Development of a gene delivery system using nano/microbubbles and ultrasound and the application to regional cancer therapy

<table>
<thead>
<tr>
<th>著者</th>
<th>堀江 佐知子</th>
</tr>
</thead>
<tbody>
<tr>
<td>学位授与機関</td>
<td>东北大学</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10097/53922">http://hdl.handle.net/10097/53922</a></td>
</tr>
</tbody>
</table>
Doctor Thesis

Development of a Gene Delivery System Using Nano/microbubbles and Ultrasound and the Application to Regional Cancer Therapy

Sachiko Horie

February 2012
Development of a Gene Delivery System Using Nano/microbubbles and Ultrasound and the Application to Regional Cancer Therapy

Sachiko Horie
(Doctoral Program in Biomedical Engineering)

Submitted to the Graduate School of Biomedical Engineering in Partial Fulfillment of the Requirements for the Degree of Philosophy in Biomedical Engineering

at the
Tohoku University
DEVELOPMENT OF A GENE DELIVERY SYSTEM USING NANO/MICROBUBBLES AND ULTRASOUND AND THE APPLICATION TO REGIONAL CANCER THERAPY
Contents

Abstract ..................................................................................................................v
Acknowledgments .................................................................................................ix

Chapter 1 Introduction .........................................................................................1
  1.1. Cancer ...........................................................................................................2
    1.1.1. What is cancer? .........................................................................................2
    1.1.2. Statistics of cancer ....................................................................................2
    1.1.3. Diagnosis and treatment in clinical settings ..............................................3
    1.1.4. Drug and gene delivery in cancer research ..............................................5
    1.1.5. Intracellular gene delivery in cancer research .........................................7
    1.1.6. Gene delivery system using nano/microbubbles and ultrasound ............8
    1.1.7. Regional gene delivery using NMBs and US .........................................9
  1.2. Thesis objectives ............................................................................................10
  1.3 Introduction to NMBs and US ......................................................................11
    1.3.1. NMBs .....................................................................................................11
    1.3.2. Dynamics of NMBs to US ......................................................................11
    1.3.3. History of NMBs as US contrast agents ...............................................14
  1.4. Application of NMBs and US in medicine ..................................................17
    1.4.1. Combination of NMBs and US as a diagnostic tool ..............................17
    1.4.2. Combination of NMBs and US as a drug delivery tool ..........................21
Chapter 2 Therapeutic gene delivery into solid tumor using nano/microbubbles and ultrasound

2.1. Summary

2.2. Introduction

2.3. Materials and Methods

2.3.1 Animal tumor models

2.3.2. Plasmid Vectors

2.3.3. Preparation of NMBs

2.3.4. US

2.3.5. Plasmid transfection

2.3.6. Monitoring gene expression and measurement of tumor volume

2.3.7. Vessel imaging and quantification of tumor vessel area using contrast enhanced US imaging

2.3.8. Blood biochemistry tests

2.3.9. Tumor tissue sampling

2.3.10. Real-time quantitative PCR

2.3.11. Histological analysis

2.3.12. Statistical analysis

2.4. Results

2.4.1. Comparison of transfection efficiency between single and repeated NMBs and US transfection
2.4.2. Comparison of treatment efficiency between single and repeated NMBs and US transfection of the TNF-α gene………………..64
2.4.3. Effectiveness of NMBs and US transfection of the TNF-α gene…65
2.4.4. Acute toxicity by NMBs and US…………………………..66
2.4.5. Change in tumor vessel density………………………..66
2.4.6. Apoptosis and tumor suppression factors…………………..67
2.5. Discussion…………………………………………………..68
2.6. Limitations of this study……………………………………71
2.7. Conclusion………………………………………………….72

Chapter 3 Application of nano/microbubbles and ultrasound to regional cancer therapy for the bladder cancer………89
3.1. Summary…………………………………………………90
3.2. Introduction ……………………………………………….91
3.3. Materials and Methods……………………………………….93
   3.3.1. Animal studies………………………………………..93
   3.3.2. Mouse bladder cancer model…………………………..93
   3.3.3. Preparation of NMBs………………………………….95
   3.3.4. Exogenous molecules…………………………………95
   3.3.5. US…………………………………………………….95
   3.3.6. Mouse preparation for molecular delivery………………96
   3.3.7. Targeted displacement and collapse of NMBs using DIUS……97
   3.3.8. Delivery of fluorescent molecules…………………….98
3.3.9. Imaging of the delivery of fluorescent molecules and analysis….98
3.3.10. Gene delivery.................................................................99
3.3.11. Imaging of gene delivery and analysis.................................99
3.3.12. Analysis of bladder inflammation.....................................100
3.3.13. Statistical analysis.........................................................100
3.4. Results and Discussion..........................................................101
  3.4.1. Establishment of mouse bladder cancer model.................101
  3.4.2. Analysis of targeted displacement and collapse of NMBs using DIUS.................................................................101
  3.4.3. Delivery of fluorescent molecules and transfection efficiency….103
  3.4.4. Gene delivery and the transfection efficiency.....................105
  3.4.5. Morphological alterations..............................................106
  3.4.6. Limitations of this method..............................................107
3.5. Conclusion...........................................................................108

Chapter 4 Conclusions..............................................................120

Bibliography.............................................................................124
Abstract

Nano/microbubbles (NMBs) as ultrasound (US) contrast agents are currently used to enhance the capabilities of diagnostic imaging. NMBs are also providing new avenues for therapeutic applications of US. A drug delivery system using NMBs and US utilizes a mechanism of sonoporation, which uses US to enhance cell permeabilization via impulsive pressures generated by either collapsing bubbles or cavitation bubbles created by the collapse. Subsequently, exogenous molecules enter nearby cells \(^{1-3}\). Thus, it allows the non-invasive delivery of therapeutic agents into specific target cells. This system is attracting increasing interest because of its advantages, including easy operation, low toxicity, low immunogenicity, low invasiveness, high tissue selectivity, and repeated applicability \(^4\).

However, the transfection efficacy is generally low with the delivery method. Routes of drug administration can be divided into two categories: systemic and regional. Drug delivery efficiency of regional administration is 1,000 times higher than that of systemic administration. Therefore, to propose a novel cancer therapy, development of a regional cancer therapy based on regional drug delivery is effective. Bladder cancer is a cancer that intravesical administration is effective. The urinary bladder is a balloon-shaped closed organ that stores urine. Therefore, therapeutic genes and NMBs can be infused through catheterization and retained in the bladder and the behavior of NMBs can be controlled by US exposure.
The objective of this dissertation is to develop an effective gene delivery system with NMBs and US for cancer therapy and to demonstrate the application of the system to regional cancer therapy evaluated in the bladder as a regional delivery target.

Chapter 1 describes a general introduction on a drug delivery system using NMBs and US and the objective of the dissertation.

Chapter 2 evaluates the effectiveness of the molecular delivery system using NMBs and US into tumor cells. Tumor necrosis factor-α (TNF-α) plasmid DNA, an anti-tumoral gene, was repeatedly transfected into mouse solid tumors using NMBs and US and the anti-tumor effects was evaluated. This study shows repeated TNF-α gene delivery using NMBs and US could maintain the TNF-α gene expression level within a tumor, leading antitumor effects including activation of p53-dependent apoptosis, decrease in vessel density in tumor, and suppression of tumor size.

Chapter 3 proposes localized gene delivery using NMBs and US, evaluated in the bladder. A dual-intensity US system (DIUS) consisting of low-/high-US intensities was used for regional molecular delivery to the mouse normal bladder wall. Low-intensity US directs NMBs to targeted cells in the bladder and high-intensity US collapses NMBs for intracellular delivery of exogenous molecules. Using the DIUS, fluorescent molecules and luciferase expressing plasmid DNA was delivered in the bladder wall successfully and transfection efficiency was increased on repeatedly applying low- and high-intensity US cycles. This system shows potential for bladder cancer as regional cancer therapy.
Chapter 4 describes conclusions.

This dissertation shows that the developed gene delivery system using NMBs and US is effective for regional cancer gene therapy and can be a clinically viable option for bladder cancer.
Acknowledgments

It would not have been possible to write this doctoral thesis without the help and support of all people around me, to only some of whom it is possible to give particular mention here.

First and foremost, I offer my sincerest gratitude to my supervisor, Professor Tetsuya Kodama, Ph. D., Molecular Delivery System Laboratory, Graduate School of Biomedical Engineering, Tohoku University, who has supported me throughout my thesis with his patience and knowledge while allowing me the room to work in my own way. His enthusiasm in embracing a new project in the lab with its own required equipments, supplies, cell culture requirements, molecular biological techniques, and care and technique for animal experiments was fundamental in allowing me to conduct this research, in addition to teaching me how to start a project from the very beginning.

I would like to express my deep and sincere gratitude to Dr. Shiro Mori, D.D.S., Maxillofacial Surgery, Tohoku University Hospital. His wide knowledge has been of great value for me. His understanding, encouraging, and guidance have provided a good basis for the present thesis.

I am deeply grateful to Professor Masao Ono, M.D., Ph.D., Department of Pathology, Tohoku University Graduate School of Medicine, for his generous encouragement and constructive comments throughout this work.

I wish to express my warm and sincere thanks to Director Yasuhiro Matsumura,
M.D., Ph.D., Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, whose achievements and concepts have had a remarkable influence on my entire career in the field of DDS research. I also would like to thank him for teaching me how to obtain a mouse bladder cancer model.

My sincere thanks are also due to Professor Kazuo Maruyama, Ph.D., and Dr. Ryo Suzuki, Ph.D., Department of Biopharmaceutics, School of Pharmaceutical Sciences, Teikyo University, for their detailed instruction of acoustic liposomes. I warmly thank, especially, to Dr. Suzuki, for his kind and warm friendship.

I owe my most sincere gratitude to Assistant Professor Apostolos G. Doukas, Ph.D., Department of Dermatology, Wellman Laboratories of Photomedicine, Massachusetts General Hospital, Harvard Medical School, for his warm encouragement and friendship.

This doctor thesis is reviewed by Professor Tetsuya Kodama, Professor Shin-ichiro Umemura, Ph. D., Professor Yoshifumi Saijo, Ph. D., Graduate School of Biomedical Engineering, Tohoku University, Professor Masao Ono, Graduate School of Medicine, Tohoku University, and Director Yasuhiro Matsumura, Investigative Treatment Division, National Cancer Center Hospital, East. I express my gratitude to them for taking time to critically review my thesis.

In my daily work, I have been blessed with a friendly and cheerful group of fellow students and colleagues. Special thanks are extended to the members of the Graduate School of Biomedical Engineering, Tohoku University, and Tohoku University Biomedical Engineering Research Organization (TUBERO), including
Dr. Yukiko Watanabe who always be my comrade to share and fight for the intense pressure toward our purchasing doctors, Mrs. Kiyoe (Konno) Funamoto who took me into this laboratory, and Dr. Atsuko (Aoi) Matoba who has been my ideal caretaker and tutor at this laboratory.

I owe my loving thanks to my parents, Kokichi and Shoko, grandmothers, Isako, who passed away on June 6, 2010, and Motoko, who passed away on December 23, 2011, and T. Ito, my fiancé. Without their encouragement and understanding, it would have been impossible for me to finish this work. They have been with me and assist me to get over with all the frustrations, anxieties, and feelings of giving up. I am so incredibly lucky to have their support.

I am also grateful to the organization that has provided funding to support this research and my graduate studies: Japan Society for the Promotion of Science, Grant-in-Aid for Young Scientists (Start-up: 19800002 and Research Fellow: 21 ・ 7271).

Finally but at most, I am sorry and thankful to the hundreds of mice that gave their lives for research.
During this Doctor thesis at Tohoku University, 2011

Tohoku earthquake and tsunami hit our town on March 11th. We lost about 20,000 people by the disaster. May their soul rest in peace.

I would like to appreciate for support, encouragement, and hope from people all over the country and the world. Thanks, my dear friends!
Chapter 1

Introduction
1.1. Cancer

1.1.1. What is cancer?

Cancer is a class of diseases characterized by out-of-control cell growth. It appears to arise from a single cell that undergoes malignant transformation \(^{(5)}\).

After the transformation, many factors are involved to tumor growth. The factors involved include changes in DNA in tumor cells, additional presence of tumor-promoting agents, individual susceptibility to malignant transformation influenced by inherit genetic factors and cancer-causing behaviors \(^{(5)}\). Cancer may remain a locally invasive process or it may additionally spread to non-contiguous sites by hematogenous or lymphatic routes \(^{(5,6)}\).

1.1.2. Statistics of cancer

Cancer is the second leading cause of death in developed countries \(^{(7)}\). According to International Agency for Research on Cancer (IARC) at World Health Organization, in 2008, 12.7 million people were diagnosed and 7.6 million people died of cancer, which constitutes 13% of the 58 million deaths worldwide. IARC estimates further increase to 21.3 million new cases and 13.3 million deaths will be achieved in the year 2030. In Japan, cancer has been the leading cause of death since 1981. According to Cancer Statistics in Japan ’10, 676,075 people were diagnosed with cancer in 2005, and about 1,000 of them die every day (total of 344,105 people) in 2009. In 1910, 32,998 people were died from cancer in Japan; since then, gradual increase in the incidence was achieved to tenfold in about 100 years (Fig. 1-1A). The increase in incidence and mortality can be attributed
because of the aging and growth of the world population alongside an increasing adaptation of cancer-causing behaviors, including smoking, physical inactivity, and consumption of calorie-dense food.

The United States of America, an advanced county in the field of cancer research, the budget for cancer research has increased steadily over 40 years since the former president Nixon pledged more than 100 million dollars at the “War on Cancer Launched by National Cancer Act of 1971” to President Obama announced $1 billion spending plan in 2011 (5,8). In fact, medical spending for cancer research increased from $15 billion in 1972 to $74 billion in 2005 in America (5,9). In Japan, the government implemented the Comprehensive 10-year Strategy for Cancer Control (1984-1993) and the New 10-year Strategy to Overcome Cancer (1994-2003). The budget for cancer control was steadily rose from ¥1.5 billion in 1984 to ¥23.6 billion in 2008 (Fig. 1-1B).

1.1.3. Diagnosis and treatment in clinical settings

Table 1-1 shows cancer diagnosis and treatment used in clinical settings (10). Cancer diagnosis and treatment vary according to the type and stage of cancer and a combination of these options are used in many cases in the course of both diagnosis and treatment.

Patients are diagnosed using easy and minimally invasive methods and, if necessary, undergo further examination using more invasive and time consuming methods to reach a conviction. Using easy and minimally invasive methods, early detection of cancer is a desirable goal in cancer diagnosis. Imaging plays an
important role by providing important diagnostic and therapeutic assessment and
intervention in cancer therapy among the listed diagnosis options (Table 1-1)\(^{(11)}\).
Imaging modalities used in everyday clinical application include mammography
magnetic resonance imaging (MRI), computed tomography (CT), positron
emission tomography (PET), single photon emission computed tomography
(SPECT), and US and Doppler imaging\(^{(7)}\). Table 1-2 shows the characteristics of
those imaging modalities.

Surgery, radiation, and chemotherapy are most commonly used for cancer
treatments\(^{(6, 10, 12)}\). Surgery is the straight and the most effective approach.
However, it is an invasive procedure involving the removal of a cancerous tumor;
is therefore traumatic\(^{(5, 6, 12)}\). Radiation is the use of high-intensity X-rays to
destroy cancerous cells and shrink tumors before surgery, to direct large doses of
radiation at a tumor during surgery, or to inhibit the growth of any remaining
cancerous cells after surgery\(^{(5, 6, 12)}\). However, because normal tissues can also be
affected by radiation, a wide variety of side effects can occur\(^{(5, 6, 12)}\). Radiation can
also increase the risk for other cancer\(^{(5, 6, 12)}\). Chemotherapy can be the first choice
for cancer treatment. Chemotherapy treats cancer with drugs that destroy cancer
cells\(^{(5, 6, 12)}\). It is also used to shrink a tumor before surgery, to help destroy cancer
cells that remain after surgery, to make radiation therapy or other treatment options
work more effectively, or to help destroy cancer that recurs or has spread for the side
of the original tumor\(^{(5, 6, 12)}\). Intravenous and oral chemotherapy are the central
cancer treatments; however, they can be associated with considerable side-effects
because they are provided to the entire body\(^{(5, 6, 12)}\). In the past years, regional
administration routes have been investigated to reduce the systemic toxicity and to increase drug concentrations with a high local efficacy \(^{(13)}\). When compared with systemic drug administration, regional delivery can potentially increase drug concentrations at tumor sites and/or lower systemic drug exposure.

1.1.4. Drug and gene delivery in cancer research

In clinical settings, cancer therapy using drug delivery includes chemotherapy, immunotherapy, and gene therapy. The serious problem is side effects by systemic administration of therapeutic agents using these therapies. In both clinical and preclinical setting, drug delivery is designed to improve the selectivity of anti-tumoral agents (Table 1-3) \(^{(14)}\). For cancer research, a vast range of drug and gene delivery has been introduced to enable high concentrations of drugs to be deposited to specific locations in the body. In the case of cancer, targeted drug delivery is achieved with three types of targeting mechanisms: passive, active, and physical targeting \(^{(15-17)}\).

Passive targeting is based on the enhanced permeability and retention (EPR) effect, which allows the preferential accumulation of therapeutic agents in solid tumors as a result of leaky blood vessels and poor lymphatic drainage of the tumor tissue caused by rapid angiogenesis \(^{(15, 16, 18, 19)}\). A study using liposomes of different mean size suggests that the liposomes smaller than 400 nm in diameter are able to extravasate into and accumulate in tumor regions \(^{(20)}\).

Active targeting involves the modification of the surface of drug carriers with certain ligands that bind specifically to tumor-associated receptors, enabling them
to be selectively attached to diseased cells \(^{(15, 17, 19)}\). Targets for active targeting of particulate drug carriers can be divided into (i) targets that are expressed on endothelial cells of tumor blood vessel (e.g. integrin-\(\alpha_v\beta_3\) \(^{(21)}\) and Vascular Endothelial Growth Factor 2: VEGFR2 \(^{(22)}\)) and (ii) targets that are overexpressed on tumor cells (e.g. Human Epidermal Growth Factor Receptor 2: HER-2 \(^{(23)}\) and Epidermal Growth Factor Receptor: EGFR \(^{(24)}\)).

In recent years, through both passive and active targeting mechanisms, nanomaterials are developed and investigated to further enhancing targeting and directing to the tumor \(^{(15)}\). A representative passive and active targeted drug delivery to tumor is shown in Fig. 1-2 \(^{(15, 25)}\). The system demonstrates nanomaterials reach tumors selectively through active targeting and ligands on the surface of nanomaterials bind to receptors expressed by cancer cells or angiogenic endothelial cells.

Physical targeting includes direct injection and catheter-mediated injection of anti-tumoral agents \(^{(26)}\). It also includes utilization of an external trigger such as ultrasound when particles contain gas (Fig. 1-3A) \(^{(27-29)}\) and magnetic field when particles are magnetic (Fig. 1-3B) \(^{(30, 31)}\). In recent researches, physical targeting is also employed with active and passive targeting to help nano/microparticles to adhere to a vessel wall \(^{(29, 31)}\).

Drug loading targeting particles have been engineered to slowly degrade and react to stimuli \(^{(14-16, 32)}\). Figure 1-4 shows examples of nano/microcarriers for cancer targeting drug/gene delivery. Nano/microcarriers include polymer
conjugates, polymeric particles, lipid-based carriers such as liposomes and micelles, dendrimers, carbon nanotubes, and gold nanoparticles.

1.1.5. Intracellular gene delivery in cancer research

It is not only important to deliver therapeutic materials in the specific tissues with passive, active, and/or physical targeting but also to delivery them within a specific cellular compartment. Cancer gene therapy is the applied consequence of the intense research with advances in understanding and manipulating gene and demonstrating potentials for treatment of cancer during the last decade (33). Gene therapy is the use of genes as medicine and the transfer of therapeutic gene materials into specific target cells, aiming at tumor cell killing or inhibition of tumor cell growth by direct targeting to the tumor cells or to the host’s tumor defense, represented by the immune system (33). Effective cancer gene therapy requires a vector that can efficiently deliver into targeted cells. Although the preclinical results of gene therapy have shown promise for some cancers, cancer gene therapy is still at an early stage of clinical development and has not yet shown a significant therapeutic benefit for patients. The intense research for suitable vector system for cancer gene therapy has led to the development of viral and non-viral vectors (Table 1-4) (34, 35).

In cancer gene therapy, adenovirus, retrovirus, and lentivirus are used as viral vectors. Viral vectors have shown to be effective with high efficiency and the possibility of long-term gene expression (36). However, viral vectors suffer from
some disadvantages including lack of site specificity, acute immune response, immunogenicity, and insertion mutagenesis\(^{(36,37)}\).

Non-viral methods present certain advantages over viral methods, with their safety, versatility, and ease of preparation and scale-up. Non-viral gene delivery has been explored using chemical and physical approaches\(^{(36)}\). Chemical approaches, such as liposomal systems (lipofection), calcium phosphate, and DEAE-dextran, use synthetic or naturally occurring compounds as carriers to transfect therapeutic genes into cells\(^{(36)}\). Physical approaches, including needle injection, particle bombardment (gene gun), hydrodynamic delivery, electroporation, and sonoporation, utilize a physical force to permeabilize cell membrane and facilitate intracellular gene delivery\(^{(36)}\). Although different approaches have been developed to overcome barriers for gene delivery, non-viral vectors are still inefficient compared to viral vectors because of their low transfection efficiencies.

### 1.1.6. Gene delivery system using nano/microbubbles and ultrasound

Drug delivery system using nano/microbubbles (NMBs) and ultrasound (US) is one of non-viral drug delivery approaches utilizing a mechanism of sonoporation. This system uses US to enhance cell permeabilization via impulsive pressure generated by either collapsing bubbles or cavitation bubbles created by the collapse, facilitating exogenous molecules including, therapeutic drugs and genes, enter nearby cells (Fig. 1-5)\(^{(38-40)}\). NMBs are small gas-filled microspheres, used as US contrast agents to enhance the capabilities of diagnostic imaging\(^{(41,42)}\).
Therefore, performing real-time imaging guidance of NMBs with adequate definition of the region of interest and accurate control over the NMB behavior, minimally invasive therapy using NMBs and US with increased cellular uptake could be achieved. The detailed concept and mechanisms of drug delivery system using NMBs and US are discussed in the later sections (1.3. Introduction to NMBs and US, and 1.4. Application of NMBs and US in medicine).

1.1.7. Regional gene delivery using NMBs and US

Routes of drug administration can be divided into two categories: systemic and regional (43, 44). With intravenously administered chemotherapy, only a fraction of the total dose reaches the tumor site and the remainder of the dose is distributed throughout healthy organs and tissues, leading to a variety of undesirable side effects. Drug delivery efficiency of regional administration is 1,000 times higher than that of systemic administration (43, 44). Therefore, regional drug delivery is an essential cancer therapy, working on selected tissues and cells. To propose a novel cancer therapy, development of a regional cancer therapy based on regional drug delivery is effective. Table 1-5 shows cancers that regional administration of drugs is effective (43-45). It includes brain tumor and liver cancer which arterial injection is effective, ovarian cancer which intraperitoneal injection is effective and bladder cancer which intravesical injection is effective. Among them, we focused on bladder cancer. The urinary bladder is a balloon-shaped closed organ that stores urine. Therefore, therapeutic molecules and NMBs can be infused through catheterization and retained in the bladder and the behavior of NMBs can be
controlled by US exposure. A dual-intensity US system (DIUS) using NMBs offers opportunities for regional drug delivery. This system consists of low-/high-US intensities. Low-intensity US direct NMBs to targeted cells in the bladder and high-intensity US intensity would collapse NMBs and increase cell membrane permeability, facilitating entry of exogenous molecules into proximate cells with increased cell-to-bubble ratio. Shortencarier et al. have delivered fluorescent molecules into blood vessel using this dual intensity US system (46). Since a NMB can be used as an US contrast agent, while a high-frequency US imaging system characterize movement and fragmentation of NMBs in the bladder real-time, image-guided US triggered drug delivery can be achieved.

1.2. Thesis objectives

This dissertation aims to develop a gene delivery system using NMBs and US and to demonstrate the application of the system to regional cancer therapy. In chapter 2, to evaluate the effectiveness of the gene delivery using NMBs and US into tumor cells, tumor necrosis factor-α (TNF-α) plasmid DNA, an anti-tumoral gene, was transfected into mouse solid tumors using NMBs and US and the anti-tumor effects was evaluated. In chapter 3, to propose a novel cancer therapy using NMBs and US, gene delivery was regionally evaluated in the bladder, used as a regional delivery target. In this dissertation, a drug delivery system using NMBs and US is proposed for both cancer diagnosis and treatment. This dissertation reports that the drug delivery system using NMBs and US is effective.
for regional cancer gene delivery and has the potential for the clinical application in cancer therapy.

1.3 Introduction to NMBs and US

1.3.1. NMBs

NMBs are small gas-filled microspheres that have specific acoustic properties that make them useful as contrast agents in the field of US with a radius of less than 5 μm \(^{(41, 42)}\).

1.3.2. Dynamics of NMBs to US

US is a sound wave with a frequency above that of the audible range of human hearing (20 kHz) \(^{(47-49)}\). Sound waves transport mechanical energy and propagate through a medium such as air, water, or any other liquid and solid matter, causing local oscillatory motion of particles of the medium \(^{(47, 48)}\). As the wave displaces NMBs, NMBs are compressed under positive pressure and expanded under negative pressure (Fig. 1-6) \(^{(50, 51)}\). The behavior of NMBs is governed by physicochemical parameters such as encapsulated gas properties, shell properties, the media, and US \(^{(52)}\). The likelihood of their oscillation patterns is frequently indicated depending on the acoustic power, which can be expressed as the mechanical index \((MI)\) \(^{(50, 52)}\). The \(MI\) has been formulated in evaluating the likelihood of cavitation-related adverse biological effects \(^{(47)}\). The \(MI\) is given by

\[
MI = \frac{P_\text{-ave}/[\text{MPa}]}{\sqrt{f}/[\text{MHz}]}
\]

\((1-1)\)
where $P_-$ [MPa] is the peak negative pressure and $f$ [MHz] is the mean frequency of the US beam\textsuperscript{(5, 14, 41, 53)}. The $MI$ is for free gas bubble models. Applying the $MI$ to shell bubbles, response of NMBs can be categorized into three phase, linear oscillation ($MI < 0.1$), non-linear oscillation ($MI = 0.1-0.3$), and bubble collapse ($MI > 0.3$), (Fig. 1-7)\textsuperscript{(10, 41, 42)}. Fig. 1-8 shows the varying ranges of $MI$s for oscillation and collapse of commercially available NMBs\textsuperscript{(47)}.

In a very low-amplitude acoustic field with very low $MI$ (< 0.1), NMBs demonstrate linear oscillation while the repetitive oscillation of bubbles stays in an equilibrium radius (Fig. 1-7-B)\textsuperscript{(10, 41, 42, 48-50)}. In a low-amplitude acoustic field with low $MI$ (0.1-0.3), NMBs increase in equilibrium size as gas dissolved in the liquid is pumped into the bubble through rectified diffusion while the bubbles can remain spherically symmetric (Fig. 1-7-C)\textsuperscript{(10, 41, 42, 50-55)}. As the result, bubbles become relatively more resistant to compression than to expansion leading non-linear oscillation of NMBs\textsuperscript{(10, 41, 42, 48)}. In a high-amplitude acoustic field with high $MI$ (> 0.3), the pulsation of bubbles become instable due to perturbations from spherical symmetry with large radial expansion and contraction. Thus, large expansion is induced and bubbles burst (Fig. 1-7-D)\textsuperscript{(5, 10, 14, 49, 51, 56)}. The threshold radius below which the undamped collapsing bubble become unstable, $R_{\text{Threshold}}$, is given by:

$$R_{\text{Threshold}} \equiv \frac{R_{\text{max}}}{10} \quad (1-2)$$

where $R_{\text{max}}$ is the maximum radius of a bubble during insonation\textsuperscript{(50)}. 
Various non-linear acoustic phenomena of bubbles are generated in an acoustic field (57-60). The non-linear phenomena of bubbles are summarized in Fig. 1-9 including bubble translation (A), clustering (B), coalescence (C), fragmentation (D), cracking (E), and jetting (F) (58). Bubble translation (A) in the direction of the sound field is caused resulting from a pressure gradient across the bubble surface (58, 61-63). Bubbles cluster (B) by aggregating each other in a standing sound wave field (58, 61-63). The pushing force to oscillating bubbles in the direction of acoustic wave propagation is called the primary Bjerknes (radiation) force and the attractive force between oscillating bubbles in an US field is called the secondary Bjerknes (radiation) force (61-63). Primary forces arise from the interaction of one bubble with the surrounding medium, and secondary forces arise between two bubbles within the medium (64). In contrast to the Bjerknes (radiation) force which acts directly on the particle, acoustic streaming, which is the movement of fluid due to a net force field in a pressure field, can also induce bubble movements (64).

Bubbles coalesce with each other (C) due to collision of bubbles by the secondary Bjerknes force to form larger bubbles (65, 66). Bubble collapse, including fragmentation (D), cracking (E), and jetting (F), is induced due to instability or inertia effect (66). Fragmentation is a phenomenon of a bubble breaking up into a number of smaller bubbles and has been observed with bubbles with thin shells (58, 67, 68). Cracking (E) is the US-induced release of gas from hard-shelled bubbles and the cracked bubbles stay acoustically active (69). Postema et al. (67) showed, at $MI > 0.6$, fragmentation (Fig. 1-9D) was observed for microbubbles with a thin shell of about 10 nm while cracking (Fig. 1-9E) was observed for those with a thick shell.
of about 250 nm. A micro-jet (Fig. 1-9F) results from the asymmetry in bubble collapse pressure near an interface \(^\textbf{(70)}\). Depending on the properties of the interface, the bubble can either generate a jet towards the interface, away from the surface, or collapse in an alternate shape. The collapse of a cavitation bubble as microjet directed toward the interface tends to occur near more rigid surface \(^\textbf{(59, 71)}\). During the collapse of bubbles, various acoustic phenomena might be generated, including heat, free radicals, shock waves and share forces \(^\textbf{(72, 73)}\).

1.3.3. History of NMBs as US contrast agents

Small bubbles, NMBs as echo-enhancing US contrast agents, came from a chance observation by a cardiologist, Dr. Charles Joiner, in the late 1960s. Dr Joiner observed transient increases in the US signal caused by small bubbles forming at a catheter tip in the left ventricle \(^\textbf{(48, 74)}\). After the discovery, technological advances over the last 20 years have allowed development of NMBs with the necessary characteristics, that are stable in the circulation, traversed the pulmonary circulation to allow recirculation, and harmless to the user.

First-generation NMBs are microbubbles that used air as the core gas (Fig. 1-10-A) \(^\textbf{(49)}\). Air microbubbles disappear in a few seconds after intravenous administration as the solubility of air in blood is high \(^\textbf{(6)}\). Transpulmonary stability was achieved by the addition of a shell surrounding and thus stabilizing the microbubble \(^\textbf{(6)}\). Microbubbles were surrounded by soft shells consisted of thin membranes or layers of phospholipids or surfactants and harder and more stable shells made of albumin or polymers to improve stability both in the vial and in the
body \(^6,49\). In addition, the average diameter of the microbubbles were set between 2 and 7 μm; they were thus about the same size of red blood cells and there is therefore no risk of capillary embolization \(^75\). Commercially available first-generation US contrast agents include Albunex, Infoson, and Levovist (Table 1-6) \(^49\). However, one of the problems with the first generation microbubbles was still the short duration of producing US contrast after intravenous injection \(^49\).

To increase further stability of microbubbles and prolong the duration in the circulation, second-generation NMB US contrast agents are coated microbubbles consisting low solubility gas, a heavy-molecular-weight gas, including perfluorocarbons and sulfur hexafluoride (Fig. 1-10-B) \(^6,49,76\). Table 1-7 shows the solubility of a gas bubble whose gas is used in UCAs with a size of 3 μm including the Ostwald coefficients and the predicted lifetime in water \(^77\). Second generation agents include Sonovist, Optison, and Sonovue (Table 1-6) \(^49\). For example, Sonovue is a phospholipid coated sulfur hexafluoride gas-containing microbubbles \(^6\). These microbubbles have smaller diameters than the first generation air-filled bubbles (about 2.5 μm), which improve passage of the pulmonary capillary bed \(^6\). Thus, the durations of contrast and Doppler enhancement increase from several seconds to several minutes \(^49\).

A third generation of NMBs imparts unique features to the bubble, i.e. active targeting of specific antigens and temperature-dependent behavior (Fig. 1-10-C) \(^49,76\). For example, Sonazoid, a clinically approved agent in Japan since 2007, is used for Kupffer cell targeting \(^78\). Kupffer cells are liver specific macrophages and the shell compound of Sonazoid, phosphatidylinerine, is the natural marker of
apoptosis. Kupffer cells in normal liver capture circulating Sonazoid bubbles but not in tumor lesion because tumors contain few or no Kupffer cells. This allows better disease characterization by “negative” target enhancement (79). The other example is EchoGen consisting a perfluoro compound which is liquid at room temperature but becomes a gas at body temperature (74). The third-generation agents also include AI-700 and CARDIOsphere, both which are now in clinical trial (Table 1-6) (49, 76). The other example would be the microbubble whose surface is decorated with ligands that bind specifically to receptors on target cells (80).

Several companies and researchers continue to develop new US contrast agents with unique properties and niche application. The forth generation of US contrast agents would be nanobubbles, in particular in cancer therapy (Fig. 1-10-D). Due to their extremely small size, nanoscale particles have unique properties to control their localization (81). As mentioned above, modern US contrast agents are primarily microbubbles that circulate in the intravascular compartment and are designed to enhance US signals in the blood pool (82). However, this size limits the agents to the blood pool. Nanobubbles would have the advantage of gaining access to areas outside the blood vessels, such as interstitial spaces in tumors. The size range enable the contrast agents to target cancer sites passively via the EPR effect, which results in exaggerated extravasation and retention of macromolecules or nanoparticles that are smaller than the pore size of tumor endothelia while avoiding particle trapping via reticuloendothelial system (RES), such as liver, spleen, and renal clearance (18,
In fact, some researches have been developed nano-sized US contrast agent including perfluorocarbon emulsion nanoparticles, echogenic liposomes, and gas-filled polylactic acid nano-dispersions \(^{(83)}\). Table 1-6 summarizes the most common US contrast agents currently licensed or in clinical development.

1.4. Application of NMBs and US in medicine

1.4.1. Combination of NMBs and US as a diagnostic tool

Until recently, US imaging technique had less place in cancer therapy because of its providing less anatomical details than other techniques such as CT or MRI regardless of its advantages including being non-invasive, cost effective, portable, and real-time \(^{(48, 86)}\). The development of NMBs as US contrast agents has increased the possibilities for diagnostic imaging \(^{(6)}\). NMBs can be used most simple introduction, by bolus injection or perfusion, to enhance the echogenicity in B-mode or Doppler images \(^{(49)}\). In general, NMBs greatly improve resolution and sensitivity of US imaging and add an additional dimension of information to CT and MRI, resulting considerable improvement of diagnostic accuracy in many cases \(^{(48)}\). High-frequency US has been a rapid growing imaging tool to study cancer in its early stages, to evaluate tumor growth and, with NMBs, to evaluate changes in blood flow \textit{in vivo} in real-time in preclinical setting \(^{(87, 88)}\).

NMBs have a high reflectivity when exposed to an US field. This is because the US image consists of reflected echoes that occur when an US pulse encounters
different materials or tissues \(^{(7, 74)}\). Tissue properties of each materials causing resistance to the propagation of US are called acoustic impedance \((Z)\) \(^{(7, 89, 90)}\). The acoustic impedance, \(Z\), is given by:

\[
Z = \rho \cdot c
\]

where \(\rho\) [kg/m\(^3\)] is the density of the medium and \(c\) [m/s] is the speed of sound in the medium \(^{(7)}\). Table 1-8 shows the acoustic impedance of some typical substances relevant to clinical sonography. The intensity of the echoes displayed in the US image, which is the degree of reflection at interface, depends on the difference in acoustic impedance between materials. In other words, the higher the difference in impedance, the stronger the reflected echoes. As shown in Table 1-8, the difference in acoustic impedance between air and other gases, and water or soft tissue is very high. This leads to high reflectivity of gas in sonography.

However, the acoustic wave reflection would not be sufficient to determine an US enhancement because NMBs are very small and sparse in the circulation \(^{(74)}\). NMBs are easily detectable in US images because reflectivity is proportional to the forth power of a particle diameter when the particle diameter is much smaller than the wavelength of the sound according to Rayleigh-scattering laws \(^{(91)}\). Since Rayleigh’s model ignores resonance, the scattering cross section of a bubble, \(\sigma_s\), is given by the following modified formula:

\[
\sigma_s = 4\pi a^2 \frac{\Omega^4}{\left(1 - \Omega^2\right)^2 + (\Omega\delta)^2}
\]

\(^{(1-4)}\)
where \( a \) is the bubble radius, \( \Omega \) is the normalized frequency \( (= \frac{\omega}{\omega_0}) \), \( \bar{\omega} \) is the angular frequency \( (= 2\pi f) \), \( \omega_0 \) the angular resonance frequency \( (= 2\pi f_0) \), \( f_0 \) is the resonance frequency, and \( \delta \) is the dimensionless damping constant \(^{(91)}\).

According to the Minneart formula \(^{(92)}\), the resonant frequency, \( f_0 \), is given by

\[
f_0 = \frac{1}{2\pi a} \sqrt{\frac{3kp}{\rho}}
\]  

\(^{(1-5)}\)

where \( \kappa \) is the polytropic coefficient of the encapsulating gas, \( p \) is the static pressure far from bubble surface, and \( \rho \) is the density of the surrounding liquid \(^{(12, 90)}\). The frequencies used for medical imaging are generally in the range of 2 to 15 MHz \(^{(13, 93)}\). Commercially available US contrast agents used in clinical practice are approximately 2-6 \( \mu \)m in diameter \(^{(13)}\). For example, if a bubble with the diameter of 4 \( \mu \)m encapsulating air is imaged with US of 3 MHz frequency with the axial resolution of 1,000 \( \mu \)m or 10 MHz with 300 \( \mu \)m resolution, the diameter of scattering cross sections for 3 MHz and 10 MHz are 1,100 \( \mu \)m and 800 \( \mu \)m, respectively. The agent can be better visualized with an US imaging modality with 10 MHz frequency.

US contrast images using NMBs detect the US backscatter from bubbles, reflection of US waves from bubbles, to produce a unique sonogram with increased contrast due to the high echogenicity difference. Transmitted and backscatter pulses are used to form one image frame (Fig. 1-11) \(^{(10)}\). If the transmitted pulses encounter a linear reflector, namely at a very low \( MI \), the response to the transmitted pulse and the backscatter pulse are identical to each other (Fig. 1-11-A). Thus, the transmitted and backscatter pulses negate one
another, so that a signal is not detected in the image. On the other hand, if transmitted pulses encounter to non-linear reflectors due to the production of non-linear-oscillating and/or collapsing bubbles in addition to the fundamental pulses at higher $MI$, each of these pulses is distorted (Fig. 1-11-B and C). As a consequence, the sum of these pulses will no longer negate and signal is detected.

However, the negating of transmitted-backscatter-pulse-imaging ignores backscatter pulses from and attenuation by the surrounding tissue; therefore, phase inversion imaging was developed to differentiate bubble echoes from tissue echoes while emphasizing non-linear signals from bubbles\(^{(80)}\). The phase inversion imaging technique uses two pulses containing a primary pulse and the inverted pulse and returned signals are summed to form one frame (Fig. 1-12)\(^{(94, 95)}\).

Harmonic imaging also differentiate echoes from bubbles and tissue. When bubbles and tissue receive transmitted US waves, both produce harmonic components at twice or multiples the fundamental frequency, but the harmonic contents from the bubbles is higher than those from the tissue (Fig. 1-13)\(^{(96)}\). By selective depiction of the second harmonic component, the signals from the bubbles can be separated from the tissue. The better separation of the undesired fundamental frequency from the harmonic frequency is achieved using the combination of the pulse inversion technique and the harmonic imaging\(^{(97)}\).

Different approaches to US imaging are designed depending on the NMB response to US in non-linear manner by changing the acoustic power applied\(^{(13, 80)}\). For example, both high and low $MI$ amplitudes can be combined, in which high
MI pulses are used to destroy the bubbles within the region being imaged and then low MI pulses are used to monitor the afterward replenishment of bubbles into the imaging region \(^{(49, 80)}\). This destruction-replenishment method is used for the assessment of regional flow such as in the heart \(^{(98)}\), kidney \(^{(99)}\), and tumor \(^{(71)}\).

### 1.4.2. Combination of NMBs and US as a drug delivery tool

NMBs as US contrast agents are currently used to enhance the capabilities of diagnostic imaging. NMBs are also providing new avenues for therapeutic applications of US in areas including drug delivery. Drug delivery system using NMBs and US utilizes a mechanism of sonoporation, which uses US to enhance cell permeabilization via the impulsive pressure generated by either collapsing bubbles or the cavitation bubbles created by the collapse. Subsequently, exogenous molecules, including therapeutic drugs and genes, enter nearby cells \(^{(38-40)}\). Thus, it allows the non-invasive delivery of therapeutic compounds into specific target cells (Fig. 1-5). This system is attracting increasing interest because of its advantages, including easy operation, low toxicity, low immunogenicity, low invasiveness, high tissue selectivity, and repeated applicability \(^{(100)}\).

The intracellular uptake of exogenous molecules is governed by passive diffusion through membrane pores with a size up to 100 nm \(^{(73)}\). Impulsive pressure including shock wave and liquid jet plays important role to temporarily permeabilize the cell membrane \(^{(57, 72)}\). The pore formation by shock wave impact is simulated numerically by Koshiyama et al. \(^{(101)}\). They show that delivery of water molecules into the hydrophobic region of lipid bilayer of cell membrane due
to shock wave impulse promotes the spontaneous pore formation in the bilayer (Fig. 1-14). On the other hand, pore formation by liquid jet is evaluated by several researchers (57, 59, 102). When a bubble exposed to US is near a cell membrane, the surface liquid around the bubble converges on a point followed by the collision between the contracting bubble surface and the radial flow. This bubble-shock-wave interaction leads to liquid jet formation. Subsequently, the liquid jet penetrates the bubble interior separating the bubble and strikes the cell membrane, creating a pore on the cell membrane (57, 102). Previous studies show the membrane recovered in 30 sec (66, 103, 104). Cell membrane repair depends on Ca\(^{2+}\)-induced fusion of the adjacent lipid molecules of the membrane for small disruption (Fig. 1-15A) or intracellular vesicles such as lysosome for large disruption (Fig. 1-15B) (66, 103, 105-108).

NMB-associated US is a safe and efficient method to deliver exogenous molecules including drugs and plasmid DNA to target cells and has been increasingly investigated in the past decade. Several cell lines including endothelial cells (109), spinal meningeal cells (110), periodontal cells (100), muscle cells (39, 40), and cancer cells (5, 111), have been successfully delivered both \textit{in vitro} and \textit{in vivo}. In our laboratory, using NMBs and US, not only an anti-tumoral drug (CDDP) but also an anti-tumoral gene (Herpes plasmid DNA) were delivered into mouse solid tumor (marine colon and breast carcinoma) and successfully enhance the treatment efficacy (5, 111). The delivery efficiency is comparable to or even higher than the results obtained by lipofection and electroporation (112).
The efficiency of NMB-associated US depends on molecules to deliver (i.e. charge, concentration, and size), acoustic parameters (i.e., frequency, acoustic pressure, and insonation time), bubbles (i.e., composition, concentration, and size), and the physiological state of the cell. Size of the molecules to deliver affects the delivery efficiency; the larger the molecular size, the harder it is for them to enter the cell\(^{113,114}\).

Various researchers optimize US parameters and suggest that increasing transmitted US energy and insonation time increase molecular uptake while those decrease cell viability\(^{115-119}\). In our laboratory, we evaluated the transfection efficiency of the molecular delivery method using three different types of NMBs and US by transfecting pGL3 luciferase encoding plasmid DNA into mouse skeletal muscle\(^{39}\). In the study, three types of NMBs, albumin microbubbles (AMs; Optison), acoustic liposomal nanobubbles (ALs), and lipid micelle microbubbles (LMs) were used. The compositions, sizes, and zeta potentials of each type of bubbles are indicated in Table 1-9. The cell viability was measured in vitro and the transfection efficiency was measured in vivo. The concentration of bubbles was determined from the cell viability by the transfection using NMBs and US in the in vitro experiment.

The concentration of AMs is \(5-8 \times 10^8\) bubbles/mL according to the manufacture’s protocol. The concentration of LM, \(N_{LM}\), was estimated by:

\[
N_{LM} = \frac{a_{PC}}{4\pi(R_{LM})^2} \times \left( \frac{C_LN_A}{M_{PC}} + \frac{C_PN_A}{M_{PEG}} \right) \quad (1-6)
\]
Where \( a_{PC} \) is head group of a phospholipid, DSPC (0.52 nm\(^2\)), \( R_{AM} \) is the radius of micelle (635 nm), \( C_L \) is lipid concentration of DSPC (2 mg/mL), \( C_P \) is concentration of PEG40distearoyl (1 mg/mL), \( N_A \) is Avogadolo \((6.02 \times 10^{23})\), \( M_{PC} \) is the molecular weight of DSPC (790.2), and \( M_{PEG} \) is the molecular weight of PEG (930).

The number of AM in 1mL solution is \( 2 \times 10^{11} \) bubbles/mL. The concentration of AL in 1mL solution, \( N_{AL} \), was estimated by:

\[
N_{AL} = \frac{C_L N_A}{4\pi[R_{AL}^2 + (R_{AL} - d)^2]} \times \frac{94a_{PC} + 6a_{PE}}{94M_{PC} + 6M_{PE}} 
\]

where \( C_L \) is lipid concentration (1 mg/mL), \( N_A \) is Avogadolo \((6.02 \times 10^{23})\), \( R_{AL} \) is the radius of liposome (100 nm), \( a_{PC} \) is the effective area of hydrophilic group for a phospholipid DSPC (0.52 nm\(^2\)), \( a_{PE} \) is the effective area of hydrophilic group for another phospholipid, DSPE (0.43 nm\(^2\)), \( M_{PC} \) is the molecular weight of DSPC (790.2), and \( M_{PE} \) is the molecular weight of DSPE-PEG2k (2750). The solution diluted at 1:8 was used. The number of AL is \( 2 \times 10^{11} \) bubbles/mL. The transfection efficiency was evaluated in the \textit{in vivo} experiment using the concentration of the NMBs determined in the \textit{in vitro} experiment.

The concentration of NMBs is a key parameter. The percentage of transfected cells and the concentration of NMBs generally show a linear relation until the transduction level reaches to a plateau level because the transfection level is stabilized by an attenuation of US waves due to the high concentration of the NMBs \((120)\). However, in the study, as the transfection efficacy by AM and US is compared with that by LM and US, there was no significant difference although
the number of bubbles applied was different (AM: $5-8 \times 10^8$ bubbles/mL vs. LB: $2 \times 10^{11}$ bubbles/mL). A previous study compared AM and lipid-shelled microbubbles (Sonovue) for the effect on gene delivery in skeletal muscle with US and showed exposure of US did increase the transfection efficacy obtained with lipid-shelled microbubbles but not with AM (121). This is because of the difference in the susceptibility of bubbles to US while a lipid shell inhibits static diffusion from bubbles after insonation but an albumin shell does not (50). The constitutive properties of the shell of NMBs are also the key parameter.

When the transfection efficacy by AL nanobubbles and US is compared with that by LM microbubbles, there was a statistically significant difference while the concentration of NMBs was the same ($2 \times 10^{11}$ bubbles/mL). The study showed the size of the bubbles was another key parameter to increase the transfection efficiency (39). In the study, reduction of the bubble size increased the cell-to-bubble ratio, resulting the increase in the transfection efficiency.

Increasing cell-to-bubble ratio with a fewer number of bubbles can build an effective delivering system using NMBs and US. In addition to reducing bubble size, changing surface charge of bubbles can also contribute to the enhancement of delivering efficiency; i.e. through charge-mediated interactions to targeting cells and delivering molecules (122-125). Moving bubbles to target site can also increase delivering efficiency by increasing cell-to-bubble ratio; i.e. using Bejerkens force for bubbles to contact cells.
1.5. Concluding remarks

In this chapter, with a detailed general introduction on a drug/gene delivery system using NMBs and US, thesis objectives and the future prospects are discussed. A drug delivery system using NMBs and US utilizes sonoporation to enhance cell permeabilization allowing delivery of therapeutic compounds non-invasively into specific target cells. This system is attracting increasing interest because the gene delivery system using NMBs and US has great potential for targeted intracellular gene delivery since NMBs can function as drug carriers with active, passive, and physical targeting features and US can control behaviors of NMBs. In the following chapters, the effectiveness of the methods for cancer gene therapy is evaluated and the application of the method to a regional delivery is introduced for the future use of this system for clinical application.
Fig. 1-1 Increase in mortality of leading causes of death and budget for cancer research in Japan. (A) Changes in number of death by cancer, heart disease, cerebrovascular disease, pneumonia, and tuberculosis in Japan since 1910. (B) Trend in the budget for cancer control of the Ministry of Health, Labour and Welfare. Source: Cancer Statistics in Japan ’10
**Fig. 1-2 A representative passive and active targeted drug delivery to tumor.**
The system demonstrates nanomaterials reach tumors selectively through active targeting and ligands on the surface of nanomaterials bind to receptors expressed by cancer cells or angiogenic endothelial cells. This figure is based on Peer et al. (15) and Danhier et al. (25).
Fig. 1-3 Examples of physical targeted drug delivery with passive and active targeted approaches to tumor. Physical targeting includes utilization of an external trigger such as (A) ultrasound when particles contain gas and (B) magnetic field when particles are magnetic.
**Fig. 1-4 Examples of nano/microcarriers for cancer targeting drug/gene delivery.** Nano/microcarriers include polymer conjugates, polymeric particles, lipid-based carriers such as liposomes and micelles, dendrimers, carbon nanotubes, and gold nanoparticles. This figure is based on Peer *et al.* (15).
Fig. 1-5 Mechanism of exogenous molecule delivery using NMBs and US. Drug delivery system using NMBs and US utilizes a mechanism of sonoporation, which uses US to enhance cell permeabilization via the impulsive pressure generated by either collapsing bubbles or the cavitation bubbles created by the collapse. Subsequently, exogenous molecules including, therapeutic drugs and genes, enter nearby cells. Thus, it allows the non-invasive delivery of therapeutic compounds into specific target cells.
Fig. 1-6 **Response of bubbles to US.** As the wave displaces bubbles, bubbles are compressed under positive pressure and expanded under negative pressure.
Without US exposure, NMBs do not oscillate. (B) In a very low-amplitude acoustic field with very low \( MI < 0.1 \), NMBs demonstrate linear oscillation while the repetitive oscillation of bubbles stays in an equilibrium radius. In a low-amplitude acoustic field with low \( MI \) (0.1 – 0.5), NMBs increase in equilibrium size as gas dissolved in the liquid is pumped into the bubble through rectified diffusion while the bubbles can remain spherically symmetric. As the result, bubbles become relatively more resistant to compression than to expansion leading non-linear oscillation of NMBs. (C) In a high-amplitude acoustic field with high \( MI > 0.3 \), the pulsation of bubbles become instable due to perturbations from spherical symmetry with large radial expansion and contraction. Thus, large expansion is induced and bubbles burst.
Fig. 1-8 Varying ranges of mechanical indexes for oscillation and collapse of commercially available nanobubbles. Commercially available US contrast agents, Levovist, Optison, Sonovue, Definity, and Sonazoid, are compared. Optison oscillates and collapse at lower MI$s$ and Levovist dose at higher MI$s$. This figure is based on Postema et al. $^{(47)}$. 
Fig. 1-9 Summarized non-linear phenomena of bubbles. Non-linear phenomena include bubble translation (A), clustering (B), coalescence (C), fragmentation (D), cracking (E), and jetting (F). This figure is based on Moriyasu et al. (58).
Fig. 1-10 History of US contrast agents. (A) First-generation NMBs are shell coated microbubbles that used air as the core gas. (B) Second-generation NMB US contrast agents are coated microbubbles consisting low solubility gas, a heavy-molecular-weight gas, including sulfur hexafluoride and perfluorocarbons. (C) A third generation of NMBs imparts unique features to the bubble, i.e. active targeting of specific antigens and temperature-dependent behavior. (D) The forth generation of US contrast agents would be nanobubbles, in particular in cancer therapy.
### Fig. 1-7 Negating of transmitted and backscatter pulse imaging

Transmitted and backscatter pulses are summed to form one image frame. (A) If the transmitted pulses encounter a linear reflector, namely at a very low MI, the response to the transmitted pulse and the backscatter pulse are identical to each other. Thus, the transmitted and backscatter pulses negate one another, so that a signal is not detected in the image. (B and C) If transmitted pulses encounter to non-linear reflectors due to the production of non-linear-oscillating and/or collapsing bubbles in addition to the fundamental pulses at higher MI, each of these pulses is distorted. As a consequence, the sum of these pulses will no longer negate and signal is detected.

<table>
<thead>
<tr>
<th></th>
<th>Transmitted pulses to a bubble</th>
<th>Backscatter pulses from resulting bubbles</th>
<th>Negating of transmitted and backscatter pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>At very low MI imaging (Linear oscillation)</td>
<td><img src="image1" alt="Transmitted pulses" /> <img src="image2" alt="Backscatter pulses" /></td>
<td><img src="image3" alt="Negating of transmitted and backscatter pulses" /></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>At low MI imaging (Non-linear oscillation)</td>
<td><img src="image4" alt="Transmitted pulses to a bubble" /> <img src="image5" alt="Backscatter pulses from resulting bubbles" /></td>
<td><img src="image6" alt="Negating of transmitted and backscatter pulses" /></td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>At high MI imaging (Non-linear oscillation and collapse)</td>
<td><img src="image7" alt="Transmitted pulses to a bubble" /> <img src="image8" alt="Backscatter pulses from resulting bubbles" /></td>
<td><img src="image9" alt="Negating of transmitted and backscatter pulses" /></td>
</tr>
</tbody>
</table>
**Fig. 1-12 Phase inversion imaging technique.** The phase inversion imaging technique uses two pulses containing a primary pulse and the inverted pulse and returned signals are summed to form one frame. This figure is based on Ferrara *et al.* (80).
Harmonic imaging also differentiates echoes from bubbles and tissue. When bubbles and tissue receive transmitted US waves, both produce harmonic components at twice or multiples the fundamental frequency, but the harmonic contents from the bubbles is higher than those from the tissue. By selective depiction of the second harmonic component, the signals from the bubbles can be separated from the tissue. This figure is based on Kitano et al. (96).

**Fig. 1-13 Harmonic imaging.** Harmonic imaging also differentiates echoes from bubbles and tissue. When bubbles and tissue receive transmitted US waves, both produce harmonic components at twice or multiples the fundamental frequency, but the harmonic contents from the bubbles is higher than those from the tissue. By selective depiction of the second harmonic component, the signals from the bubbles can be separated from the tissue. This figure is based on Kitano et al. (96).
Fig. 1-14 Pore formation of cell membrane by shock wave impact. (A) When cell membrane is exposed to shock wave, water molecules are penetrated into the hydrophobic region of lipid bilayer of cell membrane. (B) Water molecules in the hydrophobic region move into the central region of the bilayer, where the mass density of the molecules is minimum. (C) Water molecules are pushed outside of the lipid molecules to arrange themselves into a micelle-like structure and a water pore. (D) The water pore was closed with time. This figure is based on Koshiyama et al. (101).
**Fig. 1-15 Cell membrane repair.** (A) Cell membrane repair depends on Ca$^{2+}$-induced fusion of the adjacent lipid molecules of the membrane for small disruption and (B) intracellular vesicles such as lysosome for large disruption. This figure is based on McNeil et al. \(^{(107, 108)}\)
Table 1-1 Cancer diagnosis and treatment in clinical settings

<table>
<thead>
<tr>
<th>Methods</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inspection/Palpation</td>
<td></td>
</tr>
<tr>
<td>Endoscopy</td>
<td>HE staining</td>
</tr>
<tr>
<td></td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Imaging</td>
<td>X-ray, CT</td>
</tr>
<tr>
<td></td>
<td>US</td>
</tr>
<tr>
<td></td>
<td>MRI</td>
</tr>
<tr>
<td></td>
<td>PET</td>
</tr>
<tr>
<td>Biochemical diagnosis</td>
<td>Tumor marker</td>
</tr>
<tr>
<td></td>
<td>DNA diagnosis</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>Anticancer agent</td>
</tr>
<tr>
<td>Immunotherapy</td>
<td>Adaptive immunotherapy (LAK cell etc.)</td>
</tr>
<tr>
<td></td>
<td>Biological response modifier (BRM)</td>
</tr>
<tr>
<td></td>
<td>Immunomodulator</td>
</tr>
<tr>
<td>Endocrine therapy</td>
<td>Antiestrogen therapy</td>
</tr>
<tr>
<td></td>
<td>Antiandrogen therapy</td>
</tr>
<tr>
<td>Missile therapy</td>
<td>Monoclonal antibody-drug complex</td>
</tr>
<tr>
<td>Others</td>
<td>Thermotherapy</td>
</tr>
<tr>
<td></td>
<td>Embolization</td>
</tr>
<tr>
<td></td>
<td>Laser therapy</td>
</tr>
<tr>
<td></td>
<td>etc.</td>
</tr>
</tbody>
</table>

This table is based on Imura\(^2\).
Table 1-2 Characteristics of imaging modalities

<table>
<thead>
<tr>
<th>Modality</th>
<th>Imaging target</th>
<th>Spatial Resolution</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI</td>
<td>Anatomy</td>
<td>25-100 μm</td>
<td>High resolution</td>
<td>Very expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-invasive</td>
<td>Slower than CT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Patients should be put in an enclosed space with loud noises</td>
</tr>
<tr>
<td>CT</td>
<td>Anatomy</td>
<td>50-200 μm</td>
<td>High resolution</td>
<td>Relatively expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rapid</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reproducible</td>
<td></td>
</tr>
<tr>
<td>PET</td>
<td>Function</td>
<td>5 mm</td>
<td>Shows organ functionality</td>
<td>Expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fusion with CT and MRI</td>
<td>Slower than CT or MRI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>SPECT</td>
<td>Function</td>
<td>10 mm</td>
<td>Less expensive than PET</td>
<td>Limited resolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simpler application than PET</td>
<td></td>
</tr>
<tr>
<td>US</td>
<td>Anatomy</td>
<td>1-2 mm</td>
<td>Non-invasive</td>
<td>Operator dependant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inexpensive</td>
<td>Hard to see deep structures</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Portable</td>
<td>Cannot see through bone or air</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Real-time imaging</td>
<td></td>
</tr>
</tbody>
</table>

This table is based on Hayat\(^{(11)}\).
<table>
<thead>
<tr>
<th>Table 1-3 Strategies available for drug delivery in cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Direct introduction of anticancer drugs into tumor</strong></td>
</tr>
<tr>
<td>Injection directly into the tumor</td>
</tr>
<tr>
<td>Injection into the arterial blood supply of cancer</td>
</tr>
<tr>
<td>Local injection into the tumor for radiopotentiation</td>
</tr>
<tr>
<td>Localized delivery of anticancer drugs by electroporation/sonoporation</td>
</tr>
<tr>
<td>Local delivery by anticancer drug implants</td>
</tr>
<tr>
<td><strong>Systemic delivery target to tumor</strong></td>
</tr>
<tr>
<td>Heat-activated targeted drug delivery</td>
</tr>
<tr>
<td>Tissue-selective drug delivery using carrier-mediated transport systems</td>
</tr>
<tr>
<td>Tumor-activated prodrug therapy for targeted delivery of chemotherapy</td>
</tr>
<tr>
<td>Pressure-induced filtration of drug across vessels to tumor</td>
</tr>
<tr>
<td>Site-specific delivery and light-activation of anticancer proteins</td>
</tr>
<tr>
<td><strong>Drug delivery targeted to blood vessels of tumor</strong></td>
</tr>
<tr>
<td>Antiangiogenesis therapy</td>
</tr>
<tr>
<td>Angiolytic therapy</td>
</tr>
<tr>
<td>Drugs to induce clotting in blood vessels of tumor</td>
</tr>
<tr>
<td>Vascular targeting therapy</td>
</tr>
<tr>
<td><strong>Special formulations and carriers of anticancer drugs</strong></td>
</tr>
<tr>
<td>Carbohydrate-enhanced chemotherapy</td>
</tr>
<tr>
<td>Fatty acids as targeting vectors linked to active drugs</td>
</tr>
<tr>
<td>Nano/microspheres</td>
</tr>
<tr>
<td>Polyethylene glycol technology</td>
</tr>
<tr>
<td><strong>Transmembrane drug delivery to intracellular targets</strong></td>
</tr>
<tr>
<td>Cytoporter</td>
</tr>
<tr>
<td>Receptor-mediated endocytosis</td>
</tr>
<tr>
<td>Transduction of proteins and peptides</td>
</tr>
<tr>
<td>Vitamins as carriers for anticancer agents</td>
</tr>
<tr>
<td><strong>Biological therapies</strong></td>
</tr>
<tr>
<td>Antisense therapy</td>
</tr>
<tr>
<td>Cell therapy</td>
</tr>
<tr>
<td>Gene therapy</td>
</tr>
<tr>
<td>Genetically modified bacteria</td>
</tr>
</tbody>
</table>

This table is base on Jain et al. (26).
### Table 1-4 Intracellular gene delivery systems for cancer therapy

<table>
<thead>
<tr>
<th>Viral systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
</tr>
<tr>
<td>Retrovirus</td>
</tr>
<tr>
<td>Lentivirus</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>Vaccinia virus</td>
</tr>
<tr>
<td>Reovirus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-viral systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical approaches</td>
</tr>
<tr>
<td>Liposomal systems (lipofection)</td>
</tr>
<tr>
<td>Calcium phosphate</td>
</tr>
<tr>
<td>DEAE-dextran</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Physical approaches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Needle injection</td>
</tr>
<tr>
<td>Particle bombardment (gene gun)</td>
</tr>
<tr>
<td>Hydrodynamic delivery</td>
</tr>
<tr>
<td>Electroporation</td>
</tr>
<tr>
<td>Sonoporation</td>
</tr>
</tbody>
</table>

This table is based on Eggermont\(^{(34)}\), Al-Dosari \textit{et al.}\(^{(35)}\), and Gao \textit{et al.}\(^{(36)}\).
<table>
<thead>
<tr>
<th>Types of cancer</th>
<th>Route of administration</th>
<th>Incidence rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>male</td>
</tr>
<tr>
<td>Brain tumor</td>
<td>Arterial injection (internal carotid artery)</td>
<td>1.6</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>Arterial injection (hepatic artery)</td>
<td>36.4</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Intraperitoneal injection</td>
<td>-</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>Intravesical injection</td>
<td>7.2</td>
</tr>
</tbody>
</table>

This table is based on Weinberg et al. (45), Yagishita et al. (43), and Kodama (44).

The incidence rate was data in Japan in 2008 according to the data from Cancer Statistics in Japan ’10. The number was indicated per 100,000 people.
<table>
<thead>
<tr>
<th>Generation</th>
<th>Agent</th>
<th>Original developer</th>
<th>Shell</th>
<th>Gas/vapor</th>
<th>Mean diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>Albunex</td>
<td>Mallinckrodt Inc.</td>
<td>Albumin</td>
<td>Air</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Infoson</td>
<td>Mallinckrodt Inc.</td>
<td>Albumin</td>
<td>Air</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Levovist</td>
<td>Schering AG</td>
<td>Galactose/Palmitic acid</td>
<td>Air</td>
<td>2-3</td>
</tr>
<tr>
<td>2nd</td>
<td>Sonovist</td>
<td>Schering AG</td>
<td>Cyanoacrylate</td>
<td>SF₆</td>
<td>1-2</td>
</tr>
<tr>
<td></td>
<td>BR14</td>
<td>Bracco Diagnostics Inc.</td>
<td>Lipid</td>
<td>C₄F₁₀</td>
<td>2.3-3.0</td>
</tr>
<tr>
<td></td>
<td>Definity</td>
<td>Bristol-Myers Squibb Medical Imaging, Inc.</td>
<td>Lipid/surfactant</td>
<td>C₃F₈</td>
<td>1.1-3.3</td>
</tr>
<tr>
<td></td>
<td>Imagent</td>
<td>IMCOR Pharmaceuticals, Inc.</td>
<td>Lipid/surfactant</td>
<td>C₆F₁₄/N₂</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Optison</td>
<td>Molecular Biosystems Inc.</td>
<td>Albumin</td>
<td>C₃F₈</td>
<td>2.0-4.5</td>
</tr>
<tr>
<td></td>
<td>Sonovue</td>
<td>Bracco Diagnostics Inc.</td>
<td>Lipid</td>
<td>SF₆</td>
<td>2.5</td>
</tr>
<tr>
<td>3rd</td>
<td>Sonazoid</td>
<td>Amersham Health</td>
<td>Lipid/surfactant</td>
<td>C₄F₁₀</td>
<td>2.4-3.6</td>
</tr>
<tr>
<td></td>
<td>AI-700</td>
<td>Acusphere, Inc.</td>
<td>Poly-L-lactide co glycolide</td>
<td>C₄F₁₀</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>CARDIOSphere</td>
<td>POINT Biomedical Corp.</td>
<td>Polylactide/albumin</td>
<td>N₂</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>EchoGen</td>
<td>Sonus Pharmaceuticals, Inc.</td>
<td>Surfactant</td>
<td>C₃F₁₂</td>
<td>2.0-5.0</td>
</tr>
<tr>
<td></td>
<td>MicroMarker</td>
<td>VisualSonics Inc.</td>
<td>Lipid</td>
<td>C₄F₁₀/N₂</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Targestar</td>
<td>Targeson Inc.</td>
<td>Lipid</td>
<td>C₄F₁₀</td>
<td>2.5</td>
</tr>
</tbody>
</table>

This table is based on Postema et al. (126).
### Table 1-7 Solubility of a gas bubble with a diameter of 3 μm

<table>
<thead>
<tr>
<th>Gas</th>
<th>Ostwald coefficient (× 10^6)</th>
<th>Disappearance time in water (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>23168</td>
<td>0.02</td>
</tr>
<tr>
<td>Sulfur hexafluoride (SF₆)</td>
<td>5950</td>
<td>0.1</td>
</tr>
<tr>
<td>Perfluoropropane (C₃F₈)</td>
<td>583</td>
<td>1.1</td>
</tr>
<tr>
<td>Perfluorohexane (C₃H₁₄)</td>
<td>24</td>
<td>2</td>
</tr>
</tbody>
</table>

This table is based on de Jong et al. (77).
Table 1-8 Acoustic impedance

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$Z \left( \frac{\text{kg}}{\text{m}^3 \cdot \text{s}} \right)$ (× 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0.00044</td>
</tr>
<tr>
<td>SF₆</td>
<td>0.00088</td>
</tr>
<tr>
<td>C₄F₁₀</td>
<td>0.0012</td>
</tr>
<tr>
<td>Lung</td>
<td>1.80</td>
</tr>
<tr>
<td>Fat</td>
<td>1.34</td>
</tr>
<tr>
<td>Water</td>
<td>1.48</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.63</td>
</tr>
<tr>
<td>Blood</td>
<td>1.65</td>
</tr>
<tr>
<td>Liver</td>
<td>1.65</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.71</td>
</tr>
<tr>
<td>Bone</td>
<td>7.80</td>
</tr>
</tbody>
</table>

The acoustic impedances for gas were calculated by the author, where the speed of sounds were based on Estrada-Alexanders (127) and the densities were on a website at http://users.ugent.be/~mvervust/gasconversiontables.pdf. The acoustic impedances of organs are based on Kremkau et al. (7).
### Table 1-9 Characteristics of the three types of bubbles

<table>
<thead>
<tr>
<th>Bubbles</th>
<th>Abbreviation</th>
<th>shell</th>
<th>gas</th>
<th>Peak diameter (μm)</th>
<th>zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin microbubble (Optison)</td>
<td>AM</td>
<td>Human albumin</td>
<td>C₃F₈</td>
<td>1.69 ± 0.15</td>
<td>-36.9 ± 2.70</td>
</tr>
<tr>
<td>Acoustic liposomal nanobubble</td>
<td>AL</td>
<td>DSPC+DSPE-PEG2k</td>
<td>C₃F₈</td>
<td>0.20 ± 0.08</td>
<td>-2.40 ± 0.51</td>
</tr>
<tr>
<td>Lipid micelle microbubbles</td>
<td>LM</td>
<td>DSPC+PEG</td>
<td>C₃F₈</td>
<td>1.27 ± 0.16</td>
<td>-4.11 ± 0.74</td>
</tr>
</tbody>
</table>

PSPC, 1,2-distearoyl-sn-glycero-phosphatidylcholine; DSPE, 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine; PEG, polyethylene glycol. This table is based on Kodama et al. (39).
Chapter 2

Therapeutic gene delivery into solid tumor using nano/microbubbles and ultrasound
2.1. Summary

In chapter 2, to evaluate the effectiveness of the molecular delivery using NMBs and US into tumor cells, TNF-α plasmid DNA, an anti-tumoral gene, was transfected into mouse solid tumors using NMBs and US and the anti-tumor effects was evaluated. TNF-α is a cytokine that can initiate tumor cell apoptosis by disrupting vasculature and exerts cytotoxic effects on a wide range of tumor cells. Application of TNF-α has been promising in cancer treatment and the effectual use of the cytotoxicity has been studied by many researchers. However, the clinical use of TNF-α has been limited by severe systemic toxicity. Here, we show repeated TNF-α gene delivery using NMBs and US could lead the local production of TNF-α within a tumor that reduced the acute toxicity while permitting antitumor effects, including activation of p53-dependent apoptosis, decrease in vessel density in tumor, and suppression of tumor size. Since NMBs used in this study are acoustic liposomes and can be used as US contrast agents and drug carriers, TNF-α gene delivery triggered by US exposure can be achieved while imaging the gathering at tumor site. In this study, we demonstrated the effectiveness of the TNF-α gene delivery into tumor cells using NMBs and US. With great future perspectives of NMBs, spatial and temporal control of TNF-α gene delivery will be achieved. This study opens new perspectives for the TNF-α cancer gene therapy by NMBs and US.
2.2. Introduction

TNF-α is a cytokine that mediates a broad range of biological actions and it possesses a potent anti-tumor activity\(^\text{(128, 129)}\). TNF-α induces the further production of TNF-α and the anti-tumor activity is mediated by direct cytotoxicity on tumor cells and an increase in vascular permeability\(^\text{(130-133)}\). This induces apoptosis through a signaling pathway that causes caspase activation, hemorrhagic necrosis, and consequently tumor regression. Administration of TNF-α can mediate the regression of many tumors and it has, therefore, attracted much attention as a potential anti-tumor agent. However, the dose-limiting toxicity of this agent has been quite apparent in human trials\(^\text{(131, 132)}\). Severe side effects, such as systemic inflammatory response syndrome and multiple organ failure, are often observed in cancer patients receiving TNF-α treatment\(^\text{(134, 135)}\). To avoid such toxicity, TNF-α is administrated to patients though a regional drug delivery system, such as to an isolated limb perfusion or an isolated hepatic perfusion\(^\text{(136, 137)}\). Since these procedures require surgery, they are used for the treatment of locally advanced solid tumors, such as limb-threatening soft tissue sarcomas and primary or metastatic unresectable liver tumors\(^\text{(136)}\).

By induction of a high local concentration of TNF-α in a tumor via an autocrine and paracrine mechanism of TNF-α production from transfected cells, gene therapy offers the potential to advance the treatment of cancer\(^\text{(138, 139)}\). Recently, many gene therapy strategies have been explored, including use of plasmid or recombinant viral vectors carrying therapeutic genes\(^\text{(128)}\). In clinical trials, TNF-α, expressed by adenovirus, showed anti-tumor action and synergism with radiation to produce cytotoxic effects\(^\text{(140)}\). However, administration of recombinant cytokines has proven to be toxic\(^\text{(140, 141)}\).
Because of high transfection efficiency, regional bolus cytokine delivery has led to systemic toxicity\(^{(142, 143)}\). Such barriers to the use of cytokines may be overcome by the use of plasmids encoding cytokines and by controlling expression of these cytokine genes over extended time periods. To achieve the full potential of gene therapy, additional improvements in the delivery of therapeutic genes to tumor cells are required.

Gene delivery using NMBs and US is a non-viral vector method, that has certain advantages, including easy operation, low toxicity, low immunogenicity, low invasiveness, high tissue selectivity, and repeated applicability\(^{(144)}\). This system enhances cell permeabilization via the impulsive pressure generated by either collapsing bubbles or the cavitation bubbles created by the collapse. Subsequently, exogenous molecules enter nearby cells\(^{(145-147)}\). Thus, it allows the non-invasive delivery of therapeutic compounds into specific target cells. Notably, the use of nanobubbles (NBs) improves transfection efficiency because transfection efficiency is increased by increasing the number of bubbles that physically interact with cells\(^{(145, 146, 148)}\). NBs are smaller than microbubbles; therefore, the area induced by impulsive pressure from collapsing NBs can be limited and direct damage to non-targeted cells caused by the impulsive pressure is reduced. Increasing the concentration of NBs per unit volume can increase adherence to the cell membrane, resulting in an increase in transfection efficiency\(^{(148)}\). Thus, NBs are applicable for gene delivery via the EPR effect\(^{(149)}\). In addition, accumulation of NBs in tumors can be confirmed using US imaging because NBs can be used as US contrast agents, hence US triggered transfection is possible\(^{(145, 149)}\).

Our ultimate goal is to develop a gene delivery system using NMBs and US that can spatially and temporally control gene delivery. In this study, as the first step, we
have evaluated anti-tumor effects of TNF-α following transfection of TNF-α plasmid DNA into solid mouse tumors using the NMBs and US gene delivery system.

2.3. Materials and Methods

2.3.1 Animal tumor models

This study was approved by the Animal Care Committee of Tohoku University. All animals were treated in accordance with the ethical guidelines of Tohoku University.

Murine breast carcinoma (EMT6) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). EMT6 cells stably expressing the luciferase gene (EMT6-luc) were prepared by transfection of EMT6 cells with pEGFPLuc (BD Biosciences, Franklin Lakes, NJ, USA) using Lipofectin Transfer Reagent (Invitrogen, Carlsbad, CA, USA)\(^{150}\). EMT6 and EMT6-luc were cultured under standard conditions in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (BioWest, Nuaille, France) and 1% L-glutamine-penicillin-streptomycin (Sigma-Aldrich), while 1% Geneticin (G418 sulfate, 1mg/mL; Sigma-Aldrich) was added to the medium for EMT6-luc\(^{150}\). Cells cultured in a 10-cm culture dish and a T-175 culture flask was maintained in a humidified incubator at 37°C under an atmosphere of 5% CO\(_2\) and 95% air. Cells were routinely verified by morphology and growth characteristics using Trypan blue (Invitrogen). Cells were tested for mycoplasma contamination on the day of the inoculation, day 0, using MycoAlaert Mycoplasma® Detection Kit (Rockland, ME,
USA) according to the manufacturer’s protocol. Mycoplasma-negative cells were used for this study. EMT6 cells either with or without luciferase expression (1 × 10^6 cells) in 100 μL saline were injected intradermally into the right and left flanks of 6-7 week-old male CB17/Icr-Prkcsid/Crlcrj (SCID) mice (Charles River Japan Inc., Kanagawa, Japan). The mouse’s back was depilated using a commercial electric shaver and cream on the day before inoculation of the tumor cells.

2.3.2. Plasmid Vectors

pGL3-Control Vector plasmid which includes SV40 promoter and encodes the luciferase reporter (pGL3; 5256 bp; Promega, Madison, WI, USA; Fig. 2-1A), pORF9-mTNF-α containing the mouse TNF-α gene (pTNF-α; 3746 bp; Invitrogen; Fig. 2-1B), and pOFR9-mcs, the mock of pORF9-mTNF-α, (pTNF-α-MOCK; 3023 bp; Promega; Fig. 2-1C) were used in this study. Plasmid DNA was purified by an endotoxin-free QIAGEN Mega plasmid purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s protocol.

2.3.3. Preparation of NMBs

The NMBs used in this study were ALs (151). The ALs (lipid concentration, 1 mg/mL) were composed of 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC) (NOF Corporation, Tokyo, Japan) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-methoxy-polyethylene glycol [DSPE-PEG (2k)-OMe; 94:6 (m/m); NOF Corporation] containing C₃F₈ gas. Liposomes were prepared by the reverse phase evaporation method (152, 153). ALs were created from the liposome by sonication in the
presence of C₃F₈ gas for 1 min with a 20-kHz sonicator (Vibra Cell; Sonics & Materials Inc., Danbury, CT, USA) (Fig. 2-2) (145, 149). Figure 2-3 shows the morphological characteristics of ALs. AL solution includes NMBs with a diameter of up to about 30 μm showing in Fig. 2-3-A while two peaks are observed indicating diameters of 200 nm and 15,700 nm in Fig. 2-3-B (149). The mean diameter is 199 ± 84.4; < 0.01% are ALs with diameters exceeding a few micrometers (149). In this study, nano- and micro-sized mixed bubbles were used. Nano-sized bubbles were not separated with micro-sized bubbles because this study does not aim to demonstrate the effectiveness of passive targeting but of the use of bubbles and US for gene delivery into tumor cells. The zeta potential of ALs was -2.1 ± 0.9 mV (149).

2.3.4. US

A 1 MHz submersible US transducer with a diameter of 30 mm (Honda electronics, Toyohashi, Japan) was used for this study. The transducer was located in a test chamber (220 mm × 360 mm × 260 mm) filled with tap water. Signals of 1MHz were generated by a multifunction synthesizer (WF1946A; NF CO., Yokohama, Japan) and amplified with a high-speed bipolar amplifier (HSA4101, HF CO.). The pressure values were measured using a polyvinylidene fluoride (PVDF) needle hydrophone (PVDF-Z44-1000; Specialty Engineering Associates, Soquel, CA) at a standoff distance of 10 cm from the transducer surface by using a stage control system (Mark-204-MS, Sigma Koki, Tokyo, Japan) (Fig. 2-4).
2.3.5. **Plasmid transfection**

Ten microliters of pGL3 (1 μg/μL), pTNF-α (5 μg/μL) or pTNF-α-MOCK (5 μg/μL) with/without NMBs (15 μL) and saline were injected intratumorally in a total volume of 30 μL, and tumors were exposed to US on days 2, 4, 7 and 9 (2 times/week for 2 weeks). US (intensity, 3.0 W/cm²; pressure, 0.12 MPa; duty cycle, 20%; number of pulses, 200; and exposure time, 60 sec) was generated by the 1 MHz submersible US transducer (Honda electronics, Toyohashi, Japan). Mice in the control group are given intratumoral injection of 30 μL of saline on days 2, 4, 7 and 9. Each trial is independent but the mean values in the control group were used for evaluation of treatment efficiency of single or repeated NMBs and US transfection of TNF-α gene and anti-tumor effect of NMBs and US transfection of the TNF-α gene because the condition in the control group is the same. Since the focus position of the US transducer was 10 cm away from the surface (Fig. 2-5A), the tumors were positioned 10 cm from the transducer surface in tap water at 37°C and exposed to US (Fig. 2-5B). The detailed experimental procedures are based on our previous study (111, 150, 154, 155). Attenuation [dB] was given by the equation: 1/2 (frequency [MHz]) (path length [cm]) (156). US intensities, 9.5 W/cm², were attenuated to 3.0 W/cm² at the tumor.

2.3.6. **Monitoring gene expression and measurement of tumor volume**

To evaluate transfection efficiency in vivo, pGL3, expressing luciferase, was transfected to EMT6 cells (which do not express luciferase). To evaluate treatment efficiency of TNF-α delivery, TNF-α plasmid, which does not express luciferase, was
transfected to EMT6-luc cells that did express luciferase. Levels of gene expression and tumor volume were quantified on days 2, 4, 7, 9 and 11. *In vivo* bioluminescent imaging was performed using an IVIS Lumina (Xenogen, Alameda, CA, USA) and the luciferase activity was quantified according to the manufacturer’s protocol (150, 154). Briefly, mice were injected intraperitoneally (i.p.) with D-Luciferin Potassium Salt (150 μg/g body weight, Wako, Osaka, Japan) and anesthetized isoflurane (Abbott Japan, Tokyo, Japan). Ten-minute after the luciferin administration, the bioluminescence signals were monitored for 1 to 10 sec. The signal intensity was quantified as the sum of all detected photon counts within the region of interest (ROI) after subtraction of the measured background luminescence. Tumor size was measured using a mechanical caliper and tumor volume was estimated according to the formula: 0.5 × (short axis)² × (long axis) [mm³] (157).

2.3.7. Vessel imaging and quantification of tumor vessel area using contrast enhanced US imaging

Vessel imaging of tumors was performed using contrast enhanced US imaging. Tumors were imaged using a high-frequency US imaging system (Vevo770; VisualSonics, Toronto, Canada) with a 40 MHz transducer (RMV-704; VisualSonics) on day 1 (before treatment), day 5 (after two treatments), and on day 10 (after four treatments). Sonazoid® (Daiichi Sankyo, Tokyo, Japan) was used as an US contrast agent because echogenicity stability of Sonazoid® in B-mode images are longer than that of ALs *in vivo* (Fig. 2-6A). After tail vein injection of bubbles, the gray scale increases dramatically and then decreased gradually to the background level within 6
min with ALs while it increases dramatically and stable after 2 min for more than 10 min with Sonazoid® (Fig. 2-6A) \(^\text{(158)}\). Sonazoid® was prepared according to the manufacturer’s instructions. Before and 3 min after intravenous tail vein bolus injection of Sonazoid in a total volume of 100 μL, consecutive B-mode images with a slice thickness of 100 μm were captured throughout the whole tumor for 3D imaging (Fig. 2-6Ba). The difference in video intensities between pre-injection and post-injection image frames was indicated in green overlay on the B-mode anatomical images using the accompanying software (VisualSonics). The difference indicated in green area is considered as the extracted vessel image (Fig. 2-6Ba and b). From the constructive 2D images acquired with a slice thickness of 100 μm were reconstructed into a 3D image (Fig. 2-6Bb) \(^\text{(158)}\). The volume consisting of multiple polygons was calculated. The tumor boundaries were manually traced in sequential, parallel US scans according the anatomical and acoustic characteristics, and the tumor volume and intratumoral vessel area were calculated by the analysis software (VisualSonics) \(^\text{(158)}\). Intratumoral vessel area was calculated by multiplying the percentage of vessel area by the tumor volume. Throughout the imaging session, mice were kept anesthetized with 2% isoflurane in oxygen at 1 L/min on a heated stage at 38°C according to the manufacturer’s protocol. Respiratory gating was used to synchronize data acquisition with the mouse respiratory cycle to reduce motion artifact during image analysis.

### 2.3.8. Blood biochemistry tests

To evaluate the acute toxicity of this transfection method, biochemical serum tests were used. Liver and kidney injuries were evaluated by measuring blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). On day
11, each mouse in the control and treatment groups was subjected to blood sampling from the caudal vena cava under general anesthesia. Thirty minutes after sampling, blood samples were centrifuged (1,200 g, 5 min) to obtain serum to measure BUN, AST and ALT levels. Samples were measured by Oriental Yeast (Tokyo, Japan).

2.3.9. Tumor tissue sampling

On day 5 or after blood sampling on day 11, solid tumors were removed and cut perpendicularly against the tumor surface into two parts to coincident each cross section with the B-mode image; half was used for mRNA quantification and the other half was used for histological analysis. The samples for mRNA quantification were frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until use. The samples for histological analysis were mounted in optimum cutting temperature (OCT) compound (Sakura Fineteck Japan, Tokyo, Japan) carefully not to slant the sample and frozen with liquid nitrogen and stored at \(-80^\circ\text{C}\) until use.

2.3.10. Real-time quantitative PCR

For mRNA quantification, frozen tissue was ground using a mortar and pestle and total RNA was isolated with an RNeasy Mini Kit (QIAGEN) and treated with the RNase-Free DNase Set (QIAGEN) according to the manufacturer’s protocol. Reverse transcription was performed using the RNA PCR Kit (AMV; Takara Bio, Shiga, Japan) according to the manufacturer’s protocol. For quantitative real-time PCR (qPCR), duplicate reactions of 300 ng cDNA were amplified with gene-specific primers using Brilliant II Fast SYBR Green (Stratagene, Santa Clara, CA, USA) and run in an
MX3000P (Stratagene) according to the manufacturer’s protocol. In the optimization experiment for qPCR, transferin receptor (TFRC) and β-actin were chosen for housekeeping genes. There was difference in CT values with less than 0.5 cycles between the control and the treatment group for TFRC while there was with more than 2.6 for β-actin (data not shown). Expression of the genes of interest (TNF-α, caspase-3, and p53) was normalized against a housekeeping gene (TFRC) and fold change was determined relative to the control. PCR primers (forward, reverse, accession number) used were summarized in Table 2-1.

### 2.3.11. Histological analysis

With day 5 samples, duplicate serial sections cut at 8 µm were prepared: for anti-CD31+DAPI and Hematoxylin and Eosin (HE) staining. With day 11 samples, duplicate serial sections cut at 8 µm were prepared: for anti-cleaved-caspase-3+anti-p53+DAPI and HE staining. For immunohistological analysis, expression of CD31, cleaved caspase-3, and p53 were analyzed in tumor samples. Frozen sections were fixed in 4% paraformaldehyde for 15 min at room temperature and washed with PBS. The primary antibodies (Purified Rat Anti-Mouse CD31 (MED13.3), BD Pharmingen, San Diego, CA, USA, at 1:100 dilution; Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb, Cell Signaling, Danvers, MA, USA, at 1:200; and p53 (1C12) Mouse mAb, Cell Signaling, at 1:1000) diluted in PBS with 3% bovine serum albumin (BSA) and 0.1% Triton X (CD31) or with 5% BSA and 0.3% Triton X (caspase-3 and p53) were applied overnight at 4°C. After washing with PBS, slides were incubated at 4°C with the secondary antibodies: goat anti-rat Alexa 555 (Invitrogen, CD31), goat anti-rabbit Alexa Fluor 555 (Invitrogen, cleaved caspase-3), or goat anti-mouse Alexa 488 (Invitrogen, p53) diluted
in PBS with DAPI (100 ng/mL) for 40 min in a moisture chamber. After washing with PBS, sections were mounted using VECTASHIELD mounting medium (Vector, Burlingame, CA, USA). Cytotoxic area was analyzed following HE staining performed according to standard procedures. All histological images were captured using a fluorescence microscope (BX51, Olympus, Tokyo, Japan) at \( \times 100 \) magnification. For quantification of vessel density, the number of pixels that represents positive staining by CD31 was quantified using Photoshop CS3 (Adobe Systems, San Jose, CA, USA). For quantification of vessel density in the peripheral area of tumors, 2 randomly selected images (control: \( n = 7 \) (4 mice) and treatment: \( n = 6 \) (4 mice)) avoiding cytotoxic areas were captured and the percentage of vessel area from each sample was obtained. For quantification of vessel density and cytotoxic area over the entire tumor area, the images acquired with \( \times 100 \) magnification were merged using Photoshop CS3 (Adobe systems).

### 2.3.12. Statistical analysis

Data are expressed as mean ± SEM where indicated. Statistical differences were analyzed using the Kruskal-Wallis test and the Mann-Whitney U test. Differences of \( P < 0.05 \) were considered significant.
2.4. Results

2.4.1. Comparison of transfection efficiency between single and repeated NMBs and US transfection

The method with NMBs and US is able to deliver exogenous molecules repeatedly. First, we investigated the transfection efficiency between single and repeated transfection by NMBs and US. Figure 2-7A shows time-dependent gene expression of a luciferase reporter gene (pGL3) transfected into EMT6 cells using NMBs and US. With a single transfection, gene expression was maximal the day after transfection and decreased gradually to the background level by day 11. On the other hand, repeated transfection resulted in persistent gene expression. After the second transfection, the luciferase gene expression in the repeated transfection group was increased compared to that in the single transfection group. After the third transfection, the gene expression reached the maximum and became persistent by the following transfection. There was no significant difference in tumor growth in single and repeated transfection groups (Fig. 2-7B); the gene expression levels did not depend on the tumor size.

2.4.2. Comparison of treatment efficiency between single and repeated NMBs and US transfection of the TNF-α gene

Next, treatment efficiency of repeated NMBs and US transfection of the TNF-α gene was evaluated by transfecting pTNF-α into EMT6 cells expressing luciferase. Luciferase activity, which represents tumor size \(^{(150)}\), was decreased after the first
treatment (both single and repeated transfection groups) compared with that of the control group (Fig. 2-8A). However, after the second measurement, there was no significant difference in the luciferase activity between the single transfection group and the control group, while the treatment effect was sustained in the repeated transfection group. Figures 2-8A and 4B show significant tumor regression by repeated transfection of the TNF-α gene. The TNF-α protein level in tumor on day 11 both in the control and treatment group was not detected with Enzyme-Linked Immunosorbent Assay (ELISA) (data not shown). At the mRNA level, TNF-α expression in tumors on day 11 was 2.4 times higher in the single transfection group (P < 0.05) and 6.9 times higher in the repeated transfection group (P < 0.01) compared to that in the control group (Fig. 2-9).

2.4.3. Effectiveness of NMBs and US transfection of the TNF-α gene

Effectiveness of repeated NMBs and US transfection of TNF-α was evaluated by transfecting pTNF-α or pTNF-α-MOCK into EMT6 cells expressing luciferase in Fig. 2-10. Tumor volume (Fig. 2-10A) and luciferase activity (Fig. 2-10B) decreased significantly only in the TNF-α+NMB+US group compared to that in control group from day 7 and day 4, respectively. There was a difference in the number of days needed to show a statistical difference in tumor size between the measurements of luciferase activity, measured by the bioluminescent imaging system, and tumor volume, calculated from mechanical caliper measurements. This is because of the difference in measurement accuracy. Luciferase activity is measured from the expression of each single cell and the level of expression represents tumor size (159). On the other hand,
mechanical calipers measure overlying skin and surrounding non-tumor tissue in addition to the tumor itself, leading to inaccuracy of tumor volume, especially in small tumors. There were no significant differences between the tumor volume (Fig. 2-10A) and luciferase activities (Fig. 2-10B) in the control, MOCK+NMB+US or TNF-α groups.

2.4.4. Acute toxicity by NMBs and US

To determine whether there were any toxic responses with this method, blood samples and changes in body weight during the study were analyzed and compared with those of control animals (Table 2-2). Mice in the control group were injected with saline in their solid tumors while mice in the treatment group were transfected with pTNF-α in their solid tumors using NMBs and US. There was no significant difference in BUN, AST or ALT levels between groups, indicating that there was no acute toxicity to liver or kidney resulting from this method. In addition, there was no significant difference in the body weight increase (day 0-11) between the groups.

2.4.5. Change in tumor vessel density

To evaluate these anti-tumor effects, vessel area in tumors were analyzed using contrast enhanced US imaging (Fig. 2-11) and histological analysis (Fig. 2-12). Mice in the control group were injected with saline in their solid tumors while mice in the treatment group were transfected with pTNF-α in their solid tumors using NMBs and US. The same volume of saline into the tumor was injected in the control group as the treatment group. Fig. 2-11A shows vessel extraction images by the US imaging system.
Extracted vessels are indicated in green and tumor areas are enclosed in red lines. Significant reductions in normalized tumor vessel area from contrast enhanced US images in the treatment group were observed on day 5 and 10 (Fig. 2-11Aa and B). On the other hand, tumor vessel density was significantly decreased by the TNF-α treatment on day 5 but not on day 10 (Fig. 2-11Ab and C).

In this study, CD31-antibody was used to identify endothelial cells in tumors. Day 5 tumor sections stained for CD31 showed a significant decrease in vessel area in the treatment group, consistent with the contrast enhanced US imaging results (Fig. 2-11B). For histological analysis, adjacent sections of those for CD31 staining were stained with HE. Cytotoxic areas in tumors were increased by treatment on day 5 but there was not significant difference between cytotoxic area in tumor in the control group and that in the treatment group on day 10 (Fig. 2-12C).

### 2.4.6. Apoptosis and tumor suppression factors

To evaluate the cytotoxic activity of TNF-α in NMBs and US transfected tumors, levels of caspase-3 and p53 expression were evaluated by immunohistological analysis (Fig. 2-13) and by qPCR (Fig. 2-14). Mice in the control group were injected with saline in their solid tumors while mice in the treatment group were transfected with pTNF-α in their solid tumors using NMBs and US. The same volume of saline into the tumor was injected in the control group as the treatment group. Colocalization between p53 and DAPI was checked with sections stained for those in higher magnification (×600) and p53-positive cells were detected only in treatment group but not in control group (data not shown). Figures 2-13A-B are merge images of cleaved caspase-3 (C-D, red), p53 images (E-F, green), and DAPI (blue) showing the localization of expressing
cleaved caspase-3 and p53 at the center of the tumor. Cleaved caspase-3 was detected in either treated or untreated tumors (Figs. 2-13C-D). qPCR showed that caspase-3 mRNA levels in the treatment group were two times higher compared to that in the control group (Fig. 2-14A). These results indicate that apoptosis was induced in tumors in the control group and activated by the treatment. At the same time, in the treatment group, apparent increases in p53 expression, both at the protein and mRNA levels were detected (Fig. 2-13E-F and Fig. 2-14B).

2.5. Discussion

TNF-α is a cytokine that can initiate tumor cell apoptosis by disrupting vasculature and can exert cytotoxic effects on a wide range of tumor cells. TNF-α shows promise for the treatment of cancer and its efficacy has been widely studied. However, the clinical use of TNF-α has been limited because of severe systemic toxicity. Here we show that repeated TNF-α gene delivery using NMBs and US could lead to the increased gene expression of TNF-α within a tumor that permitted anti-tumor effects, including activation of p53-dependent apoptosis, decrease in tumor vessel density, and suppression of tumor size, but that reduced the acute toxic effects.

Sonoporation using NMBs and US has been reported to result in effective gene transfection of tumors; however, the transgene expression level is transient and decreases with time \(^{(150, 160)}\). Peak expression following DNA delivery was depending on tissues; i.e. gene expression reaches its peak at the next day in solid tumor \(^{(150)}\) and periodontal tissue \(^{(144)}\) and at the 4th day in skeletal muscle \(^{(146)}\) after transfection with
NMBs and US. Using ALs and US, 2.04 ± 1.48 cells per mm² was transfected when β-galactosidase encoding plasmid DNA (7.2kb) was transfected into mouse skeletal muscle (146). In Fig. 2-7, persistent transgene expression by repeated NMBs and US transfection was confirmed by the delivery of a reporter gene. Although gene expression was transient by this method, repeated transfection by NMBs and US could maintain the level of gene expression. In fact, Fig. 2-8A shows significant tumor regression by repeated transfection of the TNF-α gene. In Fig. 2-9, at the mRNA level, gene expression of TNF-α was induced even with a single transfection and was sustained in the tumor; however, the expression level was not enough to demonstrate a treatment effect. Repeated transfection of TNF-α gene is needed to induce significant tumor regression. The TNF-α protein levels in tumor on day 11 after the TNF-α treatment was lower than the detection limit of the ELISA (15 pg/mL). Regulation of protein synthesis at the translational level was depressed on day 11 because the secretion of TNF-α protein is suppressed within 48 hours of TNF-α gene transfection in tumor in vivo (142, 161) where the last treatment was on day 9. The low levels of secretion of TNF-α protein was sufficient to affect the tumor growth and to reduce the acute toxic effects.

One of the anti-tumor activities of TNF-α is mediated by cytotoxicity to endothelial cells (130-133). To evaluate these anti-tumor effects, vessel area in tumors were analyzed using contrast enhanced US imaging in Fig. 2-11 and histological analysis in Fig. 2-12. Significant reductions in normalized tumor vessel area measured with contrast enhanced US imaging in the treatment group were observed (Fig. 2-11B). This result indicates that the TNF-α treatment induced anti-angiogenic effects. On day 5, a significant reduction in tumor vessel density measured both with contrast enhanced US imaging (Fig. 2-11C) and sections stained for CD31 (Fig. 2-12A) in the treatment group were
observed. Cytotoxic area confirmed with HE staining was increased by the treatment on day 5 (Fig. 2-12C). The results on day 5 indicate the TNF-α treatment induces anti-angiogenic effects resulting cell death in tumor. However, on day 10, there was not difference between vessel density in the control group and that in the treatment group (Fig. 2-10C). In the control group, intrinsically cell death area is developed and increases as tumor grows due to lack of nutrients at the tumor center (162). On the other hand, the cytotoxic area was expanded by the TNF-α treatment. Therefore, there was not a significant difference between the cytotoxic area in the control group and that in the treatment group on day 10 (Fig. 2-12C).

Direct cytotoxicity on tumor cells through apoptosis, which is another antitumor activity of TNF-α was also evaluated (133). To evaluate the cytotoxic activity of TNF-α in NMBs and US transfected tumors, levels of caspase-3 and p53 gene expression were evaluated by immunohistological analysis (Fig. 2-13) and by qPCR (Fig. 2-14). Apoptosis induced by TNF-α is mediated through caspase activation and caspase-3 acts as initiator and executor at the end of the cascade in the apoptotic process (163). Expression in cleaved caspase-3 is increased by the absence of oxygen (164). Because cell death in the center of solid tumor was detected both in the control and treatment group (Fig. 2-12C), expression of cleaved caspase-3 was confirmed both at the mRNA and protein levels although the expression of cleaved caspase-3 was increased by the TNF-α treatment. p53, a nuclear transcription factor, regulates genotoxic stress to block cell cycle progression and induces apoptosis in TNF-α transfected cells (165, 166). p53 is involved in tumor susceptibility to the cytotoxic action of TNF-α and has a role of triggering apoptosis in response to TNF-α (165). The expression of p53 at the protein (Fig. 2-13) and mRNA (Fig. 2-14) levels were increased by the TNF-α-treatment. In this
study, TNF-α-treatment resulted in the accumulation of p53 and in the induction of apoptosis at the center of the tumor where pTNF-α was injected and transfected. Although the cellular mediator of TNF-α-promoted p53 accumulation and genotoxic stress is unknown in this paper, p53 was required to promote TNF-α-induced cell death and to help achieve the significant therapeutic effect.

2.6. Limitations of this study

In this study, antitumoral gene, TNF-α plasmid DNA and NMBs were injected directly in solid tumors and transfected into tumor cells; therefore, the gene delivery approach in this study was not clinically feasible. However, the effectiveness of this deliver method for clinical application can be assessed by targeting cancer, which regional administration is effective. In chapter 3, to propose a novel cancer therapy using NMBs and US, gene delivery was evaluated in the bladder, used as a regional delivery target.

In addition, the gene delivery approach in this study lacks selectivity to tumor cells. The NMBs used in this study were ALs, which were composed of pegylated liposomes encapsulating C₃F₈ gas and liquid (149). AL numbers can be increased at the tumor site by active accumulation using binding antibodies or ligands against tumor cells attached to PEG on the AL surface, by passive accumulation via the EPR effect because of the size distribution when systemically administered, and/or by physically guiding ALs using US as external physical force, namely using acoustic radiation force (149). Moreover, oligonucleotides can be encapsulated within ALs (167) and ALs provide enhanced echogenicity as echo contrast agents both in vitro and in vivo (145, 149). In the
next chapter, a physical targeting approach using US is evaluated while accumulation and collapse of ALs were confirmed with an US imaging modality for increased selectivity to tumor cells.

2.7. Conclusion

In this study, the effectiveness of the TNF-α gene delivery into tumor cells using NMBs and US. Utilizing the properties of NMBs effectively, therapeutic gene encapsulated in NMBs, would be transfected to tumor cells in a spatial and temporal manner as triggered by US exposure while accumulation at the tumor site is confirmed by an US imaging device. The study opens new possibilities for cancer gene therapy using the NMBs and US gene delivery system. This study opens new perspectives for the TNF-α cancer gene therapy by NMBs and US.
**Fig. 2-1 Plasmid vectors.** (A) pGL3-Control Vector plasmid which includes SV40 promoter and encodes the luciferase reporter (pGL3; 5256 bp; Promega), (B) pORF9-mTNF-α containing the mouse TNF-α gene (pTNF-α; 3746 bp; Invitrogen), and (C) pOFR9-mcs, the mock of pORF9-mTNF-α, (pTNF-α-MOCK; 3023 bp; Promega) were used in this study.
**Fig. 2-2 Liposome and acoustic liposome.** Acoustic liposomes were created from the liposome by sonication in the presence of C₃F₈ gas. This figure is based on Kodama *et al.* (149).
Fig. 2-3 Morphological characteristics of AL. (A) A representative image of Dark field image. (B) A representative scattering intensity distribution (%) and cumulative absolute frequency (%) of ALs. (C) A representative number distribution (%) and cumulative absolute frequency (%) of ALs. Both are measured using dynamic light scatting. There are two peaks indicating diameters of ~200 nm (↓) and 15700 (↓). The arrows (↓) indicate the line of the cumulative absolute frequency (%). This figure is based on Kodama et al. (149).
Fig. 2-4 Measurement of US signals. Signals of US were generated by a multifunction synthesizer and amplified with a high-speed bipolar amplifier. The pressure values were measured using a needle hydrophone by using a stage control system. This figure is used from Watanabe (168).
Fig. 2-5 US exposure to solid tumors of mice. (A) Three-dimensional US intensity profile of a 1 MHz US transducer with a diameter of 30 mm. (B) Tumors were positioned 10 cm from the transducer surface in tap water at 37°C and exposed to US (intensity, 3.0 W/cm²; pressure, 0.12 MPa; duty cycle, 20%; number of pulses, 200; and exposure time, 60 sec).
Fig. 2-6 Tumor vessel imaging using contrast enhanced US imaging. (A) Echogenicity stability of Sonazoid® and ALs in B-mode images in vivo. Mean grayscale is normalized with that before bubbles injection. After tail vein injection of bubbles, the gray scale increases dramatically and then decreased gradually to the background level within 6 min with ALs while it increases dramatically and stable after 2 min for more than 10 min with Sonazoid®. (B) Consecutive B-mode images with a slice thickness of 100 µm were captured throughout the whole tumor (a) for 3D imaging (b). The difference in video intensities between pre-injection and post-injection image frames was indicated in green overlay on the B-mode anatomical images. Tumor boundaries were traced in red. Intratumoral vessel area was calculated by multiplying the percentage of vessel area by the tumor volume.
Fig. 2-7 Evaluation of transfection efficiency of single or repeated transfection by NMBs and US. (A) Time-dependent changes in gene expression level. (B) Changes in tumor volume. Luciferase reporter plasmid DNA (pGL3 control vector) was transfected on day 2 in the single transfection group and on days 2, 4, 7, and 9 in the repeated transfection group. Gray arrows indicate the days on which tumors both in the single and repeated transfection groups were transfected (day 2). Black arrows indicate the days on which tumors in the repeated transfection group were transfected (day 4, 7, and 9). n = 12 (6 mice) in each group; mean ± SEM. *P < 0.05 and **P < 0.01.
Fig. 2-8 Evaluation of treatment efficiency of single or repeated NMBs and US transfection of the TNF-α gene. (A) Normalized luciferase activity. Control (white circles): n = 64 (32 mice), single transfection (gray circles): n = 16 (8 mice), repeated transfection (black circles): n = 42 (21 mice). Data are normalized against data on day 2. Gray arrows indicate the days on which tumors both in the single and repeated transfection groups were transfected (day 2). Black arrows indicate the days on which tumors in repeated transfection group were transfected (day 4, 7, and 9). Mean ± SEM. NS: not significant and **P < 0.01. (B) Representative images of luciferase bioluminescence in each group.
Fig. 2-9 TNF-α Gene expression. (A) Fold change of TNF-α mRNA levels in tumor quantified by qPCR, normalized against expression of the TFRC housekeeping gene. For qPCR, samples on day10 were used (n = 6: 3mice, in each group).
Fig.2-10 Anti-tumor effect of NMBs and US transfection of the TNF-α gene. (A) Normalized tumor volume. (B) Normalized luciferase activity. Plasmid DNA (Mock or TNF-α) or saline was injected on days 2, 4, 7 and 9. pTNF-α or pTNF-α-MOCK was transfected with US and NB while saline was injected in the control group on days 2, 4, 7 and 9. Control (white circles): n = 64 (32 mice), mock+NB+US (white squares): n = 24 (12 mouse), TNF-α (white triangles): n = 18 (9 mice), TNF-α+NB+US (black circles): n = 42 (21 mice); Data are normalized against data on day 2. Mean ± SEM. **P < 0.01 (Compared with data in the control group).
Fig. 2-11 Evaluation of vessel density in tumors measure with an US imaging system. (A) Vessel extraction images by the US imaging system; a: control group, b: treatment group. green: extracted vessels, red: tumor area. (B) Normalized vessel extracted area in tumors by US imaging system. US images were acquired on days 1, 5 and 10. n = 6 (6 mice) in each group. Vessel area was normalized with that on day 1. (C) Vessel extracted densities in tumors by US imaging system. US images were acquired on days 1, 5 and 10. n = 6 (6 mice) in each group. Mean ± SEM. *P < 0.05
Fig. 2-12 Evaluation of vessel density in tumors with histological analysis. (A) fluorescence images stained with anti-CD31 (a and d; red), merge images of (a) and (d) with DAPI nuclear staining (b and e; CD31 in red and DAPI in blue), and HE-stained images (c and f; dashed lines indicate boundaries to obvious cytotoxic area). pTNF-α was transfected with US and NB in the treatment group and saline was injected in the control group on days 2, 4, 7 and 9. For immunohistological analysis (for anti-CD31 staining; C and Eb-h), tumor samples on day 5 were used. For HE-staining, tumor samples on day 5 and 10 were used. Control: n = 7 (4 mice) and treatment: n = 6 (4 mice). Eb (the same section as Ec) and Ed, and Ef (the same section as Eg) and Eh are serial sections. Bars indicated 200 μm. (B) CD31 stained area in tumors on day 5. (C) Cytotoxic area in tumors from HE stained images on day 5 and day 10. Mean ± SEM. NS: not significant and *P < 0.05.
Fig. 2-13 Evaluation of apoptosis and tumor suppressor factors in tumor in response to TNF-α treatment with immunohistochemical analysis. Merge fluorescence images (A-B) of cleaved caspase-3 (C-D, red), p53 images (E-F, green), and DAPI (blue). Bars indicate 200 μm.
Fig. 2-14 Evaluation of apoptosis and tumor suppressor factors in tumor in response to TNF-α treatment with qPCR. (A) Fold change of caspase-3 mRNA levels in tumors by qPCR, normalized to the expression of the TFRC housekeeping gene, determined relative to controls. (B) Fold change of p53 mRNA levels in tumors by qPCR, normalized to the expression of the TFRC housekeeping gene, determined relative to controls. n = 6 (3 mice) in each group. Day 11 tumor samples were used. n = 3 (3 mice) in each group. Plasmid DNA (TNF-α) was transfected with US and NB in the treatment group and saline was injected in the control group on days 2, 4, 7, and 9. Mean ± SEM. *P < 0.05, and **P < 0.01.
Table 2-1 Primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence (5’ to 3’)</th>
<th>Genetic position</th>
<th>Product size (bp)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-TFRC</td>
<td>TCCGCTCGTGAGACTACTT</td>
<td>2103-2122</td>
<td>140</td>
<td>NM_008084</td>
</tr>
<tr>
<td></td>
<td>ACATAGGGCAGAGGAAGTG</td>
<td>2223-2242</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-TNF-α</td>
<td>ATGGCCTCCCTCTCATCAGT</td>
<td>353-372</td>
<td>115</td>
<td>NM_013693</td>
</tr>
<tr>
<td></td>
<td>CACTTGGTGGTTGCTACGA</td>
<td>440-459</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-caspase3</td>
<td>AATGGGCTGTTGAACTGAA</td>
<td>454-473</td>
<td>139</td>
<td>NM_009810</td>
</tr>
<tr>
<td></td>
<td>CATGGAGACAGACAGGGGA</td>
<td>576-595</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-p53</td>
<td>GAAGACAGGCAGACTTTTCG</td>
<td>767-786</td>
<td>139</td>
<td>NM_011640</td>
</tr>
<tr>
<td></td>
<td>TAAGGATAGGTCGCGGTCGTC</td>
<td>886-905</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TFRC, transferin receptor; TNF, tumor necrosis. TFRC was used as a housekeeping gene.
### Table 2-2 Evaluation of acute toxicity

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>treatment</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dL)</td>
<td>25.9 ± 2.50</td>
<td>27.6 ± 4.02</td>
<td>NS</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>46.8 ± 7.04</td>
<td>51.7 ± 7.69</td>
<td>NS</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>23.2 ± 2.48</td>
<td>26.5 ± 6.06</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight change (g)</td>
<td>+2.26 ± 0.99</td>
<td>+2.54 ± 1.25</td>
<td>NS</td>
</tr>
</tbody>
</table>

Blood sample was obtained on day 11. n = 6 (six mice) in each group. Change in body weights are for 12 days (between day 0 and 11). Values are represented as mean ± SEM. NM, not significant; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
Chapter 3

Application of nano/microbubbles and ultrasound to regional cancer therapy for the bladder cancer
3.1. Summary

In chapter 3, to propose a novel cancer therapy using NMBs and US, molecular delivery was regionally evaluated in the bladder, used as a regional delivery target. The urinary bladder is a balloon-shaped closed organ that stores urine. Therefore, therapeutic molecules and NMBs can be infused through catheterization and retained in the bladder and the behavior of NMBs can be controlled by US exposure. A DIUS using NMBs offers opportunities for regional drug delivery. This system consists of low-/high-US intensities. Low-intensity US directs NMBs to targeted cells in the bladder and high-intensity US collapses NMBs for intracellular delivery of exogenous molecules. In the chapter, fluorescent molecules and luciferase expressing plasmid DNA was delivered in the bladder using the DIUS. In the study, this approach results in high localization of molecular delivery and better transfection efficiency could be reached on repeatedly applying low- and high-intensity US cycles while a high-frequency US imaging system characterizes movement and fragmentation of NMBs in the bladder real-time. Performing real-time imaging guidance of NMBs with adequate definition of the region of interest and accurate compensation for motion, minimally invasive therapy using NMBs and US with increased cellular uptake could be achieved.
3.2. Introduction

In chapter 3, to propose a novel cancer therapy using NMBs and US, molecular delivery was regionally evaluated in the bladder, used as a regional delivery target.

Bladder cancer is a cancer of older people. The peak incidence of bladder cancer is at 85 years\(^{(169)}\) and about 70% of bladder cancer patients are over the age of 65 years\(^{(170)}\). With continued aging of the population in developed countries, concerns will certainly continue to grow. Treatment for bladder cancer generally includes conventional therapies such as surgery, radiation, chemotherapy and immunotherapy\(^{(171-176)}\). Although standard treatments have an 80% success rate, the tumor recurrence rate after treatment is high (up to 80%) and 30% of these recurrent tumors are high-grade tumors with highly invasive properties\(^{(174, 175, 177, 178)}\). An effective treatment strategy needs to be established to minimize invasiveness and reduce recurrence rates.

To minimize further compromise of the quality of life and longevity of patients, minimally invasive gene therapy may potentially be an attractive strategy for the management of bladder cancer. Recently, a growing number of preclinical studies have shown that various therapeutic genes, such as cytokine genes and tumor suppressor genes, can enhance antitumor effects and suppress pre-established tumors\(^{(176, 179)}\). The major obstacle in the use of gene therapy is the lack of safe and efficient methods for gene delivery to targeted sites in the bladder. Although researchers have examined and used many new strategies for treating bladder cancer, including virus mediated delivery, particle bombardment and lipofection, there is still a need for effective drug delivery systems that can be used to treat patients with minimal side effects\(^{(176, 180)}\).

It has been shown that NMBs in the presence of US non-invasively enhance the
uptake of exogenous molecules, including therapeutic genes, into various types of cancer cells \(^{(5, 60, 100, 111, 153)}\). Collapsing NMBs and/or the resultant cavitation bubbles in the presence of US with a certain level of acoustic pressure, induce transient membrane permeability in cells that lie within an effective radius from the NMBs \(^{(5, 60, 100, 111, 153)}\). Thus, collapsing NMBs facilitate the entry of exogenous molecules into proximate cells. Guzman et al. have estimated that the range of affected cells is between 3 and 90 times the radius of collapsed bubbles, when applying \(2\cdot817\) J/cm\(^2\)\(^{(181)}\). These findings indicate that transporting NMBs to a region proximal to the targeted cells can increase transfection efficiency.

US applied at very low acoustic pressures can be used to force NMBs to move along the axis of the US beam without disrupting the agent \(^{(182, 183)}\). This type of radiation force can facilitate the movement and accumulation of NMBs within a designated area \(^{(182, 183)}\). US that induces fragmentation of NMBs and facilitates movement of NMBs can be used in sequence to increase both specificity of delivery to the target site and transfection efficiency.

The urinary bladder is a balloon-shaped closed organ that stores urine. Therefore, therapeutic molecules and NMBs can be infused through catheterization and retained in the bladder and US can be used to control behaviors of NMBs. Because exogenous molecules are present in the bladder, they can be diffused only into transiently permeabilized cells because of a concentration gradient.

The NMBs used in this study were ALs, which were composed of liposomes encapsulating \(\text{C}_3\text{F}_8\) gas and liquid \(^{(149)}\). ALs provide enhanced echogenicity as echo contrast agents both \(\text{in vitro}\) and \(\text{in vivo}\) \(^{(145, 149)}\). Figure 3-1 shows extracted vessel images in the liver, lymph node, and solid tumor of mice using ALs. Since a ALs can be
used as an US contrast agent, while a high-frequency US imaging system characterize 
movement and fragmentation of NMBs in the bladder real-time, an image-guided US 
triggered drug delivery system can be achieved. 

In this study, the effectiveness of NMBs and a DIUS to localize molecular delivery 
to a designated area of the normal bladder wall was evaluated as a first step toward the 
development of a novel bladder cancer treatment. For this purpose, (i) \textit{in vivo} local 
delivery and fragmentation of NMBs using a high-frequency US imaging system, (ii) 
localized delivery of fluorescent molecules using confocal microscopy and 
determination of the transfection efficiency with histochemical analysis, and (iii) 
transfection of plasmid DNA and determination of transfection efficiency using a 
bioluminescence imaging system were investigated.

3.3. Materials and Methods

3.3.1. Animal studies

The study was approved by the Institutional Animal Care and Use Committee of 
Tohoku University. All animals were treated in accordance with the ethical guidelines of 
Tohoku University.

3.3.2. Mouse bladder cancer model

Human bladder tumor cells stably expressing the luciferase gene (RT-112-Luc) were 
kindly donated by Director. Yasuhiro Matsumura, Investigative Treatment Division, 
Research Center for Innovative Oncology, National Cancer Center Hospital East.
Six-week-old female BALB/c nu-nu mice (Charles River Japan Inc.) were deeply anesthetized using sodium pentobarbital (50 mg/kg i.p.; Nembutal; Dainippon Sumitomo Pharma, Osaka, Japan). A 24-gauge catheter was inserted into the bladder transurethrally and 100 μL of 0.25% trypsin-EDTA solution (Sigma Chemicals, St. Louis, MO, USA) was infused. After the trypsin was retained in the bladder for 30 min, the apex of the bladder was scratched carefully by the catheter. The bladder was recatheterized and washed with PBS (Sigma Chemicals) and 100 μL of RT-112-Luc cells (2 × 10⁷ cells/mL) was instilled into bladder. The urethra was legated with 3-0 nylon suture and the cell solution was retained for 4 hr until the suture was removed.

The tumor growth was evaluated by measuring tumor volume using a high-frequency US imaging system (VEVO770; Visual Sonics) with a 25 MHz transducer (RMV-710B; Visual Sonics) and the luciferase activity was measured using an in vivo bioluminescence imaging system (IVIS; IVIS Lumina; Xenogen Corp.) on days 8, 15, 22, 29, 36, 43, 50, and 57 after inoculation. For both measurements, mice were anesthetized with isoflurane (Abbott Japan). Using the US imaging system, B-mode images were acquired consecutively over the entire bladder by a 3D motor with a 0.1 mm step size and the tumor volumes were calculated by the manufacturer’s software (VEVO770; Visual Sonics). Using the in vivo bioluminescence imaging system, the light emitted by luciferase was measured for an integration time of 30 sec, 10 min after the i.p. injection of D-luciferin (potassium salt; Promega; 150 mg/g body weight) and the signal intensity was calculated as the sum of the photons detected in the region of interest by the manufacturer’s software (Xenogen Corp.)

The established tumor was confirmed with HE staining. On day 57, the mouse was sacrificed with diethyl ether, and the whole bladder was extracted, embedded in OCT
compound (Sakura Fineteck Japan), and rapidly frozen in a bath of liquid nitrogen. The bladder sample was sectioned at 10 μm using a cryostat microtome (CM3000; Leica, Nussloch, Germany). The sample was stained with HE using a standard procedure.

3.3.3. Preparation of NMBs

The NMBs used in this study were ALs. The materials and methods are referred in 2.3.3. In this study, nano- and micro-sized mixed bubbles were used. Nano-sized bubbles were not separated with micro-sized bubbles because this study does not aim to demonstrate the effectiveness of passive targeting but of the use of physical targeting of bubbles for localized gene delivery in the bladder. Reduction of bubble size can increase the cell-to-bubble ratio, resulting the increase in the transfection efficiency\(^{(39)}\). Therefore, it would have been preferred to separate nano-sized bubbles.

3.3.4. Exogenous molecules

Exogenous molecules, TOTO-3 fluorescent molecules (T-3604; Molecular Probes, Eugene, OR, USA) and the pGL3-control plasmid DNA encoding luciferase (5256 bp; Promega; Fig. 2-1), were used as molecular markers and delivered to the normal bladder wall. The plasmid was propagated and purified according to a previously described method\(^{(5, 60, 100, 111)}\).

3.3.5. US

Figure 3-2 shows a diagram of the condition under which US was applied to move and fragment the NMBs \textit{in vitro}. NMBs were exposed to 0.05 W/cm\(^2\) (0.03 MPa) US
for 10 sec to induce NMB movement to the designated area, whereas they were exposed to 37 W/cm$^2$ (0.75 MPa) for 0.5 or 1.0 sec to destroy the NMBs. A 2.2 MHz 14-mm diameter submersible piezoceramic transducer (Fuji Ceramics, Fujinomiya, Japan) was used for this study. The US pressure generation and measurement at a stand off distance of 1 mm are referred in section 2.3.4. The half width of each pressure profile was 11.3 mm. The US transducer was positioned on the mouse urinarius meatus in the direction of the apex. The distances between the meatus urinarius and the internal urethral, and between the meatus urinarius and the apex were 1.0 cm and 1.6 cm, respectively. The distances were measured with the high-frequency US imaging system (Visual Sonics).

Attenuation [dB] was given by the equation: $1/2 \times \text{(frequency [MHz])} \times \text{(path length [cm])}$ \cite{156}. US intensities, 0.05 and 37 W/cm$^2$, were attenuated to 0.03 and 25 W/cm$^2$ at the apex and to 0.04 and 29 W/cm$^2$ at the internal urethral, respectively. The $MI$ was calculated to be 0.41 for 25 W/cm$^2$, which was above the threshold values ($MI > 0.3$) known to collapse gas-filled bubbles \cite{5,184}.

### 3.3.6. Mouse preparation for molecular delivery

We used 6- to 10-week-old female BALB/c nu-nu mice (Charles River Japan Inc.). Mice were deeply anesthetized using sodium pentobarbital (50 mg/kg i.p.; Nembutal; Dainippon Sumitomo Pharma) and a 24-gauge catheter was inserted and washed with PBS (Sigma Chemicals) before NMBs and exogenous molecules were infused into the bladder through the catheter.
3.3.7. Targeted displacement and collapse of NMBs using DIUS

To achieve local delivery of fluorescence molecules and plasmid DNA, 25% (v/v) of acoustic liposomes (25 mL) diluted with PBS (75 mL) to a total volume of 100 mL was infused into the bladder. The high frequency US imaging system allowed observation of the movement and fragmentation of NMBs within the bladder. An ultrasonic transducer for manipulating NMBs was positioned on the meatus urinarius and the US transducer for imaging was placed on the abdomen. The apex of the bladder was chosen for molecular delivery because the bladder cancer was intended to form on the apex of the bladder in the mouse bladder cancer model. We carried out bubble movement to the designated area, i.e., from the neck to the apex of the bladder using low intensity US (0.05 W/cm\(^2\) for 10 sec) and bubble collapse using high-intensity US (37 W/cm\(^2\) for 1.0 sec).

The translational velocity of an individual NMB was measured by frame-by-frame analysis of B-mode US imaging (frame rate, 29 Hz). The velocity was averaged over 10 randomly selected NMBs using images obtained with the high-frequency US imaging system.

Using images obtained with the high-frequency US imaging system, we also examined changes in B-mode contrast enhancement produced by NMBs with exposure to three sets of low-intensity US (10 sec) and high intensity US (1.0 sec) sequences. The B-mode brightness was measured using the high-frequency US imaging system after each exposure to low- and high-intensity US (at 10, 11, 21, 22, 32 and 33 sec). Supposing that the bladder was an ellipsoid, the X-axis direction was from the apex to the internal urethral and the Y-axis was from the dorsal to the ventral (Fig. 3-3). The third quadrant was defined as the bubble accumulation area (A), whereas the first
quadrant was defined as the non accumulation area (N) (Fig. 3-3). The mean grayscale values in both areas were calculated using the histogram function of Photoshop CS3 (Adobe Systems). Each mean grayscale value at each time was normalized with respect to that of the bladder containing NMBs before exposure to US (at 0 sec).

3.3.8. Delivery of fluorescent molecules

For the delivery of fluorescent molecules, 100 mL of 10 mM TOTO-3 (Molecular Probes) and 25% (v/v) of acoustic liposome (25 mL) diluted with PBS (65 mL; Sigma Chemicals) to a total volume of 100 mL were infused into the bladder. TOTO-3 molecules were delivered by applying 0.05 W/cm² (low-intensity US) for 10 sec and 37 W/cm² (high-intensity US) for 0.5 or 1.0 sec. The movement of NMBs to the designated area and the collapse of NMBs were monitored using the high frequency US imaging system. The acoustic sequences used in this experiment are shown in Fig. 3-2. Immediately after the delivery, the bladders were washed with saline. The anesthetized mice were euthanized with diethyl ether and whole bladders were extracted, embedded in OCT compound (Sakura Fineteck Japan) and rapidly frozen in a bath of liquid nitrogen.

3.3.9. Imaging of the delivery of fluorescent molecules and analysis

The bladder samples were sectioned at 10 μm using a cryostat microtome (CM3000; Leica). Nuclei were counterstained with 100 ng/mL DAPI-staining solution for 5 min at room temperature. Images showing DAPI (excitation, 405 nm; emission, 400 to 450
nm) and TOTO-3 (excitation, 635 nm; emission, 660 nm) fluorescence were captured on a confocal laser scanning microscope (Fluoview FV1000; Olympus) with × 100 magnification. Because the × 100 magnification field alone was not enough to capture the entire bladder, the field view was extended using the photomerge function of Photoshop CS3 (Adobe Systems). The different fluorophores were quantified by Photoshop based image analysis (pixel analysis). Confocal images of the two different chromogens were separately transformed into grayscale and the color of the stained area was changed to white. The total pixel count, at levels 40-255, was determined using a brightness level histogram because the pixel count indicates the surface area of the image. The total pixel count for the DAPI chromogen was defined as the entire tissue section, whereas that for the TOTO-3 chromogens was defined as the area where the fluorescent molecules were delivered.

### 3.3.10. Gene delivery

For gene delivery, 33 mL of 1 mg/mL luciferase expressing plasmid DNA and 25 mL of acoustic liposome diluted with 42 mL PBS in a total volume of 100 mL were infused into the bladder. The NMBs were delivered to the designated area and the luciferase-expressing plasmid DNA was transfected by applying 0.05 W/cm² for 10 sec (low-intensity US) and 37 W/cm² for 1 sec (high-intensity US). After transfection, the bladders were washed with saline and the mice were kept warm until required.

### 3.3.11. Imaging of gene delivery and analysis

The activity of the luciferase gene was measured by using the in vivo
bioluminescence imaging system (IVIS; IVIS Lumina; Xenogen Corp.). D-luciferin (potassium salt; Promega) was intraperitoneally injected at a dose of 150 mg/g body weight. All mice were anesthetized with isoflurane (Forane; Abbott Japan) and at 10 min after the infusion, the light emitted by luciferase was measured for an integration time of 30 sec. The signal intensity was calculated as the sum of the photons detected in the region of interest using IVIS analysis software (Xenogen Corp.).

3.3.1.2. Analysis of bladder inflammation

The local inflammatory reaction in the bladder in response to this method was characterized by an influx of inflammatory cells including polymorphonuclear neutrophils (PMNs) and lymphocytes, hemorrhage, and enhanced submucosal edema (185). Bladder inflammation was evaluated with the bladder samples for the delivery of fluorescent molecules. The bladder samples were sectioned at 10 μm using a cryostat microtome (CM3000; Leica). Sections were stained with HE using a standard procedure. The number of PMNs and leukocytes were counted in three random fields per slides at × 200 magnification. The area of edema was determined from the area of the mucosa/submucosa and normalized per cross-sectional area of the bladder area. The sections for analysis of edema were acquired at × 100 magnification and the cross-section of the entire bladders was extended using the photomerge function of Photoshop CS3 (Adobe Systems).

3.3.1.3. Statistical analysis

All measurements are expressed as the mean ± SEM. The Kruskal-Wallis test and
the Mann-Whitney U test were used to analyze the results. The differences were considered to be significant at $P < 0.05$.

3.4. Results and Discussion

3.4.1. Establishment of mouse bladder cancer model

Since our goal in this section is to establish a NMBs and DIUS delivery system in the bladder, it is critical in animal studies to obtain a suitable bladder tumor model which tumors were formed on the apex of the bladder. Figure 3-4 shows an establishment of a tumor in the mouse bladder. Longitudinal images of a mouse bladder obtained by the high frequency US imaging system show increase in the tumor volume (Fig. 3-4A and C). Longitudinal images of the mouse obtained by the in vivo bioluminescent imaging system show increase in the luciferase activities (Fig. 3-4B and C). Both the HE stained bladder and the US image show the establishment of a tumor formed on the apex of the bladder (Fig. 3-4A and D). Thus, the apex of the bladder was chosen for the target of the molecular delivery.

3.4.2. Analysis of targeted displacement and collapse of NMBs using DIUS

Using B-mode imaging, we captured the movement of NMBs to the designated area and NMB fragmentation. Figure 3-5 shows the accumulation of NMBs to a designated area, i.e., the apex of the bladder, upon the application of low-intensity US and the
collapse of the NMBs upon the application of high-intensity US. Figure 3-5Aa shows a bladder containing NMBs at 0 sec, Fig. 3-5Ab shows the movement of NMBs toward the apex of the bladder upon the application of low intensity US for 10 sec at 10 sec, and Fig. 3-5Ac shows the collapse of the NMBs in the bladder upon the application of high intensity US for 1 sec at 11 sec.

To evaluate the system, we measured the translational velocity and compared it with a theoretical open medium estimation. On performing B-mode US (frame rate, 29 Hz), the NMBs’ absolute velocity as a result of low-intensity US along the x-axis (see Fig. 3-3) was $5.3 \pm 0.8$ mm/sec ($n = 10$; mean $\pm$ SEM). The velocity of NMBs, $U_{rad}$, is given by:

$$U_{rad} = \frac{I_{spta} \omega R_0^2}{\eta c^2}$$

(3-1)

where $I_{spta}$ the spatial-peak temporal-average intensity (0.04 W/cm$^2$ at the internal urethral), $\omega$ the angular driving frequency ($2\pi f$), $R_0$ the NMB equilibrium radius (100 nm), $\eta$ the viscosity of the host water (0.001 Pa $\cdot$ s) and $c$ the speed of sound in water (1497.3 m/sec) (186). The velocity of the NMBs is calculated to be 24 nm/sec. As seen in Fig. 2-3, the mean diameter of ALs is 200 nm (187); however, there are large ALs with a diameter larger than 1571 nm. If the $R_0 = 50$ $\mu$m, then $U_{rad}$ = 6 mm/sec. This value accords with the experimental results.

The calculated scattering cross section of a bubble with a diameter of 200 nm is 0.03 $\mu$m$^2$ (equation 1-4). Because the bubbles size is within the spatial resolution of the US imaging system, the dramatic increase in brightness in the bladder due to the presence of ALs in the US images was contributed because of the aggregation of ALs.

Figure 3-5B indicates the changes in brightness in the bubble accumulation (Fig.
3-3A) and non-accumulation areas (Fig. 3-3N) in the bladder at 3 sets of low- and high-intensity US sequences, normalized with respect to that of the bladder containing NMBs before exposure to US (at 0 sec). Bubble displacement was defined as the decrease in the brightness value in the non-accumulation area. Bubble destruction was defined as the decrease in the brightness value in the bubble accumulation area. A significant decrease was seen in the brightness in the non-accumulation area after the first exposure of low-intensity US for 10 sec (at 10 sec) and in the targeted area after the first exposure of high-intensity US for 1 sec (at 11 sec). This result indicates that NMBs were moved and accumulated in the designated area and destroyed with the DIUS. However, after subsequent cycles of DIUS, there was no significant decrease in either the accumulation or the non-accumulation area.

### 3.4.3. Delivery of fluorescent molecules and transfection efficiency

Fluorescent imaging analysis of TOTO-3 localization was performed and the nuclei were counterstained with DAPI. Figure 3-6A shows representative confocal images of the bladder with the delivery sequences; the fluorescent molecules were delivered in cells in which the NMBs were accumulated and in contact. A confocal image with a higher magnification shows that the fluorescent molecules were delivered to the layer beyond the submucosa in the bladder but not to the muscularis of the bladder (Fig. 3-6B). The accumulation of NMBs with low-intensity US at the designated area in the bladder was shown to be necessary for the efficient transfer of a fluorescent dye to the site.
Figure 3-8 shows the determination of the transfer efficiency of TOTO-3 molecules with DIUS. On repeatedly applying low- and high-intensity US cycles, the area emitting fluorescence caused by TOTO-3 molecules increased (Fig. 3-8). The fluorescence expressing area increases with increasing duration of fragmentation pulses; it increases with increasing acoustic energy. In Fig. 3-5B, the brightness values were decreased after each instance of exposure to high-intensity US, although the decrease was slight. This indicates that the NMBs did not collapse effectively with exposure to high-intensity US with 2.25-MHz US transducers for 1 sec. The reasons for this are: (i) bubbles collapse effectively under optimized US conditions \(^{(188, 189)}\); and (ii) US is reflected at bubble phases consisting of a number of NMBs and is attenuated by NMB oscillation. At the same time, in Fig. 3-5B, it can be seen that there was no statistically significant decrease in the brightness in either the accumulation area or the non-accumulation area as a result of exposure to high-intensity US after the second set of DIUS, although the transfection efficiency increased with repeated exposure to DIUS. It is assumed that this is because shell-coated NMBs collapsed with exposure to high-intensity US into fine, stable gas bubbles acting as cavitation nuclei, which the high-frequency US imaging system could not capture. The transfection efficiency increased because the subsequent sets of low-intensity US sequences resulted in the accumulation of the remaining NMBs and cavitation bubbles created by the stable gas bubbles in the designated area, and high-intensity US oscillated and/or collapsed those bubbles, which then permeabilized the bladder wall.

Shortencarier et al. analyzed delivery of fluorescent molecules to a designated area of the vascular endothelium with and without the accumulation of bubbles \(^{(183)}\). Their findings agree with the results, indicating that bubbles in contact with target cells are
required for the delivery of exogenous molecules to the target site.

### 3.4.4. Gene delivery and the transfection efficiency

Next, the transfection efficiency of the combined use of NMBs and DIUS by monitoring the expression of the luciferase gene in the bladder was investigated. The luciferase gene expression in the bladder with NMBs and one set of DIUS was at a maximum on the first day after transfection and was reduced to the background level on the second day (Fig. 3-9). Therefore, on the day after gene transfection with DIUS, the activity of the luciferase gene was measured using an *in vivo* bioluminescent imaging system (Fig. 3-10). The luciferase activity of treated groups was normalized by that of the control group (mean ± SEM). The luciferase activity was at the background level in mice that were not exposed to US, regardless of whether the NMBs were absent (Fig. 3-10a) or present (Fig. 3-10b). The luciferase activity increased as the number of DIUS cycles increased (Fig. 3-10c–e). As mentioned before (Figs. 3-5 and 3-8), repeated exposure to US facilitates the destruction and oscillation of uncollapsed NMBs and cavitation bubbles during exposure to the first few sets of US sequences, thereby increasing the transfection efficiency. The luciferase activity was higher in the group transfected with NMBs than in the group transfected without NMBs (Fig. 3-10e and f). It is assumed that a gene delivery approach involving NMBs can more easily permeabilize cells with fewer cycles of DIUS compared with the case in the absence of NMBs (Fig. 3-10d and f). The luciferase activity was lower in the group transfected with high-intensity US than that in the group transfected with low-intensity US (Fig. 3-10e and g). This result indicates that low-intensity US is required to deliver NMBs to a designated area to increase transfection efficiency, as shown in the delivery of
fluorescent molecules (Fig. 3-8). NMBs and five cycles of DIUS were needed to demonstrate significant differences in the induction of gene expression from the control group; a positive correlation between the acoustic energy and transfection efficiency in the delivery of both plasmid DNA and fluorescent molecules is brought about by NMBs and DIUS (Fig. 3-11), where the linear curves were fitted with high R-square values of 0.82 for delivery of plasmid DNA and 0.86 for delivery of fluorescent molecules.

3.4.5. Morphological alterations

Figure 3-11 shows morphological changes in the bladder wall by the delivery method. The local inflammatory reaction in the bladder in response to this method was characterized by an influx of inflammatory cells including PMNs and lymphocytes, hemorrhage, and enhanced submucosal edema (185). Tissue damage was evaluated by counting inflammatory cells, presence of bleeding, and appearance of edema in HE-stained sections (Fig. 3-11). Infiltration of both PMNs and lymphocytes were not induced by the delivery method and bladder hemorrhage was not found in the HE section (Fig. 3-11B). In fact, there was no associated hematuria when the targeted delivery system was used. However, the area of the mucosa/submucosa of the treated bladders was significantly increased compared to that of untreated bladders, where the area of edema was determined from the osmotic swelling area of the mucosa/submucosa (Fig. 3-11D). Acute damage to the bladder wall was detected. Immediately after the delivery, the bladders were extracted for histological analysis; therefore, it is not clear if the edema was invertible or non-invertible. Long-term follow-up was needed to determine if the acute damage was instant or permanent.
3.4.6. Limitations of this method

There are some limitations to this method. First, non-invasive, low-intensity US from one direction can accumulate NMBs only in a limited area. However, it is desirable for NMBs to be thrust toward the whole bladder wall because in one-third of patients with bladder cancer, several tumors form simultaneously within the bladder at different locations \(^{(190)}\). A transducer tipped US catheter can be used to manipulate NMBs intravesically. A catheter-delivered transducer-tipped US has already been developed for the treatment of vascular thrombosis and can be used to develop an intravesical US catheter \(^{(191, 192)}\).

Another limitation is the non-specificity of this method toward cancer cells, which can be overcome by using modified NMBs. In this study, the NMBs used were liposomes encapsulating gas and liquid. With liposome technology, NMBs can be modified by (i) introducing a targeted ligand to enhance the selective uptake of therapeutic agents by targeted cells and (ii) encapsulating therapeutic agents so that the agents are released only at the site where the NMBs collapse \(^{(193)}\). This can greatly reduce side effects and the risk of the uptake of therapeutic molecules by normal cells. The encapsulated therapeutic gene and gas, modified with a superficial bladder-targeting ligand, can facilitate localized gene delivery with DIUS and NMBs.

In this chapter, the effectiveness of physical targeting using NMBs and US was evaluated in the bladder; however, when combination of passive targeting through systemic administration with physical targeting using NMBs and US is considered, this system using nano-sized bubbles and a high-frequency US imaging system can only apply for small animals, such as mice. To overcome the limitation of low acoustic reflectivity of nano-sized contrast agents in a clinically used low-frequency US imaging
system, several approaches are introduced. One of the approaches is the use of US-sensitive nanodroplets, whose liquid core can be vaporized into gas, or US-sensitive nanobubbles, which coalesce into larger bubbles, in the field of US are introduced (194-198). For example, if US sensitive nanobubbles are moved to a designated location, coalesced, and collapsed by carefully controlled three different intensities of US, this delivery system can be a clinically viable option for cancer therapy.

3.5. Conclusion

In this study, the ability to deliver molecules to a designated area using NMBs and a DIUS was demonstrated, and the transfection efficacy was shown to depend on acoustic energy. The effectiveness of localized gene delivery by NMBs and DIUS in bladder cancer was described. This method can be a new clinical tool, with applications that will help reduce the recurrence of bladder cancer and mortality. At the same time, this study proposed a regional gene delivery system using NMBs and US, integrating therapeutic interventions with imaging and treatment for regional drug delivery for cancer therapy.
Fig. 3-1 Vessel image extraction in various organs of mice using ALs and a high frequency US imaging system. (A) A brief method for vessel extraction. From b-mode images of before and after tail vein injection of ALs, vessel images in targeted organs are constructed. (B) Extracted vessel images in the liver, lymph node, and solid tumor of mice.
Fig. 3-2 Diagram showing the optimum experimental set-up of a DIUS. For low-intensity US, 0.05 W/cm² was applied for 10 sec and for high-intensity US, 37 W/cm² was applied for 0.5 sec or 1.0 sec.
Fig. 3-3 **Illustration of the experimental set up.** Supposing that the bladder was an ellipsoid, the X-axis direction was from the apex to the internal urethral and the Y-axis was from the dorsal to the ventral. The third quadrant was defined as the bubble accumulation area (A), whereas the first quadrant was defined as the non-accumulation area (N).
Fig. 3-4 Establishment of a mouse bladder cancer model. (A) Longitudinal images of a mouse bladder obtained by a high frequency US imaging system. The white bars indicate 5 mm. (B) Longitudinal images of the mouse obtained by an *in vivo* bioluminescent imaging system. (C) Changes in the volume and the luciferase activities of the mouse. (D) An HE-stained section of the mouse bladder on day 57.
Fig. 3-5 Analysis of targeted displacement and collapse of NMBs using DIUS. (A) A schematic representation of B-mode US imaging scans of the bladder showing NMB behavior on exposure to low- and high-intensity US. After the bladder was filled with NMBs (a, at 0 sec), NMBs were moved toward the designated area with low-intensity US for 10 sec (b, at 10 sec), and the NMBs were collapsed with high-intensity US for 1 sec at (c, 11 sec). (B) Quantification of changes in the brightness value in the bladder with three sets of low- and high-intensity US sequences. n = 4; mean ± SEM. The brightness value in the accumulation and non-accumulation areas at 10, 11, 21, 22, 32, and 33 sec were normalized by that of an image of a bladder containing NMBs but not exposed to US at 0 sec. Open arrows indicate exposure to low-intensity US and closed arrow indicate exposure to high-intensity US (*P < 0.05)
Fig. 3-6 Localization of the delivered fluorescence molecules in the bladder. (A) A representative fluorescent image (cross-section) of a bladder in which TOTO-3 fluorescence molecules were delivered using NMBs and DIUS. For this sample, three sets of low-intensity US (0.05 W/cm² for 10 sec) and high-intensity US (37 W/cm² for 1.0 sec) sequence were used. DAPI stain (blue) shows the location of nuclei in bladder cells. TOTO-3 (red) fluorescence shows the location where the molecules were delivered. (B) A fluorescent image of the bladder wall where fluorescent molecules were delivered with higher magnification. The fluorescent molecules were delivered to the layer beyond the submucosa in the bladder but not to the muscularis of the bladder.
Fig. 3-7 Determination of the transfection efficiency of TOTO-3 molecules using NMBs and DIUS. The area emitting fluorescence increased with an increase in the number of DIUS cycles. n = 4; mean ± SEM.
Fig. 3-8 Time-dependent changes of gene expression with NMBs and one set of DIUS in the bladder. The luciferase gene expression in the bladder with NMBs and one set of DIUS was at a maximum on the first day after transfection and was reduced to the background level on the second day. n = 4; mean ± SEM. *P < 0.05
Fig. 3-9 Quantification of the transfection efficiency of luciferase-expressing plasmid DNA using NMBs and DIUS. The activity of the luciferase gene was measured the day after gene delivery. The luciferase activities of the treated groups were normalized by those of the control group (control, a, b and f: n = 3; c and g: n = 5; and d and f: n = 6; mean ± SEM). *P < 0.05 (Compared with data in the control group).
Fig. 3-10 Relationship between the acoustic energy and transfection efficiency using NMBs and DIUS. The solid and dashed lines are the linear curve fits to the data for transfection efficiency of plasmid DNA and fluorescent molecules, respectively. The luciferase activities of the treated groups were normalized by those of the control group. Acoustic energy (J/cm$^2$) was calculated using the formula:

\[
\text{Acoustic energy (J/cm}^2\text{)} = \text{US intensity (W/cm}^2\text{)} \times \text{exposure duration time (sec)}
\]

\(n = 3\) for fluorescent molecules, white circle; \(n = 6\) for plasmid DNA, black circles; mean ± SEM.
**Fig. 3-11 Cell damage by the delivery method.** The local inflammatory reaction in the bladder in response to this method was characterized by an influx of inflammatory cells including PMNs and lymphocytes, hemorrhage, and enhanced submucosal edema. HE stained sections of untreated (A) and treated (B) bladder wall at × 200 magnification. The sections for analysis of edema were acquired at × 100 magnification and the cross-section of the entire bladders was extended. The area of edema was determined from the area of the mucosa/submucosa and normalized per cross-sectional area of the bladder area.
Chapter 4

Conclusions
Over recent decades, the incidence and mortality rate of cancer have been increased in developed countries, including Japan, and the budget for cancer control has been steadily rose. Although we have been facing such a serious situation and spending a lot of money to tackle cancer, cancer is still a menace.

A gene delivery system using NMBs and US utilizes a mechanism of sonoporation. It uses US to enhance cell permeabilization via the impulsive pressure generated by either collapsing bubbles or the cavitation bubbles created by the collapse, leading entry of therapeutic genes into nearby cells \(^{(38-40)}\). Thus, it allows the non-invasive delivery of therapeutic genes non-invasively into specific target cells. In addition to the non-invasiveness, this system is attracting increasing interest for cancer therapy because of its advantages, including easy operation, low toxicity, low immunogenicity, high tissue selectivity, and repeated applicability \(^{(100)}\). One of the challenges of the gene delivery using NMBs and US is the low transfection efficiency. In this dissertation, a regional gene delivery system using NMBs and US was proposed to increase the transfection efficiency for cancer therapy.

Regional drug delivery is essential cancer therapy because drug delivery efficiency of regional administration is higher than that of systemic administration. To propose a novel cancer therapy, development of a regional cancer therapy based on regional drug delivery is effective. Cancers that regional administration of drugs is effective are brain tumor and liver cancer which arterial injection is effective, ovarian cancer, which intraperitoneal injection is effective, and bladder cancer, which intravesical injection is effective \(^{(43-45)}\). Among them, we focused on bladder cancer.

In the bladder, NMBs can be retained and the behavior of NMBs can be controlled by US exposure. A DIUS using NMBs offers opportunities for regional drug delivery.
This system consists of low-/high-US intensities. Low-intensity US direct NMBs to targeted cells in the bladder and high-intensity US collapse NMBs and increase cell membrane permeability, facilitating entry of exogenous molecules into proximate cells with increased cell-to-bubble ratio. Because the gene delivery method using NMBs and US is non-immunogenic, gene expression in tumor can be maintained to induce therapeutic effects by repeated transfection. On repeated applying low- and high-intensity US cycles, bubbles not collapsed with one cycle of DIUS can again move to and collapse at a designated area, leading increased transfection efficiency with high localization of molecular delivery.

Chapter 2 evaluated the effectiveness of the gene delivery into tumor cells by transfecting an anti-tumoral gene, TNF-α plasmid DNA, repeatedly into mouse solid tumors using NMBs and US. The chapter shows repeated delivery of TNF-α gene using NMBs and US could prolong the TNF-α gene expression within a tumor permitting antitumor effects, demonstrating the effectiveness of the approach to tumor cells.

Chapter 3 evaluated the application to regional cancer therapy by achieving localized delivery of fluorescent molecules and luciferase expressing plasmid DNA in the mouse normal bladder, used as a control of the regional delivery target. This chapter demonstrates the application to regional cancer therapy using NMBs and US by presenting image-guided US triggered drug delivery where a high-frequency US imaging system characterizes movement and fragmentation of NMBs in the bladder, and the transfection efficiency.

In this dissertation, an effective regional gene delivery system with NMBs and US for cancer therapy was proposed with increased delivery efficiency and localization
while performing real-time imaging guidance of NMBs to an adequate region of interest.

The proposed gene delivery system using NMBs and US gives potential for future cancer treatment technology. That is, (1) low-intensity US which moves to target area can be used for targeting technique, (2) high-intensity US which collapse NMBs can be used for drug controlled release technique, and (3) drug delivery using NMBs and US has some advantages, including non-immunogenicity. The development of this system requires the combined use of engineering techniques for the development of procedures, pharmaceutical techniques for the preparation of NMBs, and biomedical research for understanding of the mechanisms behind gene delivery and, in fine, elimination of cancer.
Bibliography


9. Leighton TG. What is ultrasound? *Progress in Biophysics and Molecular


43. Yagishita Y, Kodama T. Delivery of genes/therapeutic molecules using acoustic


2008; 345-348.


86. Mehrmohammadi M, Oh J, Mallidi S, Emelianov SY. Pulsed magneto-motive ultrasound imaging using ultrasmall magnetic nanoprobe. Molecular Imaging


104. Reddy A, Caler EV, Andrews NW. Plasma membrane repair is mediated by


146. Kodama T, Aoi A, Watanabe Y, Horie S, Kodama M, Li L, Chen R, Teramoto N, Morikawa H, Mori S, Fukumoto M. Evaluation of transfection efficiency in


