

Fig. 24. C-CPE修飾リポソームのL-CL-4細胞への結合評価  
 L-CL-4細胞にNBD標識したC-CPE修飾リポソームを添加し、4℃、1時間培養した。その後、細胞の蛍光をフローサイトメトリーにより測定した。

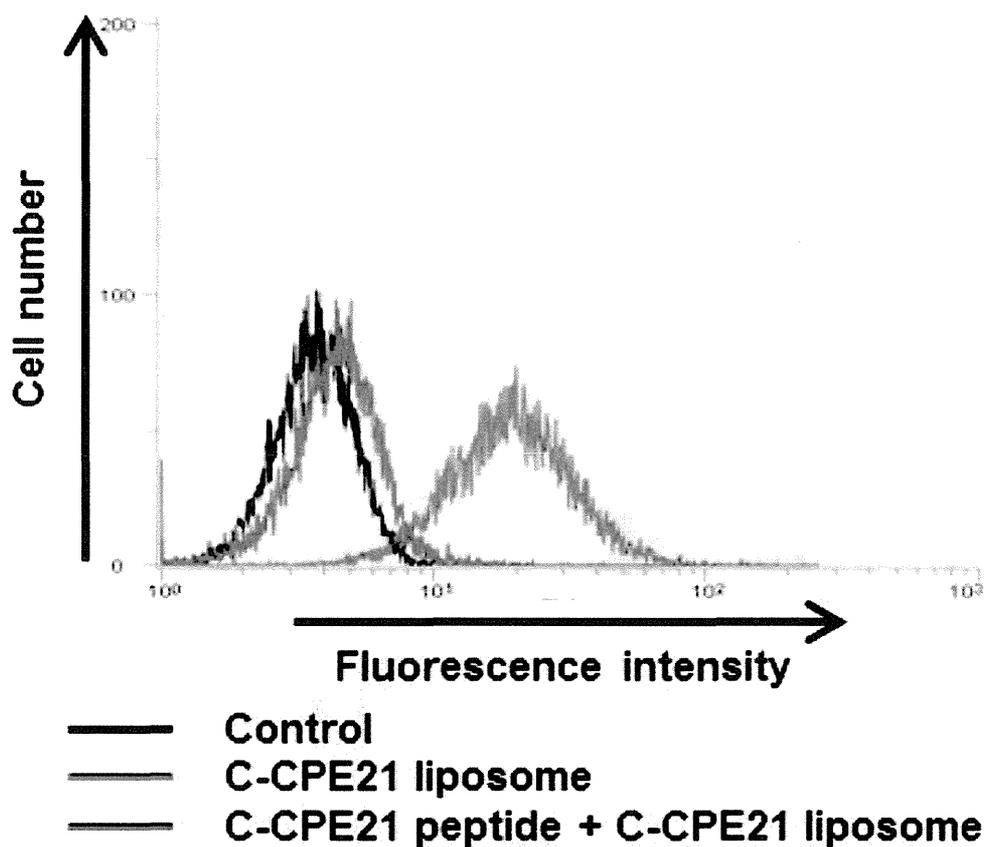


Fig. 25 競合阻害実験  
 L-CL-4細胞にC-CPE 194N309A/S313AペプチドおよびNBD標識したC-CPE 194N309A/S313A修飾リポソームを添加し、4℃、1時間培養した。その後、細胞の蛍光をフローサイトメトリーにより測定した。

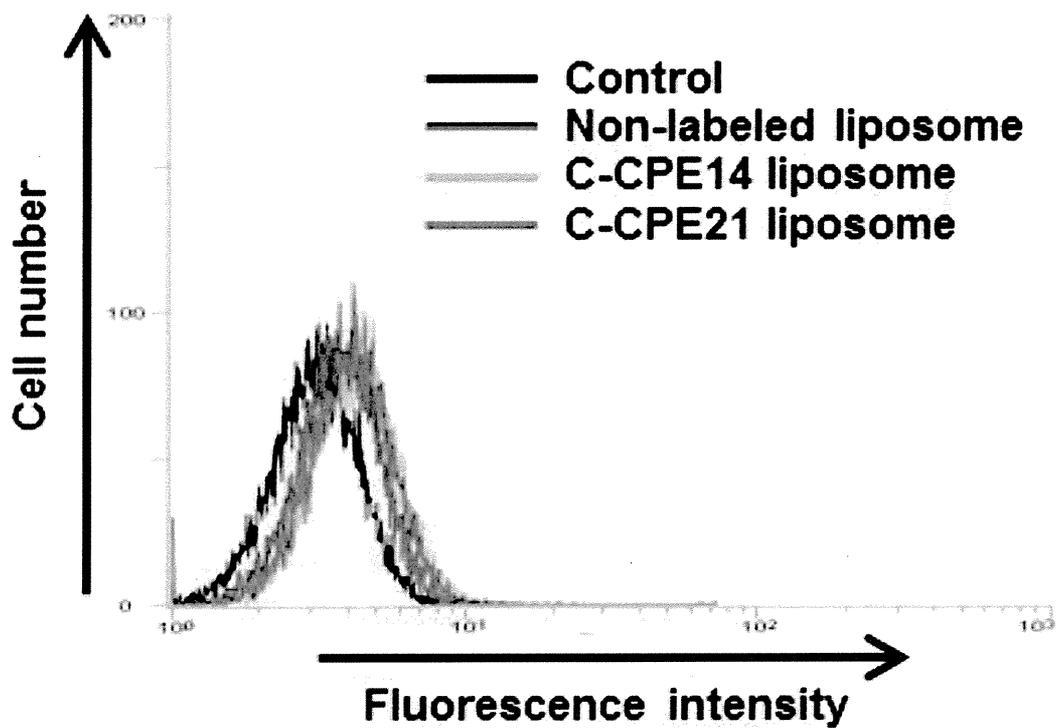


Fig. 26 凍結乾燥後のC-CPE修飾リポソームのL-CL-4細胞への結合評価  
 L-CL-4細胞に凍結乾燥後のNBD標識したC-CPE修飾リポソームを添加し、  
 4℃、1時間培養した。その後、細胞の蛍光をフローサイトメトリーにより  
 測定した。

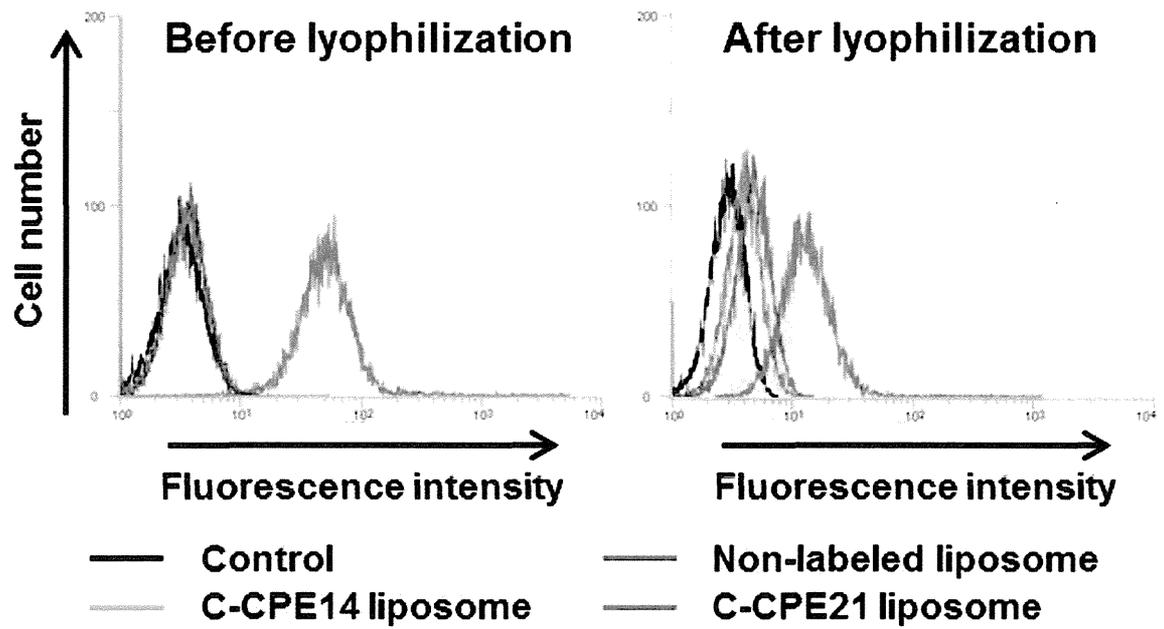


Fig. 27 C-CPE修飾リポソームのL-CL-4細胞への結合に及ぼす凍結乾燥操作の影響  
 凍結乾燥前および凍結乾燥後のNBD標識したC-CPE修飾リポソームをL-CL-4細胞に添加し、4℃、1時間培養した。その後、細胞の蛍光をフローサイトメトリーにより測定した。

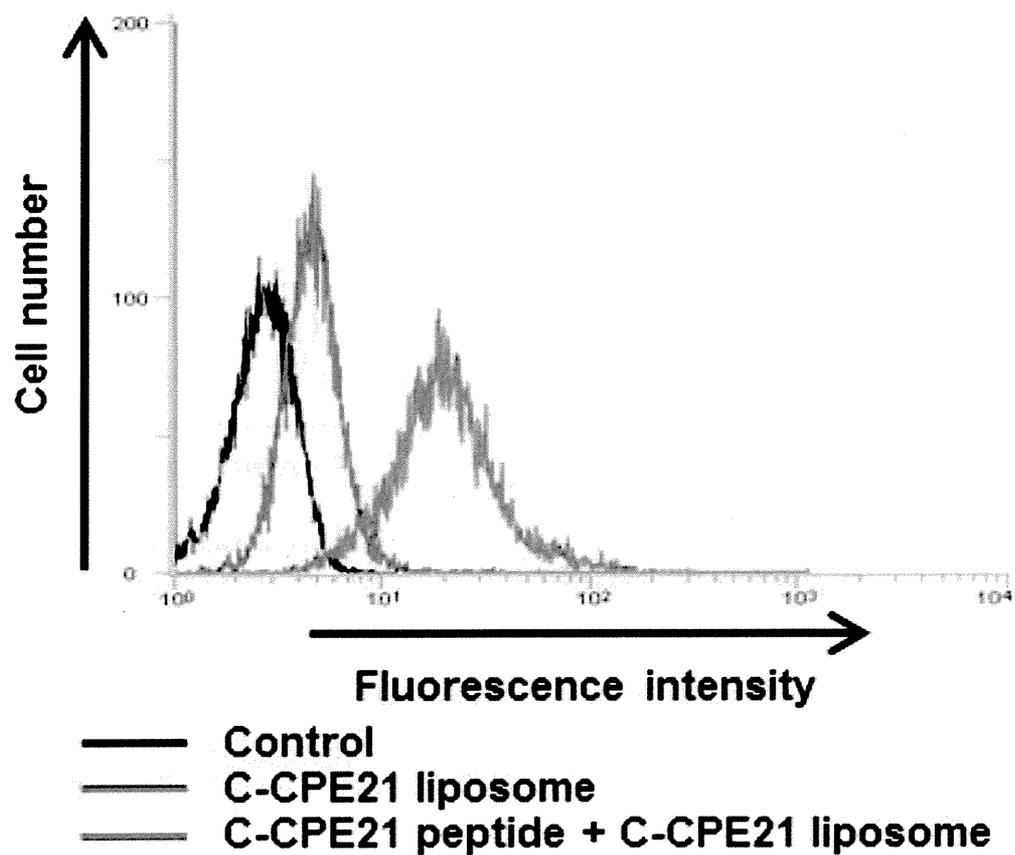


Fig. 28 凍結乾燥後のC-CPE修飾リポソームにおける競合阻害実験  
 凍結乾燥後のNBD標識したC-CPE 194 N309A/S313A修飾リポソームおよびC-CPE 194 N309A/S313AペプチドをL-CL-4細胞に添加し、4℃、1時間培養した。その後、細胞の蛍光をフローサイトメトリーにより測定した。

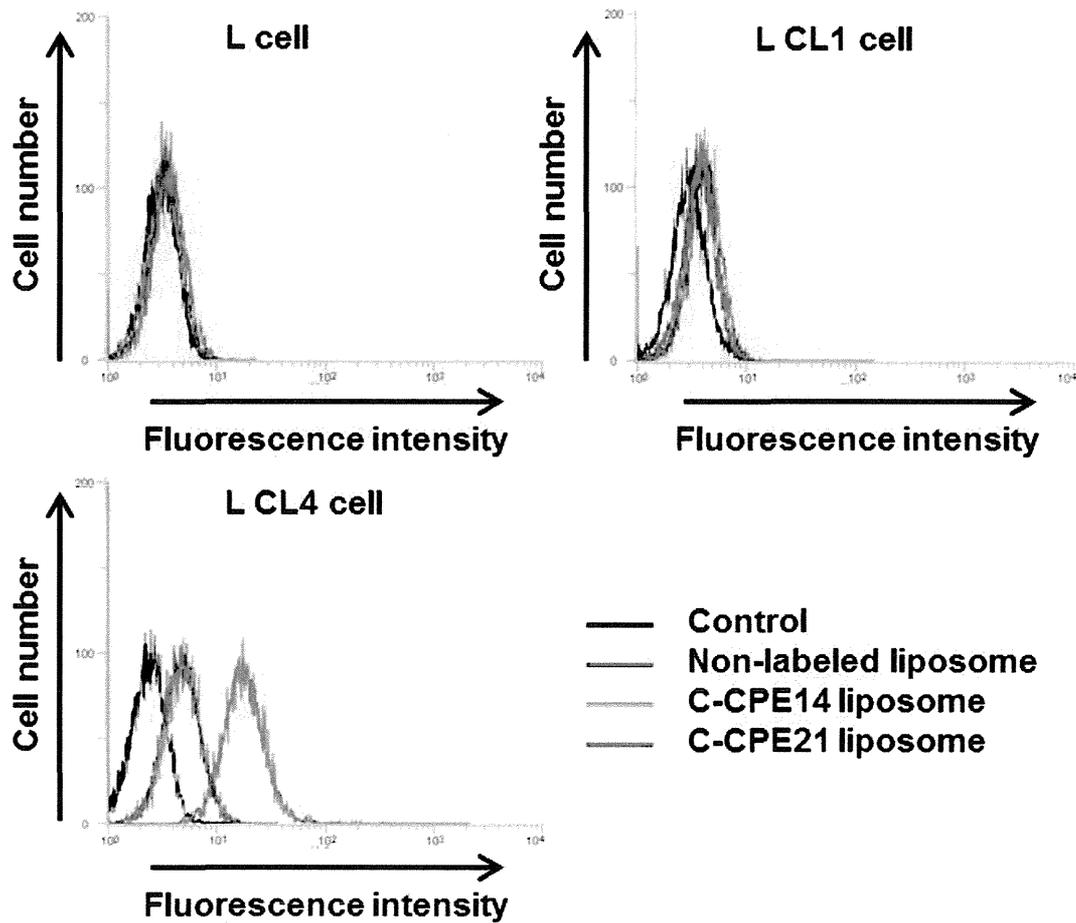


Fig. 29 C-CPE修飾リポソームのCL-4発現細胞への結合評価  
 L、L-CL-1、L-CL-4細胞にNBD標識したC-CPE修飾リポソームを添加し、4℃、1時間培養した。その後、細胞の蛍光をフローサイトメトリーにより測定した。

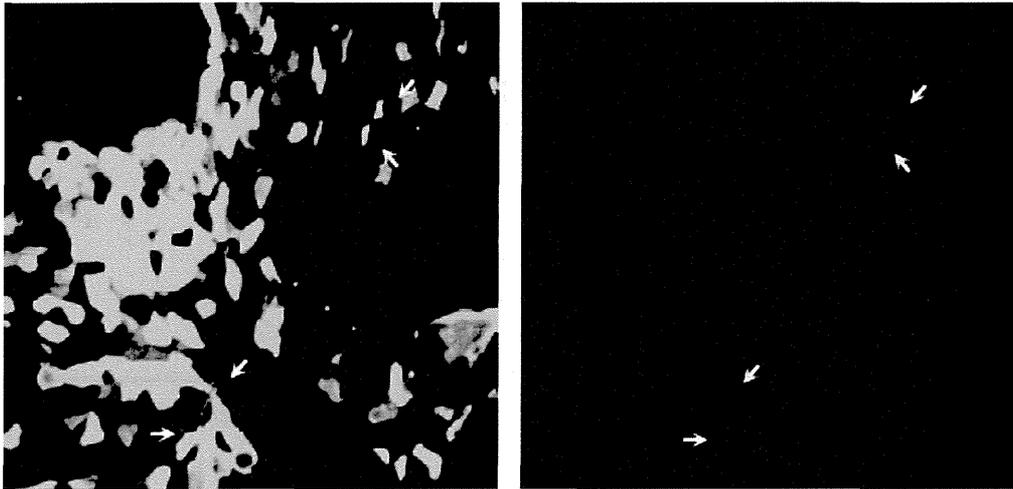


Fig. 30 C-CPE修飾リポソームのPPへの移行性評価  
Rhodamine標識C-CPE 194N309A/S313Aリポソームを十二指腸より投与し、2時間後、PPを回収した。FITC標識UEA1によりM細胞を染色し、共焦点顕微鏡によるZ-stack解析を行った。(緑：M細胞、赤：リポソーム)

<b>Liposome</b>	<b>Particle size</b>
<b>Non-labeled</b>	<b>119.6 nm</b>
<b>C-CPE14</b>	<b>146.2 nm</b>
<b>C-CPE21</b>	<b>166.6 nm</b>

Table 1 C-CPE修飾リポソームの粒子径

<b>Liposome</b>	<b>Particle size</b>
<b>Non-labeled</b>	<b>193.5 nm</b>
<b>C-CPE14</b>	<b>170.5 nm</b>
<b>C-CPE21</b>	<b>155.1 nm</b>

Table 2. 凍結乾燥後のC-CPE修飾リポソームの粒子径

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
	該当事項なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Omata D., Negishi Y., Hagiwara S., Yamamura S., Endo-Takahashi Y., Suzuki R., Maruyama K., Nomizu M., Aramaki Y.	Bubble Liposomes and Ultrasound Promoted Endosomal Escape of TAT-PEG Liposomes as Gene Delivery Car- riers.	<i>Mol. Pharm.</i>	8	2416-2423	2011
Un K., Kawakami S., Higuchi Y., Suzuki R., Maruyama K., Yamashita F., Hashida M.	Involvement of activat- ed transcriptional pro- cess in efficient gene transfection using un- modified and mannose- modified bubble lipop- lexes with ultrasound exposure.	<i>J. Control Release</i>	156	355-363	2011
Shiraishi K., Endoh R., Furuhata H., Nishihara M., Suzuki R., Maruyama K., Oda Y., Jo J., Tabata Y., Yamamoto J., Yokoyama M.	A facile preparation method of a PFC-containing nano-sized emulsion for theranostics of solid tumors.	<i>Int. J Pharm.</i>	421	379-387	2011
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Hagisawa K., Nishioka T., Suzuki R., Takizawa T., Maruyama K., Takase B., Ishihara M., Kurita A., Yoshimoto N., Ohsuzu F., Kikuchi M.	Enhancement of ultrasonic thrombus imaging using novel liposomal bubbles targeting activated platelet glycoprotein IIb/IIIa complex-in vitro and in vivo study.	<i>Int. J. Cardiol.</i>	152	202-206	2011
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Oda Y., Suzuki R., Otake S., Nishiie N., Hirata K., Koshima R., Nomura T., Utoguchi N., Kudo N., Tachibana K., Maruyama k.	Prophylactic immunization with Bubble liposomes and ultrasound-treated dendritic cells provided a four-fold decrease in the frequency of melanoma lung metastasis.	<i>J. Control. Release</i>	160	362-366	2012

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Iida M., Yoshida T., Watari A., Yagi K., Hamakubo T., Kondoh M.	A baculoviral display system to assay viral entry.	<i>Biol. Pharm. Bull.</i>	36(11)	1867-9	2013
Sugano M., Negishi Y., Endo Takahashi Y., Hamano N., Usui M., Suzuki R., Maruyama K., Aramaki Y., Yamamoto M.	Gene delivery to periodontal tissue using Bubble liposomes and ultrasound.	<i>J. Periodontal Res.</i>			2013
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Negishi Y., Tsunoda Y., Hamano N., Omata D., Endo Takahashi Y., Suzuki R., Maruyama K., Nomizu M., Aramaki Y.	Ultrasound-mediated gene delivery systems by AG73-modified bubble liposomes.	<i>Biopolymers</i>	100(4)	402-7	2013

EndoTakahashi Y., Negishi Y., Nakamura A., Suzuki D., Ukai S., Sugimoto K., Moriyasu F., Takagi N., Suzuki R., Maruyama K., Aramaki Y.	pDNA-loaded Bubble liposomes as potential ultrasound imaging and gene delivery agents.	<i>Biomaterials</i>	34(11)	2807-13	2013
Hamano N., Negishi Y., Omata D., Takahashi Y., Manandhar M., Suzuki R., Maruyama K., Nomizu M., Aramaki Y.	Bubble liposomes and ultrasound enhance the antitumor effects of AG73 liposomes encapsulating antitumor agents.	<i>Mol. Pharm.</i>	10(2)	774-9	2013
Negishi Y., Hamano N., Tsunoda Y., Oda Y., Choijamts B., EndoTakahashi Y., Omata D., Suzuki R., Maruyama K., Nomizu M., Emoto M., Aramaki Y.	AG73-modified Bubble liposomes for targeted ultrasound imaging of tumor neovasculature.	<i>Biomaterials</i>	34(2)	501-7	2013
Li X., Saeki R., Watari A., Yagi K., Kondoh M.	Tissue distribution And safety evaluation of a claudin-targeting molecule, the C-terminal fragment Of Clostridium perfringens enterotoxin.	<i>Eur. J. Pharm. Sci.</i>	14(52)	132-7	2014

# Bubble Liposomes and Ultrasound Promoted Endosomal Escape of TAT-PEG Liposomes as Gene Delivery Carriers

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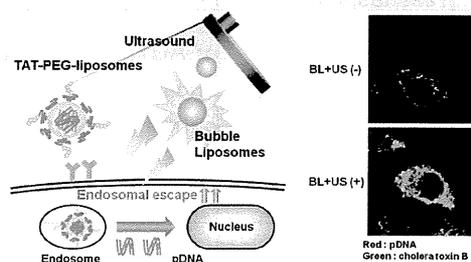
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**ABSTRACT:** We have previously developed laminin-derived AG73 peptide-labeled poly(ethylene glycol)-modified liposomes (AG73-PEG liposomes) for selective cancer gene therapy and reported that Bubble liposomes (BLs) and ultrasound (US) exposure could accelerate the endosomal escape of AG73-PEG liposomes, leading to the enhancement of transfection efficiency; however, it is still unclear whether BLs and US exposure can also enhance the transfection efficiency of other vectors. We therefore assessed the effect of BLs and US exposure on the gene transfection efficiency of *trans*-activating transcription factor (TAT) peptide modified PEG liposomes. Although TAT-PEG liposomes were efficiently internalized into cells, the efficacy of endosomal escape was insufficient. The transfection efficiencies of TAT-PEG liposomes were enhanced by about 30-fold when BLs and US exposure were used. We also confirmed that BLs and US exposure could not enhance the direct transportation of TAT-PEG liposomes into cells. Confocal microscopy showed that BLs and US exposure promoted endosomal escape of TAT-PEG liposomes. Our results suggested that BLs and US exposure could enhance transfection efficiency by promoting endosomal escape, which was independent of modified molecules of carriers. Thus, BLs and US exposure can be a useful tool to achieve efficient gene transfection by improving endosomal escape of various carriers.

**KEYWORDS:** Bubble liposomes, gene delivery, TAT peptide, ultrasound



## INTRODUCTION

Successful gene therapy depends on the efficient and safe delivery of genes into the desired tissues and cells. It is therefore necessary to develop efficient delivery vectors or methods for gene therapy. Nonviral vectors, such as cationic lipids or polymers, continue to be an attractive alternative to viral vectors due to their safety and convenient large-scale production, but their relatively low transfection efficiency compared with viral vectors is a major disadvantage.<sup>1</sup> In nonviral gene therapy, high transfection activity is required to improve the rate-limiting steps such as cellular internalization, endosomal escape, nuclear transfer, and intranuclear transcription.<sup>2,3</sup> In these steps, endosomal escape is considered one of the most important steps. When the vector cannot overcome this process, the cargo is degraded in lysosomes, leading to decreased gene transfection efficiency. For efficient endosomal escape, some studies have developed carriers equipped with functions such as pH sensitivity,<sup>4,5</sup> temperature dependence,<sup>6</sup> or photosensitivity.<sup>7</sup>

We have previously developed laminin-derived AG73 peptide-labeled poly(ethylene glycol) modified liposomes (AG73-PEG liposomes) for selective cancer gene therapy.<sup>8</sup> We also reported that echo-contrast gas-entrapping PEG liposomes, called "Bubble liposomes" (BLs), and ultrasound (US) exposure could accelerate the endosomal escape of AG73-PEG liposomes, leading to

enhanced transfection efficiency.<sup>9</sup> It is expected that BLs and US exposure may promote the endosomal escape of various carriers and enhance their transfection efficiency; however, it is still unclear whether BLs and US exposure can enhance the transfection efficiency of vectors other than AG73-PEG liposomes, and the effect of BLs and US exposure on the transfection efficiency and endosomal escape of other functional molecule modified gene delivery carriers is not clearly understood.

Cell-penetrating peptides (CPPs), such as TAT and R8 peptides, are able to facilitate penetration through cell membranes and translocate different cargo into cells.<sup>10</sup> The TAT peptide, derived from a human immunodeficiency virus trans-acting transcriptional activator, has been studied to achieve highly efficient gene delivery and to develop TAT-modified liposomes and a polyplex.<sup>11,12</sup> The majority of these carriers were internalized via endocytosis and were required to achieve endosomal escape for efficient gene transfection.<sup>13</sup> Additionally, TAT-modified carriers equipped with components enhancing endosomal escape have been developed.<sup>14</sup>

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We therefore prepared TAT-modified liposomes as a model to evaluate the effect of BLs and US exposure on gene transfection efficiency via endosomal escape.

In this study, to assess the utility of BLs and US exposure for efficient gene delivery in general, we focused on TAT peptide and evaluated the effect of BLs and US exposure on the gene transfection efficiency of TAT-modified liposomes.

## ■ EXPERIMENTAL SECTION

**Materials.** The plasmid pCMV-Luc is an expression vector encoding the firefly luciferase gene under the control of cytomegalovirus promoter. Fluorescein isothiocyanate-conjugated cholera toxin B subunit (FITC-CTB), chloroquine, chlorpromazine and protamine were purchased from Sigma (St. Louis, MO). Cy3-labeled pDNA was purchased from Mirus Bio, LLC (Madison, WI). Genistein was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Amiloride was purchased from Calbiochem (San Diego, CA).

**Cell Lines and Cultures.** HeLa cells (human cervical cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM; Kohjin Bio Co. Ltd., Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS; Equitech Bio Inc., Kerrville, TX), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

**Preparation of TAT-PEG Liposomes.** The Cys-TAT peptide (CGG-GRKKRRQRRRPPQ) was synthesized manually using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase strategy, prepared in the COOH-terminal amide form and purified by reverse-phase high performance liquid chromatography. Liposomes were prepared by the hydration method. pDNA diluted in 10 mM HEPES buffer (pH 7.4) was condensed using protamine ( $N/P = 5.0$ ). The complex of pDNA and protamine was added to a lipid film composed of 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (DOPG) (AVANTI Polar Lipids Inc., Alabaster, AL), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (AVANTI Polar Lipids, Inc.), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-polyethyleneglycol-maleimide (DSPE-PEG<sub>2000</sub>-Mal) (NOF Corporation, Tokyo, Japan) in a molar ratio of 2:9:0.57, followed by incubation for 10 min at room temperature to hydrate the lipids. The solution was sonicated for 5 min in a bath-type sonicator (42 kHz, 100 W) (2510J-DTH; Branson Ultrasonic Co., Danbury, CT). For coupling, TAT peptide, at a molar ratio of 5-fold DSPE-PEG<sub>2000</sub>-Mal, was added to the PEG liposomes, and the mixture was incubated for 6 h at room temperature to conjugate the cysteine of the Cys-TAT peptide with the maleimide of the PEG liposomes using a thioether bond. The resulting TAT-peptide conjugated PEG liposomes (TAT-PEG liposomes) were dialyzed to remove any excess peptide. TAT-PEG liposomes were modified with 5 mol % PEG and 3 mol % peptides of total lipid. The particle size and ζ-potential of prepared liposomes were measured by NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA).

**Preparation of Bubble Liposomes.** PEG liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (NOF Corporation) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG<sub>2000</sub>-OMe) (NOF Corporation) in a molar ratio of 94:6 were prepared by the reverse-phase evaporation method. In brief, all reagents were dissolved in 1:1 (v/v) chloroform/diisopropyl ether. Phosphate-buffered saline was added to the lipid solution, and the mixture was sonicated and then evaporated at 47 °C. The organic solvent was completely

removed, and the size of the liposomes was adjusted to less than 200 nm using extruding equipment and a sizing filter (pore size: 200 nm) (Nuclepore Track-Etch Membrane; Whatman Plc, UK). The lipid concentration was measured using a phospholipid C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). BLs were prepared from liposomes and perfluoropropane gas (Takachio Chemical Ind. Co. Ltd., Tokyo, Japan). First, 2 mL sterilized vials containing 0.8 mL of liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressurized with a further 3 mL of perfluoropropane gas. The vial was placed in a bath-type sonicator (42 kHz, 100 W) (2510J-DTH; Branson Ultrasonics Co.) for 5 min to form BLs.

**Transfection of pDNA into Cells Using TAT-PEG Liposomes.** The day before the experiments, HeLa cells ( $3 \times 10^4$ ) were seeded in a 48-well plate. The cells were treated with TAT-PEG liposomes (encapsulated pDNA: 3 µg/mL) in serum-free medium for 4 h at 37 °C. After replacement with fresh medium, the cells were cultured for 20 h, and then luciferase activity was measured.

**Transfection of pDNA into Cells by Combination of TAT-PEG Liposomes with BLs and US Exposure.** The day before the experiments, HeLa cells ( $3 \times 10^4$ ) were seeded in a 48-well plate. The cells were treated with TAT-PEG liposomes (encapsulated pDNA: 3 µg/mL) in serum-free medium for 4 h at 37 °C. After incubation, the cells were washed twice within 10 min to remove any excess TAT-PEG liposomes that were not associated with the cells, and BLs (120 µg/mL) were added. Within 2 min, US exposure was applied through a 6 mm diameter probe placed in the well (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1.0 W/cm<sup>2</sup>; time, 10 s). A Sonopore 3000 (NEPA GENE Co. Ltd., Chiba, Japan) was used to generate the US exposure. The cells were cultured for 20 h; then luciferase activity was determined, and cell viability was measured using a WST-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan).

**Measurement of Luciferase Expression.** Cell lysate was prepared with lysis buffer (0.1 M Tris-HCl (pH 7.8), 0.1% Triton X-100, and 2 mM EDTA). Luciferase activity was measured using a luciferase assay system (Promega) and a luminometer (LB96 V; Berthold Japan Co. Ltd., Tokyo, Japan). Activity is indicated as relative light units (RLU) per milligrams of protein.

**Flow Cytometry Analysis.** The day before the experiments, HeLa cells were seeded in a 12-well plate. Then, 0.2 mol % DiI-labeled TAT-PEG liposomes (pDNA: 3 µg/mL) were added to the cells and incubated for 1 h at 37 °C. The cells were collected, and the fluorescence intensities were measured by flow cytometry (FACSCanto; BD Biosciences, Franklin Lakes, NJ) to evaluate the cellular association of liposomes.

To examine the effect of BLs and US exposure on cellular uptake of pDNA, TAT-PEG liposomes (encapsulating Cy3-labeled pDNA: 3 µg/mL) were added to cells and incubated for 4 h at 37 °C. After incubation, the cells were washed twice, and BLs (120 µg/mL) were added. Then, US exposure was applied (frequency, 2 MHz; duty, 50%; burst rate, 2 Hz; intensity, 1.0 W/cm<sup>2</sup>; time, 10 s). Subsequently, the cells were incubated for 10 or 60 min, and then the cells were collected by trypsinization and washed with PBS supplemented with heparin (50 µg/mL) three times to remove TAT-PEG liposomes and pDNA bound to the cell surface. The fluorescence intensity was measured by flow cytometry.

**Confocal Laser Scanning Microscopy (CLSM).** HeLa cells were seeded a day before the experiments. HeLa cells were

treated with TAT-PEG liposomes (Cy3-labeled pDNA: 3  $\mu\text{g}/\text{mL}$ ) and FITC-CTB (10  $\mu\text{g}/\text{mL}$ ) for 1 h at 37  $^{\circ}\text{C}$ . After incubation, the cells were washed, and BLs (120  $\mu\text{g}/\text{mL}$ ) were added. US exposure was then applied (frequency, 2028 kHz; duty, 50%; burst rate, 2.0 Hz; intensity, 1.0 W/cm<sup>2</sup>; time, 10 s). Subsequently, the cells were incubated for 10, 60, or 180 min and then fixed with 4% paraformaldehyde for 1 h at 4  $^{\circ}\text{C}$ . CLSM was then performed (FV1000D; Olympus Corporation, Tokyo, Japan).

## RESULTS

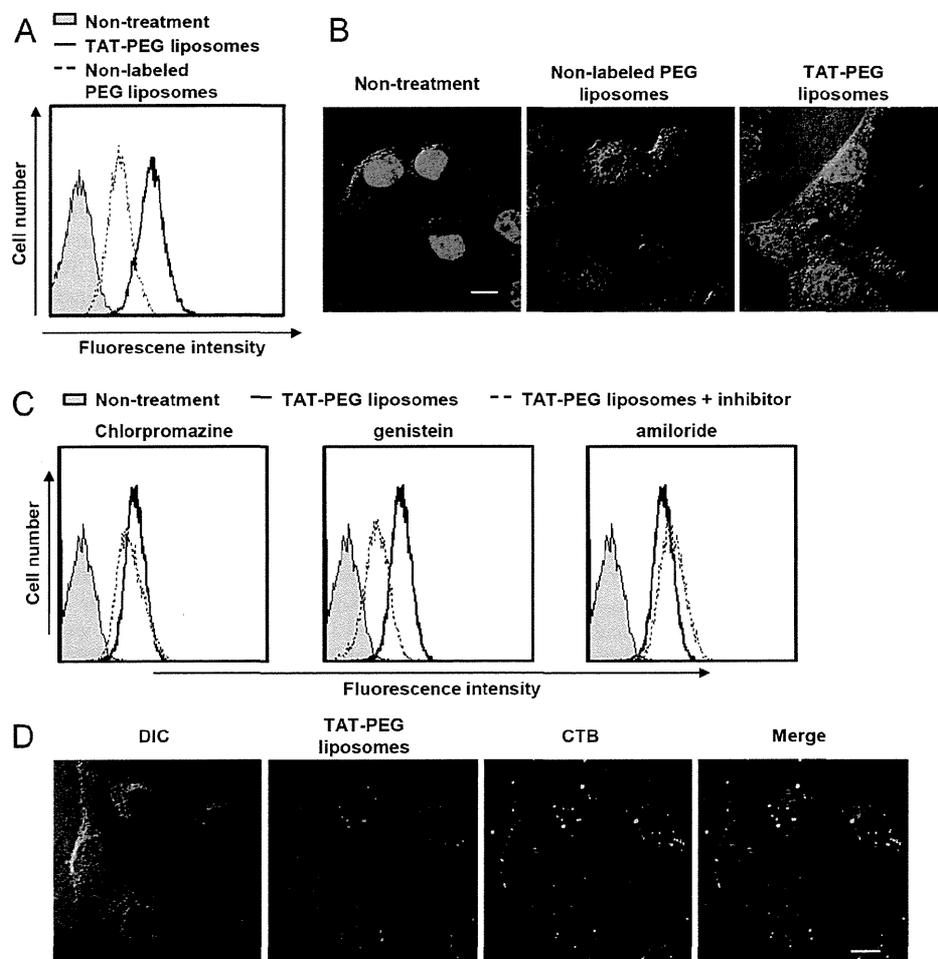
**Characterization of Prepared TAT-PEG Liposomes.** We evaluated the average size and zeta potential of prepared TAT-PEG liposomes, which were about 130 nm with a slight positive charge (Table 1).

**Table 1. Characteristics of Prepared Liposomes<sup>a</sup>**

prepared liposomes	PEG liposomes	TAT-PEG liposomes
particle size (nm)	132.1 $\pm$ 6.5	122.5 $\pm$ 10.5
$\zeta$ potential (mV)	3.17 $\pm$ 1.7	7.91 $\pm$ 1.6

<sup>a</sup>Data are the means and SD of three different determinations.

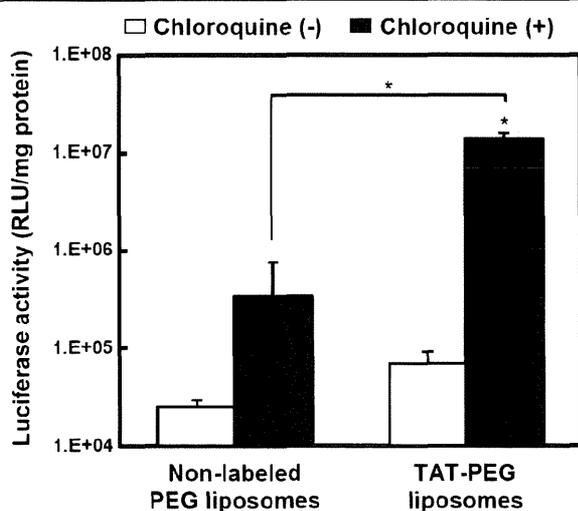
**Cellular Association of TAT-PEG Liposomes.** We first confirmed the effect of TAT peptide coating on the cellular association of liposomes and examined the association of TAT-PEG liposomes with HeLa cells. The cells were incubated with DiI-labeled liposomes for 1 h at 37  $^{\circ}\text{C}$ , and fluorescence intensity was determined by flow cytometry. The cellular internalization of TAT-PEG liposomes was observed by confocal laser scanning microscopy (CLSM). The cells treated with TAT-PEG liposomes showed increased fluorescence intensities compared with nonlabeled PEG liposomes (Figure 1A). In cells treated with TAT-PEG liposomes, the fluorescence of liposomes was observed in the cytoplasm, whereas it was weak in the cytoplasm of cells treated with nonlabeled PEG liposomes (Figure 1B). Furthermore, we investigated the cellular uptake pathway of TAT-PEG liposomes. Inhibitors that block clathrin-mediated endocytosis, raft-dependent endocytosis, and macropinocytosis were used to determine the cellular uptake pathway of TAT-PEG liposomes. Clathrin-mediated endocytosis was inhibited by chlorpromazine, which prevents the assembly of coated pits at the plasma membrane.<sup>15</sup> Raft-dependent endocytosis was inhibited by genistein, which is a tyrosine



**Figure 1.** Cellular association of TAT-PEG liposomes. (A, B) HeLa cells were treated with DiI-labeled nonlabeled or TAT-PEG liposomes for 1 h at 37  $^{\circ}\text{C}$ . (A) The fluorescence intensity was measured by flow cytometry. (B) Cells were observed by CLSM. The scale bar represents 10  $\mu\text{m}$ . (C) Cells were incubated with chlorpromazine (10  $\mu\text{g}/\text{mL}$ ), genistein (400  $\mu\text{M}$ ), or amiloride (1 mM) for 30 min and then treated with DiI-labeled TAT-PEG liposomes in the presence of an endocytic inhibitor for a further 1 h at 37  $^{\circ}\text{C}$ . Fluorescence intensities were measured by flow cytometry. (D) Cells were treated with DiI-labeled nonlabeled or TAT-PEG liposomes in the presence of FITC-CTB (10  $\mu\text{g}/\text{mL}$ ) for 1 h at 37  $^{\circ}\text{C}$ . Cells were observed by CLSM. The scale bar represents 10  $\mu\text{m}$ .

kinase inhibitor.<sup>16</sup> We also used amiloride, a specific inhibitor of the  $\text{Na}^+/\text{H}^+$  exchange required for macropinocytosis.<sup>17</sup> Flow cytometry analysis showed that the fluorescence intensity of TAT-PEG liposomes in the cells was decreased when cells were treated with genistein. In contrast, the fluorescence intensity of TAT-PEG liposomes in the cells was not changed when cells were treated with chlorpromazine or amiloride (Figure 1C). Furthermore, to elucidate the intracellular localization of TAT-PEG liposomes, the cells were treated with DiI-labeled TAT-PEG liposomes and FITC-cholera toxin B subunit (FITC-CTB), a marker of raft-dependent endocytosis,<sup>18</sup> and then observed by CLSM. As a result, the fluorescence of TAT-PEG liposomes was colocalized with the fluorescence of CTB in cells treated with TAT-PEG liposomes and CTB for 1 h (Figure 1D).

**Gene Transfection by TAT-PEG Liposomes.** Although TAT-PEG liposomes could be internalized efficiently into cells via raft-dependent endocytosis, it was necessary to achieve high gene expression so that genes in the endosome were delivered into the cytoplasm. To assess the ability of endosomal escape in TAT-PEG liposomes, the cells were treated with TAT-PEG liposomes in the presence of chloroquine, which is recognized as an endosomolytic agent.<sup>19</sup> Luciferase activity was 100-fold higher than that following treatment with TAT-PEG liposomes in the absence of chloroquine (Figure 2). It was suggested that



**Figure 2.** Gene transfection by TAT-PEG liposomes. Cells were preincubated with chloroquine (100  $\mu\text{M}$ ) for 30 min before transfection and then treated with nonlabeled or TAT-PEG liposomes in the presence of chloroquine for a further 4 h at 37 °C. After replacement with fresh medium, the cells were cultured for 20 h, and then luciferase activity was determined. Scale bars represent 10  $\mu\text{m}$ . Data are the means  $\pm$  SD ( $n = 4$ ). \* $p < 0.05$  compared with treatment in the absence of chloroquine.

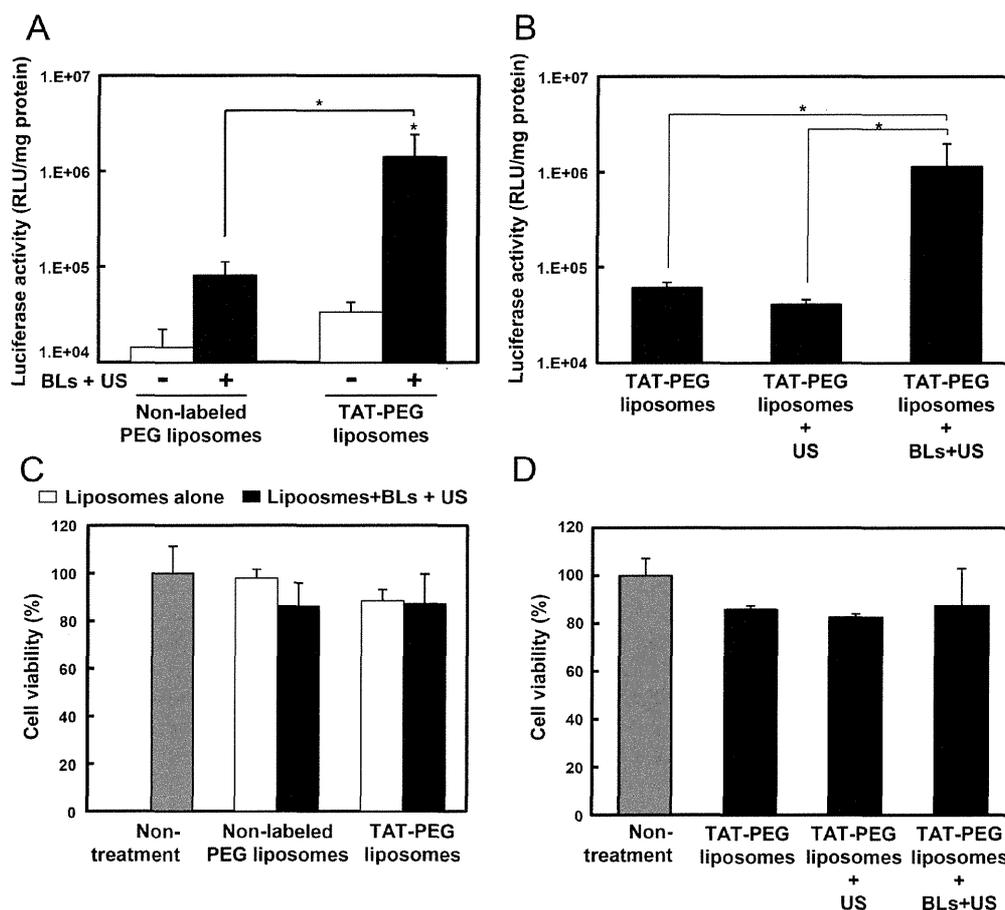
the TAT-PEG liposomes prepared in this study could be efficiently internalized into cells but might not release genes into the cytoplasm from endosomes.

**Effects of BLs and US Exposure on the Transfection Efficiency of TAT-PEG Liposomes.** To investigate the effect of BLs and US exposure on TAT-mediated liposomal gene transfection, HeLa cells were treated with TAT-PEG liposomes for 4 h at 37 °C in a serum-free medium, and then the cells were treated with BLs and US exposure. After treatment with TAT-PEG liposomes, luciferase activity was enhanced up to

30-fold by BLs and US exposure compared with TAT-PEG liposomes alone. Furthermore, the combination of TAT-PEG liposomes with BLs and US exposure had about 10-fold higher luciferase activity than nonlabeled PEG liposomes with BLs and US exposure (Figure 3A). We also examined the transfection efficiency by treating TAT-PEG liposomes with US in the absence of BLs. As a result, the transfection efficiency was barely enhanced by treatment with TAT-PEG liposomes with US compared with TAT-PEG liposomes alone (Figure 3B). The cytotoxicity of the combination of TAT-PEG liposomes with or without BLs and US exposure was determined using a WST-8 assay. The cell viability was more than 80% even after each transfection (Figure 3C,D). It was suggested that BLs and US exposure could enhance the transfection efficiency of TAT-PEG liposomes without significant cytotoxicity.

**Mechanism of Gene Transfection by TAT-PEG Liposomes with BLs and US Exposure.** We examined the effects of BLs and US exposure on the cellular uptake of TAT-PEG liposomes. Flow cytometry analysis was performed to measure the fluorescence intensity of Cy3-labeled pDNA in cells transfected by TAT-PEG liposomes with or without BLs and US exposure. As a result, the cellular uptake of pDNA showed almost no difference in the presence of TAT-PEG liposomes with or without BLs and US exposure (Figure 4A). To evaluate the involvement of the direct induction of TAT-PEG liposomes into cells, the cells were transfected with TAT-PEG liposomes with or without BLs and US exposure at 37 or 4 °C. Twenty-three hours after transfection, luciferase activity was measured. When the cells were transfected by TAT-PEG liposomes with BLs and US exposure at 37 °C, the luciferase activity increased compared with that of cells treated with TAT-PEG liposomes alone. In contrast, luciferase activity did not change in cells treated with TAT-PEG liposomes with BLs and US exposure at 4 °C compared with TAT-PEG liposomes alone (Figure 4B). We also confirmed the effect of temperature on the cellular uptake of TAT-PEG liposomes. The cells were treated with DiI-labeled TAT-PEG liposomes for 1 h at 37 °C or at 4 °C, and then fluorescence intensity was measured by flow cytometry. In cells treated at 4 °C, fluorescence intensity decreased compared with cells treated at 37 °C (data not shown). To evaluate the intracellular distribution of pDNA, HeLa cells were treated with TAT-PEG liposomes containing Cy3-labeled pDNA in the presence of FITC-CTB for 1 h, and then the cells were treated with BLs and US exposure. After US exposure, the cells were incubated for 10, 60, or 180 min and observed by CLSM. In cells treated with TAT-PEG liposomes alone, the fluorescence of pDNA colocalized with the fluorescence of CTB. In contrast, when cells were treated with BLs and US exposure, the fluorescence of CTB was dispersed widely in cells that were incubated for 10 min after US exposure (Figure 4C). We also confirmed the intracellular distribution of pDNA and CTB in living cells. As a result, the distribution of pDNA and CTB in living cells was similar to that of fixed cells (data not shown). Furthermore, we examined the intracellular distribution of pDNA and CTB treated by TAT-PEG liposomes with US in the absence of BLs. The intracellular distribution of pDNA and CTB showed almost no difference between the treatment of TAT-PEG liposomes alone and TAT-PEG liposomes with US exposure without BLs (data not shown).

It was suggested that BLs and US exposure could affect the intracellular trafficking of pDNA and enhance the transfection efficacy of TAT-PEG liposomes.



**Figure 3.** Effect of BLs and US exposure on TAT-mediated liposomal gene transfection. HeLa cells were treated with nonlabeled or TAT-PEG liposomes for 4 h. The cells were then washed and treated with or without BLs (120  $\mu$ g/mL) and US exposure. They were incubated for 20 h; then (A, B) luciferase activity was determined, and (C, D) cell viability was measured using a WST-8 assay. Data are the means  $\pm$  SD ( $n = 4$ ). \* $p < 0.05$  compared with TAT-PEG liposomes alone.

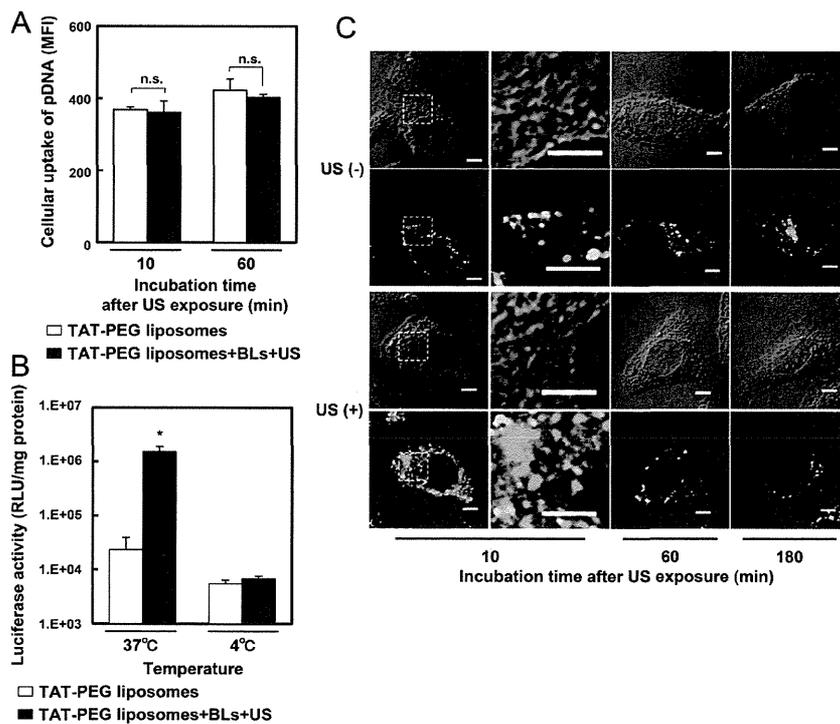
## DISCUSSION

Recent studies have suggested that endosomal escape is important to achieve efficient gene delivery.<sup>2,3</sup> We have previously reported that BLs and US exposure could improve the transfection efficiency of laminin-derived AG73-PEG liposomes containing pDNA by promoting endosomal escape.<sup>9</sup> In this report, we demonstrated that BLs and US exposure could enhance not only the transfection efficiency of AG73-PEG liposomes but also that of TAT-PEG liposomes.

For efficient gene delivery, various moieties were used to develop carriers which enhance cellular internalization or selectivity. CPPs, such as TAT, R8, or penetratin, were used to achieve efficient gene internalization.<sup>12,20,21</sup> On the other hand, for selective gene delivery, folate, transferrin, RGD, or anisamide was used as a ligand.<sup>22–25</sup> These moieties were associated with a specific receptor and internalized via several endocytoses. TAT peptide was associated with heparan sulfate proteoglycan, which has been controversial.<sup>26</sup> In addition, some studies have developed TAT peptide-modified carriers, which were equipped with components enhancing endosomal escape;<sup>14</sup> therefore, we focused on TAT peptide and evaluated whether BLs and US exposure can enhance the transfection efficiency of TAT peptide-modified carriers to demonstrate the utility of BLs and US exposure in general. The present results showed that BLs and US exposure could enhance the gene

transfection efficiency of TAT-PEG liposomes (Figure 3A). Furthermore, although we have previously reported that AG73-PEG liposomes were partially internalized via clathrin-mediated endocytosis,<sup>9</sup> the TAT-PEG liposomes prepared in this study were mostly internalized via a raft-dependent endocytic pathway (Figure 1C). These results suggested that BLs and US exposure could enhance the transfection efficiency of vectors, which were internalized via various receptor and endocytic pathways; however, further studies are needed to evaluate the effect of BLs and US exposure on the transfection efficiency of vectors, which were internalized via various endocytic pathways, such as macropinocytosis.

For successful gene therapy, nonviral vectors could be needed to overcome rate-limiting steps, such as cellular internalization, endosomal escape, and nuclear transfer.<sup>2,3</sup> Endosomal escape is considered to be one of the most important steps. Although PEG modification was considered a useful component to increase the stability of vectors in vivo, it also inhibited endosomal escape, leading to decreased gene expression.<sup>27,28</sup> Our results also showed that TAT-PEG liposomes could not escape from endosomes to the cytosol efficiently (Figures 1D, 2). We previously reported that BLs and US exposure could enhance the endosomal escape of AG73-PEG liposomes.<sup>9</sup> We therefore further confirmed the effect of BLs and US exposure on endosomal escape of TAT-PEG liposomes. We and other



**Figure 4.** Mechanism of accelerated TAT-mediated liposomal gene transfection by BLs and US exposure. (A) HeLa cells were incubated with TAT-PEG liposomes encapsulating Cy3-labeled pDNA for 4 h at 37 °C. After incubation, the cells were washed, and BLs were added. Then the cells were exposed to US and incubated for 10 or 60 min. The cells were then collected and washed with heparin-containing PBS three times. The fluorescence intensity was measured by flow cytometry. Data are shown as the means  $\pm$  SD ( $n = 3$ ). (B) Cells were preincubated for 30 min at either 37 or 4 °C before transfection and then treated with TAT-PEG liposomes for a further 1 h at 37 or 4 °C. After incubation, the cells were washed, and BLs were added. The cells were then exposed to US and cultured for 23 h. Luciferase activity was determined. Data are the means  $\pm$  SD ( $n = 4$ ). \* $p < 0.05$  compared with TAT-PEG liposomes alone. (C) Cells were treated with TAT-PEG liposomes encapsulating Cy3-labeled pDNA and FITC-CTB (10  $\mu$ g/mL) for 1 h at 37 °C. After incubation, the cells were washed, and BLs were added. The cells were then exposed to US, incubated for 10, 60, 180 min, and then fixed with 4% paraformaldehyde for 1 h at 4 °C and observed by CLSM. The areas surrounded by dotted line are shown as enlarged images. Scale bars represent 5  $\mu$ m.

groups have reported that the combination of BLs or microbubbles with US exposure could increase cell membrane permeability and deliver genes into the cytosol directly;<sup>29–33</sup> however, our results indicated that enhanced transfection efficiency did not rely on the increase of the direct cellular uptake of TAT-PEG liposomes, which is associated with the cell membrane (Figure 4A). In addition, CLSM analysis showed that BLs and US exposure could affect intracellular trafficking of TAT-PEG liposomes. Although endocytic vesicles labeled with FITC-CTB were observed as punctuate structures, when BLs and US exposure was applied, it was observed that FITC-CTB diffused into the cytosol (Figure 4B). These results suggested that BLs and US exposure could accelerate endosomal escape of TAT-PEG liposomes. We also examined whether sonazoid (Daiichi-Sankyo Pharmaceuticals, Tokyo, Japan) and US exposure could enhance the transfection efficiency of TAT-PEG liposomes. Sonazoid consists of perfluorobutane gas microbubbles stabilized by a monolayer membrane of hydrogenated egg phosphatidyl serine.<sup>34</sup> As a result, the transfection efficiency of TAT-PEG liposomes was enhanced by sonazoid and US exposure (data not shown). This result suggested that microbubbles and US exposure could enhance the gene transfection efficiency of gene delivery carriers.

We also prepared folate-PEG liposomes containing pDNA and examined whether BLs and US exposure could enhance the transfection efficiency of folate-PEG liposomes. Folate,

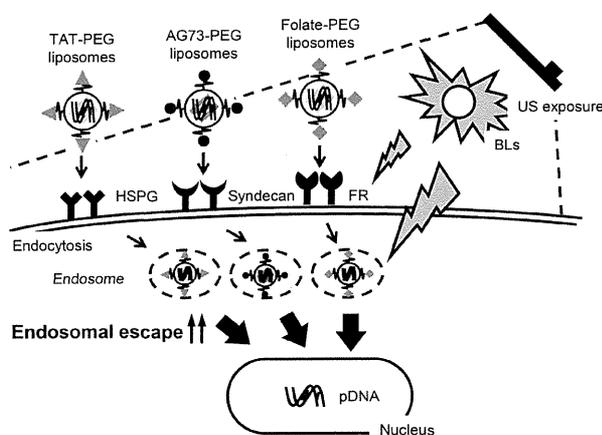
a high-affinity ligand for folate receptor, has been widely used as a ligand for selective gene delivery, and folate-modified carriers required various components enhancing endosomal escape to achieve high gene transfection efficiency.<sup>35</sup> We confirmed that folate-PEG liposomes had relatively low transfection efficiency because of the lower ability of endosomal escape, but when BLs and US exposure was used with folate-PEG liposomes, the transfection efficiency of folate-PEG liposomes was enhanced (data not shown). These findings also suggested that BLs and US exposure could enhance the endosomal escape of gene delivery vectors, leading to increased gene expression.

We have reported that the transfection efficiency of AG73-PEG liposomes using BLs and US exposure was enhanced 60-fold,<sup>9</sup> whereas that of TAT-PEG liposomes was up-regulated 12-fold (Figure 3A). These results suggested that BLs and US exposure could easily influence the intracellular trafficking of AG73-PEG liposomes compared to TAT-PEG liposomes. The different efficacy of endosomal escape between AG73-PEG liposomes and TAT-PEG liposomes might be dependent on the difference of the receptor, endocytic pathway of carrier, and the type of cells. The responsibility of BLs and US exposure to individual cells might also affect the efficiency of endosomal escape, leading to different transfection efficiencies; therefore, it is important to clarify the mechanism of the different efficacy of endosomal escape of these carriers. However, we expect that this method of promoting endosomal escape using BLs and US

exposure may also be applied to existing carriers for drug, peptide, or protein delivery, which have low intracellular delivery efficacy due to poor endosomal escape.

In further studies, we will attempt to demonstrate the detailed mechanism of enhanced endosomal escape of carriers by treatment with BLs and US exposure. It has been demonstrated that microbubbles and US exposure induce several biological effects, such as influx of calcium ions or generation of reactive oxygen species.<sup>36–39</sup> It has been also reported that endosomal acidification is adjusted by calcium ions;<sup>40</sup> therefore, we will assess whether the influx of calcium ions induced by BLs and US exposure affects endosomal acidification and function, leading to the destabilization of endosomes and enhancement of endosomal escape. On the other hand, we may also need to elucidate more clearly the effect of BLs and US exposure on transcription and other organelles. Although it is possible that BLs and US exposure may induce several biological effects involved in gene expression, BLs and US exposure could affect the intracellular distribution of pDNA and CTB (Figure 4); therefore, our results suggested that BLs and US exposure could certainly improve at least the endosomal escape of TAT-PEG liposomes.

In conclusion, as schematically shown in Figure 5, TAT-PEG liposomes were internalized into cells via HSPG and raft-



**Figure 5.** Diagram of enhanced gene delivery by BLs and US exposure. Several moiety-modified gene delivery carriers were internalized into cells via receptors and the endocytic pathway. When BLs and US exposure were applied, endosomal escape was enhanced, leading to increased transfection efficiency, which was independent of the receptor and endocytic pathway of carriers. HSPG, heparan sulfate proteoglycan; FR, folate receptor; US, ultrasound; BLs, Bubble liposomes; PEG, poly(ethylene glycol).

dependent endocytosis. On the other hand, AG73-PEG liposomes and folate-PEG liposomes were internalized via syndecan-2 and folate receptor, respectively. When BLs and US exposure were applied, endosomal escape was enhanced, leading to increased transfection efficiency of these carriers. These results suggested that BLs and US exposure could enhance transfection efficiency by promoting endosomal escape, which was independent of the receptors and endocytic pathway of carriers. Thus, BLs and US exposure can be useful tools to achieve efficient gene transfection by improving endosomal escape using various carriers.

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## ■ ABBREVIATIONS

BLs, Bubble liposomes; CTB, cholera toxin B subunit; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine; FBS, fetal bovine serum; Fmoc, fluorenylmethoxycarbonyl; Mal, maleimide; pDNA, plasmid DNA; PEG, poly(ethylene glycol); US, ultrasound

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