(Form4-2)

JOINT RESEARCH PROJECT

FINAL REPORT
For Japan-Korea Joint Research Project

AREA	1. Mathematics & Physics 2. Chemistry & Material Science 3. Biology 4. Informatics & Mechatronics 5. Geo-Science & Space Science (6.) Medical Science 7. Humanities & Social Sciences

1. Research Title:

A study of cancer therapy using nanobubbles and ultrasound: Biophysical aspect

2. Term of Research: From July 1, 2010 To June 30, 2012

3. Total Budget

a. Financial Support by JSPS: Total amount: 2,390.640 thousand yen

1st Year 1,000.00 thousand yen 2nd Year 1,200.00 0 thousand yen

3rd Year 190.640 thousand yen

b. Other Financial Support : Total amount: 0 thousand yen

4. Project Organization

a. Japanese Principal Researcher	
Name	Tetsuya KODAMA
Institution / Department Position	Graduate School of Biomedical Engineering Tohoku University Professor
b. Korean Principal Researcher	
Name	Min Joo CHOI
Institution / Department Position	Biomedical Engineering Department, School of Medicine, Jeju National University

c. List of Japanese-side Participants (Except for Principal Researcher)

Name	Institution/Department	Position
Yukio TOMITA	Graduate School of Education/ Hokkaido University of Education	Professor
Hiroyuki TAKAHIRA	Osaka Prefecture University/ Graduate School of Enginering	Professor
Yoko YAGISHITA	Tohoku University/ Graduate School of Dentistry	PhD student
Takashi KOCHI	Tohoku University/ Graduate School of Medicine	PhD student
Takuma SATO	Tohoku University/ Graduate School of Medicin	PhD student
Shota SATO	Tohoku University/ Graduate School of Biomedical Engineering	Master student
Shigeki KATO	Tohoku University/ Graduate School of Biomedical Engineering	Master student
Yuriko HATAKEYAMA	Tohoku University/ Graduate School of Biomedical Engineering	Master student
Tomoharu KOISO	Tohoku University/ Graduate School of Biomedical Engineering	Master student
Mamoru MIKADA	Tohoku University/ Graduate School of Biomedical Engineering	Master student
Yoshinobu MIURA	Tohoku University/ Graduate School of Biomedical Engineering	Master student
Tatsuki OKUNO	Tohoku University/ Graduate School of Engineering	Master student
Tomoaki TAKEMURA	Tohoku University/ Graduate School of Engineering	Master student

d. List of Korean-side Participants (Except for Principal Researcher)

Name	Institution/Department	Position
Kwan Suk KANG	Biomedical Engineering Department, School of Medicine, Jeju National University	PhD Student
Jee Hun OH	Biomedical Engineering Department, School of Medicine, Jeju National University	MSC student

5. Number of Exchanges during the Final Fiscal Year*

a. from Japan to Korea *Japanese fiscal year begins April 1.

Name	Home Institution	Duration	Host Institution

For Final Fiscal Year(FY2012)	For Final Fiscal Year(FY2012)
Total: 0 persons	Total: 0 man-days

Numbers of Exchanges during the Past Fiscal Years

FY2010: Total 2 persons
FY2011: Total 7 persons

b. from Korea to Japan

Name	Home Institution	Duration	Host Institution
Min Joo CHOI	Tohoku University	13-15 June, 2012	Tohoku University
Kwan Suk KANG	Tohoku University	13-15 June, 2012	Tohoku University

For Final Fiscal Year(FY2012)	For Final Fiscal Year(FY2012)
Total: 2 persons	Total: 6 man-days

Numbers of Exchanges during the Past Fiscal Years

FY2010: Total 0 persons
FY2011: Total 2 persons

6. Objective of Research

Sonoporation with low-intensity ultrasound (US) has recently been recognized as a new, safe and noninvasive delivery method using nano/microbubbles (NMBs). When NMBs are collapsed by US, mechanical forces are generated most likely via two different mechanisms. One mechanism is the collapse of the NMBs themselves and the other is bubble cavitation resulting from the collapse of NMBs. The resulting mechanical forces produced by these mechanisms can directly permeabilize the cell membrane, thereby allowing the delivery of exogenous molecules into to the cell/tissue. Therefore, it is likely that the efficiency of molecular delivery can be improved by optimizing the physicochemical parameters of NMBs and the ultrasonic wave characteristics and subsequent dynamics of bubble cavitation.

However, it is hard to establish *in vivo* acoustic detection techniques, theoretical model of *in vivo* wave propagation, animal experiments in a single institute with limited research budget, therefore a collaborative multi-center research system is required.

The aim of the present Japan-Korea Joint Research Project was to establish theoretical models of controlling impulsive pressures of bubble collapse and to develop cancer therapy using US and NMBs. The subjects of the research as follows.

(1) Observation of bubble collapse in the field of ultrasound.
(2) Theoretical analysis of pressure filed in a cell culture plate.
(3) Delivery of fluorescent molecules into cells.

7. Methodology

1. Observation of bubble collapse in the field of ultrasound.

Figure 1 shows a block diagram schematically illustrating the experimental setup. Sinusoidal waves with the frequency of 1 MHz were generated with a multi-purpose synthesizer and amplified through a bipolar amplifier (NF Corporation, WF 1946 A & HAS 4101). They were sent to a concave US probe (Fuji ceramic HPP 1038-SUS) whose geometric focal length was 80 mm. Both continuous waves and burst waves with the duty ratio D (= T_1/T_2) were employed, where T_1 is the duration of sinusoidal waves and T_2 the period of a square wave. As shown in Figure 2, we conducted several experiments by using two types of test sections, termed A and B. Case A is the situation where expanding ultrasonic waves interacted with Sonazoid microbubbles in a suspension with the volume of 1 ml which was injected above a 2 ml agar gel with the mass concentration of 1 %, placing on the bottom of a cuvette. In Case B, on the other hand, ultrasonic waves were focused near the surface of an agar gel which was inserted from above.

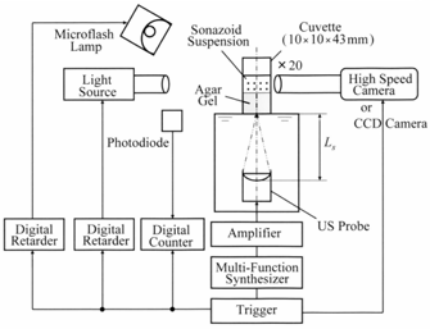


Figure 1: Schematic illustration of experimental setup.

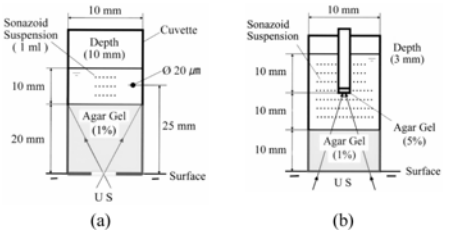


Figure 2: Two types of test section, (a) Case A and (b) Case B.

To visualize phenomena with high magnification, we set a focal plane in a suspension at 1 mm away from the inner observation wall in Case A where the field depth was 10 mm. In Case B, the field depth was 3 mm and the best focus was positioned at 1.5 mm. We should notice that a pressure distribution in a cuvette (Case A) was not uniform. Using distilled water as a test liquid, pressure measurement was conducted by employing a hydrophone (ONDA HNC - 0400). A pressure mainly used in the present experiment was $p_{max,c}$ = 0.92 MPa at the center, while it was $p_{max,e}$ = 0.40 MPa at the observation area. The mechanical index *MI* (= $p_{-,max}/f^{1/2}$) for the latter was determined as 0.4, where $p_{-,max}$ is a negative peak pressure. However it was difficult to measure any pressures precisely in Case B, because the field depth of the vessel was comparable to the diameter of the hydrophone. The ultrasound intensity I (= $p_{max}{}^2/(2\rho c)$) was 5.4 W/cm^2 for the observation area and 28.3 W/cm^2 for the central region in Case A, where ρ is the water density and c the sound velocity in water. The ultrasound energy density E in J/cm^2 can be expressed as

$$E = I \cdot D \cdot T_{ex} \qquad (1)$$

where T_{ex} is the ultrasound irradiation time. Test liquid was a Sonazoid suspension with the volumetric concentration of *C*s, which was diluted with distilled water. Sonazoid contrast agent (Daiichi-Sankyo Co., Ltd.) was used as microbubbles, each of which is a 4 nm shell-encapsulated bubble containing C_4F_{10} gas inside it, and they commonly apply to visualize blood flow around the liver. Sonazoid particles of 16 μL were mixed with 2 mL water for injection to make an undiluted solution of 2.016 mL which was defined as a 100% Sonazoid suspension (*Cs* = 100%) and a range of concentrations from 1 % to 20 % were examined here. The modal diameter of Sonazoid microbubbles was determined as 2.3 μm by analyzing 1408 snapshots taken before US irradiation. This can easily lead to the estimation of the initial Sonazoid particle number per unit volume, *N100%*, to be 0.92×106 particles/mm^3 in a Sonazoid suspension with the volumetric concentration of 100 %. In the case of *Cs* = 5 %, therefore, the Sonazoid particle number per unit volume reduces to 4.61×10^4 particles/mm^3. Two methods were applied to measure the number of Sonazoid microbubbles after US irradiation. One is a method using a microscope and the other is a direct observation using high speed photography. For the purpose of counting Sonazoid survival number *N* precisely, we determined the depth of field according to each optical setup. For the case of high-speed photography using a CASIO EX-F1, we obtained the observation volume of 8.80×10-4 mm3 and we have 3.74×10^{-4} mm^3 for the images taken with a Nikon Coolpix 995. In addition a relatively deep field depth was measured when taking shadowgraph by means of high-speed photography using a Photron FASTCAM SA5 with the maximum framing rate of 300,000 frames/sec. A Xenon lamp with the duration of 2 μs was flashed at 70 μs after US irradiation in the Case A and 54 μs in the Case B, respectively. These are the indications of the timing when the first ultrasound wave started to interact with Sonazoid microbubbles.

2. Theoretical analysis of pressure filed in a cell culture plate.

According to IEC61102, an instantaneous intensity I (t) is written by

$$I(t) = \frac{p(t)^2}{\rho c} \qquad (2)$$

where P(t) is the atmospheric pressure, ρ is the density of water, and c is the sound speed of water.

For an ultrasonic pulse, temporal peak intensity I_{tp} is

$$I_{tp} = \frac{1}{\rho c}\frac{1}{(T2-T1)}\int_{T1}^{T2} p(t)^2 dt \quad (3)$$

For a tone burst sine wave which would be in your experimental case,

$$p(t) = P_o \sin(2\pi f t) \quad (4)$$

$$I_{tp} = \frac{P_o^2}{\rho c}\frac{1}{T}\int_0^T (\sin(2\pi f t))^2 dt \quad (5)$$

where T=1/f and since

$$\frac{1}{T}\int_0^T (\sin(2\pi f t))^2 dt = 1/2,$$

$$I_{\text{tp}} = \frac{1}{2}\frac{P_0^2}{\rho c}, \quad (6)$$

$$P_{\text{rms}} = \frac{P_0}{\sqrt{2}},$$

since

$$I_{\text{tp}} = \frac{P_{\text{rms}}^2}{\rho c} \quad (7)$$

Accordingly we may use the equation (5) for the intensity (which would be temporal peak) in the manuscript. The peak acoustic pressures for the intensity you considered are given in Table 1. Mechanical Index MI is defined by

$$MI = \frac{P_-}{\sqrt{f}} \quad (8)$$

where *P-* is the peak pressure, *f* is the center frequency

Table 1. Ultrasound intensity

I_{tp} (W/cm^2)	P_o (MPa)
1	0.17
2	0.24
3	0.30
4	0.35

The (temporal peak) intensity considered in the experiment and the corresponding peak acoustic pressure calculated by equation (6). Note that, if frequency is 1 MHz and the intensity is low, *MI* =*P-* ≈ *P*o. Suppose the temporal peak intensity I_{tp} = 3 W/cm^2, the peak acoustic pressure in the well

would be 0.3 MPa. (see Appendix below).

The acoustic field in the well is characterized by well developed standing wave field. The structure of the acoustic field in the well which were calculated by a FEM method using COMSOL. The boundary condition is described in Fig.3.

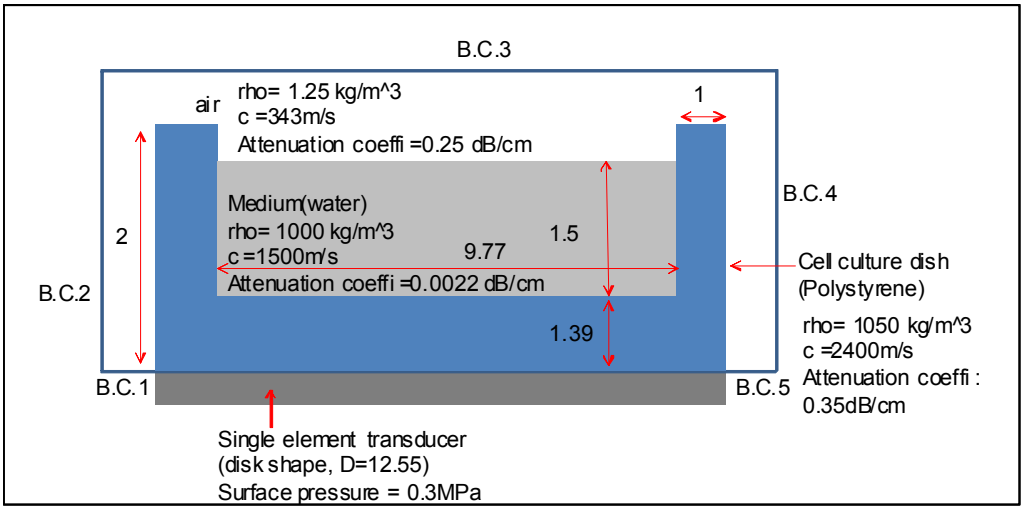


Figure 3. Boundary conditions of theoretical model.

3. In vitro experiments

Cell culture

Human kidney (293T) cells were obtained from M. ONO, Tohoku University. Cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 1% penicillin/glutamine/streptomycin. Cells were incubated at 37°C in a mixture of 5% carbon dioxide and 95% air until 80% confluence was achieved. Cells were routinely verified by morphology and growth characteristics using Trypan blue. 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.

Plasmid

The luciferase reporter vector pGL3-control (pGL3), which expresses luciferase under control of the SV40 promoter, was purchased from Promega (Madison, WI, USA). Pladmid DNA was extracted by EndoFree Plasmid Mega Kit (QIAGEN, Hilden, Germany). Adjusted to 1 mg/mL concentration of the plasmid, the experiment was carried out. The luminescence spectrum ranged from 510 nm to 650 nm when the luciferase gene was expressed.

Acoustic liposome (AL)

ALs were prepared as described in a previous publication. The shell composition was DSPC (NOF Co., Tokyo, Japan) and N-(carbonyl- methoxypolyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3- phosphoethanolamine (DSPE-PEG2000-OMe) (PEG molecular weight, 2000; NOF

Co.) [94:6 (mol/mol)]. Since ALs displayed a capability to include liquid as well as gas, they could be used as a drug carrier. Size was measured using dynamic light scattering. Approximately 100% of ALs were ~ 200 nm in diameter. ALs with diameters larger than a few micrometers accounted for < 0.01%. The zeta potential was −2.1 ± 0.9 mV (n = 4). It is supposed that gas sealted in the AL exist in either between the lipid bilayer or inside the micelle in the liquid phase of AL. Adjusted to 1 mg/mL concentration of the plasmid, the experiment was carried out.

Ultrasound (US)

Pulsed US at 1MHz (BFC Applications. Inc. Fujisawa, Japan) was used. To measure the pressure field generated by the probe, the transducer was placed in the test chamber (length, 300 mm; width, 450 mm; and height, 300 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; NF Co.). The peak negative pressure P_- and intensity I_A of US were measured by a PVDF needle hydrophone (PVDFZ44-1000; Specialty Engineering Associates, Soquel, CA, USA) in the two dimensional surface (39 mm × 39 mm) at a 1-mm stand-off distance from the transducer surface by using a three-dimensional (3-D) stage control system (Mark-204-MS; Sigma Koki, Tokyo, Japan). The signals from both the amplifier and hydrophone were recorded and stored in a digital phosphor oscilloscope [Wave Surfer 454; 500 MHz, 1 MΩ (16 pF); LeCroy Co., Chestnut, NY, USA].

Transfection by ultrasound and acoustic liposome

Cells (5×10^4 cells/ well) were seeded in 48-well plates in complete media at 37° C in a 5% CO_2 incubator. The next day, the medium was replaced with either fresh medium (110μL) containing either 2 μL pGL3 and 10μL NMB for luficerase gene expression measurement or fresh medium (110μL) containing 10μL NMB for cell viability. Both hight of the medium was 1.5 mm. The 48-well plates were located just above the US transducer in a test chamber filled with tap water and exposed to the US. The US parameters were shown in Table 2. The surface of the media was disturbed by US, thus we ignored the effect of standing waves on gene expression. Because cells were seeded into wells alternately, neighboring wells were not exposed to ultrasound at the same time. The plates were incubated for 1 h at 37° C in a 5% CO_2 incubator, supplemented with 390 μL of complete media and then incubated for another 24 h at 37° C in a 5% CO_2 incubator.

Luciferase assay

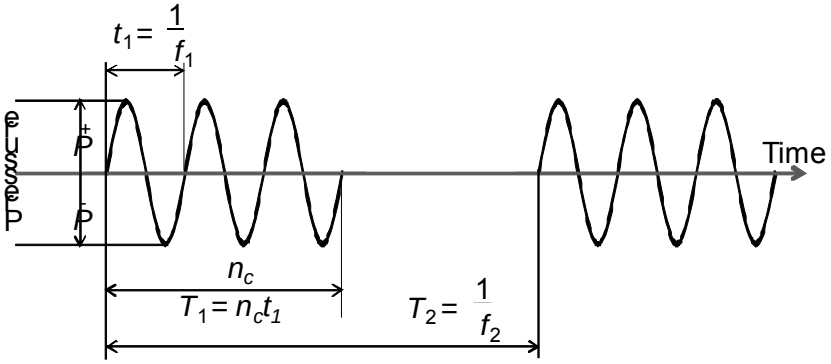
Twenty-four hours after ultrasound treatment, the cells were washed (PBS), lysed with 200 μL of Reporter Lysis Buffer (Promega), and subsequently frozen at -80°C until required. When required, the cells were defrosted on ice. Each lysate was centrifuged to pellet cell debris at 12,000 g for 2 min. Ten μL of the lysate was examined for luciferase activity with 50 μL of luciferase assay reagent containing D-Lucifer (Promega). Luminescence emissions were measured as relative light units (RLU) at 25°C for 10 s using a luminometer (TD-20/20, Turner BioSystems, Inc., Sunnyvale, CA, USA). Total protein content was calculated using albumin standard curves (BCA protein assay kit, Pierce, Rockford, IL, USA). All standards and samples were performed in duplicate. In all cases, mean protein absorbance was measured at 562 nm using a plate reader (SpectraMax, Molecular Device Corp., Sunnyvale, CA). Luciferase activity was converted to RLU/mg protein.

Cell viability

After 24 h of US exposure, cell viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] assay, as described previously . The cell survival fractions were calculated relative to the cells untreated by NMBs or US.

Statistical analysis

All measurements were represented as mean ± SE (standard error). Comparison of the two groups was made by Student's *t* test. An overall difference between the groups was determined by one-way analysis of variance (one-way ANOVA). Following this, the significance of the difference was determined by Turkey-Kramer test. A *P* value of < 0.01 (**) and < 0.05 (*) was considered to be statistically significant.



$$DC\ (\%) = \frac{T_1}{T_2} = n_c \frac{f_2}{f_1}$$

$$E_A\ (\mathrm{J/cm^2}) = DC \times T_E \times I_A$$

Figure 4. Ultrasound parameters