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Development of cholesteryl peptide micelles for siRNA delivery

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Abstract

Despite of the rapid progress in the siRNA field, developing a safe and efficient delivery system of siRNA remains to be an obstacle in the therapeutical application of siRNA. The purpose of this study is to develop an efficient peptide-based siRNA delivery system for cancer therapy. To this end, cholesterol was conjugated to a series of peptides composed of lysine and histidine residues. The resultant cholesteryl peptides were characterized, and their potential for siRNA delivery was evaluated. Our results indicate that short peptides (11–21 mer) composed of various numbers of lysine and histidine residues alone are not sufficient to mediate efficient siRNA delivery. However, the amphiphilic cholesteryl peptides can self-assemble to form a micelle-like structure in aqueous solutions, which significantly promotes the siRNA condensation capability of the peptides. The cholesteryl peptides form stable complex with siRNA and effectively protect siRNA from degradation in rat serum up to three days. Furthermore, the cholesteryl peptides efficiently transfect siRNA into different cancer cells and trigger potent gene silencing effect, whereas peptides without cholesterol modification cannot deliver siRNA into the cells. In addition, one of the cholesteryl peptides Chol-H3K2s displays comparable cellular uptake and gene silencing effect but less cytotoxicity compared with branched polyethylenimine (bPEI) and Lipofectamine-2000. Our results reveal that the cholesteryl peptides possess great potential as an efficient siRNA delivery system.

Keywords

siRNA; cholesterol; cholesteryl peptide; micelle; histidine; CMC

1. Introduction

Since its discovery in 1998, RNAi has been explored as a powerful research tool for gene function study (1, 2). Meanwhile, due to the potent and specific knockdown activity against target genes, synthetic siRNAs also hold great promise as therapeutic agents for a variety of incurable diseases, such as HIV infection, genetic disorders and cancers (3–5). Unfortunately, lack of efficient and safe delivery systems remains to be a major obstacle for the application of siRNA in the clinic. Due to its susceptibility to plasma nuclease, siRNA's half-life in the plasma is within several minutes after intravenous injection (6). Moreover, naked siRNAs have poor cellular entry due to the high molecular mass and polyanionic

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feature. To circumvent these barriers, numerous siRNA delivery systems, including viral and non-viral vectors, have been extensively investigated. Although viral vectors provide high transduction efficiency and stable inhibition of gene expression, their application is restricted due to safety concerns, such as immunogenicity and potential mutagenicity. As a result, growing efforts have been devoted to the development of non-viral systems.

Currently, the most common non-viral delivery strategy is to pack siRNAs into nanoscale polycation complexes, which are formed through electrostatic interaction between nucleic acids and cationic carriers, such as cationic lipids, polymers or peptides. The formation of polycation complexes not only facilitate the cellular uptake of siRNA, but also protect siRNA against nuclease degradation. In general, siRNA complexes are internalized into cells via endocytosis. To reach the site of action in the cytoplasm, siRNA must escape from the endosome compartments and avoid lysosomal degradation. Therefore, a functional moiety that can disrupt endosome integrity is essential in a siRNA delivery system.

Among various non-viral siRNA delivery systems, peptide-based carriers have gained considerable attention because of the versatility of side-chain groups, ease of synthesis and chemical modification, and biodegradability. For example, cell-penetrating peptides (CPPs) have been utilized to form complex with siRNA via electrostatic interaction. Significant gene silencing effects were achieved by different CPP sequences, such as MPG (7, 8), CADY (9) and Endoporter (8, 10). Compared with cationic polymers, such as polyethylenimine (PEI) and PLL, short cationic peptides are relatively less efficient in condensing siRNA to form stable complexes. Linear or branched peptide polymers have therefore been utilized to overcome this problem. Stevenson et al. reported a group of reducible peptide polycations that were prepared from the oxidation of histidine-rich $CH_6K_3H_6KC$ monomers. The resultant peptide polymers (36–80 kDa) are able to form stable complex with siRNA and readily release the siRNA under a reducing environment (11). In another study, Liang et al. synthesized a series of branched peptide polymers composed of histidine and lysine and identified one branched peptide, H3K8b, that can efficiently deliver siRNA into cells (12).

Among the peptide-based siRNA delivery systems, only a few of them possess high potency as lipid-based delivery systems without inducing cytotoxicity. In this work, we developed a novel strategy by attaching cholesterol to the N-termini of hydrophilic peptides. The cholesteryl peptides are able to form a micelle-like structure spontaneously in aqueous environments, which significantly promotes the siRNA condensation capability in comparison with unmodified peptides.

2. Materials and Methods

2.1 Materials

PC-3, MCF-7, SK-BR-3 and LNCaP cell lines were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% Fatel Bovine Serum (FBS) plus penicillin (100 unit/mL) and streptomycin (100 μg/mL). DU145 cells were maintained in DMEM supplemented with 10% FBS plus penicillin (100 unit/mL), and streptomycin (100 μg/mL). Branched PEI (bPEI, 25 kDa) and filipin were purchased from Sigma-Aldrich (St Louis, MO). Amiloride was obtained from Fisher Scientific Inc (Pittsburgh, PA). Lipofectamine-2000 and Block-IT FITC-labeled siRNA were purchased from Invitrogen (Carlsbad, CA). The VEGF siRNA was ordered from Invitrogen (Carlsbad, CA) and the scrambled siRNA was obtained from Ambion (Austin, TX). All the starting reagents listed below were obtained from commercial sources and used without further purification. Piperidine and cholesteryl chloroformate were obtained from Sigma-Aldrich (St Louis, MO). Triisopropylsilane (TIPS), trifluoroacetic acid (TFA),

triethylamine (TEA) and *N*,*N*-diisopropylethylamine (DIPEA) were ordered from Acros Organics (Morris Plains, NJ). All the amino acids used in solid phase peptide synthesis and 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were obtained from Anaspec Inc. (Fremont, CA). All the organic solvents used for synthesis and HPLC were ordered from Fisher Scientific Inc (Pittsburgh, PA). The human VEGF Quantikine ELISA Kit was purchased from R&D system (Minneapolis, MN).

2.2 Synthesis of peptides and cholesteryl peptides

All peptides were manually synthesized by Fmoc solid-phase peptide synthesis method. 2-chlorotrityl resin was used as the solid phase for the peptide synthesis. DIPEA was used as the base and HCTU was served as the coupling reagent. Cholesterol was coupled to the -NH_2 end of the peptides before cleaving the peptides from the resin and deprotection. Cholesteryl chloroformate (1.55 mmol) was dissolved in 20 mL DMF. The peptidyl-resin (~0.155 mmol -NH_2) was subsequently suspended in the solution, followed by adding TEA (1.55 mmol). After gentle stirring at room temperature for 48 hr under N_2 protection, the resin was harvested and washed with DMF. The cholesteryl peptides were then cleaved from the resin by acetic acid/ trifluoroethanol/DCM (v/v/v, 10/20/70) at room temperature for 2 hr. The resin was removed by filtration and the solvent was evaporated. After deprotection in cocktails (TFA/phenol /water /TIPS, 88:5:5:2) at room temperature for 2 hr, the solution was concentrated and added into ice-cold diethyl ether for precipitation. The final products were purified by reverse phase HPLC. The molecular weight was confirmed by LC/MS. The results are listed in Table 1.

2.3 Critical micelle concentration (CMC)

CMC of the cholesteryl peptides was determined by fluorescence spectroscopy using pyrene as a probe (13). Briefly, a series of cholesteryl peptides solutions ranging from 0.002 to 2 mg/mL were prepared in acetonitrile. One hundred microliters of the solution were transferred into Eppendorf tubes and mixed with 20 μL of pyrene (2 mg/mL in acetonitrile). Acetonitrile was removed by air-dry overnight. Each tube was reconstituted with 50 μL of distilled water. The undissolved pyrene was precipitated by high-speed centrifugation for 1 min. The supernatants were carefully transferred to a black 96-well plate for fluorescence assay. The fluorescence intensity was recorded using Spectramax Gemini XPS spectrofluorometer (Molecular Devices, Sunnyvale, CA) with an E_x=330 nm, $E_m(I_1)$ =372 nm and $E_m(I_2)$=392 nm. The intensity ratio ($I_{392\ nm}/I_{372nm}$) was plotted as a function of logarithm of peptide concentration. The CMC value was calculated from the intersection of two tangents drawn to the curve at high and low concentrations, respectively.

2.4 Gel mobility retardation assay

siRNAs were mixed with increasing amounts of cholesteryl peptides and incubated at room temperature for 15 min. The complexes were electrophoresed through 1% agarose gel. The siRNAs were stained with GelRed™ and visualized under UV light.

2.5 Characterization of the cholesteryl peptide/siRNA complex

The particle size and zeta potential of the cholesteryl peptide/siRNA complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Westborough, MA).

The morphology of the cholesteryl peptide/siRNA complexes was examined using a Philips CM12 transmission electron microscopy (TEM). One drop of the siRNA complexes was placed onto a circular copper grid and stained with 1% uranyl acetate. The grid was then dried in air and examined by the TEM.

2.6 Heparin competition study

The chol-H3K2s/siRNA complexes were formed as described above at a weight ratio of 7:1. The complexes were then incubated with increasing concentrations of heparin sodium at room temperature for 30 min and subsequently analyzed by agarose gel mobility assay.

2.7 siRNA Transfection

Cells were transfected with siRNA as described before (14). Briefly, PC-3 cells (5×10^4 cells per well) were cultured in 24-well plates 12 hr before transfection. The transfection mixture was prepared by mixing siRNA and cholesteryl peptides in Opti-MEM (Invitrogen) and then incubated at room temperature for 15 min to form the complex. The cells were washed with DPBS, and 50 μL of the transfection mixture was added to each well along with 450 μL of Opti-MEM. Twenty-four hours after the transfection, the cells were harvested using Trizol reagent for RNA isolation.

2.8 Real-time PCR

Total RNA was isolated from cells using TRIzol reagent according to the manufacturer's protocol and converted to cDNA using random hexamer primer and MultiScribe Reverse Transcriptase Reagent. One hundred nanograms of the cDNA were amplified by real-time PCR using SYBR Green-1 dye universal Master mix on a Light Cycler 480 system (Roche, Indianapolis, IN). To confirm the PCR specificity, PCR products were subjected to a melting-curve analysis. The comparative threshold (Ct) method was used to calculate the relative amount of mRNA in treated samples relative to the control group. Each treatment was performed in triplicate, and the mean values were calculated. The primers used for the study were as follows: hVEGF: 5'-AGGGCAGAATCATCACGAAGTGGT-3' (forward primer) and 5'-TCTGCATGGTGATGTTGGACTCCT-3' (reverse primer). We used 18s ribosomal RNA as an internal control with the following primers: 5'-GTCTGTGATGCCCTTAGATG-3' (forward primer) and 5'-AGCTTATGACCCGCACTTAC -3' (reverse primer).

2.9 Enzyme-linked immunosorbent assay (ELISA)

LNCaP cells were transfected with siRNA as described above. Briefly, LNCaP cells (1×10^4 cells per well) were cultured in 96-well plates 12 hr before transfection. The transfection mixture was prepared by mixing siRNA and cholesteryl peptides in Opti-MEM (Invitrogen) and then incubated at room temperature for 15 min to form the complex. Fifty microliters of the transfection mixture was added to each well along with 50 μL of Opti-MEM. The culture medium was collected at 24 and 48 post-transfection for VEGF assay. The secretion of VEGF in the cell culture medium was determined using human VEGF Quantikine ELISA Kit (R&D system, Minneapolis, MN) according to the manufacturer's instruction.

2.10 Cytotoxicity assay

Cytotoxicity of the cholesteryl peptide/siRNA complexes was evaluated using the CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) as described before (15). PC-3 cells seeded in a 96-well plate were transfected with 50 nM and 100 nM scrambled siRNA using bPEI, Lipofectamine-2000 and chol-H3K2s. Twenty-four hours after the transfection, 100 μl of the CellTiter-Glo® reagent was added to each well. The cells were lysed by shaking on an orbital shaker for 2 min, followed by incubation at room temperature for 10 min to stabilize the luminescent signal. The luminescent intensity was detected using a Beckman DTX 880 multimode Detector (Beckman Coulter, Inc., Fullerton, CA).

2.11 Confocal microscopic study

PC-3 cells were seeded onto Lab-Tak® 1-chamber culture slides and transfected with FITC-labeled siRNA using different transfection reagents (chol-H3K2s, lipofectamine-2000 and bPEI). Six hours post-transfection, the cells were washed with DPBS containing heparin sulfate (1 mg/mL) and DPBS containing 0.1% Trypan Blue to quench any fluorescence on the cell membrane. The cells were fixed with 10% buffered formalin and treated with TO-PRO®-3 for nucleus staining. All the samples were dried at room temperature before sealing the cover slip with mounting medium. The cells were then imaged with a Nikon Eclipse E800 fluorescence microscopy attached with Argon and krypton lasers providing excitation energy at 488 nm for the double-labeled specimens (FITC on siRNA and TO-PRO®-3).

2.12 Flow cytometry

PC-3 cells (2×10^5 per well) were cultured in 12-well plates 12 hr before transfection. The cells were transfected with FITC-labeled siRNA using different transfection reagents (chol-H3K2s, Lipofectamine-2000 and bPEI) as described above. Six hours post-transfection, the cells were washed with DPBS containing heparin sulfate (1 mg/mL). The cells were then trypsinized, washed again with DPBS, and examined by a BD FACSCanto II flow cytometer (BectoneDickinson Instruments, Franklin Lakes, NJ).

2.13 Serum stability

Naked siRNA or the chol-H3K2s /siRNA complexes were incubated in 70% rat serum at a final siRNA concentration of 2 μM. The solutions were incubated at 37 °C for their indicated time interval. The samples were separated by a 20% native polyacrylamide gel, followed by visualization with GelRed™ staining under UV light. To visualize all the siRNA, the samples (either naked siRNA or complexes) were treated with heparin and subsequently analyzed via agarose gel mobility assay.

2.14 Cellular entry mechanism study

Before transfection, cells were treated in serum-free medium for 1 hr with different inhibitors (0.5 mM amiloride, 5 μg/mL filipin or 100 mM sucrose) at 37 °C, or in the absence of inhibitors at 4 °C. The cells were subsequently incubated with chol-H3K2s/VEGF siRNA complexes (7:1, w/w) for 5 hr in the presence of endocytosis inhibitors (37 °C), or in the absence of inhibitors at 4 °C. After 5 hr of transfection, the cells were washed with DPBS and incubated in Opti-MEM. After another 19 hr, the cells were harvested using Trizol reagent, and the silencing effect on VEGF was measured by real-time PCR.

2.15 Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The difference between any two groups was determined by ANOVA. $P<0.05$ was considered statistically significant.

3. Results

3.1 Cholesteryl peptide can efficiently condense siRNA

In the present study, we have designed a series of peptide sequences consisting of histidine and lysine residues (Table 1). Each repeating unit contains five peptide residues in which the ratio of histidine to lysine varies from 2:3 (HHKKK) to 0:5 (KKKKK). The sequences H3K2s, H2K3s, H1K4s and K5s have two repeating units, while the sequence H3K2l and H3K2b have four repeating units. The sequence H3K2b contains a branched structure, and thus both N-termini are conjugated with cholesterol. All the cholesteryl peptides were synthesized using solid-phase method and purified by HPLC (Figure 1).

The capability of unmodified peptides and cholesteryl peptides to condense nucleic acids was evaluated by gel mobility shift assay. The condensation capability of different cholesteryl peptides is listed in Table 2. Compared with their unmodified counterparts, the cholesteryl peptides exhibit dramatically enhanced capability to condense siRNA. For instance, the complete retardation of 1 μL siRNA (20 μM) was achieved by 0.8 μg of chol-H3K2s, while more than 20 μg of the unmodified peptide were needed to condense the same amount of siRNA. The improvement of condensation capability was also observed in other peptide sequences containing the cholesterol modification.

3.2 Cholesteryl peptides form micelle-like structure

Having shown that cholesteryl peptides are able to efficiently condense siRNA, we next evaluated the characteristics of the cholesteryl peptide/siRNA complexes by dynamic light scattering (DSL) and zeta potential analysis. As displayed in Table 3, the hydrodynamic diameters of cholesteryl peptide/siRNA complexes (14:1, w/w) are around 100–200 nm, regardless of the peptide sequence. In contrast, complexes made from unmodified peptides and siRNA with the same ratio exhibited larger particle sizes ranging from 400 to 550 nm. This suggests that the cholesteryl peptides can form more compact complexes with siRNA in comparison with their unmodified counterparts. The zeta potential measurement demonstrate that the surface charges of the siRNA/cholesteryl peptides complexes, are ~12–40 mV, whereas the zeta potential values of unmodified peptide/siRNA complexes are less than 3 mV and even become slightly negative in the sequence $(HHHKK)_2C$. Zeta potential is an important parameter used to evaluate the stability of colloidal dispersion system. These results indicate that the cholesteryl peptides are able to form more compact and stable siRNA complexes than unmodified peptides.

We also used TEM to characterize the morphology of the cholesteryl peptide/siRNA complex. As shown in Figure 2, the complexes are spherical particles of uniform size. The observed particle size, around 200 nm, is consistent with the particle size obtained by DSL analysis.

All the designed peptide sequences are hydrophilic and thus the resultant cholesteryl peptides are amphiphilic molecules. To further confirm the formation of micelle, the CMC value of the cholesteryl peptide chol-H3K2s was determined by fluorescence spectroscopy. Pyrene is one of the commonly used fluorescence probes to evaluate the CMC of amphiphilic molecules because the fluorescence intensity ratio of peak III (λ=392 nm) and peak I (λ=372 nm) of pyrene is very sensitive to the hydrophobicity change of local environment. The I_{392nm}/I_{372nm} is almost constant when the concentration of amphiphilic molecules is below CMC. However, this ratio increases dramatically when pyrene is entrapped in the hydrophobic interior of micelle once surfactant concentration exceeds CMC (16). The ratio of $I_{392\ nm}/I_{372nm}$ was plotted against the concentration of chol-H3K2s. The CMC of chol-H3K2s is 0.030 mg/mL and 0.027 mg/mL in the absence of siRNA and in the presence of 200 nM siRNA, respectively (Figure 3).

3.3 Cholesterol peptides deliver siRNA into cells

To examine if the cholesteryl peptides are able to deliver siRNA into cells and trigger gene silencing effect, we used the cholesteryl peptides to transfect VEGF siRNA into cancer cells. The reason for choosing VEGF as the target gene is because it is extensively expressed in a variety of tumors and has been recognized as an important target for anticancer therapy (17). The comparison of the transfection efficiency of different cholesteryl peptides and unmodified peptides is displayed in Figure 4 A&B. All the cholesteryl peptides deliver siRNA into the cells and exhibit significant gene silencing effect (at least 59.4% knockdown), while none of the unmodified peptides mediates gene suppression. It should be

noted that the physical mixture of peptide H3K2s and cholesterol does not induce gene silencing effect (Figure 4C), indicating that formation of the micelle-like structure plays an essential role in siRNA delivery. Among these cholesteryl peptides, chol-H3K2s exhibits better silencing effect than other sequences with the same peptide length, including chol-H2K3s, chol-H1K4s, chol-K5s, possibly due to its higher histidine content. There is no significant difference between the silencing effect of chol-H3K2s and the 21-mer cholesteryl peptide chol-H3K2l, indicating that increasing the length of peptide from 11-mer to 21-mer without changing the composition does not further increase the transfection efficiency. Interestingly, chol-H3K2b bearing two cholesterol moieties did not show higher silencing effect compared to other peptides with mono-cholesterol (Figure 4 A&B). Based on these results, chol-H3K2s was selected for the following studies. A time course study of the silencing effect of the cholosteryl peptide on the VEGF gene was conducted over a 72-hr period. The maximum gene knockdown effect (~85.7%) was obtained at 24 hr post-transfection, while the silencing effect decreased to 46.9% and 51.8% at 48 hr and 72 hr post-transfection (Figure 4 D). This is accordance with our previous studies that the silencing effect at the mRNA level peaks at 12–24 hr post-transfection (18, 19). Furthermore, significant reductions of VEGF mRNA level by as much as 59.6–92.2% were achieved by chol-H3K2s in several other cancer cell lines (Figure 4 E). The VEGF knockdown effect of chol-H3K2s at the protein level was shown in Figure 4F. The VEGF protein expression was inhibited by 29.5% and 43.7% at 24 and 48 hr post-transfection, respectively.

3.4 Transfection efficiency of chol-H3K2s in comparison with other transfection reagents

Lipofectamine-2000 is a commercial available reagent that has been widely used for *in vitro* siRNA transfection in a wide variety of cells. bPEI (25 kDa) is a cationic polymer that is regarded as the “gold-standard” for nucleic acid delivery in the past few decades. In this study, we compared the transfection efficiency of the chol-H3K2s with these two transfection reagents. FITC-labeled siRNA was used for complex preparation and transfected to PC-3 cells. Figure 5A shows the flow cytometric analysis of PC-3 cells treated with chol-H3K2s, Lipofectamine-2000 and bPEI. Six hours post-transfection, chol-H3K2s, bPEI and Lipofectamine-2000 transfect 77.7±1.5%, 97.3±1.5% and 71.6±2.4% of the cells, respectively, while FITC-labeled siRNA alone shows negligible transfection. A confocal microscopic study (Figure 5B, d, e&f) of the transfected cells further supports the observation in flow cytometry analysis. The intercellular localization of the FITC-siRNA, in either bPEI or chol-H3K2s transfected cells, is in a punctate staining pattern. In contrast, dispersed fluorescence in the cytosol and nucleus was observed in a small proportion of Lipofectamine-2000 treated cells. These results indicate that siRNA complexes made from different cationic carriers may have distinct cellular trafficking pathways. Furthermore, the VEGF siRNA with different concentrations were transfected into PC-3 cells using chol-H3K2s, Lipofectamine-2000 and bPEI, respectively. As illustrated in Figure 6, chol-H3K2s displays a similar gene silencing effect as Lipofectamine-2000 and bPEI at all siRNA concentrations (1 nM, 10 nM and 50 nM), suggesting that chol-H3K2s possesses a comparable siRNA transfection capacity as Lipofectamine-2000 and bPEI.

3.5 Cellular toxicity of cholesteryl peptide/siRNA complexes

The cytotoxicity of various siRNA formulations was assessed in PC-3 cells after transfection. To exclude the potential effect of VEGF knockdown on cell proliferation, a scrambled siRNA was used as the model siRNA to form complex with bPEI, Lipofectamine-2000 or different cholesteryl peptides. Twenty-four hours after the transfection, the cytotoxicity was determined using CellTiter-Glo® reagent. As shown in Figure 7, all of the cholesteryl peptide/siRNA complexes exhibit negligible cytotoxicity in the conditions in which significant gene silencing effect can be achieved (Figure 4A).

Moderate cytotoxicity was induced by Lipofectamine-2000 at high concentration, while severe toxicity was observed in bPEI treatment, which resulted in as much as 42.4%–83.7% cell death.

3.6 Cholesteryl peptides enhance serum stability of siRNA

The half-life of naked siRNA is very short in blood stream. Therefore, an efficient siRNA delivery system must be able to protect siRNA from nuclease degradation after intravenous administration. To determine if cholesteryl peptides can improve the serum stability of siRNA, the chol-H3K2s/siRNA complex and naked siRNA were incubated in rat serum for different time intervals (2, 4, 8, 24, 48 and 72 hr). As illustrated in Figure 8A, a significant amount of the naked siRNA is degraded into small fragments at 2 hr post-incubation. After 4 hr incubation, nearly all of the naked siRNA is degraded. In the case of the chol-H3K2s/siRNA complex, there is a strong band at the interface of the stacking and running gels, which corresponds to the siRNA molecules encapsulated in the complex. After incubation for 2 hr, we observed the dissociation of intact siRNA from the complex. The degradation of the dissociated siRNA is negligible until 48 hr, indicating that the complex efficiently protects siRNA from nucleases in the serum. To further elucidate the integrity of the siRNA encapsulated in the complex, heparin sodium, an anionic polymer, was used to dissociate siRNA from the complex after incubation in the serum. First, we optimized the concentration of heparin to completely dissociate all the encapsulated siRNA. As Figure 8B shows, the heparin/siRNA ration of 2.3:1 (w/w) is sufficient to release all the encapsulated siRNA from the chol-H3K2s/siRNA complex (Figure 8B). We next incubated the chol-H3K2s/siRNA complex and naked siRNA with rat serum for 72 hr and then incubated them with heparin at the optimized concentration. Upon heparin treatment, intact siRNA was dissociated from the chol-H3K2s/siRNA complex, while no intact siRNA was detected in the naked siRNA (Figure 8C). Taken together, these results indicate that cholesteryl peptide is able to effectively prevent siRNA from enzymatic degradation in the serum.

3.7 Cell entry mechanism of cholesteryl peptide/siRNA complexes

To better understand the cellular entry mechanism of the cholesteryl peptide/siRNA complexe, the cellular uptake of the complex was investigated in the presence of three different inhibitors of endocytic pathway. Filipin, sucrose, and amiloride are known to inhibit the caveolin-mediated process, the clathrin-associated pathway, and the micropinocytosis pathway, respectively. First, the effect of these inhibitors on cell viability was examined because it has been reported that the treatment of endocytosis inhibitors can cause cytotoxicity (20). As illustrated in Figure 9A, no significant cell death or morphology change was observed under treatment with these inhibitors. Knockdown effect of the VEGF siRNA was determined by real-time PCR (Figure 9B). All of the inhibitors barely affect the gene silencing activity. We also investigated the effect of temperature on the cellular entry process. Incubating cells at 4°C results in ~ 40% depletion of the knockdown effect (Figure 9B).

4. Discussion

In general, to efficiently translocate siRNA into cells and mediate gene silencing activity, two essential components, including cationic moieties and fusogenic moieties, must be incorporated into a siRNA delivery system. Keeping this notion in mind, we designed a series of 11–21 mer peptides consisting of histidine and lysine residues. The ε amino group of lysine is positively charged under neutral pH conditions, which can form electrostatic interaction with negative charged nucleic acids. On the other hand, the imidazole group of histidine residue (pKa: ~6) possesses a proton sponge activity for endosomal disruption because it can be protonated and become cationic in acidic medium. Indeed, partial

substitution with histidine residue has been reported to promote DNA transfection efficiency of cationic polymers (21, 22). To ensure a buffering effect for endosome release, the histidyl content in the carriers must exceed a certain extent (21). Previous studies demonstrated that more than 70% of histidine content is sufficient for a peptide-based reducible polycation to mediate efficient nucleic acid delivery without the requirement of chloroquine, a lysosomotropic reagent (23). In this study, all of the four 11-mer cholesteryl peptides (chol-H3K2s, chol-H2K3s, chol-H1K4s and chol-K4s) with different histidine contents (0, 18.2, 36.4 and 54.5%) exhibit significant gene silencing effect (66.6–87.6%) (Figure 4A), indicating that histidyl moiety is not a prerequisite for siRNA delivery using these cholesteryl peptides. The cholesteryl peptides with higher histidine content only slightly improve the silencing effect. On the other hand, unmodified oligopeptides, regardless of the sequence, failed to mediate efficient gene silencing effect (Figure 4B). This is in agreement with our previous study, in which protamine alone cannot deliver siRNA into prostate cancer LNCaP cells.(24) The results reveal that short peptide containing positive charges and histidines are not sufficient to trigger an efficient intracellular uptake of siRNA. Other components, such as targeting ligand and cell-penetration moiety, are usually required for an efficient delivery system. For example, Mo et al. reported that the model amphipathic peptide (MAP)/siRNA complex can silence a target gene, while oligoarginine (R6)/siRNA did not induce gene knockdown effect although the MAP peptide contains less positive charges than the R6 peptide (25). Similarly, Bartz et al. evaluated 11 cationic peptides and found only two of them can deliver siRNA into cells (26).

Unlike DNA, siRNA molecules are much more difficult to be condensed due to their short length and the rigid two-turn helical structure (27). Several approaches, such as polymerization of cationic oligopeptides (11, 23) or siRNA (27–29), have been utilized to improve siRNA condensation. Polymerization of peptides via disulfide reaction increases positive charge in the carriers, while polymerization of siRNA can increase the flexibility and length of siRNA, thus making them easy to be condensed. However, these two approaches are always associated with heterogeneous products (11, 29). In this study, cholesterol modification to the N-terminus of peptides is an effective method to enhance the siRNA condensation capability of short peptides (Table 1). This improvement can be attributed to two possible mechanisms. First, cholesterol can self-aggregate to form micelles in aqueous solution (30). Once cholesterol moiety is coupled to the hydrophilic peptides, the resultant amphiphilic molecules are able to form a micelle-like structure in water, leading to increased positive-charge density. In agreement with this hypothesis, the CMC value of the cholesteryl peptide chol-H3K2s is 0.030 mg/mL. TEM study reveals that the chol-H3K2s/ siRNA complex has nanoscale particle size with uniform sphere-shape (Figure 2 and 3). Second, the hydrophobic interaction between lipid moieties may facilitate the condensation process and result in the formation of more compact and stable complex. Oba et al. reported that introduction of cholesterol into the ω-terminus of PEG-PAsp(DET) micelles can increase the association of block copolymer with DNA, and thereby enhancing the stability of the micelle in the blood stream (31). Consistent with their finding, we observed that the cholesteryl peptide/ siRNA complexes exhibit smaller particle size and greater zeta potential compared to the native peptide/siRNA complexes (Table 2). In addition, the cholesteryl peptide/ siRNA complexes could protect siRNA against nuclease degradation in the serum (Figure 8), suggesting that the cholesteryl peptides can form a very compact and stable complex with siRNA.

In contrary to the unmodified oligopeptides, all peptides with cholesteryl modification efficiently transfect VEGF siRNA into cells and mediate significant RNAi activity (Figure 4). Several favorable properties of the cholesteryl peptide, such as efficient condensation with siRNA and formation of stable complexes contribute to functional delivery of siRNA. Besides, the hydrophobic nature of cholesterol may promote the interaction of the

complexes with cell membrane and enhance cellular uptake. For example, lipid moieties, such as stearyl and cholesteryl moieties have been coupled to CPPs to enhance the cellular penetration capability (32). One of the possible mechanisms for the enhancement is that lipid moiety can facilitate CPPs to insert into the lipid monolayer of the cells (33). On the other hand, since CPPs emerged as promising carriers for nucleic acid delivery, many researchers have applied stearyl or cholesteryl modification to enhance gene delivery efficacy of CPPs (34–37). Our results demonstrate that cationic peptides without cell penetrating capability can also be used for siRNA delivery after cholesterol modification.

Branched PEI and Lipofectamine-2000 have been previously shown to successfully transfect siRNA into a variety of cell lines (14, 19, 38, 39). Here we demonstrate that the cholesteryl peptide chol-H3K2s is equally effective in transfecting siRNA. Although potent gene knockdown can be achieved by bPEI mediated siRNA delivery, it is always accompanied with nonspecific cytotoxicity (40–42). The cholesteryl peptides display less cytotoxicity *in vitro* compared with bPEI and Lipofectamine-2000 (Figure 7). Degradation of cholesteryl peptides by intercellular trypsin-like protease could be one of the reasons accounting for their low cytotoxicity. Trypsin predominantly cleaves peptides at the carboxyl side of the arginine and lysine residues, and the cholesteryl peptides are composed of multiple lysine residues (43).

A variety of factors, such as cellular entry pathway, endosomal disruption efficiency and interaction between siRNA and carriers, could influence the cellular uptake, intracellular distribution and trafficking of siRNA complexes. In the present work, the cellular uptake of chol-H3K2s/siRNA complexes, lipoplexes and polyplexes was visualized and compared using fluorescence confocal microscopy. The cholesteryl peptide/siRNA complexes exhibited distinct intracellular distribution compared with lipoplexes and polyplexes made from Lipofectamine-2000 and bPEI, respectively (Figure 5B). Despite of the differences, these three systems displayed similar silencing effect (Figure 6). In fact, the cellular uptake rate of an siRNA is not always correlated with its silencing effect. A recent study from Lu et al. suggested that although the majority of siRNA lipoplexes are internalized though endocytic pathway, the functional gene silencing effect is triggered only by a small portion (~5%) of the siRNA molecules that enters the cells via membrane fusion process (44). A similar result was also observed in functional delivery of antisense oligonucleotides via lipoplexes (45).

The cellular internalization mechanisms of DNA delivery mediated by cationic lipoplex or polyplex have been extensively studied. Upon binding to cell membrane, the cellular entry of DNA complex is primarily through endocytosis. However, the functional delivery of siRNA complexes remains to be elucidated (44). In this study, we investigated the influence of transfection temperature on gene silencing effect. Low temperature reduces the fluidity and flexibility of the plasma membrane, and block the endocytic pathway (46, 47). Our results showed that the cholesteryl peptide-mediated siRNA delivery is significantly impaired at 4 °C, suggesting that an energy-dependent uptake process is involved in the internalization of the cholesteryl peptide/siRNA complex. Meanwhile, we determined the effect of different endocytosis inhibitors on the gene knockdown activity of cholesteryl peptide/siRNA complexes. We analyzed the effect of filipin, which can remove cholesterol from the plasma membrane and thereby disrupt the caveolin-mediated endocytosis and membrane fusion (48, 49). No significant difference was observed in the RNAi effect after the treatment with filipin. Similar results were also observed in the treatment with sucrose, which can unselectively block clathrin-dependent endocytosis, and the potent macropinocytosis inhibitor amiloride. The possible explanation for these seemingly-controversial results may be that multiple pathways are involved in the cellular entry process of the cholesteryl peptide/siRNA complex. Inhibition of a single pathway may result in

shifting the uptake to other unblocked pathways. In accordance with these finding, Bartz et al reported that the silencing effect of the Endoporter peptide/siRNA complex was diminished at 4°C, while none of the endocytotic inhibitors treatment can block the delivery (10). On the other hand, many aspects of the cellular internalization pathway are still unknown (9, 10, 50), we should not exclude the possibility that cholesteryl peptide/siRNA complexes might be taken up into cells via other unknown mechanisms.

In conclusion, our results demonstrate that short peptides containing positive charges and endosomal disruption moieties are not sufficient to mediate efficient siRNA delivery. Cholesterol modification of the peptides containing lysine and histidine residues can form a micelle-like structure and dramatically enhance the condensation capability with siRNA. Most importantly, the cholesteryl peptides not only deliver siRNA into cells, but also trigger gene silencing effect without inducing toxicity in the cells. The present approach may provide a novel platform for furture development of RNAi therapeutics.

Acknowledgments

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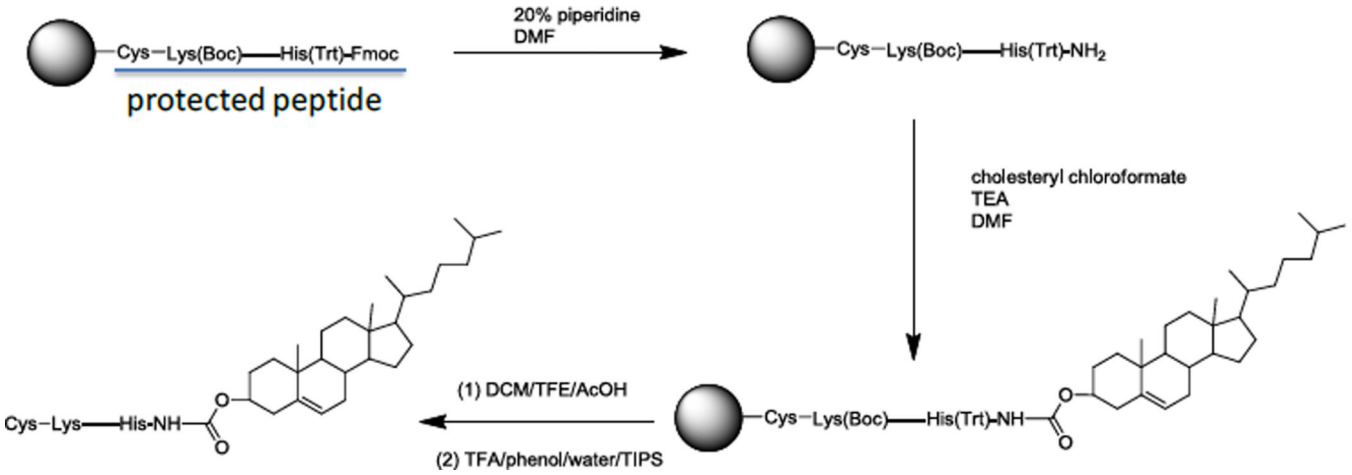


Figure 1.
Synthetic scheme of the cholesteryl peptides

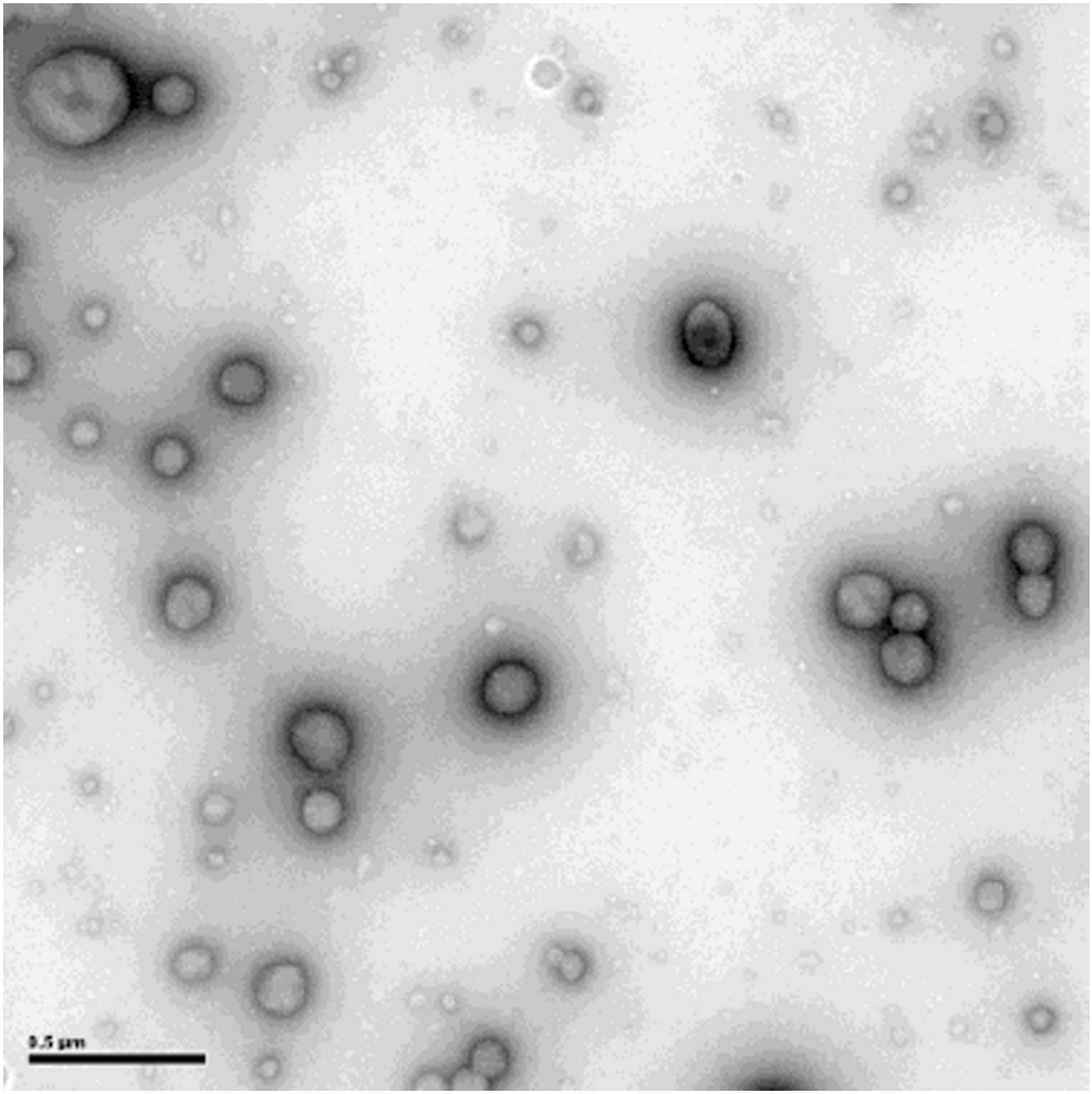


Figure 2.
TEM of the chol-H3K2s/siRNAcomplex

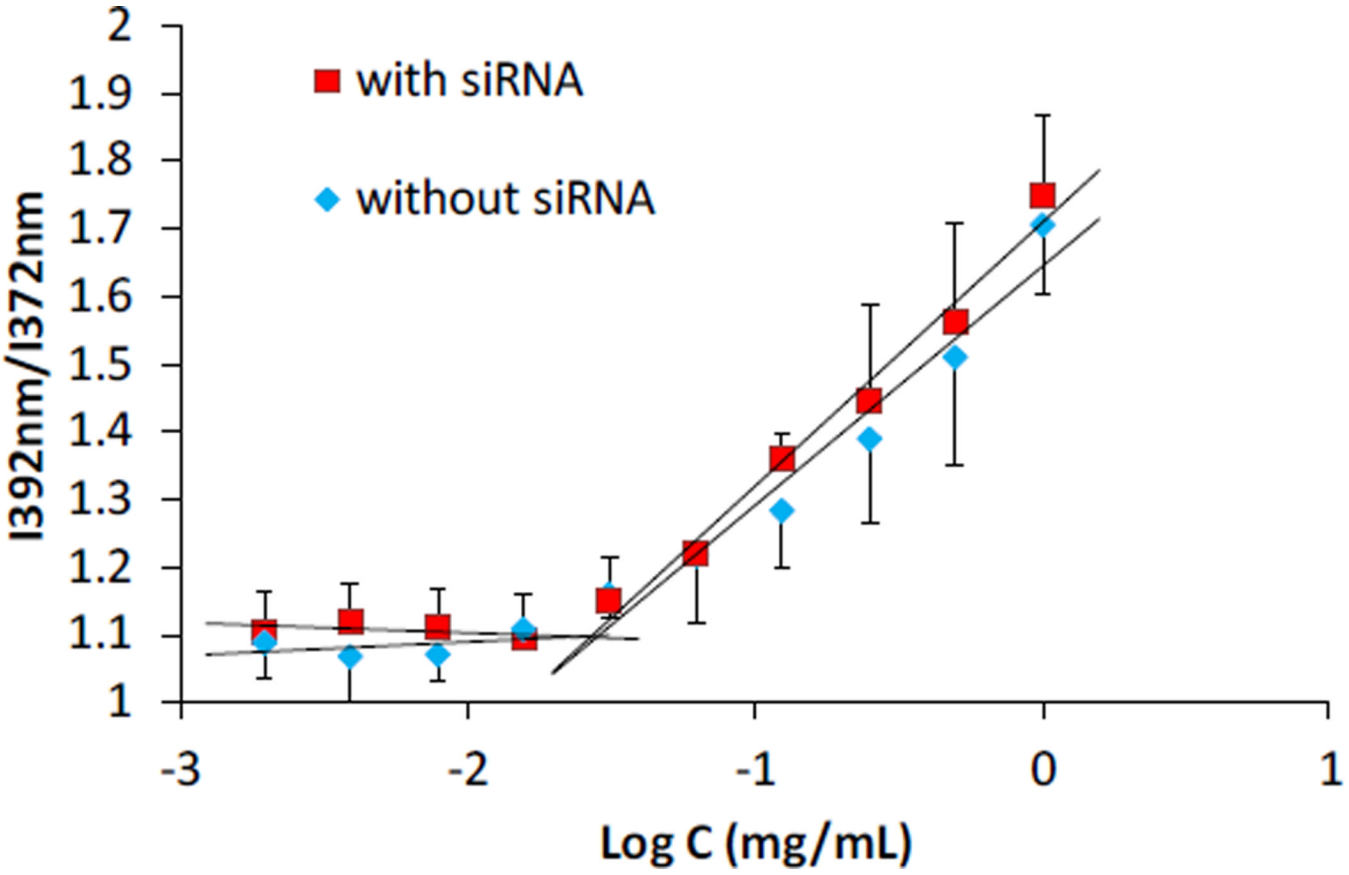


Figure 3.
The CMC value of chol-H3K2s. The CMC value of chol-H3K2s, either in the absence or presence of 200 nM siRNA, was determined with fluorescence spectroscopy using pyrene as a probe. The intensity ratio (I_{392}/I_{372}) was plotted against the logarithm of the peptide concentration. The CMC value was calculated from the intersection of two tangents drawn to the curve at high and low concentrations.

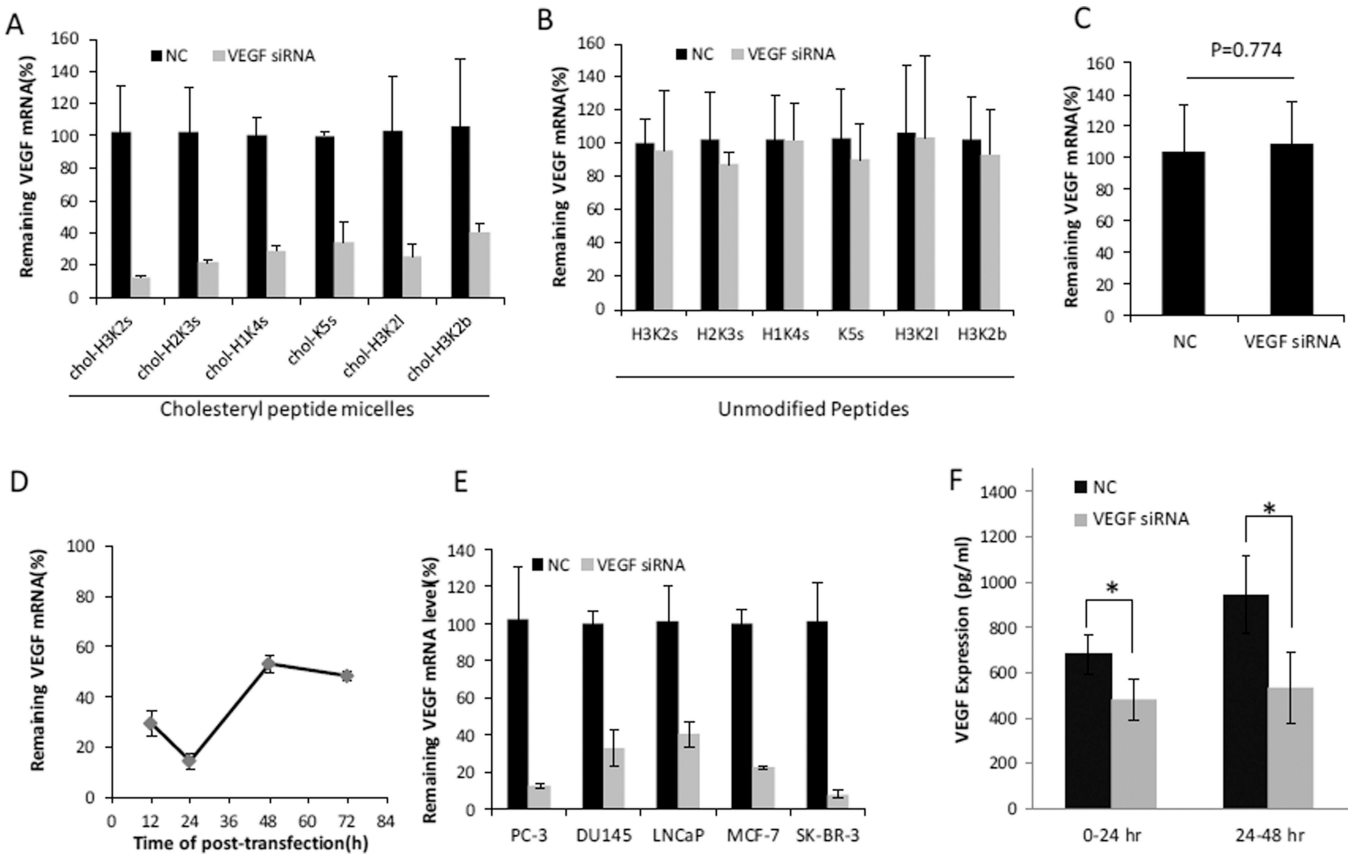


Figure 4.

Cholesteryl peptides, rather than unmodified peptides, trigger knockdown effect of the VEGF siRNA. PC-3 cells were transfected with the VEGF siRNA condensed by different cholesteryl peptides (A), unmodified peptides (B) or the physical mixture of equal amounts of peptide H3K2s and cholesterol (C). Scrambled siRNA was used as the negative control (NC). The weight ratio of the peptide to siRNA is 7:1. (D) The time-course of the silencing effect. PC-3 cells were transfected with chol-H3K2s/VEGF siRNA complexes (w/w, 7:1), and the silencing effect was determined at 12, 24, 48 and 72 hr post-transfection. (E) Silencing effect of the chol-H3K2s/VEGF siRNA complexes (w/w, 7:1) in various cancer cell liens. (F) Silencing effect of the chol-H3K2s/VEGF siRNA complexes (w/w, 7:1) at the protein level in LNCaP cells. The results are represented as mean ± SD (n=3). (* $p<0.05$)

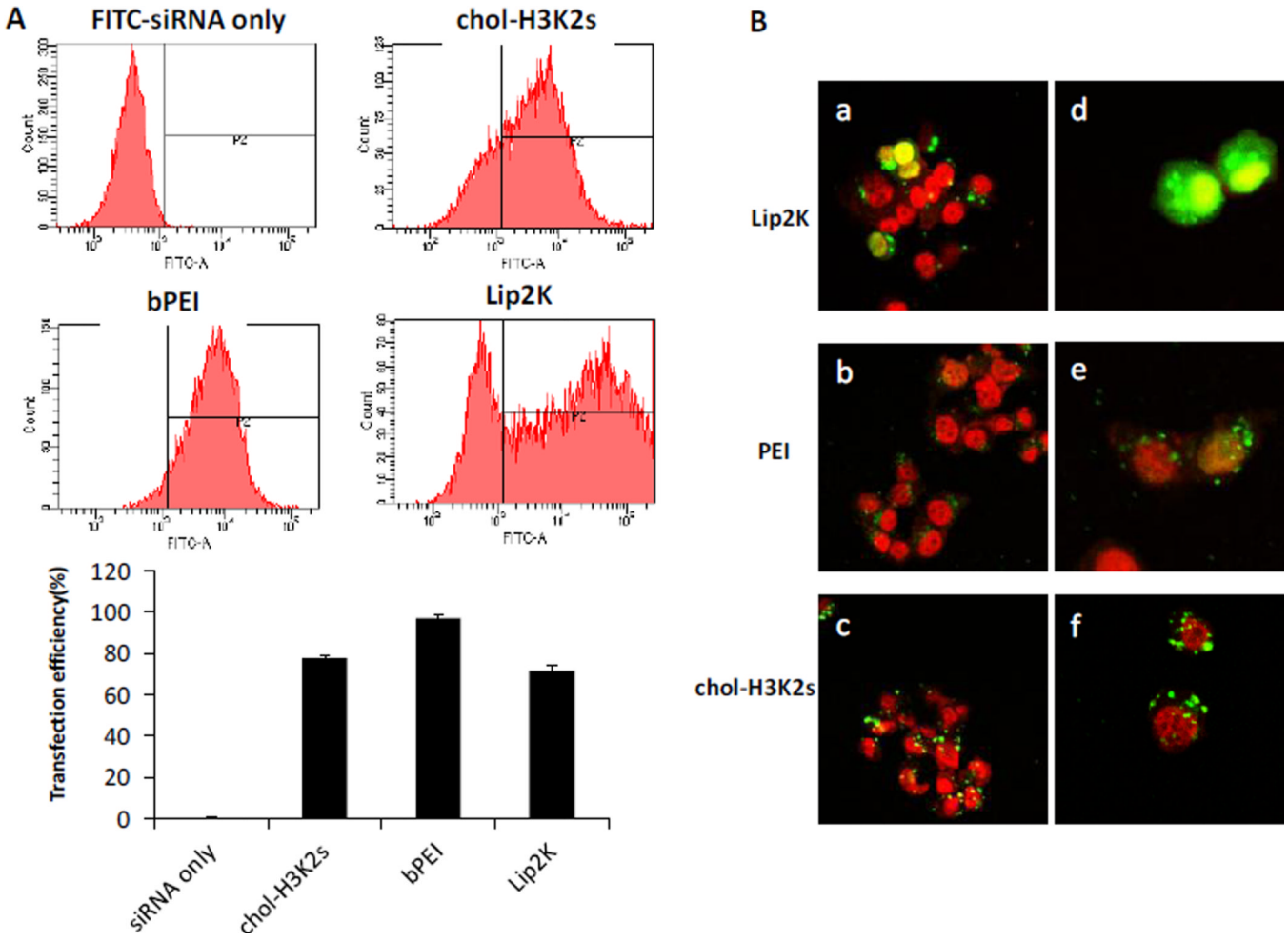


Figure 5.

Transfection efficiency of the cholesteryl peptides and other transfection reagents. (A) Flow cytometry histogram profiles of the fluorescence intensity of the PC-3 cells transfected with FITC-labeled siRNA complexed with Lipofectamine-2000 (Lip2K), bPEI or chol-H3K2s. (B) Confocal microscopic analysis at 6 hr post-transfection with FITC labeled-siRNA complexed with Lip2K (a, d), bPEI (b, e) or chol-H3K2s (c, f) (Red: TO-PRO-3, Green: FITC-labeled siRNA).

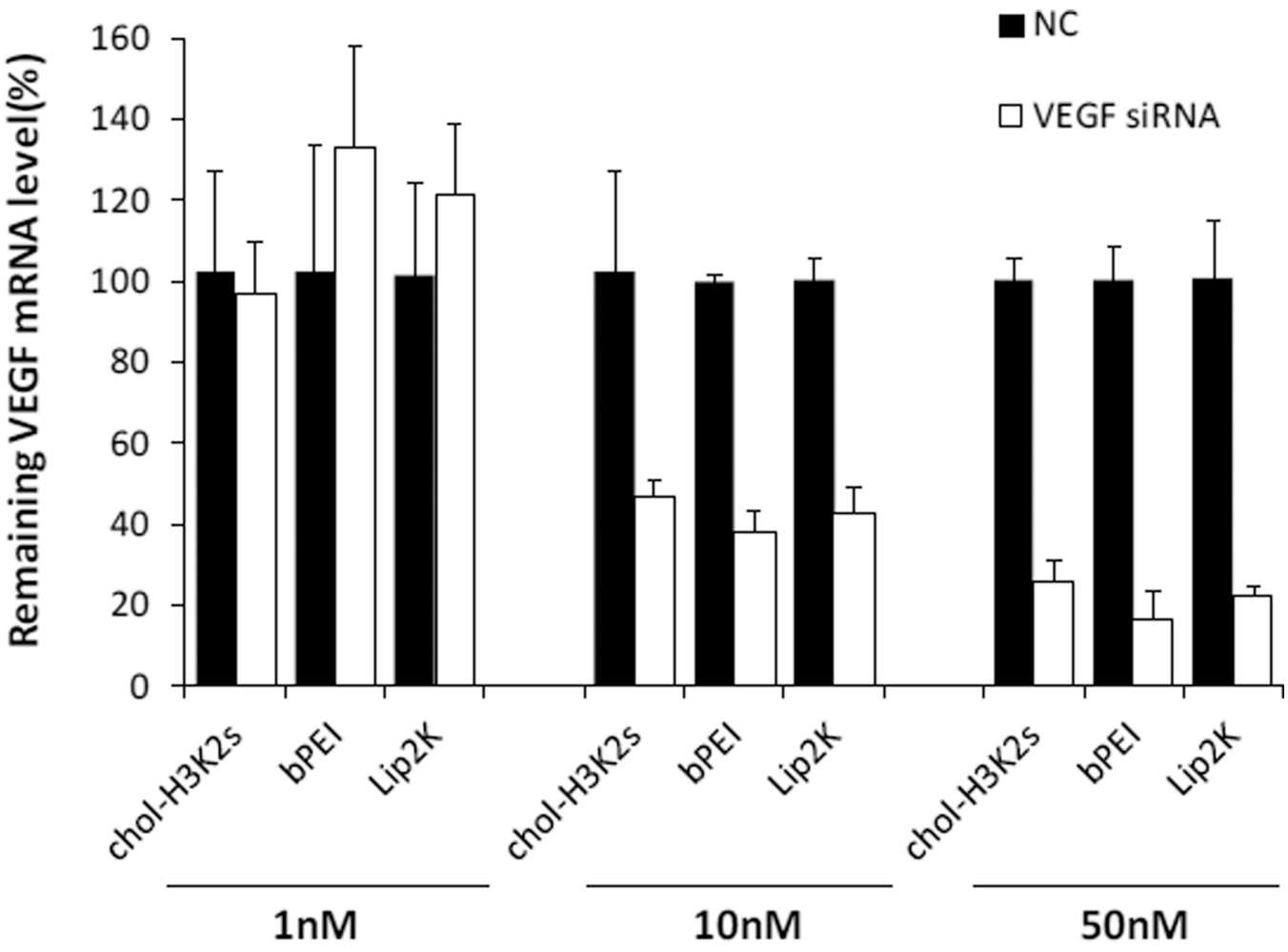


Figure 6.

The effect of siRNA concentration on the gene silencing effect of the VEGF siRNA complexed with Lip2K, bPEI and chol-H3K2s. The cells were transfected with at three different siRNA concentrations: 1 nM, 10 nM and 50 nM). The results are represented as mean ± SD (n=3).

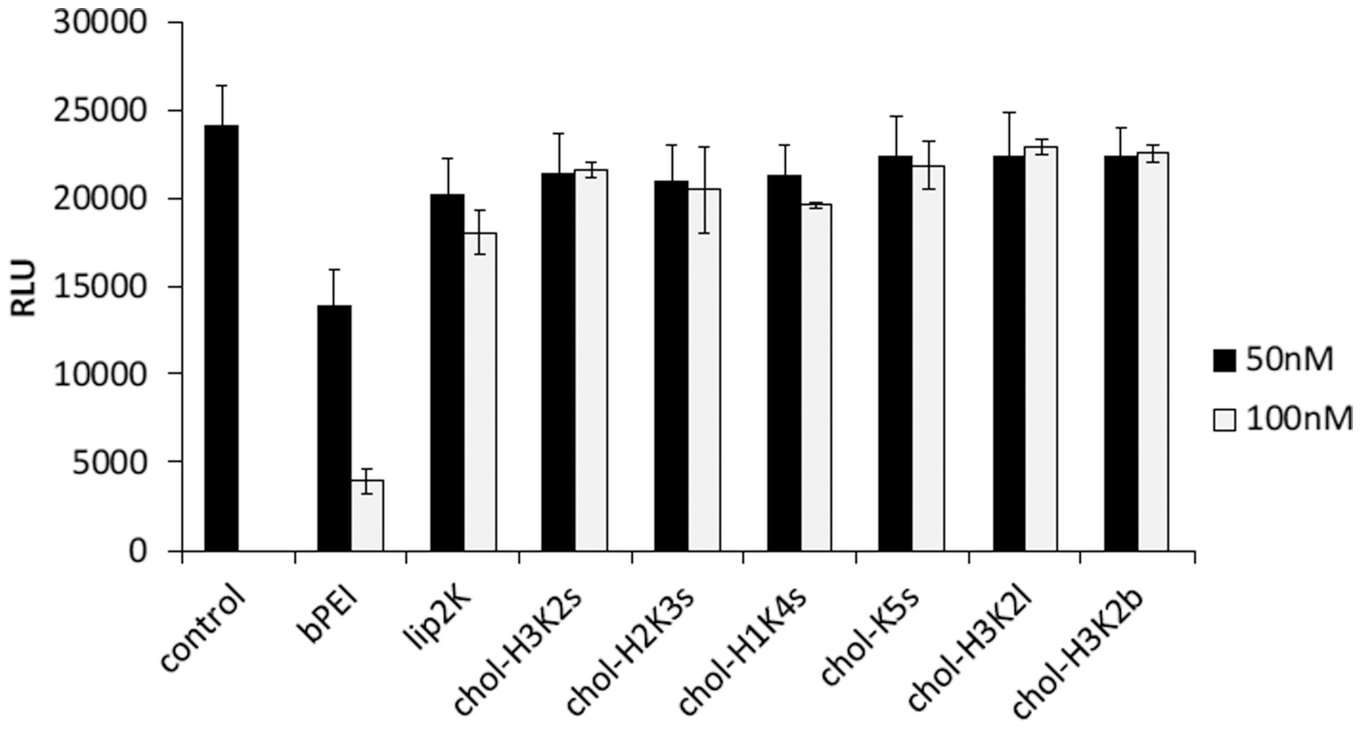


Figure 7.

Cytotoxic effects of cholesteryl peptide/siRNA complexes. PC-3 cells were transfected with 50 nM and 100 nM scrambled siRNA using bPEI (1:7, w/w), Lip2K (1:4.5, w/v (μg/μl)) and different cholesteryl peptides (1:7, w/w). Twenty-four hours after the transfection, cytotoxicity was determined using CellTiter-Glo Luminescent Cell Viability Assay Kit. The results are represented as mean ± SD (n=3)

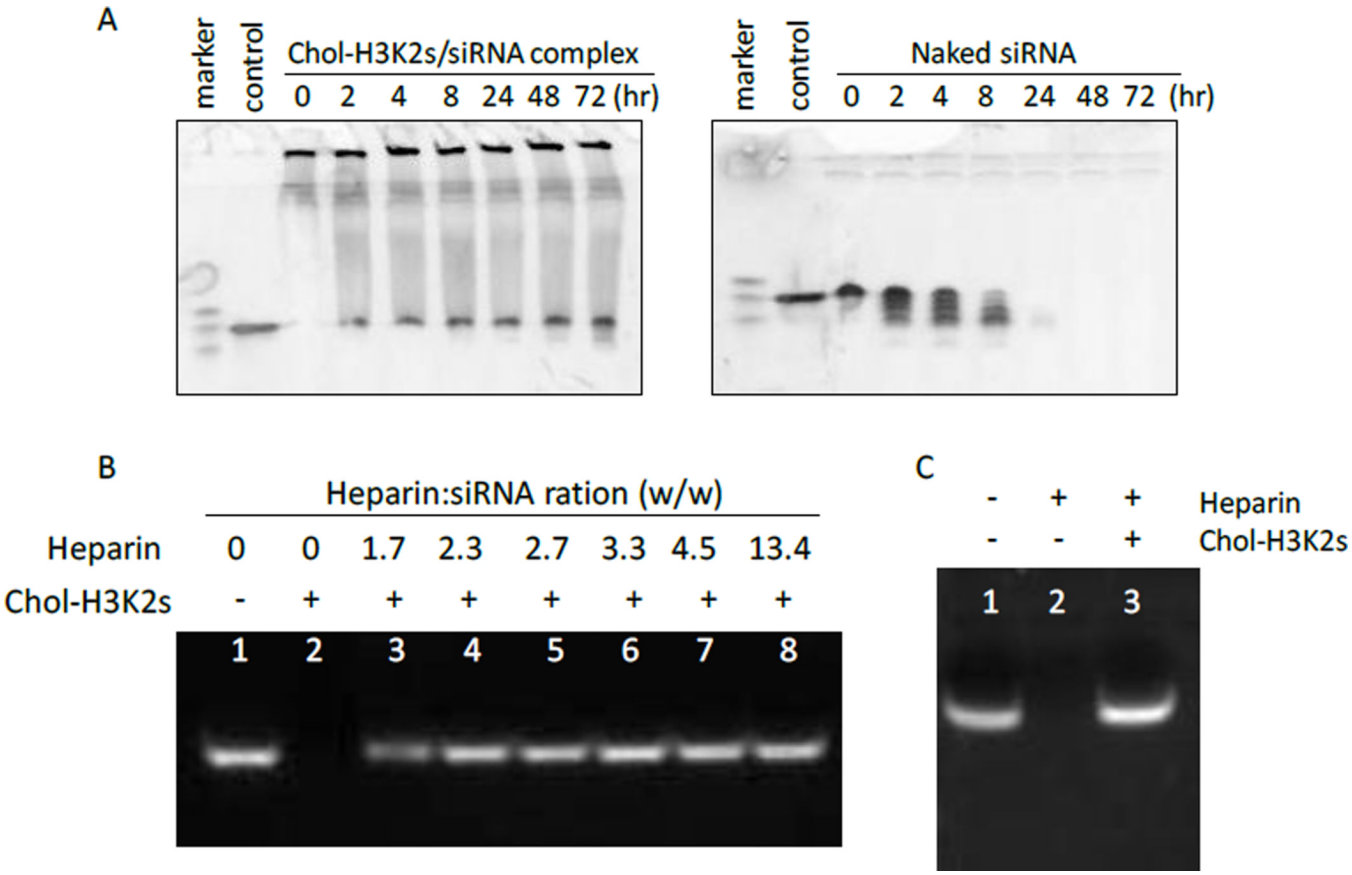


Figure 8.

Cholesteryl peptides protect siRNA from serum nuclease. (A) chol-H3K2s/ siRNA complexes (7:1, w/w) or naked siRNA (2 μM) were incubated in 70% rat serum at 37 C for different time intervals. The samples were separated by a 20% native polyacrylamide gel, followed by visualization with GelRed staining under UV light. (B) chol-H3K2s/ siRNAcomplexes (7:1, w/w) were incubated with increasing concentrations of heparin at room temperature for 30 min and then separated on 1% agarose gel. (C) chol-H3K2s/ siRNA complexes (7:1, w/w) or naked siRNA (final concentration: 2 μM) were incubated in 70% rat serum at 37 C for 72 hr. The samples were incubated with heparin at room temperature for 30 min and then separated on 1% agarose gel

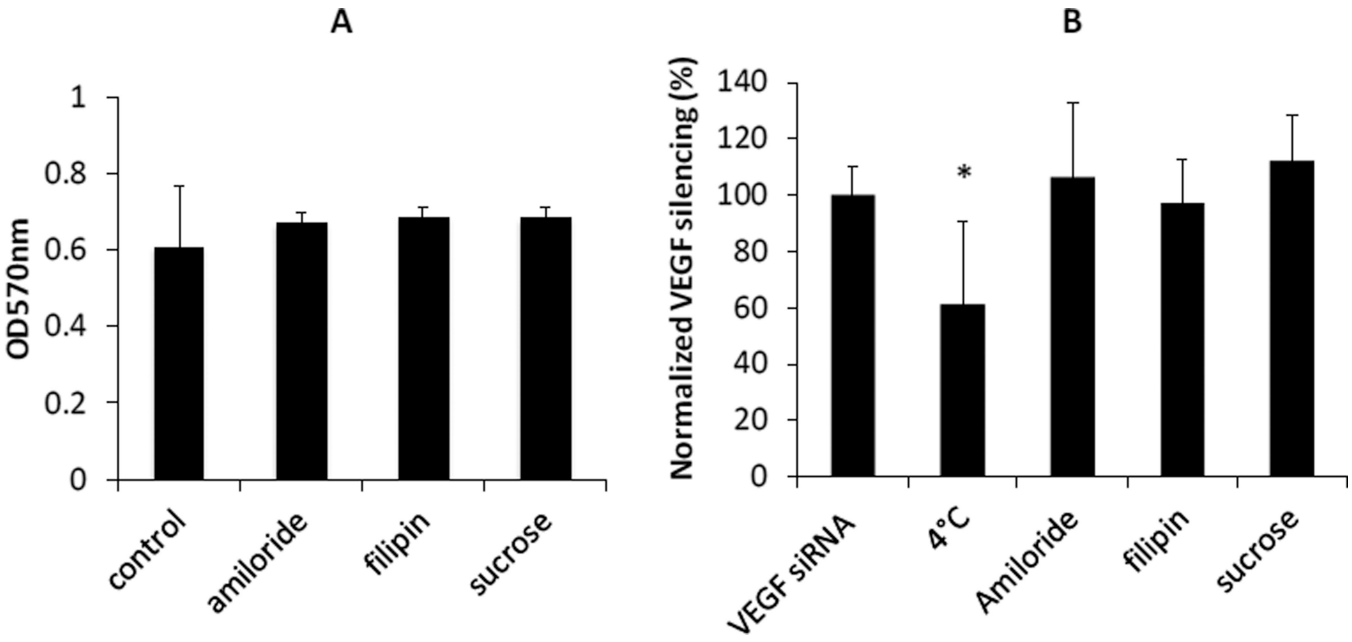
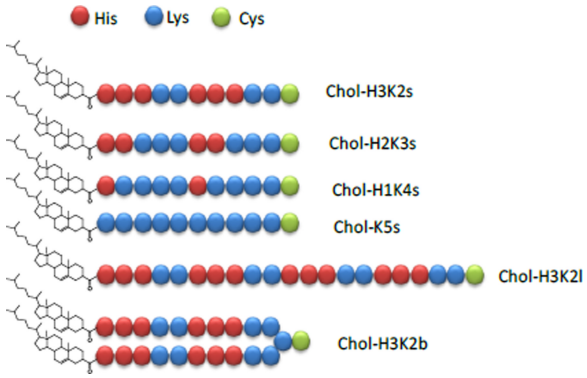


Figure 9.

The effect of endocytosis inhibitors and temperature on chol-H3K2s mediated siRNA delivery. (A) The cytotoxic effect of the endocytosis inhibitors treatment was determined by MTT assay. (B) Before transfection, the cells were treated in serum-free medium for 1 hr with inhibitors (0.5 mM amiloride, 5μg/mL filipin or 100mM sucrose) at 37°C, or in the absence of inhibitors at 4 °C. The cells were then subsequently incubated with chol-H3K2s/siRNAcomplex for 5 hr in the presence of endocytosis inhibitors (37°C), or in the absence of inhibitors at 4°C. After the transfection, the cells were washed with DPBS and incubated in OptiMem for another 19 hr. The results are represented as mean ± SD (n=5–6). (*: $p<0.05$)

Table 1

Sequence of cholesteryl peptides



name	Sequence	Peptide length	Histidine content (%)*	Charge at neutral pH	Average hydrophobicity$	Molecular weight
Chol-H3K2s	Chol-$(HHHKK)_2$C	11	54.5	+4	−5.88	1869.3
Chol-H2K3s	Chol-$(HHKKK)_2$C	11	36.4	+6	−6.99	1851.4
Chol-H1K4s	Chol-$(HKKKK)_2$C	11	18.2	+8	−8.10	1833.4
Chol-K5s	Chol-$(KKKKK)_2$C	11	0	+10	−9.21	1815.5
Chol-H3K2l	Chol-$(HHHKK)_4$C	21	57.1	+8	−6.05	3204.8
Chol-H3K2b	[Chol-$(HHHKK)_2]_2$KC	branched	54.5	+8	−6.23	3745.7

*histidine content calculated according to the number of histidiyl residue in the entire peptide;

$Hydrophobicity and hydrophobic moment calculated using the CCS scale.

Table 2

SiRNA condensation capability of unmodified and cholesteryl peptides

Peptide sequence	Unmodified (μg)[Ψ]	N-terminal Cholesteryl modification (μg)[Ψ]
$(HHHKK)_2C$	>20	0.8
$(HHKKK)_2C$	5	0.8
$(HKKKK)_2C$	5	0.8
$(KKKKK)_2C$	1	0.6
$(HHHKK)_4C$	5	0.8
$[(HHHKK)_2]_2KC$	5	1.2

[Ψ]: amount of peptide (μg) to completely condense 1μl siRNA(20 μM).

The values were determined by gel mobility shift assay

Table 3

Particle size and zeta potential of the peptides

Peptide sequence	Unmodified		N-terminal Cholesteryl modification	
	Particle size (nm)*	Zeta potential (mV)	Particle size (nm)*	Zeta potential (mV)
(HHHKK)2C	449.6±10.4	−1.76±0.14	219.5±18.7	40.0±2.0
(HHKKK)2C	441.3±33.1	0.99±0.55	163.4±8.3	30.1±8.3
(HKKKK)2C	443.5±36.2	1.88±0.83	202±20.9	30.5±6.8
(KKKKK)2C	499.0±62.2	1.72±0.78	198.5±19.9	31.5±1.2
(HHHKK)4C	419.7±30.6	2.60±0.47	89.6±27.4	12.7±2.0
[(HHHKK)2]2KC	532.9±15.9	1.96±0.51	170.9±6.2	15.57±1.63

All the complexes used in the study were prepared with a ratio of 14:1 (peptide: siRNA, w/w)

*Reported as the Z-average cumulant mean diameter (dnm)