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Efficient siRNA delivery using novel siRNA-loaded Bubble liposomes and ultrasound

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ABSTRACT

Recently, we developed novel polyethyleneglycol (PEG)-modified liposomes (Bubble liposomes; BLs) entrapping an ultrasound (US) imaging gas and reported that the combination of BLs and US was useful for the delivery of siRNA directly into the cytoplasm. However, the results were obtained using a mixture of BLs and naked siRNA. With systemic injections, it is important to control the biodistribution of both BLs and siRNA. In addition, the delivery of siRNA is affected by nuclease degradation after intravenous administration. In this study, we prepared novel siRNA-loaded BLs (si-BLs) using a cationic lipid, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). We demonstrated that siRNA could be loaded onto BLs containing DOTAP and that siRNA-loaded BLs were stable in serum. A specific gene-silencing effect was also achieved by transfection with si-BLs. Thus, the combination of si-BLs with US exposure can be used for delivery of siRNA to a specific tissue via systemic injection.

1. Introduction

RNA interference (RNAi) has potential application in the development of new therapies for malignant, infectious, and autoimmune diseases. Indeed, synthetic siRNAs are capable of knocking down targets *in vivo* (Frank-Kamenetsky et al., 2008; Halder et al., 2006; Kim et al., 2008; McCaffrey et al., 2002; Morrissey et al., 2005; Niu et al., 2006; Sato et al., 2008; Song et al., 2003; Takeshita et al., 2005; Xia et al., 2007). However, effective and nontoxic delivery is the major challenge to its implementation in a clinical setting.

One novel approach to the administration of a drug or gene is ultrasound (US)-enhanced delivery, which exploits cavitation bubbles produced by the pressure oscillations of US. US pressures above a certain threshold can cause oscillating bubbles to collapse violently, a process known as inertial cavitation. Inertial cavitation is believed to temporarily improve the permeability of cell membranes, enabling the transport of extracellular molecules into viable cells (Delius and Adams, 1999; Duvshani-Eshet and Machluf, 2005; Greenleaf et al., 1998; Holmes et al., 1992; Schratzberger et al., 2002). Furthermore, in combination with microbubbles, contrast agents for medical US imaging improve siRNA transfection efficiency (Du et al., 2011; Kinoshita and Hynynen, 2005; Otani et al., 2009; Tsunoda et al., 2005). However, microbubbles have problems with size, stability, and targeting functionality.

Polyethyleneglycol (PEG)-modified liposomes have excellent biocompatibility, stability, and a long circulation time and can be easily prepared in a variety of sizes and modified to add a targeting function. For these reasons, they are widely used as carriers of drugs, antigens, and genes (Allen et al., 1991; Blume and Cevc, 1990; Harata et al., 2004; Maruyama et al., 1992, 2004). Therefore, PEG-liposomes containing a US imaging gas could be used as novel gene delivery agents. We recently reported that "Bubble liposomes" (BLs) were suitable for gene delivery *in vitro* and *in vivo* (Negishi et al., 2011b,c; Suzuki et al., 2007, 2008a,b). Furthermore, we showed that the combination of BLs and US was also useful for the delivery of siRNA *in vitro* and *in vivo* and that siRNA was introduced directly into the cytoplasm (Negishi et al., 2008). However, the results were obtained using a mixture of BLs and naked siRNA. With systemic injections, transfection efficiency is reduced if the BLs and siRNA are not colocalized in blood vessels. Therefore, it is important to control the biodistribution of both BLs and siRNA. In addition, siRNA is degraded by nuclease and removed rapidly from the circulation after intravenous administration. To overcome these problems, the loading of siRNA onto BLs could be effective for siRNA delivery. Recently, it has been reported that PEGylated lipoplexes (PEG-siPlex) bound to microbubbles led to an increase in the local lipoplex concentration near the cell membrane and resulted in much higher transfection with siRNA in the presence of US (Lentacker et al., 2009; Vandenbroucke et al., 2008). It was also shown that the delivery of siRNA by siRNA-microbubble complexes

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was effective for transfection into arteries (Suzuki et al., 2010). However, microbubbles were used in these reports. Microbubbles have problems with size, stability, and targeting functionality as mentioned above. Therefore, we developed nanosized, siRNA-loaded BLs using cholesterol-conjugated siRNA (chol-si-BLs) and demonstrated that using chol-si-BLs led to the stability of siRNA (Negishi et al., 2011a). In this study, we prepared siRNA-loaded BLs (si-BLs) using a cationic lipid. Novel si-BLs were able easily prepared compared with chol-si-BLs. Additionally, this method may have widespread utility for drug delivery systems because it is applicable to various materials possessing negative electrical charges. We also investigated the effects of the amount of PEG in the BLs on their interaction with siRNA, the stability of siRNA in serum, and the gene-silencing effects of transfection with si-BLs and US.

2. Materials and methods

2.1. Cell lines and cultures

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Kohjin Bio Co. Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Equitech Bio Inc., Kerrville, TX), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Preparation of liposomes and BLs

To prepare liposomes for conventional BLs, 1,2-dipalmitoyl-sn-glycero-phosphatidylcholine (DPPC) and 1,2-distearoylphosphatidylethanolamine-methoxy-polyethylene glycol (PEG_{2000}) were mixed at a molar ratio of 94:6. Both lipids were purchased from NOF Corporation (Tokyo, Japan). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-750] (PEG_{750}) from Avanti Polar Lipids (Alabaster, AL) were also used. Liposomes with various lipid compositions were prepared by a reverse-phase evaporation method, as described previously (Negishi et al., 2008). In brief, all reagents were dissolved in 1:1 (v/v) chloroform/diisopropylether. 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 (Nuclepore Track-Etch Membrane, 200 nm pore size, Whatman plc, UK). After being sized, the liposomes were passed through a sterile 0.45-μm syringe filter (Asahi Techno Glass Co., Chiba, Japan) to sterilize them. The lipid concentration was measured using the Phospholipid C test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). BLs were prepared from liposomes and perfluoropropane gas (Takachiho Chemical Inc., Co., Ltd., Tokyo, Japan). First, 5-mL sterilized vials containing 2 mL of liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane gas, capped, and then pressurized with 7.5 mL of perfluoropropane gas. The vials were placed in a bath-type sonicator (42 kHz, 100 W, Bransonic 2510J-DTH, Branson Ultrasonics Co., Danbury, CT) for 5 min to form BLs. The zeta potential and mean size of the BLs were determined using the light-scattering method with a zeta potential/particle sizer (Nicomp 380ZLS, Santa Barbara, CA).

2.3. Ultrasound imaging of BLs

BLs diluted with PBS were dispensed into 6-well plates. B-mode recordings were made using a high-frequency ultrasound imaging system (NP60R-UBM, Nepa Gene, Co., Ltd., Chiba, Japan).

2.4. Plasmid DNA and siRNA

The plasmid pCMV-GL3, derived from pGL3-basic (Promega, Madison, WI), is an expression vector encoding the firefly luciferase gene under the control of a cytomegalovirus promoter. Small interfering RNA targeting luciferase (Luciferase GL3 siRNA; siGL3) and a nontargeting siRNA (Control (non-sil.) siRNA; siCont) were purchased from Qiagen K.K. (Tokyo, Japan). Their sequences were as follows: siGL3, *5′-CUUACGCUGAGUACUUCGAdTdT-3′* and *5′-UCGAAGUACUCAGCGUAAGdTdT-3′*; siCont, *5′-UUCUCCGAACGUGUCACGUdTdT-3′* and *5′-ACGUGACACGUUCGGAGAAdTdT-3′*. Nontargeting fluorescein-labeled siRNA (BLOCK-iT Fluorescent Oligo) was purchased from Invitrogen Japan K.K. (Tokyo, Japan).

2.5. Preparation of si-BLs

For the preparation of si-BLs, adequate amounts of siRNA were added to BLs and gently mixed. FITC-labeled siRNA and flow cytometry were used to examine the interaction between siRNA and BLs. The fluorescence intensity of si-BLs was analyzed using a FACSCanto (Becton Dickinson, San Jose, CA). To quantify the amount of siRNA loaded onto the BL surfaces, the BLs were centrifuged at 2000 rpm for 1 min and the unbound siRNA was removed. The BL solution and the aqueous solution containing the unbound siRNA were then boiled for 5 min after which the optical density was measured at 260 nm using a spectrophotometer.

2.6. Stability of siRNA in serum

The BLs, siRNA, and si-BLs were incubated in 50% serum for 15, 30, and 60 min. Serum was used without heat inactivation. The stability of the siRNA was confirmed by 15% polyacrylamide gel electrophoresis. The gel was stained with SYBR SAFE (Invitrogen Japan K.K., Tokyo, Japan) and visualized under ultraviolet light.

2.7. Transfection of siRNA into cells using BLs or si-BLs

The mixture of siRNA (final concentration 100 nM) and BLs or si-BLs (60 μg) in culture medium containing 10% FBS was added to the cells transfected with pDNA on the previous day. The cells were immediately exposed to US (frequency, 2 MHz; duty, 50%; burst rate, 2.0 Hz; intensity 2.0 W/cm^2) for 10 s through a 6-mm diameter probe placed in the well. A Sonopore 3000 (NEPA GENE, Co., Ltd., Chiba, Japan) was used to generate the US. The cells were washed twice with culture medium and cultured for two days.

To measure luciferase activity after transfection, cell lysate was prepared with a 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, Madison, WI) and a luminometer (LB96 V, Belthold Japan Co., Ltd., Tokyo, Japan). The activity is reported in as relative light units (RLU) per mg of protein.

2.8. Statistical analyses

All data are reported as the mean ± SD ($n = 4$). Data were considered significant when $P < 0.05$. The t-test was used to calculate statistical significance.

3. Results

3.1. Preparation of BLs containing DOTAP

Initial experiments were performed to investigate whether liposomes containing a cationic lipid, DOTAP, could entrap a US imaging gas as well as conventional BLs. We prepared liposomes containing DOTAP in various amounts and attempted to entrap the gas. The

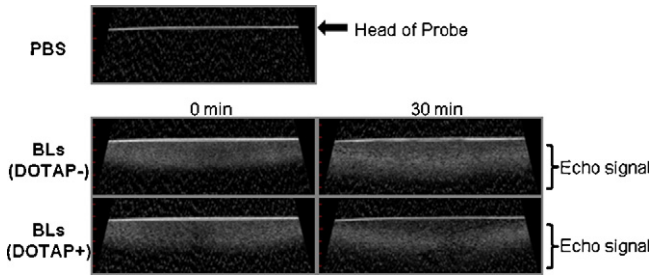

Fig. 1. Ultrasonographic images of a plate containing BLs with or without DOTAP.

liposomes containing up to 15 mol% DOTAP became cloudy, and we concluded that they could effectively entrap the imaging gas (data not shown). The liposomes containing more than 15 mol% DOTAP had difficulty entrapping the gas. We also examined BLs containing DOTAP using a high-frequency US imaging system. The system is a two-dimensional US image display composed of bright dots representing the US echoes. The brightness of each dot is determined by the amplitude of the returned echo signal. As shown in Fig. 1, the US echo signal was detected even 30 min later.

3.2. Effects of polyethyleneglycol on the interaction of siRNA with BLs

To assess whether siRNA could be loaded onto the surface of BLs, we used a fluorescence-activated cell sorter, the FACSCanto. We also prepared BLs containing different lengths of PEG to assess the effect of PEG on BL interactions with siRNA. As shown in Fig. 2, BLs not containing DOTAP were successfully loaded with siRNA. Approximately 40% of the BLs were FITC positive. Approximately 45% of the BLs containing DOTAP but not containing PEG_{750} were FITC positive. In contrast, BLs containing DOTAP and PEG_{750} were more heavily loaded with siRNA. Approximately 80% were FITC positive. Thus, in all subsequent experiments, BLs composed of DPPC, DOTAP, PEG_{2000}, and PEG_{750} (in a 79:15:3:3 molar ratio) were used.

Table 1
Size (nm) and zeta potential (mV) of BLs and si-BLs.

Lipid composition of BLs (molar ratio)	BLs	si-BLs
DPPC:PEG_{2000} = 94:6	528.3 nm	587.9 nm
DPPC:DOTAP:PEG_{2000}:PEG_{750} = 79:15:3:3	749.0 nm	862.2 nm
DPPC:PEG_{2000} = 94:6	−0.81 mV	−0.42 mV
DPPC:DOTAP:PEG_{2000}:PEG_{750} = 79:15:3:3	−0.20 mV	−0.13 mV

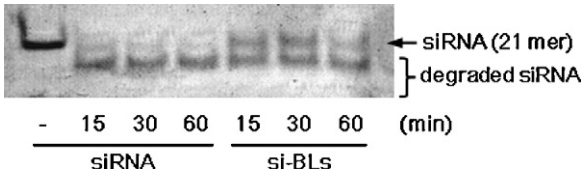

Fig. 3. Stability of siRNA in the presence of serum. Naked siRNA or si-BLs (DOTAP (+), PEG_{2000} and PEG_{750} (3:3)) were subjected to 50% serum degradation at 37 °C for 0.5 or 1 h and confirmed by 15% acrylamide gel electrophoresis.

As shown in Table 1, there was almost no change in the size and zeta potential of the BLs after siRNA was added.

We investigated the stability of siRNA in serum. Small interfering RNA held by BLs showed increased stability in 50% serum compared with free siRNA, although some siRNA was degraded (Fig. 3). We also examined the change in the amount of siRNA bound to BLs when the concentration of the siRNA was increased. As shown in Fig. 4, the amount siRNA loaded increased in a dose-dependent manner. We finally estimated that 60 μg of BLs could be loaded with at least 100 pmol of siRNA and that approximately 30% of the siRNA was bound to the lipid surface.

3.3. Transfection of siRNA into cells using BLs or si-BLs

Before the transfection experiments, we investigated the destruction efficiency of si-BLs under the US exposure. The solution of si-BLs was exposed to the same conditions used for *in vitro* transfection and was analyzed using the FACSCanto. Unlike the solution of si-BLs before US exposure, no fluorescence was detected in the

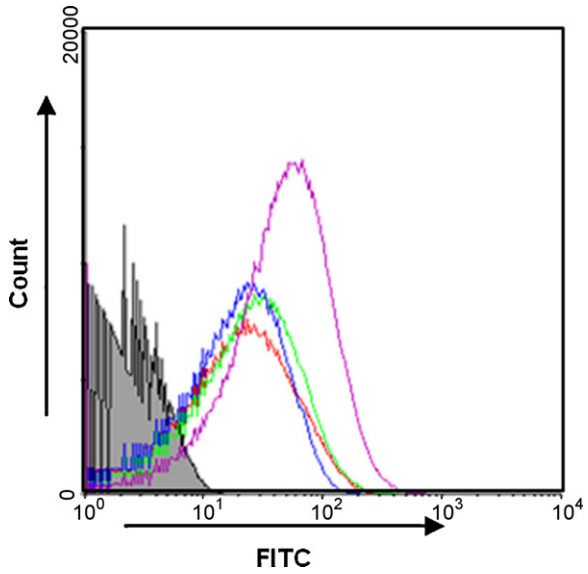

Fig. 2. Interaction of siRNA with BLs and the effects of PEG chain length on the interaction. The interaction was examined by analyzing a mixture of FITC-siRNA (50 pmol) and various BLs (60 μg) with the FACSCanto; gray area: BLs only; red curve: si-BLs (DOTAP (−), PEG_{2000} and PEG_{750} (molar ratio, 6:0)); green curve: si-BLs (DOTAP (+), PEG_{2000} and PEG_{750} (6:0)); blue curve: si-BLs (DOTAP (−), PEG_{2000} and PEG_{750} (3:3)); purple curve: si-BLs (DOTAP (+), PEG_{2000} and PEG_{750} (3:3)).

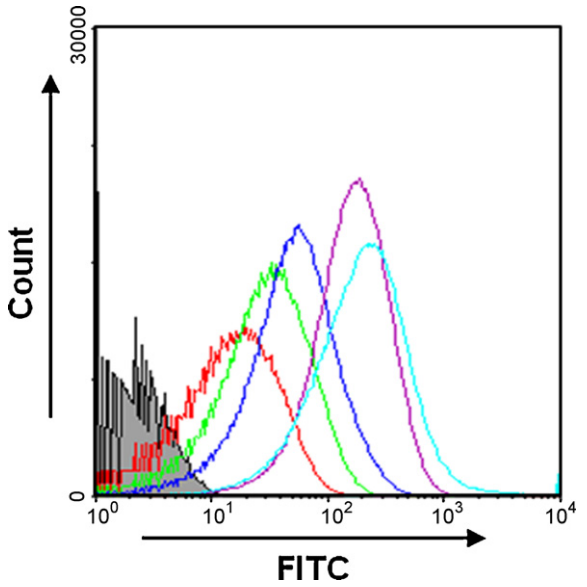

Fig. 4. Loading of siRNA onto BLs. The interaction was examined by analyzing a mixture of FITC-siRNA (12.5–200 pmol) and BLs (60 μg) containing DPPC, DOTAP, PEG_{2000} and PEG_{750} (79:15:3:3) with FACSCanto; gray area: BLs only; red curve: si-BLs (siRNA 12.5 pmol); green curve: si-BLs (siRNA 25 pmol); blue curve: si-BLs (siRNA 50 pmol); purple curve: s si-BLs (siRNA 100 pmol); light blue curve: si-BLs (siRNA 200 pmol).

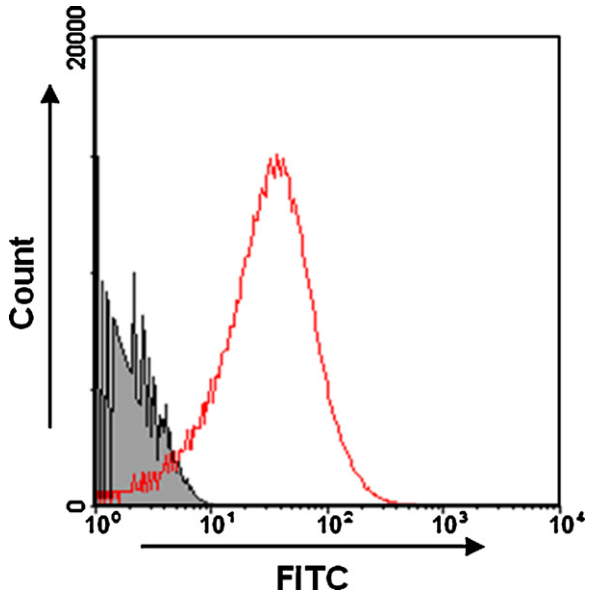

Fig. 5. Effects of US on si-BLs. The interaction was examined by analyzing a mixture of FITC-siRNA (50 pmol) and BLs (60 μg) containing DPPC, DOTAP, PEG_{2000}, and PEG_{750} (79:15:3:3) with the FACSCanto; gray area: BLs only; red curve: si-BLs; green curve: solution of si-BLs after US exposure (frequency, 2 MHz; duty, 50%; burst rate, 2.0 Hz; intensity, 2.0 W/cm^2; time, 10 s). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

solution (Fig. 5). This result suggested that the US caused the release of siRNA from the surface of the BLs.

To investigate the gene-silencing effects of siRNA transfection with si-BLs and US, cells transfected with pDNA encoding firefly luciferase (pCMV-GL3) on the previous day were added to BLs loaded with nontargeting control or luciferase-targeting siRNA (siCont or siGL3) and exposed to US (Fig. 6). Approximately 80% of luciferase expression was specifically blocked by siGL3 in the si-BLs-treated group and in the group treated with conventional BLs. Cytotoxicity was absent after the transfection with si-BLs and US (data not shown).

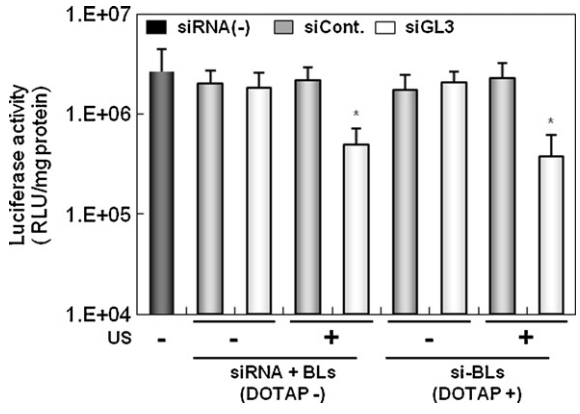

Fig. 6. Down-regulation of luciferase expression by siRNA with BL and US. COS-7 cells transfected with pCMV-GL3 on the previous day were added to siRNA (100 nM) and conventional BLs (DOTAP (−), PEG_{2000}, and PEG_{750} (molar ratio, 6:0)) or si-BLs (DOTAP (+), PEG_{2000}, and PEG_{750} (molar ratio, 3:3)) and applied. At 2 days posttransfection, luciferase expression was measured. siRNA(−); the group not transfected with siRNA, siCont; the group transfected with nontargeting siRNA (siCont), siGL3; the group transfected with siRNA targeting luciferase (siGL3). *P values <0.05 compared with the group transfected with siCont. All data are reported as the mean ± SD (n = 4).

4. Discussion

RNAi therapeutics have great potential for treating intractable diseases ranging from acquired diseases, such as viral infections, to purely genetic disorders. However, inefficient delivery into specific organs has hindered their clinical application.

Recently, a combination of microbubbles and US has been proposed as a less invasive and tissue-specific method of gene delivery. The combination produces transient changes in the permeability of the cell membrane and allows for the site-specific intracellular delivery of molecules such as dextran, pDNA, peptides, and siRNA both *in vitro* and *in vivo* (Du et al., 2011; Kinoshita and Hynynen, 2005; Li et al., 2003; Otani et al., 2009; Sonoda et al., 2006; Taniyama et al., 2002a,b; Tsunoda et al., 2005; Unger et al., 2004). However, because existing microbubbles have problems with size, stability, and targeting functionality, we developed liposomal bubbles (BLs). BLs are an effective and novel tool for gene and siRNA delivery *in vitro* and *in vivo* (Negishi et al., 2008, 2011b,c; Suzuki et al., 2007, 2008a,b). Our method using BLs and US did not involve endocytosis, and siRNA was directly introduced into the cytoplasm within a fairly short time. Thus, it seems unnecessary to consider the escape of siRNA from the endosome and the degradation of siRNA in lysosomes, although the endosomal escape is an important issue in other delivery tools. Furthermore transfection methods using physical energy other than US are expected and are currently being developed (Endoh and Ohtsuki, 2009; Kong et al., 2004; Oliveira et al., 2007; Schiffelers et al., 2005; Takei et al., 2008). These methods are difficult to apply to deep tissue. In contrast, US is able to control the accessible tissue sites by changing of the frequency and to reach the deep tissues. However, our previous results were obtained using a mixture of BLs and naked siRNA, which do not colocalize in blood vessels after intravenous administration. Additionally, siRNA is susceptible to degradation by nucleases and rapid removal from circulation. Consequently, these factors may cause a reduction in transfection efficiency. In this study, we prepared siRNA-loaded BLs (si-BLs) using a cationic lipid as a more effective, efficient delivery tool for systemic injections.

We initially attempted to entrap a US imaging gas in BLs containing DOTAP, a cationic lipid often used for gene delivery. Liposomes containing up to 15 mol% DOTAP did entrap the gas and could be used as ultrasound contrast agents. However, liposomes containing more than 15 mol% DOTAP had difficulty maintaining the gas. We also tested BLs containing DOTAP using a high-frequency US imaging system. An echo signal was detected as well as for BLs without DOTAP, although the imaging effect was slightly reduced 30 min later (Fig. 1). These results were assumed to be due to differences in the response to US and the stability of each BL type.

We used flow cytometry to examine the interaction between siRNA and BLs. The BLs were detectable, although the fluorescence intensity was low. The fluorescein-labeled siRNA molecules were too small to be detected with flow cytometry. However, the siRNA-loaded BLs exhibited strong fluorescence. We determined that siRNA could interact with BLs, and the interaction was due to the cationic charge of DOTAP. The amount of siRNA bound to the BLs was slightly increased in the presence of DOTAP. Furthermore, BLs containing both DOTAP and PEG_{750} could be loaded with much more siRNA (Fig. 2). BLs containing neither DOTAP nor PEG_{750} also loaded successfully with a certain amount of siRNA. These results suggested that siRNA could be loaded not only by the electrostatic interaction, but also by the fixed aqueous layer formed with PEG. It has been reported that the modification of liposomes with short and long PEG chains increases the fixed aqueous layer thickness (Sadzuka et al., 2002). We considered that the structural changes in the PEG chain facilitated interaction between the cationic lipid and anionic siRNA. Moreover, there were no significant changes in size after adding siRNA (Table 1). The data suggested that siRNA

was bound to the surface of BLs and that BLs did not aggregate. We also investigated the stability of siRNA interacting with BLs in 50% serum. Although some siRNA was degraded, siRNA held by BLs showed increased stability in 50% serum compared with free siRNA (Fig. 3). In the solution of si-BLs, free siRNA was present with si-BLs. Therefore, the siRNA not held by BLs was degraded. We examined the change in the amount of bound siRNA by adding various amounts of siRNA to BLs. As shown in Fig. 4, the amount of siRNA loaded onto BLs (60 μg) increased with siRNA addition in a dose-dependent manner up to 100 pmol.

We also investigated the effects of US exposure on si-BLs by analyzing the si-BL solution after exposure under the same conditions used for the *in vitro* transfection. No fluorescence was detected. Moreover, there were only a few detectable molecules in the solution of si-BLs after US exposure, and the histogram representing the results was almost parallel to the horizontal axis, similar to the solution of free siRNA (Fig. 5). This result suggested that US exposure collapsed si-BLs, releasing siRNA from the surface of the BLs. We confirmed that there was no damage to siRNA from US exposure by electrophoresis (data not shown). Undetectable fluorescence does not necessarily mean that siRNA were released from BLs: it is also possible that siRNA interacted with lipids or BLs that reverted to liposomes by degassing. However, the gene-silencing effects of siRNA transfection via si-BLs and US were comparable to those of siRNA transfection with conventional BLs and US (Fig. 6). Therefore, it appears that the exposure to US-induced cavitation, the release of siRNA from BLs, and the delivery of siRNA into the cytoplasm. We are currently developing BLs composed of lipids other than DPPC or DOTAP in attempts to form more stable and effective BLs. In the future, we will also examine siRNA delivery and disease-associated gene-silencing effects.

The preparation method of si-BLs developed in this study was easier than that of chol-si-BLs reported previously (Negishi et al., 2011a). Furthermore, BLs containing cationic lipid are expected to have widespread application to delivery tools of various molecules possessing negative electric charges. We confirmed that not only siRNA but also pDNA can be loaded onto BLs (p-BLs). Additionally, microbubbles conjugated to an antibody and having a targeting function have been developed recently (Behm et al., 2008; Leong-Poi et al., 2005; Palmowski et al., 2008). Liposomes can be easily modified to add a targeting function. Thus, the development of targeting si-BLs or p-BLs using an antibody or peptide is expected to lead to beneficial clinical applications for various diseases.

5. Conclusion

In this study, we showed that si-BLs could deliver siRNA as well as conventional BLs, although there remains room for improvement. Additionally, BLs containing a cationic lipid interacted with siRNA and protected the siRNA against nuclease degradation. These results suggest that si-BLs combined with US exposure may be useful for delivering siRNA to a tissue or organ via systemic injection.

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