon:
resources, future, development and colonization,” Praxis Publishing Ltd. Chichester.

CHAPTER 5

CONCLUSIONS

The purpose of the research described in this dissertation was to satisfy the following research objectives: (1) to study the behavior of liposome in comparison with an ultrasound contrast agent (Optison®) in the sonoporation process; (2) to investigate the feasibility of inducing drug release from nanometer-sized drug encapsulated liposomes by ultrasound; (3) to investigate the feasibility of using acoustic levitation method to remove fine dust particles from a dust-covered-surface with minimal surface abrasion. As described in chapter 2, 3 and 4, these investigations have been carried out and the conclusions are summarized below.

Firstly, our preliminary experimental results have shown that liposomes synthesized in our laboratory were quite stable under the sonication; liposomes can sustain 40 s of ultrasound exposure (2.2 MHz and the in situ spatially averaged and temporally averaged intensity \( I_{SATA} \) was 80W/cm\(^2\)), while many Optison® spheres would rupture and disappear under 20s of the same ultrasound exposure. It has also been experimentally shown that liposomes like Optison® were able to assist ultrasound to deliver antibodies into Jurkat cells. The sub-harmonic component of the scattering signal from the liposome samples during the sonication suggests acoustic cavitation did occur in sonoporation process with the presence of liposomes and liposomes in this case might serve as cavitation nuclei. Although the antibody delivery efficiency by using liposomes is lower than that when
Optison® spheres were used, the improvement of liposome preparation method and optimizing acoustic parameters may enhance the efficiency in future. Further studies on the delivery mechanisms are certainly needed as well.

Secondly, a method to synthesize nanometer-sized liposome (<300nm) is introduced. Our *in vitro* study demonstrated that high intensity focused ultrasound (HIFU) is capable of effectively inducing leakage of contents from this type of nanometer-sized 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 $I_{SPTA} = 900 \text{ W/cm}^2$. The transmission electron microscopy (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-sized liposomes and the presence of a strong subharmonic spectrum suggest inertial cavitation might dominate in the sonication process and liposomes might play a role as cavitation nuclei. The 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.

The potential clinical applications of the targeting drug delivery via liposomes using HIFU may integrate with HIFU surgery in treatment of cancers. The $I_{SPTA}$ used here is lower than that ($I_{SPTA} \sim 1000$ to 4660 W/cm$^2$) used to cause tissue necrosis for cancer treatment for cancers. But the above-mentioned $I_{SPTA}$ in this study was determined from data obtained from a water tank (free-field). To get the in situ value of $I_{SPTA}$ equal to 900
W/cm², depending on the depth of the target in tissue, the free-field intensity should be much higher. Further in vivo studies are certainly needed to determine the in situ value of ISPTA required to release the content of liposomes in vivo. The great enhancement of HIFU-mediated release in the nanometer-sized liposomes may prove useful for preclinical and clinical applications.

Thirdly, it has been experimentally shown that the acoustic levitating radiation force produced by a 13.8 kHz 128 dB sound-level standing wave between a 3 cm-aperture tweeter and a reflector separated by 9 cm is strong enough to overcome the van der Waals adhesive force between the dust-particles and the reflector-surface. Significant reduction of Mars dust simulants lodged on the target surfaces of silicon wafer, solar panel and synthetic leather few minutes after using the acoustic technique incorporated with air flow was observed. The removal efficiency deteriorates for particles of less than 2μm in size, because acoustic radiation force is proportional to the \( a^3 \) (\( a \) is particle radius), for smaller particles, the acoustic radiation force becomes too weak compared to the strong adhesive van der Waals force between the particles and the target-surface. This study was performed under the Earth atmosphere condition. The condition on the moon and Mars are quite different, thus, the technique may be limited to be applied in a space-stations or other enclosures for habitation.


Wu, J. (2002) Theoretical study on shear stress generated by microstreaming surrounding...


APPENDIX A

LIPOSOME SYNTHESIS

A.1 Routine liposome

Phosphatidylcholine (PC), stearylamine (SA), and cholesterol (CH) were purchased from Sigma Chemicals (Sigma Chemical Co., St. Luis MO). Routine reagents and solvent were of analytical grade.

Component lipids of PC, SA and CH, totaling 350mg, were mixed in the mole percent ratio of 65:5:30 and dissolved in 10ml of chloroform in a round-bottomed flask. The chloroform solvent was removed by evaporation under reduced pressure using a rotary vacuum evaporator (Buchi Rotavapor R-200, BÜCHI Labortechnik AG Meierseggstrasse 40 Postfach CH-9230 Flawil 1 Switzerland). Lipid film was hydrated with 35 ml deionized water to yield a concentration of 10 mg lipid/mL by continuous vortex-mixing of the flask. The rehydrated mixture in a plastic culture tube of 10 mm diameter and 75 mm length (Kimble, Owens-Illinois, Toledo, OH) was sonicated with 20 kHz ultrasound for 2 minutes with a power of 12W (reading from the system) using the microprobe of a sonicator (Misonix Sonicator 3000, Misonix, Inc. New Highway Farmingdale, NY, USA). After this process the lipid suspension formed a milky solution of liposomes.

A.2 FITC-encapsulated liposome

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 N₂ using a rotary evaporator to generate a lipid film attached to the inner flask 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 70°C 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 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. 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.
Dynamic light scattering (DLS) is also known as Quasi-Elastic Light Scattering (QELS) or Photo Correlation Spectroscopy (PCS). Its fundamental physical principle is to measure the intensity of light scattered by the molecules/microscopic particles in the sample as a function of time. When light generated by a laser is scattered by a molecule or particle some of the incident light is scattered. If the molecule was stationary then the amount of light scattered would be a constant. However, since all molecules in solution diffuse with Brownian motion in relation to the detector there will be interference (constructive or destructive) which causes a change in light intensity (Figure B. 1). By measuring light intensity fluctuations as a function of time, DLS can provide information regarding the average size, size distribution, and polydispersity of molecules and particles in solution.

Figure B. 1. Left: Constructive Interference; Right: Destructive Interference. Adapted from http://www.chemeurope.com.

Normally DLS is concerned with measurement of particles suspended within a
Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them. The larger the particle, the slower the Brownian motion will be. An accurately known temperature is necessary for DLS because knowledge of the viscosity is required (because the viscosity of a liquid is related to its temperature). The temperature also needs to be stable, otherwise convection currents in the sample will cause non-random movements that will ruin the correct interpretation of size. The velocity of the Brownian motion is defined by a property known as the translational diffusion coefficient \( D \). The size of a particle is calculated from the translational diffusion coefficient by using the Stokes-Einstein equation;

\[
d(H) = \frac{kT}{3\pi \eta D}
\]

where:
- \( d(H) \) = hydrodynamic diameter
- \( D \) = translational diffusion coefficient
- \( k \) = Boltzmann’s constant
- \( T \) = absolute temperature
- \( \eta \) = viscosity

Note that the diameter that is measured in DLS is a value that refers to how a particle diffuses within a fluid so it is referred to as a hydrodynamic diameter. The diameter that is obtained by this technique is the diameter of a sphere that has the same translational diffusion coefficient as the particle. The translational diffusion coefficient will depend not only on the size of the particle, but also on any surface structure, as well as the concentration and type of ions in the medium.

If the particles are small compared to the wavelength of the laser used, then the
scattering from a particle illuminated by a vertically polarized laser will be essentially isotropic, i.e. equal in all directions. The Rayleigh approximation tells us that $I \propto d^6$ and also that $I \propto 1/\lambda^4$, where $I$ = intensity of light scattered, $d$ = particle diameter and $\lambda$ = laser wavelength. The $d^6$ term tells us that a 50nm particle will scatter $10^6$ or one million times as much light as a 5nm particle. Hence there is a danger that the light from the larger particles will swamp the scattered light from the smaller ones. This $d^6$ factor also means it is difficult with DLS to measure, say, a mixture of 1000nm and 10nm particles because the contribution to the total light scattered by the small particles will be extremely small. The inverse relationship to $\lambda^4$ means that a higher scattering intensity is obtained as the wavelength of the laser used decreases.

For a system of particles undergoing Brownian motion, a speckle pattern is observed where the position of each speckle is seen to be in constant motion. This is because the phase addition from the moving particles is constantly evolving and forming new patterns. The rate at which these intensity fluctuations occur will depend on the size of the particles. Figure B. 2 schematically illustrates typical intensity fluctuations arising from a dispersion of large particles and a dispersion of small particles. The small particles cause the intensity to fluctuate more rapidly than the large ones.
It has been seen that particles in a dispersion are in a constant, random Brownian motion and that this causes the intensity of scattered light to fluctuate as a function of time. The correlator used in a DLS instrument will construct the correlation function $G(\tau)$ of the scattered intensity:

$$G(\tau) = \langle I(t) \cdot I(t + \tau) \rangle$$

where $\tau$ is the time difference (the sample time). Basically a correlator is a signal comparator. It is designed to measure the degree of similarity between two signals, or one signal with itself at varying time intervals. For a large number of monodisperse particles in Brownian motion, the correlation function ($G(\tau)$) is an exponential decaying function of the correlator time delay $\tau$.
\[
G(\tau) = A[1 + B \exp(-2Dq^2\tau)]
\]

where \(A\) is the baseline of the correlation function, \(B\) is the intercept of the correlation function, \(D\) is the diffusion coefficient, \(q = (4\pi n / \lambda_0)\sin(\theta/2)\), where \(n\) is refractive index of dispersant, \(\lambda_0\) is wavelength of the laser, \(\theta\) is the scattering angle. For polydisperse samples, the equation can be written as:

\[
G(\tau) = A[1 + BG_s(\tau)^2]
\]

where \(G_s(\tau)\) is the sum of all the exponential decays contained in the correlation function. Once the correlation function \(G(\tau)\) has been plotted (normally on a log scale time base), then the hydrodynamic diameter can be determined by the Stokes-Einstein equation

\[
d(H) = kT/(3\pi \eta D).
\]

The size information can be obtained from the correlation function by using two approaches: (1) fit a single exponential to the correlation function to obtain the mean size (z-average diameter) and an estimate of the width of the distribution (polydispersity index), (2) fit a multiple exponential to the correlation function to obtain the distribution of particle sizes. The size distribution obtained is a plot of the relative intensity of light scattered by particles in various size classes and is therefore known as an intensity size distribution.

The process to determine the hydrodynamic diameter and size distribution of particles in a solution by using DLS technique is shown in a flowchart (Figure B.3). The theoretical limit of this technique is that species of less than 40% difference in size cannot be resolved. However, in practice a factor of 2-3x can be required. If full resolution is
necessary the sample must be physically separated by centrifugation, filtration, HPLC or field flow fractionation techniques.

Figure B. 3. Determination of the size information of particles in solution by DLS technique