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Abstract	Gene delivery with a physical mechanism using ultrasound (US) and nano/microbubbles is expected as an ideal system in terms of delivering plasmid DNA noninvasively into a specific target site. We developed novel liposomal bubbles (Bubble liposomes (BLs)) containing the lipid nanobubbles of perfluoropropane which were utilized for contrast enhancement in ultrasonography. BLs were smaller in diameter than conventional microbubbles and induced cavitation upon exposure ultrasound. In addition, when coupled with US exposure, BLs could deliver plasmid DNA into various types of cells in vitro and in vivo. The transfection efficiency with BLs and US was higher than that with conventional lipofection method. Therefore, the combination of BLs and US might be an efficient and novel nonviral gene delivery system.	
Key words (separated by '-')	Liposomes - Nanobubbles - Gene delivery - Ultrasound - Noninvasive - Nonviral vector	

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Chapter 33 1

Effective In Vitro and In Vivo Gene Delivery by the Combination of Liposomal Bubbles (Bubble Liposomes) and Ultrasound Exposure 2 3 4

Ryo Suzuki and Kazuo Maruyama 5

Abstract 6

Gene delivery with a physical mechanism using ultrasound (US) and nano/microbubbles is expected as an ideal system in terms of delivering plasmid DNA noninvasively into a specific target site. We developed novel liposomal bubbles (Bubble liposomes (BLs)) containing the lipid nanobubbles of perfluoropropane which were utilized for contrast enhancement in ultrasonography. BLs were smaller in diameter than conventional microbubbles and induced cavitation upon exposure ultrasound. In addition, when coupled with US exposure, BLs could deliver plasmid DNA into various types of cells in vitro and in vivo. The transfection efficiency with BLs and US was higher than that with conventional lipofection method. Therefore, the combination of BLs and US might be an efficient and novel nonviral gene delivery system. 7 8 9 10 11 12 13 14 15

Key words: Liposomes, Nanobubbles, Gene delivery, Ultrasound, Noninvasive, Nonviral vector 16

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1. Introduction 17

Ultrasound (US) has been utilized as a useful tool for in vivo imaging, destruction of renal calculus and treatment for fibroid of the uterus. It was reported that US was proved to increase permeability of the plasma membrane and reduce the thickness of the unstirred layer of the cell surface, which encourages the DNA entry into cells (1, 2). The first studies applying ultrasound for gene delivery used frequencies in the range of 20–50 kHz (1, 3). However, these frequencies, along with cavitation, are also known to induce tissue damage if not properly controlled (4–6). To improve this problem, many studies using therapeutic ultrasound for gene delivery, which operates at frequencies of 18 19 20 21 22 23 24 25 26 27 28

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1–3 MHz, intensities of 0.5–2.5 W/cm^2, and pulse-mode have emerged (7–9). In addition, it was reported that the combination of therapeutic US and microbubble echo contrast agents could enhance gene transfection efficiency (10–14). In the sonoporation with microbubbles, it was reported that estimates of pore size based on the physical diameter of maker compounds were most commonly in the range of 30–100 nm, and estimates of membrane recovery time ranged from a few seconds to a few minutes (15). Therefore, it is thought that plasmid DNA is effectively and directly transferred into the cytosol via these pores. Conventional microbubbles including US contrast agents based on protein microspheres and sugar microbubbles are commercially available, the size of these bubbles being about 1–6 μm (16). For example, although the mean diameter of Optison microbubbles is about 2.0–4.5 μm, and they contain bubbles of up to 32 μm in diameter. Tsunoda et al. reported that some mice died immediately after the i.v. injection of Optison without ultrasound exposure due to lethal embolisms in vital organs (17). The same problem has not been reported in humans, but there is the possibility that Optison can not pass through capillary vessels. Therefore, microbubbles should generally be smaller than red blood cells. From this stand point of view, it is necessary to develop novel bubbles which are smaller than conventional microbubbles. Using liposome technology, we developed novel liposomal bubbles containing perfluoropropane gas. We called these bubbles "Bubble liposomes (BLs)." BLs were smaller than Optison (18–21). In addition, BLs could effectively deliver plasmid DNA by the combination with US exposure in vitro and in vivo.

2. Materials

2.1. Preparation of BLs (18)

1. 1,2-distearoyl-sn-glycero-phosphatidylcholone (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-methoxypolyethyleneglycol (DSPE-PEG(2 k)-OMe) (NOF corporation, Tokyo, Japan).
2. Chloroform.
3. Diisopropyl ether.
4. Phosphate buffered saline (pH 7.4) (PBS): 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4 (Wako Pure Chemical Industries).
5. Perfluoropropane (Takachiho Chemical Industries, Tokyo, Japan).
6. Rotary evaporator (TOKYO RIKAKIKAI, Co. Ltd. (EYELA), Tokyo, Japan).

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7. Extruding apparatus (Northern Lipids Inc., Vancouver, BC).
8. Bath-type sonicator (42 kHz, 100 W) (Branson Ultrasonics Co., Danbury, CT).
9. Liposome sizing filters (pore sizes: 100 and 200 nm) (Nuclepore Track-Etch Membrane, Whatman plc, UK).
10. 0.45 μm pore size filter (MILLEX HV filter unit, Durapore PVDF membrane) (Millipore Corporation, MA).
11. Dynamic light scattering (ELS-800) (Otsuka Electronics Co., Ltd., Osaka, Japan).
12. Phospholipid C-test wako (Wako Pure Chemical Industries).

2.2. Transmission Electron Microscopy of BLs (20)

1. Sodium alginate (500-600cP).
2. Calcium chloride.
3. Glutaraldehyde.
4. Cacodylate buffer.
5. Osmiumtetroxide.
6. Ethanol.
7. Epan812.
8. Uranyl acetate.
9. Electron microscope: JEOL JEM12000EX at 100 kV.

2.3. In Vitro Ultrasonography with BLs (19)

1. Ultrasound imaging equipment: UF-750XT (Fukuda Denshi Co. Ltd., Tokyo, Japan).
2. 9 MHz linear probe (9 MHz, Fukuda Denshi Co. Ltd.)

2.4. Gene Delivery with BLs and US In Vitro and In Vivo

1. Cells: COS-7 cells (the African green monkey kidney fibroblast cell line), S-180 cells (mouse sarcoma), Meth-A fibrosarcoma cells (mouse fibrosarcoma), Jurkat cells (human T cell line), Colon 26 cells (mouse colon adenocarcinoma), B16BL6 cells (mouse meranoma), Human umbilical vein endothelial cells (HUVEC) (Kurabo Industries, Osaka, Japan).
2. Culture media: Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, Eagle's medium (MEM) and medium 199 (Sigma Chemical Co., St. Louis, MO), Supplements: Fetal bovine serum (FBS, GIBCO, Invitrogen Co., Carlsbad, CA), HEPES and heparin (Wako Pure Chemical Industries), endothelial cell growth supplement (ECGS) (Sigma Chemical Co.), Antibiotics: Penicillin and Streptomycin (Wako Pure Chemical Industries).
3. COS-7 cells and S-180 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. Meth-A fibrosarcoma cells and Jurkat cells were cultured with RPMI-1640 supplemented with 10% heat inactivated FBS. Colon 26 cells

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were cultured with RPMI-1640 supplemented with 10% heat-inactivated FBS and 2.5% HEPES. B16BL6 cells were cultured with MEM supplemented with 10% heat-inactivated FBS. HUVECs were cultured in a DMEM and medium 199 mixture with 15% heat-inactivated FBS, heparin (3.25 U/mL) and ECGS. All culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

4. Animals: ddY mice (4–6 weeks age, male), Anesthetic agent: NEMBUTAL (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), Adhesive agent (Aron Alpha) (Daiichi Sankyo Co., Ltd., Tokyo, Japan).
5. Ultrasound equipments and probes for gene delivery – Ultrasound equipments: Sonopore 3000 and Sonopore 4000 (NEPAGENE Co. Ltd.), Probe: KP-T6 (diameter: 6 mm) and KP-T8 (diameter: 8 mm), KP-T20 (diameter: 20 mm) (NEPAGENE Co., Ltd.)
6. Assessment of cytotoxicity: MTT [3-(4,5-s-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Dojindo, Kumamoto, Japan), Sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries), Microplate reader (POWERSCAN HT; Dainippon Pharmaceutical, Osaka, Japan).
7. Luciferase assay: Cell lysis buffer (0.1 M Tris–HCl (pH 7.8), 0.1% Triton X-100, 2 mM EDTA), Luciferase assay system (Promega, Madison, WI), Luminometer (TD-20/20) (Turner Designs, Sunnyvale, CA).
8. In vivo luciferase imaging: Escain (Mylan Inc., Tokyo, Japan), D-luciferin and In vivo luciferase imaging system (IVIS) (Caliper Life Sciences, MA).

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3. Methods

3.1. Preparation of BLs (18)

1. DSPC and DSPE-PEG(2 k)-OMe were dissolved in 8 mL of 1:1 (v/v) chloroform/diisopropyl ether.
2. Four milliliter of PBS (pH 7.4) was added into the lipid solution. The mixture was sonicated to make suspension, and evaporated at 65° (water bath) to remove solvent.
3. After evaporation, liposome suspension was passed through sizing filters (pore sizes: 100 and 200 nm) using an extruding apparatus. And the size of liposomes was adjusted to less than 200 nm.
4. The liposomes suspension was sterilized by passing them through a 0.45 μm pore size filter. (see Fig. 1a)
5. Finally, size of the sterilized liposomes was measured with dynamic light scattering (ELS-800). The average diameter of

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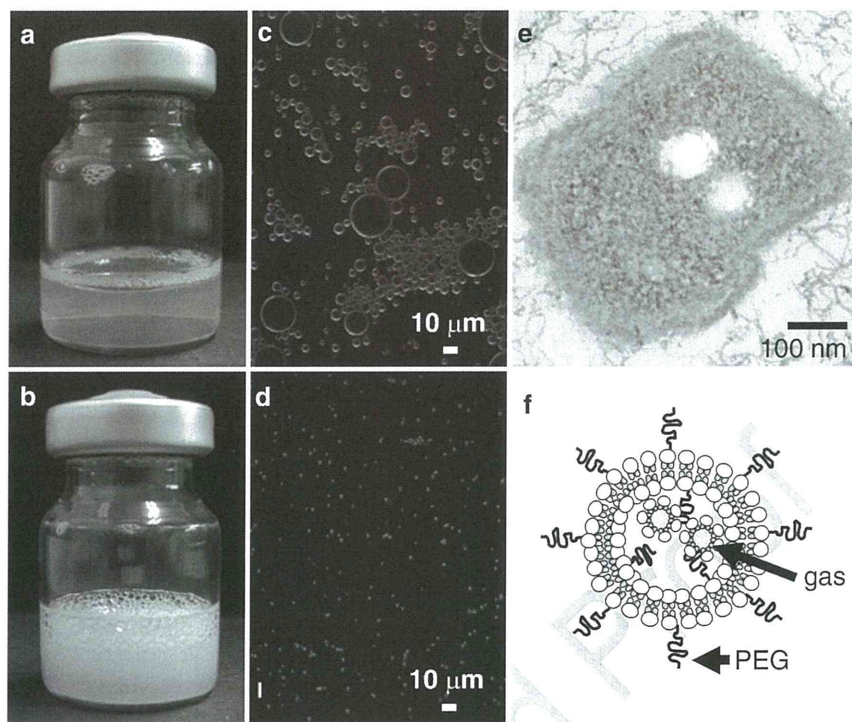

Fig. 1. Aspect and structure of BLs. PEG-liposomes (**a**) were sonicated with supercharged perfluoropropane gas. After that, they became to BLs (**b**). Optison (**c**) and BLs (**d**) were observed with microscope using the darklite illuminator (NEPAGENE, Co., Ltd). (**e**): Transmission electron microscopy (TEM) of BLs. (**f**): Scheme of structure of BLs

these liposomes were about 150–200 nm. In addition, lipid concentration was measured with the Phospholipid C-test wako. 153 154

6. The lipid concentration of liposomes suspension was adjusted to 1 mg/mL with PBS. 155 156
7. Two milliliter of the liposomes suspension (lipid conc. 1 mg/mL) was entered into sterilized vial (vial size: 5 mL). 157 158
8. The vial was filled with perfluoropropane, capped and then supercharged with 7.5 mL of perfluoropropane. 159 160
9. The vial was placed in a bath-type sonicator (42 kHz, 100 W) for 5 min to form BLs (see Fig. 1 and Note 1). 161 162

3.2. Transmission Electron Microscopy of BLs (20)

1. BLs were suspended into sodium alginate (500-600cP) solution (0.2% (w/v) in PBS). 163 164
2. This suspension was dropped into calcium chloride solution (100 mM in PBS) to hold BLs within calcium alginate gel. 165 166
3. The beads of calcium alginate gel containing BLs were prefixed with 2% glutaraldehyde solution in 0.1 M Cacodylate buffer. 167 168
4. The beads were postfixed with 2% OsO_4, dehydrated with an ethanol series, and then embedded in Epan812 (polymerized at 60°). 169 170 171

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5. Ultrathin sections were made with an ultramicrotome at a thickness of 60–80 nm.
6. Ultrathin sections were mounted on 200 mesh copper grids.
7. They were stained with 2% uranyl acetate for 5 min and Pb for 5 min.
8. The samples were observed with JEOL JEM12000EX at 100 kV (see Fig. 1e; Notes 2 and 3).

3.3. In Vitro Ultrasonography with BLs (19)

1. BLs were placed into latex tube filled with degassed PBS (10 mL) in a water bath.
2. The probe (9 MHz) of an ultrasound imaging equipment was positioned under the water bath.
3. BLs in the tube were imaged (see Fig. 2 a, b).

3.4. In Vitro Gene Delivery with BLs and US

3.4.1. Transfection of Plasmid DNA into Cells with BLs and US (21)

1. Plasmid DNA, cells and BLs were suspended in culture medium with 10% FBS (final volume; 500 μL) in 2 mL polypropylene tubes.
2. The probe (KP-T6) (2 MHz, diameter: 6 mm) of US was placed into the suspension.
3. US was exposed to the suspensions with Sonopore 3000 or 4000 under the condition of various US parameters (Duty, Intensity, Exposure time, Burst rate) (see Fig. 2c).

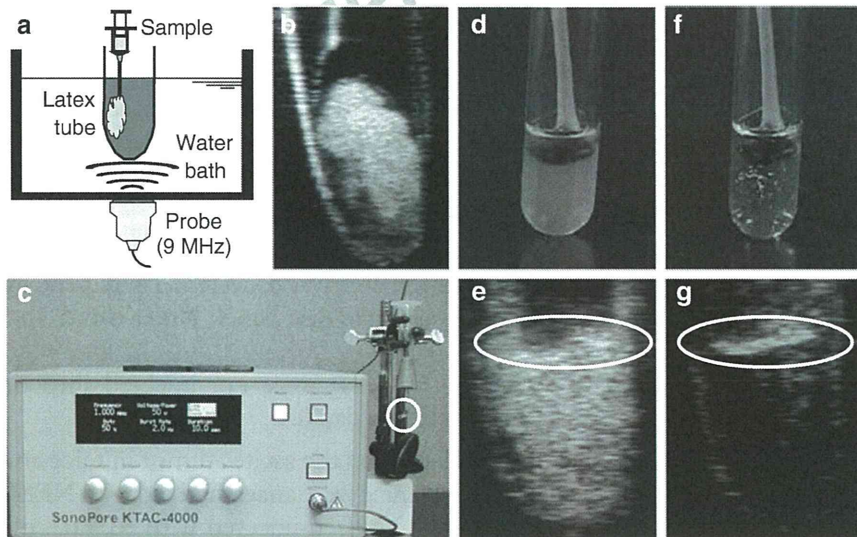

Fig. 2. In vitro Ultrasonography with BLs. The Method of ultrasonography for observation of BLs was shown in (**a**). BLs were injected into PBS filled latex tube in the water bath. Then, the samples were observed with ultrasonography (**b**). To confirm the disruption of BLs by US exposure using Sonopore 4000 (**c**), BLs were observed with naked image (**d**, **f**) and ultrasonography (**e**, **g**) before (**d**, **e**) and after (**f**, **g**) US exposure (2 MHz, 2.5 W/cm^2, 10 s). *Circle* in (**c**, **e**, **g**) shows US probe

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4. After US exposure, the cells were washed twice with PBS and then resuspended in fresh culture medium.
5. The cells were cultured in culture plate or wells.
6. After 2 days culture of cells, the expression of transgene was measured (see Fig. 3; Notes 4 and 5).

3.4.2. Assessment of Cytotoxicity by the Treatment of BLs and US to Cells (18)

1. Cells (1×10^5) and BLs were suspended in culture medium with 10% FBS (final volume; 500 μL) in 2 mL polypropylene tubes.
2. US was exposed to cells using Sonopore 3000 or 4000 with a probe (KP-T6) (2 MHz, diameter: 6 mm).
3. After US exposure, the cells were washed twice with PBS and then resuspended in fresh culture medium.
4. One hundred microliter of the cells suspension were cultured in 96 well plates for 24 h.
5. Cell viability was assayed using MTT, as described by Mosmann, with minor modifications (22). Briefly, MTT (5 mg/mL, 10 μL) was added to each well, and the cells were incubated at 37°C for 4 h. The formazan product was dissolved in 100 μL of 10% SDS containing 15 mM HCl. Color intensity was measured using a microplate reader at test and reference wavelengths of 595 and 655 nm, respectively.

3.5. In Vivo Gene Delivery with BLs and US

3.5.1. Gene Delivery for Femoral Artery (18)

1. The femoral artery was exposed by operation.
2. BLs (250 μg) and plasmid DNA (10 μg) suspension (300 μL) was slowly injected into the femoral artery of ddY mice (6 weeks age, male) using 30-gauge needle (M-S Surgical MFG. Co. Ltd., Tokyo, Japan).
3. In the same time, US (frequency: 1 MHz, duty: 50%, intensity: 1 W/cm^2, time: 2 min) was transdermally exposed to downstream of injection site using Sonopore 3000 or 4000 with a probe (KP-T8) (diameter: 8 mm). [AU1]
4. After 2 days of injection, the mice were sacrificed and the femoral artery of US exposure area was collected. Then, gene expression in the artery was measured (see Fig. 4; Notes 6 and 7).

3.5.2. Gene Delivery for Ascites Tumor (20)

1. S-180 cells (1×10^6 cells) were i.p. injected into ddY mice (4 weeks age, male) on day 0.
2. When S-180 cells grew as the ascites tumor in mice after 8 days of the injection, the mice were anaesthetized with NEMBUTAL Injection (50 mg/kg), then injected with 510 μL of plasmid DNA and BLs (500 μg) in PBS.
3. US (frequency: 1 MHz, duty: 50%, intensity: 1 W/cm^2, time: 1 min) was transdermally exposed to the abdominal area using Sonopore 300 or 4000 with a probe (KP-S20) (diameter: 20 mm).

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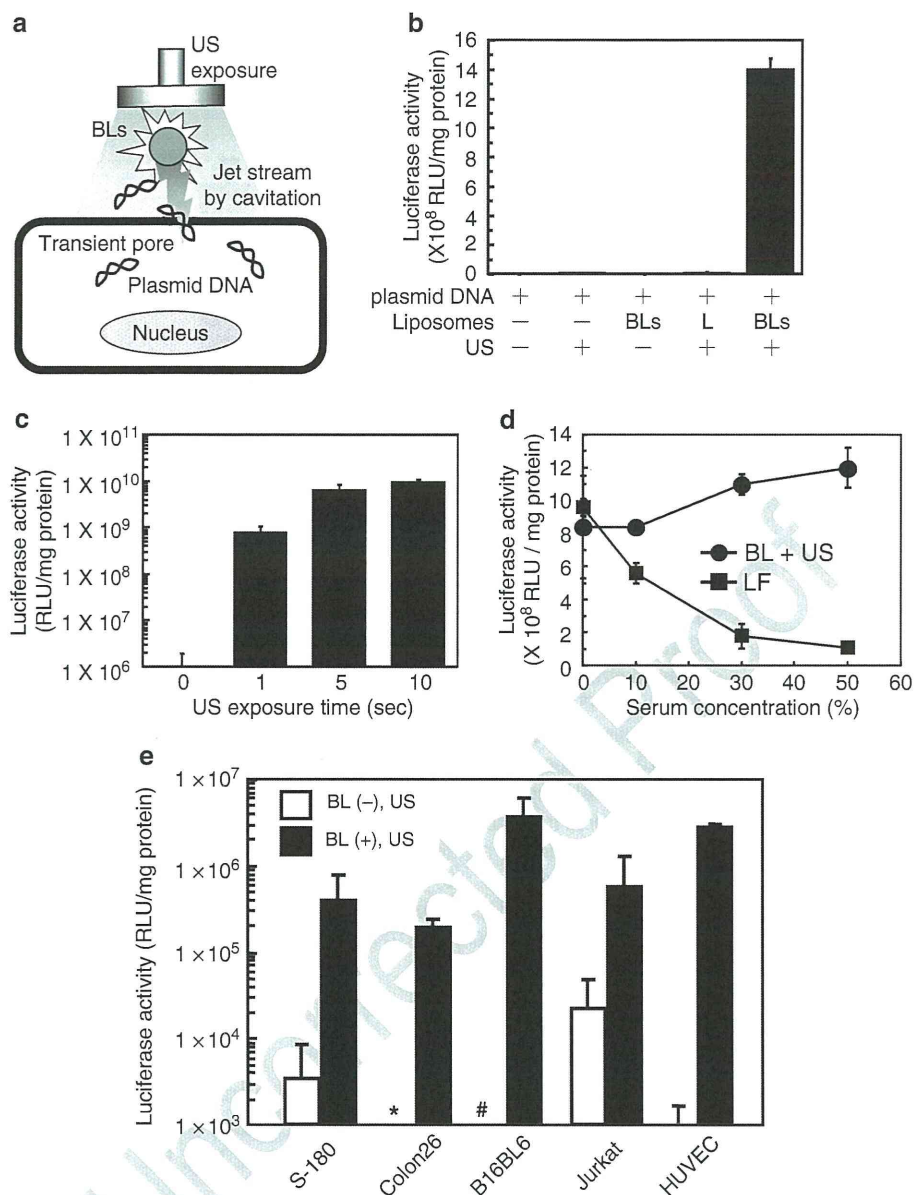

Fig. 3. Property of gene delivery with BLs and US exposure (**a**) Schema of transfection mechanism by BLs and US. The mechanical effect based on the disruption of BLs by US exposure, which results in generation of some pores on plasma membrane, is associated with direct delivery of extracellular plasmid DNA into cytosol. (**b**) Luciferase expression in COS-7 cells transfected by BLs and US. COS-7 cells (1×10^5 cells/500 μL/tube) were mixed with pCMV-Luc (5 μg) and BLs (60 μg). The cell mixture was exposed with US (Frequency: 2 MHz, Duty: 50%, Burst rate: 2 Hz, Intensity: 2.5 W/cm^2, Time: 10 s). The cells were washed and cultured for 2 days. After that, luciferase activity was measured. (**c**) Effect of US condition on transfection efficiency with BLs. COS-7 cells were exposed with US (Frequency: 2 MHz, Duty: 50%, Burst rate: 2 Hz, Intensity: 2.5 W/cm^2, Time: 0, 1, 5, 10 s) in the presence of pCMV-Luc (0.25 μg) and BLs (60 μg). Luciferase activity was measured as above. (**d**) Effect of serum on transfection efficiency of BLs. COS-7 cells in the medium containing FBS (0, 10, 30, 50% (v/v)) were treated with US (Frequency: 2 MHz, Duty: 50%, Burst rate: 2 Hz, Intensity: 2.5 W/cm^2, Time: 10 s), pCMV-Luc (0.25 μg) and BLs (60 μg) or transfected with lipoplex of pCMV-Luc (0.25 μg) and lipofectin (1.25 μg). (**e**) In vitro gene delivery to various types of cell using BLs and US. The method of gene delivery was same as above. S-180: mouse sarcoma cells, Colon26: mouse colon adenocarcinoma cells, B16BL6: mouse melanoma cells, Jurkat: human T cell line, HUVEC: human umbilical endothelial cells. Luciferase activity was measured as above. * $<10^3$ RLU/mg protein, # $<10^0$ RLU/mg protein Each data represents the mean ± S.D. ($n = 3$). L: PEG-liposomes, LF: Lipofectin

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