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Agitation during lipoplex formation improves the gene knockdown effect of siRNA

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ABSTRACT

The successful delivery of therapeutic siRNA to the designated target cells and their availability at the intracellular site of action are crucial requirements for successful RNAi therapy. In the present study, we focused on the siRNA-lipoplex preparation procedure and its effect on the gene-knockdown efficiency of siRNA *in vitro*. Agitation (vortex-mixing) during siRNA-lipoplex (vor-LTsiR) preparation and its effect on the gene-knockdown efficiency of stably expressed cell GFP was investigated, and their efficiency was compared with that of spontaneously formed lipoplex (spo-LTsiR). A dramatic difference in size between lipoplexes was observed at the N/P ratio of 7.62 (siRNA dose of 30 nM), even though both lipoplexes were positively charged. With the siRNA dose of 30 nM, vor-LTsiR accomplished a 50% gene-knockdown, while spo-LTsiR managed a similar knockdown effect at the 120 nM level, suggesting that the preparation procedure remarkably affects the gene-knockdown efficacy of siRNA. The uptake of vor-LTsiR was mainly via clathrin-mediated endocytosis, whereas that of spo-LTsiR was via membrane fusion. In addition, by inhibiting clathrin-mediated endocytosis, the gene-knockdown efficiency was significantly lowered. The size of the lipoplex, promoted by the preparation procedure, is likely to define the entry pathway, resulting in an increased amount of siRNA internalized in cells and an enhanced gene-knockdown efficacy. The results of the present study definitively show that a proper siRNA-lipoplex preparation procedure makes a significant contribution to the efficiency of cellular uptake, and thereby, to the gene-knockdown efficiency of siRNA.

1. Introduction

RNA interference (RNAi), a naturally occurring biological process of gene regulation conserved in mammalian cells (Elbashir et al., 2001), has recently shown great potential as a novel therapeutic strategy (Li et al., 2006; Behlke, 2006). The target sites of the negatively charged small interfering RNA (siRNA) used in RNAi therapy are inside the cytoplasm. Therefore, it is essential that these molecules traverse the plasma membrane to reach their target sites (Li et al., 2006; Behlke, 2006). The plasma membrane of living cells is a dynamic structure that is relatively lipophilic and negatively charged in nature, and it restricts the entry of large, hydrophilic, or negatively charged, molecules (Leung and Whittaker, 2005). Thus, an appropriate delivery system is required to achieve an efficient cellular uptake and to release the siRNA inside the cell, cytoplasm (Soutschek et al., 2004; Leung and Whittaker, 2005).

Several delivery methodologies based on viral and nonviral vectors have been developed to circumvent this problem and have been successfully used for the introduction of siRNA into cells both *in vitro* and *in vivo* (Leung and Whittaker, 2005; Li et al., 2006; Behlke, 2006). Among these methodologies, cationic liposome has shown simplicity of use and ease of large-scale production, which makes it particularly promising and potentially useful for the delivery of siRNA (Behlke, 2006; Khalil et al., 2006a). The current cationic liposomes used for siRNA delivery have been adapted from those developed to deliver plasmid DNA (pDNA) and oligodeoxynucleotide to cells. Thus, conventional siRNA-lipoplex is formed through the spontaneous electrostatic interaction between the positively charged lipid in the liposome membrane and the negatively charged phosphate backbone of the siRNA (Lasic and Templeton, 1996). Despite a certain degree of success, spontaneous

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formation is a static method that allows little control over the interaction process, leading to a very heterogeneous lipoplex size distribution and to excessive size (Lasic and Templeton, 1996; Kawakami et al., 2002; Faneca et al., 2004). However, although some data demonstrating the gene-knockdown efficiency of siRNA-lipoplex has been accumulated, little is known about the effect of varying the condition during lipoplex formation and to what extent this affects the gene knockdown efficiency of siRNA.

Therefore, in the present study, we focused on the preparation procedure for siRNA-lipoplex. The effect of agitation (vortexing) during siRNA-lipoplex formation on the knockdown efficiency against green fluorescence protein (GFP) stably expressed in HT-1080 cells was investigated. Also, the size distribution of lipoplex, its attached amount of siRNA, and its uptake mechanism were investigated, along with the amount of siRNA internalized in cells. Herein, we show that the uptake and promoting pathway of siRNA-lipoplex, which is modulated by the preparation procedure, influences the gene-knockdown efficiency of siRNA *in vitro*.

2. Material and methods

2.1. Materials

The cationic liposome, LipoTrust™-SR (LT), composed of *O*,*O*′-ditetradecanoyl-*N*-(α-trimethyl ammonioacetyl) diethanolamine chloride (DC-6-14), dioleoylphosphatidylethanolamine (DOPE) and cholesterol in the molar ratio of 1.00/0.75/0.75 was purchased from Hokkaido System Science (Hokkaido, Japan). The Hoechst 33342 was purchased from Molecular Probes (OR, USA). Z-Phe-Phe-Gly (ZfFG) and filipin complex were purchased from Sigma–Aldrich (MO, USA). The 1% Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine-containing LipoTrust™-SR (RhLT) was a generous gift from Daiichi-Sankyo Pharmaceutical (Tokyo, Japan). All other chemicals were reagent grade and used as received.

2.2. siRNA preparation

All RNA sequences were chemically synthesized and purified with HPLC by Hokkaido System Science. The siRNA for GFP (siGFP) composed of the sense strand 5′-GGCUACGUCCAGGAGCGCACC-3′ and the antisense strand 5′-UGCGCUCCUGGACGUAGCCUU-3′ (Song et al., 2003) and the siRNA against firefly luciferase (siLuc) composed of a sense strand 5′-AGCUUCAUAAGGCGCAUGCTT-3′ and an antisense strand 5′TTUCGAAGUAUUCCGCGUACG-3′ were used as the control siRNA (Elbashir et al., 2001). The siGFP labeled at the 5′-end of the sense strand with carboxyfluorescein (FAM) (siFAM) and labeled at the 5′ end of the antisense strand with alexa Fluor 488 (siALEXA) were used as fluorescent siRNA probes, respectively. For siRNA preparation, the complementary antisense and sense strands were mixed in TE buffer (1×10^{-2} mM Tris–HCl, 1×10^{-3} mM EDTA, pH 8.0, DNase and RNase free grade, Nippon Gene, Tokyo, Japan) in a 1:1 molar ratio followed by heating at 95 °C for 1 min. The reaction was then allowed to cool at room temperature. The quality of siRNA was checked by 15% PAGE. The final concentration of the duplexes was 50 μM in TE buffer.

2.3. Preparation of siRNA lipoplexes

Various aliquots (0.1875–6.000 μl) of the siRNA solution (50 μM) were diluted to a final volume of 100 μl with fresh Opti-MEM (Invitrogen, CA, USA). A 25 μl aliquot of LT suspension (2.4 mM) was also diluted to a final volume of 100 μl with fresh Opti-MEM. The diluted siRNA solutions were then mixed with the diluted LT suspension. The N/P ratios were set at 1.90, 3.81, 7.62, 15.24, or 30.45. The LT-siRNA lipoplexes (LTsiR) were allowed to form in two ways: the lipoplex was formed spontaneously (spo-LTsiR) by standing samples for 10 min, and the lipoplex was formed under application of a high vortex-mixing (2500 rpm) (vor-LTsiR) (Vortex-Genie 2, Scientific Industries, NY, USA) for 10 min.

2.4. Particle size and zeta potential of siRNA lipoplexes

The particle size and zeta-potential of siRNA lipoplexes formed at the N/P ratio of 7.62 (9.6 μM/30 nM of cationic lipid/siRNA, respectively) were determined on a Nicomp 380 Submicron Particle Sizer (Particle Sizing System, CA, USA). To determine the particle size, LTsiR lipoplexes (200 μl) were prepared in either Opti-MEM or 9% sucrose, as described above, and were diluted with 200 μl of the same medium (Opti-MEM or 9% sucrose). The mean particle size represents the average of three different preparations of the same lipoplex. For zeta-potential determination, lipoplexes were formed in 9% sucrose, and the volume was adjusted with the same medium to 2.2 ml. The zeta potential represents the average of three different preparations of the same lipoplex. Zeta-potential could not be determined in Opti-MEM due to the large amount of salts this medium contains.

2.5. Correlation between the relative size of the siRNA lipoplex and the amount of siRNA attached to the lipoplexes

To examine the correlation between the relative size of the siRNA lipoplex and the amount of siRNA attached to the lipoplex, LT-siFAM lipoplexes (200 μl) were prepared at an N/P ratio of 7.62 (9.6 μM/30 nM per well), as described above in Opti-MEM. Then, a flow cytometer Guava EasyCyte mini system (Guava Technology, CA, USA) equipped with an argon laser exciting at a wavelength of 488 nm was used to analyze 20,000 lipoplexes. Forward scatter and fluorescence emission was centered at 525 nm (Green fluorescence). The fluorescence was collected using a logarithmic scale. Data were analyzed using WinMDI 2.7 software (Scripps Institute, CA, USA).

2.6. Cells and cell culture

A human fibrosarcoma (HT-1080) cell line was purchased from Dainippon-Sumitomo Pharmaceutical (Osaka, Japan). The stably expressed green fluorescence protein (GFP) in HT-1080 cells (HT-1080GFP) was established previously by Drs. T. Asai and N. Oku (Department of Medical Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, Japan) (Yamakawa et al., 2000). The cells were cultured in DMEM (Sigma, St. Louis, MO, USA) and supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Bioserum, Tokyo, Japan), 100 IU/ml penicillin and 100 μg/ml streptomycin (ICN Biomedical, OH, USA) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were maintained in exponential growth.

2.7. In vitro GFP gene knockdown

HT-1080GFP cells were seeded in 24-well plates at a density of 1.25×10^4 cells/well 24 h prior to siRNA lipofection. For lipofection, the amount of the lipoplex was fixed at 9.6 μM per well while the siRNA amounts were varied at 7.5, 15, 30, 60, and 120 nM per well. The growth medium was removed and replaced with 410 μl of Opti-MEM, 50 μl of FBS and 40 μl of LT-siGFP or LT-siLuc, followed by incubation for 24 h. Then, the medium was replaced with 500 μl of fresh DMEM containing 10% FBS and the cells again were incubated for another 24 h. To assess gene knockdown efficiency, HT-1080GFP cells were lysed in 2% Triton X-100 in PBS (100 μl/well) for 1 h on ice, following removal of the incubation media. Plates were then gently agitated in a shaker for 10 min

and 1 ml of PBS/well was added. The samples were transferred to microtubes and centrifuged at 10,000 rpm for 5 min at 4 °C. The fluorescence of the GFP in the clear lysates was measured using a standard fluorimetric method for GFP (excitation at 495 nm and emission at 510 nm) in a F-4500 Fluorescence Spectrophotometer (HITACHI, Tokyo, Japan).

2.8. Confocal microscopy

HT-1080 cells were seeded in a 35-mm glass-bottom dish (Iwaki Glass, Tokyo, Japan) at a density of 5×10^3 cells/dish and incubated for 24 h. For lipofection, the growth medium was replaced with 164 μl of Opti-MEM, 20 μl of FBS and 16 μl of RhLT-siFAM at the N/P ratio of 7.62 (LT/siFAM = 9.6 μM/30 nM) per well. Cells were then incubated for 4 h with RhLT-siFAM in OptiMEM. After lipofection, the cells were washed with 200 μl of PBS and then were incubated with Hoechst 33342 DMEM (10 μM) supplemented with 10% FBS for 20 min at room temperature. Confocal images were acquired using a Zeiss LSM5 inverted confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) without fixation. LSM510 (Version 3.2 SP2) software was used to process and analyze the images.

2.9. Interaction and internalization of siRNA lipoplexes with cells

To investigate the cellular association and internalization of both vor-LTsiR and spo-LTsiR in HT-1080 cells, RhLT-siGFP and LT-siFAM were prepared spontaneously or with high-vortex mixing at an N/P ratio of 7.62 (9.6 μM/30 nM, respectively). HT-1080 cells were seeded at a density of 5.0×10^4 cells/well in 6-well plates and were incubated for 24 h at 37 °C. After 4 h of lipofection with LTsiR, the lipoplexes were removed and the cells were washed twice with PBS. Then, the cells were trypsinized and collected in a 1.5 ml tube. After removal of the supernatant by centrifugation (1,000 rpm, 5 min, 4 °C), the cell pellet was resuspended in 0.5 mM EDTA-PBS. The total fluorescence intensity of siRNA lipoplexes (surface-bound and internalized ones) in a cell was directly analyzed in a flow cytometer Guava EasyCyte mini system. The fluorescence intensity-related to the internalized lipoplex was analyzed after quenching the extracellular fluorescence by incubating the cells in a 0.3% trypan blue PBS solution (Zuhorn et al., 2002).

2.10. The uptake mechanism of siRNA lipoplexes

To examine the uptake mechanism of vor-LTsiR and spo-LTsiR by cells, the effect of the following uptake inhibitors on the internalization of the lipoplexes was investigated: a hypertonic sucrose medium, which can inhibit clathrin-mediated endocytosis through dissociation of the clathrin lattice; amiloride, which can specifically inhibit macropinocytosis by inhibiting the Na^+/H^+ exchange required for macropinocytosis; filipin complex, which can specifically inhibit caveolar uptake through cholesterol depletion; and, the tri-peptide ZfFG, which can inhibit membrane fusion (Khalil et al., 2006a,b). Cells were incubated with Opti-MEM supplemented with 10% FBS in the absence or presence of the inhibitors for 30 min: sucrose (0.45 M), ZfFG (200 μM), amiloride (0.5 mM), or filipin complex (5 μg/ml). LT-siFAM lipoplex (LT/siFAM = 7.2 μM/22.5 nM) was then added, followed by incubation for 1 h. The cells then were trypsinized, centrifuged at 1,000 rpm for 5 min and 4 °C, and collected in 0.3% trypan blue PBS solution to quench the extracellular fluorescence (Zuhorn et al., 2002). Trypan blue-treated cells were then washed twice with PBS, and resuspended in 0.5 mM EDTA-PBS. Samples (5,000 cells) were analyzed in a flow cytometer Guava EasyCyte mini system.

2.11. Influence of the uptake pathway on the gene knockdown effect of siRNA

This experiment was designed to examine the influence of the uptake pathway on the gene knockdown effect of siRNA. In this experiment, an exogenous gene instead of an endogenous gene model was used in order to shortening the time for siRNA transfection, since some of the inhibitors we used are toxic for the cell, and could lead to cell death. HT-1080 cells were seeded in a 12-well plate at a density of 2.5×10^4 cells/well in DMEM supplemented with 10% FBS prior to the experiment, then were cultured for 24 h at 37 °C. One microgram of pDNA (pEGFP-N1 vector) was transfected for 1 h with 2 μl of LipofectAMINE 2000 (Lf 2000, Invitrogen, CA, USA), according to the manufacturer's instruction. The transfection medium was then replaced with DMEM supplemented with 10% FBS and incubated for 30 min at 37 °C. After removal of the medium, the cells were incubated with OptiMEM supplemented with 10% FBS in the absence or presence of the uptake inhibitors sucrose (0.45 M), amiloride (0.5 mM), and ZfFG (200 μM) for 30 min at 37 °C. After removal of the medium, the cells were transfected with LT-siGFP or LT-siLuc (LT/siRNA = 4.8 μM/15 nM) for 2 h. The lipofection medium was then removed and replaced with fresh DMEM containing 10% FBS, and cells were incubated for a further 42 h. The cells were trypsinized, transferred to a 1.5 ml tube and centrifuged at $100 \times g$ for 5 min at 4 °C. The collected cells were resuspended in 0.5 mM EDTA-PBS. A flow cytometer Guava EasyCyte mini system was then used to analyze 5,000 cells in each sample.

2.12. Statistical analysis

Statistical analyses (Unpaired *t*-test with Welch correction) were performed using Graph Pad Stat View software (Abacus Concepts, Inc., CA). For the GFP gene silencing effect data, a non-parametric ANOVA (Kruskal–Wallis test) with post hoc Dunn's multiple comparisons was applied using the same software. The level of significance was set at $p < 0.05$.

3. Results

3.1. The effect of agitation (vortexing) on the gene knockdown efficiency of siRNA

A different method to achieve knockdown efficiency of the GFP gene in an HT-1080GFP cell was investigated during lipoplex formation. For spo-LTsiR, an effective gene knockdown was observed at the higher dose of 120 nM of siRNA (Fig. 1A). Interestingly, for vor-LTsiR, prepared by applying vortex-mixing during lipoplex formation, a significant gene knockdown effect was observed at a dose of only 30 nM of siRNA (Fig. 1B). Alteration of the viability of HT-1080GFP cells after lipoplex treatment was not observed for any siRNA concentration we tested (data not shown). These indicate that application of agitation during lipoplex formation effectively improves the gene knockdown efficiency of siRNA.

3.2. Particle size and zeta-potential of siRNA lipoplexes

The mean particle size and the zeta-potential of vor-LTsiR and spo-LTsiR are summarized in Table 1. The mean sizes for the lipoplexes vor-LTsiR and spo-LTsiR when prepared in 9% sucrose were 0.300 ± 0.045 and 0.270 ± 0.008 μm, respectively. The zeta-potential of lipoplexes prepared in the same medium was 22.4 ± 3.9 and 18.5 ± 0.4 for vor-LTsiR and spo-LTsiR, respectively. In both cases, no significant difference was observed between the preparation procedures. On the other hand, when lipoplexes were formed in OptiMEM, the transfection medium that contains counter ions, there was an abrupt increase in the population of larger

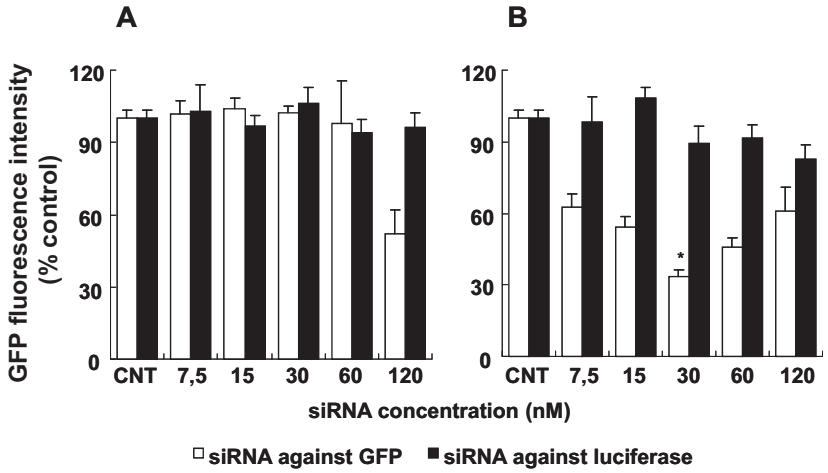

Fig. 1. Effect of siRNA-lipoplex preparation method on gene knockdown efficiency. (A) Lipoplex formed spontaneously (spo-LTsiR) and (B) lipoplex formed under agitation (vortex-mixing) (vor-LTsiR). Amount of cationic liposome was fixed at 9.6 μM while the N/P charge ratios were changed at 15.24, 7.62, 3.81, 1.90 and 0.95 corresponding to siRNA doses. *Legend:* CNT, control (untreated cells). *$P < 0.05$, significant difference in the mean knockdown effect of vor-LTsiR compared to spo-LTsiR at an siRNA dose of 120 nM.

lipoplexes for spo-LTsiR ($p < 0.001$). It appears that lipoplex formation under vortex-mixing allows control over the association process of cationic liposome with siRNA, leading to lipoplexes of less aggregative properties than those formed spontaneously.

3.3. Correlation between the size of siRNA lipoplex and the amount of siRNA attached to it

Despite the wide distribution of particle size, the preparation procedure seems to induce siRNA association to lipoplex populations of a distinct size. In vor-LTsiR (Fig. 2A), the fluorescence intensity of siRNA was detected in a narrow population of small lipoplexes (30–400 nm), indicating that each lipoplex contains a similar amount of associated siRNA. However, in spo-LTsiR (Fig. 2B), the fluorescence of siRNA was detected in relatively larger lipoplexes (150–1500 nm). Interestingly, in spo-LTsiR, the siRNA fluorescence increased in proportion to an increase in lipoplex size, indicating that larger lipoplexes contain larger amounts of siRNA. Agitation during lipoplex formation may somehow alter the association behavior of siRNA and cationic liposomes, resulting in lipoplexes that are homogeneous in terms of size distribution and amount of associated siRNA.

3.4. The effect of agitation (vortexing) on the internalization of lipoplexes

The cellular association of both vor-LTsiR and spo-LTsiR in HT-1080 cells was visualized by confocal microscopy (Fig. 3). Both lipoplexes were internalized, and the green fluorescence related to siRNA was co-localized with the red fluorescence related to the LT. In addition, the red fluorescence of the LT internalized in the cells was proportional between the lipoplexes, whereas the green fluorescence of the siRNA was more intense from the cells treated with vor-LTsiR than from those treated with spo-LTsiR. In addition, the amount of siRNA associated and internalized in the cells was evaluated by flow cytometry (Fig. 4). In spite of the LT and siRNA, the total (surface-bound + internalized) fluorescence intensity in the cells was comparable between vor-LTsiR and spo-LTsiR. In the internalized fraction of lipoplexes, the fluorescence intensity of the LT was also comparable between lipoplexes, while that of the siRNA was 3-fold higher for vor-LTsiR in comparison with spo-LTsiR. These findings indicate that a large amount of siRNA transfected with the spo-LTsiR simply was associated with the cellular surface, and was not being internalized in the cells.

Table 1
Effect of the lipoplex formation method on particle size and zeta potential of the siRNA lipoplex.

	Size		Zeta-potential (mV)
	Opti-MEM (μm)	Sucrose 9% (μm)	
LTrust alone	0.233 ± 0.006	0.239 ± 0.003	21.6 ± 2.0
spo-LTsiR	8.290 ± 1.560	0.270 ± 0.008	18.5 ± 0.4
vor-LTsiR	2.040 ± 0.500*	0.300 ± 0.045	22.4 ± 3.9

The cationic liposome/siRNA ratio was 9.6 μM/30.0 nM and corresponds to an N/P ratio of 7.62. The data represent the mean ± S.D. of three independent experiments performed in triplicate.

* $P < 0.001$, significant difference between the mean particle size of vor-LTsiR and spo-LTsiR.

3.5. The uptake mechanism of siRNA transfected by cell lipoplexes

The internalization pathways of both vor-LTsiR and spo-LTsiR were evaluated as an indication of fluorescence intensity for siRNA in the presence of various inhibitors (Fig. 5). The uptake of vor-LTsiR was inhibited at around 42% by the 0.45 M sucrose (clathrin-mediated endocytosis inhibitor), 22% by the ZfFG (fusion inhibitor), and 22% by the amiloride (macropinocytosis inhibitor). The uptake of spo-LTsiR was inhibited 16% by 0.45 M sucrose, 45% by ZfFG, and 22% by amiloride. The treatment with filipin complex (caveolae-mediated endocytosis inhibitor) did not inhibit the uptake of either lipoplex. These results clearly indicate that vor-LTsiR is mainly internalized via clathrin-mediated endocytosis, whereas spo-LTsiR is mainly internalized through membrane fusion.

3.6. Effect of the uptake pathway on the gene knockdown efficiency of vor-LTsiR

The contribution of each uptake pathway of vor-LTsiR to the gene knockdown effect was evaluated (Fig. 6). The knockdown efficiency of the GFP gene was inhibited 25.7 ± 3.5% by 0.45 M sucrose (clathrin-mediated endocytosis) and 10.4 ± 2.8% by amiloride (macropinocytosis), but was not affected by the filipin complex (caveolae-mediated endocytosis inhibitor). The ZfFG, however, enhanced the knockdown efficiency of vor-LTsiR (membrane fusion). These suggest that clathrin-mediated endocytosis and macropinocytosis is the major contributing pathway of

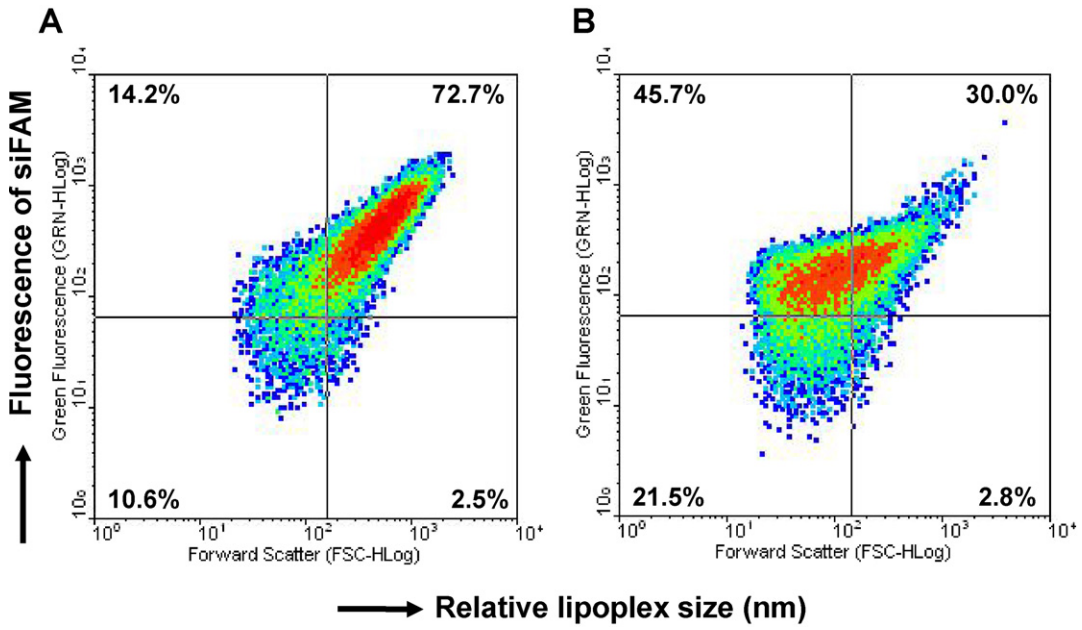

Fig. 2. Effect of lipoplex preparation method on siRNA-lipoplex size distribution. (A) Lipoplex formed spontaneously (spo-LTsiR) and (B) lipoplex formed under agitation (vortex-mixing) (vor-LTsiR). Cationic liposome/siRNA ratio was 9.6 μM/30.0 nM and corresponds to an N/P ratio of 7.62. The size distribution and fluorescence intensity of the lipoplexes were measured by flow cytometry.

vor-LTsiR to achieve effective gene knockdown in HT-1080GFP cells.

4. Discussion

siRNA lipoplex usually forms spontaneously, and allows for little control over the process of cationic liposome interaction, which leads to both a wide size distribution and excessive sizes (Lasic and Templeton, 1996; Kawakami et al., 2002; Faneca et al., 2004). In the present study, the initial focus was on investigating the effect of agitation during siRNA lipoplex formation (vor-LTsiR) on the *in vitro* knockdown efficiency of GFP gene stably expressed in HT-1080 cells. This system can partially simulate the downregulation of an endogenous gene. The present study demonstrated that vor-LTsiR could efficiently knockdown the GFP gene using a dose of siRNA against GFP that was 4-fold lower than the dose of spo-LTsiR (Fig. 1).

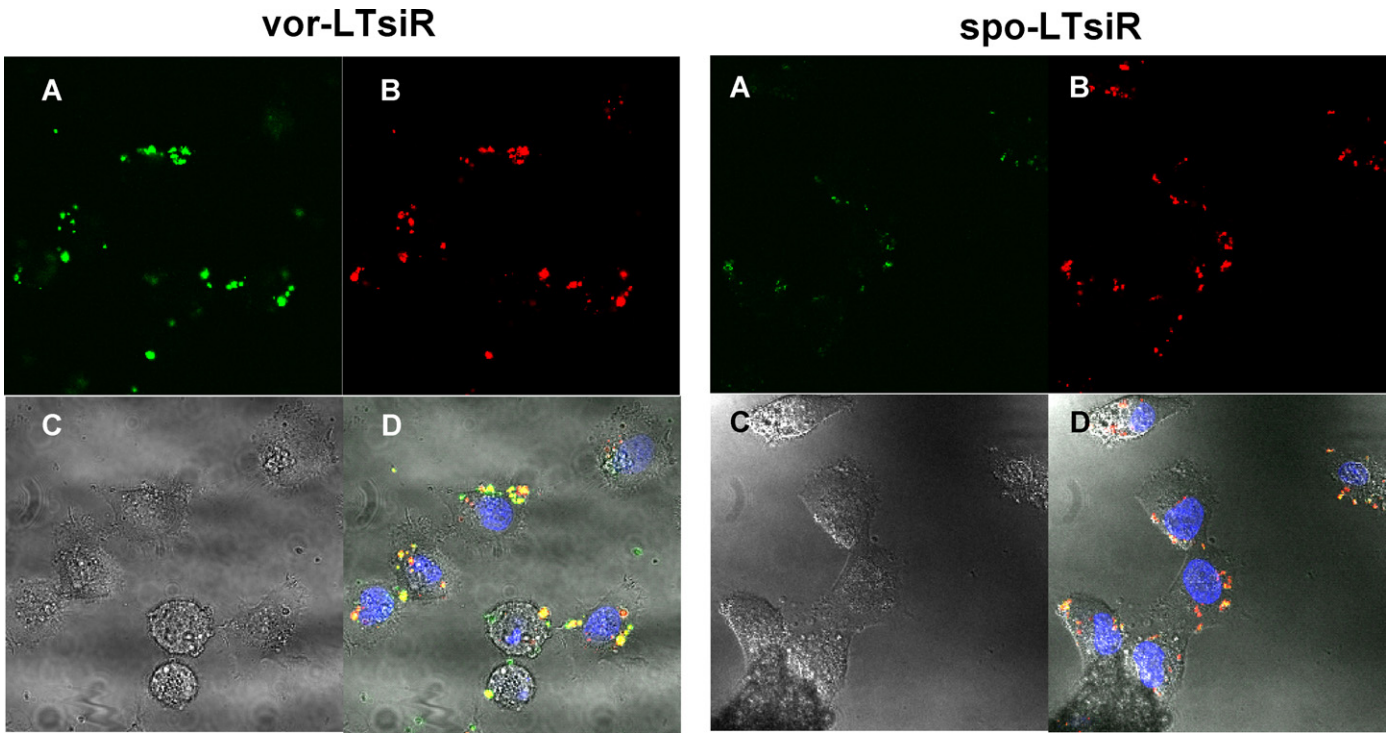

Fig. 3. Visualization of the intracellular vor-LTsiR (left) and spo-LTsiR (right) in the cell. Cationic liposome/siRNA amount was 9.6 μM/30.0 nM and corresponds to a charge ratio of 7.62. (A) siRNA (green), (B) LT (red), (C) phase-contrast image, and (D) merged image of A, B and C. Blue corresponds to the nucleus. (Magnification: ×630.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

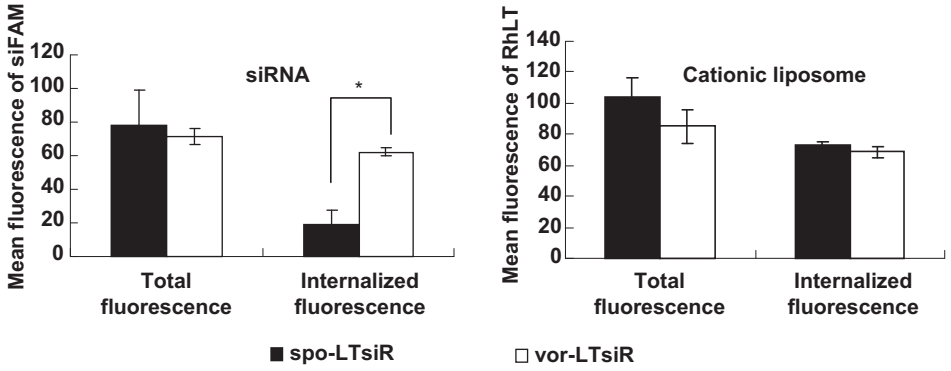

Fig. 4. Amount of siRNA associated with lipoplexes internalized by the cells. Total fluorescence represents the siRNA which was surface-bound and internalized in the cells. Cationic lipid/siRNA amount was 9.6 μM/30.0 nM and corresponds to an N/P ratio of 7.62. Data represent the mean ± S.D. of three independent experiments performed in triplicate. $^{*}P < 0.05$ was considered significant.

The results of the present study clearly showed that the preparation procedure affects not only the physicochemical property of siRNA lipoplex but also their gene knockdown efficiency.

The interaction of particles with cells is significantly influenced by particle size (Rejman et al., 2004; Khalil et al., 2006b; Gratton et al., 2008) and surface charge (Lima et al., 2001). The surface charges of both lipoplexes were positive at the siRNA concentration (N/P ratio of 7.62 (9.6 μM/30 nM; L^{+}/siRNA^{-})) where the most distinct difference in the RNAi effect was observed. The size of both lipoplexes did not vary significantly when prepared in 9% sucrose, a medium free of counter ions. However, when prepared in Opti-MEM, the medium most routinely used for lipofection, the lipoplexes displayed a heterogeneous size distribution (Table 1) due to the effect of counter ions that stimulate the aggregative properties of lipoplexes (Spagnou et al., 2004). The mean particle size of spo-LTsiR was at least 3-fold larger than that of vor-LTsiR. In addition, the lipoplexes prepared by different procedures displayed a very heterogeneous association of siRNA to distinct lipoplex size population. In vor-LTsiR, approximately 80% of the siRNA molecules attached to the lipoplex smaller than 200 nm, whereas in spo-LTsiR,

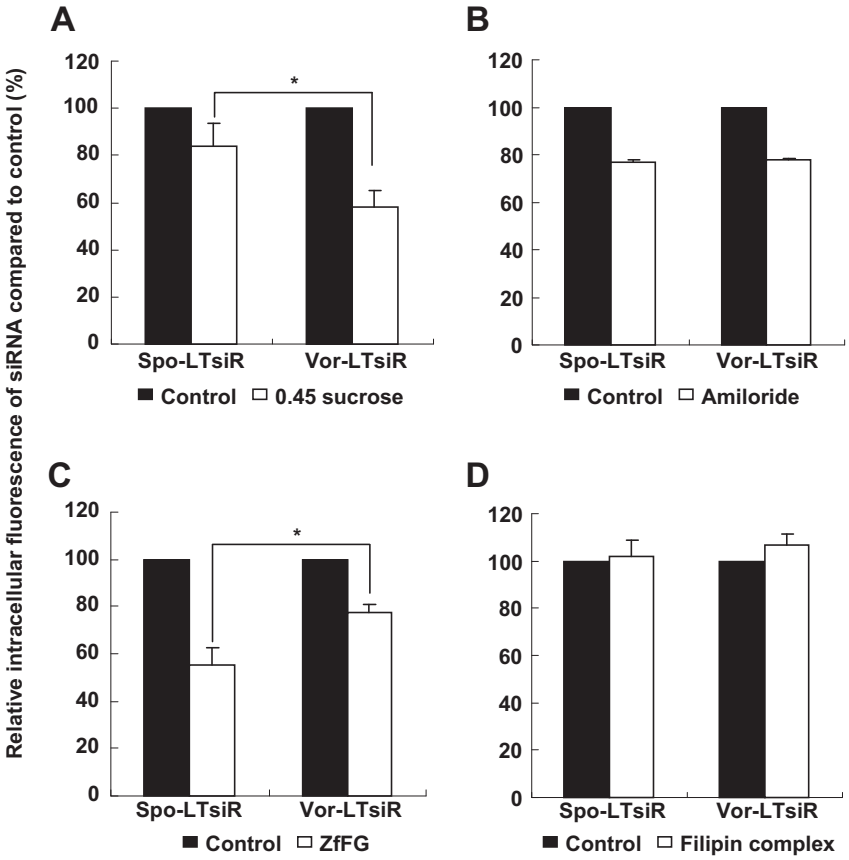

Fig. 5. Uptake mechanism of vor-LTsiR (left) and spo-LTsiR (right) by the cells. siRNA lipoplexes were incubated with the cells, which were treated with various uptake inhibitors. The fluorescence intensity relating to siRNA in the cells was determined by flow cytometry. Data represent the mean ± S.D. of three independent experiments performed in triplicate. $^{*}P < 0.05$ was considered significant. Inhibition of (A) clathrin-mediated endocytosis, (B) macropinocytosis, (C) membrane fusion, and (D) caveolae-mediated endocytosis.

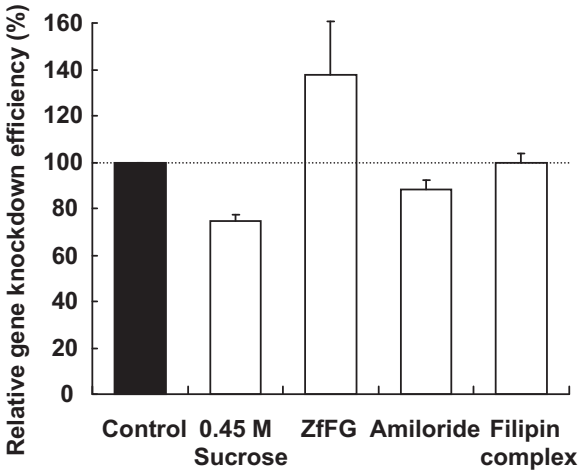

Fig. 6. The major uptake route contributing to the gene knockdown effect of vor-LTsiR. Data represent the mean ± S.D. of three independent experiments performed in triplicate. Cells were transfected with vor-LTsiR in the absence (control) or in the presence of sucrose, ZfFG, amiloride or filipin complex. GFP fluorescence was measured at 22 h after transfection by flow cytometry.

only around 36% of the siRNA attached to a relative lipoplex size (Fig. 2). Given the same lipid concentration, a smaller lipoplex size would equate to a larger total surface area. A larger total surface area for lipoplex can promote extensive interactions with target cells. Hence, the higher gene knockdown efficiency induced by vor-LTsiR at a relatively lower siRNA dose (Fig. 1) might be due to the efficient delivery of siRNA into the cells as a result of the enhanced interaction of the lipoplex with the cells. This assumption is strongly supported by the results of qualitative and quantitative analysis, as described in Figs. 3 and 4.

From the mechanism of RNAi, the intracellular presence of siRNA molecules complementary to the target mRNA is of crucial importance for inducing gene knockdown (Behlke, 2006; Li et al., 2006). Therefore, it is essential that the siRNA lipoplex is taken up intact by the cells, and that the lipoplex efficiently releases the siRNA molecule into the cytoplasm (Khalil et al., 2006a). As shown in Fig. 3, in the vor-LTsiR, LT (red) was extensively co-localized with siRNA (green), indicating that the lipoplex associated with siRNA was efficiently internalized in the cells. In the spo-LTsiR, however, the LT (red) was less co-localized with siRNA (green), indicating either that the lipoplex were associated with fewer siRNA or that empty LT were internalized. In addition, the result of semi-quantitative evaluation (Fig. 4) indicated that vor-LTsiR improved 3-fold the amount of siRNA internalized in the cells compared with spo-LTsiR, although there was no significant difference in the total amount of siRNA and LT that interacted with the cells between both formulations. Accordingly, the superior gene knockdown efficacy of vor-LTsiR observed in Fig. 1 may be explained by the following scenario: the smaller sized (≤200 nm) vor-LTsiR contained a large amount of siRNA (Fig. 2A) and were extensively taken up by the targeted cells (Fig. 4 left). However, the smaller sized (≤200 nm) spo-LTsiR contained fewer siRNA (Fig. 2B) that were taken up by the cells and the remaining spo-LTsiR (≥200 nm) simply associated with the cell surface, which limited or delayed their internalization. Several studies have demonstrated that the uptake of viral particles (Matlin et al., 1982), polyplexes (Godbey et al., 1999), and beads (Rejman et al., 2004) by cells is delayed in a size-dependent manner.

Particle size determines the pathway of cellular entry, and therefore, also determines the kinetics of internalization and the intracellular trafficking of the particles (Rejman et al., 2004; Gratton et al., 2008). There is convincing evidence that endocytosis represents the major pathway of entry into cells for particles <1 μm in size (Rejman et al., 2004; Spagnou et al., 2004; Khalil et al., 2006a; Hoekstra et al., 2007; Gratton et al., 2008). These pathways include phagocytosis, clathrin-dependent endocytosis and clathrin-independent endocytosis, the latter including macropinocytosis and internalization via caveolae (Rejman et al., 2004; Khalil et al., 2006a; Sahay et al., 2010). In the present study, three pathways were found in the uptake of both lipoplexes by cells. As shown in Fig. 5, the internalization of vor-LTsiR was mainly via clathrin-mediated endocytosis while macropinocytosis and cell fusion were secondary routes. On the contrary, the internalization of spo-LTsiR was mainly through membrane fusion while macropinocytosis and clathrin-mediated endocytosis were secondary routes. At present, the majority of reports suggest that positively charged nanomaterials predominantly internalize through clathrin-mediated endocytosis with some fraction utilizing macropinocytosis (Sahay et al., 2010). Our current results are consistent with the previous reports.

Clathrin-mediated endocytosis and macropinocytosis have been suggested as the major routes of cellular entry for lipoplexes containing nucleic acids such as pDNA and siRNA (Thomsen et al., 2002; Zuhorn et al., 2002; Zhang et al., 2003; Nakase et al., 2004; Spagnou et al., 2004; Wadia et al., 2004; Kaplan et al., 2005; Rejman et al., 2005; Khalil et al., 2006a; Sahay et al., 2010). The present study also clearly showed that both routes are major inducements of the vor-LTsiR-mediated gene silencing effect (Fig. 6). Under normal conditions, the formation of clathrin-coated pits is very rapid (within 28 s), with the entire population of coated pits being turned over approximately every 6 min, making this structure highly mobile (Kawakami et al., 2002). Particles of 50 and 100 nm in size are internalized in a relatively rapid process by this pathway (Rejman et al., 2004). Hence, it is assumed that the lipoplex of vor-LTsiR (≤200 nm), which contains 80% siRNA, are quickly and extensively internalized (Figs. 3 and 4), resulting in an efficient gene-knockdown effect (Fig. 1). Macropinocytosis is known as an efficient route for the nonselective endocytosis of solute macromolecules and provides some advantageous aspects on siRNA-based gene-knockdown such as the avoidance of lysosomal degradation and an easing of the escape from macropinosomes (Khalil et al., 2006a). However, in the present study, it appears that macropinocytosis only partly contributes to vor-LTsiR-mediated gene silencing.

Cationic liposome, including lipoplex, is known to interact and fuse with cell membranes (Leventis and Silvius, 1990). Fusion was one of the major cellular entry routes for spo-LTsiR, which agrees with previous reports (Fig. 5). Size is known to be one of the most important factors affecting the dynamics of vesicle sedimentation onto the cells (Faneca et al., 2004). In the present study, lipoplexes formed spontaneously in Opti-MEM, the transfection medium, and presented a wider size distribution due to the relatively uncontrolled process of association between siRNA and LT. Accordingly, in spo-LTsiR, approximately 64% of siRNA preferentially attached to lipoplexes larger than 200 nm in size (Fig. 2). Assuming that spo-LTsiR (≥200 nm) is taken up only to a limited extent by macropinocytosis, the remaining lipoplexes bound to the cell surface are assumed to be held at the cell surface for a relatively longer period. Such a long period on the cell surface might lead to the progressive dilution of the lipoplexes as the cell divide (Leung and Whittaker, 2005). In addition, extensive leakage of contents from phosphatidylethanolamine-based liposomes is known to occur during fusion (Brown and Silvius, 1989). This may expose the siRNA associated with, or released from, the spo-LTsiR to potential enzymatic or physical degradation before it can reach the cytoplasm (Spagnou et al., 2004). Consequently, spo-LTsiR might have shown a lower gene knockdown effect in the present study (Fig. 1).

5. Conclusion

The present study showed that the preparation procedure for siRNA-lipoplex remarkably affects the *in vitro* gene-knockdown efficiency of siRNA. Smaller (≤200 nm) and relatively homogeneous lipoplexes containing a large amount of siRNA (80% of dose), produced by agitation (vortex-mixing) during the preparation, allowed for the efficient cellular interaction of lipoplex and a shift in their entry pathway from fusion to clathrin-mediated endocytosis, resulting in an increased amount of siRNA internalized in cells, and thereby, an enhanced gene-knockdown efficacy. A proper siRNA-lipoplex preparation procedure was strongly related to both the efficiency of cellular uptake and the gene-knockdown efficiency of siRNA. The results of the present study may have implications for designing a more efficient and successful siRNA delivery system.

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