

# Evaluation of Small Interfering RNA Delivery into Cells by Reverse Transfection in Suspension with Cationic Liposomes

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## Abstract

Successful gene silencing by small interfering RNA (siRNA) requires efficient uptake of siRNA into targeted cells. For *in vitro* transfection of siRNA using cationic liposomes, two types of transfection method are currently being used: conventional (forward; Fw) and reverse (Rev) transfections. Here, to investigate an efficient siRNA transfection method using cationic liposomes, we compared the transfection efficiency of siRNA between Fw-transfection and Rev-transfection methods with various types of cationic liposomes. In Fw-transfection, siRNA/cationic liposomes complex (siRNA lipoplexes) was added to pre-plated cells. In contrast, Rev-transfection was performed by co-incubation of cells with siRNA lipoplexes in suspension. As a result, Rev-transfection with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-based or cationic cholesterol derivative-based liposomes could deliver siRNA into the cells *via* efficient cellular association, and induce an improved gene silencing effect by siRNA compared with Fw-transfection. Furthermore, Rev-transfection did not show increased cytotoxicity compared with Fw-transfection. These findings suggested that Rev-transfection in suspension has better potential for efficient transfection of siRNA into cells with minimal toxicity.

## Keywords

Cationic Liposome, siRNA Delivery, Reverse Transfection, Tumor Cells

## 1. Introduction

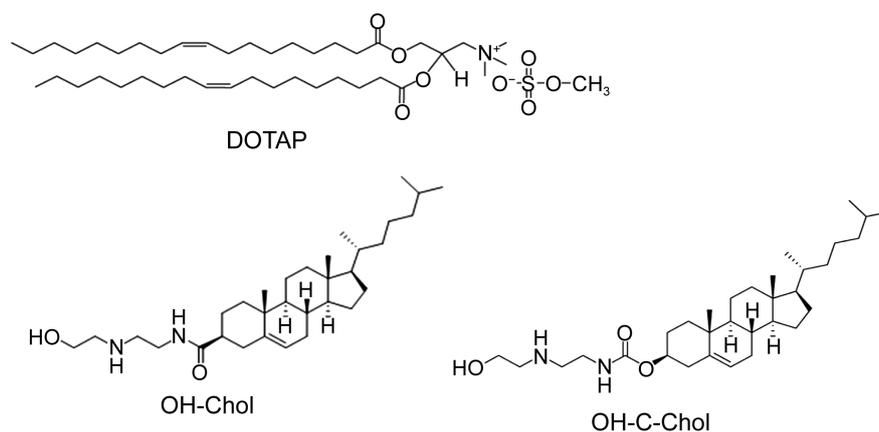
Synthetic small interfering RNAs (siRNAs), which are small, double-stranded RNAs, are substrates for the RNA-induced silencing complex [1]. Successful gene silencing by siRNA requires efficient uptake of siRNA into targeted cells.

For *in vitro* experiments, cationic liposomes have often been used for siRNA transfection into the cells [2] [3]. In siRNA transfection by cationic liposomes, two types of transfection methods are currently being used: conventional (forward; Fw) and reverse (Rev) transfections. They differ in the order and timing of the addition of the three necessary components of transfection: siRNA, cationic liposomes, and cells. In the Fw-transfection method, siRNA and cationic liposomes are complexed and then the siRNA lipoplexes are added to pre-plated cells. In the Rev-transfection method, all three components are added to the wells essentially at the same time. Compared with Fw-transfection, Rev-transfection enables direct and sufficient contact between cells and siRNA lipoplexes, thereby improving transfection efficiency [4]. In particular, solid-phase Rev-transfection, also known as surface-mediated transfection, is used as a high-throughput method for the parallel transfection of cells on microarrays [5] [6] or microplates [7]. In solid-phase Rev-transfection, siRNA complexes using cationic liposomes such as commercially available Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and Lipofectamine RNAiMax (Invitrogen) are attached to the bottom of cell culture plates by freeze-drying [4] [8] [9], and cells take up these complexes after plating on the culture plate. However, solid-phase Rev-transfection has been restricted so far to microarray- or microplate-based analyses. Reverse transfection can be also performed by co-incubation of cells with siRNA lipoplexes in suspension (Rev-transfection in suspension). Previously, it has been reported that transfection activities by commercially available Lipofectamine 2000 and RNAiFect (Qiagen, Hilden, Germany) could be improved by mixing siRNA complexes with cells in suspension and allowing the cells to attach in the presence of the complex [10]. However, to the best of our knowledge, there are still few reports on the application of Rev-transfection in suspension with cationic liposomes for the delivery of siRNA into cells. Therefore, in this study, we investigated Rev-transfection of siRNA in suspension with various type of cationic liposomes, and demonstrated that Rev-transfection in suspension with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-based or cationic cholesterol-derivative based liposomes could increase gene silencing activity in the cells *via* efficient cellular association.

## 2. Materials and Methods

### 2.1. Materials

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) methyl sulfate salt (**Figure 1**) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol (Chol) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *N*-(2-(2-Hydroxyethylamino)ethyl)cholesteryl-3-carboxamide (OH-Chol) (**Figure 1**) and cholesteryl 2-((2-hydroxyethyl)amino)ethyl)carbamate (OH-C-Chol) (**Figure 1**) were synthesized as described previously [11]. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was obtained from NOF Co. Ltd. (Tokyo, Japan). All other chemicals were of the finest grade available.



**Figure 1.** Structure of cationic lipids used in this study: 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) methyl sulfate salt, (3 $\beta$ )-*N*-(2-(2-hydroxyethylamino)ethyl)cholesteryl-3-carboxamide (OH-Chol) and cholesteryl 2-((2-hydroxyethyl)amino)ethylcarbamate (OH-C-Chol).

## 2.2. Small Interfering RNAs

siRNAs targeting nucleotides of *firefly* pGL3 luciferase (Luc siRNA) and nonsilencing siRNA (control [Cont] siRNA) as a negative control were synthesized by Sigma Genosys (Tokyo, Japan). The siRNA sequences of the Luc siRNA were: sense strand: 5'-GUGGAUUUCGAGUCGUCUAAA-3' and antisense strand: 5'-AAGACGACUCGAAAUCCACAU-3'. The siRNA sequences of the Cont siRNA as a negative control for Luc siRNA were: sense strand: 5'-GUACCGCACGUCAUUCGUAUC-3' and antisense strand: 5'-UACGAAUGACGUGCGGUACGU-3'. Alexa Fluor<sup>®</sup>488-labeled AllStars Negative Control siRNA (AF-siRNA) was obtained from Qiagen (Valencia, CA, USA).

## 2.3. Preparation of Cationic Liposomes and siRNA Lipoplexes

Cationic liposomes were prepared from DOTAP/Chol or DOTAP/DOPE at a molar ratio of 1:1 using a thin-film hydration method, as reported previously [12]. Cationic cholesterol derivative-based liposomes were prepared from OH-Chol/DOPE or OH-C-Chol/DOPE at a molar ratio of 3:2 using a thin-film hydration method [11]. The thin film was hydrated with water at 60°C by vortex mixing for 1 min. The liposomes were sonicated in a bath-type sonicator for 10 min.

To prepare complexes of siRNA/cationic liposome (siRNA lipoplexes), each liposome preparation was added to 50 pmol siRNA at a charge ratio (+:–) of 4:1 of cationic lipid to siRNA for DOTAP/Chol and DOTAP/DOPE liposomes or 7:1 for OH-Chol/DOPE and OH-C-Chol/DOPE liposomes with gentle shaking and left at room temperature for 15 min. The charge ratio (+:–) of liposomes: siRNA is expressed as the molar ratio of cationic lipid to siRNA phosphate.

## 2.4. Size and $\zeta$ -Potential of Cationic Liposomes and siRNA Lipoplexes

The particle size distributions of cationic liposomes and lipoplexes were meas-

ured by the cumulant method using a light-scattering photometer (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan) at 25°C after diluting the dispersion with an appropriate volume with water. The  $\zeta$ -potentials were measured using the ELS-Z2 at 25°C after diluting the dispersion with an appropriate volume of water.

## 2.5. Cell Culture

Human breast cancer MCF-7-Luc (TamR-Luc#1) cells stably expressing *firefly* luciferase (pGL3) were donated by Dr. Kazuhiro Ikeda (Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100  $\mu\text{g}/\text{mL}$  kanamycin, and 0.5  $\text{mg}/\text{mL}$  G418 at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere.

## 2.6. Transfection

For Fw-transfection, MCF-7-Luc cells were detached with TrypLE™ Express (Invitrogen), plated into 6-well (35 mm) culture dishes at a density of  $2.5 \times 10^5$  cells per well and then incubated for 1 h at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere. Each lipoplex with 50 pmol siRNA was diluted in 1 mL of DMEM (50 nM siRNA) supplemented with 10% FBS, and then added to the cells (**Figure 2**).

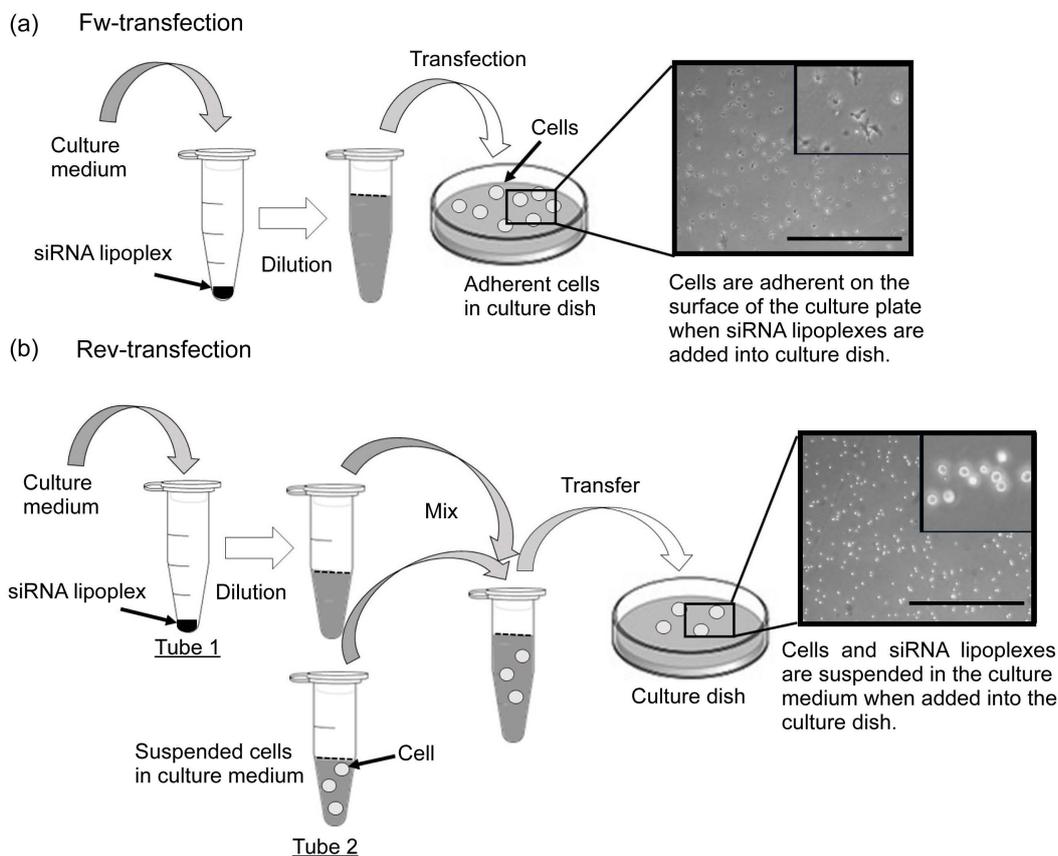
For Rev-transfection, MCF-7-Luc cells were detached with TrypLE™ Express, and  $2.5 \times 10^5$  cells were suspended in 0.5 mL of DMEM supplemented with 10% FBS (**Figure 2**). Each lipoplex with 50 pmol siRNA was diluted in 0.5 mL of DMEM supplemented with 10% FBS, and then added to the cells suspended in 0.5 mL of the medium. The mixtures (1 mL) of siRNA lipoplexes (50 nM siRNA) and suspended cells were transferred into 6-well culture dishes.

## 2.7. Luciferase Activity

Each lipoplex with 50 pmol Luc siRNA or Cont siRNA was transfected by the Fw-transfection or Rev-transfection methods into MCF-7-Luc cells in 6-well culture dishes (final 50 nM siRNA concentration). Forty-eight hours after transfection, luciferase activity was measured as counts per sec (cps)/ $\mu\text{g}$  protein using a luciferase assay system (Pica Gene, Toyo Ink Mfg. Co. Ltd., Tokyo, Japan) and BCA reagent (Pierce, Rockford, IL, USA), as reported previously [13]. Luciferase activity (%) was calculated relative to the luciferase activity (cps/ $\mu\text{g}$  protein) of untransfected cells.

## 2.8. Flow Cytometric Analysis

For Fw-transfection, MCF-7-Luc cells were detached with TrypLE™ Express, plated into 6-well culture dishes at a density of  $5.0 \times 10^5$  cells per well and then incubated for 1 h. Each lipoplex with 50 pmol AF-siRNA was diluted in 1 mL of medium (50 nM siRNA) supplemented with 10% FBS, and then added to the cells. For Rev-transfection, each lipoplex with 50 pmol AF-siRNA was diluted in



**Figure 2.** Schematic diagram of the reverse transfection (Rev-transfection) method with siRNA lipoplexes. In conventional transfection (Forward (Fw)-transfection), siRNA/cationic liposome complexes (siRNA lipoplexes) are diluted in culture medium, and then added to the pre-plated adherent cells (a). In Rev-transfection, siRNA lipoplexes are diluted in culture medium, and are then mixed with an equal volume of medium-diluted cells, followed by transfer to a culture dish (b). Scale bar = 1,000  $\mu\text{m}$ .

0.5 mL of medium supplemented with 10% FBS, and then added to  $5.0 \times 10^5$  cells suspended in 0.5 mL of medium. The mixtures (1 mL) of siRNA lipoplex (50 nM AF-siRNA) and suspended cells were transferred into 6-well culture dishes. After 3 h incubation, the cells were washed twice with 1 mL phosphate-buffered saline (PBS) to remove any unbound lipoplexes. The amount of AF-siRNA in the cells was determined by examining fluorescence intensity using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), as described previously [14].

### 2.9. Cytotoxicity

For Fw-transfection, MCF-7-Luc cells were plated into 96-well culture dishes at a density of  $2.5 \times 10^4$  cells per well and incubated for 1 h. Each lipoplex with 50 pmol Cont siRNA was diluted in 1 mL of medium supplemented with 10% FBS, and then the mixture (100  $\mu\text{L}$ ) was added to the cells (final 50 nM siRNA concentration).

For Rev-transfection, each lipoplex with 50 pmol Cont siRNA was diluted in 0.5 mL of medium supplemented with 10% FBS, and then added to  $2.5 \times 10^5$

cells suspended in 0.5 mL of the medium. The mixtures (100  $\mu$ L) of the lipoplexes and  $2.5 \times 10^4$  cells were transferred into 96-well culture dishes (final 50 nM at siRNA concentration).

After a 24 h incubation period, cell numbers were determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cell viability was expressed as relative to the absorbance at 450 nm of untransfected cells.

### 2.10. Statistical Analysis

Data were compared using analysis of variance and evaluated with Student's *t* test. A *p* value of 0.05 or less was considered significant.

## 3. Results and Discussion

First, we prepared DOTAP-based and cationic cholesterol derivative-based liposomes for siRNA delivery by Fw- and Rev-transfections, respectively. In this study, we used DOTAP, OH-Chol, or OH-C-Chol (**Figure 1**) as a cationic lipid, and DOPE or Chol as a neutral lipid. Because we reported previously that cationic liposomes composed of OH-Chol or OH-C-Chol and DOPE could efficiently deliver siRNA into tumor cells [11] [14]. In the formulations with DOTAP-based liposomes, cationic liposomes composed of DOTAP and cholesterol (Chol) or DOPE were prepared, because their formulations were often used for siRNA delivery [12] [15] [16]. The sizes of DOTAP/Chol, DOTAP/DOPE, OH-Chol/DOPE, and OH-C-Chol/DOPE liposomes were approximately 100 - 130 nm, and their  $\zeta$ -potentials were 47 - 56 mV (**Table 1**). When the lipoplexes were prepared with siRNA, their sizes were approximately 190 - 220 nm and their  $\zeta$ -potentials were about 42 - 47 mV.

Next, we examined the effect of transfection methods on the gene knockdown effect by the lipoplexes with 50 nM siRNA using a luciferase assay system with MCF-7-Luc cells. Conventional transfection (Fw-transfection) is a common technique for delivering siRNA into cells for gene silencing (**Figure 2**). In this standard approach, siRNA lipoplexes are added to adherent cells on the surface of culture plate. Generally, siRNA lipoplexes are added into cells plated 24 h prior to transfection; however, in this study, siRNA lipoplexes were added into the cells plated 1 h prior to transfection, because the number of cells between

**Table 1.** Formulae, size, and  $\zeta$ -potentials of cationic liposomes and siRNA lipoplexes.

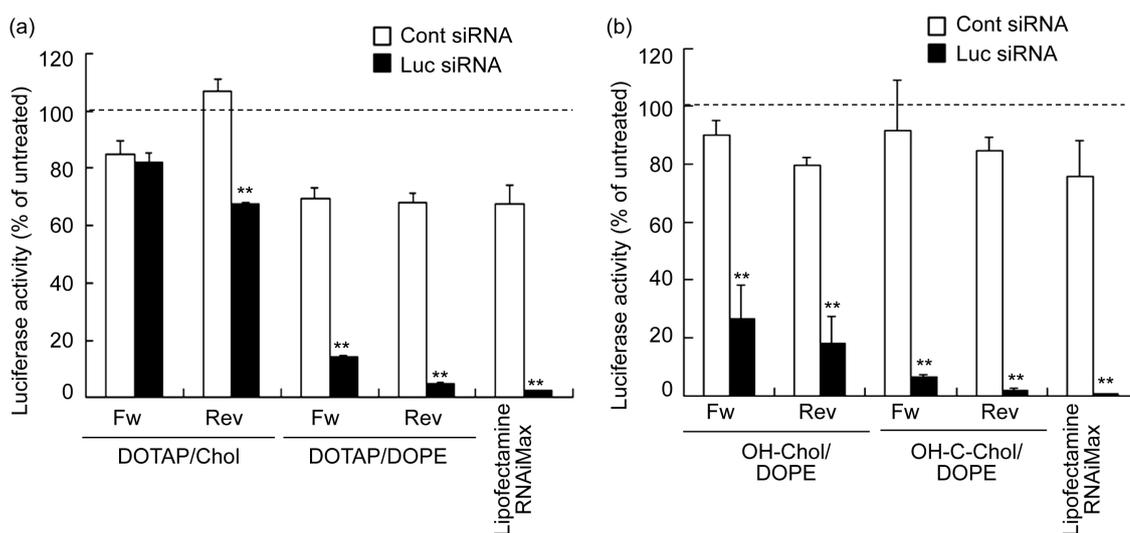
Formulation	Molar ratio	Liposome <sup>a)</sup>		Lipoplex of siRNA <sup>a,b)</sup>	
		Size (nm)	$\zeta$ -potential (mV)	Size (nm)	$\zeta$ -potential (mV)
DOTAP/Chol	1/1	101.2 $\pm$ 2.5	56.0 $\pm$ 1.0	189.5 $\pm$ 5.0	41.7 $\pm$ 0.4
DOTAP/DOPE	1/1	116.2 $\pm$ 2.9	49.7 $\pm$ 0.6	189.0 $\pm$ 6.0	44.4 $\pm$ 0.8
OH-Chol/DOPE	3/2	126.0 $\pm$ 2.9	52.8 $\pm$ 0.9	220.1 $\pm$ 1.9	46.7 $\pm$ 0.8
OH-C-Chol/DOPE	3/2	120.5 $\pm$ 2.7	47.5 $\pm$ 1.8	195.3 $\pm$ 5.2	46.1 $\pm$ 1.8

<sup>a)</sup>In water. <sup>b)</sup>Charge ratio (+/-) of cationic liposome/siRNA = 4/1 for DOTAP/Chol and DOTAP/DOPE lipoplexes, and 7/1 for OH-Chol/DOPE and OH-C-Chol lipoplexes, respectively. Values represent means  $\pm$  S.D. (n = 3).

Fw-transfection and Rev-transfection must be uniform. In contrast, in Rev-transfection, siRNA lipoplexes were diluted in culture medium, and mixed with an equal volume of medium diluted with MCF-7 cells (Figure 2).

For DOTAP-based formulations, DOTAP/Chol lipoplexes with Luc siRNA did not suppress luciferase activity by Fw-transfection; however, moderate suppression of luciferase activity was observed by Rev-transfection (Figure 3(a)). In contrast, DOTAP/DOPE lipoplexes with Luc siRNA significantly suppressed luciferase activity by Fw-transfection, and its gene silencing effect was increased by Rev-transfection (Figure 3(a)). For cationic cholesterol derivative-based formulations, OH-Chol/DOPE and OH-C-Chol/DOPE lipoplexes could strongly suppress luciferase activities by both Fw-transfection and Rev-transfection methods, respectively, although Rev-transfection slightly improved gene silencing activities compared with Fw-transfection (Figure 3(b)). From the results of these gene silencing effects, Rev-transfection in suspension with siRNA lipoplexes could improve gene knockdown by siRNA.

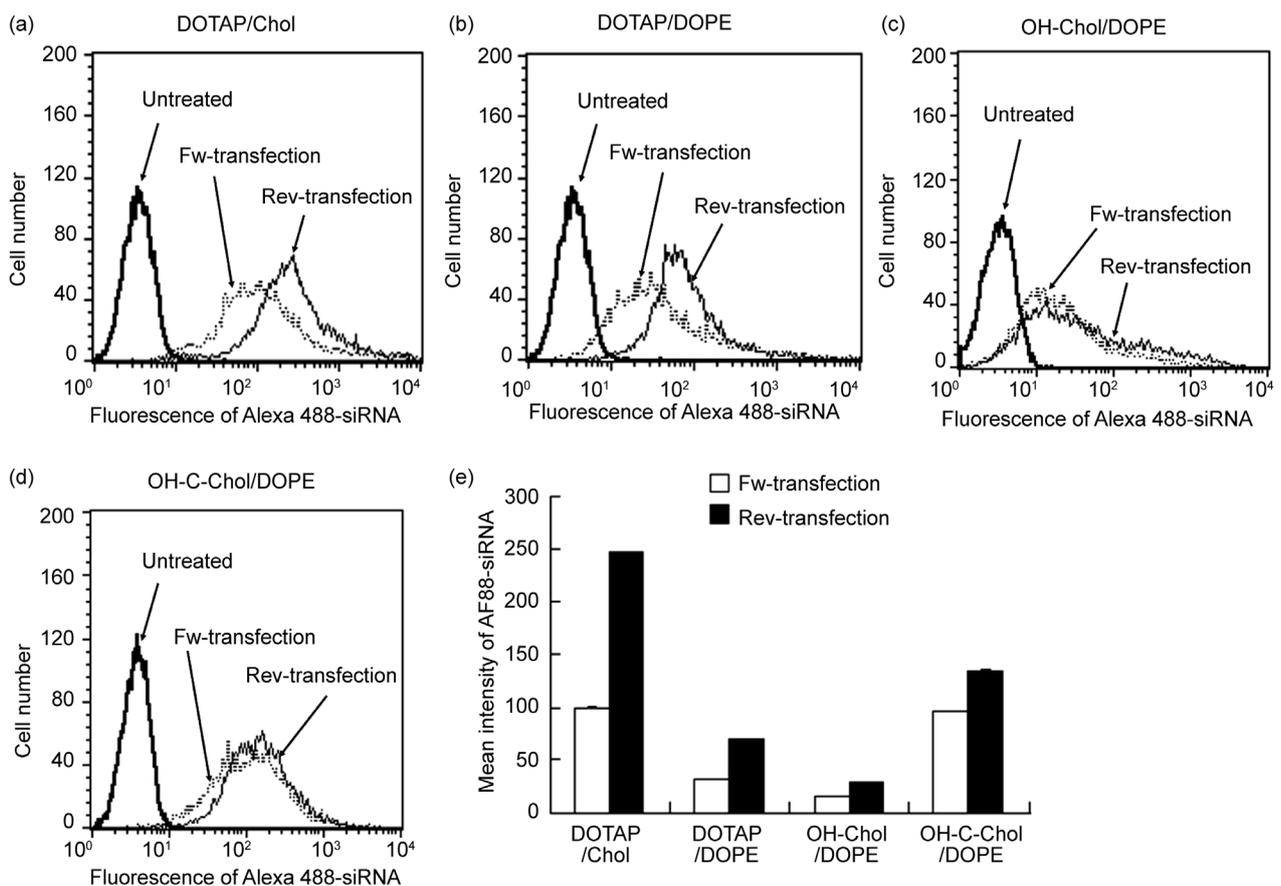
DOTAP/DOPE lipoplexes exhibited higher gene silencing activity than DOTAP/Chol lipoplexes (Figure 3(a)). It is generally known that cationic lipoplexes need to escape from endosomes after cellular internalization for the induction of gene knockdown. DOPE has been widely used as a co-lipid for cationic liposome-mediated transfection of siRNA and plasmid DNA [17]. The role of DOPE is not fully understood, but it might affect the structural transition of cationic liposomes at acidic pH in late endosomes in cells [18] [19], which promotes endosomal escape of siRNA lipoplexes. From these findings, DOTAP/DOPE lipoplexes might be able to induce high gene silencing effects in the cells compared with DOTAP/Chol lipoplexes.



**Figure 3.** Effect of transfection method on gene silencing activity in MCF-7-Luc cells 48 h after transfection. (a) DOTAP/Chol and DOTAP/DOPE lipoplexes were transfected into the cells using the Fw-transfection or Rev-transfection methods. (b) OH-Chol/DOPE and OH-C-Chol/DOPE lipoplexes were transfected into the cells by Fw-transfection or Rev-transfection methods. These lipoplexes were added to cells with 50 nM small interfering RNA (siRNA). Commercially available transfection reagent Lipofectamine RNAiMax was used as a control. Each column represents the mean  $\pm$  S.D. (n = 3). \*\* $p < 0.01$  compared with control (Cont) siRNA.

To clarify the relationship between the gene silencing effect and cellular uptake in Fw-transfection and Rev-transfection with siRNA lipoplexes, we examined the cellular association of lipoplexes by flow cytometric analysis. In Fw-transfection, DOTAP/Chol lipoplexes exhibited higher cellular association than DOTAP/DOPE lipoplexes (**Figure 4(a)** and **Figure 4(b)**), indicating that the inclusion of cholesterol in a formulation with cationic liposomes resulted in good uptake by the cells, but could not induce a gene silencing effect. Furthermore, cellular associations with DOTAP/Chol (**Figure 4(a)**), DOTAP/DOPE (**Figure 4(b)**), OH-Chol/DOPE (**Figure 4(c)**), and OH-C-Chol/DOPE (**Figure 4(d)**) lipoplexes after Rev-transfection were higher than those after Fw-transfection, respectively (**Figure 4(e)**). This result corresponded to those of gene knockdown efficacy (**Figure 3**). From the results, Rev-transfection with siRNA lipoplexes could deliver siRNA into the cells *via* efficient cellular association and induce a large gene silencing effect using siRNAs.

Finally, we investigated cytotoxicity after Fw- and Rev-transfections with siRNA lipoplexes. As a result, in any siRNA lipoplexes, Rev-transfection did not

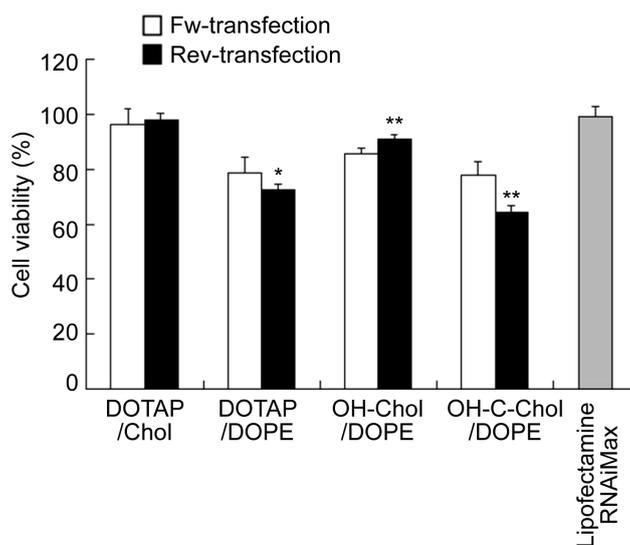


**Figure 4.** Cellular association at 3 h after Fw-transfection or Rev-transfection of siRNA lipoplexes. (a) DOTAP/Chol lipoplexes, (b) DOTAP/DOPE lipoplexes, (c) OH-Chol/DOPE lipoplexes, and (d) OH-C-Chol/DOPE lipoplexes were formed by mixing with Alexa Fluor<sup>®</sup>488-labeled small interfering RNA (AF-siRNA). These lipoplexes were added to cells with a final concentration of 50 nM siRNA. The association of siRNA lipoplexes with MCF-7 cells was determined on the basis of Alexa Fluor<sup>®</sup>488-fluorescence by flow cytometry. (e) Each column represents the mean fluorescent intensity  $\pm$  S.D. ( $n = 3$ ).

strongly increase cytotoxicity compared with Fw-transfection (Figure 5). These findings suggested that the Rev-transfection method has potential for efficient transfection of siRNA into cells with minimal toxicity.

In solid-phase Rev-transfection, the siRNA complex must be freeze-dried on a culture plate in small volumes. The one of advantages for Rev-transfection in suspension is the ability to easily prepare a large number of plates in larger volumes. However, the reasons for the improvements in gene silencing (Figure 3) and cellular uptake (Figure 4) observed at the Rev-transfection in suspension are not clear. Amarzguioui described that in Rev-transfection in suspension, a more favorable cell surface environment for the attachment of siRNA lipoplexes might be generated after partial removal of surface proteins by treatment with trypsin [10]. However, in our study, Fw-transfection was performed with cells treated with TrypLE™ Express for detachment and plated 1 h prior to transfection. TrypLE™ Express is an enzyme that cleaves peptide bonds on the C-terminal sides of lysine and arginine as well as trypsin. This indicated that treatment of the cells with trypsin was not a critical factor for the improvement of transfection efficiency by the Rev-transfection method. We speculated that transfection efficiency by Rev-transfection might be improved through an increased area of cellular surface that could associate with siRNA lipoplexes in suspension. Alternatively, morphological changes associated with attachment to the surface of the culture plate might affect cellular uptake of siRNA lipoplexes.

In conclusion, Rev-transfection with siRNA lipoplexes in suspension could deliver siRNA into the cells *via* efficient cellular association and induce an improved gene silencing effect by siRNA compared with Fw-transfection. These findings suggested that the Rev-transfection method in suspension has potential for efficient transfection of siRNA into cells with minimal toxicity.



**Figure 5.** Cell viability 24 h after Fw-transfection or Rev-transfection with siRNA lipoplexes into MCF-7 cells. Each column represents the mean  $\pm$  S.D. ( $n = 6$ ). Lipofectamine RNAiMax was used as a control. \* $p < 0.05$ , \*\* $p < 0.01$  compared with Fw-transfection.

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