Review

DOI: 10.5582/ddt.2016.01063

Gene delivery systems by the combination of lipid bubbles and ultrasound

Yoichi Negishi[2], Yoko Endo-Takahashi[2], Kazuo Maruyama[1,*]

[1] *Department of Drug and Gene Delivery Research, Faculty of Pharma-Sciences, Teikyo University, Itabashi, Tokyo, Japan;*
[2] *Department of Drug Delivery and Molecular Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan.*

Summary **Gene therapy is promising for the treatment of many diseases including cancers and genetic diseases. From the viewpoint of safety, ultrasound (US)-mediated gene delivery with nano/microbubbles was recently developed as a novel non-viral vector system. US-mediated gene delivery using nano/microbubbles are able to produce transient changes in the permeability of the cell membrane after US-induced cavitation while reducing cellular damage and enables the tissue-specific or the site-specific intracellular delivery of gene both *in vitro* and *in vivo*. We have recently developed novel lipid nanobubbles (Lipid Bubbles). These nanobubbles can also be used to enhance the efficacy of the US-mediated genes (plasmid DNA, siRNA, and miRNA *etc*.) delivery. In this review, we describe US-mediated delivery systems combined with nano/microbubbles and discuss their feasibility as non-viral vector systems.**

Keywords: Ultrasound, lipid bubbles, gene delivery, cavitation

1. Introduction

Gene therapy has potential for treating genetic diseases and cancer. Viral vector-directed gene transfers show high gene transfer efficiency but are deficient in several areas. For example, some viral vectors could randomly integrate DNA into host genomes, which poses potential risks, including neoplastic transformation (*1-3*). Delivery vectors that are highly efficient for gene transduction must also be safe and easy to use. Non-viral vectors have recently received attention as gene carriers, but their transduction efficiency is very low, although efforts have been made to address this (*4-6*). Towards this end, ultrasound (US) has been investigated for improving the efficiency of transgene delivery and holds promise as a means of generating a non-invasive gene delivery system.

US has been utilized as useful tool for *in vivo* imaging, destruction of renal calculus and treatment of fibroid. Recently, it was reported that US was proved to increase permeability of the plasma membrane and reduces the thickness of the unstirred layer of the cell surface, which encourage the DNA entry into cells (*7,8*). The first studies applying US for gene delivery used frequencies in the range of 20-50 kHz. However, these frequencies, along with cavitation, are also known induce tissue damage if not properly controlled. To improve this problem, many studies using therapeutic US for gene delivery, which operates at frequencies of 1-3 MHz, intensities of 0.5-2.5 W/cm^2 and pulse-mode have emerged. In addition, it has been reported that microbubbles, used as US contrast agents, play an important role in enhancing the efficiency of gene delivery without causing cell damage (*9*). In general, cell damage is dependent on the US intensity, the exposure time, the concentration of microbubbles, and the cell type, with US intensity and exposure time being particularly important. Therefore, effective US-mediated gene delivery requires optimization of the US exposure conditions (*10-13*). Some researchers studied about the cell damage by the disruption of microbubbles with US exposure (*14-19*).

Microbubbles are cavitated by exposure to US, generating microstreams or microjets which result in

Released online in J-STAGE as advance publication October 28, 2016.

*Address correspondence to:
Dr. Kazuo Maruyama, Department of Drug and Gene Delivery Research, Faculty of Pharma-Sciences, Teikyo University, 2-11-1 Kaga, Itabashi, Tokyo 173-8605, Japan.
E-mail: maruyama@pharm.teikyo-u.ac.jp

shear stress to cells and the generation of transient holes in the cell membrane (*20*). Since this approach can be used to deliver extracellular material such as genes into cells, microbubbles could facilitate US-mediated gene delivery. In addition, submicron-sized bubbles (nanobubbles), which are smaller than conventional microbubbles, were recently reported (*21,22*), and we have developed novel lipid nanobubbles (Lipid Bubbles) (*13,23-31*). These nanobubbles can also be used to enhance the efficiency of US-mediated gene delivery. In this review, we describe US-mediated delivery systems combined with nano/microbubbles and discuss their feasibility as non-viral vector systems.

2. Microbubbles and US

The behavior of microbubbles depends on the amplitude of the US used. Very low acoustic pressure (mechanical index (MI) < 0.05-0.1) induces linear oscillation of the microbubble, and the reflected frequency is equal to the transmitted frequency (Figure 1a). An increase in acoustic pressure (0.1 < MI < 0.3), referred to as low-power imaging, causes non-linear expansion and compression of the microbubble (Figure 1b). The bubble is somewhat more resistant to compression than to expansion, a phenomenon known as stable or non-inertial cavitation, and results in the emission of non-linear harmonic signals at multiples of the transmitted frequency (*32*). Harmonic imaging with microbubbles enhances the bubble-to-tissue backscatter signal ratio due to insignificant harmonic backscatter from tissue in this range of MI. Therefore, this technique can improve the signal-noise ratio and be useful in left ventricular pacification imaging (*33*). In addition, stable or non-inertial cavitation can enhance transient cell membrane permeability (Figure 2a) (*34*). Machluf *et al*. reported that the exposure of cells to US (0.16 MI, 1 MHz) in the presence of microbubbles resulted in the delivery of plasmid DNA (pDNA) into the cells (*35,36*).

Higher acoustic pressure (MI > 0.3-0.6) causes forced expansion and compression of the microbubble and results in bubble disruption (collapse) (Figure 1c). Bubble disruption by this inertial cavitation is utilized as flash-replenishment in reperfusion study of diagnosis (*37*). This inertial cavitation induces microstreams/microjets around the bubbles. These microstreams/microjets can enhance the permeability of the cell membrane due to the formation of transient pores (Figure 2b) (*20*). In the presence of nano-/microbubbles, the threshold for cavitation decreases, allowing the destruction of the microbubbles at lower US energies.

3. Lipid bubbles

As mentioned above, there are many important information for US-mediated gene delivery utilizing

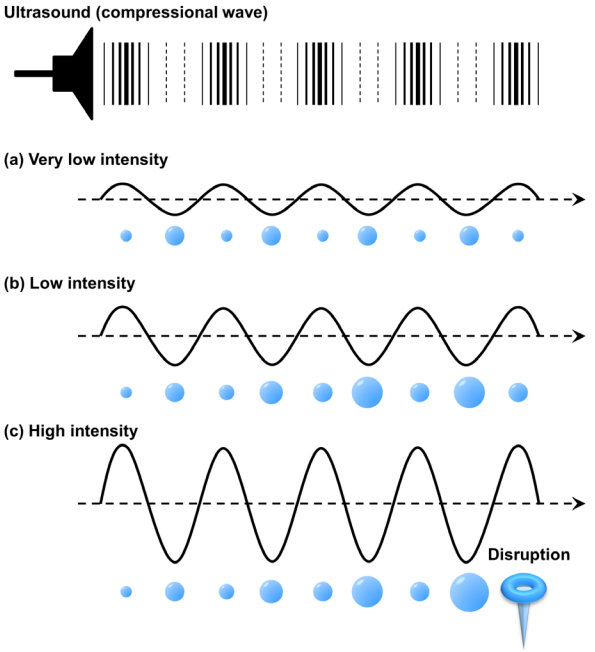

Figure 1. Schematics showing microbubble behavior in acoustic fields. (a) Very low intensity ultrasound induces linear oscillation of the microbubble. **(b)** Low intensity ultrasound induces oscillation of the microbubble, with a gradual increase in the diameter of the microbubble. Stable oscillation occurs when the microbubble reaches its resonant diameter. **(c)** High intensity ultrasound causes a rapid increase in the diameter of the microbubble for a few cycles, which induces bubble disruption.

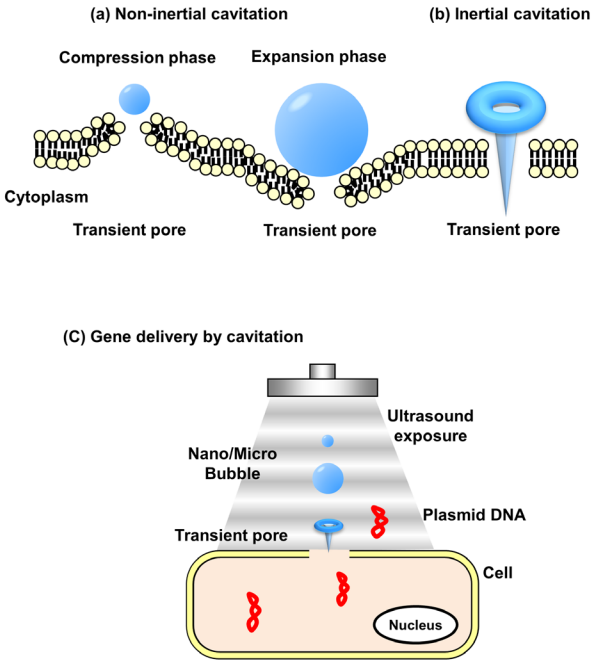

Figure 2. Schematics showing pore formation in the cell membrane by oscillating or disrupting microbubbles. (a) The pushing and pulling behavior (non-inertial cavitation) of microbubbles and **(b)** the collapse of microbubbles (inertial cavitation) rupturing the cell membrane and creating pores allowing trans-membrane flux of fluid and macromolecules such as plasmid DNA and oligonucleotides **(c)**.

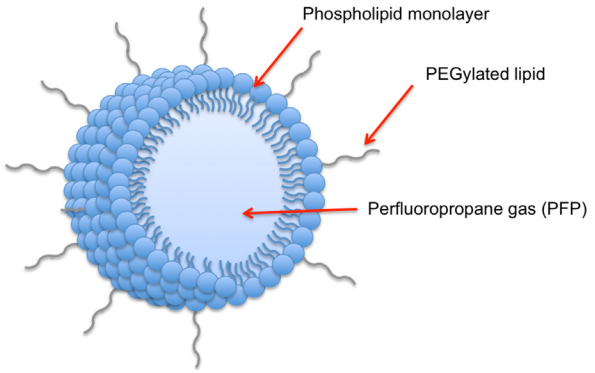

Figure 3. Illustration of a Lipid Bubble.

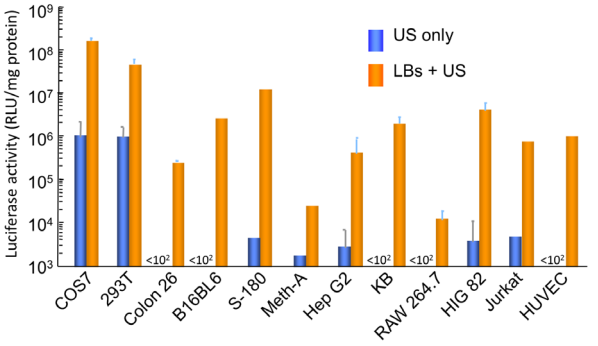

Figure 4. pDNA delivery with LBs and US *in vitro*. Luciferase activities in various types of cells transfected with LBs and US.

microbubbles. Microbubbles are contrast agents used in US imaging. Microbubbles can also be used to improve transfection efficiency when combined with US. Microbubbles are generally unstable and have a mean diameter of between 1-6 μm, making them too large for intravascular applications (*38*). Moreover, it is difficult to modify their surface with functional molecules. Therefore, microbubbles should be small and stable, and their surface should be easily modified with functional molecules for targeting.

Liposomes have several advantages as drug, antigen and gene delivery carriers as their size can be easily controlled, and they can be modified with targeting molecules. Therefore, we used liposome technology to develop novel lipid bubbles (LBs) containing the US gas, perfluoropropane (Figure 3). LBs are about 600-800 nm in diameter, making them smaller than Sonazoid® which is US contrast agent (about 2 μm).

4. *In vitro* pDNA delivery with LBs

We investigated *in vitro* whether the combination of LBs and US could provide a gene delivery tool. First, we attempted to transfect pDNA encoding firefly luciferase (pCMV-Luc) into COS-7 and 293T cells with LBs and US. Luciferase activity in COS-7 and 293T cells treated with LBs and US was higher than in cells treated with LBs or US alone (Figure 4). The gene expression efficiency was very high with the US exposure for 10 seconds though it was so short. We also confirmed that LBs could effectively deliver plasmid DNA into cells even for 1 second of US exposure (Data not shown). Thus, LBs were novel gene delivery tools that could instantaneously transfect extracellular plasmid DNA into cells. Heat and jet streams are generally induced with cavitation, which might damage cells. We therefore examined the effects of US on cells with or without LBs. Ultrasound did not damage COS-7 cells in the absence of LBs and only slightly affected the cells even when the amount of US was sufficient to induce cavitation of LBs. Though it was natural, we also confirmed that the cavitation induced with LBs did not damage plasmid DNA (data not shown).

Next, we demonstrated that LBs and US could be used to transfect pDNA into various cell lines such as Colon 26, B16BL6, S-180, Meth-A, Hep G2, KB cells, mouse macrophase-like cell line RAW 264.7 cells, Jurkat cells derived from T cell lines, and human umbilical vein endothelial cells (HUVEC) (Figure 4). Since it is difficult to transfect lymphocytes with pDNA using non-viral vectors, the combination of LBs and US hold promise as tools for gene transfection *in vitro*. Considering *in vivo* gene delivery with Bubble liposomes, it is necessary to deliver plasmid DNA into cells in presence of serum. Then, we examined about the effect of serum on gene delivery with LBs. Gene expression with LBs was not affected even in the presence of serum.

5. *In vivo* pDNA delivery with LBs

Cancer gene therapy requires the delivery of genes into tumor tissue with high efficiency, safety and minimal invasiveness. We attempted to deliver pDNA into tumor tissue using LBs and US. B6C3F1 tumor model mice were intradermally inoculated with OV-HM cells in the flank. After 7 days, a mixture of LBs (2.5 μg of lipid) and pCMV-Luc (10 μg) was injected into the tumor, then US (1 MHz, 0.7 W/cm^2, 1 min) was transdermally applied to the tumor tissue. Transfection efficiency using a conventional lipofection method was also investigated. Complexes of Lipofectamine 2000 (20 μg) and pCMV-Luc (10 μg) were injected into the tumors. Luciferase activity was measured two days after LBs with US or conventional transfection. Tumors treated with LBs and US showed higher luciferase activity compared to tumors treated with LBs or US alone, or with conventional lipofection. This result indicates that LBs and US can efficiently deliver pDNA into tumor tissue *in vivo*, and motivated us to examine whether LBs and US could be used for cancer gene therapy. Interleukin-12 (IL-12) exhibits immunomodulatory antitumor effects and is considered an effective antitumor agent (*39,40*), but its short

half-life and systemic toxicity following intravenous injection are major obstacles to its therapeutic use (*41,42*). Therefore, we transfected pDNA encoding the IL-12 gene (pCMV-IL-12) into tumor tissue using LBs and US with the aim of achieving high local expression of IL-12. When pCMV-IL-12 was transfected using LBs, US or Lipofectamine 2000, tumor growth was not suppressed. In contrast, transfection of pCMV-IL-12 with LBs and US suppressed tumor growth significantly (Figure 5). To investigate the mechanism behind the anti-tumor effects of pCMV-IL-12 transfected using LBs and US, we assessed the involvement of $CD4^+$ and $CD8^+$ T cells and NK cells. The depletion of $CD8^+$ T cells effectively blocked the anti-tumor effect of pCMV-IL-12 transfected using LBs and US. These results suggest that the combination of LBs and US can effectively induce sufficient IL-12 expression to cause anti-tumor immune responses.

Systemic gene delivery techniques are ideal for cancer therapy because they can deliver genes to tumors deep in the body. We confirmed that LBs act as US imaging agents for several minutes following intravascular administration. We attempted to deliver genes into tumor tissue using LBs and US by intravascular administration. S-180 cells were inoculated into the left footpad of ddY mice; after 4 days, 100 μL of pCMV-Luc (10 μg) and LBs (100 μg) were injected into the femoral artery and US (0.7 MHz, 1.2 W/cm^2, 2 min) was immediately applied transdermally to the tumor tissue. Transfection with LBs, US or Lipofectamine 2000 alone was also conducted. Two days following transfection, the luciferase activity of the tumor tissues was measured. The luciferase activity of tumors treated with LBs and US was higher than that of tumors treated with LBs, US, or Lipofectamine 2000, suggesting that LBs and US can efficiently deliver genes into tumor tissue following intravascular administration. Thus, LBs and US hold promise for cancer gene delivery by local or intravenous administration.

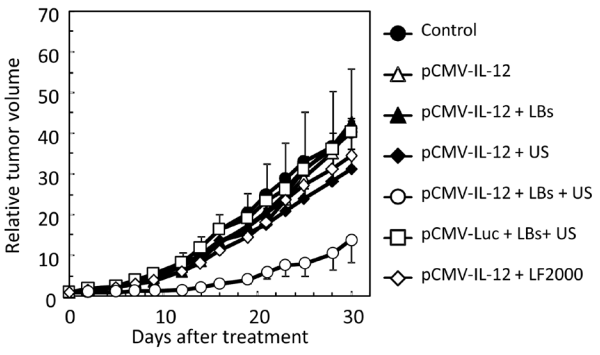

Figure 5. Antitumor effect of IL-12 gene delivery with LBs and US. Antitumor effect was evaluated by measuring tumor volume. BL: Lipid Bubbles, US: Ultrasound, LF2000: Lipofectamine 2000, pCMV-IL-12: pDNA encoding the IL-12 gene, pCMV-Luc: pDNA encoding the Luciferase gene.

6. *In vitro*/*In vivo* siRNA delivery with LBs

Small interfering RNA (siRNA) can silence specific gene expression. Therefore, siRNA is expected to be a novel therapeutic drug, however, efficient and robust delivery systems have been required for its clinical application. Recently, we have developed novel polyethyleneglycol modified liposomes (Lipid bubbles; LBs) entrapping an US (US) imaging gas, which can function as a gene delivery tool in the combination of US exposure. So, we tried to know the ability of LBs in the combination of US to deliver siRNA *in vitro* and *in vivo* (*23*). We first examined to transfect pDNA and siRNA into COS-7 cells. Cells were cotransfected with pDNA encoding firefly luciferase (pCMV-GL3) and a nontargeting control or luciferase-targeting siRNA (siCont or siGL3). The luciferase activity was downregulated even in the presence of a high serum concentration. This is in accordance with our previous data concerning pDNA delivery with LBs and US (*44*). These results might be owing to the mechanism of transfection enabling siRNA to be directly and instantaneously delivered into the cytoplasm. In fact, the intracellular localization of fluorescence labeling siRNA just behind the transfection was homogeneously dispersed, primarily in the cytoplasm. The intracellular localization of pDNA transfected with LBs and US also showed the same tendency. These results differed from an observation made regarding the endosomal pathway of the lipoplex. Furthermore, the gene-silencing efficiency of the transfected siRNA using LBs and US was independent of the culture conditions (4°C or 37°C) and the presence of an endosomolytic agent. Thus, endocytosis minimally participates in the transfection process with LBs and US and siRNA is able to be directly and instantaneously delivered into the cytoplasm. Recent studies have reported that siRNAs are capable of activating innate immunity in mammalian cells and the immune response was mainly caused *via* Toll-like receptors (TLRs) in the endosome (*44-46*). It may be possible that the transfection method with Lipid bubbles and US ignores the activation of an immune response *via* TLRs. Therefore, the combination method of LBs and US enabling siRNA delivery directly into the cytoplasm of target cells may be suitable for siRNA delivery.

When pDNA encoding luciferase and its siRNA were cotransfected into the muscles of mice using LBs and US, the suppression of luciferase expression was observed, and the effect persisted for up to 7 days, suggesting that LBs could also be a useful tool *in vivo*. Furthermore, when the siRNA targeted to the endogenous gene (GSK-3β) was transfected into the muscle using LBs and US, protein suppression could be observed in US exposed area. As a result, gene-silencing effect was also observed in the skin. In contrast, in kidney, gene-silencing effect was not observed and

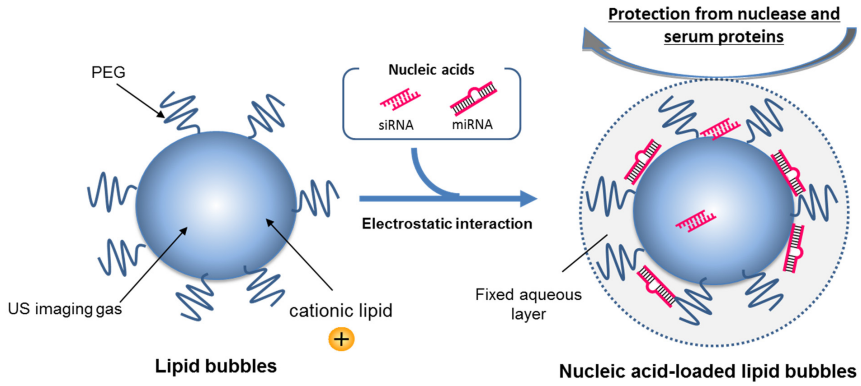

Figure 6. Scheme of nucleic acid-loaded lipid bubbles.

the luciferase expression was not even observed in the group not transfected with siRNA. These results were assumed to be due to the direct injection into the parenchymal tissue of kidney, the excretory organ of pDNA and siRNA (*47,48*). Therefore, by the application of intravascular administration or other methods suited for target tissue, LBs and US might be a useful tool for the delivery of siRNA into widespread tissue (*49*).

7. siRNA-loaded lipid bubbles

LBs as referred to above are composed neutral lipid; therefore, it is difficult to colocalize LBs in blood vessels after intravenous administration. In addition, if siRNA is delivered *via* systemic injection, it suffers from nuclease degradation, plasma protein interaction, and rapid removal from circulation, leading to a reduction in transfection efficiency *in vivo*. To solve these issues, we next tried to load siRNA to LBs by two methods in an attempt to make a more effective delivery tool for systemic injections (Figure 6).

7.1. *SiRNA-loaded LBs using the electrostatic interaction between cationic lipid and siRNA*

To load siRNA to LBs, cationic lipids are also useful because the lipids are able to interact with siRNA possessing negative electric charges. We initially attempted to prepare LBs containing 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), a cationic lipid often used for gene delivery (*50*). This LBs was composed of DPPC, DOTAP, PEG2000, and PEG750 in a 79:15:3:3 (molar ratio). Using a high-frequency US imaging system, we confirmed that LBs containing DOTAP could function as an echo-contrast reagent (*51*). By flow cytometry, we also confirmed that siRNA could interact with LBs, the interaction was due to the cationic charge of DOTAP, and LBs containing DOTAP, PEG_{750}, and PEG_{2000} could be loaded with much more siRNA compared to that of LBs containing DOTAP and PEG_{2000}. We considered that the structural changes in the PEG chain by the presence of both short and long PEG chains facilitated interaction between the cationic lipid and anionic 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. We also showed that siRNA held by LBs showed increased stability in 50% serum compared with free siRNA. We next attempted to transfect siRNA to cells previously transfected luciferase gene using si-LBs and US exposure. The gene-silencing effect of siRNA was comparable to those of siRNA transfected using conventional LBs and US. Therefore, it appears that the exposure to US induced cavitation, the release of siRNA from LBs, and the delivery of siRNA into the cytoplasm. The siRNA could be bound to only outside of LBs containing cationic lipids; therefore, the loadable amount of siRNA might be fewer than that by chol-si-LBs. However, the preparation method of si-LBs was easier than that of chol-si-LBs, and si-LBs had the advantage because there was no need to reprepare LBs whenever siRNA was changed. For these reasons, LBs containing DOTAP could be a convenient siRNA carrier. Furthermore, LBs containing cationic lipid are expected to have widespread application to delivery tools of various molecules possessing negative electric charges. Indeed, we showed that LBs containing DOTAP were also useful delivery tool of pDNA *in vitro* and *in vivo* study (*50,51*). It is known that short-chain and unsaturated fatty acids increase membrane fluidity (*52*). DOTAP is unsaturated fatty acid and is thought to destabilize the membrane of LBs. In fact, the increased DOTAP content made it difficult to entrap the gas (*9*). We therefore expected that LBs using other saturated cationic lipids would improve the stability of the liposomal membrane. Indeed, LBs containing other three types of saturated cationic lipids (DSTAP, DSDAP, DDAB) were more effective as a contrast agent compared to LBs containing DOTAP (*53*). Especially, LBs containing DSDAP could work well in the US imaging ability and transfection efficacy. These results suggested that changing of the cationic lipid led to membrane stabilization and improvement of gas retention ability.

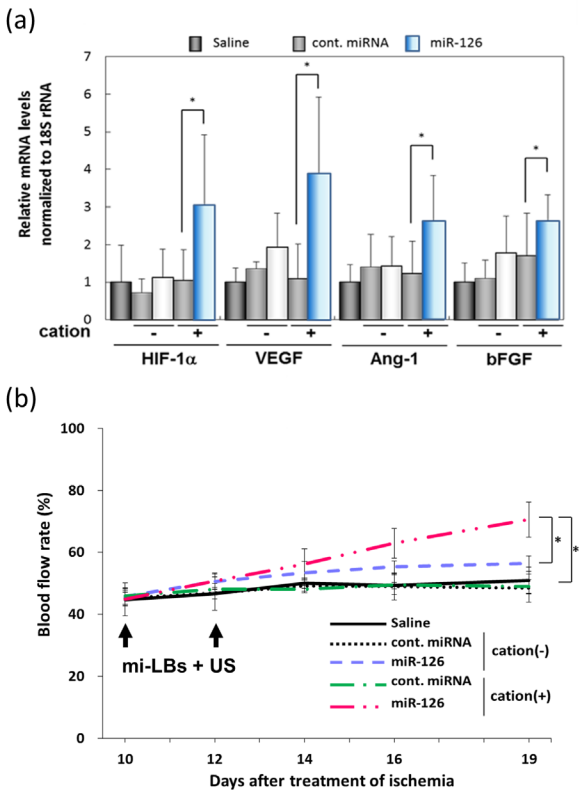

Figure 7. The therapeutic effects of miR-126 transfer by mi-LBs and US exposure on mice with hindlimb ischemia. Ten days after femoral artery ligation, mice were treated with mi- LBs and US. The treatment was administered *via* tail vein injection twice daily every two days to mice with hindlimb ischemia. We injected a solution of miRNA (40 μg of miCont or miR-126) and LBs (200 μg). **(a)** The effect of miR-126 transfer by mi- LBs and US on mRNA expression for angiogenic genes. Seven days after the second transfection, RNA was isolated from the thigh muscle and analyzed using real-time PCR. **(b)** The effect of miR-126 transfer using mi- LBs and US on the recovery of blood flow. After the second transfection, blood flow was measured using a laser Doppler blood flow meter. All data are reported as the mean ± S.D. (n = 4-6). *indicates $p < 0.05$ using a one-way ANOVA with Tukey's post-hoc test. Reprinted from Endo-Takahashi *et al*. with permission (*57*).

The combination of LBs and US is effective tool *via* local injection. However, it is useful that siRNA are transfected using novel efficient siRNA carrier like chol-si-LBs or si-LBs *via* systemic injection to kidney in which gene-silencing effects were not observed *via* local injection or to deep tissues which were difficult for directly injection. Taken together, it is expected that tissue- or organ-specific delivery of siRNA and the specific gene-silencing effect may be achieved by the destruction technologies of LBs combined with targeted US exposure leading to clinical applications for various diseases.

7.2. *microRNA-loaded lipid Bubbles*

MicroRNAs (miRNAs) are involved in biological functions and their dysregulations often lead to human diseases (*54*). Recently, a large number of miRNAs have been identified as a key target for therapeutic intervention. However, the efficient delivery vehicle of a therapeutic RNA to its target tissue is required to develop miRNA-based therapeutics (*55*). So, we attempted to prepare miRNA-loaded LBs (mi-LBs) and evaluated the utility of mi-LBs using a hindlimb ischemia model and miR-126 (*56*), which promotes angiogenesis *via* the cancellation of negative regulators of VEGF signaling (*57-59*). Before *in vivo* transfection experiments, we confirmed the interaction between miRNA and LBs containing the cationic lipid, which was previously reported to be an effective cationic lipid for the preparation of pDNA loaded- LBs (*53*). We found that the amount of miRNA bound to the LBs increased in the presence of cationic lipid. Next, the delivery of mi-LBs to the hindlimb ischemia mouse model was performed *via* intravascular injection. The mi-LBs reached an ischemic site after intravascular injection, were visualized by diagnostic US, and delivered miR-126 following therapeutic US. The delivery of miR-126 led to the increase of angiogenic gene expressions and followed by the improvement of blood flow (Figure 7). Thus, the combination of mi-LBs and US exposure may serve as a theranostic agent with combined properties of a diagnostic and therapeutic system (*56*).

8. Conclusions

US has long been used as a useful diagnostic tool. Therapeutic US was recently developed and is being used in clinical settings. The combination of therapeutic US and nano/microbubbles is an interesting and important system for establishing a novel and non-invasive gene delivery system. Genes are delivered more efficiently using this system compared with a conventional non-viral vector system such as the lipofection method, resulting in higher gene expression. This higher efficiency is due to the gene being delivered into the cytosol and bypassing the endocytosis pathway. Many *in vivo* studies have demonstrated US-mediated gene delivery with nano/microbubbles, and several gene therapy feasibility studies for various diseases have been reported. In addition, nano/microbubbles can deliver genes site-specifically by the control of US exposure site. Therefore, it is expected that this technology could be used clinically as a novel gene delivery system.

Acknowledgements

This study was supported by MEXT-Supported Program for the Strategic Research Foundation at Private Universities 2013-2016.

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(Received September 27, 2016; Revised October 24, 2016; Accepted October 25, 2016)