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Novel cationic cholesterol derivative-based liposomes for serum-enhanced delivery of siRNA

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Abstract

Most cationic liposomes used for gene delivery suffer from reduced transfection efficiency in the presence of serum. In this study, we report serum-enhanced delivery efficiency of siRNA via the use of newly synthesized liposomes that contain cationic lipids. Two cholesterol derivatives, cholesteryloxypropan-1-amine (COPA) and cholesteryl-2-aminoethylcarbamate (CAEC), were synthesized. A fluorescein label was then used to visualize cellular uptake of small interfering RNA (siRNA) via COPA or CAEC-based liposomes. The presence of serum had different effects on the cellular delivery of siRNA when siRNA was complexed to different cationic liposomes. CAEC-based liposomes showed significantly reduced cellular delivery of siRNA in serum-containing media as compared to serum-free media. Conversely, COPA-based liposomes (COPA-L) provided serum-enhanced delivery of siRNA in Hepa1–6, A549, and Hela cell lines. Following delivery of the oncogene survivin-specific siRNA, COPA-L reduced the mRNA expression levels of the target gene more efficiently than did Lipofectamine 2000. The delivery of green fluorescent protein-specific siRNA with COPA-L reduced the expression of green fluorescent protein in 293T stable cell lines. The apoptosis of Hepa1–6 significantly increased by delivery of survivin-specific siRNA by COPA-L. Additionally, Hepa1–6, A549, and Hela cells were >80% viable after treatment with COPA-L. These results suggest that the newly synthesized cholesterol derivative, COPA-L, could be further developed as a serum-enhanced delivery system of siRNA.

Keywords: Cationic lipids; Cholesterol derivatives; Serum stability; siRNA delivery

1. Introduction

Small interfering RNA (siRNA) is emerging as a new therapeutic class of drugs, and several clinical trials are currently in progress to develop siRNA for the treatment of cancer (Zaffaroni et al., 2007), viral infections (Palliser et al., 2006), and various diseases (Dykxhoorn et al., 2006). Development of effective delivery vectors is essential for siRNA therapy to be successful, therefore, several viral and non-viral delivery systems have been studied (Gilmore et al., 2006). Non-viral gene delivery systems have advantages over viral vectors with regard to convenience of complex formation, ease of large production and reduced risk of immunogenicity (Simoes et al., 2005).

Cationic liposomes are the most widely studied non-viral vectors for the delivery of negatively charged gene medicines by formation of charge complexes (Simoes et al., 2005; Wasungu and Hoekstra, 2006). Among cationic liposomes, commercially available Lipofectamine 2000 has been widely used for delivery studies of siRNA (Dalby et al., 2004). However, despite progress in cationic liposome-mediated delivery, the reduced cellular uptake efficiency of siRNA in the presence of serum is still considered a major drawback for in vitro and vivo application. Since siRNA is known to form less condensed complexes with cationic liposomes than plasmid DNA (Spagnou et al., 2004), it may be more vulnerable to attack by nucleases in the serum.

Therefore, in this study, we focused on development of new cationic lipid-based delivery systems of siRNA that can increase

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serum stability and transfection efficiency. Here, we report that a newly synthesized cholesterol-derivative might be useful for enhanced cellular delivery of siRNA via liposomes in a serum-stable manner.

2. Material and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). The synthetic cationic lipids were prepared as described below. Commercially available Lipofectamine 2000 (LA, Invitrogen, Carlsbad, CA, USA) was used as a control. All other chemicals used in this study were of reagent grade.

2.2. Synthesis of cationic lipids

The procedures for the synthesis of cholesteryloxypropan-1-amine (COPA) and cholesteryl-2-aminoethylcarbamate (CAEC) are described in Fig. 1A and B, respectively. COPA and CAEC were synthesized as described below for formulation of the new cationic liposomes.

2.2.1. Synthesis of cholesteryl propanenitrile (lipid 2)

Cholesterol (lipid 1, 772 mg) was dissolved in 15 ml dichloromethane (DCM) in a two-necked 100 ml round-bottomed flask. Next, 40% aqueous KOH (0.4 ml) and 52 mg of 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6) (0.2 mmol) were added. The reaction mixture was stirred at room temperature for 3 h. Evaporation followed by column chromatographic purification (using silica and 10%, v/v ethyl acetate (EtOAc) in hexane as an eluant) of the residue yielded lipid 2 as a white solid (783 mg, $R_f = 0.4$, 1:4 v/v, EtOAc:hexane). Lipid 2 was then analyzed by ^{1}H NMR at 300 MHz in $CDCl_3$. The results of this analysis revealed the following: $\delta = 0.60$–2.30 [m, 43H, cholesteryl skeleton], 2.60 [t, 2H, NC–C*H*$_2$–CH$_2$–O], 3.10 [m, 1H, $H_{3\alpha}$(Chol)], 3.80 [t, 2H, NC–CH$_2$–C*H*$_2$–O–], 5.30 [m, 1H, H_6(Chol)] FABMS (EI) $m/z = 439$.

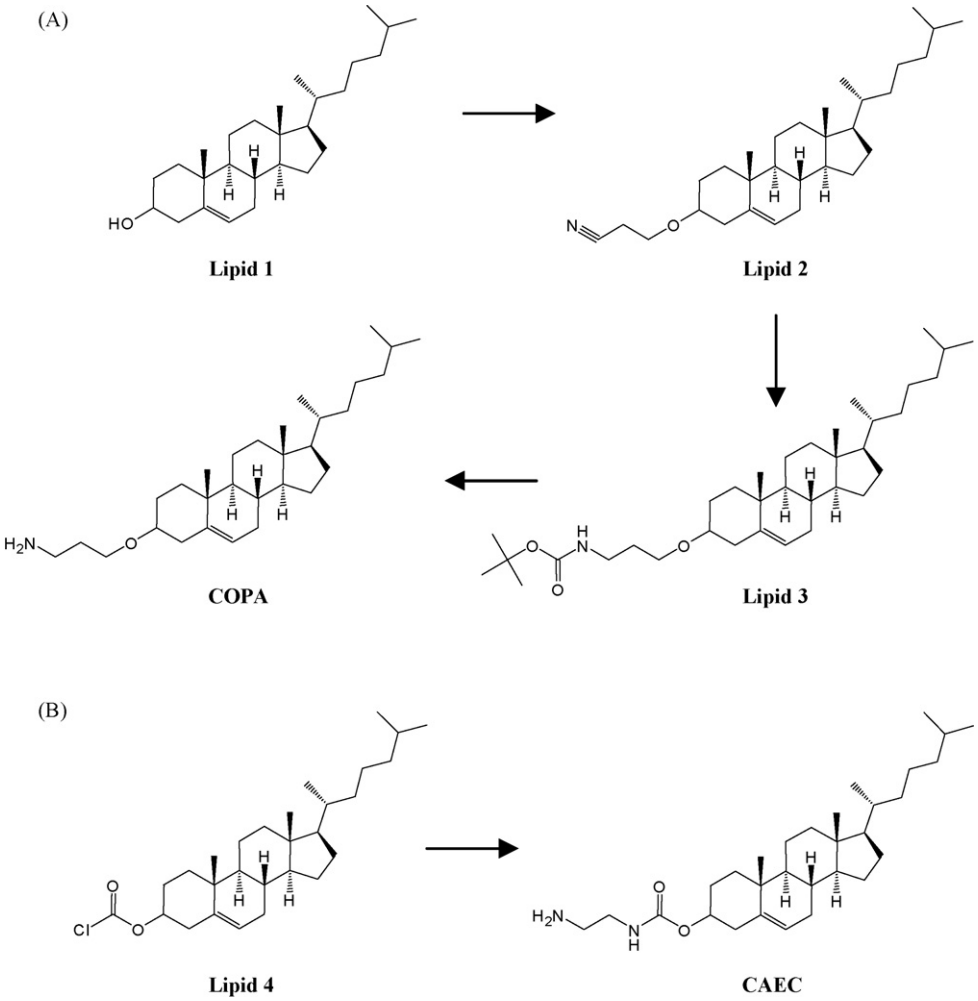

Fig. 1. Synthesis scheme of cationic lipids. (A) Using cholesterol (lipid 1) as a starting material, COPA was synthesized via the intermediate products cholesteryl propanenitrile (lipid 2) and *tert*-butyl 3-cholesteryl oxypropylcarbamate (lipid 3). (B) CAEC was synthesized from cholesteryl chloroformate (lipid 4).

2.2.2. Synthesis of t-butyl 3-cholesteryloxypropylcarbamate (lipid 3)

Lipid 2 (486 mg, 1.1 mmol) was dissolved in methanol (85 ml) and added with 260 mg $NiCl_2{\cdot}6H_2O$ (1.1 mmol) and 287 mg di-*tert*-butyldicarbonate (1.32 mmol). The reaction was allowed to proceed at room temperature for 5 min while stirring, and 285 mg $NaBH_4$ (7.7 mmol) was added. The reaction mixture was placed in a rotary evaporator to remove the solvent, and then 200 ml EtOAc was added and the solution was washed with brine. The residue was purified by column chromatography using C18 silica and 10% (v/v) EtOAc in hexane as an eluant, which produced lipid 3 as a white solid (430 mg, $R_f = 0.4$, 1:4 v/v, EtOAc:hexane). ^{1}H NMR analysis of lipid 3, the immediate precursor of COPA, was performed at 300 MHz in $CDCl_3$. The results of the analysis revealed the following: $\delta = 0.60$–2.30 [m, 43H, cholesteryl skeleton], 1.40 [s, 9H, $(CH_3)_3$COCONH–], 1.70 [m, 2H, Boc–NH–CH_2–CH_2–CH_2–O–], 2.60 [t, 2H, Boc–NH–CH_2–CH_2–CH_2–O–], 3.10 [m, 1H, $H_{3\alpha}$(Chol)], 3.40 [t, 2H, Boc–NH–CH_2–CH_2–CH_2–O–], 5.15 [bs, 1H, BOC–N*H*–CH_2–CH_2–CO–], 5.30 [m, 1H, H_6(Chol)] FABMS (EI) $m/z = 543$.

2.2.3. Synthesis of COPA

Lipid 3 (277 mg, 0.51 mmol) was dissolved in 5 ml of a trifluoroacetic acid (TFA) and DCM mixture (1:4, v/v). The reaction was allowed to proceed at room temperature while stirring for 50 min. Next, the mixture of TFA and DCM was completely removed by co-evaporation with ethanol. The residue was purified by column chromatography using C18 silica and 10% (v/v) methanol in chloroform as an eluant, which resulted in the production of COPA in a white solid form (203 mg, $R_f = 0.1$, 10%, v/v methanol in chloroform). ^{1}H NMR analysis of COPA was performed at 300 MHz in $CDCl_3$. The results of the analysis revealed the following: $\delta = 0.60$–2.30 [m, 43H, cholesteryl skeleton], 2.20 [m, 4H, NH_3^+–CH_2–CH_2–CH_2–O–], 3.30–3.40 [m, 4H, NH_3^+–CH_2–CH_2–CH_2–O–], 3.15 [m, 1H, $H_{3\alpha}$(Chol)], 5.30 [m, 1H, H_6(Chol)] 8.00–8.10 [brs, 3H, NH_3^+–CH_2–CH_2–CH_2–O–], FABMS (EI) $m/z = 444$.

2.2.4. Synthesis of CAEC

Cholesteryl chloroformate 449 mg (lipid 4, 1.0 mmol, Sigma–Aldrich, St. Louis, MO, USA) was dissolved in 15 ml DCM followed by slow addition of 60.1 mg ethylenediamine (10 mmol) pre-dissolved in 5 ml of DCM. Next, the reaction was allowed to proceed at room temperature for 100 min while stirring, and the reactant was completely removed by co-evaporation with ethanol. The residue was dissolved in a 5 ml mixture of TFA and DCM (1:4, v/v), and the reaction was then allowed to proceed at room temperature for 50 min. Next, the mixture of TFA and DCM was removed by co-evaporation with ethanol. Column chromatographic purification of the residue was conducted using C18 silica and 10% (v/v) methanol in chloroform as an eluant. The purification resulted in the formation of CAEC as a white solid (564 mg, $R_f = 0.2$, 10%, v/v methanol in chloroform). ^{1}H NMR analysis of CAEC was performed at 300 MHz in $CDCl_3$. The results of the analysis revealed the following: $\delta = 0.60$–2.30 [m, 43H, cholesteryl skeleton], 2.78–2.81 (2H, t, CH_2–NH_3^+), 3.15 [m, 1H, $H_{3\alpha}$(Chol)], 3.45–3.48 (2H, m, CH_2NH), 4.60–4.64 (1H, m, H-3), 5.04–5.08 (1H, m, N*H*), 5.30 [m, 1H, H_6(Chol)], 8.00–8.10 [brs, 3H, NH_3^+–CH_2–], FABMS (EI) $m/z = 472$.

2.3. Liposome preparation and complexation

Liposomes of multilamella vesicles were prepared using a slight modification of a previously described method (Oh and Straubinger, 1997). Briefly, synthetic cationic lipids (COPA or CAEC) were dissolved in chloroform and mixed with DOPE at a weight ratio of 3:1. The lipid mixtures were placed in a rotary evaporator under reduced pressure to remove the chloroform, which resulted in the formation of thin lipid films. Next, HEPES buffer (20 mM, pH 7.4) was added to the films and vigorously vortexed. The resulting suspension of liposomes was passed through an extruder (Northern Lipids Inc., Canada) equipped with double-layered 0.2 μm polycarbonate membrane filters 3 times. The COPA-based liposomes (COPA-L) or CAEC-based liposomes (CAEC-L) were complexed with siRNA at various molar ratios and the formation of these complexes was confirmed by a gel retardation assay.

2.4. Quantitative determination of phospholipids

The concentration of phospholipid contents in the liposome solutions was determined using the inorganic phosphate assay method (Oh et al., 2006). Briefly, liposome suspensions were digested with 400 μl 5 M H_2SO_4 at 170 °C. After cooling the samples, 100 μl of 30% H_2O_2 was added. The mixtures were heated to 170 °C until the H_2O_2 was completely removed, which was confirmed using H_2O_2 detection strips. An aliquot (4.6 ml) of 0.2% ammonium molybdate solution and 100 μl of 15% fresh ascorbic acid were sequentially added to the cooled sample with vortexing. The resulting mixtures were boiled for 10 min at 100 °C, cooled to room temperature, and the absorbance at 830 nm was read. The concentration of phospholipids was then determined using calibration curves generated using phosphorus standard solutions (Sigma).

2.5. Particle size measurements

The sizes of the cationic liposomes or the complexes of liposome and siRNA were measured using a dynamic light scattering technique. The samples were diluted with HEPES buffer (pH 7.4) in the absence or presence of 10% serum, and then transferred into a quartz cuvette in an ELS-8000 dynamic light scattering instrument (ELS-8000, Photal, Osaka, Japan). Next, the hydrodynamic diameters of the particles were determined via dynamic He-Ne laser (10 mW) light scattering at an angle of 90° at 24.1 °C. Data were analyzed using a software package (ELS-8000 software) supplied by the manufacturer.

2.6. Cell culture and treatment of siRNA

The transfection efficiencies of various liposomes were determined using three different cell lines. Murine hepatoma cell line

Hepa1–6, human lung carcinoma cell line A549, and human cervical carcinoma cell line Hela cells were cultured at 37 °C in complete culture medium (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 100 units/ml penicillin plus 100 μg/ml streptomycin (Sigma). Before the transfection experiment, cells were seeded in 24-well plates (16 mm in diameter/well, Corning, USA) at a density of 1×10^5 cells/well. After overnight incubation, the culture medium was replaced in each well. To visualize delivery of siRNA into the cells, Block-It™ Fluorescent Oligo (Invitrogen, Carlsbad, CA, USA) was complexed to COPA-L, CAEC-L, or LA according to the manufacturer's instructions. Block-It™ Fluorescent Oligo (Invitrogen) is a fluorescein-labeled, non-targeted dsRNA compound that has been used to assess the cellular uptake of siRNA (Hough et al., 2006). For cellular delivery, COPA-L or CAEC-L was complexed with siRNA at a complexation molar ratio of 5:1 (total lipid:siRNA). The complexes of fluorescein-labeled siRNA and cationic liposomes were diluted to a final volume of 100 μl with OPTI-MEM®I medium (Gibco, Grand Island, NY, USA) and added to each well. After 24 h of incubation, cells were washed and then observed under a fluorescence microscope (Axiovert 200, Zeiss, Germany) to evaluate the delivery of siRNA into the cells.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

The RNA degradation of specific genes in the Hepa1–6 cells was tested after delivery of survivin-specific siRNA using various liposomes. The siRNA for the targeted silencing of murine survivin (gene bank accession number M13994) was provided by Samchully Pharm Inc. (Seoul, South Korea). Before transfection with the siRNA and cationic liposome complexes, Hepa1–6 cells were seeded into 24-well plates at a density of 1×10^5 cells/well, and incubated overnight. Next, the cells were treated with the siRNA and liposome complexes and incubated for 24 h. To determine the levels of survivin mRNA in the cells, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and transcribed into cDNA using AccuPower RT PreMix (Bioneer, Daejeon, Korea). Polymerase chain reaction (PCR) amplification of survivin was then conducted using the following primers: 5′-ATCCACTGCCCTACCGAGAA-3′ for the sense, and 5′-CTTGGCTCTCTGTCTGTCCAGTT-3′ for the antisense. The PCR was conducted under the following conditions: 32 cycles of denaturation (94 °C, 40 s), annealing (55 °C, 40 s), and extension (72 °C, 1 min). PCR yielded a 229-bp PCR product, which was separated on a 1.5% agarose gel, and visualized by ethidium bromide staining. Expression of the target gene, survivin, was normalized to that of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.8. Fluorescence microscopy of green fluorescent protein-expressing cell lines

The knockdown of a target protein by siRNA and COPA-L complexes was tested by fluorescence microscopy. Green fluorescent protein (GFP)-expressing 293T stable cell lines, GFP-293T cells, were kindly provided from Dr. Sung Man Kang (Korea University, Seoul, South Korea). The siRNA for the targeted silencing of GFP was purchased from Bioneer Co. (Daejeon, South Korea). The sequence of siRNA was 5′-GCAUCAAGGUGAACUUCAA-3′ (sense), and 5′-UUGAAGUUCACCUUGAUGC-3′ (antisense). Before transfection with the GFP-specific siRNA, 293T-GFP cells were seeded into 24-well plates at a density of 1×10^5 cells/well and incubated overnight in complete DMEM culture medium supplemented with 10% fetal bovine serum. Cells were treated with 30 pmol of the siRNA in naked form or complexes with COPA-L at the molar ratio of 5:1, and incubated for 72 h. The GFP-293T cells were then washed and observed under a fluorescence microscope (Axiovert 200, Zeiss, Germany).

2.9. siRNA-mediated apoptosis detection assay

The siRNA-mediated apoptotic effect was tested after delivery of survivin-specific siRNA into Hepa1–6 cells using various liposomes. Before transfection with the survivin-specific siRNA in naked form or complexes with cationic liposomes, Hepa1–6 cells were seeded into 6-well plates at a density of 3×10^5 cells/well, and incubated overnight. Next, the cells were treated with the complexes of siRNA and liposomes, and incubated for 96 h. Total cells were harvested and washed twice, and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) using Annexin V-FITC Apoptosis Detection kit™ (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The stained cells were then measured by dual color FACS analysis (BD FACS Calibur, BD Biosciences). Annexin V-positive fractions were defined as apoptotic cell populations.

2.10. Assessment of cytotoxicity

To assess the cytotoxicity of COPA-L, commercially available scrambled siRNA (GL2, Samchully Pharm Inc.) was complexed to the liposomes. The sequences of the 21-mer scrambled siRNA were 5′-CGUACGCGGAAUACUUCGATT-3′ for the sense, and 5′-UCGAAGUAUUCCGCGUACGTT-3′ for the antisense. COPA-L was complexed with siRNA at a complexation molar ratio of 5:1. LA was complexed with siRNA according to the manufacturer's instructions. After treatment of the various cells with the complexes, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were used to measure the cytotoxicity (Noh et al., 2007). Briefly, cells were seeded in 96-well plates at an initial concentration of 1×10^4 cells per well for 12 h, at which time the growth medium was removed and replaced with 0.1 ml of fresh growth medium. Each complex was then added to the cells, followed by incubation for 24 h. Next, 10 μl of MTT stock solution (5 mg/ml in sterile phosphate-buffered saline, pH 7.4) was added to each well. After the cells were incubated for 2 h in the presence of MTT, the medium was removed, and 300 μl of extraction buffer (0.04N HCl/isopropanol solution) was added to each well to solubilize the dark violet formazan crystals. The optical densities at 579 nm were then measured using a microplate reader (Sunrise-

Basic TECAN, Männedorf, Switzerland). Cell viability was expressed as a percentage relative to the untreated control cells.

2.11. Statistical analysis

Statistical analysis of data was performed using ANOVA, with the Student–Newman–Keuls test employed as a post hoc test. SigmaStat software (version 3.5, Systat Software, Richmond, CA, USA) was used for all analyses and a p value of less than 0.05 was considered significant.

3. Results

3.1. H NMR identification of cationic cholesterol derivative lipids

The synthesis schemes of COPA and CAEC are summarized in Fig. 1. As shown in Fig. 1A, COPA was synthesized from lipid 1 in three steps through the synthesis of two intermediate compounds, lipid 2 and lipid 3. The alcohol group of lipid 1 was cyanoethylated with 1,4,7,10,13,16-hexaoxacyclooctadecane to produce lipid 2 at an 89% yield. Lipid 2 was then reacted with di-*tert*-butyldicarbonate using $NaBH_4$ as a reducing agent, which produced lipid 3 at a 72% yield. Deprotection of the di-*tert*-butyldicarbonate group of lipid 3 with TFA then produced COPA at a 92% yield. CAEC (Fig. 1B) was produced by coupling ethylenediamine to lipid 4, which gave a 97% yield. The chemical structures of the cationic intermediate lipids as well as COPA and CAEC were identified using ^{1}H NMR.

3.2. Complexation of cationic liposomes and siRNA

To confirm the complex formation between cationic liposomes and negatively charged siRNA, we employed an agarose gel retardation assay. COPA-L (Fig. 2A) and CAEC-L (Fig. 2B) showed gel retardation upon complex formation with siRNA. When the complexation molar ratios of total lipids:siRNA were less than 0.05:1, no retardation of siRNA was observed, however gel retardation was clearly indicated when the complexation molar ratio of total lipids:siRNA was greater than 0.05:1.

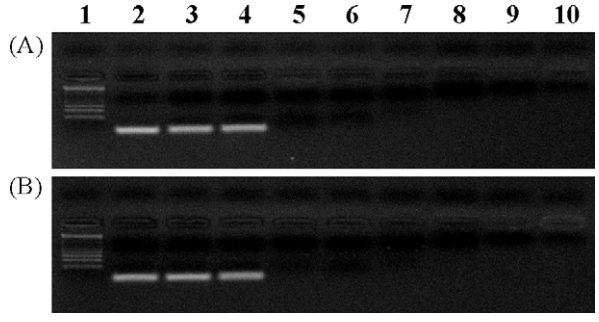

Fig. 2. Gel retardation patterns of cationic liposomes and siRNA complexes. COPA-L (A) or CAEC-L (B) was complexed with siRNA at various molar ratios, and then run through a 1% agarose gel. The mobility of siRNA complexed with cationic liposomes was visualized by ethidium bromide staining. Lane 1, 1 kb plus ladder; lane 2, siRNA alone; lane 3, lipid/siRNA molar ratio 0.005:1; lane 4, lipid/siRNA ratio 0.05:1; lane 5, lipid/siRNA ratio 0.5:1; lane 6, lipid/siRNA ratio 1:1; lane 7, lipid/siRNA ratio 2.5:1; lane 8, lipid/siRNA ratio 5:1; lane 9, lipid/siRNA ratio 10:1; lane 10, lipid/siRNA ratio 25:1.

The size distribution patterns of the cationic liposome/siRNA complexes were affected by the complexation ratios, but not by the presence of serum. The mean diameters of COPA-L (Fig. 3A) and CAEC-L (Fig. 3B) were 147.12 ± 20.51 and 141.60 ± 9.11, respectively. Regardless of which serum was present, the mean diameters of the CAEC-L/siRNA complexes peaked at a ratio of 1:1, and then decreased as the molar complexation ratios

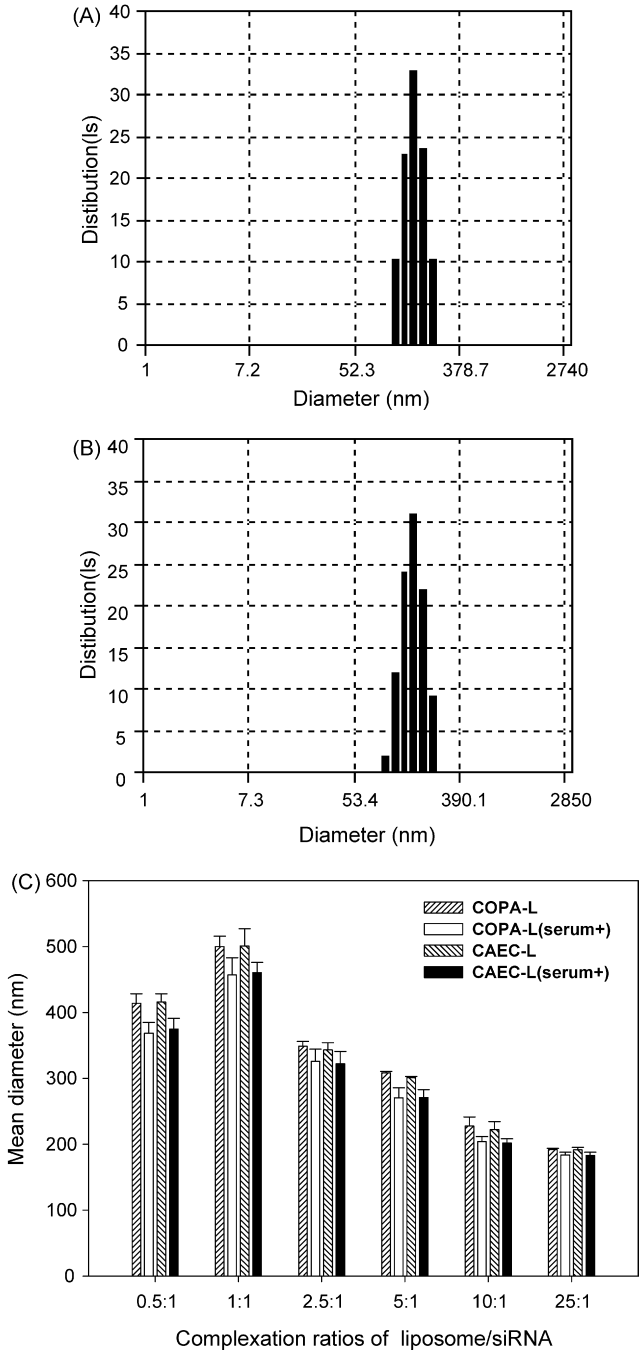

Fig. 3. The sizes of cationic liposomes and siRNA complexes. COPA-L (A) or CAEC-L (B) was suspended in HEPES buffer, and the sizes of the liposome samples were measured by light-dynamic scattering techniques. (C) COPA-L or CAEC-L was complexed with siRNA at various molar ratios. The sizes of the complexes were measured in the absence or presence of 10% serum.

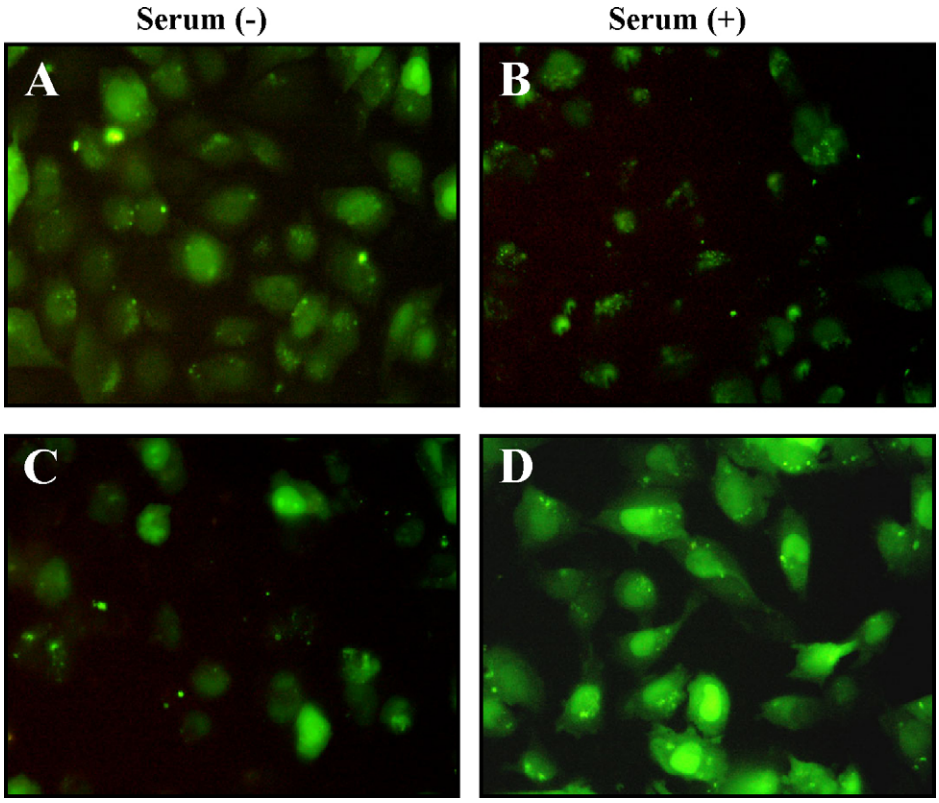

Fig. 4. Cellular uptake of fluorescein-labeled siRNA following liposome-mediated delivery. Hepa1–6 cells were incubated with complexes composed of fluorescein-labeled siRNA and CAEC-L (A and B) or COPA-L (C and D) in media with or without serum. The cellular uptake of siRNA was observed under fluorescence microscopy.

increased (Fig. 3C). Similar to the CAEC-L/siRNA complexes, the COPA-L/siRNA complexes did not show any significant size changes upon the addition of serum.

3.3. Effect of liposome compositions on cellular delivery of siRNA in serum

The compositions of cationic liposomes differentially affected cellular delivery of siRNA in the presence and absence of serum. A fluorescein-labeled siRNA marker was used to visualize the delivery of siRNA into cells that occurred at a 5:1 molar ratio of total cationic lipids to siRNA. Following delivery by CAEC-L, the cellular uptake of the fluorescein-labeled marker of siRNA by Hepa1–6 cells was lower in the presence of the serum (Fig. 4B) than under serum-free conditions (Fig. 4A). Conversely, delivery by COPA-L in the presence of serum revealed higher levels of siRNA uptake (Fig. 4D) than under serum free conditions (Fig. 4C). Additionally, a greater amount of fluorescein-labeled siRNA marker was delivered in the presence of serum than under serum-free conditions when A549 and Hela cells were tested (Fig. 5A, B and C).

3.4. RNAi effect of survivin-specific siRNA delivered in cationic liposomes

Next, to test whether siRNA delivered by COPA-containing cationic liposomes could reduce the mRNA levels of the target gene, specific RNA inhibitory function (RNAi effect) was studied. Murine survivin-specific siRNA was used as a marker to study the gene-specific reduction of mRNA expression levels in murine hepatoma Hepa1–6 cells. After delivery of survivin-specific siRNA in naked form or in complexes with cationic lipid carriers such as LA and COPA-L, the relative expression levels of survivin over the housekeeping gene, GAPDH, were compared by semi-quantitative RT-PCR (Fig. 6A). COPA-L had relative mRNA levels of 0.23 ± 0.08, which represented a 1.87-fold reduction in cellular expression of survivin compared to that of LA (Fig. 6A and B).

3.5. GFP silencing of GFP-specific siRNA delivered in cationic liposomes

siRNA delivered by COPA-L reduced the expression of the specific target gene at protein levels. GFP-293T cells either untreated (Fig. 7A) or treated with GFP-specific siRNA in naked form (Fig. 7B) showed the fluorescence of GFP. In contrast, GFP-293T cells treated with GFP-specific siRNA in complexes with COPA-L revealed significantly reduced levels of GFP fluorescence (Fig. 7C).

3.6. Apoptotic effect of survivin-specific siRNA delivered in cationic liposomes

Survivin-specific siRNA delivered by COPA-L significantly induced apoptosis in Hepa1–6 cells. Most of the cells treated with naked siRNA were not positively stained with annexin V-

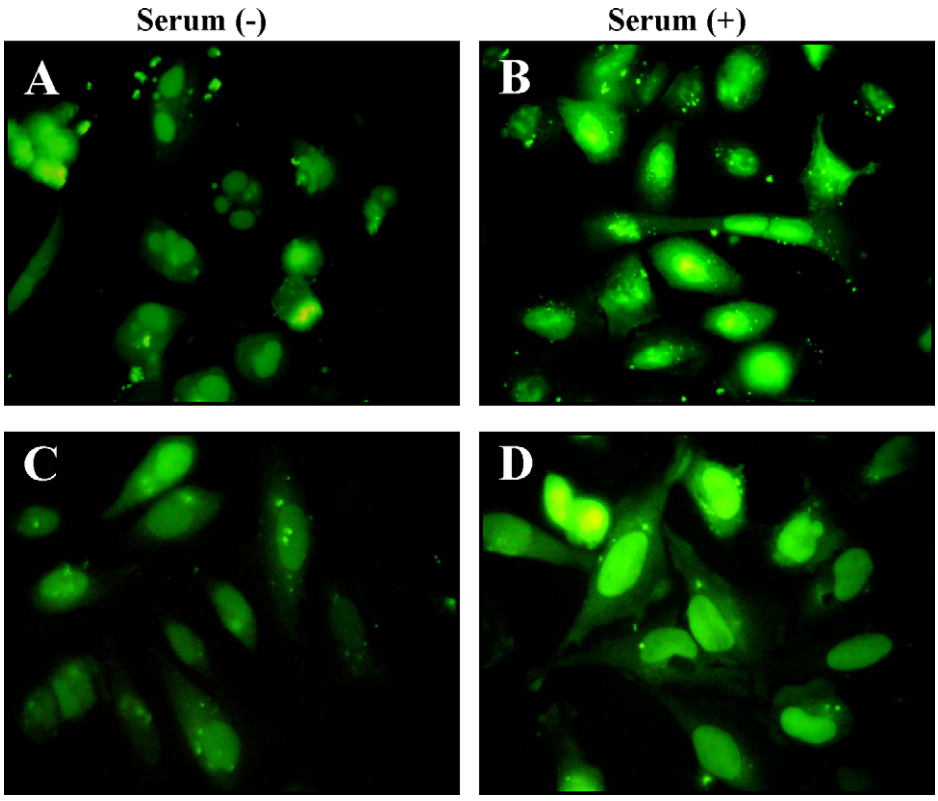

Fig. 5. Serum-dependent cellular uptake of fluorescein-labeled siRNA following delivery with COPA-L. A549 cells (A and B) or Hela (C and D) cells were incubated with complexes comprised of fluorescein-labeled siRNA and COPA-L in media with or without serum. The cellular uptake of siRNA was observed under fluorescence microscopy.

FITC, indicating low fraction (8.8%) of apoptotic cells (Fig. 8A). When the cells were treated with survivin-specific siRNA complexed to LA, the apoptotic population of siRNA and LA treated cells was 60.2% (Fig. 8B). The delivery of survivin-specific siRNA in complexes with COPA-L provided 78% of apoptotic cell populations (Fig. 8C).

3.7. Cell viability after delivery of siRNA in complexes with cationic liposomes

The cytotoxicity of cationic liposomes (LA, COPA-L) and scrambled siRNA complexes was tested in various cell lines, including Hepa1–6 (Fig. 9A), A549 (Fig. 9B), and Hela cells (Fig. 9C). Scrambled siRNA was used in this study to eliminate the possible cytotoxic effects of functional siRNA due to suppression of specific genes. There was no significant reduction in cell viability observed between the untreated group and the COPA-L-treated group when Hepa1–6 (Fig. 9A) and A549 cells (Fig. 9B) were tested. However, COPA-L and siRNA complexes produced significantly lower levels of cell viability than the untreated group in Hela cells (Fig. 9C). Groups treated with the COPA-L siRNA complex showed >80% cell viability in all cell lines.

4. Discussion

In this study, we demonstrated that cationic liposomes containing the newly synthesized cholesterol derivative, COPA, showed increased cellular siRNA delivery in the presence of serum.

To formulate the cationic liposomes, we synthesized two cholesterol derivatives of cationic charges. Cholesterol was used to synthesize the new cationic lipids since cholesterol itself has little toxicity due to its endogenous nature (Lv et al., 2006). Cholesterol has previously been used as the major lipid component of liposomes for the delivery of various genes (Li and Huang, 2006; Tagami et al., 2007) and chemical drugs (Maestrelli et al., 2006; Al-Jamal and Kostarelos, 2007), and recently, cholesterol derivatives have been studied for their ability to deliver siRNA. Cholesteryl oligoarginine was used to deliver vascular endothelial growth factor siRNA in a mouse tumor model (Kim et al., 2006). Additionally, cholesterol has been used to modify the structure of siRNA and cholesterol-linked siRNA has been studied to evaluate how useful it is for in vivo systemic application of siRNA (Soutschek et al., 2004).

In this study, DOPE was used to formulate cationic liposomes containing COPA or CAEC. DOPE has been widely used as a fusogenic lipid component of cationic liposomes used to deliver plasmid DNA and siRNA. The addition of DOPE to cationic liposomes has been reported to enhance cellular delivery of siRNA and reduce expression of the target genes of the siRNA when compared to the effect of liposomes composed of only cationic lipids (Hassani et al., 2005). Moreover, the addition of DOPE to cationic liposomes has been shown to provide higher transfection efficiency in vivo (Hattori et al., 2005). Further, it has been suggested that the presence of DOPE in cationic

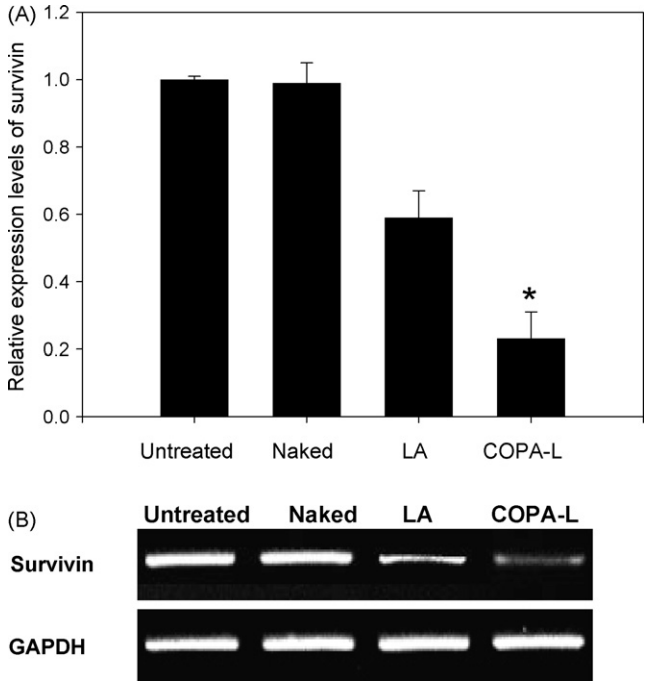

Fig. 6. mRNA expression levels of survivin after delivery of siRNA in complexes with cationic liposomes. Hepa1–6 cells were treated with survivin-specific siRNA alone or in complexes with cationic liposomes. Lipofectamine 2000 (LA) or COPA-L was used for complexation with siRNA. (A) The mRNA expression levels of survivin were normalized to those of GAPDH. The results are expressed as the mean ± S.E.M. of four independent experiments. (B) Representative pictures of survivin or GAPDH expression levels at various conditions are presented. *Significantly lower than the other groups ($p < 0.05$).

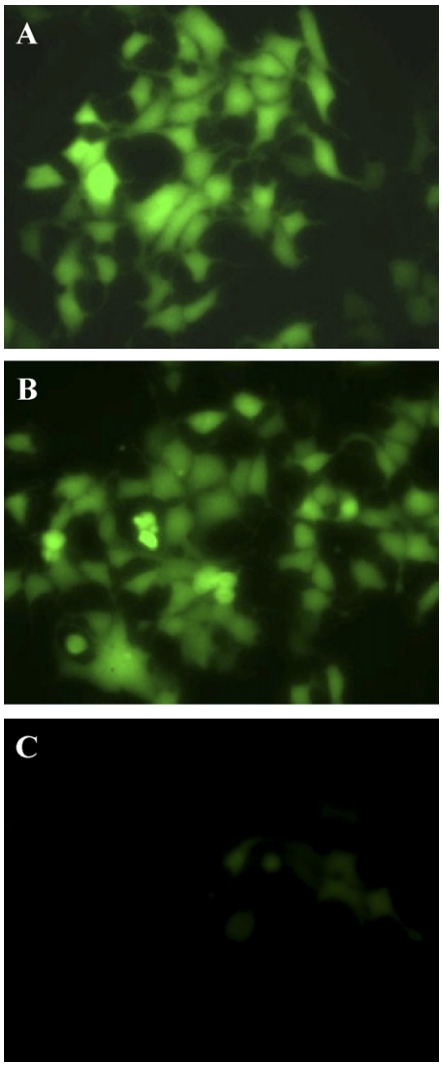

Fig. 7. Protein expression levels of GFP after delivery of GFP-specific siRNA in complexes with cationic liposomes. GFP-293T cells were treated with GFP-specific siRNA alone or in complexes with COPA-L. As a control, untreated GFP-293 T cells were used. GFP-293 T cells untreated (A), treated with naked siRNA (B) or with siRNA/COPA-L complexes (C) were observed under fluorescence microscopy, respectively.

liposomes might contribute to enhanced biodistribution to the target site, as well as intracellular sorting in target cells under in vivo conditions (Hattori et al., 2005). In this study, although both COPA-L and CAEC-L contained DOPE, only COPA-L showed enhanced delivery of siRNA in the presence of serum. Therefore, it is unlikely that the presence of DOPE in COPA-L significantly contributed to serum-enhanced cellular delivery of siRNA.

Although COPA and CAEC are both cationic cholesterol derivatives, the cellular siRNA delivery patterns of COPA-L and CAEC-L differed significantly in the presence of serum. This observation indicates that structures of lipids and the composition of cationic liposomes may be crucial for delivery of siRNA. It has been suggested that biophysical properties, such as size, charge density, and morphology of the resulting DNA complexes, determine the transfection efficiencies of non-viral vectors in serum (Esposito et al., 2006; Zhang and Anchordoquy, 2004). Non-viral vectors producing larger particle sizes with nucleic acids were reportedly effective at protecting DNA from attack by DNAse I (García et al., 2007) and increasing serum resistance (Almofti et al., 2003). Given the similarity of the sizes of the complexes formed between siRNA and COPA-L and those between siRNA and CAEC-L (Fig. 2A and B), it is unlikely that the size of the complex attributed to the different delivery efficiencies between the two liposomes. Moreover, the absence of a significant effect of serum on the sizes of the siRNA and COPA-L complexes (Fig. 2C) indicates that the higher rate of siRNA delivery in the presence of serum might not be due to the size changes of the complexes.

Although the underlying mechanisms of the serum-enhanced delivery of siRNA via COPA-L need further study, we cannot exclude the possibility that the cholesterol moiety of COPA may bind to certain ligand components of serum, enhancing cellular delivery via ligand-mediated endocytosis. In the case of cholesterol-linked siRNA, it has been suggested that the interaction of cholesterol-linked siRNA with serum albumin enhances tissue distributions via low-density lipoprotein receptors on cellular surfaces (Kim et al., 2006). Alternatively, the enhanced delivery of siRNA via COPA-L in the presence of serum might be in part due to the strong electrostatic interaction between COPA-L and siRNA against the serum-mediated dissociation (Lian and Ho, 2003).

Our results indicate that it may be possible to develop COPA-L for the delivery of siRNA under in vivo conditions. Low

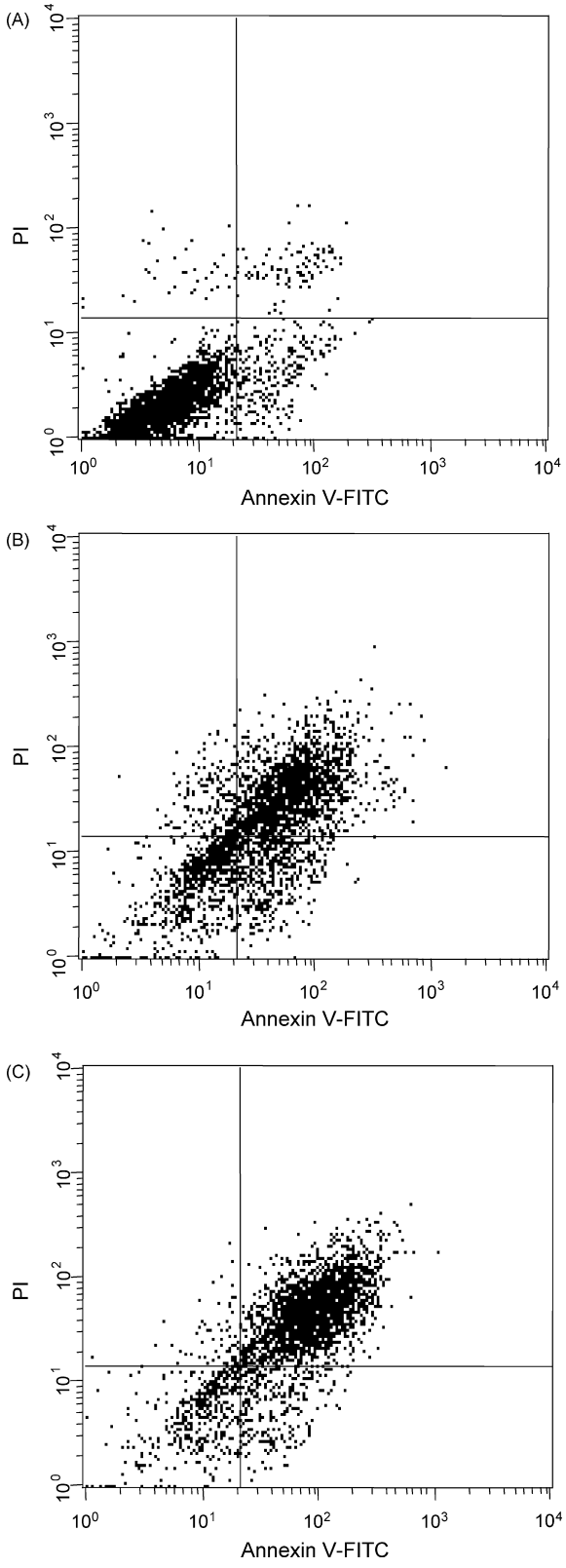

Fig. 8. Apoptotic effects of survivin-specific siRNA delivered in complexes with cationic liposomes. Hepa1–6 cells were treated with survivin-specific siRNA in naked form or complexes with cationic liposomes. COPA-L and LA were used for complexation with siRNA. The apoptotic populations of the cells treated with survivin-specific siRNA in a naked form (A), complexes with LA (B), or complexes with COPA-L (C) were analyzed by flow cytometry.

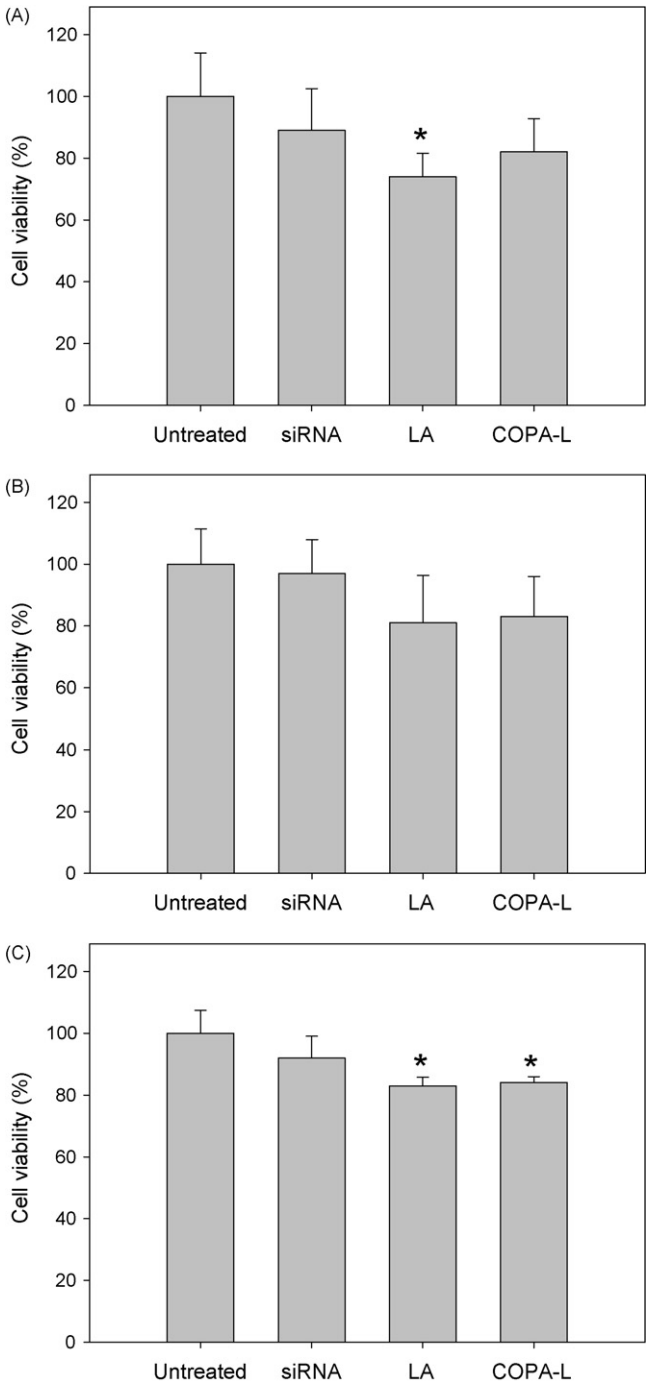

Fig. 9. Cytotoxicity of cationic liposomes in various cell lines. Hepa1–6 (A), A549 (B), or Hela (C) cells were treated with scrambled siRNA alone or in complexes with LA or COPA-L. After treatment of the cells under various conditions for 24 h, cell viability was measured by MTT assay. The results are expressed as the mean ± S.E.M. of four independent experiments. *Significantly lower than the untreated group ($p < 0.05$).

serum stability is a major obstacle of cationic liposome-mediated gene delivery and serum proteins are known to interact with cationic liposomes to disturb in vitro and in vivo transfection (Simberg et al., 2005). Pegylation has been employed to reduce the interaction between charged molecules in the serum and the liposome/gene complexes to increase serum stability. Although pegylation on liposome surfaces has been shown to prolong the circulation time of siRNA (Zimmermann et al., 2006), there is little information regarding the in vivo behavior of cationic liposomes of various compositions. Further studies to determine whether COPA-L could affect the pharmacokinetics and biodistributions of siRNA are needed. Additionally, the reduction of oncogenic survivin mRNA and the increased apoptosis of Hepa1–6 cells by complexes comprised of survivin-specific siRNA and COPA-L suggest that the newly synthesized cationic lipid, COPA, may be a useful component for cationic delivery vehicles of anticancer gene-based medicines.

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