Efficient delivery of Notch1 siRNA to SKOV3 cells by cationic cholesterol derivative-based liposome

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Abstract: A novel cationic cholesterol derivative-based small interfering RNA (siRNA) interference strategy was suggested to inhibit Notch1 activation in SKOV3 cells for the gene therapy of ovarian cancer. The cationic cholesterol derivative, N-(cholesterylhemisuccinoylamino-3-propyl)-N, N-dimethylenamine (DMAPA-chems) liposome, was incubated with siRNA at different nitrogen-to-phosphate ratios to form stabilized, near-spherical siRNA/DMAPA-chems nanoparticles with sizes of 100–200 nm and zeta potentials of 40–50 mV. The siRNA/DMAPA-chems nanoparticles protected siRNA from nuclease degradation in 25% fetal bovine serum. The nanoparticles exhibited high cell uptake and Notch1 gene knockdown efficiency in SKOV3 cells at an nitrogen-to-phosphate ratio of 100 and an siRNA concentration of 50 nM. They also inhibited the growth and promoted the apoptosis of SKOV3 cells. These results may provide the potential for using cationic cholesterol derivatives as efficient nonviral siRNA carriers for the suppression of Notch1 activation in ovarian cancer cells.

Keywords: siRNA, cationic cholesterol derivative, Notch1, ovarian cancer cells

Introduction

Epithelial ovarian cancer is the most lethal gynecological malignancy.¹ Because of its high incidence and mortality rates, ovarian cancer is a lethal and underrecognized detriment to women’s health all over the world. Despite the improvement in therapy, patients with advanced ovarian cancer have a low 5-year survival rate of only 10%–20%.² Traditionally, ovarian cancer is treated with platinum-based chemotherapy followed by surgical tumor debulking; however, cisplatin resistance is a major obstacle to the successful treatment of ovarian cancer. Several mechanisms have been reported to be related to the development of cisplatin resistance, including reduced uptake, enhanced efflux, increased inactivation, and evasion of apoptosis.³ Thus, developing a novel therapeutic approach is extremely important for the treatment of ovarian cancer.

In recent years, RNA interference approaches, especially utilizing small interfering RNA (siRNA), have received considerable attention in the treatment of ovarian cancer.⁴–⁶ The siRNA approach, as a therapeutic method of blocking neovascularization, was reported to have incomparable long-term effects as well as safety effects.⁶–⁷ The Notch signaling pathway plays a pivotal role in cellular development, proliferation, differentiation, and apoptosis. It has been shown to be involved in the carcinogenesis, progression, invasion, and neovascularization of many solid tumors.⁸ Notch1 can function as a tumor suppressor or as an oncogene depending on the cell type and context. It acts as a tumor inhibitor in murine skin tumors¹¹ and non-small-cell
lung cancer.\textsuperscript{12} In contrast, it acts as an oncogene in many other kinds of tumors, such as renal cancer, pancreatic cancer, cervical cancer, breast cancer, and prostate cancer.\textsuperscript{13} Notch1 is considered to act as an oncogene in ovarian cancer, and high Notch1 protein expression was discovered in ovarian cancers and corresponding ovarian carcinoma cell lines, including SKOV3 and A2780 cells.\textsuperscript{14,15} Notch1 expression has been reported to correlate to tumor differentiation status in ovarian cancer.\textsuperscript{16,17}

Although siRNA is small in size, siRNA transportation across the cytomembrane is hindered by its hydrophility and negative electric charge and is easily eliminated during in vivo circulation before arrival at the target site.\textsuperscript{18} Therefore, effective siRNA-mediated therapy relies heavily on the improvement of siRNA delivery carriers with high efficiency of transfection, the ability to easily escape the endosome, and minimal cytotoxicity.\textsuperscript{19} Nonviral gene delivery vectors, particularly cationic polymers (eg, chitosan, polyethyleneimine, and polylysine peptides), liposomes, and micelles, have attracted increasing attention in gene therapeutic stratagies.\textsuperscript{20–23} A typical cationic lipid generally consists of three parts: cationic head groups, linkers/linkages, and hydrophobic lipid tails. However, the exact combination of these three parts to achieve lower cytotoxicity and higher transfection performance is still a major challenge as it relies on the good understanding of the structure–activity relationships of lipid carriers. Compared with cationic lipid-based liposomes, the cationic cholesterol derivative-based liposomes are cheaper to get and also have lower cytotoxicity.\textsuperscript{24,25} Liu et al\textsuperscript{26} used nanoparticles based on hyper-forked cationic polysaccharide derivatives to investigate the delivery of siRNA into human retinal pigment epithelial (hRPE) cells to silence NF-κB gene expression. They found that a 3-(dimethylamino)-1-propylamine-conjugated glycogen (DMAPA-Glyp) derivative could condense siRNA efficiently owing to its higher branching structure. The DMAPA-Glyp derivative showed less toxicity to hRPE cells and facilitated better protection of siRNA from degradation by nucleases. Due to their small sizes and high zeta potentials, the DMAPA-Glyp/siRNA nanoparticles effectively transferred siRNA to the hRPE cells. This led to significant suppression of the expression of NF-κB p65 mRNA and protein in those cells transfected with DMAPA-Glyp/siRNA nanoparticles. In a study by Hattori et al,\textsuperscript{27} they synthesized a new cationic cholesteryl triamine (OH-N-Chol, (3S)-N-(2-(2-hydroxyethylamino) ethylamino) ethyl) cholesteryl -3-carboxamide) with an ethylenimine extension and prepared cationic nanoparticles (NP-N) composed of OH-N-Chol and Tween 80 which could form an siRNA/NP-N nanoplex 200 nm in size on vortex mixing. The NP–N nanoplex was internalized via macroinocytosis-mediated endocytosis and produced a high level of gene knockdown. We recently reported that N-(cholesterylhemisuccinoyl-amino-3-propyl)-N, N-dimethylamine (DMAPA-chems) liposomes showed good blood compatibility and low cytotoxicity and could efficiently deliver plasmid DNA in vitro.\textsuperscript{28} However, whether DMAPA-chems cationic cholesterol derivatives can effectively deliver siRNA into SKOV3 cells and efficiently knockdown the target gene have not yet been elucidated.

In this study, DMAPA-chems liposomes were successfully synthesized, and the interference strategy based on cationic cholesterol derivative liposomes was used to develop an excellent siRNA delivery system for efficient cellular uptake and knockdown of the Notch1 gene in SKOV3 human ovarian cancer cells. The siRNA/DMAPA-chems complexes were estimated for cytotoxicity, stability, special gene silencing efficiency, cell proliferation, and apoptosis at different nitrogen-to-phosphate (N/P) ratios and at different doses of siRNA in SKOV3 cells.

Materials and methods

Materials

N, N-dimethyl propylene diamine (DMAPA) was purchased from Aladdin (Shanghai, People’s Republic of China), N-hydroxysuccinimide (NHS) was supplied by Sigma-Aldrich (St Louis, MO, USA), cholesterol was procured from Sinopharm (Shanghai, People’s Republic of China), soybean phospholipid S100 was bought from Lipoid (Ludwigshafen, Germany), polymerase chain reaction (PCR) primers were supplied by Generay (Shanghai, People’s Republic of China), and negative control (NC) siRNA and Notch1-homo-siRNA or a green fluorescent agent FAM at the 3’ end of the sense strand or antisense strand were procured from GenePharma (Shanghai, People’s Republic of China). Lipofectamine 2000 and Trizol were obtained from Thermo Fisher Scientific (Waltham, MA, USA). PrimeScript RT Reagent Kit and SYBR Premix Ex Tap Reagent Kit were obtained from Takara (Dalian, People’s Republic of China). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo (Tokyo, Japan). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Multi Sciences (Shanghai, People’s Republic of China). HRP-conjugated goat anti-mouse IgG (H + L), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer, and anti-β-actin antibody were procured from Auragene (Hunan, People’s Republic of China), and anti-Notch1 antibody was provided by Abcam (Cambridge, MA, USA). The SKOV3 cells were obtained...
from the American Type Culture Collection (ATCC, HTB-77), McCoy’s 5A, trypsin/ethylenediaminetetraacetic acid (EDTA), and phosphate buffer solution (PBS) were bought from Genom (Hangzhou, People’s Republic of China). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific.

Preparation of the siRNA/DMAPA-chems nanoparticles

A novel kind of cholesterol cationic lipids (DMAPA-chems) was designed and synthesized, in which natural cholesterol was employed as a hydrophobic block, and DMAPA served as a kind of cationic head group; the hydrophobes and head groups were connected through flexible 1,4-succinic anhydride linkers with biodegradable carbonate ester linkage bonds. The synthesis of DMAPA-chems is accomplished in several simple and easy steps as previously described.20 Forty milliliters of triethylamine was added to a mixture of 5.0 g commercially available cholesteryl and 4.0 g succinic anhydride in 150 mL tetrahydrofuran (THF) at 80°C. After 48 hours, the solvent was removed by vacuum distillation at 40°C. The reactant was dissolved with 60 mL ethanol. The combined organic phase was extracted with 100 mL deionized water and recrystallized twice from acetone to obtain the cholesteryl hemisuccinate. The obtained cholesteryl hemisuccinate was added to NHS to excite the cholesteryl hemisuccinate to a semistable amine-reactive activated NHS-ester. DMAPA-chems was prepared by reacting 0.5 g activated ester with 10 mL DMAPA (1.2% dichloromethane [CH₂Cl₂] solution) in 20 mL CH₂Cl₂ on ice for 1 hour, then precipitated in cold isopropanol, purified by cold ether, and lyophilized.

To produce liposomes, 19 mg DMAPA-chems and 50 mg phospholipid S100 were combined using the film dispersion method. The cationic cholesterol derivative DMAPA-chems liposomes were at a concentration of 3.8 mg/mL. The lyophilized siRNA was dissolved into RNase-free water, forming a 20 mM stock solution. The siRNA stock solution in part was diluted in Opti-MEM reduced serum media (Thermo Fisher Scientific) of 200 μL and incubated for 5 minutes at room temperature. Then, the DMAPA-chems solution was added at different N/P ratios. The mixtures were gently agitated and then incubated at room temperature for 20 minutes before use. Nanoparticles containing FAM-siRNA were obtained in the same way in the darkroom.

Particle size, zeta potential, and morphology

The particle size and zeta potential of siRNA/DMAPA-chems nanoparticles at different N/P ratios were measured using dynamic light scattering with a laser Doppler anemometry (Delsa Nano C A53878, Beckman Coulter, Brea, CA, USA). The diameter and zeta potential values were measured in triplicate. The morphology of siRNA/DMAPA-chems complexes (N/P =100) was observed using transmission electron microscopy (JEM-1230, JEOL, Tokyo, Japan), and the nanoparticles were negatively stained using 2 wt% aqueous uranyl acetate.

Agarose gel electrophoresis

Electrophoresis tests were carried out to observe the condensation ability of the cationic cholesterol derivatives to siRNA. Ten microliters of the siRNA/DMAPA-chems nanoparticles with various N/P ratios in the range of 2–80 and naked siRNA were loaded onto 1.5% agarose gels containing 1 μL of 2% bromophenol blue loading buffer and run with Tris/Borate/EDTA buffer at 100 V for 20 minutes. siRNA retardation was then detected and photographed with a gel document imaging system (Gel DocTMXR+, Bio-Rad Laboratories Inc., Hercules, CA, USA).

Stability analysis

To estimate the protective property of the cationic cholesterol derivative against siRNA degradation, the siRNA/DMAPA-chems nanoparticles (N/P=50) containing 1 μg siRNA were incubated with 25% FBS and 0.1 mg/mL RNase A at 37°C for 24 hours. At the same time, naked siRNA was incubated with 25% FBS (the NC). Samples were incubated for 1 hour with excess heparin solution at a siRNA/heparin weight ratio of 1:5 to ensure siRNA released from the nanoparticles completely.26 The samples were assessed using an agarose gel document imaging system, as described earlier, and the integrity of siRNA was detected.

Cell culture

SKOV3 cells were cultured in McCoy’s 5A, which was supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin; the cells were then incubated at 37°C in a 5% CO₂ atmosphere. After rinsing with PBS, the cells were detached using trypsin/EDTA and then resuspended in a fresh medium for subsequent experiments.

Cytotoxicity assay

To evaluate the cytotoxicity of the cationic cholesterol derivatives and the siRNA/DMAPA-chems nanoparticles, a CCK-8 assay was performed. SKOV3 cells (6×10⁴ per well) were plated into 96-well plates and incubated in a humidified incubator (37°C, 5% CO₂) for 24 hours for adherence. The medium
was then replaced with 100 μL of fresh medium containing DMAPA-chems liposomes at different concentrations ranging from 10 to 800 μg/mL. siRNA/DMAPA-chems nanoparticles with various N/P ratios from 1 to 120 were added for the cytotoxicity assay. After the cells were incubated for 24, 48, and 72 hours, 10 μL of the CCK-8 solution was added to each well of the experiment plate, and incubated for 1.5 hours. The absorbance was measured at 450 nm using a microplate reader (Varioskan Frash, Thermo Fisher Scientific). All experiments were repeated in quintuplicate.

Quantification of cellular uptake
Flow cytometry analysis was used to analyze the cellular uptake of siRNA/DMAPA-chems nanoparticles. SKOV3 cells were plated into six-well plates and incubated in complete McCoy’s 5A until they reached 50% confluence. The cells were cultured with FAM-siRNA/DMAPA-chems complexes (N/P =20, 40, 60, 80, 100, or 120) at a FAM-siRNA dose of 50 nM. After incubation about 5 hours, the medium was discarded and the cells were rinsed twice with cold PBS twice. The cells were then trypsinized, washed with cold PBS three times, and subjected to analysis using a flow cytometer (BD FACSVerse, BD Biosciences, San Jose, CA, USA).

Quantitative real-time PCR (qRT-PCR) analysis
SKOV3 cells were seeded into six-well plates (2×10^5 cells/well), incubated for 24 hours, then transfected with Notch1 siRNA/DMAPA-chems complexes (N/P =80, 100, or 120) at an siRNA dose of 50 nM. In addition, different siRNA doses (30, 50, 80, or 100 nM) of Notch1 siRNA/DMAPA-chems complexes at a fixed N/P ratio of 100 were also investigated. SKOV3 cells without any disposure were used as the control, while SKOV3 cells cultured with the nonspecific siRNA/Lipo complex were grouped as the NC. Notch1 siRNA/lipofectamine 2000 served as a positive control. The cells were then cultured at 37°C for 5 hours and rinsed with PBS solution. The medium was substituted with fresh serum-supplemented medium and further cultured at 37°C. After incubation for 48 hours, Trizol reagent was used to extract total RNA from SKOV3 cells. qRT-PCR was performed as described in the SYBR Premix Ex Tap kit procedure instructions: denaturation, 1 cycle at 95°C for 2 minutes; amplification, 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds; final extension, 70 cycles at 95°C for 1 minute, 60°C for 1 minute, 60°C to 95°C (10 s/ cycle, 0.5°C/cycle), using a sequence detection system (ABI VIIA 7DX, Thermo Fisher Scientific). A value of 2^ΔΔCT was figured out to estimate Notch1 mRNA expression.

Western blot analysis
SKOV3 cells were seeded into six-well plates and incubated for 24 hours, which was followed by incubation with the Notch1 siRNA/DMAPA-chems nanoparticles (N/P =100) at an siRNA dose of 50 nM for 5 hours. Afterward, the medium was replaced with fresh McCoy’s 5A supplemented with 10% FBS. After an additional 24, 48, and 72 hours of incubation, the medium was discarded. After washing with PBS twice, the cells were collected for protein extraction. To each well, 150 μL of radioimmunoprecipitation assay lysis buffer was added and the six-well plates were shaken on an ice-bath for 45 minutes. The lysate was gathered and centrifuged at 14,000×g at 4°C for 10 minutes. Forty micrograms of total protein per lysate were separated on an 8% SDS-PAGE gel and then transferred to a nitrocellulose membrane. The membrane was immersed in 5% nonfat milk Tris buffer for 1 hour and then cultured overnight with an anti-Notch1 antibody. An anti-β-actin antibody acted as the Western blot loading control. The membrane was incubated with a secondary antibody for 1 hour. After rinsing with Tris buffer for 30 minutes, a near-infrared two-color laser scanner imaging system (Image Quant LAS 4000 mini, GE Healthcare Life Sciences, Chicago, IL, USA) was used to scan and photograph the membrane.

Cell proliferation assay
CCK-8 assay was also used to observe the cell proliferation of the Notch1 siRNA/DMAPA-chems at various N/P ratios (80, 100, and 120), with NC siRNA/DMAPA-chems (N/P =80, 100, or 120) as a NC. SKOV3 cells were plated into 96-well plates at an initial density of 6×10^3 cells/well, incubated in complete McCoy’s 5A for 24 hours, and then transfected with 50 nM of siRNA. After transfection for 24, 48, and 72 hours, 10 μL of the CCK-8 solution was added and incubated for 1.5 hours. The absorbance was measured at 450 nm using a microplate reader (as described earlier).

Cell apoptosis assay
An Annexin V-FITC apoptosis detection kit was utilized for evaluation of the percentage of apoptosis induced by Notch1 siRNA/DMAPA-chems nanoparticles (N/P =100) at an siRNA dose of 50 nM, according to the protocol provided by the manufacturer. After transfection for 48 hours, floating and trypsinized adherent cells were gathered separately and rinsed with cold PBS three times. The cells were resuspended in 500 μL of 1× Annexin V binding buffer containing 5 μL Annexin V-FITC and 5 μL propidium iodide and incubated in
a darkroom at 4°C for 15 minutes. Analysis was immediately carried out by using a flow cytometer (described earlier).

**Statistical analysis**

All experiments were performed three or more times independently. Statistical analyses were carried out using one-way analysis of variance (GraphPad Prism 5, La Jolla, CA, USA) and Student’s two-tailed t-test to analyze statistically significant differences between the data of various experimental groups. A value of \( P < 0.05 \) was considered statistically significant.

**Results and discussion**

**Preparation of the siRNA/DMAPA-chems nanoparticles**

Recently, novel cationic cholesterol derivatives have received attention as efficient nonviral gene delivery vectors due to their high gene transfection efficiency.\(^{29,30}\) In this study, DMAPA-chems was successfully synthesized from cholesteryl, succinic anhydride, NHS, and DMAPA (data has been reported). DMAPA-chems and phospholipid S100 were used to produce liposomes by the film dispersion method. DMAPA-chems liposomes were added to siRNA solution at different N/P ratios for delivery of siRNA into SKOV3 cells in an effort to downregulate Notch1 gene expression (Figure 1).

**Particle size, zeta potential, and morphology assay**

The particle size and zeta potential of siRNA/DMAPA-chems complexes were measured using dynamic light scattering. As shown in Figure 2A, the zeta potentials were found to be positive when siRNA/DMAPA-chems complexes formed at N/P \( > 10 \). Meanwhile, the zeta potentials of the siRNA/DMAPA-chems complexes increased in the range of +40 to +50 mV when their N/P ratios increased, which is attributed to the effective

![Figure 1](https://www.dovepress.com/)

**Figure 1** The synthetic mechanism of action of the cationic cholesterol derivative DMAPA-chems and the formulation of siRNA/DMAPA-chems complexes.

**Abbreviations:** DMAPA-chems, N-(cholesterylhemisuccinoyl-amino-3-propyl)-N,N-dimethylamine; siRNA, small interfering RNA.
condensation of siRNA. The particle size of the siRNA/DMAPA-chems complexes decreased following the N/P ratios increasing, but was still in the range of 100–200 nm. This suggested that the complexes became progressively smaller the further the siRNA condensation processed.

The morphology of siRNA/DMAPA-chems complexes was investigated by transmission electron microscopy. As shown in Figure 2B, the complexes at an N/P ratio of 100 were observed to have a near-spherical shape. There was excellent cellular uptake owing to the positive zeta potential, ideal particle size, and spherical shape of the siRNA/DMAPA-chems complexes.

Agarose gel electrophoresis and stability analysis

Agarose gel electrophoresis assay was utilized to observe the formation of the siRNA/cholesterol derivative nanoparticles, utilizing naked siRNA as a control. As shown in Figure 3A, the migration of siRNA was entirely arrested when the siRNA/DMAPA-chems N/P ratios exceeded 30. This indicated that cholesterol derivative DMAPA-chems liposomes could condense siRNA to the complexes, which probably aids in cellular uptake, elevates serum stability, and provides better protection from nuclease degradation.

Higher serum stability as well as lower cytotoxicity are pivotal for successful siRNA delivery, both in vitro and in vivo.31 The stability of siRNA/DMAPA-chems complexes (N/P =100) was evaluated in 25% FBS (Figure 3B). Naked siRNA control group was totally degraded within 24 hours of cultivation, while the migration of siRNA was still observed after siRNA/DMAPA-chems complexes treated with heparin solution were cultivated in 25% FBS. To analyze the stability of siRNA/DMAPA-chems complexes (N/P =100) against nuclease, both naked siRNA and siRNA/DMAPA-chems complexes were exposed to 0.1 mg/mL RNase A at 37°C for 24 hours. The naked siRNA was completely degraded in RNase A solution within 0.5 hour, whereas the migration of siRNA could be still be observed after siRNA/DMAPA-chems complexes were incubated in RNase A solution for 24 hours (Figure 3B). These results indicate that siRNA/DMAPA-chems complexes exhibit an excellent protective effect against RNase A for siRNA in serum.

Cytotoxicity assay

CCK-8 assay was used to observe the cytotoxicity of the cationic cholesterol derivative DMAPA-chems liposome in SKOV3 cells. As shown in Figure 4A, cytotoxicity increased with increasing concentrations of DMAPA-chems liposome. At a DMAPA-chems liposome dose less than 80 μg/mL, the derivative showed little cytotoxicity against SKOV3 cells. The viability of SKOV3 cells remained above 80%. The low toxicity may be attributable to the lower molecular weight of the DMAPA-chems derivative as well as better biocompatibility of the cationic cholesterol derivative.20,32

A positive charge on nanoparticles is known to facilitate cellular uptake via a nonspecific electrostatic interaction between the positively charged nanoparticles and negatively charged cell membranes, but it is also considered a major cause of cytotoxicity.33 Thus, evaluating the cytotoxicity of the siRNA/cholesterol derivative nanoparticles at different N/P ratios was very important. As shown in Figure 4B, the siRNA/cholesterol derivative nanoparticles exhibited low cytotoxicity in the SKOV3 cells owing to the low cytotoxicity of the cholesterol derivative liposome. As the N/P ratio reached 120, cells treated

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**Figure 2** Particle size, zeta potential, and morphology assessment of siRNA/DMAPA-chems nanoparticles with different N/P ratios.

**Notes:** (A) Size distribution and zeta potential of siRNA/DMAPA-chems nanoparticles with different N/P ratios by DLS. (B) TEM images of siRNA/DMAPA-chems nanoparticles (N/P =100) (scale bar =500 nm, 80 kW).

**Abbreviations:** DLS, dynamic light scattering; TEM, transmission electron microscope; siRNA, small interfering RNA; DMAPA-chems, N-(cholesterylhemisuccinoyl-amino-3-propyl)-N,N-dimethylamine; N/P, nitrogen-to-phosphate.
Cholesterol derivative liposome for siRNA

with the siRNA/cholesterol derivative nanoparticles for 72 hours maintained a relatively high viability of about 80%.

Quantification of cellular uptake

Cellular uptake efficiency of gene/cationic polymers complexes is considered to depend on the surface properties and particle sizes.\(^{34,35}\) It has been reported that gene/cationic polymer complexes with positive charge and particle sizes of fifty to several hundred nanometers would be suitable for the endocytosis of complexes and for superior gene delivery.\(^{36}\) The zeta potentials and particle sizes of the prepared siRNA/DMAPA-chems complexes (N/P >20) were in the range of +40 to +50 mV and 100–200 nm, respectively. These properties might facilitate the entry of the siRNA/DMAPA-chems nanoparticles into the SKOV3 cells as expected. The SKOV3 cell uptake of the siRNA/DMAPA-chems nanoparticles was evaluated using flow cytometry and compared with the FAM-siRNA/Lipo 2000 complex as the positive control and the naked FAM-siRNA as the NC. The siRNA/DMAPA-chems nanoparticles displayed significantly more fluorescent cells than the naked FAM-siRNA cells group, and an increase in the N/P ratio resulted in an increasing percentage of fluorescence in Figure 5A. At an N/P ratio of 100, the cellular uptake efficiency was 93.95%±0.19%, which was not significantly different from that observed for the siRNA/Lipo-positive control group (97.66%±1.02%).

Cellular uptake was further detected by the accumulation and spread of green fluorescence of FAM-siRNA in the cytoplasm, as observed in the inverse fluorescent and confocal microscopy images (Figure 5B and C). The SKOV3 cells were incubated for 24 hours. After 6 hours of incubation with the FAM-siRNA/DMAPA-chems nanoparticles

Figure 3 Agarose gel electrophoresis and stability analysis of siRNA/DMAPA-chems complexes.

Notes: (A) Agarose gel electrophoresis retardation assay of siRNA/DMAPA-chems complexes at different N/P ratios at a fixed siRNA dose of 1 μg. (B) Protection and release assay of siRNA/DMAPA-chems nanoparticles (N/P =100, at an siRNA dose of 1 μg) in FBS (25%) and RNase A solution (0.1 mg/mL), visualized on a 1.5% agarose gel.

Abbreviations: siRNA, small interfering RNA; FBS, fetal bovine serum; DMAPA-chems, N-(cholesterylhemisuccinoyl-amino-3-propyl)-N, N-dimethylamine; N/P, nitrogen-to-phosphate.

Figure 4 Cytotoxicity assay of DMAPA-chems liposomes and siRNA/DMAPA-chems complexes.

Notes: (A) Viability of SKOV3 cells incubated with the DMAPA-chems liposomes at various concentrations in the range from 10 to 800 μg/mL (n=3) for 24, 48, and 72 h. (B) Viability of SKOV3 cells incubated with the siRNA/DMAPA-chems complexes at various N/P ratios from 1 to 120 (n=3) for 24, 48, and 72 h.

Abbreviations: siRNA, small interfering RNA; DMAPA-chems, N-(cholesterylhemisuccinoyl-amino-3-propyl)-N, N-dimethylamine; N/P, nitrogen-to-phosphate.
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(N/P = 100), the spread of green fluorescence of FAM-siRNA in the cytoplasm indicated endocytosis and endosome escape of the siRNA/DMAPA-chems nanoparticles, which suggested the DMAPA-chems derivative has a good buffering capability.

qRT-PCR analysis
Notch1 gene inhibition by Notch1 siRNA/DMAPA-chems complexes (N/P = 80, 100, or 120) at an siRNA dose of 50 nM as well as different siRNA doses (30, 50, 80, or 100 nM) of Notch1 siRNA/DMAPA-chems complexes at an N/P ratio of 100 were evaluated at the mRNA level using qRT-PCR analysis. As shown in Figure 6A, at an siRNA dose of 50 nM, the expression efficiency of Notch1 mRNA was significantly reduced by transfection with Notch1 siRNA/DMAPA-chems complexes with different N/P ratios and was not significantly different from lipofectamine 2000. As shown in Figure 6B, at an N/P ratio of 100, Notch1 siRNA/DMAPA-chems complexes at an siRNA dose of 50 nM displayed the most efficient target gene silencing with a Notch1 mRNA expression
of 0.22±0.07. These results indicate that the gene expression suppression efficacy relies heavily on cellular uptake, which is closely associated with the size, charge, and morphology of the siRNA/DMAPA-chems nanoparticles.

Western blot analysis
Western blot assay was used to measure the protein expression level of Notch1 in SKOV3 cells. As shown in Figure 7, SKOV3 cells incubated with the Notch1 siRNA/DMAPA-chems nanoparticles had lower levels of Notch1 protein with increasing incubation times, as compared with the NC group (P<0.05). At 72 hours, the Notch1 protein expression efficiency decreased to 26.47±6.23%. There was no significant difference between the Notch1 siRNA/DMAPA-chems nanoparticles group and the positive control group (P>0.05).

Cell proliferation and apoptosis assay
SKOV3 cells were transfected with Notch1 siRNA/DMAPA-chems nanoparticles at various N/P ratios (80, 100, and120) and NC siRNA/DMAPA-chems nanoparticles (N/P=80, 100, and 120) were examined 24 hours later to confirm that the cell line was stable. As shown in Figure 8A, the cell growth was significantly inhibited at 24, 48, and 72 hours after treatment with Notch1 siRNA/DMAPA-chems nanoparticles.
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Figure 8 Proliferation and apoptosis assay of SKOV3 cells.

Notes: (A) The viability of SKOV3 cells after transfection with NC siRNA/DMAPA-chems complexes (N/P = 80, 100, and 120) and Notch1 siRNA /DMAPA-chems complexes (N/P = 80, 100, and 120) with 50 nM of siRNA. The results represent the mean ± SD (n=5). (B) Annexin V-FITC apoptosis detection kit was used to measure the percentage of apoptosis induced by NC siRNA/DMAPA-chems complexes (N/P = 100) and Notch1 siRNA /DMAPA-chems complexes (N/P = 100) with 50 nM of siRNA. All the data are presented as mean ± SD, n=4. **P, 0.01.

Abbreviations: NC, negative control; siRNA, small interfering RNA; n, number; SD, standard deviation; DMAPA-chems, N-(cholesterylhemisuccinoyl-amino-3-propyl)-N,N-dimethylamine; N/P, nitrogen-to-phosphate; FITC, fluorescein isothiocyanate; ns, no significant differences; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

(N/P =80, 100 and 120), while SKOV3 cells treated with NC siRNA/DMAPA-chems nanoparticles (N/P =80, 100, and 120) showed no statistical significant change in growth, indicating that siRNA/DMAPA-chems nanoparticles have minimal toxic effects on the cells.

We have reported that imbalances between cell proliferation and the rate of apoptosis allow cancer to grow uncontrolled.23 Thus, we analyzed the effects of NC siRNA/DMAPA-chems nanoparticles and Notch1 siRNA/DMAPA-chems nanoparticles at the same N/P ratio (N/P =100). As shown in Figure 8B, treatment with Notch1 siRNA/DMAPA-chems nanoparticles increased the percentage of apoptotic cells (P<0.01), whereas treatment with NC siRNA/DMAPA-chems nanoparticles did not show any significant differences compared with the blank cell group. The data suggested that Notch1 siRNA/DMAPA-chems nanoparticles could influence
both growth and survival of SKOV3 cells. Furthermore, these findings were similar to other reports that indicated that blocking active Notch1 leads to apoptosis of cells and inhibition of cell proliferation.\textsuperscript{16,37–39}

**Conclusion**

In this study, we utilized a novel cationic cholesterol derivative-based liposome, DMAPA-chems, for efficient delivery of siRNA into human SKOV3 ovarian cancer cells to inhibit Notch1 gene expression. The siRNA/DMAPA-chems nanoparticles with a size of 100–200 nm, a positive charge of 40–50 mV, and a near-spherical morphology are very stable in serum and RNase solution. Furthermore, the siRNA/DMAPA-chems nanoparticles showed low toxicity against SKOV3 cells and had excellent cellular uptake and gene silencing efficiency. This study also demonstrated that siRNA/DMAPA-chems nanoparticles could inhibit the growth of SKOV3 cells and promote apoptosis. These in vitro results imply that the cationic cholesterol derivative-based DMAPA-chems liposomes may be a good vector for siRNA delivery in vivo.

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**Disclosure**

The authors report no conflicts of interest in this work.

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