

Development of anionic bubble lipopolyplexes for efficient and safe gene transfection with ultrasound exposure in mice

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Abstract

Anionic bubble lipopolyplexes have been developed as anionic ultrasound (US)-responsive gene delivery carriers with biocompatible compounds for efficient and safe transfection in mice. The particles of the anionic bubble lipopolyplexes were approximately 450–600 nm with an anionic surface charge. In the absence of US exposure, the bubble lipopolyplexes showed extremely low gene expression in the human vascular endothelial cell line EAhy926. The anionic bubble lipopolyplexes, however, delivered pDNA into cells without endocytosis and showed markedly high gene expression following US exposure. The anionic bubble lipopolyplexes showed little cytotoxicity in EAhy926 cells and little aggregation with erythrocytes. Following intravenous administration into mice, the anionic bubble lipopolyplexes showed high levels of gene expression in the liver, kidney, and spleen only after US exposure to the abdominal area. The level of gene expression in liver non-parenchymal cells was significantly higher than that in parenchymal cells. In addition, the anionic bubble lipopolyplexes did not show any severe hepatic toxicity and did not enhance the production of proinflammatory cytokines. Overall, we have succeeded in preparing anionic bubble lipopolyplexes for efficient and safe transfection with US exposure in mice.

Keywords: DNA; transfection; self assembly; biocompatibility; liposomes; sonoporation

1. Introduction

It is largely expected that gene therapy will provide a range of novel approaches for the treatment of genetic disorders and intractable diseases such as cancer [1-3]. Advances in this area, however, have been limited by the fact that naked plasmid DNA (pDNA) is readily degraded *in vivo* and barely taken up by cells. To date, a variety of different gene delivery methods have been developed to take full advantage of gene therapy, and these gene delivery methods can be categorized as either viral or non-viral methods [4, 5]. Non-viral gene delivery methods have several advantages over the viral methods, such as providing the flexibility to design a vehicle with well-defined structural and chemical properties capable of mass production [6, 7]. Non-viral gene delivery methods can also be split into two sub-categories, with one involving the use of gene delivery carriers such as cationic polymers and liposomes, and the other involving the use of naked pDNA molecules according to the electroporation, hydrodynamic, pressure, and suction methods [8-12]. Most of the gene delivery carriers deliver the pDNA into cells through endocytosis. Unfortunately, however, intracellular delivery from endosomes to the cytosol provides a major obstacle to gene expression [13].

Sonoporation, which is a combination of ultrasound (US) exposure and microbubbles containing US imaging gas, has recently been reported to deliver pDNA directly into the cytosol without endocytosis and show high levels of gene expression [14, 15]. US exposure disrupts the microbubbles and generates cavitation energy [16], which can create transient pores in the cellular membrane that allow pDNA to be delivered into the cytosol [15, 17]. In addition, some novel approaches have been developed to allow for the incorporation of non-viral gene delivery carriers with microbubbles using covalent binding, as well as avidin-biotin and electrostatic interactions [18-24].

The microbubbles are generally several micrometers in size, which generally prevents these materials from reaching the peripheral tissues following intravenous administration. Maruyama et al. recently succeeded in developing a novel formulation capable of producing bubble liposome particles of approximately 500 nm in size [25, 26]. In our previous study, we reported the development of mannosylated bubble lipoplexes for the targeted delivery of pDNA and siRNA into macrophages [27-29]. With regard to the surface charge of the bubble lipoplexes, those possessing an anionic or neutral surface charge were suitable for clinical application because cationic charged lipoplexes have been reported to interact with biogenic substances such as serum albumin and erythrocytes [30]. Given that neutral bubble lipoplexes can readily aggregate through the neutralization of their electric charge, it was envisaged that the anionic bubble lipopolyplexes could be prepared without aggregation even under high concentration conditions. There have, however, been very few reports concerning the development or application of

US responsive gene delivery carriers with an anionic surface charge. With all of this in mind, we prepared bubble lipopolyplexes as novel anionic US responsive gene delivery carriers from a ternary pDNA complex, cationic polymers, and anionic bubble liposomes with biocompatible compounds for efficient and safe transfection.

In this study, we have investigated the physicochemical properties, transfection efficiencies, and toxicity of the anionic bubble lipopolyplexes before and after US exposure.

2. Materials and methods

2.1. Chemicals

Protamine sulfate (PS), poly-L-lysine (PLL), poly-L-arginine (PLA), and polyethylenimine (PEI, branched form, average molecular weight 25,000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dendrigrift poly-L-lysine (DPLL) was purchased from Colcom (Montpellier, France). Distearoyl phosphatidylglycerol (DSPG), distearoyl phosphatidyl acid (DSPA), distearoyl phosphatidylserine (DSPS), distearoyl phosphatidylcholine (DSPC), and dioleoyl trimethylammoniumpropane (DOTAP) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol (Chol) and methoxy-polyethyleneglycol 2000-distearoylphosphatidylethanolamine (PEG-DSPE) were purchased by Nacalai Tesque (Kyoto, Japan) and NOF Co. (Tokyo, Japan), respectively. All of the other chemicals used in the current study were purchased as the highest purity grades available.

2.2. Construction of pDNA

pCMV-Luc was used as described previously [31]. The pDNA was amplified using an EndoFree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany) and dissolved in sterile dH₂O. Fluorescein-labeled pDNA was prepared using a Label IT Tracker Fluorescein Kit (Mirus Co., Madison, WI, USA).

2.3. Preparation of the anionic bubble lipopolyplexes

DSPG, DSPC, and PEG-DSPE were mixed in chloroform in a molar ratio of 7:2:1 for the construction of the anionic liposomes (AL). The resulting lipid mixture was then dried by evaporation before being desiccated in a vacuum to give a lipid film, which was subsequently suspended in sterile dH₂O. Following a 30-min period of hydration at 65°C, the dispersion was sonicated for 10 min using a bath sonicator, and then sonicated in a tip sonicator for 3 min to produce liposomes, which were sterilized using a 0.45- μ m filter (Nihon-Millipore, Tokyo, Japan).

Various cationic polyplexes were prepared by gently mixing a pDNA solution with an appropriate amount of a cationic polymer solution such as PS, PLL, PLA, or DPLL, with the resulting mixture being incubated for 15 min. The resulting cationic polyplexes were then mixed with the appropriate amount of the AL to allow for the construction of the anionic lipopolyplexes. Various anionic lipopolyplexes were also constructed at weight ratios of 1.0:1.5:17.6 (pDNA:PLL, PLA, or DPLL:AL) or 1.0:1.25:2.5 (pDNA:PS:AL). The isotonicity properties of the solutions were then adjusted via the addition of 10 \times phosphate buffered saline (PBS). For the preparation of the

bubble lipopolyplexes (pDNA/PS, PLL, PLA, and DPLL/BL), US imaging gas captured within the anionic lipopolyplexes using a method described previously in the literature [32]. Briefly, the anionic lipopolyplexes were added to 5-mL sterilized vials, which were subsequently capped and pressurized with 7.5 mL of perfluoropropane gas (Takachiho Chemical Industries Co., Ltd., Tokyo, Japan). To enable the US imaging gas to become encapsulated within the anionic lipopolyplexes, the vial was sonicated in a bath-type sonicator (AS ONE Co., Osaka, Japan) for 5 min. For the optimization of the anionic lipids, three anionic liposomes containing DSPG, DSPA, or DSPS were prepared and pDNA/PS/AL and pDNA/PS/BL were developed for each of the anionic liposomes.

2.4. Physicochemical properties of the anionic bubble lipopolyplexes

The particle sizes and zeta-potentials of the bubble lipopolyplexes were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom).

Ten-microliter aliquots of different complex solutions containing 1 µg of pDNA were mixed with 2 µL of loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 1% agarose gel. Electrophoresis (Mupid-2X; Cosmo Bio, Tokyo, Japan) experiments were carried out at 100 V in a running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM EDTA). The pDNA was visualized with a GelRed Nucleic Acid Gel Stain (Wako, Osaka, Japan) using an ImageQuant LAS4000 System (GE Healthcare Life Sciences, Fairfield, CT, USA). Transmission electron microscope (TEM) images of the pDNA/PS, pDNA/PS/AL, and pDNA/PS/BL were recorded on a H-7650 system (Hitachi Co., Tokyo, Japan) with negative staining using uranyl acetate.

2.5. In-vitro gene expression and intracellular distribution experiments

The human vascular endothelial cell line EAhy926 was purchased from American Type Culture Collection (Manassas, VA, USA) and the cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 100 µM non-essential amino acids at 37°C in 5% CO₂. Following a pre-incubation period of 24 hr, the culture medium was replaced with Opti-MEM I containing different complexes (10 µg pDNA). The EAhy926 cells were then exposed to US (frequency, 2.0 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm²) for 20 seconds using a 6-mm diameter probe, which was placed in the well 10 min after the addition of the different complexes. The US was generated using a Sonopore-4000 sonicator (Nepa Gene, Co., Ltd., Chiba, Japan). The incubation medium was then replaced with the culture medium and the resulting mixture was incubated for an additional 24 hr. Following

the incubation period, the cells were suspended in lysis buffer (0.05% Triton X-100, 2 mM EDTA, and 0.1 M Tris; pH 7.8), and the lysate was mixed with luciferase assay buffer (Picagene, Toyo Ink Co., Ltd., Tokyo, Japan). The luciferase activity was then measured using a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany) and normalized with respect to the protein content of the cells using a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan).

The intracellular distribution of the pDNA was determined using fluorescein-labeled pDNA and LysoTracker Red DND-99 (Invitrogen, Carlsbad, CA, USA). Briefly, the cells were transfected with the pDNA/PS/BL or Lipofectamine 2000 (Invitrogen) using fluorescein-labeled pDNA. The cells were subsequently treated with LysoTracker Red DND-99 6 hr after the transfection process, and then fixed using a 10% formalin solution before being stained with DAPI and observed under a confocal microscope (Nikon A1RMP, Nikon, Tokyo, Japan).

2.6. WST-1 assay

The cytotoxicities of the bubble lipopolyplexes towards the EAhy926 cells were determined using a WST-1 Cell Proliferation Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA). Following a pre-incubation period of 24 hr, the different bubble lipopolyplexes containing 10 μ g of pDNA in the culture medium were added to the EAhy926 cells and incubated for 10 min before being exposed to US in the same manner as the cells described above. Following their exposure to the US, the cells were incubated for 24 hr with the different bubble lipopolyplexes. The medium was replaced with the culture medium and the WST-1 Cell Proliferation Reagent was added to each well. The cells were then incubated for 2 hr at 37°C and the absorbance in each well was measured at a wavelength of 450 nm with a reference wavelength of 630 nm using an EON Microplate Spectrophotometer (BioTek, Winooski, VT, USA). The results are shown as the percentage of untreated cells (control).

2.7. Erythrocyte aggregation experiment

Erythrocytes from mice were washed three times with PBS at 4°C by centrifugation at 5000 \times g for 5 min and 2% (v/v) stock suspensions of the resulting erythrocytes were then prepared in PBS. A 40- μ L of the erythrocyte suspension were mixed with 40 μ L of the cationic polyplexes or the anionic bubble lipopolyplexes solution containing 5 μ g of pDNA, and the resulting mixture was incubated for 15 min at ambient temperature. Lipofectamine 2000 and PEI were used for the positive control. A 30- μ L of pDNA solution containing 5 μ g of

pDNA was mixed with 10 μ L of Lipofectamine 2000 or PEI solution (0.5 mg/mL) and the resultant solution was mixed with the erythrocyte suspension as described above. A 10- μ L sample of the mixture was then collected and placed on a glass plate to allow for the aggregation to be observed by microscopy.

2.8. *Animals*

All of the animal experiments were performed in accordance with the Principles of Laboratory Animal Care, as adopted and promulgated by the US National Institutes of Health, and the guidelines for animal experiments of Kyoto University. Female ICR mice (5–6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Following their shipping, the mice were acclimatized to the environment for at least 1 day before each experiment.

2.9. *In-vivo gene expression experiment*

The mice were intravenously injected with 400 μ L of the bubble lipopolyplexes via the tail vein using a 26-gauge syringe needle at a dose of 50 μ g of pDNA. Immediately after the injection of the bubble lipopolyplexes, US (frequency, 1.0 MHz; duty, 50%; burst rate, 10 Hz; intensity, 0.5 W/cm²; time, 1 min) was applied transdermally to the abdominal area using a Sonopore-4000 sonicator with a probe (diameter: 20 mm). Six hours after the injection, the mice were sacrificed and their organs, including their liver, kidney, and spleen, were collected for each experiment. The collected organs were then washed twice with cold saline and homogenized in the lysis buffer. The lysis buffer was added in a weight ratio of 5 mL/g for the liver or 4 mL/g for the other organs. The homogenates were centrifuged at 15,000 \times g for 10 min at 4°C. The luciferase activities of the resulting supernatants were then determined.

2.10. *Separation of hepatic parenchymal cells and non-parenchymal cells*

The separation of mouse hepatic parenchymal cells and non-parenchymal cells was performed as previously described in the literature [33]. The parenchymal and non-parenchymal liver cells were resuspended separately in the lysis buffer and their luciferase activity was determined. Furthermore, Kupffer cells and liver endothelial cells were separated from the non-parenchymal cells with an immunomagnetic cell isolation system (Robosep, Veritas Corp., Tokyo, Japan) using FITC labeled anti-CD14 and anti-CD31 antibodies (Abcam, Tokyo, Japan) with a RoboSep Mouse FITC Selection Kit (Veritas Corp., Tokyo, Japan). The total RNA was extracted from the cells using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA).

Reverse transcription of the mRNA to cDNA was carried out using a PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan). Luciferase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression were determined with a Lightcycler 2.0 real-time PCR system (Roche Diagnostics, Indianapolis, IN, USA), using an SYBR Premix Ex Taq (Takara Bio Inc.). Primers for the luciferase and GAPDH were constructed according to the following procedure. Primer for luciferase, 5'-TTCTTCGCCAAAAGCACTC-3' (forward) and 5'-CCCTCGGGTGTAATCAGAAT-3' (reverse); primer for GAPDH, 5'-TCTCCTGCGACTTCAACA-3' (forward) and 5'-GCTGTAGCCGTATTCATTGT-3' (reverse) (Invitrogen).

2.11. Hepatic toxicity of the pDNA/PS/BL with or without US exposure

The mice were transfected with pDNA/PS/BL containing 25 µg of pDNA and subsequently exposed to US, as described above. Blood samples were then collected from the vena cava under pentobarbital anesthesia at predetermined time points. A hydrodynamic transfection experiment was also performed as a positive control for liver toxicity involving the administration of pDNA dissolved in 2.5 mL of saline into the tail vein of mice within 5 seconds [10]. Cationic liposomes composed of DOTAP and cholesterol (Chol) were prepared as previously described [34]. DOTAP/Chol liposomes were mixed with pDNA at charge ratios (-:+) of 1.0:2.3. pDNA-DOTAP/Chol complexes (DOTAP/Chol lipoplexes) were also constructed as a positive control for cytokine production. The serum aspartate transaminase (AST) and alanine transaminase (ALT) activities were then determined using a Transaminase CII-Test Wako kit (Wako Pure Chemical Industries, Ltd.) according to the manufacturer's instructions. The levels of TNF- α and IL-6 in the serum were then evaluated using a BD OptEIA ELISA Set (BD, Franklin Lakes, NJ, USA).

2.12. Statistical analysis

Statistically significant differences between the two groups were identified by the Mann-Whitney U test. Multiple comparisons were made between the different groups using Scheffe's test with ANOVA, whereas multiple comparisons with the control results were made using Dunnett's test. $P < 0.05$ indicated significance.

3. Results

3.1. Physicochemical properties of the anionic bubble lipopolyplexes

The particles of the cationic polyplexes were approximately 130 to 150 nm in size, with zeta-potentials in the range of 25 to 55 mV (Table 1). Addition of anionic liposomes to the cationic polyplexes extensively decreased zeta-potentials with partial effect on the particle sizes. The trapping of the perfluoropropane gas led to an increase in the particle size of the anionic lipopolyplexes, with the particles becoming 450 to 600 nm in size. The trapping of the gas, however, had little effect on the zeta-potential. Following the trapping of the gas, the lipopolyplex solutions became cloudy in appearance, as shown Fig. 1a. TEM images were then taken to allow for a detailed view of the structure of the pDNA/PS, pDNA/PS/AL, and pDNA/PS/BL (Fig. 1b). Those TEM images revealed that AL coated on the surface of pDNA/PS and the pDNA/PS/BL existed as a cluster of bubble liposomes. The formation of the complex through electrostatic interactions with or without US exposure was clarified by gel electrophoresis (Fig. 2).

3.2. In-vitro gene expression experiments

The cationic polyplexes, anionic lipopolyplexes, and anionic bubble lipopolyplexes were added to the EAhy926 cells to determine their transgene efficiencies (Fig. 3). The results revealed that the transgene efficiencies of the cationic polyplexes and anionic lipopolyplexes were not affected by exposure to the US. In contrast, US exposure significantly increased the level of gene expression in the anionic bubble lipopolyplexes, including the pDNA/PS/BL, pDNA/PLL/BL, pDNA/PLA/BL, and pDNA/DPLL/BL systems ($P < 0.01$), where the levels of gene expression were significantly higher than those in the cationic polyplexes and anionic lipopolyplexes ($P < 0.01$ or $P < 0.05$). Of the anionic bubble lipopolyplexes tested in the current study, the pDNA/PS/BL with US exposure showed the highest level of gene expression.

3.3. Intracellular distribution experiments

The intracellular distribution of the anionic bubble lipopolyplexes composed of pDNA/PS/BL with or without US exposure was determined by confocal microscopy (Fig. 4). The nucleus, pDNA, and lysosomes were indicated as blue, green, and red fluorescein, respectively. As shown in Fig. 4a, the pDNA was mainly localized with the lysosomes as yellow dots following transfection using Lipofectamine 2000. The co-localization of the pDNA and lysosomes (yellow dots) was also observed in the cells transfected with pDNA/PS/BL without US exposure (Fig. 4b). In contrast, the pDNA and lysosomes were found to be separate from each other in the cells

transfected by pDNA/PS/BL with US exposure (Fig. 4c).

3.4. Cytotoxicity and erythrocyte aggregation

The cytotoxicities of the anionic bubble lipopolyplexes towards the EAhy926 cells were determined using a WST-1 assay (Fig. 5). Although US exposure did not affect the cell viability, significant levels of cytotoxicity ($P < 0.05$ or $P < 0.01$) were observed in the cells treated with the anionic bubble lipopolyplexes, except for pDNA/PS/BL.

The cationic lipoplexes and polyplexes, and anionic bubble lipopolyplexes were mixed with erythrocytes and the aggregation levels were determined (Fig. 6). The cationic lipoplexes that had been prepared with Lipofectamine 2000 caused aggregation of the erythrocytes and hemolysis (Fig. 6b). The cationic polyplexes such as pDNA/PEI, pDNA/PS, pDNA/PLL, pDNA/PLA, and pDNA/DPLL showed extensive aggregation (Fig. 6c, d, f, h, and j, respectively). In contrast, no significant levels of aggregation or hemolysis were determined in the anionic bubble lipopolyplexes (Fig. 6e, g, i, and k).

3.5. *In-vivo* gene expression experiments

Various anionic bubble lipopolyplexes were intravenously administered and the abdominal areas of the mice were then exposed to US. The levels of gene expression in the liver, kidney, and spleen were then determined (Fig. 7a, b, and c, respectively). The anionic bubble lipopolyplexes without US exposure showed extremely low levels of gene expression, whereas US exposure in these cases led to a significant increase ($P < 0.05$ or $P < 0.01$) in gene expression in the liver, kidney, and spleen.

3.6. Effect of anionic lipids constructing anionic liposomes in the anionic bubble lipopolyplexes (pDNA/PS/BL)

The effects of the anionic lipids of the anionic liposomes in the anionic bubble lipopolyplexes on the *in-vitro* transfection efficiency, *in-vivo* transfection efficiency, and cytotoxicity were determined (Fig. 8a, b, and c, respectively). The DSPA containing the anionic bubble lipopolyplexes (pDNA/PS/BL) with US exposure showed the lowest transfection efficiency of all of the systems tested, both *in-vitro* and *in-vivo* (Fig. 8a and b). The DSPA and DSPS containing the anionic bubble lipopolyplexes (pDNA/PS/BL) with US exposure showed a slight reduction in cell viability (Fig. 8c). In contrast, the DSPG containing the anionic bubble lipopolyplexes (pDNA/PS/BL) with US exposure showed the highest level of gene expression with no discernible impact on the cell viability.

3.7. Intrahepatic gene expression resulting from the anionic bubble lipopolyplex (pDNA/PS/BL)

Liver parenchymal cells and non-parenchymal cells were separated by collagenase perfusion and their levels of luciferase expression were determined. The anionic bubble lipopolyplex (pDNA/PS/BL) with US exposure showed significantly higher levels of gene expression in liver non-parenchymal cells than parenchymal cells ($P < 0.05$) (Fig. 9a). Determination of the levels of luciferase mRNA revealed that the anionic bubble lipopolyplex with US exposure showed significantly higher levels of expression in liver non-parenchymal cells (Kupffer cells and liver endothelial cells) than parenchymal cells ($P < 0.05$). Furthermore, the levels of mRNA expression in the Kupffer cells and liver endothelial cells were almost identical in the liver non-parenchymal cells (Fig. 9b).

3.8. In-vivo toxicity by the anionic bubble lipopolyplex (pDNA/PS/BL)

The serum AST and ALT activities of the anionic bubble lipopolyplex (pDNA/PS/BL) were determined with or without US exposure (Fig. 10a and b, respectively). Very little hepatic toxicity was observed for the anionic bubble lipopolyplex (pDNA/PS/BL) with or without US exposure. The use of the hydrodynamic method as a positive control led to significant increases in the serum AST and ALT activities. The effects of the anionic bubble lipopolyplex (pDNA/PS/BL) with or without US exposure on the level of serum inflammatory cytokines such as TNF- α (Fig. 11a) and IL-6 (Fig. 11b) were also evaluated. The results revealed that the anionic bubble lipopolyplex (pDNA/PS/BL) with or without US exposure had no discernible impact on cytokine production. The use of DOTAP/Chol lipoplexes as a positive control under the same conditions led to high levels of cytokine production.

4. Discussion

For clinical applications, it is important that non-viral carrier materials are composed of biocompatible and/or biodegradable constituents. To date, various biodegradable cationic polymers have been developed, including PS, PLL, and octa-arginine. In all of these cases, however, the transfection efficiencies were poor because materials did not exhibit any endosomal escape properties following endocytosis [35-37]. Endosomolytic gene delivery carriers such as PEI and cationic liposomes have recently been developed for effective transfection [38, 39]. Unfortunately, however, these polyplexes and lipoplexes possess a cationic charge and can interact with biogenic substances to become cytotoxic at high concentrations [30]. One promising approach for overcoming these safety issues involves the construction of neutral or anionic charged carriers from ternary complexes, and ternary complexes of this type have been reported to possess an anionic surface charge and show lower levels of toxicity than cationic gene delivery carriers [40, 41]. In the current study, we have developed for the first time anionic bubble lipopolyplexes that are ternary complexes of pDNA, cationic polymers, and anionic bubble liposomes with biocompatible compounds to be used for efficient and safe transfection processes.

The sonoporation method has been used in the current study because this method has been reported to deliver genes directly into the cytosol [14-17, 25, 27]. To prepare an anionic bubble formulation, the pDNA was initially condensed by the biodegradable cationic polymers. The anionic lipopolyplexes were subsequently prepared by coating the resulting cationic polyplexes with AL. In the TEM images, AL was observed on the surface of the pDNA/PS/AL and the zeta-potentials of those cationic polyplexes were turned from positive to negative by additions of AL (Fig. 1b and Table 1). Those results support that the AL is coating those cationic polyplexes. The bubble lipopolyplexes were then formed through the encapsulation of perfluoropropane gas. The particles of the anionic lipopolyplexes, including pDNA/PS/AL, pDNA/PLL/AL, pDNA/PLA/AL, and pDNA/DPLL/AL, were approximately 160 to 200 nm in size with zeta-potentials in the range of -20 to -40 mV (Table 1). The encapsulation of the perfluoropropane gas led to an increase the particle size of the lipopolyplexes, which became approximately 450 to 600 nm in size, although the encapsulation process had very little impact on the zeta-potential values (Table 1). Given that the use of the saturated lipids of liposomes is essential to the encapsulation of perfluoropropane gas in bubble lipoplexes [27, 42], we prepared AL using a range of saturated lipids, such as DSPG, DSPA, DSPE, PEG-DSPE, and DSPC. The particle sizes of the anionic bubble lipopolyplexes were found to be almost identical to those of the mannosylated cationic bubble lipoplexes reported in our previous paper [27]. Electron microscopy was then used to confirm the structure of the anionic bubble lipopolyplex (pDNA/PS/BL). To date, there have been very few reports in the literature concerning the use of bubble lipoplexes. As shown in Fig. 1b, the TEM image of the

pDNA/PS/BL system revealed that this material existed as a cluster of anionic bubble liposomes. The particle sizes of these anionic bubble lipopolyplexes were also very similar to those measured using a Zetasizer Nano ZS (Table 1). The anionic liposomes on the surface of the pDNA/PS/AL were crowded as a consequence of electrostatic interactions with the cationic polyplex (pDNA/PS), and steric hindrance from the anionic liposomes could potentially complicate any expansion. Further study is needed, however, to clarify the detailed structure of these anionic bubble lipopolyplexes.

Some polyanions such as heparin sulfate have been reported to dissociate polyplexes, with the pDNA being released from the polyplexes as a consequence [43]. To examine complex formation in the bubble lipopolyplexes, the release of pDNA from the anionic bubble lipopolyplexes was evaluated by gel electrophoresis (Fig. 2). The migration of pDNA was not detected in the polyplexes, lipopolyplexes, bubble lipopolyplexes, or bubble lipopolyplexes with US exposure. These results suggested that the pDNA is retained in the anionic bubble lipopolyplexes regardless of the addition of anionic liposomes, the encapsulation of perfluoropropane gas or the US exposure procedure.

Polyplexes constructed from biodegradable polymers such as PS and PLL have been reported to show low levels of gene expression [35, 36]. In general, anionic nanoparticles are not taken up by cells that possess an anionic charge because of electric repulsion. In contrast, the use of microbubbles in conjunction with US exposure can deliver pDNA directly into the cytosol by creating transient pores on the cellular membrane, and this technique shows high levels of gene expression [15, 17]. The anionic bubble lipopolyplexes with US exposure were therefore expected to give high gene expression potency. The transfection efficacy of the anionic bubble lipopolyplexes with US exposure was much higher than those without US exposure in EAhy926 cells (Fig. 3) and in mice (Fig. 7). In case of the absence of US exposure, the anionic bubble lipopolyplexes showed lower gene expression than the anionic lipopolyplexes (Fig. 3). It might be due to sizes of the anionic bubble lipopolyplexes which were approximately four times bigger than the anionic lipopolyplexes (Table 1). To assess the intracellular localization of the anionic bubble lipopolyplexes, we observed the intracellular distribution of the anionic bubble lipopolyplex (pDNA/PS/BL) in EAhy926 cells with or without US exposure (Fig. 4). When the cells were transfected with Lipofectamine 2000, 47.4% of the pDNA was reported to have remained in the lysosomes [44]. We also observed that the pDNA was highly localized in the lysosomes following transfection with Lipofectamine 2000 (Fig. 4a). The co-localization of pDNA and lysosomes was also observed in cells transfected with the anionic bubble lipopolyplex (pDNA/PS/BL) without US exposure (Fig. 4b). In contrast, barely any pDNA was observed in the lysosomes in cells transfected with the anionic bubble lipopolyplex (pDNA/PS/BL) with US exposure (Fig. 4c).

These intracellular distribution characteristics supported the high gene expression of the anionic bubble lipopolyplex (pDNA/PS/BL) with US exposure.

It has been reported that the coating of cationic lipoplexes or polyplexes with anionic polymers markedly reduces the toxicity properties of cationic lipoplexes or polyplexes such as their cytotoxicity towards cultured cells, hepatic toxicity, and proinflammatory cytokine production properties [40, 45-47]. With this in mind, we evaluated the safety profile of the current anionic bubble lipopolyplexes, including their aggregation with erythrocytes, cytotoxicity, liver toxicity, and cytokine production. As shown in Fig. 6e, g, i, and k, the anionic bubble lipopolyplexes exhibited low levels of interaction with erythrocytes even when the transfection reagent (Lipofectamine 2000, Fig. 6b) and cationic polyplexes such as pDNA/PEI, pDNA/PS, pDNA/PLL, pDNA/PLA, and pDNA/DPLL (Fig. 6c, d, f, h, and j) showed high levels of aggregation, with the transfection reagent in particular also showing hemolysis (Fig. 6b). In contrast, the use of anionic bubble lipopolyplexes with US exposure led to a slight reduction in cell viability. pDNA/PS/BL in particular showing the lowest cytotoxicity of the four types of anionic bubble lipopolyplexes tested in the current study (Fig. 5). Furthermore, the lowest cytotoxicity was observed in the anionic bubble lipopolyplex (pDNA/PS/BL) containing DSPG (Fig. 8c). In addition, the anionic bubble lipopolyplex (pDNA/PS/BL) containing DSPG showed the highest gene expression in *in-vitro* and *in-vivo* (Fig. 8a and b). On the basis of these results, we decided to use pDNA/PS/BL containing DSPG in the subsequent experiments as an optimized formulation for efficient and safe gene transfer. We then proceeded to evaluate the hepatic toxicity and production of proinflammatory cytokines by the anionic bubble lipopolyplex (pDNA/PS/BL) with US exposure (Figs. 10 and 11). The anionic bubble lipopolyplex had no discernible impact on the serum AST and ALT activities, and did not give rise to an increase in the serum TNF- α and IL-6 levels. When pDNA is taken up by cells through the endocytosis, TLR9 on the endosomal membranes recognizes the CpG motif in pDNA and produces proinflammatory cytokine [48]. The anionic bubble lipopolyplex (pDNA/PS/BL) is considered to have a core-shell structure capable of shielding pDNA in the core (Figs. 1b and 2). Uchida and Kataoka et al. reported the development of a PEGylated polyplex with optimized PEG shielding that minimized the inflammatory response [49]. These results therefore lead us to believe that the core-shell structure of the anionic bubble lipopolyplex (pDNA/PS/BL) would result in the low TLR9 recognition and low cytokine production.

As far as the gene expression in the liver is concerned, the anionic bubble lipopolyplex (pDNA/PS/BL) with US exposure showed significantly higher levels of gene expression in liver non-parenchymal cells (i.e., Kupffer cells and liver endothelial cells) than in parenchymal cells ($P < 0.05$) (Fig. 9). These results can be explained in terms of the size of the anionic bubble lipopolyplex (approximately 500 nm) being much larger than

that of sinusoidal fenestrae (< 150 nm) [50]. The anionic bubble lipopolyplexes could therefore not pass efficiently through the sinusoidal fenestrae. As a consequence, the pDNA would be transferred to the liver non-parenchymal cells rather than the liver parenchymal cells.

It would be necessary to prepare the anionic bubble lipopolyplexes from biocompatible materials for their clinical application. In the current study, the anionic bubble lipopolyplex (pDNA/PS/BL) was constructed from pDNA, PS, and bubble liposomes (AL and perfluoropropane gas). The PS has been used clinically to neutralize the anticoagulant effect of heparin [51]. The lipids of AL, including DSPG, DSPC, and PEG-DSPE, have been used clinically as additives for a number of different drugs, including Ambisome, DaunoXome, and Doxil, respectively [52-54]. Perfluoropropane gas is also clinically used for microbubble contrast agents such as Definity [55].

5. Conclusion

In the current study, we have successfully constructed an anionic bubble lipopolyplex as a novel gene delivery carrier. This material was prepared as a ternary complex of pDNA, cationic polymer, and an anionic bubble liposome using biocompatible materials for efficient and safe gene transfection with US exposure. The anionic bubble lipopolyplex showed high levels of gene expression *in-vitro* and *in-vivo* only after US exposure. Furthermore, this material did not give rise to any liver toxicity or proinflammatory cytokine production. Anionic bubble lipopolyplex of this type could be used as novel formulation tools for effective and safe transfection processes, and we believe that the information provided in this report could provide a platform for the development of the anionic charged bubble formulation strategies for efficient and safe gene transfection with US exposure.

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Tables

Table 1. Particle size and zeta-potential data for the bubble lipopolyplexes

Complex	Size (nm)			ζ -Potential		
pDNA/PS	136.33	\pm	3.18	25.87	\pm	0.15
pDNA/PS/AL	202.3	\pm	3.8	-39.8	\pm	0.2
pDNA/PS/BL	563.0	\pm	40.7	-39.2	\pm	1.8
pDNA/PLL	137.3	\pm	2.5	55.0	\pm	0.2
pDNA/PLL/AL	161.0	\pm	1.0	-32.0	\pm	0.1
pDNA/PLL/BL	586.7	\pm	27.6	-44.4	\pm	0.7
pDNA/PLA	149.3	\pm	2.8	52.4	\pm	1.3
pDNA/PLA/AL	179.6	\pm	3.6	-32.0	\pm	0.2
pDNA/PLA/BL	457.2	\pm	140.0	-28.5	\pm	0.6
pDNA/DPLL	145.7	\pm	5.3	51.0	\pm	2.2
pDNA/DPLL/AL	169.5	\pm	1.2	-21.3	\pm	0.5
pDNA/DPLL/BL	448.6	\pm	0.6	-20.7	\pm	0.7

Data represent the mean \pm S.E..

Captions

Fig. 1. Photographs and TEM image of bubble lipopolyplexes

(a) Bubble lipopolyplexes (right) were prepared by sonication of the lipopolyplexes (left) with supercharged perfluoropropane gas. (b) The pDNA/PS, pDNA/PS/AL, and pDNA/PS/BL were observed by TEM with negative staining using uranyl acetate.

Fig. 2. Agarose gel electrophoresis of various complexes.

Each complex was loaded onto agarose gel for electrophoresis. Retardation of pDNA was visualized using a GelRed Nucleic Acid Gel Stain.

Fig. 3. *In-vitro* transgene efficiency of various complexes.

EAhy926 cells were transfected with different complexes with or without US exposure. The cells were then incubated for 24 hr after transfection and the level of luciferase gene expression was determined. Each bar represents the mean + S.E.. **: $P < 0.01$ vs all other groups; ##: $P < 0.01$; #: $P < 0.05$.

Fig. 4. Intracellular distribution of the pDNA.

Cells were transfected with fluorescein-labeled pDNA using Lipofectamine 2000 (a), pDNA/PS/BL (b), and pDNA/PS/BL with US (c). Six hours after transfection, the nuclei and lysosomes were stained by DAPI and LysoTracker Red DND-99, respectively. The merged images of the nuclei (blue), pDNA (green), and lysosomes (red) using confocal microscopy are shown.

Fig. 5. Cytotoxicity tests of the different complexes towards EAhy926 cells.

The viabilities of the cells treated with different complexes were measured by WST-1 assay. The different complexes were added to the cells and the cells subsequently exposed to US. The cells were incubated with complexes for 24 hr and the cell viability was measured. Data represents the percentage to untreated cells. Each bar represents the mean + S.E.. **: $P < 0.01$; *: $P < 0.05$ vs control.

Fig. 6. Aggregation of complexes with erythrocytes.

PBS (a), pDNA/Lipofectamine 2000 (b), pDNA/PEI (c), pDNA/PS (d), pDNA/PS/BL (e), pDNA/PLL (f),

pDNA/PLL/BL (g), pDNA/PLA (h), pDNA/PLA/BL (i), pDNA/DPLL (j), and pDNA/DPLL/BL (k) were added to the erythrocytes, and the aggregation was observed by phase microscopy.

Fig. 7. *In-vivo* transgene efficiency of bubble lipopolyplexes

ICR female mice were intravenously injected with bubble lipopolyplexes via the tail vein at a dose of 50 µg of pDNA. US was applied transdermally to the abdominal area immediately after the injection of the bubble lipopolyplexes. Six hours after administration, the mice were sacrificed and the luciferase activities in the liver (a), kidney (b), and spleen (c) were determined. Each bar represents the mean + S.E. **: $P < 0.01$; *: $P < 0.05$ vs each US (-) group.

Fig. 8. Effect of anionic lipids on the luciferase expression and cytotoxicity of pDNA/PS/BL

(a) EAhy926 cells were transfected with the different complexes with or without US exposure. Cells were incubated for 24 hr after transfection and the luciferase gene expression was determined. Each bar represents the mean + S.E. **: $P < 0.01$. (b) ICR female mice were intravenously injected with bubble lipopolyplexes via the tail vein at a dose of 50 µg of pDNA. US was then applied transdermally to the abdominal area immediately after the injection of the bubble lipopolyplexes. Six hours after administration, the mice were sacrificed and the luciferase activities in their liver, kidney, and spleen were determined. Each bar represents the mean + S.E. *: $P < 0.05$. (c) Viability of EAhy926 cells treated with the different complexes was measured by WST-1 assay. Different complexes were added to cells and the cells were subsequently exposed to US. Cells were incubated with complexes for 24 hr and the cell viability was measured. Data represent the percentage to untreated cells. Each bar represents the mean + S.E. *: $P < 0.05$ vs control.

Fig. 9. Hepatic cellular localization of luciferase expression by pDNA/PS/BL

pDNA/PS/BL was intravenously administrated into mice. US was then applied transdermally to the abdominal area immediately after the injection of the bubble lipopolyplexes. Six hours after administration, liver parenchymal cells and non-parenchymal cells were separated by the collagenase perfusion. Luciferase activity in the cells was determined (a). Kupffer cells and endothelial cells were separated by the immunomagnetic cell isolation system from non-parenchymal cells and the luciferase mRNA expression was determined (b). Each bar represents the mean + S.E.. *: $P < 0.05$ vs parenchymal cells.

Fig. 10. Hepatic toxicity of pDNA/PS/BL

pDNA/PS/BL was intravenously administrated into mice. US was then applied transdermally to the abdominal area immediately after injection of bubble lipopolyplexes. Serum was collected at 6, 12, and 24 hr after administration. The serum AST (a) and ALT (b) activities were determined using a Transaminase CII-Test Wako kit. Each bar represents the mean \pm S.E. **: $P < 0.01$ (Hydrodynamic methods); #: $P < 0.05$ (bubble lipopolyplexes US (-)); ††: $P < 0.01$ (bubble lipopolyplexes US (+)) vs group of non-treated.

Fig. 11. Proinflammatory cytokine induction by pDNA/PS/BL

pDNA/PS/BL was intravenously administrated into mice. US was then applied transdermally to the abdominal area immediately after injection of bubble lipopolyplexes. Serum was collected at 6, 12, and 24 hr after administration. The serum TNF- α (a) and IL-6 (b) levels were determined by ELISA. Each bar represents the mean \pm S.E. **: $P < 0.01$ vs group of non-treated.

Fig. 1

(a)



Bubble (-) Bubble (+)
pDNA/PS/AL



Bubble (-) Bubble (+)
pDNA/PLL/AL



Bubble (-) Bubble (+)
pDNA/PLA/AL



Bubble (-) Bubble (+)
pDNA/DPLL/AL

(b)

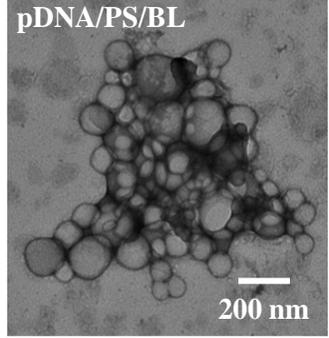
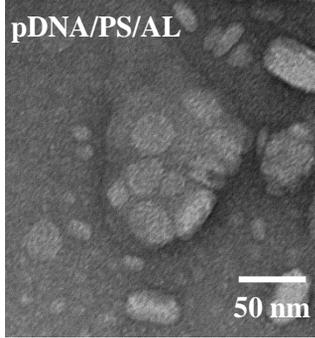
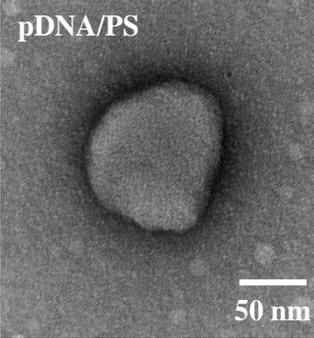


Fig. 2

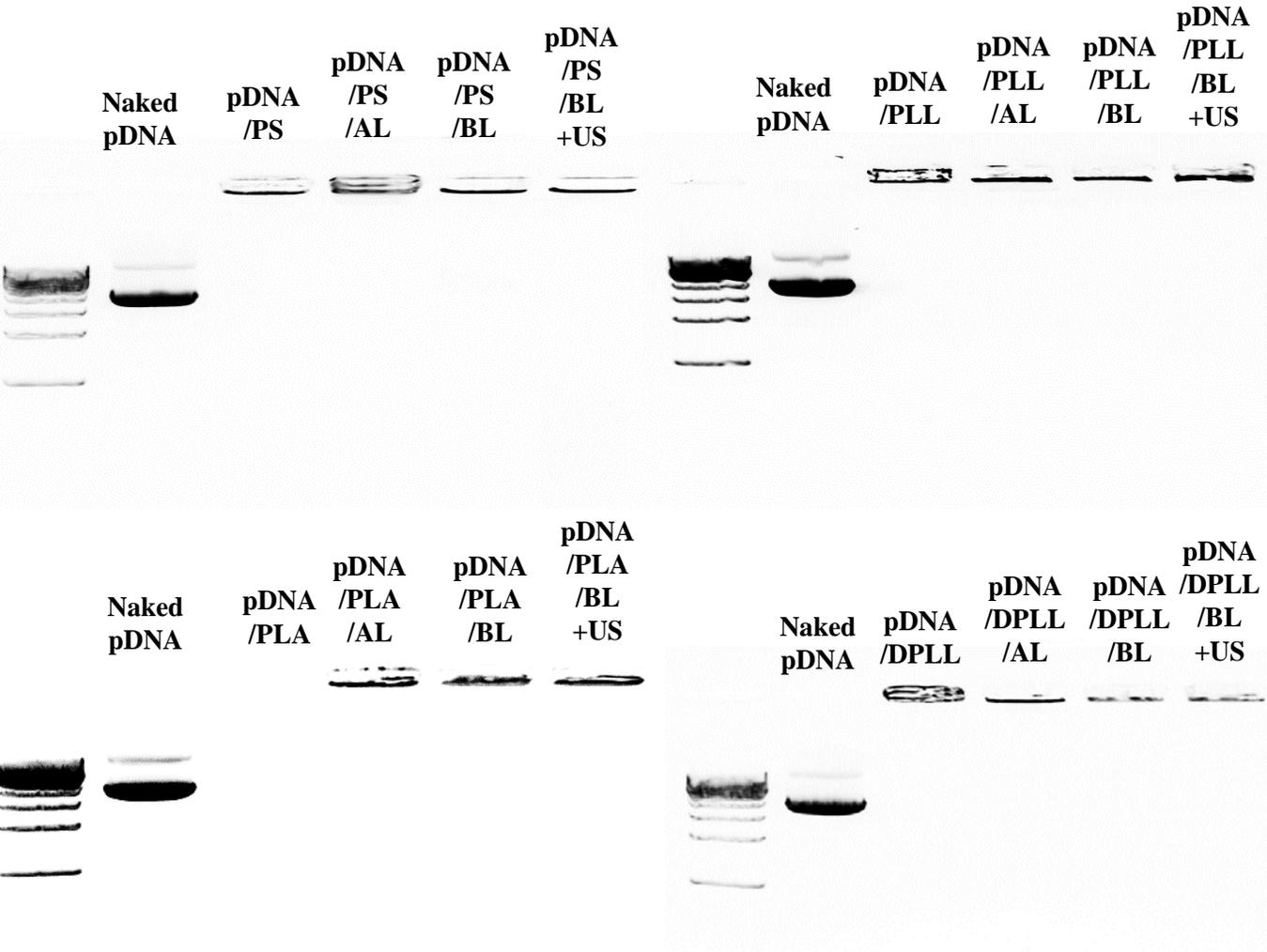


Fig. 3

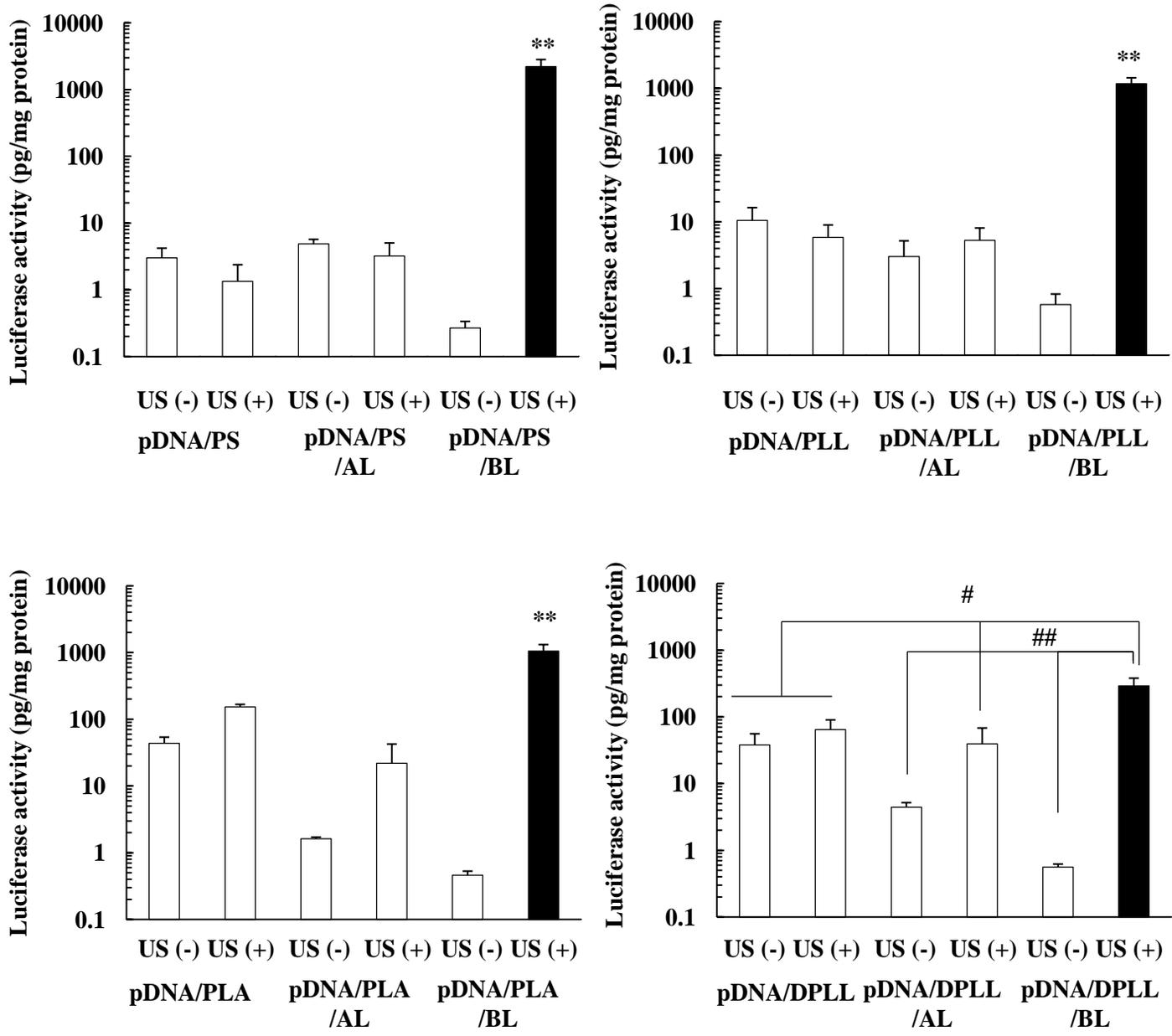


Fig. 4

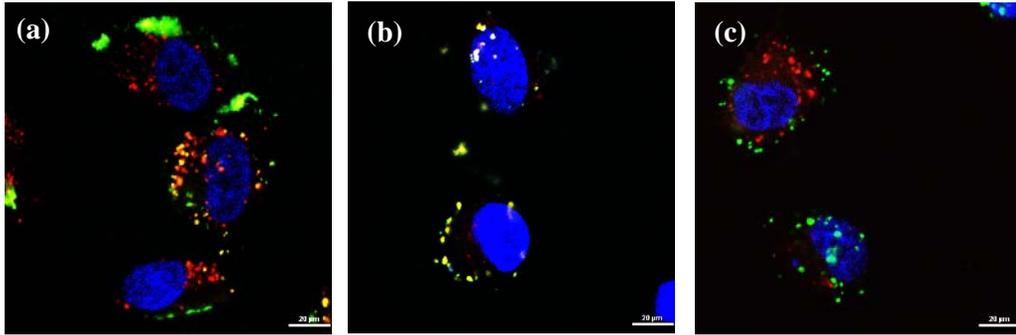


Fig. 5

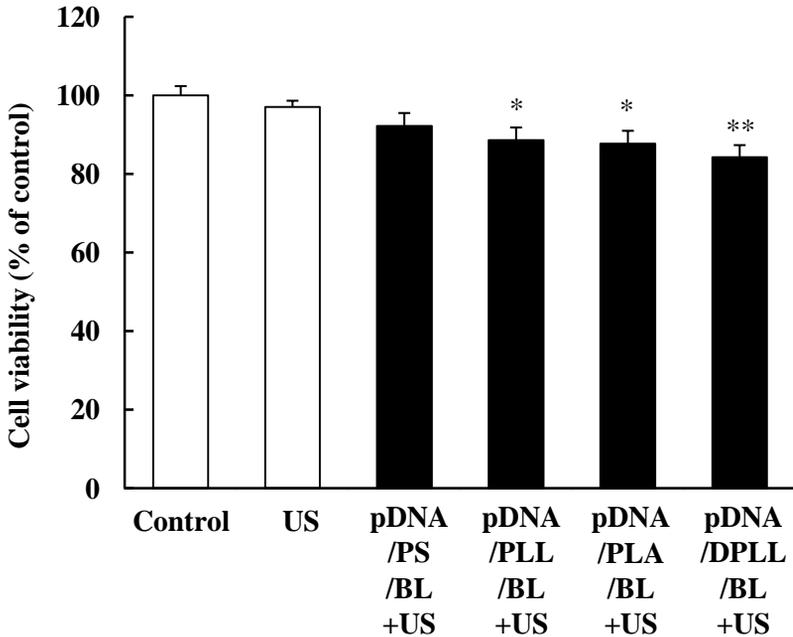


Fig. 6

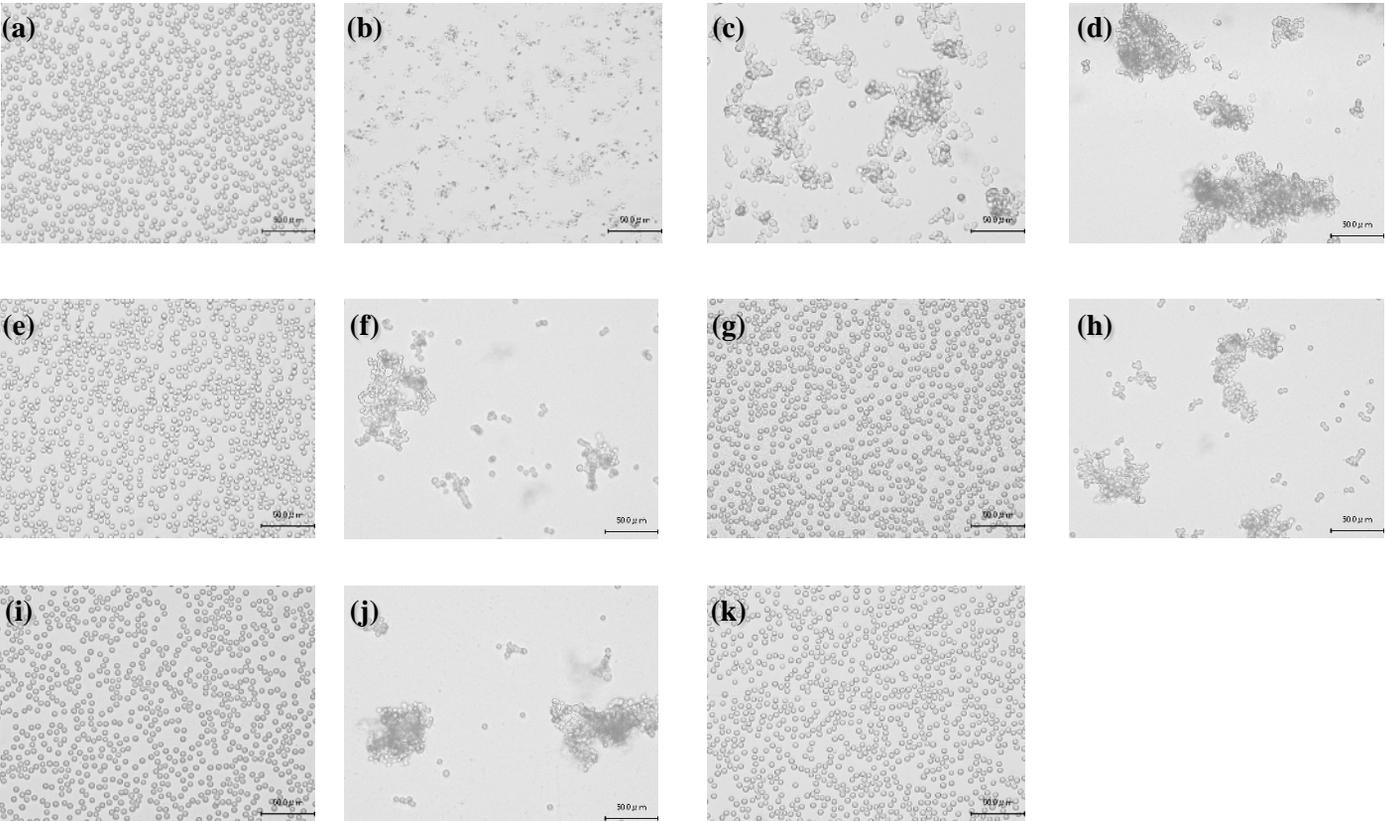


Fig. 7

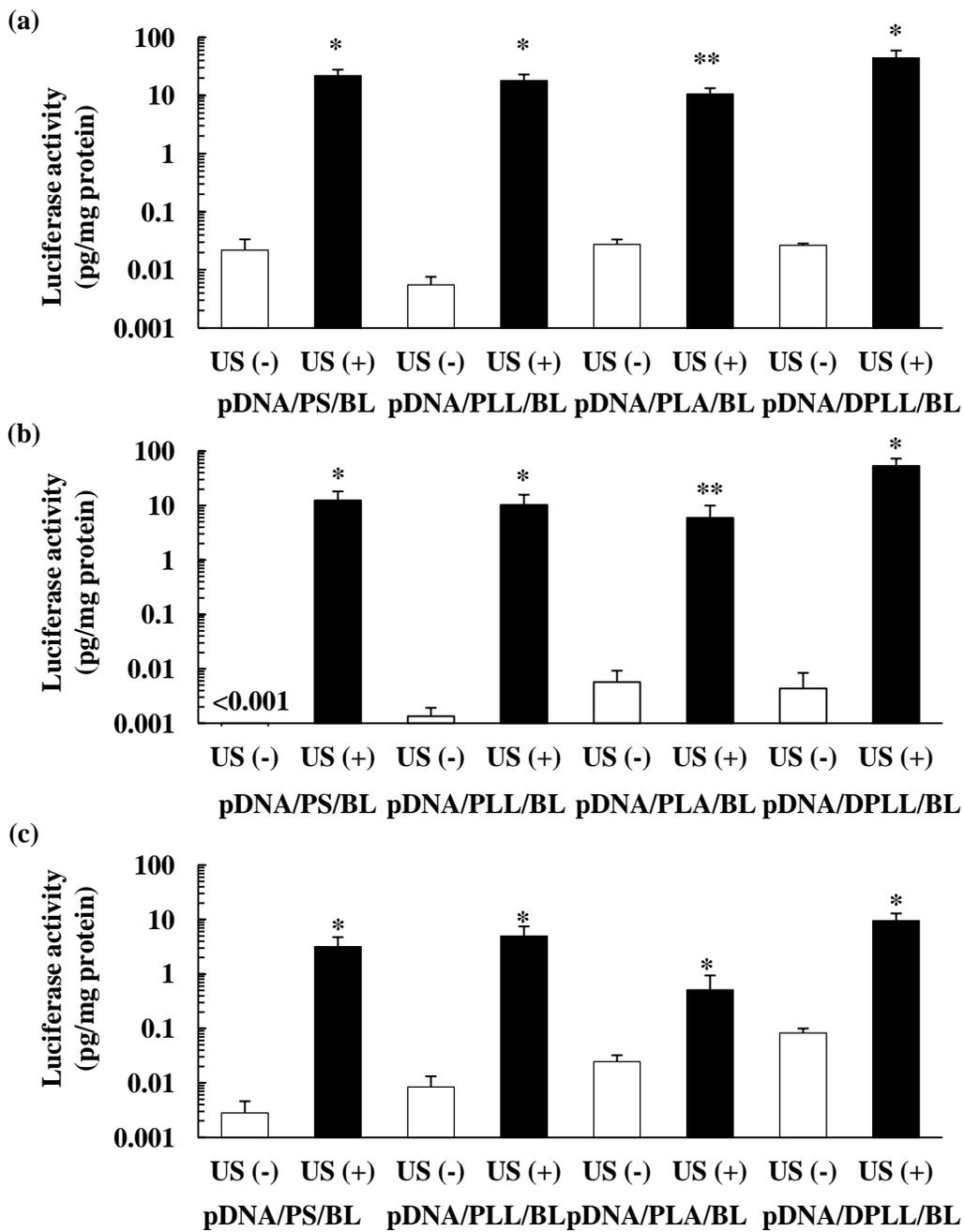


Fig. 8

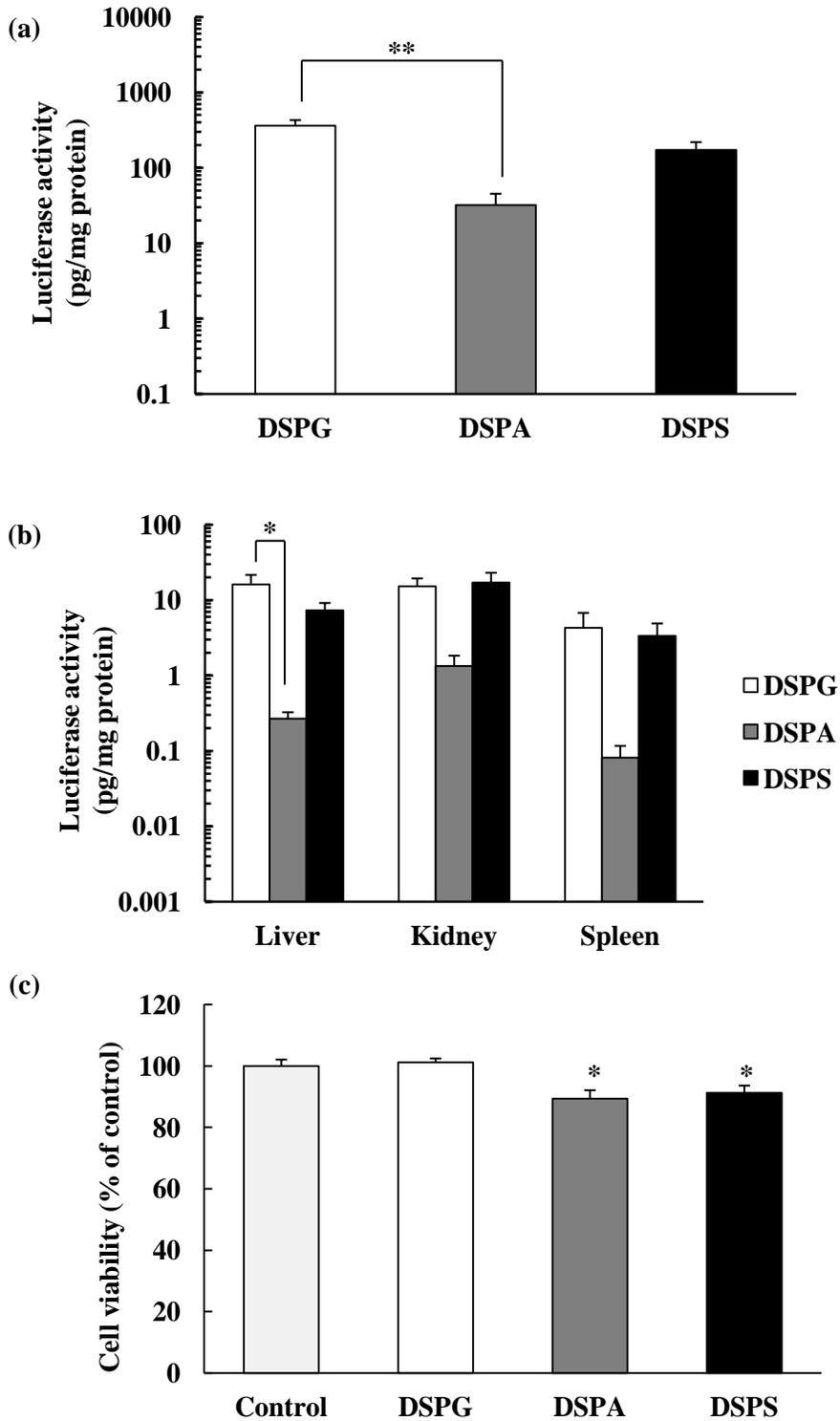


Fig. 9

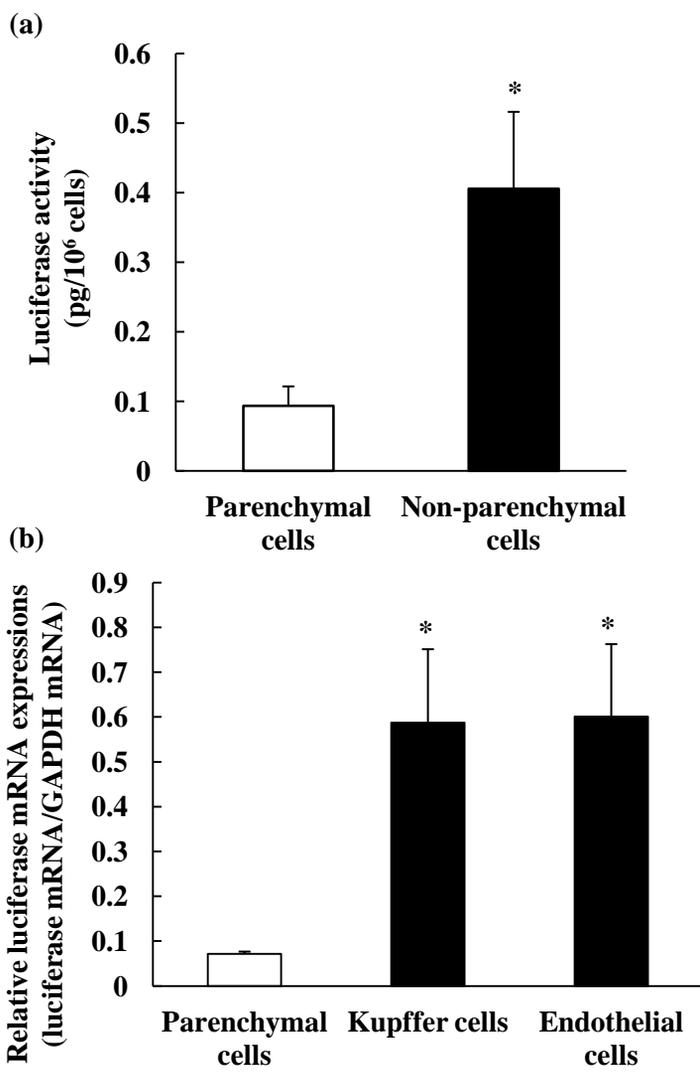


Fig. 10

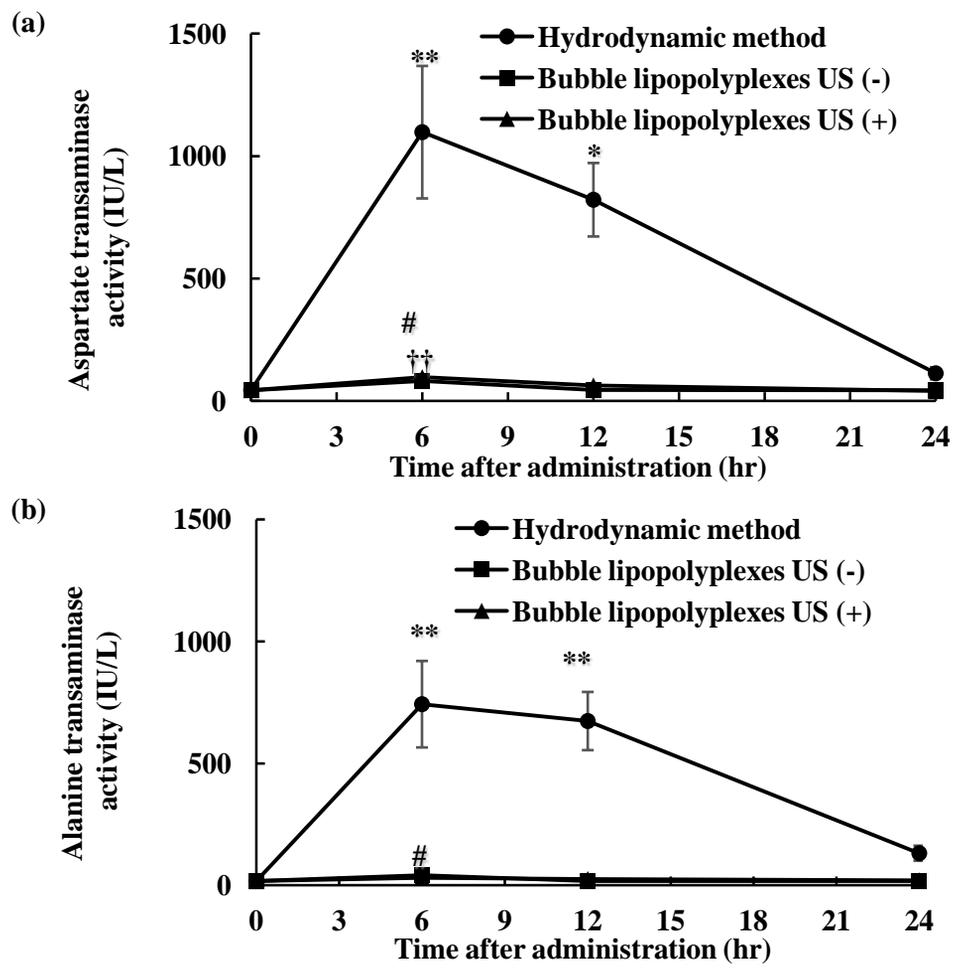


Fig. 11

