Ago2 gene knockdown suppresses cellular proliferation in human fibrosarcoma (HT-1080) and HUVEC [28]. We here confirm that Ago2 knockdown induces inhibition of proliferation of LLCC (Supplementary Figure 2). This might be due to transient inhibition of miRNA-targeted mRNAs resulting in the induction of apoptosis and cell cycle arrest in G0/G1 phase. In the present study, we found the potent antitumor activity of the combination of S-1 dosing and PEG-coated siAgo2-lipoplexes to be synergistic (Fig. 5b). This synergistic antitumor activity might be due to the following tentative mechanism: pretreatment with metronomic S-1 dosing results in a preferential intratumoral accumulation, along with cytotoxic action on tumor cells and endothelial cells, and permits efficient delivery of siAgo2-lipoplexes into the tumor tissue and thereby potentiates the apoptotic effects of the accumulated siAgo2-lipoplexes as a result of significant down-regulation of Ago2 protein in the tumor tissue (Fig. 5d). Interestingly, no body weight changes were observed with the combination therapy of S-1 and PEG-coated siAgo2-lipoplexes (Fig. 5c). This might be attributed to the targeted delivery of siAgo2-lipoplexes to tumor tissues which allows enhanced accumulation of siAgo2-lipolexes in tumor tissues (Table 1).

One of the major hurdles for intratumoral drug delivery is the heterogeneity of tumor tissue. The abnormalities in vessel and microenvironment of solid tumors will often result in insufficient drug delivery and therapeutic efficiency [36]. To improve drug delivery to the disordered tumor microenvironment and increase therapeutic efficacy, various approaches have been applied. For example, it has been reported that the combination of physical power (radiation [37], ultrasound [38], mild hyperthermia [39], and chemical drug (TβR-1 [40], TNF-α [41])) improved the intratumoral distribution of nanoparticles. In the current study, we utilized a novel approach for improving the delivery of siRNA-lipoplexes to tumor tissue. This approach using a clinically approved anticancer drug, S-1, is considered a breakthrough in gene delivery strategies and is hopefully translated into clinical settings.

Conclusion

In this study, we showed that the metronomic S-1 dosing improved the intratumoral accumulation and distribution of PEG-coated siAgo2-lipoplexes and, as a result, significantly improved the overall therapeutic efficacy of the combination of these two treatments. The improved delivery of PEG-coated siRNA-lipoplexes was presumably mediated by alterations in tumor microenvironment brought about by the S-1 treatment. The current study serves as a positive proof-of-concept demonstration for the enhanced systemic delivery of PEG-coated siRNA-lipoplexes upon combination with metronomic S-1 chemotherapy we assumed recently [20]. Moreover, combination of a chemotherapeutic agent and siRNA-based therapy, two therapeutic strategies with different mechanisms of action, constitutes a promising approach for the development of novel strategies in cancer therapy.

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Agitation during lipoplex formation harmonizes the interaction of siRNA to cationic liposomes

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ABSTRACT

We recently demonstrated that agitation during lipoplex formation (vorLTsiR) improves the gene knockdown effect of siRNA because the resultant decrease in lipoplex size leads to an enhanced uptake by cells. In furthering this line of research, the present study was focused on the interaction of siRNA to cationic liposomes during lipoplex preparation. A fluorescence resonance energy transfer (FRET) study indicated that the application of agitation in the presence of siRNA effectively reorganized positively charged lipids (DC-6-14 and DOPE) in an order that effectively promoted further electrostatic interaction between the negatively charged phosphate backbone of siRNA and the positively charged lipids in the cationic liposome membrane. A circular dichroism (CD) study indicated that the agitation did not bring about a change in the A-form helix of siRNA, therefore the interactions between the lateral anionic groups of siRNA – responsible for the characteristic bands of the A-form helix – and cationic liposomes were effectively promoted. Factorial design coupled with response surface methodology was used to statistically analyze the influence of vortex speed and time and siRNA dose on the *in vitro* gene knockdown effects of siRNA-lipoplex that were spontaneously formulated (spoLTsiR) along with that formulated under agitation (vorLTsiR). The analysis indicated that vortex speed plays the most important role in enhancing the gene knockdown effect of siRNA among the three variables, although all three are important. It was concluded that the high energy transmitted by applying agitation during lipoplex formation harmonized the interaction of siRNA to positively charged lipids (DC-6-14 and DOPE) in cationic liposomes, resulting in a superior gene knockdown efficacy of vorLTsiR compared to spoLTsiR. Our study suggests that the preparation procedure is one of the critical factors in producing the enhanced gene knockdown effect of siRNA.

1. Introduction

RNA interference (RNAi), the naturally occurring biological process of gene silencing in mammalian cells, has emerged as a promising therapeutic strategy for treating malignant, infectious and autoimmune diseases (Li et al., 2006). Cationic liposomes are non-viral vectors that can complex with negatively charged siRNA duplexes, allowing these molecules to overcome the electrostatic repulsion of the cell membrane and to be taken up by the targeted cells (Lasic and Templeton, 1996; Lima et al., 2001).

It is well known that the preparation procedure strongly determines the final physicochemical features of the pDNA–cationic liposome complex (lipoplex) that modulate its biological activity (Lasic and Templeton, 1996; Lima et al., 2001). Lipoplexes are generally formed spontaneously through the electrostatic interaction between the positively charged lipids of liposomes and the negatively charged phosphate backbones of nucleic acids (Lasic and Templeton, 1996). We recently demonstrated that the application of vortex-mixing (agitation) during lipoplex formation considerably improves the gene knockdown effect of siRNA. The efficacy of the lipoplex formulated by agitation (vorLTsiR) was superior to the lipoplex formed spontaneously (spoLTsiR) in the same siRNA dose (Barichello et al., 2011). Vortex mixers are often used in bioscience laboratories to mix reagents in solution or suspension. They quickly create a vigorous vortex, a spiral flow, inside the liquid mixture. However, when the lipoplex preparation procedure is varied from

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static (spontaneous formation) to dynamic (under vortex-mixing) it is unknown what the associated behavior of siRNA to a cationic liposome will be, nor do we know the implications for the gene knockdown effect of siRNA.

In an expansion of our recent study, in this study, we investigated the effect of the lipoplex preparation procedure on the behavior of siRNA as it associates with a cationic liposome by using fluorescence resonance energy transfer (FRET) and circular dichroism (CD). Then, we determined the effect of the three variables (vortex speed and time and siRNA dose) on the gene knockdown effect of siRNA lipoplex using an *in vitro* luciferase gene knockdown assay system with HeLa cells. To determine which was the most important variable among the three, a statistical analysis was undertaken with a 3^3 full factorial design and response surface methodology, which is a widely used statistical tool for the systematic and effective evaluation of influences from differences among variables (Dillen et al., 2004; Gonzalez-Rodriguez et al., 2007). Herein, we show that the preparation procedure has far-reaching implications for the behavior of siRNA as it associates with a cationic liposome, and, therefore, for the final physicochemical features of a lipoplex and for the gene knockdown effect of siRNA in target cells.

2. Materials 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 a molar ratio of 1.00/0.75/0.75, was purchased from Hokkaido System Science (Hokkaido, Japan). The 1% Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rho-DOPE)-containing LipoTrust™-SR (Rho-LT) was a generous gift from Daiichi-Sankyo Pharmaceutical (Tokyo, Japan). All other chemicals were of reagent grade and used as received.

2.2. siRNA preparation

A siRNA for firefly luciferase (siLuc) (sense sequence, 5′-CUUACGCUGAGUACUUCGATT-3′; antisense sequence 5′-UCGAAGUACUCAGCGUAAGTT-3′) and an unrelated siRNA (sense sequence, 5′-AGCUUCAUAAGGCGCAUGCTT-3′; antisense sequence 5′-GCAUGCGCCUUAUGAAGCUTT-3′) (Elbashir et al., 2001) were chemically synthesized and purified with HPLC by Hokkaido System Science (Hokkaido, Japan). The siLuc labeled at the 5′-end of the sense strand with carboxyfluorescein (FAM) (FAM-siLuc) was used as a fluorescent siRNA probe. For siRNA preparation, the complementary antisense and sense strands were mixed in TE buffer (10 μM Tris–HCl, 1 μM EDTA (pH 8.0), DNase and RNase free grade, Nippon Gene, Tokyo, Japan) in a 1:1 molar ratio followed by heating at 90 °C for 1 min. The reaction was then allowed to cool to room temperature. The quality of siRNA was checked by 15% PAGE. The final concentration of the duplexes was 50 μM in a TE buffer.

2.3. Preparation of siRNA lipoplex

Various aliquots of the siRNA solution (50 μM) were diluted to a final volume of 100 μl with fresh Opti-MEM (Invitrogen, CA, USA) or 9% sucrose. A 25 μl aliquot of LT suspension (2.4 μM) was also diluted to a final volume of 100 μl with fresh Opti-MEM or 9% sucrose. The diluted siRNA solutions were then mixed with the diluted LT suspension. The N/P ratios were set at 7.62 (9.6 μM/30.0 nM; cationic lipid$^+$/siRNA$^-$) for FRET and CD experiments, while for the 3^3 full-factorial design, the N/P ratios were set at 0.95, 1.90 and 3.81 (9.6 μM/1.88, 3.75 and 7.50 nM; cationic lipid$^+$/siRNA$^-$; respectively) to evaluate the effect of LTsiRNA lipoplex (LTsiR) preparation. The LTsiR was allowed to form in 2 ways: the lipoplex was formed spontaneously (spoLTsiR) by allowing samples to stand for 10 min, and it also was formed under application of high vortex-mixing (2500 rpm) (vorLTsiR) (Vortex-Genie 2, Scientific Industries, NY, USA) for 10 min (Barichello et al., 2011).

2.4. Fluorescence resonance energy transfer (FRET)

FRET was determined by monitoring the decrease in fluorescence of FAM-siLuc (donor) in the presence of Rho-DOPE in LT (acceptor) using a fluorescence spectrophotometer Jasco FP6600 (JASCO, Tokyo, Japan). Emission spectra were recorded between 500 and 650 nm with excitation at 505 nm at 25 °C immediately after the complexes were formed. Data are reported as the efficiency of FRET (E) (which is calculated as follows: $E = [1 - (F_{DA}/F_D)] \times 100$, where F_{DA} and F_D are the fluorescence intensity of the FAM-siLuc (excitation at 505 nm and emission at 525 nm) in the presence of Rho-LT and LT, respectively (Zelphati and Szoka, 1996). The Rho fluorescence value (I) was computed as $I = [F'_{DA}/(F'_D + F'_A)] \times 100$, where F' is the fluorescence intensity of the Rho-DOPE in LT (excitation at 505 nm and emission at 588 nm) in the presence of Rho-LT and FAM-siLuc (F'_{DA}), LT and FAM-siLuc (F'_D), and Rho-LT and siLuc (F'_A) (Zelphati and Szoka, 1996).

2.5. Circular dichroism (CD)

CD spectra were collected at 25 °C using a spectropolarimeter Jasco J600 (Tokyo, Japan). Spectra were measured from 200 to 300 nm with a resolution of 1 nm using a 2 mm path length cuvette and were expressed as the average of 8 scans at a 20 nm/min scan rate. The spectrum of siRNA (30 nM) and LT (9.6 μM) alone, and the spectra of their complex were taken. The complexes formed in 9% sucrose and the spectrum of 9% sucrose were recorded as a control, which was subtracted. Data analysis was performed using Excel 2003 (Microsoft, Redmond, WA, USA).

2.6. The in vitro luciferase gene knockdown assay

The human cervical cancer (HeLa) cell line was obtained from Dr. Y. Shinohara (Division of Gene Expression, Institute of Genome Research, The University of Tokushima, Japan). HeLa cells were cultured in DMEM (Sigma, MO, USA) supplemented with 10% (v/v) heat-inactivated FBS, 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. The cells were maintained in exponential growth.

Two luciferase plasmids (*Photinus* (firefly), luciferase pGL-3 and *Renilla* (sea pansy) luciferase, pRL-TK) were used as reporter and control genes, respectively, by a reported transfection protocol with modifications (Elbashir et al., 2001). Typically, the complex of pDNA-LT was prepared at a ratio of 1 μg of pDNA to 5 μmol of cationic lipid (the molar charge ratio (N/P) was 1.33) in Opti-MEM medium as previously described (Li et al., 2004). The cells were seeded in 24-well plates at a density of 5.0×10^4 cells/well for 24 h prior to transfection. The growth medium was removed and replaced with 146.7 μl of Opti-MEM, 20 μl of FBS and 33.3 μl of pDNA-LT to transfect. After incubation for 1 h, the transfection medium was removed and replaced with 410 μl of Opti-MEM, 50 μl of FBS and 40 μl of LTsiR. The plates were incubated for another 4 h. The lipofection medium was then removed and replaced with 500 μl of fresh DMEM containing 10% FBS, and the plates were

Table 1
The experimental design.

Trial	Variable levels			F-luc/R-luc (% control)
	X_1	X_2	X_3	
1	3.75	0	2	92.0 ± 13.2
2	3.75	0	5	89.1 ± 4.4
3	3.75	0	10	89.1 ± 14.9
4	3.75	800	2	84.2 ± 6.6
5	3.75	800	5	78.3 ± 4.1
6	3.75	800	10	73.4 ± 5.1
7	3.75	2500	2	75.3 ± 12.0
8	3.75	2500	5	60.5 ± 1.9
9	3.75	2500	10	38.2 ± 1.8
10	7.50	0	2	82.9 ± 4.9
11	7.50	0	5	76.2 ± 4.3
12	7.50	0	10	70.9 ± 1.9
13	7.50	800	2	81.0 ± 9.9
14	7.50	800	5	63.9 ± 4.7
15	7.50	800	10	59.9 ± 10.2
16	7.50	2500	2	72.1 ± 12.0
17	7.50	2500	5	48.9 ± 5.1
18	7.50	2500	10	28.9 ± 1.8
19	15.00	0	2	64.3 ± 2.7
20	15.00	0	5	64.5 ± 0.9
21	15.00	0	10	58.9 ± 3.9
22	15.00	800	2	76.3 ± 10.0
23	15.00	800	5	52.1 ± 8.7
24	15.00	800	10	42.7 ± 6.9
25	15.00	2500	2	58.2 ± 3.2
26	15.00	2500	5	48.3 ± 5.7
27	15.00	2500	10	31.9 ± 2.3

X_1 = dose of siRNA (nM); X_2 = vortex speed (rpm); X_3 = vortex time (min).

incubated for another 20 h. Cells were then harvested at 24 h post-lipofection of siRNA using a passive lysis buffer (100 μl/well), according to instructions, with minor modifications, that was provided with the Dual-Luciferase Reporter Assay System (Promega, WI, USA). The luciferase activities of the samples were measured using a BLR-301 luminometer (Aloka, Tokyo, Japan), with a delay time of 2 s and an integrate time of 10 s. The inhibitory effect on luciferase activities by siLuc was expressed as the percentage of the normalized ratios between the luciferase activities of the reporter and control genes (F-Luc/R-Luc) (Elbashir et al., 2001; Xu et al., 2003). All experiments were performed in quadruplicate and were repeated at least twice.

2.7. Statistical analysis with a 3^3 full-factorial design

Elements of the experimental design of *in vitro* luciferase gene knockdown, siRNA dose (nM), vortex speed (rpm), and vortex time (min), are summarized in Table 1. Calculations of the effects and their statistical interpretation, as well as the response surface, were performed using the software STATISTICA 6.1 (StatSoft, OK, USA). To determine the relationship between the 3 controlled variables and the luciferase gene knockdown effect, the experimental data was adjusted to a second-order model shown by the following Eq. (1):

$$Y = b_0 + \Sigma b_i X_i + \Sigma b_{ij} X_i X_j + \Sigma b_{ii} X_i^2 \quad (1)$$

For the 3 variables, the following equation model (Eq. (2)) applies:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}(X_1)^2 + b_{22}(X_2)^2 + b_{33}(X_3)^2 \quad (2)$$

where b_0 is the arithmetic mean response of 27 runs and $b_1 \ldots b_{33}$ are the estimated coefficients for the 3 factors (X_1, dose of siRNA; X_2, vortex speed and X_3, vortex time).

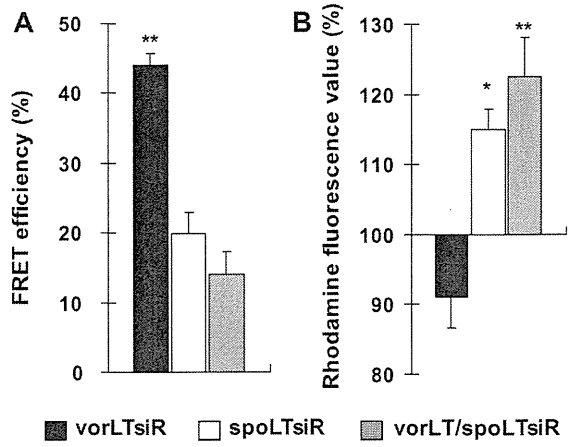

Fig. 1. Effect of the lipoplex preparation procedure on FRET efficiency (*E*) and rhodoamine fluorescence values (*I*). *Legend*: vorLT/spoLTsiR, LT alone was vortexed for 10 min followed by spontaneous lipoplex formation after addition of siRNA. Cationic lipid$^+$/siRNA$^-$ ratio was 9.6 μM/30 nM, which corresponds to a charge ratio of 7.62. Data represent the mean ± S.D. of 5 independent experiments. $^{**}P < 0.01$; significant difference in the mean *E* of vorLTsiR compared with that of spoLTsiR and vorLT/spoLTsiR. $^{**}P < 0.01$ and $^{*}P < 0.05$; significant difference in the mean *I* of vorLTsiR compared with that of spoLTsiR and vorLT/spoLTsiR, respectively.

The regression analysis was carried out using Eq. (3):

$$\begin{aligned}\text{RNAi effect(F-luc/R-luc)} = {} & 67.08 - 10.16[X_1] - 12.54[X_2] \\ & -10.69[X_3] + 3.91[X_1][X_2] \\ & -1.20[X_1][X_3] - 7.17[X_2][X_3] \\ & +0.42[X_1]^2 - 2.04[X_2]^2 + 0.91[X_3]^2 \quad (3)\end{aligned}$$

where X_1, X_2 and X_3 represent the main effects, dose of siRNA, vortex speed and vortex time, respectively.

Calculations were performed using the least-square method and validation was performed through ANOVA, multiple-correlation coefficient and estimation of the lack of fit using the criteria proposed by Wherle et al. (1995). Response surface methodology was used to understand as fully as possible the effects and levels of the valuables, and to predict the response inside the experimental domain.

2.8. Statistical analysis

Statistical analyses (one-way ANOVA and unpaired *t*-test) were performed using Graph Pad Stat View software (Abacus Concepts Inc., CA, USA). This evaluation was carried out at the N/P ratio of 7.62 (9.6 μM/30 nM; cationic lipid (L)$^+$/siRNA$^-$) wherein the most distinct difference in the RNAi effect between the lipoplex preparation procedures was observed (Barichello et al., 2011).

3. Results

3.1. FRET study to evaluate the behavior of the association of siRNA to a cationic liposome

FRET was employed to evaluate the behavior of the association of siRNA to a cationic liposome during the preparation process. As shown in Fig. 1A, the FRET between FAM-siRNA and Rho-labeled cationic liposome occurred regardless of the preparation procedure (Fig. 1A). The FRET efficiency of vorLTsiR was 2.2-fold higher than that of spoLTsiR, indicating that application of agitation further promoted the effective interaction of FAM-labeled siRNA with Rho-labeled cationic liposome. Interestingly, the addition of siRNA following application of agitation to cationic liposome for 10 min

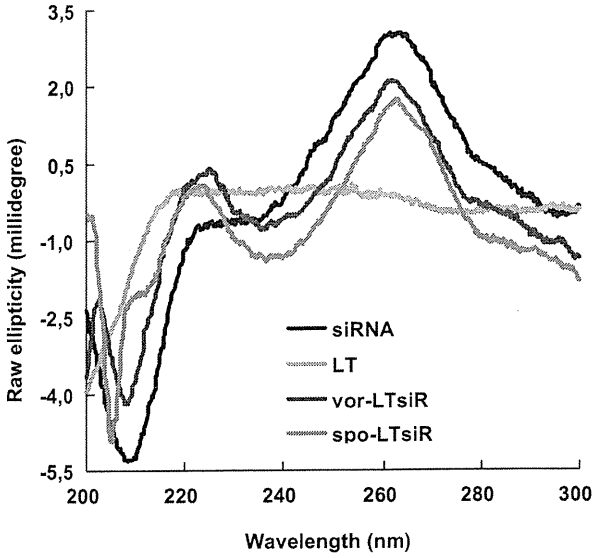

Fig. 2. Effect of preparation procedure on the CD spectra of siRNA in the lipoplex. Cationic lipid$^+$/siRNA$^-$ ratio was 9.6 μM/30 nM, which corresponds to a charge ratio of 7.62. The data are typical of 3 independent experiments.

did not increase the FRET efficiency. This demonstrates the importance of the presence of siRNA in promoting an efficient membrane lipid mixture and consequent efficient interaction between siRNA and the surface of a cationic liposome.

It is generally believed that nucleic acids associate with cationic lipids in a cationic liposome. In the present study, it was assumed that DC-6-14 is the major positively charged lipid that associates with siRNA. However, the Rho fluorescence value was decreased for vorLTsiR, while that of spoLTsiR was increased (Fig. 1B). Because Rho is covalently bound to DOPE, the Rho fluorescence value might reflect free DOPE in the membrane of cationic liposome. Thus, not only DC-6-14 but also DOPE in the membrane participated with siRNA interaction in vorLTsiR (Fig. 1A). The addition of siRNA following application of agitation to cationic liposome for 10 min slightly increased the Rho fluorescence value (Fig. 1B). This indicates the importance of the present of siRNA during agitation to promote the homogeneous distribution of DOPE and DC-6-14 in the membrane.

3.2. CD study for evaluation of the behavior of siRNA association to a cationic liposome

The spectrum of siRNA alone presented a large positive band at 260 nm and a large negative band at 208 nm (Fig. 2), which readily characterized the A-form helix of a double-stranded RNA. The CD spectrum of a cationic liposome (LT) alone showed no band at these wavelengths. In the spectrum of vorLTsiR, a small reduction in intensity of both bands at 260 and 208 nm was observed (Fig. 2), suggesting that the characteristic A-form helix that is characteristic of siRNA in vorLTsiR was maintained. In the spectrum of spoLTsiR, however, the reduction in intensity of the band at 260 nm was more prominent with a drastic change in the large negative band at 208 nm to an acute signal at approximately 205 nm (Fig. 2). These results indicate that application of agitation caused less perturbation to the A-form helix of siRNA during lipoplex formation.

Table 2
Statistical evaluation and validation tests applied to the mathematical model.

Factor	SS	DF	MS	F
X_1 (lineal)	7433.87	1	7433.87	144.09*
X_1 (quadratic)	4.28	1	4.28	0.083
X_2 (lineal)	11,327.63	1	11,327.63	219.57*
X_2 (quadratic)	399.89	1	399.89	7.75*
X_3 (lineal)	8236.86	1	8236.86	159.66*
X_3 (quadratic)	19.86	1	19.86	0.39
X_1 *vs.* X_2 (interaction)	733.99	1	733.99	14.23*
X_1 *vs.* X_3 (interaction)	69.84	1	69.84	1.35
X_2 *vs.* X_3 (interaction)	2469.64	1	2469.64	47.87*
Lack of fit	1220.16	17	71.77	1.39
Pure error	4178.81	81	51.59	
Total	36,094.83	107		
R^2	0.85042			

X_1 = dose of siRNA (nM); X_2 = vortex speed (rpm); X_3 = vortex time (min); SS, sum of squares; DF, degrees of freedom; MS, mean square; F = test F (Fisher).
* Significant for $\alpha = 0.05$.

3.3. In vitro siRNA gene knockdown

Twenty-seven vorLTsiR were prepared by changing siRNA doses and vortex conditions (speed and time). *In vitro* luciferase gene knockdown effects by these vorLTsiRs were determined as described above (Table 1). The gene knockdown effect of prepared vorLTsiRs was strongly dependent on all 3 variables: the efficacy correlated with increases in siRNA, vortex speed and vortex time.

3.4. Statistical analysis to identify the most important variable in vorLTsiR-mediated gene knockdown

The *in vitro* gene knockdown data (Table 1) were applied to the generation of a second-order model for the dependent variable (Eqs. (1) and (2)). The regression analysis relating to *in vitro* gene knockdown data was carried out with Eq. (3) and a summary of the analysis is shown in Table 2. The gene silencing effect estimated from the model agreed well with the *in vitro* gene knockdown data (coefficient of determination [R^2] = 0.8504)

Table 3
Statistics for the regression coefficients and estimated effects.

Factor	Coefficient	SE	t-Test$_{(coeffic.)}$	Effect	t-Test$_{(effect)}$
Mean	67.08	1.829	36.69*	–	–
X_1 (lineal)	−10.16	0.846	−12.01*	−20.32	−12.01*
X_1 (quadratic)	0.42	1.466	0.29	−0.42	−0.29
X_2 (lineal)	−12.54	0.846	−14.82*	−25.09	−14.82*
X_2 (quadratic)	−2.04	0.733	−2.78*	4.08	2.78*
X_3 (lineal)	−10.70	0.846	−12.64*	−21.39	−12.64*
X_3 (quadratic)	0.91	1.466	0.62	−0.91	−0.62
X_1 *vs.* X_2 (interaction)	3.91	1.036	3.77*	7.82	3.77*
X_1 *vs.* X_3 (interaction)	−1.20	1.036	−1.16	−2.41	−1.16
X_2 *vs.* X_3 (interaction)	−7.17	1.036	−6.92*	−14.35	−6.92*

X_1 = dose of siRNA (nM); X_2 = vortex speed (rpm); X_3 = vortex time (min); SE, standard error.
* Significant for $\alpha = 0.05$.

(Table 2). An insignificant result for the lack of fit confirmed that the experimental variability is due mainly to the variables studied, and no violations of the model assumptions occurred (Wherle et al., 1995).

Considering the results for the statistical analysis by *t*-test, it was clear that the linear contributions of the factors showed important and quite similar effects (Table 3) and had a negative effect on the *in vitro* luciferase gene knockdown effect (Table 1). The contribution of the second-order term was interpreted as the presence of a curvature and represents the nature of the response surface system (maximum, minimum, or saddle system). The positive signal thus observed with the speed (quadratic) term revealed the convex form of the curve. However, only a slight influence could be imputed to this term when compared to linear terms. In the same way, the interactions between vortex speed and the other variables were also found to be statistically significant but they had just a minor importance in the response behavior (Table 3).

According to the *t*-test, non-significant terms of the model should be withdrawn. The model was recalculated and only the significant terms ($\alpha < 0.05$) were selected for the equation. The results obtained for the statistical validation of the model were presented in Table 4. Thus, the experimental behavior could be satisfactory described by Eq. (4):

Table 4
Statistical analysis of the simplified model.

Factor	Coefficient	t-Test$_{(coeffic.)}$	F-test$_{(ANOVA)}$
Mean	67.08	94.41*	–
X_1 (lineal)	−10.16	−12.00*	144.09*
X_2 (lineal)	−12.54	−14.82*	219.57*
X_2 (quadratic)	−2.04	2.78*	7.75*
X_3 (lineal)	−10.70	−12.64*	159.66*
X_1 *vs.* X_2 (interaction)	3.91	3.77*	14.23*
X_2 *vs.* X_3 (interaction)	−7.17	−6.92*	47.87*
Lack of fit	–	–	1.27
R_2	0.8478		

X_1 = dose of siRNA (nM); X_2 = vortex speed (rpm); X_3 = vortex time (min).
* Significant for $\alpha = 0.05$.

$$\text{RNAi effect(F-luc/R-luc)} = 67.08 - 10.16[X_1] - 10.69[X_3] + 3.91[X_1][X_2] - 7.17[X_2][X_3] + 0.42[X_1]^2 - 2.04[X_2]^2 \quad (4)$$

Validation experiments were carried out by ANOVA and multiple-correlation coefficient to verify the availability and the accuracy of

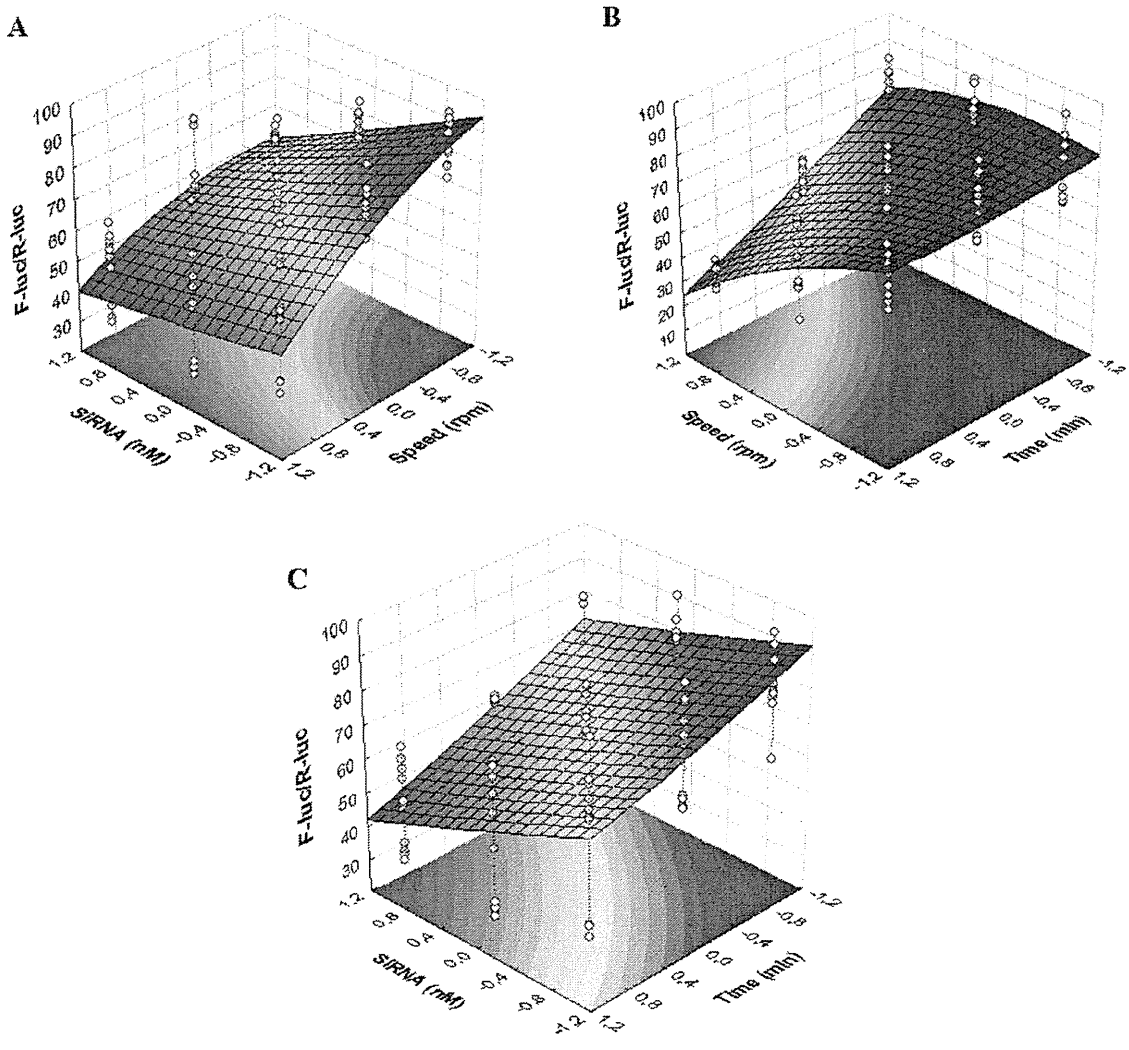

Fig. 3. Response surface plots showing the effect of siRNA dose and vortex speed (A), the effect of vortex speed and vortex time (B), and the effect of siRNA dose and vortex time (C) on the *in vitro* luciferase gene knockdown effect.

the models showed that the predict values agreed well with the experimental values (Table 4).

The estimated response surface plots of the effect of the variables on the *in vitro* luciferase gene knockdown effect are shown in Fig. 3. The response surface plots demonstrated that an increase in the siRNA dose expectedly results in an increase in the gene silencing effect. On the other hand, the plots demonstrated that an increase in the vortex speed promotes an efficient *in vitro* luciferase gene silencing effect at a lower siRNA dose. Concerning the association of the vortex speed and vortex time, the luciferase gene silencing effect is assumed to increase abruptly when both variables, *i.e.* vortex speed and vortex time, are at their highest level. This indicates that for vorLTsiR, vortex time contributes, to some extent, to vortex speed on the effective gene knockdown effect of siRNA. Nevertheless, it also assumes that an effective gene knockdown with the variable vortex time at a constant vortex speed is dependent on the siRNA dose. Under optimal conditions, *i.e.* 2500 rpm for 10 min, an induced luciferase gene knockdown could be achieved with a 4-fold lower siRNA dose (Fig. 3B). This estimation agreed well with the experimental data, as shown in Table 1. These results suggest that all 3 variables are significantly important, but statistically the vortex speed is the most important for the *in vitro* gene knockdown effect of vorLTsiR.

4. Discussion

Lipid molecules are known to interact with nucleic acids, either electrostatically *via* hydrophobic interaction or *via* hydrogen bonding (Lasic and Templeton, 1996). The FRET study (Fig. 1) indicated that in spoLTsiR, siRNA preferentially interacts with a cationic liposome through DC-6-14, while in vorLTsiR, siRNA preferentially interacts with a cationic liposome *via* DC-6-14 as well as DOPE. Our cationic liposome, LT, was composed of three lipids: the monocationic lipid DC-6-14, cholesterol, and DOPE. Cholesterol and DOPE are the most common helper lipids of the cationic liposomes that are used for lipofection (Ramezani et al., 2009). DOPE intensively affects the physicochemical properties of lipoplexes containing monocationic lipids such as *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTAP) or 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (DMRIE) (Felgner et al., 1994; Zuidam and Barenholz, 1997). DOPE is zwitterionic at near neutral pH (Ellens et al., 1986; Siegel and Epand, 1997) and also contributes to lower the overall positive charge and surface potential of a cationic liposome through the formation of a salt bridge (ion pairing) between its phosphate group and the quaternary amine group on the monocationic lipid. The chemical structure of DC-6-14 closely resembles the chemical structure of DOTAP and DMRIE. It is very likely that the balance between the acyl chain (length, unsaturation) and headgroup moieties (charge, hydrophilicity) could modulate the interactions guiding the formation of a salt bridge between the phosphate group on DOPE and the quaternary amine group on DC-6-14 (Sugahara et al., 2002; Shaikh et al., 2004, 2006). In such a case, because DOPE would contribute to lower the overall positive charge in the membrane, the location and order of the positive charges in the membrane are not in a good distribution, and the surface potential of the LT therefore becomes entirely lowered. Accordingly, it is likely that, in spoLTsiR, the electrostatic association of siRNA to cationic charges in the LT occur disorderedly. On the other hand, in vorLTsiR, the constant and intense agitation by vortexing would function as a driving force to gain entropy separating the interactions between the cationic lipid DC-6-14 and DOPE, and thereby promoting interactions between these cationic lipids and siRNA (Zuidam and Barenholz, 1997; Zuidam et al., 1999). The dynamic environment created by vortexing seems to promote an interaction of siRNA with the surface of LT and, consequently, helps siRNA achieve a better distribution on it (Wang and MacDonald, 2004; Koynova et al., 2007), although this is one of factors to gain better gene knockdown effect by vorLTsiR.

siRNA is a small tri-dimensional nanometric rod with an average single-molecule size below 10 nm in length and approximately 2 nm in diameter (Kim et al., 2009). Since its anionic groups are disposed at the side of the molecule, they must interact laterally with positively charged lipids in the membrane of a cationic liposome (Spagnou et al., 2004). In a CD experiment, the lateral anionic groups of an siRNA molecule in the lipoplexes is expected to cause either of the 2 polarizations to absorb more, and this wavelength-dependent difference can yield an interaction of siRNA with a cationic liposome during the lipoplex preparation procedure. A CD study (Fig. 2) showed that the intensity of the characteristic bands of the A-form helix of siRNA was reduced following its interaction with cationic liposome. The reduction in intensity was more prominent in spoLTsiR than in vorLTsiR, which caused an important shift in the large negative band at 208 nm to an acute band near 205 nm.

Previous studies have shown that electrostatic interaction between cationic liposomes and nucleic acids causes each component to dehydrate during lipoplex formation (Zhang et al., 2003; Hirsch-Lerner and Barenholz, 1999). The dehydration of the polar head groups implies a reduction in the number of water molecules separating positive and negative charges after complexation (Lasic and Templeton, 1996; Hirsch-Lerner and Barenholz, 1999). It thus might be that the closer the molecules, the more efficient and strong must be the interaction between siRNA and cationic lipids in vorLTsiR. In addition, under such conditions, other forces, such as elastic (bending and stretching) and hydrogen bonding forces, could contribute to the complementary and harmonious association of siRNA and cationic liposomes (Chong and Sugar, 2002; Janas et al., 2006). Their harmonious association might result in the formation of smaller and homogeneous lipoplexes ($\leq$200 nm), loading a larger amount of siRNA (80% of the dose) (Barichello et al., 2011). However, the larger number of dehydrated polar head groups in spoLTsiR could indeed trigger a relatively uncontrolled interactive process between lipoplexes in the presence of counter ions (Opti-MEM), leading to lipoplexes of excessive size and poor stability (Barichello et al., 2011). Such differences in physicochemical properties between spoLTsiR and vorLTsiR might be a major cause in the shift of the entry pathway increasing the internalization of siRNA and resulting in the increased *in vitro* gene knockdown efficacy of vorLTsiR (Barichello et al., 2011).

The factorial design and response surface plots (Fig. 3) demonstrated that an increase in siRNA dose expectedly increased the gene knockdown effect. However, the plots also indicated that an increase in vortex speed achieved an efficient gene knockdown effect at a lower siRNA dose. In addition, the vortex time cooperatively contributed to the vortex speed, but at a constant vortex speed, the vortex time did not contribute. Collectively, it appears that among the variables we studied, vortex speed was the most important variable in achieving the highest gene knockdown effect by vorLTsiR. The higher energy transmission induced by agitation likely increased reorganization of the positively charged cationic lipids DC-6-14 and DOPE in the liposomal membrane to harmoniously accommodate the negatively charged siRNA on a cationic liposome.

5. Conclusion

The high energy transmitted by the application of agitation during lipoplex formation harmonized the interaction of siRNA with the positively charged lipids (DC-6-14 and DOPE) in cationic liposomes, resulting in a superior gene knockdown efficacy of vorLTsiR

compared with spoLTsiR. Our results indicate that control over the variables contributing to lipoplex formation is required in order to obtain reliable and reproducible siRNA-lipoplexes and a superior *in vitro* gene knockdown effect. The selection of the correct lipoplex preparation procedure will avoid drawbacks that could jeopardize the potential use of non-viral vectors for siRNA therapy.

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