Delivery of siRNA Using Cationic Liposomes Incorporating Stearic Acid-modified Octa-Arginine

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Abstract. *Cationic liposomes incorporating stearic acid-modified octa-arginine (StA-R8) were evaluated for survivin small interfering RNA (siRNA) delivery. StA-R8 was synthesized and incorporated into liposomes. The composition of liposomes was optimized. Physicochemical properties, cytotoxicity, cellular uptake and gene silencing activity of the liposomes complexed to survivin siRNA were investigated. The results showed that StA-R8-containing liposomes had reduced cytotoxicity and improved delivery efficiency of siRNA into cancer cells compared with StA-R8 by itself.*

High molecular weight agents, such as oligonucleotides (1), peptides (2, 3) and proteins (4, 5), are promising for therapeutic applications. However, poor stability and low membrane permeability limit their clinical application. Cell-penetrating peptides (CPPs) can facilitate transmembrane delivery of biomolecules (6). Many CPPs have been reported to date, including HIV-Tat (7), penetratin (8), transportan (9) and octa-arginine (R8) (10). R8 is highly cationic and can facilitate electrostatic interaction with the plasma membrane and facilitate membrane translocation (11). Many delivery systems have been developed for nucleic acid therapeutics (12), including viral vectors (13), liposomes (14, 15), nanoparticles (16), dendrimers (17, 18) and electroporation (19, 20). These methods suffer from problems, such as cytotoxicity, immunogenicity and low efficiency. CPP-based drug delivery strategies have recently gained attention (21), especially for small interfering RNA (siRNA) delivery. In this article, we report synthesis and evaluation of cationic liposomes incorporating a stearic acid-modified R8 (StA-R8) for delivery of a survivin siRNA into cancer cells.

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Materials and Methods

Materials. 2-Chlorotrityl chloride resins and Fmoc-L-arginine were obtained from JiEr Biochemical Company (Shanghai, China). Stearic acid ((StA), 98.5%), cholesterol (Chol), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Egg phosphatidylcholine (ePC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were from HyClone (Logan, UT, USA). Anti-survivin siRNA, FAM and Cy3 fluorescence-labeled siRNA were synthesized by Ribo Biochemistry (Guangzhou, China). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Invitrogen Molecular Probes (Eugene, OR, USA). All other reagents were commercially purchased in reagent grade.

Synthesis of StA-R8. StA-R8 was prepared by Fmoc solid-phase peptide synthesis on a 2-chlorotrityl chloride resin as reported previously (22). Fmoc-L-arginine was loaded onto the resin one by one to synthesize R8 (23) and, then, stearic acid was coupled to R8 *via* an amide bond using the same approach (24). Next, the target peptide was purified and structurally confirmed by reverse-phase high-performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The scheme of StA-R8 synthesis is shown in Figure 1A.

Preparation of StA-R8 liposomes and transfection complexes. An ethanol injection method was used to prepare StA-R8 liposomes. StA-R8, ePC and Chol were dissolved in ethyl alcohol at different molar ratios. This solution was injected at a constant speed into 20 mM HEPES buffer (pH 7.4) with vortex mixing and at an ethanol/water ratio of 1:3 v/v. Then, the resulting liposomes were sonicated for 30 sec. siRNA complexes were synthesized by combining StA-R8

liposomes with anti-survivin siRNA. The composition of the complexes was optimized by varying the charge ratios of StA-R8-to-siRNA from 0.5:1 to 8:1. A schematic diagram of the preparation of StA-R8 liposomes and transfection complexes is shown in Figure 1B.

Particle size and zeta potential measurements. The particle size and zeta potential of StA-R8-modified liposomes were determined on a Zetasizer Nano ZS 90 (Malvern Instruments, Ltd., Malvern, UK). The measurement of each complex was repeated three times.

Agarose gel retardation assay. StA-R8 liposomes are capable of forming electrostatic complexes with siRNA, which was measured by gel retardation. The siRNA/StA-R8 complexes at varying charge ratios ranging from 0.5:1 to 8:1 were analyzed by electrophoresis on a 2% (w/v) agarose gel in TAE buffer, containing 0.5 μg/ml ethidium bromide (Sigma, St. Louis, MO, USA). The gel was run for 20 min at 80 V and visualized with a UV lamp using a Vilber Lourmat imaging system (Marne La Vallée, France). The pictures were digitized and analyzed using the Image J software (National Institutes of Health, Bethesda, MD, USA) to compute the mean density of siRNA band.

Cell culture. HeLa and HepG2 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotics/antimycotics in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cytotoxicity assay. A549, HepG2 and 293T cells were seeded in a 96-well plate at 1×10^4 cells/well and cultured for 24 h. Then, siRNA complexes of StA-R8 or StA-R8 liposomes at different concentrations were added into the plate. The serum-free medium was replaced with fresh medium with FBS after 4 h incubation and the cells were incubated for another 20 h. Next, 15 μl MTT stock solution (5 mg/ml) was added into each well and the cells were incubated for 4h at 37˚C. Finally, the culture medium was removed and 100 μl/well DMSO was added to dissolve the formazan converted from MTT. Cell viability data were obtained by measuring OD490 on a BioTek Synergy™ 4 Hybrid Microplate Reader (Winooski, VT, USA).

Cellular uptake analysis. Cells (1×10^5/well) were seeded onto a 24-well plate. The StA-R8 liposomes/siRNA^{FAM} or StA-R8/siRNA^{FAM} complexes diluted in 1 ml of medium without FBS were added to the cells and the cells were incubated for 4 h in dark at 37˚C. Subsequently, cells were washed three times with 1×PBS, trypsinized and fixed in 4% paraformaldehyde at 4˚C overnight. Fluorescence intensity of the cells was measured on a flow cytometer (BD Biosciences, San Jose, CA, USA).

Confocal microscopy. Cells (1×10^5/well) were incubated with StA-R8 liposomes/siRNA^{Cy3} or StA-R8/ siRNA^{Cy3} complexes for 4 h in the dark, washed three times with 1×PBS and fixed with 4% paraformaldehyde for 20 min. DAPI, 2μg/ml, was used to stain the cellular nuclei for 5 min at room temperature. Then, the cells were observed on a Zeiss 710 LSMNLO Confocal Microscope (Carl Zeiss, Jena, Germany).

Bioactivity of the siRNA complexes. HepG2 cells (2×10^5 cells/well) seeded in a 6-well plate were treated with various transfection complexes as described above. The incubation medium was remove after 4 h and fresh medium with 10% FBS was added. The cells were cultured for another 44 h, washed and harvested. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot were used to determine survivin mRNA and protein expression.

Table I. *Particle size and Zeta potential of liposomes of different compositions*.

Formulation composition	Ratio of components	Average particle size (nm)	Zeta potential (mV)
StA-R8/ePC/Chol	50/15/35	546.6±10.8	28.1±1.2
	40/25/35	202.2±12.3	25.3±1.4
	30/25/45	136.1±4.9	22.4±0.8
	20/35/45	126.4±6.2	18.3±1.3
	10/35/55	128.4±5.4	10.8±0.6
	5/45/55	130.4±5.7	7.8±1.2

Data are shown as mean±SD. StA-R8, Stearic acid-modified octa-arginine; ePC, egg phosphatidylcholine; Chol, cholesterol.

Results

Characterization of StA-R8. Octa-arginine is hydrophilic and is coupled to lipophilic stearic acid (StA) to yield StA-R8. Formation of StA-R8, with a molecular weight (Mw) of 1,534, was confirmed by MALDI-TOF/MS. Formulation optimization of StA-R8 liposomes. A series of StA-R8 liposomes with different compositions were synthesized and their particle size and zeta potential are shown in Table I. With decrease of StA-R8 content, the average particle sizes of the liposomes decreased. When the content of StA-R8 was below 30%, the average particle size remained constant. Zeta potential also decreased along with the content of StA-R8 but remained positive in value. Although the particle size was small at low StA-R8 content, the siRNA binding efficiency would be adversely affected. Therefore, we chose the formulation StA-R8/ePC/Chol (20:35:45) for subsequent studies.

Formation of StA-R8 liposome/siRNA complexes. StA-R8 liposome/siRNA complexes were prepared with 0.1nmol siRNA at a series of (+/–) charge ratios ranging from 0.5:1 to 8:1. The complexation efficiency was tested by gel retardation assay. As shown in Figure 2, the first lane was naked siRNA in which a corresponding band was observed. With increase of charge ratio, more and more siRNA was complexed to the liposomes. When the charge radios reached above 4:1, there were no free siRNA bands detectable, indicating that all siRNA was completely incorporated into the liposome complex.

Assessment of cytotoxicity. MTT assay was used to evaluate the viability of HepG2 and A549 cells treated with StA-R8 and StA-R8 liposomes (Figure 3). A series of different concentrations of StA-R8 and StA-R8 liposomes, each

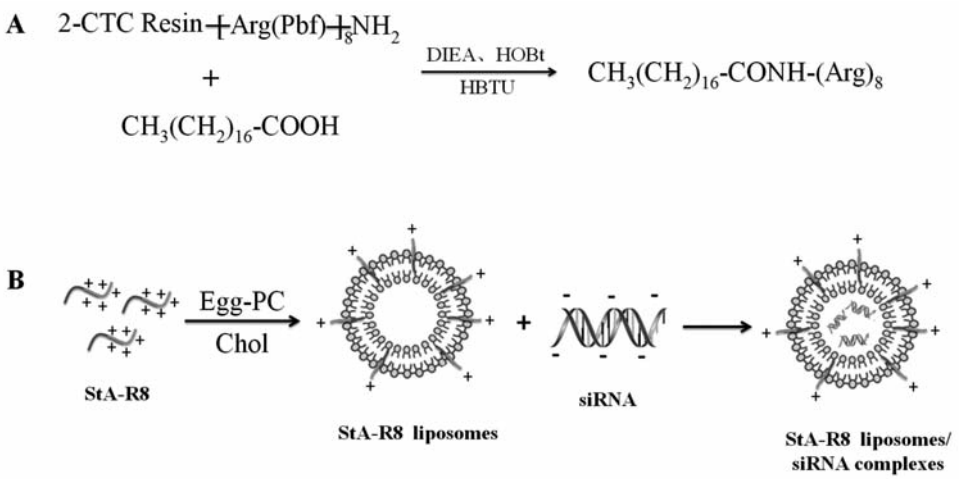

Figure 1. *Synthetic schemes of StA-R8 and siRNA complexes. (A) Synthesis of StA-R8; (B) preparation of StA-R8 liposome/siRNA complexes.*

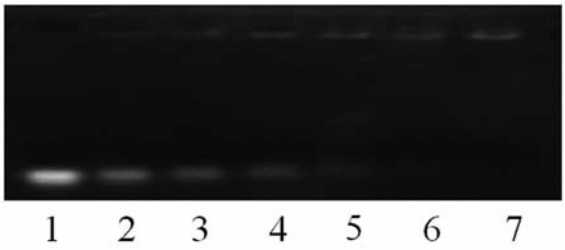

Figure 2. *Agarose gel analysis of StA-R8 liposome/siRNA complexes at different N/P ratios. Lanes: 1, naked siRNA; lanes 2-7, StA-R8 liposome/siRNA complexes formed at different N/P ratios, 0.5:1 (lane 2), 1:1 (lane 3), 2:1 (lane 4), 4:1 (lane 5), 6:1 (lane 6) and 8:1 (lane 7).*

containing the same amount of StA-R8, were added to the cells. As shown in Figure 3, at all concentrations of StA-R8 liposomes, cell viabilities were over 80% compared to untreated controls. Conversely, StA-R8 at high concentration showed greater cytotoxicity than the corresponding StA-R8 liposomes. Thus, StA-R8 liposomes displayed reduced cytotoxicity as a cationic vector compared to StA-R8 itself.

Study of Cy3-siRNA uptake by flow cytometry. Fluorescence intensity was used to quantify uptake of FAM-labelled siRNA by HepG2 and A549 cells. The cells were treated with StA-R8/siRNA or StA-R8 liposome/siRNA complexes at the charge ratio of 4:1. As shown in Figure 4A and B, the fluorescence peaks of StA-R8 liposomes and StA-R8 siRNA complex-treated cells migrated to the right, showing that the cellular uptake of Cy3-siRNA complexed to StA-R8 liposomes and StA-R8 was much higher than that of naked siRNA in HepG2 and A549 cells. Meanwhile, the shift in peak position of complexes StA-R8 liposomes was greater

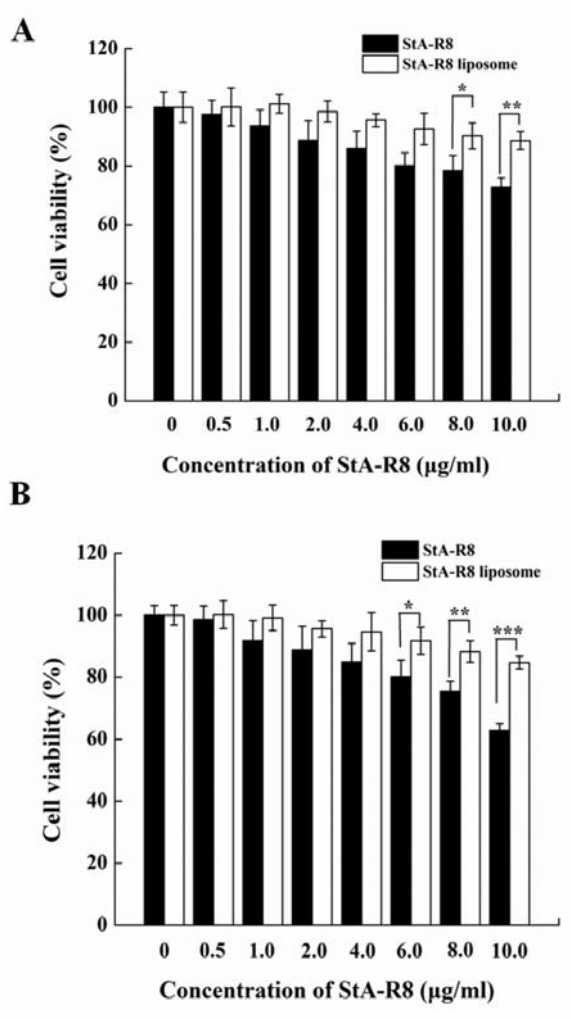

Figure 3. *Cytotoxicity of various cationic agents on HepG2 and A549 cells. (A) Effect on viability of HepG2 cells; (B) effect on viability of A549 cells. *p<0.05; **p<0.01; ***p<0.001.*

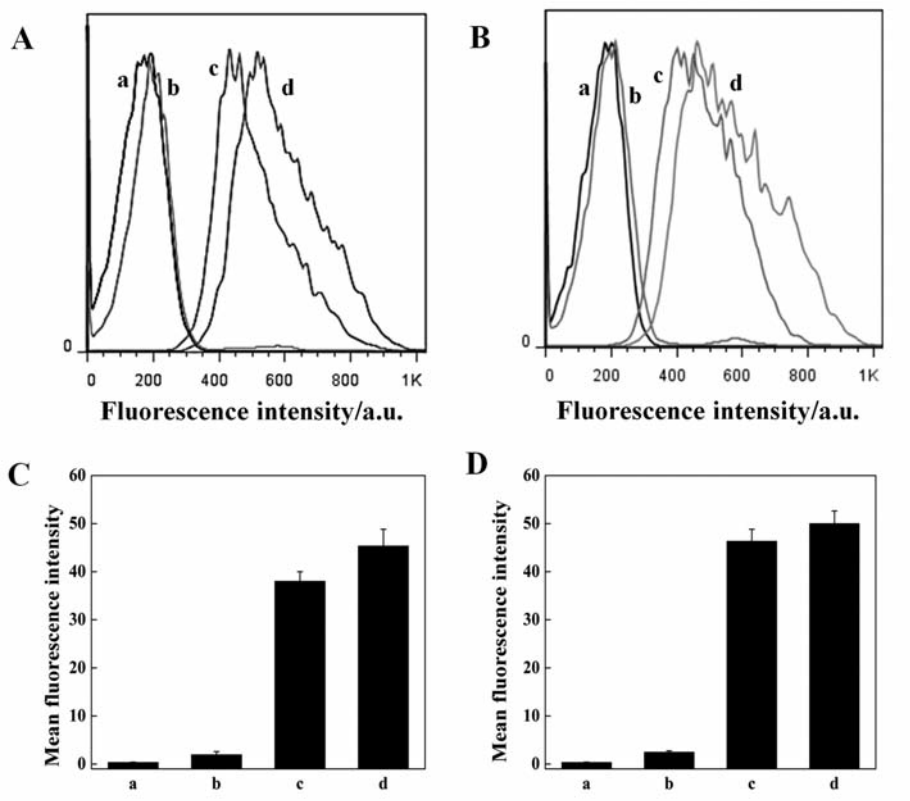

Figure 4. *Cellular uptake in vitro measured by flow cytometry. A and B, flow cytometry histograms of HepG2 cells (A) and A549 cells (B) treated with siRNA complexes; C and D, mean fluorescence values of HepG2 (C) and A549 cells (D) treated with siRNA complexes. The treatments were: a, untreated control; b, free siRNA; c, StA-R8/siRNA complexes; d, StA-R8 liposome/siRNA complexes. a.u., Arbitrary units of fluorescence.*

than that of StA-R8, hence the uptake efficiency of Cy3-siRNA complexed StA-R8 liposomes was greater than that of StA-R8. The mean fluorescence intensities of the treated cells were also measured (Figure 4C and D). The values for cells treated with StA-R8 liposome/siRNA complexes were about 40-times higher than those treated with naked siRNA. In A549 cells, StA-R8 liposomes/siRNA complexes also showed higher cellular uptake than that of StA-R8/siRNA complexes. These data indicated that StA-R8 present in the liposomes mediated more efficient uptake of siRNA *in vitro*.

Cellular internalization analysis by confocal microscopy. The internalization of Cy3-labeled siRNA mediated by StA-R8 liposome or StA-R8 was also determined in HepG2 cells by confocal microscopy. In Figure 5, red fluorescence of Cy3-siRNA was found to be extensively distributed in the cytosol and the nucleus. In addition, fluorescence intensity of HepG2 cells treated with StA-R8 liposome/siRNA complexes was higher than those treated with StA-R8/siRNA complexes.

Survivin protein levels were determined by western blot. Densitometric analysis was used to calculate relative survivin expression. The results showed that StA-R8 liposome/siRNA complexes more efficiently decreased survivin protein levels compared to StA-R8/siRNA complexes (Figure 6A). The results showed that the expression levels of survivin protein in cells treated with StA-R8 /siRNA and StA-R8 liposome/siRNA complexes were 47.7% and 12.7%, respectively, of the control. Therefore, delivery efficiency of siRNA by StA-R8 liposomes was significantly higher than StA-R8 in the cell lines tested (Figure 6).

Discussion

In this study, we synthesized a modified cell-penetrating peptide, StA-R8. Introduction of a hydrophobic stearic acid moiety makes the peptide conjugate amphiphilic. StA-R8 can self-assemble into cationic micelles and form complexes with siRNA. It has shown high efficiency in delivering siRNA into cells.

Here, we demonstrated that cationic liposomes containing StA-R8 mediated even better cellular survivin siRNA delivery *in vitro* than the peptide conjugate itself, as shown by increased siRNA uptake by tumor cells and greater down-regulation of survivin. The modified R8 has the characteristics

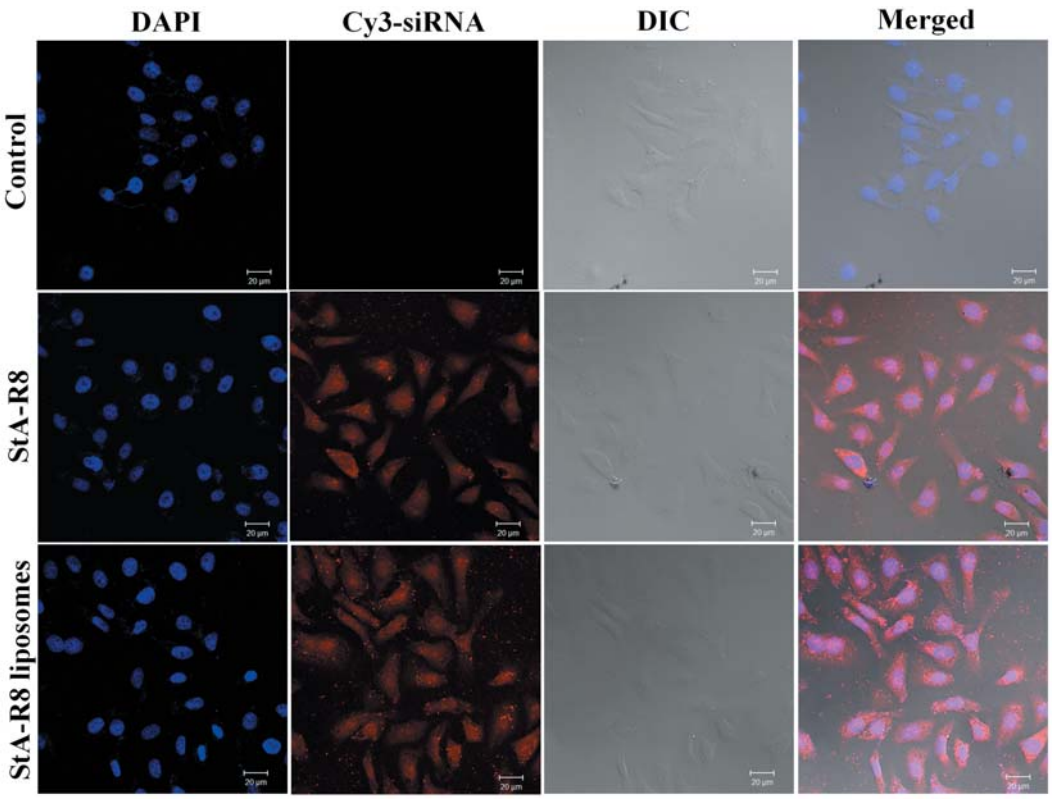

Figure 5. *Intracellular localization of StA-R8/siRNA and StA-R8 liposome/siRNA complexes. The cells were treated with siRNA complexes and stained with DAPI, as described in Materials and Methods. They were then examined by confocal microscopy.*

of multivalent polyamine-based cationic lipid, which can contribute to efficient endosomal escape for siRNA. At the same time, the hydrophobic groups can enhance membrane interactions by the StA-R8 compared to underivatized R8, thereby enhancing siRNA delivery. Incorporation of StA-R8 into liposomes reduced its cytotoxicity. Therefore, StA-R8 liposome/siRNA complexes represent a novel formulation with improved safety and efficacy.

Conclusion

Cationic liposomes based on StA-R8 is highly efficient *in vitro* and warrant further evaluation as an siRNA delivery vehicle.

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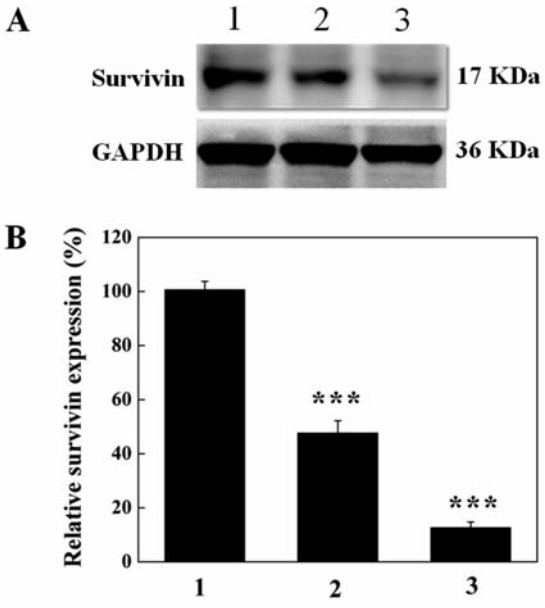

Figure 6. *Effect on siRNA on survivin protein expression in HeLa cells. (A) Protein expression determined by western blot. (B) Densitometric analysis of the western blot shown in (A). Lane 1, untreated control; Lane 2, StA-R8/siRNA complexes; Lane 3, StA-R8 liposome/siRNA complexes. Each bar is the mean±SD of three experiments. ***p<0.01 vs. control.*

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